12-14-2018

Behavioral Deficits and Associated Alterations in the Proteome in the Amygdala of Adolescent Rats Exposed to Delta9-Tetrahydrocannabinol as Juveniles

Afzaal Nadeem Mohammed

Follow this and additional works at: https://scholarsjunction.msstate.edu/td

Behavioral deficits and associated alterations in the Proteome in the Amygdala of

adolescent rats exposed to delta9-tetrahydrocannabinol as juveniles

By

Afzaal Nadeem Mohammed

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Environmental Toxicology
in the College of Veterinary Medicine

Mississippi State, Mississippi

December 2018

Behavioral deficits and associated alterations in the Proteome in the Amygdala of

adolescent rats exposed to delta9-tetrahydrocannabinol as juveniles

By

Afzaal Nadeem Mohammed

Approved:

_____
Russell Carr
(Major Professor)

_____
Bindu Nanduri
(Co-Major Professor)

_____
Barbara L. Kaplan
(Committee Member)

_____
Jeffery B. Eells
(Committee Member)

_____
Mathew K. Ross
(Committee Member)

_____
Mark L. Lawerence
Dean
College of Veterinary Medicine

Name: Afzaal Nadeem Mohammed

Date of Degree: December 14, 2018

Institution: Mississippi State University

Major Field: Environmental Toxicology

Major Professor: Russell Carr

Title of Study: Behavioral deficits and associated alterations in the Proteome in the Amygdala of adolescent rats exposed to delta9-tetrahydrocannabinol as juveniles

Pages in Study 161

Candidate for Degree of Doctor of Philosophy

Abstract: Δ9-tetrahydrocannabinol (Δ9-THC) is an active component of marijuana. During recent years, the popularity of marijuana in the United States has increased tremendously. Marijuana edibles are a form of marijuana that has become very popular in recent years. These are easily accessible not only to adolescents but also to young children. According to recent statistical data, the consumption of marijuana edibles by children below the age of 5 has increased 600% in the states that have legalized marijuana. This has led to an adverse impact on children's health as evidenced by a sudden increase in the number of children seeking emergency assistance in hospitals. In the current research, we addressed the issue of possible persistent effects on children's behavior due to an earlier exposure to Δ9-THC. Juvenile rats were treated with 10 mg/kg of Δ9-THC from postnatal day 10 through 16. Once they reached adolescence, these rats were tested using several behavioral paradigms. To evaluate the biological basis for the behavioral deficits observed, brain samples obtained from these rats were subjected to proteomic analysis to determine any altered pathways related to the behavior. Our behavioral data indicated that juvenile exposure to Δ9-THC has no effect on anxiety-

related behavior in adolescents. However, we observed a significant effect of treatment on multiple parameters related to social interactions. Of these, episodes and time of social play were significantly increased in the Δ9-THC treated rats suggesting alterations in the reward circuit function occurring as a result of developmental Δ9-THC exposure. In the proteomics, we observed a significant effect on relevant canonical pathways such as the changes in thrombin and opioid signaling. Thrombin signaling in neurons is associated with processes involved in the connection between neurons and opioid signaling is involved in the activation processes of the reward circuit suggesting that juvenile Δ9-THC exposure alters these processes in adolescence which could have detrimental effects on behavior. Overall, our data suggest that consumption of edibles by juveniles leads to altered behavioral and biochemical outcomes in adolescence. This may be detrimental in terms of the appropriate acquisition of skills necessary for meeting the challenges in future life.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

DELTA9-TETRAHYDROCANNABINOL: MOLECULAR TARGETS, EFFECTS ON

EMOTIONAL AND SOCIAL BEHAVIOR AND INCREASING EXPOSURE

THROUGH MARIJUANA EDIBLES.

1.1 Background

Marijuana refers to the dried leaves, flowers, seeds or stems of plants known as *Cannabis sativa* or *Cannabis indica*. Marijuana resins can be obtained from these plants in the form of hash oil, wax, budder, or shatter which are widely smoked by the adolescent and adult population in order to get 'high' (NIDA: Marijuana facts 2017). Even though marijuana is currently among the most widely used illicit drug, it has been historically used for many commercial and medicinal purposes. Marijuana plants were commonly grown in the 17th century for the production of rope, sails and clothing. The medical applications for marijuana originated from Chinese folk medicine that included the use of marijuana for a variety of treatments as early as the 27th century BC (McKenna 2014). In the United States, the use of marijuana for medical and recreational purposes increased in the early 19th century and marijuana was an active ingredient in more than 20 drugs. During the same period, the prohibitionist attitudes towards drugs was increasing and marijuana was perceived as a dangerous drug able to induce sudden violent and murderous actions in intoxicated individuals (Carliner et al., 2017). In 1937, the federal Marijuana Tax Act was

passed which prohibited the use of marijuana for recreational purposes. Since this law did not ban medical uses, marijuana continued to be used for medicinal purposes under this act. However, this law discouraged the use of marijuana for medical purposes by placing the burden of detailed reporting on physicians who were in favor of prescribing marijuana (McKenna 2014). According to the Control Substance Act of 1970, the United States Drug Enforcement Administration (DEA) categorized marijuana as schedule 1 drug (i.e. a drug with no medical application and high potential of abuse). This categorization made both recreational and medical use of marijuana illegal. Despite the federal stance against marijuana use, many Americans are in favor of marijuana being used for medical purposes. Owing to the favorable attitude of public towards marijuana, states across the nation have been eliminating the legal restrictions against marijuana. California was the first state to allow marijuana for medical uses in 1996. Following that, more states allowed marijuana for medical purposes. These changes have had consistent public support due to the inclination of the majority of the population to live in jurisdictions where marijuana is legal. By 2017, 28 states have legalized marijuana for medical purposes and 7 of them allow recreational use as well (Carliner et al., 2017; Murphy and Carnevale 2016).

While legalization has alleviated the restrictions against using marijuana for medical purposes, it has also increased voluntarily self-intoxication among adolescents (Hopfer 2014). This increase in self-intoxication can be attributed not only to the increased ease of availability but also to the positive or permissible attitudes and diminished perception of marijuana-associated risks of adolescents (Walker et al., 2017). Adolescents appear to be unaware of the neurological and/or psychotic problems associated with the early-onset use of marijuana as reported by number of studies. According to the Diagnostic and Statistical

Manual of Mental Disorders, 4th edition (DSM-IV), abuse and dependence are two major problems associated with marijuana and account for 75-80% of total marijuana use disorder cases. In a statistical study, marijuana abuse and dependence cases among the US adult population occurring during 1991-1992 were compared with those occurring during 2001-2002. There was a significant increase in the level of abuse and dependence cases in the latter period despite no significant increase in marijuana use among the adult population. However, the comparatively higher potency of marijuana available in the latter period might be an additional factor in the increased level of disorders (Compton et al., 2004). Another consequence of marijuana legalization has been an increase in the popularity of marijuana among school children. A study in Monitor the Future (MTF) reported that marijuana smoking has been a common practice among 12th graders but since the mid-1990s, it has become increasingly popular among 10th graders and 8th graders because of the lowered perceived risk and increase in the ease of availability during that period (MTF 2015).

Early onset of marijuana use is associated with certain structural abnormalities in the brain that eventually lead to behavioral deficits. Neuronal pruning is one of the essential processes by which the developing brain forms into a mature brain by establishing proper connections across the brain regions. Disrupted pruning can lead to mental disorders such as autism, schizophrenia, depression and others (Boksa 2012). Neuroimaging studies have reported that neuronal pruning can be drastically affected in cannabis users (Filbey et al., 2015). In addition to neuronal pruning, heavy cannabis consumption is also related to a reduction in the area of white matter that is associated with increased impulsivity (Gruber et al., 2014), and increased activity in cortical areas that is associated with attention

3

difficulties (Abdullaev et al., 2010). Currently, the available literature is limited to addressing the adverse effects of marijuana use in adolescents. Despite the documented increase in the availability of and exposure to marijuana in children, there have been no studies that have addressed the long-term effects of marijuana exposure in children.

Edible marijuana is one of the choice methods for marijuana intake along with smoking and vaping. Starting in 2014, Colorado allowed the commercial production of edible marijuana and marijuana is now available in a form that mimics popular candies or sweets. These products can be legally purchased in retail stores in four states; Colorado, Washington State, Alaska, and Oregon (MacCoune et al., 2015). In California, marijuana edibles are available in dispensaries but only those who possess a medical marijuana card which can be obtained by anyone including those below 18 years as long as they are under a parent's supervision (Borodovsky et al., 2016). According to sales (NIDA 2014) and social networking surveys (Borodovsky et al., 2016; Lamy et al., 2016), the popularity of marijuana edible products is increasing in states with legalized marijuana as compared to non-legalized states. This has created serious health concerns because of the enhanced risk of edible marijuana being consumed by children even though current laws restrict recreational marijuana use to those under the age of 21 (MacCoun et al., 2015). In addition, the packaging of these edibles in bright colors is more likely to attract children who are unaware of the risk. This 'child friendly' packaging is increasing either deliberate or unintentional consumption of marijuana among children and there has been increased reports of cases of childhood intoxication especially in states with legalized marijuana (MacCoun et al., 2015). The data from poison control centers indicates higher rates of emergency calls and an increase in the number of patients seeking care at children's

hospitals in states with legalized marijuana because of the unintentional ingestion of marijuana in children of age below 9. Soon after the commercialization of marijuana edibles in Colorado in 2014, there was 70% increase in the calls related to marijuana exposure compared to the prior year (Ghosh et al., 2013). In another study conducted at a tertiary-care children's hospital in Colorado, there were 14 cases, all below the age of 12, that involved intoxication related to marijuana ingestion during 2009-2011 as compared to none during 2005-2009 (Wang et al., 2013). Marijuana edibles were originally introduced for medical purpose and meant for medical-prescribing to those who are not comfortable with smoking marijuana or children who can take them under caregiver's supervision. However, this has led to unintentional unwanted childhood exposure.

The main psychoactive component of marijuana is Δ9-tetrahydrocannabinol commonly known as Δ9-THC. When marijuana is consumed through any route of exposure, the concentration of Δ9-THC increases in the blood and is mainly responsible for the marijuana-related effects (NIDA 2017). Δ9-THC was first isolated in 1964 (Gaoni and Mechoulam 1964) and has been frequently studied for its pharmacological and psychoactive effects. In rodents, Δ9-THC is known to produce myriad of behavioral effects of which the most common ones are catalepsy, analgesia, hypothermia and hypolocomotion. These effects are commonly known as 'tetrad model' of cannabimimetic activity (Martin et al., 1991). Catalepsy is muscular rigidity or fixity of posture regardless of external stimuli. Several studies reported that Δ9-THC could induce catalepsy in rodent models by using bar catalepsy test (Marchese et al., 2003; Monory et al., 2007; Sano et al., 2008). Analgesia is reduced response to painful stimuli and has been detected following Δ9-THC exposure by measuring reaction time using the hot plate analgesia test (Monory

et al., 2007) or the tail-flick latency test (Fuentes et al., 1999). Similarly, Δ9-THC exposure is also associated with reductions in body temperature and locomotor activity as detected by measuring the rectal temperature and performance in the open field test, respectively (Monory et al., 2007).

To relate the route of exposure and the change in Δ9-THC levels in the blood, Lemberger and colleagues (1972) compared the plasma concentration of Δ9-THC following intravenous, ingestion, or inhalation administration of marijuana. The plasma Δ9-THC level was at its peak 70 min following inhalation and 3 hours following ingestion. Ingestion could be more dangerous route of exposure because of increased Δ9-THC bioavailability since it does not require combustion, which is the case for inhalation. Around 50% of Δ9-THC is lost in the burning process and the amount of uptake of the remainder depends upon the lung capacity and the amount of time that the smoker retains the smoke inside the lungs before exhalation (Elzinga et al., 2015). The onset of effects is rapid following inhalation. However, the onset of effects following ingestion is much slower than smoking because of delay of entrance of Δ9-THC into the blood stream. This factor is a prominent cause of marijuana-related overdoses because of the misinterpretation that initial dose did not induce the desired effect and the continued intake of more of the edible marijuana product (Brodovsky et al., 2016).

The Δ9-THC content in marijuana edibles is another major concern that could be related to the increase in intoxications. Over the time course of marijuana legalization, the Δ9-THC content in the products has increased to more than 20% from 15% (Ghosh et al., 2015). Often, underestimation of the Δ9-THC content on labeling is worsening this

problem. In a sample of 75 marijuana edibles collected from four different states, more than half of these edibles contained a label that underestimated the Δ9-THC content of the product (Vandrey et al., 2015). This could be a significant contributor towards overdose. The Δ9-THC content of marijuana edibles are calculated by number of Δ9-THC servings present in the edibles. Considering the increasing intoxication cases related to Δ9-THC ingestion, states that allow edible sales are implementing the restrictions on the amount of Δ9-THC in each serving. Δ9-THC content is set to be 10 mg/serving in Colorado and Washington State (MacCoun et al., 2015), and 5 mg/serving in Alaska and Oregon (Gourdet et al., 2017). However, there is not a policy that instructs manufacturer to limit the number of servings in each edible or to include on the label, the maximum daily intake of number of servings and/or edibles (Ghosh et al., 2015).

The primary target of Δ9-THC in the brain is the type I cannabinoid receptors (CB1; Devane 1988; Belue et al., 1994) which is a component of the endocannabinoid system. Most of the behavioral effects induced by Δ9-THC in laboratory studies are due to its action on CB1 receptors. The behavioral effects induced by Δ9-THC are because of its detrimental effects on developing neurons. Tyrosine Hydroxylase (TH) is an enzyme responsible for maturation of developing dopaminergic neurons (Fernandez-Ruiz et al., 1999). Gestational exposure to Δ9-THC has been reported to reduce the mRNA level of TH during the gestational period (Bonin et al., 1995), the postnatal/juvenile period, and the adult stage of life in rats (Suarez et al., 2000). Cholecystokonin is involved in the growth cone formation and axonal migration in developing neurons and prenatal Δ9-THC exposure reduces the density of cholecystokonin containing interneurons in hippocampus of juvenile rats (Vargish et al., 2017). Synapsin is a synaptic protein present in the axonal terminal and

important in establishing synaptic functions and exposure to Δ9-THC is associated with reduction in the mRNA levels of synapsin and CB1 receptor in adolescent rats (Steel et al., 2011). Exposure to Δ9-THC during adolescence is also related to hypofunction of GABA neurotransmission in prefrontal cortex of adult rats and this decrease in GABA neurotransmission was accompanied by an increase in the dopaminergic neurotransmission in ventral tegmental area (Renard et al., 2017).

1.2   The Endocannabinoid System

The endocannabinoids are a group of neuro-modulatory lipids involved in a variety of physiological processes such as appetite, mood, sensation, and memory. These are not stored in vesicles but are produced through an on-demand basis when neurons are in excited state. This excitation of neurons leads to increase in intracellular calcium. The increase in intracellular calcium activates calcium-dependent N-acetyl transferase (NAT) which catalyzes the rate limiting step in the production of anandamide (Kano et al., 2009). This leads to increased production of the endocannabinoids that are then released from neurons and bind to the CB1 receptors (Lutz 2004). The mechanism by which the endocannabinoids exert their function was described by Alger and Marty (2001) who studied cerebellar purkinje neurons and hippocampal neurons. They observed that depolarization in the postsynaptic GABA cell leads to release of retrograde messengers that travel to presynaptic locus to suppress the release of neurotransmitter in that neuron. They called it "depolarization induced suppression of inhibition" or DSI.   They also observed that this retrograde messenger was performing an analogous function in glutamatergic neurons by inhibition of excitation which they called as "depolarization induced suppression of excitation" or DSE. Both DSI and DSE can be blocked by CB1

receptor antagonists and enhanced by CB1 agonists indicating that the retrograde messenger inducing DSE and DSI is an endogenous cannabinoid or an endocannabinoid (Alger 2004). There are two main endogenous lipids, which bind to the CB1 receptors that are Arachidonoyl ethanolamine or Anandamide (AEA), and 2-Arachidonoyl glycerol (2-AG). These are produced in the postsynaptic neuron, cross the synapse, and stimulate CB1 receptors that are present on the presynaptic neuronal membrane. After stimulating the CB1 receptor, these lipids are taken up into the neurons through specific reuptake transporters and then degraded. The enzymes fatty acid amid hydrolase (FAAH) degrades anandamide and monoacylglycerol lipase (MAGL) degrades 2-AG (Kano et al., 2009).

1.2.1 Functions of CB1 receptors in developing brain

Being a part of the endocannabinoid system, the CB1 receptor plays a vital role in brain development. Receptor dimerization is a critical phenomenon during neuronal development. Diverse receptors, such as the neurotrophic and growth factor receptors, are recruited to the CB1 receptor which implicates a role for the CB1 receptor in neuronal development (Harkany et al., 2008). Gestational day (GD) 11-14 is an important period in rats because this is the period where formation of the neural tube from ectodermal cells occurs. The brain and spinal cord are subsequently differentiated from the neural tube (Semple et al., 2013). CB1 mRNA is detected during this period suggesting that the cannabinoid receptors are important for these developmental processes. In addition to presence of CB1 mRNA, an increase in GTP-binding protein occurs in the presence of a cannabinoid agonist. This GTP-binding was mainly observed during the gestational period in brain regions where the CB1 receptors are scarcely distributed or practically absent in the adult brain. These brain regions are enriched in neuronal fibers and include white

9

matter areas of the midbrain and brainstem (Romero et al., 1997). Later, it was reported that in white matter areas, the CB1 receptors are located in the migrating axons whose cell bodies are located at significant distance from the site of the functioning receptor. This emphasizes the role of CB1 receptor in synaptogenesis (Fernandez-Ruiz et al., 2000). This role is also supported by the fact that the expression of the CB1 receptor is positively correlated with expression of Brain Derived Neurotropic Factor (BDNF), which is involved in axonal growth and migration during development (Maison et al., 2009).

Other studies have observed that activation of CB1 receptors by external cannabinoids during pregnancy can be detrimental to key proteins involved the brain development. Neural cell adhesion molecule (L1) is responsible for cell-cell and cell-matrix interactions allowing proteins to perform their functions such as cell proliferation, migration, synaptogenesis, and myelin formation. Mutation in the L1 genes leads to malformation of brain. Activation of the CB1 receptors by Δ9-THC exposure during pregnancy resulted in down regulation of genes responsible for L1 in rat brain during the fetal (Gomes et al., 2003) and early postnatal period (Gomes et al., 2007).

Irregular activation of CB1 receptors leading to abnormalities in the brain development is not only restricted to fetal period. Activation of the CB1 receptors as a result of exposure to Δ9-THC during the perinatal ages can also be harmful enough to cause certain behavioral and neurochemical abnormalities which remain into the later stages of life. For example, decreased locomotor activity and increased stereotypic behaviors such as self-grooming and shaking have been observed in Δ9-THC treated rats and these were accompanied by decreased presynaptic activity of niagro-striatal dopaminergic neurons, decreased striatal

dopamine D1 receptor intensity, and increased dopamine turnover (Novarro et al., 1994). Another long-term effect of Δ9-THC exposure is a change in the pattern of socio-sexual and social interactions. This change is accompanied by an increase in the turnover of mesolimbic dopamine (Navarro et al., 1996). Both of these studies involved adult rats who were exposed to hashish extracts during postnatal day (PND) 1-24. These studies establish a link between behavioral alterations in adults and the dopamine neurotransmitter system. Dopamine is mainly produced by the action of the enzyme tyrosine hydroxylase (TH). The CB1 receptors are present in the neurons containing TH and activation of the CB1 receptors is associated with an increase in the level and activity of TH. Interestingly, the CB1 receptors were detected in neurons containing TH during developmental stage and disappear from these neurons in adult brain (Hernandez et al., 2000). TH contributes to many different aspects of brain development such as differentiation of dopaminergic neurons, formation of connectivity, axonal guidance, and synaptogenesis (Fernandez-Ruiz et al., 1999). Therefore, behavioral abnormalities due to perinatal Δ9-THC exposure reported in the previously mentioned studies may be occurring because of effects of CB1 activation on TH.

The complexities of brain development are not limited to fetal and perinatal ages. The neuronal maturation and synaptogenesis continue to occur during the adolescent ages as evidenced by the reduction of grey matter. This reduction is mainly due to neuronal pruning, synaptogenesis (Whitford et al., 2007) and neuronal maturation of different neurotransmitter systems such as the endocannabinoid, GABA, dopaminergic, serotonergic, and adrenergic systems which mature to adult levels by pruning (Sturman and Moghaddam 2011). Exposure to Δ9-THC during adolescence is associated with certain

neurological disorders in later life such as long lasting cognitive deficits and impaired spatial and non-spatial learning (Cha et al., 2006). Interestingly, the learning deficits observed by Cha et al were specific to adolescent exposure whereas adults were more tolerant to the Δ9-THC induced learning impairments in a water maze task.

In light of the above studies concerning the CB1 receptors, it is obvious that there is a defined pattern of CB1 expression during brain development. Any alteration in this pattern due to exposure to exogenous substances like Δ9-THC may cause persistent changes in this brain development due to effects on certain neuronal activities which would consequently lead to adverse behavioral outcomes.

1.2.2   Mechanism of CB1 receptor function

CB1 receptors are the most abundant G-protein coupled receptors in the brain and are mostly located on inhibitory neurons (GABA) and excitatory neurons (glutamate). They are differentially distributed among the different brain regions potentially because of the diverse functions that these receptors are involved in. Dense populations of the CB1 receptors were located in regions such as the basal ganglia, substantia niagra pars reticulata, globus padillus (Herkenham et al. 1990). Later, using immunohistochemical analysis and ligands specific for CB1 receptors, the sub-cellular localization of CB1 receptors in the brain areas like hippocampus and amygdala was revealed that they are present on presynaptic GABA interneurons (Katona et al., 2001). As demonstrated by Hewlett and colleagues (1984), Δ9-THC-mediated CB1 activation leads to inhibition of adenylyl cyclase activity and a decrease in cAMP production. This inhibition was not observed in the presence of pertussis toxin which prevents the binding of Gi/o subunit to G-protein

couple receptors. This identified the role of Gi/o subunit in the functioning of the CB1 receptor. It has been demonstrated that both cannabinoid agonist-mediated inhibition of adenylyl cyclase and CB1-mediated adenylyl cyclase inhibition requires GTP. This suggests a pharmacological similarity in agonist-mediated and receptor-mediated action on adenylyl cyclase (Pacheo et al., 1991). This is further strengthened by the fact that agonist-mediated inhibition of adenylyl cyclase is blocked by selective CB1 antagonists (Rinaldi-Carmona et al., 1994).

Functioning of the CB1 receptors is not only mediated through Gi/o signaling and additional mechanism have been proposed involving binding of CB1 receptors to the Gs subunit instead of the Gi/o subunit (Hewlett et al., 2004, 2005). Binding of Gs to the G-protein receptor involves intracellular calcium signaling. One study reported that activation of the CB1 receptors following administration of an agonist inhibits the N-type and Q-type calcium channels. These channels normally allow the entry of calcium ions inside the neurons, increase in the calcium current, and facilitate the release of neurotransmitters stored in small vesicles in presynaptic neurons (Mackie and Hille 1992). It was also reported that agonist-mediated CB1 activation can cause stimulation of inwardly rectifying potassium channels and potassium current (Mackie et al., 1995). This study also confirmed that CB1-mediated effects on calcium and potassium channels were dependent on GTP-binding protein, but were not consequences of cAMP depletion, because the CB1-mediated inhibition of calcium channels was not reversed in presence of an external cAMP analogue (Mackie et al., 1995). Later, it was proposed that the cannabinoid-mediated effects on potassium and calcium channels occurs through binding of the G beta-gamma subunit which dissociated from the Gi sununit following CB1 activation (Ibsen et al., 2017). This

suggested the functioning of CB1 receptors through alternate subunits rather than only through Gi/o subunit. Several other studies support these findings in that CB1 receptors function by stimulating, rather than inhibiting the activity of adenylyl cyclase and increase the production of cAMP (Maneuf and Brotchie 1997; Glass and Felder 1997; Bonhaus et al., 1998; Felder et al., 1998). These studies observed that the CB1-mediated increase in adenylyl cyclase activity and cAMP accumulation could be due to the recruitment of the Gs subunit instead of the Gi/o subunit. These studies have observed that in the presence of pertussis toxin, CB1 activation can block the inhibition of adenylyl cyclase and increase cAMP accumulation that can be reversed by administration of CB1 antagonist (Felder et al., 1998). This stimulatory effect of the CB1 receptors on cAMP implies the participation of the Gs subunit. However, the high abundance of the Gi/o subunit lowers the affinity of the Gs subunit to bind to the CB1 receptor, thus making the stimulatory action less likely to occur (Glass and Felder, 1997). Another proposed mechanism of stimulatory action by the CB1 receptor is the association with specific isoforms of adenylyl cyclase in target tissues. While the activation of the CB1 can inhibit the 5/6 or 1/3/8 isoform families of adenylyl cyclase, it can also activate the 2/4/7 isoform family of adenylyl cyclase resulting in a stimulatory action (Rhee et al., 1998). Alternatively, the differential binding of subunits to CB1 receptors may be due to the different agonists used in previous studies. Different agonists can cause different conformational changes in CB1 receptors and these conformational changes dictate the binding of specific subunits (Ibsen et al., 2017).

1.2.3   Function of CB1 receptor in mediating behavior

After the discovery that the CB1 receptor was a primary target of the cannabinoids, they have been studied for their role in memory, as well as in other types of behaviors. Stress is

one of the primary factors that plays a fundamental role in mental illnesses like depression and anxiety. When exposed to the same type of stress repeatedly, it leads to habituation which is a process of progressive decrease in the stress response after repeated exposure to same stress (Patel and Hillard, 2008). Stress habituation is controlled by glutamate release and has a prominent effect on memory encoding because it determines the ability to distinguish between stimuli that are worthy of memory consolidation and stimuli that are not. Stress habituation and memory are two interlinked processes that are controlled by the CB1 receptor through glutamate release (Litvin et al., 2013).

Social discrimination is a behavioral paradigm used to test social memory in animals. Social memory is predicated by the discriminative indices such as amount of time the animal spends with a novel or a familiar conspecific. Both genetic and pharmacological blockage of CB1 receptor in mice caused a significant increase in the discrimination indices indicating that CB1 receptor have a role in social memory (Litvin et al., 2013). Improved performance of CB1 knockout mice in an active avoidance task has been reported (Martin et al., 2002). Blockage of the CB1 receptor in adult mice is associated with improvement in working memory tasks observed in an avoidance task in the elevated T-maze suggesting significant improvement in memory acquisition and consolidation due to the suppression of the CB1 receptors (Takahashi et al., 2005). On the other hand, activation of CB1 by an agonist either by systemic administration or by direct injection into the hippocampus induced memory impairments in a water maze task and in a T-maze (Cha et al., 2006; Abush and Akirav 2010). Activation of CB1 receptors in the medial-prefrontal cortex via adeno-associated virus vector-mediated gene transfer induced impairment in the recognition memory (Klugmann et al., 2011).

The negative effects of CB1 activation on learning and memory could be due to changes in the level of anxiety mediated by CB1 activation because anxiety facilitates learning and memory (Litvin et al., 2013). Anxiety is a type of emotional response that involves the release of diverse neurotransmitters from different brain regions. CB1 receptor control anxiety because of their retrograde function that inhibits the release of neurotransmitters (Ruehle et al., 2012). Genetic or pharmacological manipulation of the CB1 receptors is associated with prominent effects on anxiety and the high density of expression of CB1 receptors in the brain areas, such as the amygdala that mediates emotions, strengthens the concept of the involvement of CB1 receptors in anxiety behavior (Kathuria et al., 2002). CB1 activation has a biphasic effect on anxiety as demonstrated by low doses of agonists inducing an anxiolytic response whereas high doses of agonists induce an anxiogenic response (Vivors et al., 2005). In addition, CB1 receptors in different brain regions differentially regulate anxiety. In the medial prefrontal cortex and dorsal hippocampus (Rubino et al., 2008; Libosa et al., 2015), CB1 activation induced by administration of either direct or indirect agonists produced anxiolytic effects in the elevated plus maze. Whereas, administration of direct agonist in the amygdala produced anxiogenic effects (Rubino et al., 2008). Anxiogenic effects were also observed following systemic administration of indirect agonists (Manduce et al., 2015). In contrast, Libosa and colleagues (2015) reported anxiolytic effects following CB1 activation by an indirect agonist in the amygdala. The basis for this disparity may be due to the fact that anxiety behavior is sensitive to the state of emotionality of the animal where anxiolytic behavior is displayed when tested under low emotional arousal but a state of high emotional arousal masks the anxiety (Morena et al., 2016).

Resident intruder tests and chronic unpredictable mild stress models (CMS) are two valid animal behavioral paradigms used to measure aggression and depression, respectively. To investigate the role of the CB1 receptors in emotional responses, CB1 knockout mice were tested in the resident intruder test and the CMS. Anhedonia, a loss of responsiveness to a pleasurable event, is a measure of depressive behavior exhibited by animals during CMS. The number of attacks made by an intruder when it is placed into a resident's cage measures aggressive behavior. CB1 knockout mice exhibited decreased preference for sucrose water in the CMS and increased intruder attacks in the resident intruder test (Martin et al., 2002).

The CB1 receptors are also associated with social behaviors that are an integral part of learning and developing skills necessary for survival of a group or species. Several studies have addressed the role of CB1 receptors on social behaviors by examining the effects of CB1 agonists or antagonists on play behavior, the earliest form of social behavior (Trezza et al., 2010). CB1 activation by systemic administration of either direct or indirect agonists was found to be associated with a reduction in play as demonstrated by decreased frequency of play solicitation and reciprocation (Trezza and Vanderschuren 2008a, 2008b). In contrast, another study reported an increase in the overall play activity induced by CB1 activation. Pinning is one of the characteristic activities performed by a pair of rats when they are subjected to play. This activity is an important measure of play behavior because execution of pinning happens by equal involvement of both partners. CB1 activation by an indirect agonist induced an increase in the number of pinning in playing rats and this increase was masked in presence of CB1 antagonist (Trezza and Vanderschuren 2009). A similar finding was also reported by Manduce et al. (2015) where CB1 activation by endogenous cannabinoids increased the amount of pinning and pouncing during play

sessions in adolescent rats. Another line of thought concerning the mechanistic basis of social play is the hypothesis that there is a co-activation of the cannabinoid, opioid, and dopamine receptor system during the social play. Social play has been reported to be reduced in rats that were treated with a direct CB1 agonist. However, administration of an indirect agonist enhanced social play and this involved co-activation of the dopamine and opioid receptor systems along with CB1. This enhancement of play induced by the activation of CB1 disappeared in the presence of either an opioid or a dopamine receptor antagonist. In contrast, the presence of a CB1 antagonist can suppress the enhancing effect of the opioid agonist morphine on social play (Review: Trezza et al., 2008).

Considering that CB1 activity is strongly influenced by Δ9-THC exposure and that there is a significant role for CB1 receptors in brain development as well as in mediating anxiety and social behavior, it is possible that exposure to Δ9-THC during a critical window of brain development can have a long-term impact on these types of behaviors. This research will address this issue by testing the following central hypothesis: **Repeated exposure of juveniles to Δ9-THC alters the CB1 signaling pathways leading to long-term effects on behaviors related to anxiety and social interactions.**

1.3   Review of Literature

1.3.1   Elevated plus maze (EPM)

The EPM is a widely accepted and validated animal model designed to measure the anxiety level in rats and mice (Hogg 1996). The EPM apparatus consists of two closed arms and two open arms of equal size and is elevated to a considerable height above the ground. The open arms have a higher illumination compare to the closed arms. The anxiety level in rats

or mice is predicated based on their natural tendency to avoid illuminated areas. Both forced and voluntary passages into the open arm are associated with increased plasma corticosterone levels and increased freezing which are hormonal and behavioral changes indicative of increased anxiety, respectively (Pellow et al., 1985). The natural exploratory behavior of rat and mice normally occurs more in the closed arms and the time spent in the closed arms increases when the animals are exposed to anxiogenic compounds. However, administration of anxiolytic compounds reduces the natural aversion to the open arms and increase the exploration and time spent in the open arms (Hogg 1996). In recent studies, the EPM has also been used to measure locomotor activity which is indexed by the exploration within the closed arm (Brujinzeel et al., 2016) and the number of closed arm entries (Huang et al., 2010). Table 1.1, below, indicates different types of behaviors reflected by exploration of animal in different parts of EPM.

Table 1.1   Specific behaviors represented by level of exploration performed by rats or mice in different arms of elevated plus maze

EMP Parameters	Associated Behavior	Reference
Fraction of time spent in open arm exploration	Anxiety behavior	Hogg 1996
Fraction of time spent in closed arm exploration	Anxiety behavior	Hogg 1996
	Locomotor activity	Brujinzeel et al., 2016

Table 1.1 (continued)

Fraction of number of open arm entries	Anxiety behavior	Komada et al., 2008
Number of closed arm entries	Locomotor activity	Huang et al., 2010

There are a fair number of studies examining the effect of Δ9-THC on anxiety by using the elevated plus maze. One study reported that administration of Δ9-THC at a dosage of 0.75 mg/kg (i.p) 30 min before the trial in adolescent male rats can significantly induce anxiolytic effects as demonstrated by increased open arm exploration. This study further examined the potential involvement of serotonin receptors (5HT1A) in the Δ9-THC-induced anxiolytic effects and found attenuation of Δ9-THC-induced anxiolytic effects in presence of a 5HT1A antagonist (Braida et al., 2007). Similar findings were also reported by another study which observed anxiolytic behavior in adolescent rats receiving Δ9-THC (i.p) in the dosage range of 0.075-1.5 mg/kg 30 min prior to testing in the EPM (Rubino et al., 2007). This study suggested the mechanistic basis of CB1-mediated anxiety involves the activity of several kinases.

cFOS is an activator protein that forms transcriptional factor by dimerization with members of jun family and can be used as a mapping tool to resolve the neuronal circuits underlying the behaviors induced by stress including anxiety (Kovacs 1998). CREB is a transcriptional factor that is activated in response to cAMP and associated with depressive or emotional behaviors (Barrot et al., 2002). CREB-mediated gene expression is closely

related to the activity of kinases such as protein kinase A (PKA) which phosphorylates CREB and calcium/calmodulin protein kinase II (CaMKII) which inhibits CREB by preventing dimerization and binding of CREB-related proteins (Rubino et al., 2007). In the EPM, cFOS phosphorylation was increased in behavioral control rats compared to rats that did not go through testing. Δ9-THC treatment altered this scenario in that there was a decrease in the level of phosphorylated cFOS in the prefrontal cortex (PFC) and amygdala. In the hippocampus, testing in the EPM did not have any effect on CREB, CaMKII, PKA, and extracellular-signal-regulated kinase (ERK) signaling. Δ9-THC treatment also altered this scenario by reducing the activity of CaMKII which, in turn, triggers CREB activation. All of the Δ9-THC-induced effects observed in this study were reversed in the presence of the CB1 antagonist AM251 which suggests CB1-mediated effects on all of these kinases and transcription factors. In PFC, there was an inhibitory effect of EPM on CaMKII, but there was no prominent effect of Δ9-THC (Rubino et al., 2007). This disparity of effects in different regions could be due the fact that these three regions differentially modulate the anxiety induced by Δ9-THC. Direct administration of Δ9-THC into the hippocampus and PFC produced anxiolytic effects whereas, direct administration of Δ9-THC into the amygdala led to anxiogenic effects (Rubino et al., 2008b). On the other hand, an anxiogenic response was observed at the dosage of 2.5 mg/kg Δ9-THC (i.p) given to adolescent and adult rats 30 min before the EPM trial with adults showing significantly more effects at the same dosage (Schramm-Sapyta et al., 2007). An anxiogenic response was also observed in adolescent rats who were given gradually increasing doses of Δ9-THC on the scale of 2.5-10 mg/kg for 11 consecutive days. This anxiogenic response persisted for seven days following the last exposure (Stopponi et al., 2014). However, no effects were observed 30

days following the administration using the same dosing scale and regimen (Rubino et al., 2008a).

The most common route of exposure to Δ9-THC in humans at adolescent ages is through inhalation in the form of smoke by Δ9-THC cigarettes or pipes. Recently, Brujinzeel and colleagues (2016) simulated this exposure paradigm by exposing 55 days old male rats to cannabis smoke containing 5.7% Δ9-THC. The concentration of Δ9-THC in the serum following the exposure was found to be 225 ng/ml which is similar to the level detected in the humans following smoking cannabis. This study observed no significant effects on open arm activity but did observe an increase in exploration of closed arm in treated rats as compared to control rats. This suggests that Δ9-THC-induced increase in locomotor activity which was then confirmed by using open field test. This increased locomotion was only observed when tested immediately after the exposure and the effects were no longer detected at 4 hours following exposure. However, this time-line of appearance and disappearance of effect does not always hold true because other studies reported contradictory findings. Chronic administration of 3 mg/kg (i.p) Δ9-THC to male mice at adolescent or adult ages for three weeks did not change the level of anxiety in any group when the EPM test was performed immediately following treatment. When the EPM test was performed six weeks following the cessation of exposure, there was an increased aversion to the open arms in the treated rats (Murphy et al., 2017). Another study investigated the effect of 10 mg/kg Δ9-THC administered subcutaneously in male mice daily for 10 consecutive days then followed by administration of CB1 antagonist SRA141716 (i.p) on the last day in sub-samples of both Δ9-THC and vehicle mice. Mice that received Δ9-THC alone performed in the same way as controls in terms of percent

22

open arm entries and percent time in the open arms. Δ9-THC-treated mice that were given a challenge of 3 mg/kg of SRA141716 exhibited a sharp decrease in the percent time in the open arms and percent open arm entries as compared to their respective controls. These data suggest the anxiety inducing effect of Δ9-THC withdrawal (Huang et al., 2010), which is also observed in humans (Winstock and Lea 2009). However, rats and mice may have a different time-line of anxiogenic effects following Δ9-THC withdrawal because Rubino and colleagues (2008a) observed no effects in rats 30 days after the last exposure but Murphy and colleagues (2017) observed effects in mice following 42 days after the last exposure (Murphy et al., 2017).

1.3.2   Social play

Play is a deceptive term as it not so easily defined as it sounds because of the ambiguity in the categorizing the behavior as "playful behavior". Play is associated with many aspects of development and especially important is the fact that it provides a supportive environment in which children can develop the skills to interact with others. In most mammals, play is generally observed between the ages of weaning and sexual maturation. This phenomenon is very important in juvenile animals and one of the determining factors in establishing adult behavior (Anne and Einon, 1981). There are many theories concerning the concept of play behavior but the empirical evidence to actually define the boundaries of actions involved in play are still questionable. This may be because actions involved in play are so random and seem to be performed without any purpose (Panksepp et al., 1984). Because of the ambiguity in these actions and the absence of a specific purpose in performing them, the biochemical mechanism that regulate the programming of this behavior is not well characterized (Blake and McCoy, 2015). However, play is thought to

be tightly regulated by some unidentified brain circuits not only in the short term but throughout the life span of an organism (Panksepp et al., 1984).

1.3.2.1   Social play in developmental disorders

It has also been determined in human juveniles that play behavior is necessary for the development of normal social interactions. Play behavior therapy is currently being used as a treatment in several neurodegenerative disorders (Dawson 2008). Social play has a prominent role in identification and diagnosis of the autism spectrum disorder. Autism is a developmental disorder characterized by dysfunctions in specific aspects of behavior including the ability to participate in social interactions, the ability to communicate, and characterized by repetitive behaviors and lack of interests (Hille 2004). These deficits are similar to alterations in social play as evidenced by the significant differences in social play activities observed in children diagnosed with autism. In addition, a modified form of play that would facilitate autistic children to engage in play could be an effective therapy in autism (Restall and Magill-Evan 1993). Alterations in play behavior is also associated with Attention Deficit Hyperactivity Disorder (ADHD). When observing patterns of play, pre-school children with ADHD exhibited different patterns of play as compared to non-ADHD children. In ADHD children, play activities were decreased while non-play activities were increased. There was also a decrease in the sustained attention of these children and an increase in the frequency of transition from one activity to another (Alessandri 1992).

1.3.2.2   Structure and neurobiology of play

Based on our present knowledge about most behaviors, the molecular mechanisms controlling a specific behavior are not well characterized and there is no single gene

directly involved in the control of certain behaviors (Peills and Peills 1993, Popova 2006, Blake and McCoy 2015). This is especially true for play. Play is a complex form of behavior involving a variety of random actions performed by the individuals involved that are not only difficult to capture in words but the boundaries between the different forms of these random actions are even difficult to define. Despite play being a very complex and random phenomenon, it does have a distinct role in the establishment of normal social, sexual, and aggressive behavior in adults and it is one of those essential tasks required for the normal development of the brain (Panksepp et al., 1984).

In an experimental setup, social play in rats can be observed by documenting the occurrence of certain parameters such as pouncing, chasing, grooming wrestling, napping, pinning, sniffing and crawl over. A bout of play generally starts by one rat approaching or soliciting another to play. The soliciting rat attempts to sniff or rub the neck of the play partner and from this, chasing, boxing, wrestling crawling, and/or pinning may follow. The time that two rats spend involved in these activities is considered as the time of play. Each of these activities has been validated to represent specific type of behavior (Table 1.2). From the neurobiological point of view, social play is influenced by multiple centrally acting neurotransmitters such as acetylcholine, adenosine, catecholamine, dopamine, serotonin, opioids, and noradrenaline (Vanderchuren et al., 1997).

Table 1.2   Different types of activities performed during the act of play and associated
           behaviors represented by each activity

Play Activities	Behavior	Reference
Sniffing/anogenital sniffing	Contact behavior	Peills and McKenna 1995.
Self-Grooming	Aggressive behavior	Mickzek 1974
Crawling over/under	Unfamiliar situation	Peills and McKenna 1995.
Chase	Appetitive play	Panksepp and Beatty 1980.
Nape attack	Play solicitation	Peills and McKenna 1995.
Pinning	Play variable	Panksepp and Beatty 1980.
Wrestling/Time of wrestling	Consummatory play	Panksepp and Beatty 1980.

1.3.2.3   Effect of Δ9-THC on play behavior

Miczek (1974) first reported the effect of Δ9-THC on aggressive interactions by observing

a dominant adult male rat and a subordinate adult male rat inside a dark chamber. When

the subordinate rats were administered 4 mg/kg (i.p) of Δ9-THC, the defensive behaviors

of the submissive rats were decreased as evidenced by the increased incidence of severe

injuries inflicted by the dominant rat during the test. When the dominant rat was

administered the same dosages of Δ9-THC, there was reduction in the frequency of

aggressive attacks towards the subordinate conspecific. In a subsequent study, injection of

5 mg/kg of Δ9-THC (i.p) into adult mice resulted in a reduction in exploratory behavior

and an increase in immobility in male-to-male encounters. In male-female encounters,

there was a reduction in sexual behaviors as indicated by decrease in the number of mounts and chases followed by sniffs (Cutler and Mackintosh 1984).

Active social interaction of two rats placed into the same arena can be measured by the time it takes for the rats to approach each other or for one rat to move towards a stationary conspecific. Higher latency indicates a reduced level of interaction. As reported by Malone and colleagues (2009), the latency of initial interaction was reduced in adolescent rats treated with 1 mg/kg of Δ9-THC (ip) but this reduction was not observed at higher dosages (3 or 10 mg/kg). Similarly, an increase in investigative behavior was observed in adult rats (PND90) receiving 2 mg/kg of Δ9-THC (sc) during PND2-10 (Newsome and Kelley, 2008). However, during exposure, no effect on total social interactions were observed in adult and adolescent rats receiving a chronic dosages of 5 mg/kg Δ9-THC (ip). However, when a social interaction test was performed 16 days following the last administration of Δ9-THC, there was a decline in social interactions in the treated rats (Quinn et al., 2008). A decline in social interactions as evidenced by reduced body sniffing and allo-grooming and increased rearing was observed in adult rats receiving 2 mg/kg of Δ9-THC as compared to saline-control rats. Interestingly, this study also reported no differences in social interactions between Δ9-THC-treated rats and ethanol-control rats (Slamberova et al., 2016) suggesting that it is possible that ethanol, which is the most frequently used solvent in Δ9-THC studies, may have some effect on behavioral performance in rats.

1.4   Proteomic Approach to Study Behavior

Proteomics involves the study of complete protein complement of the genome of an organism. Recently, this technology has been employed to study the protein profiles in

rodent brain to study behavioral and/or developmental changes (Richard et al., 2002). The gel-based proteomics is one of the classic methods used for protein identification and quantification. This method involves the separation of proteins by using two dimensional polyacrylamide gel electrophoresis (2D PAGE) and identification of proteins by using Mass Spectrometry (MS) or Tandem Mass Spectrometry (MS/MS; Chevalier 2010). Gel-based proteomics has been employed to study the changes in the protein expression in mice with anxiety like behavior and psychomotor deficits. The differentially expressed proteins were then analyzed in Ingenuity Pathway Analysis (IPA; Qiagen Inc., https://www.qiagenbioinformatics.com) to predict the biofunctional categories in which these differentially expressed proteins were involved in (Abdullah et al., 2011). Similarly, the gel-based proteomic technique was used to evaluate the differentially expressed proteins in developing brains of rats and mice (Quinn et al., 2008; Taoka et al., 2000; Fountoulakis et al., 1999) or to quantify the differentially expressed proteins in different brain regions obtained from human Alzheimer's Disease patients (Schonberger et al., 2001). The gel-based proteomics method can also be modified to specifically identify oxidatively modified proteins or phosphorylated proteins in tissues to obtain more specific information about experimental or disease conditions (Butterfield et al., 2006). Another method utilized in proteomics is the label free proteomic approach, which involves digestion of individual protein samples followed by separation of digested peptides by using liquid chromatography. The separated peptides are then introduced into a mass spectrometer to generate the MS/MS spectra for each identified peptide (Zhu et al., 2010). The number of spectra matched to specific peptide in a protein is a linear measure for relative abundance of the protein (Zhang et al., 2006). In this study, we employed the label free shotgun

proteomic approach to study the expression pattern of proteins in the amygdala of adolescent rats exposed to Δ9-THC as juveniles.

Considering the increased availability of marijuana edibles and the adverse effects of its active component (Δ9-THC) on brain development, this research was designed to investigate the long-lasting effects of Δ9-THC ingestion at early stage of life in rat model. Chapter II will investigate the Δ9-THC-mediated effects on anxiety level and performance during social interaction test at adolescent stage. Chapter III will not study behavior but will address proteins and canonical pathways affected following repeated Δ9-THC ingestion. Chapter IV will analyze the canonical pathways involved in the social behaviors, and alterations in these canonical pathways following repeated Δ9-THC ingestion. Chapter V will be the overall conclusion emphasizing the adverse behavioral outcomes, and alterations in canonical pathways at adolescent ages due to unwanted activation of CB1 receptors at juvenile ages.

1.5   Reference:

Abdullaev, Y., Posner, M.I., Nunnally, R., Dishion, T.J., 2010. Functional MRI evidence for inefficient attentional control in adolescent chronic cannabis abuse. Behavioral brain research 215, 45–57.

Abdullah, L., Crynen, G., Reed, J., Bishop, A., Philips, J., Ferguson, S., Mouzon, B., Mullen, M., Mathura, V., Mullen, M., Ait, Ghazala, G., Crawford, F. 2011. Proteomics CNS profile of delayed cognitive impairment in mice exposed to gulf war agents. Neuromolecular methods, 13, 274-288.

Abush, H., Akirav, I., 2010. Cannabinoids modulate hippocampal memory and plasticity. Hippocampus 20, 1126–1138.

Alessandri, 1992. Journal of abnormal child psychology, 20, 289-302.

Alger, B, E. 2005. Endocannabinoids and their implications in epilepsy. Epilepsy currents, 2, 27–36.

Amirav, I., Luder, A., Viner, Y., Finkel, M., 2011. Decriminalization of cannabis - Potential risks for children? Acta paediatrica international journal of paediatrics, 100, 618–619.

Anne PH and Einon FD: Play as a reinforcer in maze learning in juvenile rats, 1981. Animal behavior 29, P: 259-270

Barrot, M., Olivier, J.D.A., Perrotti, L.I., DiLeone, R.J., Berton, O., Eisch, A.J., Impey, S., Storm, D.R., Neve, R.L., Yin, J.C., Zachariou, V., Nestler, E.J., 2002. CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proceedings of national academy of science 99, 11435–11440.

Barthelemy, O.J., Richardson, M.A., Cabral, H.J., Frank, D.A., 2016. Prenatal, perinatal, and adolescent exposure to marijuana: Relationships with aggressive behavior. Neurotoxicollgy and teratology 58, 60–77.

Becker, B., Wagner, D., Gouzoulis-Mayfrank, E., Spuentrup, E., Daumann, J., 2010. The impact of early-onset cannabis use on functional brain correlates of working memory. Progress in neuro-Psychopharmacology biological psychiatry 34, 837–845.

Belue, R.C., Howlett, A.C., Westlake, T.M., Hutchings, D.E., 1995. The ontogeny of cannabinoid receptors in the brain of postnatal and aging rats. Neurotoxicology and teratology 17, 25–30.

Blake, B.E., McCoy, K.A., 2015. Hormonal programming of rat social play behavior: Standardized techniques will aid synthesis and translation to human health. Neuroscience and biobehavioral reviews 55, 184–197.

Bonin, A., de Miguel, R., Hernandez, M, L., Ramos, J, A., Fernandez-Ruiz, J, A. 1995. Prenatal exposure ot delta9-tetrahydrocannabinol alters the gene expression and the activity of tyrosine hydroxylase during early brain development. Life science, 56, 2177-2184.

Boksa, P., 2012. Abnormal synaptic pruning in schizophrenia: Urban myth or reality? Journal of psychiatry and neuroscience 37, 75–77.

Bonhaus, D.W., Chang, L.K., Kwan, J., Martin, G.R., 1998. Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. Journal of pharmacology and experimental therapeutics 287, 884–888.

Braida, D., Limonta, V., Malabarba, L., Zani, A., Sala, M., 2007. 5-HT1A receptors are involved in the anxiolytic effect of Δ9-tetrahydrocannabinol and AM 404, the anandamide transport inhibitor, in Sprague-Dawley rats. European journal of pharmacology 555, 156–163.

Brodovsky, J,D., Crossier, B,S., Lee, D,C., Sargent, J,D., Budney, A.J. 2016. International journal of drug policy, 36,141-147.

Bruijnzeel, A.W., Qi, X., Guzhva, L. V., Wall, S., Deng, J. V., Gold, M.S., Febo, M., Setlow, B., 2016. Behavioral characterization of the effects of cannabis smoke and anandamide in rats. PLoS One 11, 1–24.

Buckner, J.D., Ecker, A.H., Cohen, A.S., 2010. Mental health problems and interest in marijuana treatment among marijuana-using college students. Addictive Behaviors 35, 826–833.

Butterfield, A, D., Abdul, A, M., Newman, S., Reed, T., 2006. Redox proteomics in some age-related neurodegenerative disorders or model thereof. The journal of American society for experimental neurotherapeutics, 3, 344-357.

Campolongo, P., Trezza, V., Ratano, P., Palmery, M., Cuomo, V., 2011. Developmental consequences of perinatal cannabis exposure: Behavioral and neuroendocrine effects in adult rodents. Psychopharmacology 214, 5–15.

Carliner, H., Brown, Q.L., Sarvet, A.L., Hasin, D.S., 2017. Cannabis use, attitudes, and legal status in the U.S.: A review. Preventive medicine 104, 13–23.

Caulkins, J.P., 2017. Recognizing and regulating cannabis as a temptation good. International Journal of drug policy 42, 50–56.

Cha, Y.M., White, A.M., Kuhn, C.M., Wilson, W.A., Swartzwelder, H.S., 2006. Differential effects of delta9-Δ9-THC on learning in adolescent and adult rats. Pharmacology biochemistry and behavior 83, 448–455.

Charilaou, P., Agnihotri, K., Garcia, P., Badheka, A., Frenia, D., Yegneswaran, B., 2017. Trends of Cannabis Use Disorder in the Inpatient: 2002 to 2011. American journal of medicine 130, 678–687

Claudet, I., Le Breton, M., Bréhin, C., Franchitto, N., 2017. A 10-year review of cannabis exposure in children under 3-years of age: do we need a more global approach? European journal of pediatrics 176, 553–556.

Claudet, I., Mouvier, S., Labadie, M., Manin, C., Michard-Lenoir, A.-P., Eyer, D., Dufour, D., 2017. Unintentional Cannabis Intoxication in Toddlers. Pediatrics 140.

Compton, W.M., Grant, B.F., Colliver, J.D., Glantz, M.D., Stinson, F.S., 2004. Prevalence of Marijuana Use Disorders in the United States. Journal of American medical association 291, 2114-2121

Dawson G: Early behavioral intervention, brain plasticity, and the prevention of autism spectrum disorder,2008. Developmental Psychopathology, 20, P: 775–803.

Devane, W,A., Dysarz III, F,A., Johnson, M, R., Melvin, L,S., Howlett, A,C. 1988. Determination and characterization of cannabinoid receptors in rat brain. Molecular pharmacology, 34, 605-613.

Dugré, J.R., Dellazizzo, L., Giguère, C. édouard, Potvin, S., Dumais, A., 2017. Persistency of cannabis use predicts violence following acute psychiatric discharge. Frontiers in psychiatry 8, 1–8.

Elzinga, S., 2015. The Conversion and Transfer of Cannabinoids from Cannabis to Smoke Stream in Cigarettes. Natural products chemistry and research 3, 1–5.

Felder, C.C., Joyce, K.E., Briley, E.M., Glass, M., Mackie, K.P., Fahey, K.J., Cullinan, G.J., Hunden, D.C., Johnson, D.W., Chaney, M.O., Koppel, G. a, Brownstein, M., 1998. LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. Journal of pharmacology and experimental therapeutics 284, 291–297.

Fernandez-Ruiz, J., Berrendoro, F., Hernandez, M, L., Romero, J., Ramos, J,A., 1999. Role of endocannabinoids in brain development. Life sciences, 65, 725-736.

Fernandez-Ruiz, J., Berrendero, F., Hernandez, M.L., Ramos, J.A., 2000. The endogenous cannabinoid system and brain development. Trends in neuroscience 23, 14–20.

Filbey, F.M., McQueeny, T., DeWitt, S.J., Mishra, V., 2015. Preliminary findings demonstrating latent effects of early adolescent marijuana use onset on cortical architecture. Developmental cognitive neuroscience 16, 16–22.

Fontes, M.A., Bolla, K.I., Cunha, P.J., Almeida, P.P., Jungerman, F., Laranjeira, R.R., Bressan, R.A., Lacerda, A.L.T., 2011. Cannabis use before age 15 and subsequent executive functioning. British journal of psychiatry 198, 442–447.

Fountoulakis, M., Schuller, E., Hardmeier, R., Berndt, P., Luber, G. 1999. Electrophoresis. 20, 3572-3579.

Fuentes, J, A., Ruiz-Gayo, M., Manzarnas, J., Vela, G., Reche, I., Corchero, J. 1999. Cannabinoids as potential new analgesics. Life sciences, 65, 675-685.

Gaoni, Y., Mechoulam, R., 1964. Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. Journal of American chemical society 86, 1646–1647.

Glass, M., Felder, C.C., 1997. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. Journal of neuroscience 17, 5327–5333.

Goldschmidt, L., Richardson, G.A., Larkby, C., Day, N.L., 2016. Early marijuana initiation: The link between prenatal marijuana exposure, early childhood behavior, and negative adult roles. Neurotoxicology and teratology 58, 40–45.

Gomes, M., Hernandez, M., Fernandez-Ruiz, J. 2003. Activation of cannabinoid receptors during early postnatal development reduces the expression of cell adhesion moleculr L1 in the rat brain. Brain research, 1145, 48-55.

Gomes, M., Hernandez, M., Fernandez-Ruiz, J. 2007. The activation of cannabinoid receptors during early postnatal development reduces the expression of cell adhesion molecule L1 in the rat brain.

Gourdet, C., Giombi, K.C., Kosa, K., Wiley, J., Cates, S., 2017. How four U.S. states are regulating recreational marijuana edibles. International journal of drug policy 43, 83–90.

Gruber, S.A., Dahlgren, M.K., Sagar, K.A., Gönenç, A., Lukas, S.E., 2014. Worth the wait: Effects of age of onset of marijuana use on white matter and impulsivity. Psychopharmacology 231, 1455–1465.

Guttmannova, K., Kosterman, R., White, H.R., Bailey, J.A., Lee, J.O., Epstein, M., Jones, T.M., Hawkins, J.D., 2017. The association between regular marijuana use and adult mental health outcomes. Drug and alcohol dependence 179, 109–116.

Harkany, T., Keimpema, E., Barabás, K., Mulder, J., 2008. Endocannabinoid functions controlling neuronal specification during brain development. Molecular and cellular endocrinology 286, 84–90.

Herkenham, M., Lynn, A.B., Litrle, M.D., Johnsont, M.R., Melvin, L.S., De Costa, B.R., Riceo, K.C., 1990. Cannabinoid receptor localization in brain. Neurobiology 87, 1932–1936.

Hernandez, M., Berrendoro, F., Suarez, I., Garcia-Gila, L., Cebeirra, M., Mackie, K., Ramosa, J, A., Fernandez-Ruiz, J. Cannabinoid receptors are co-localized with tyrosine hydroxylase in cultured fetal mesencephalic neurons and their activation increases the levels of this enzyme. Brain research, 857, 56-65.

Hille, E, L. 2004. Executive dysfunction in autism. Trends in cognitive sciences, 8, 26-32.

Hogg, S., 1996. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacology biochemistry and behavior 54, 21–30.

Hopfer, C. 2014. Implication of marijuana legalization for adolescent substance abuse. Substance abuse, 35, 331-335.

Howlet, A, C. 2005. Cannabinoid receptor signaling. Hep, 168, 53-79.

Howlett, A,C.1984. Inhibition of neuroblastoma adenylate cyclase by cannabinoid and nantradol compounds. Life science, 35, 1803-1810.

Howlett, A.C., Breivogel, C.S., Childers, S.R., Deadwyler, S.A., Hampson, R.E., Porrino, L.J., 2004. Cannabinoid physiology and pharmacology: 30 Years of progress. Neuropharmacology 47, 345–358.

Huang, P., Liu-Chen, L.Y., Kirby, L.G., 2010. Anxiety-like effects of SR141716-precipitated delta9-tetrahydrocannabinol withdrawal in mice in the elevated plus-maze. Neuroscience letters 475, 165–168.

Humphreys, A.P., Einon, D.F., 1981. Play as a reinforcer for maze-learning in juvenile rats. Animal behavior 29, 259–270.

Johnston, L.D., Malley, P.M.O., Miech, R.A., Bachman, J.G., Schulenberg, J.E., 2015. Monitoring the FUTURE: Key Findings on Adolescent Drug Use - 2015. Institute of social research 1–104.

Kano, M., 2009. Endocannabinoid-mediated control of synaptic transmission. Physiological reviews 89, 309–380.

Kathuria, S., Gaetani, S., Fegley, D., Valiño, F., Duranti, A., Tontini, A., Mor, M., Tarzia, G., La Rana, G., Calignano, A., Giustino, A., Tattoli, M., Palmery, M., Cuomo, V., Piomelli, D., 2003. Modulation of anxiety through blockade of anandamide hydrolysis. Nature medicine 9, 76–81.

Katona, I., Rancz, E.A., Acsády, L., Ledent, C., Mackie, K., Hajos, N., Freund, T.F., 2001. Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. Journal of neuroscience 21, 9506–9518.

Klugmann, M., Goepfrich, A., Friemel, C.M., Schneider, M., 2011. AAV-Mediated Overexpression of the CB1 Receptor in the mPFC of Adult Rats Alters Cognitive Flexibility, Social Behavior, and Emotional Reactivity. Frontiers in behavioral neurosci. 5, 1–10.

Kovács, K.J., 1998. c-Fos as a transcription factor: A stressful review from a functional map. Neurochemistry international 33, 287–297.

Lamy, F.R., Daniulaityte, R., Sheth, A., Nahhas, R.W., Martins, S.S., Boyer, E.W., Carlson, R.G., 2016. "Those edibles hit hard": Exploration of Twitter data on cannabis edibles in the U.S. Drug and alcohol dependence 164, 64–70.

Levine, A., Clemenza, K., Rynn, M., Lieberman, J., 2017. Evidence for the Risks and Consequences of Adolescent Cannabis Exposure. Journal of American academy of child and adolescent psychiatry 56, 214–225.

Lisboa, S.F., Borges, A.A., Nejo, P., Fassini, A., Guimarães, F.S., Resstel, L.B., 2015. Cannabinoid CB1 receptors in the dorsal hippocampus and prelimbic medial prefrontal cortex modulate anxiety-like behavior in rats: Additional evidence. Progress in neuro-Psychopharmacology biological psychiatry 59, 76–83.

Litvin, Y., Phan, A., Hill, M.N., Pfaff, D.W., McEwen, B.S., 2013. CB$_1$ receptor signaling regulates social anxiety and memory. Genes brain and behavior 12, 479–489.

Litvin, Y., Phan, A., Hill, M.N., Pfaff, D.W., McEwen, B.S., 2013. CB$_1$ receptor signaling regulates social anxiety and memory. Genes brain and behavior 12, 479–489.

Lutz, B., 2004. On-demand activation of the endocannabinoid system in the control of neuronal excitability and epileptiform seizures. Biochemical pharmacology 68, 1691–1698.

MacCoun, R,J., Mello, M,M. 2015. Half baked – The retail promotion of marijuana edibles. New England journal of medicine, 363, 989-991.

MacCoun, R.J., Mello, M.M., 2015. Half-Baked — The Retail Promotion of Marijuana Edibles. New England journal of medicine 372, 989–991.

Mackie, K., Hille, B., 1992. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proceedings of the national academy of science 89, 3825–3829.

Mackie, K., Lai, Y., Westenbroek, R., Mitchell, R., 1995. Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. Journal of neuroscience 15, 6552–6561.

Malone, D.T., Jongejan, D., Taylor, D.A., 2009. Cannabidiol reverses the reduction in social interaction produced by low dose Delta9-tetrahydrocannabinol in rats. Pharmacology biochemistry and behavior 93, 91–96.

Maneuf, Y.P., Brotchie, J.M., 1997. Paradoxical action of the cannabinoid WIN 55,212-2 in stimulated and basal cyclic AMP accumulation in rat globus pallidus slices. British journal of pharmacology 120, 1397–1398.

Marchesse, G., Casti, P., Ruiu, S., Saba, P., Sanna, A., Casa, G-L., Pani, L. 2003/ Haloperidol but not clozapine produces dramatic catalepsy in THC treated rats: possible clinical implications. British journal of pharmacolog, 140, 520-526.

Martin, M., Ledent, C., Parmentier, M., Maldonado, R., Valverde, O., 2002. Involvement of CB1 cannabinoid receptors in emotional behaviour. Psychopharmacology 159, 379–387.

Martin, B, R., Compton, D, R., Thomas, B, F., Prescott, W, R., Little, P, J., Razdan, R, K., Johnson, M, R., Melvin, L, S., Mechoulam, R., Ward, S, J. 1991. Behavioral, biochemical and molecular modeling evaluation of cannabinoid analogs. Pharmacology, biochemistry and behavior, 40, 471-478.

McKenna, G.J., 2014. The current status of medical marijuana in the United States. Hawaii journal of medicine and public health 73, 105–8.

Meier, M.H., Hill, M.L., Small, P.J., Luthar, S.S., 2015. Associations of adolescent cannabis use with academic performance and mental health: A longitudinal study of upper middle class youth. Drug and alcohol dependence 156, 207–212.

Melissa, J, K., Sowles, S,J., Stelzer-Manhan, H,E., Beirut, T., Cavozos-Rehg, P, A. 2017. It takes longer, but when it hits you it hits you! Videos about marijuana edibles on youtube. Substance use misuse, 52, 709-716.

Miczek, K.A., Barry, H., 1974. ??9-Tetrahydrocannabinol and Aggressive Behavior in Rats. Behavior biology, 11, 261–267.

Monory, K., Blaudzun, H., Massa, F., Kaiser, N., Lemberger, T., Schutz, G., Wotjak, C, T., Lutz, B., Marsicano, G. 2007. Genetic dissection of behavioral and autonomic effects of)9-tetrahydrocannabinol in mice. PLOS biology, 5, e269.

Monte, A, A., Zane, R, D., Heard, K, J. 2016. The implication of marijuana legalization in Colorado. Journal of American medical association, 313, 241-242.

Morena, M., Leitl, K.D., Vecchiarelli, H.A., Gray, J.M., Campolongo, P., Hill, M.N. 2016. Emotional arousal state influences the ability of amygdalar endocannabinoid signaling to modulate anxiety. Neuropharmacology 111, S397–S402.

Morrison, R., Kinoshifat, Y., Johnson, M, D., Uo, T., Ho, J, T., McBee, J, K., Conrads, T, P., Veenstra, T, D., 2002. Proteomics analysis in neuroscience. Molecular and cellular proteomics, 8, 553-560.

Murphy, M., Mills, S., Winstone, J., Leishman, E., Wager-Miller, J., Bradshaw, H., Mackie, K., 2017. Chronic Adolescent Δ^9-Tetrahydrocannabinol Treatment of Male Mice Leads to Long-Term Cognitive and Behavioral Dysfunction, Which Are Prevented by Concurrent Cannabidiol Treatment. Cannabis and cannabinoid research 2, 235–246.

Murphy, P., Carnevale, J., 2016. Regulating Marijuana in California. Public policy institute of California.

National Institute of Drug Abuse (NIDA): Marijuana. 2017.

National institute of drug abuse. Drug facts: Marijuana. August 2017, Page 1 1–9.

Navarro, M., Rodriguez De Fonseca, F., Hernandez, M,L., Ramos, J, A., Fernandez-Ruiz, J,J. Motor behavior and niagrostriatal dopaminergic activity in adult rats perinatally exposed to cannabinoids. Pharmacology biochemistry and behavior, 47, 47-58.

Navarro, M., de Miguel, R., Rodriguez De Fonseca, F., Ramos, J, A., Fernandez-Ruiz, J, J. Perinatal cannabinoid exposure modifies the sociosexual approach behavior and

the mesolimbic dopaminergic activity in adult male rats. Behavior brain research, 75, 91-98.

Newsom, R.J., Kelly, S.J., 2008. Perinatal delta-9-tetrahydrocannabinol exposure disrupts social and open field behavior in adult male rats. Neurotoxicology and teratology 30, 213–219.

Onders, B., Casavant, M.J., Spiller, H. a., Chounthirath, T., Smith, G. a., 2015. Marijuana Exposure Among Children Younger Than Six Years in the United States. Clinical pediatrics 1–9.

Pacheco, M.A., Ward, S.J., Childers, S.R., 1993. Identification of cannabinoid receptors in cultures of rat cerebellar granule cells. Brain research 603: 102–110.

Panksepp, J. 1980. Social deprivation and play in rats. Behavioral and neural biology, 30:197-206.

Panksepp, J., Sivity, S., Normshell, L. 1984. The psychobiology of play: Theoretical and Methodological perspectives. Neuroscience and behavioral reviews, 2:465-492.

Pellis, S.M., McKenna, M., 1995. What do rats find rewarding in play fighting? - an analysis using drug-induced non-playful partners. Behav. Brain Res. 68, 65–73.

Pellis, S.M., Pellis, V.C., 1998. Play fighting of rats in comparative perspective: A schema for neurobehavioral analyses. Neuroscience biobehavioral reviews, 23: 87–101.

Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. Journal of neuroscience methods 14, 149–167.

Pope, H.G., Gruber, A.J., Hudson, J.I., Huestis, M.A., Yurgelun-Todd, D., 2001. Neuropsychological performance in long-term Cannabis users. Archives of general psychiatry 58, 909–915.

Popova, N.K., 2006. From genes to aggressive behavior: The role of serotonergic system. BioEssays 28, 495–503.

Quinn, H.R., Matsumoto, I., Callaghan, P.D., Long, L.E., Arnold, J.C., Gunasekaran, N., Thompson, M.R., Dawson, B., Mallet, P.E., Kashem, M. a, Matsuda-matsumoto, H., Iwazaki, T., Mcgregor, I.S., 2008. Adolescent Rats Find Repeated D9 -Δ9-THC Less Aversive Than Adult Rats but Display Greater Residual Cognitive Deficits and Changes in Hippocampal Protein Expression Following Exposure. Neuropharmacology, 33, 1113–1126.

Renard, J., Krebs, M.O., Le Pen, G., Jay, T.M., 2014. Long-term consequences of adolescent cannabinoid exposure in adult psychopathology. Frontiers in neuroscience 8, 1–14.

Restall, G., Magill-Evans, J., 1994. Play and Preschool children with autism. American journal of occupational therapy 48, 113–120.

Rhee, M.-H., Bayewitch, M., Avidor-Reiss, T., Levy, R., Vogel, Z., 2002. Cannabinoid Receptor Activation Differentially Regulates the Various Adenylyl Cyclase Isozymes. Journal of neurochemistry 71, 1525–1534.

Renard, J., Szkudlarek, H, J., Kramer, C, P., Jobson, C, E, L., Moura, K., Rushlow, W, J., Laviolette, S, R. 2017. Adolescent THC exposure causes enduring prefrontal cortical disruption of GABAergic inhibition and dysregulation of subcortical dopamine function. Scientific reports, 7, 114-120.

Rinaldi-Carmona, M., Barth, F., Héaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Néliat, G., Caput, D., Ferrara, P., Soubrié, P., Brelière, J.C., Le Fur, G., 1994. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS letters, 350, 240–244.

Romero, J., Garcia-Palomero, E., Berrendoro, F., Garcia-Gil, L., Hernandez, M, L., Ramos, J, A., Fernandez-Ruiz, J, J. 1997. Atypical location of cannabinoid receptors in white matter areas during rat brain development. Synapse, 26, 317-323.

Rubino, T., Guidali, C., Vigano, D., Realini, N., Valenti, M., Massi, P., Parolaro, D., 2008. CB1 receptor stimulation in specific brain areas differently modulate anxiety-related behaviour. Neuropharmacology 54, 151–160.

Rubino, T., Sala, M., Viganò, D., Braida, D., Castiglioni, C., Limonta, V., Guidali, C., Realini, N., Parolaro, D., 2007. Cellular mechanisms underlying the anxiolytic effect of low doses of peripheral Δ9-tetrahydrocannabinol in rats. Neuropsychopharmacology 32, 2036–2045.

Rubino, T., Vigano', D., Realini, N., Guidali, C., Braida, D., Capurro, V., Castiglioni, C., Cherubino, F., Romualdi, P., Candeletti, S., Sala, M., Parolaro, D., 2008. Chronic delta 9-tetrahydrocannabinol during adolescence provokes sex-dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. Neuropsychopharmacology 33, 2760–2771.

Ruehle, S., Rey, A.A., Remmers, F., Lutz, B., 2012. The endocannabinoid system in anxiety, fear memory and habituation. Journal of psychopharmacology 26, 23–39.

Sano, K., Mishima, K., Koushi, E., Orito, K., Egashira, N., Irie, K., Takasaki, K., Katasurabayashi, S., Iwasaki, K., Uchida, N., Egawa, T., Kitamura, Y., Nishimura,

R., Fujiwara, M. 2008. Delta9-tetrahydrocannabinol-induced catalepsy-like immobilization is mediated by decreased 5-HT neurotransmission in the nucleus accumbens due to the action of glutamate containing neurons. Neuroscience, 151, 320-328.

Schauer, G.L., King, B.A., Bunnell, R.E., Promoff, G., McAfee, T.A., 2016. Toking, Vaping, and Eating for Health or Fun: Marijuana Use Patterns in Adults, U.S., 2014. American Journal of preventive medicine 50, 1–8.

Schramm-Sapyta, N.L., Cha, Y.M., Chaudhry, S., Wilson, W.A., Swartzwelder, H.S., Kuhn, C.M., 2007. Differential anxiogenic, aversive, and locomotor effects of Δ9-THC in adolescent and adult rats. Psychopharmacology 191, 867–877.

Schroberger, S, J., Edger, P, F., Kydd, R., Faull, R, L, M., Cooper, S, J, S. 2001. Proteomics, 1, 1519-1528.

Semple, B.D., Blomgren, K., Gimlin, K., Ferriero, D.M., Noble-Haeusslein, L.J., 2013. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Progress in neurobiology 106–107, 1–16.

Šlamberová, R., Mikulecká, A., Macúchová, E., Hrebíčková, I., Ševčíková, M., Nohejlová, K., Pometlová, M., 2016. Morphine decreases social interaction of adult male rats, while Δ9-THC does not affect it. Physiological research 65, S547–S555.

Steel, R, W.. Miller, J, H., Sim, D, A., Day, D, J. 2014. Delta9-tetrahydrocanabinol disrupts the hippocampal neuroplasticity and neurogenesis in trained but not in untrained adolescent Sprague-dawley rats. Brain research, 1548, 12-19.

Stopponi, S., Soverchia, L., Ubaldi, M., Cippitelli, A., Serpelloni, G., Ciccocioppo, R., 2014. Chronic Δ9-THC during adolescence increases the vulnerability to stress-induced relapse to heroin seeking in adult rats. Eur. Neuropsychopharmacoly 24, 1037–1045.

Sturman DA and Moghaddam B: The neurobiology of adolescence: changes in the brain architecture, functional dynamics and behavioral tendencies , 2011. Neuroscience and behavior reviews, 35, 1704-1712.

Suarez, I., Bodega, G., Ramos, J, A., Fernandez-Ruiz, J, J., Fernandez, B. 2000. Neuronal and astroglial response to pre- and perinatal exposure to delta9-tetrahydrocannabinol in the rat substantia nigra. Developmental neuroscience, 22, 253-263.

Taoka, M., Wakamiya, A., Nakayama, H., Isobe, T. 2000. Electrophoresis. 21, 1872-1879.

Takahashi, R.N., Pamplona, F.A., Fernandes, M.S., 2005. The cannabinoid antagonist SR141716A facilitates memory acquisition and consolidation in the mouse elevated T-maze. Neuroscience letters 380, 270–275.

Thomas, A.A., Mazor, S., 2017. Unintentional Marijuana Exposure Presenting as Altered Mental Status in the Pediatric Emergency Department: A Case Series. Journal of emergency medicine 53, e119–e123.

Toomey, R.B., Mitchell, K.J., 2016. Teen usage of marijuana edibles: A focus group study of an emerging issue. Journal of primary prevention, 51, 87–100.

Trezza, V., Baarendse, P.J.J., Vanderschuren, L.J.M.J., 2010. The pleasures of play: Pharmacological insights into social reward mechanisms. Trends in pharmacological sciences 31, 463–469.

Trezza, V., Cuomo, V., Vanderschuren, L.J.M.J., 2008. Cannabis and the developing brain: Insights from behavior. European journal of pharmacology 585, 441–452.

Trezza, V., Damsteegt, R., Manduca, A., Petrosino, S., Van Kerkhof, L.W., Pasterkamp, R.J., Zhou, Y., Campolongo, P., Cuomo, V., Di Marzo, V., Vanderschuren, L.J., 2012. Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. Journal of neuroscience 32, 14899–14908.

Trezza, V., Vanderschuren, L.J.M.J., 2008. Cannabinoid and opioid modulation of social play behavior in adolescent rats: Differential behavioral mechanisms. European Neuropsychopharmacoly 18, 519–530.

Trezza, V., Vanderschuren, L.J.M.J., 2009. Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. Journal of pharmacology and experimental therapeutics 328, 343–350.

Vanderschuren, L.J., Niesink, R.J., Van Ree, J.M., 1997. The neurobiology of social play behavior in rats. Neuroscience and biobehavioral reviews 21, 309–326.

Vandrey, R., Raber, J.C., Raber, M.E., Douglass, B., Miller, C., Bonn-Miller, M.O., 2015. Cannabinoid dose and label accuracy in edible medical cannabis products. JAMA – Journal of American medical association, 313: 2491–2493.

Vargish, G, A., Peikey, K, A., Yuan, X., Chittajalu, R., Collins, D., Fang, C., McBain, C, J. 2017. Persistent inhibitory circuit defects and disrupted social behavior following in utero exogenous cannabinoid exposure. Molecular psychiatry, 22, 56-67.

Vo, K.T., Horng, H., Li, K., Ho, R.Y., Wu, A.H.B., Lynch, K.L., Smollin, C.G., 2017. Cannabis Intoxication Case Series: The Dangers of Edibles Containing Tetrahydrocannabinol. Annuals of emergency medicine (In press).

Volkow, N.D., Baler, R.D., Compton, W.M., Weiss, S.R.B., 2014. Adverse Health Effects of Marijuana Use. New England journal of medicine, 370, 2219–2227.

Walker, D.D., 2017. Legalization of cannabis: Considerations for intervening with adolescent consumers. Preventive medicine, 104, 37–39.

Wang, G.S., Le Lait, M.C., Deakyne, S.J., Bronstein, A.C., Bajaj, L., Roosevelt, G., 2016. Unintentional pediatric exposures to marijuana in Colorado, 2009-2015. JAMA Pediatrics 170, e160971.

Wang, G.S., Roosevelt, G., Heard, K., 2013. Pediatric marijuana exposures in a medical Marijuana state. JAMA Pediatrics 167, 630–633.

Whiteford, T, J., Rennie, C, J., Grieve, S, M., Clark, S, R., Gordon, E., Williams, L, M. Brain maturation in adolescence: concurrent changes in neuroanatomy and neurophysiology. Human brain mapping, 28, 228-237.

Wright, N.E., Scerpella, D., Lisdahl, K.M., 2016. Marijuana Use Is Associated with Behavioral Approach and Depressive Symptoms in Adolescents and Emerging Adults. PLoS One 11.

Zhange, B., VerBerkmoes, N, C., Langston, M, A., Uberbacher, E., Hettich, B, L., Samatova, N, F. 2006. Detecting differential and correlated protein expression in lable free shotgun proteomics. Journal of proteome research, 5, 2909-2918.

CHAPTER II

EFFECT OF REPEATED JUVENILE EXPOSURE TO

Δ9-TETRAHYDROCANNABINOL ON ANXIEYT-RELATED BEHAVIORS AND

SOCIAL INTERACTIONS IN ADOLESCENT RATS

2.1   Introduction

Cannabinoids are a group of terpenophenols present in the granular trichomes of the plant
Cannabis sativa. Because of the psychoactive properties, cannabis preparations, such as
marijuana and hashish, have been used recreationally for centuries and remain one of the
most widely used illicit drugs (Brujzeel et al., 2016; Substance Abuse and Mental Health
Services Administration, 2014; Fellermeier et al., 2001). After the discovery of the
potential medical application of marijuana, there has been much debate about its
legalization for medical use. The debate was initiated in 1972 when the National
Organization for the Reform of the Marijuana Laws (NORML) filed a petition with the
Bureau of Narcotics and Dangerous Drugs to reschedule marijuana under the Controlled
Substances Act.   However, crude marijuana remains classified with the Schedule I drugs,
which are described as 'drugs with no accepted medical applications in treatment in
United States' (Zeese, 1999). Despite the strict regulation of marijuana, its use has
increased dramatically in the past few decades. This widespread use is a growing concern

especially because of the abuse of the drug by pregnant women and by adolescents. In fact, there are many behavioral and neurological disorders in human and animal studies associated with marijuana exposure either during the prenatal period (Higuera-Mates et al., 2015) or during adolescent ages (Fontes et al., 2011; Fried et al., 2002; Meier et al., 2012; Pope et al., 2001). However, exposure to marijuana is also increasing in a very unlikely population. From 2006 through 2013, the rate of marijuana exposure among children 5 years old or less rose 147.5% in the United States. In states that have legalized marijuana, the exposure rate increased almost 610% during the same period (Onders et al., 2016). An even earlier study reported that marijuana exposure among children 9 years old or less increased by 30.3% per year between 2005 and 2011 in decriminalized states but did not change in nonlegal states (Wang et al., 2013). In both studies, these data were obtained from reports to Poison Control Centers and it is therefore likely that many additional exposures are occurring that go unreported. While literature has expanded in recent years with respect to the behavioral and neuroanatomical effects of adolescent and prenatal exposure to marijuana in laboratory animals, very little information is available on the persistent effects of marijuana exposure during developmental periods that mimic exposure in young children.

The main psychoactive component of marijuana is Δ9-tetrahydrocannabinol (Δ9-THC) (Gaoni & Mechoulam 1964) which activates the cannabinoid receptors, including type I cannabinoid receptor (CB1). CB1 receptors are abundant in the brain (Devane et al., 1988), appear during fetal development (Berrendoro et al., 1998), and the highest density of CB1 receptors occur in brain regions which are directly involved in brain developmental processes (Fernandez-Ruiz et al., 2000). During development, the CB1

receptor is involved in synaptogenesis, the formation of connections across the neurons which is essential for efficient processing of adult cognition (Basavarajappa et al., 2009). Synaptogenesis peaks during the postnatal period suggesting that any inappropriate activation of the CB1 receptor during this postnatal period, such as that which occurs during Δ9-THC exposure, could result in altered synaptic connections. This could lead to neurobehavioral abnormalities that may not be observed until later stages of life. In fact, many behavioral abnormalities have resulted from developmental Δ9-THC exposure in animal studies. Most of these studies have focused on the behavioral abnormalities observed following Δ9-THC exposure either during pregnancy (Hutchings et al., 1991; Newsome & Kelly 2008; Rubio et al., 1995, 1998) or adolescence (Fleming et al., 2016; Keeley et al., 2015; Zamberletti et al., 2012; Realini et al., 2011; Rubino et al., 2011; Quinn et al., 2008; Rubino et al., 2008; Cha et al., 2006; Jarbe et al., 1998; Miczek & Barry 1974; Ueki et al., 1972).

Since the unintentional exposure of marijuana in children is increasing dramatically (Onders et al., 2016; Wang et al., 2013), it is imperative that research focus on investigating whether or not this pediatric exposure scenario can lead to persistent abnormalities. The majority of pediatric exposures result from the ingestion of Δ9-THC-infused edibles which are indistinguishable from their equivalent non-infused food product with respect to look, smell, and taste (Wang et al., 2017). For commercial products, several states with legalized marijuana (Alaska, Oregon, Colorado, and Washington, California) have limited the amount of Δ9-THC that can be in a single serving (5-10 mg; Gourdet et al., 2017). However, multiple servings of Δ9-THC are allowed in a single edible (i.e., brownie, cookie, chocolate) and children could easily

consume multiple servings and even multiple edibles (Gosh et al., 2015). Even with limitations placed on the number of servings, the total Δ9-THC concentration in one edible can reach as high as 100 mg. Some individual edibles contain up to 1000 mg (Richards et al., 2017). In addition, the exposure levels from homemade Δ9-THC-infused edibles, which are not restrained by regulations, could be even higher. Thus, the range of exposure levels in children can be very wide. For this investigatory project, we selected 10 mg/kg/day which has been utilized in previous studies exposing developing rodents (Burston et al., 2010; Moore et al., 2010; Philippot et al., 2016). The human equivalent dose for this dosage in a 5 year old child (17-20 kg) calculates to be 40-48 mg/day. However, it is not clear if this dosage recapitulates exposure levels in children. This research project was designed to determine if exposure to the psychoactive component of marijuana Δ9-tetrahydrocannabinol during the postnatal period altered anxiety and social behaviors in adolescent rats. Since rat brain development at postnatal day (PND) 7 has historically been used as a rough estimate of human brain development at birth (Clancy et al., 2007), the exposure treatment period utilized in this project was from PND10-16 which should be equivalent to early childhood ages in humans (Beck et al., 2011).

2.2   Materials and Methods

2.2.1   Chemicals

Δ9-tetrahydrocannabinol was provided by the National Institute on Drug Abuse. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2.2 Animals

A breeding colony of adult male and female Sprague Dawley rats (CD IGS; Harlan Laboratories, Indianapolis, IN) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility with temperature control (22 $\pm$ 2˚C) and with lights on between 0700 and 1900 for a 12-h dark-light cycle. Tap water and LabDiet rodent chow were freely available during the experimentation. The procedures used in this project were approved by the Mississippi State University Institutional Animal Care and Use Committee. From this colony, male and female rat pups were obtained and the day of birth was considered as PND0. On PND10, rat pups within the same litter were assigned to different treatment groups. There were always a representative control animal of the same sex present in each litter to match the Δ9-THC-treated animal to ensure equal distribution of animals to different treatment groups.

2.2.3 Exposure

Beginning on PND10, rats were exposed daily for 7 days to either corn oil (vehicle) or 10 mg/kg Δ9-THC by oral gavage at a volume of 0.5 ml/kg. Solutions were delivered to the back of the throat using a 50-ul tuberculin syringe equipped with a 1-inch 24-gauge straight intubation needle (Popper and Sons, Inc., New Hyde Park, NY). At weaning, the rats were weaned and remained housed with littermates throughout behavioral testing.

2.2.4 Elevated plus maze (EPM)

The elevated plus maze was constructed of black-painted wood and consisted of four arms (50 X 10 cm), two open and two enclosed (40 cm wall), intersecting at perpendicular distances to each other to form a 90° angle and elevated to a height of 50

cm above the floor. On PND29, the rats were transported to the testing room, placed in the center of the maze and allowed to explore the maze for 5 min. After each test, the maze was cleaned with 70% ethanol and dried. Each test session was recorded using a remotely operated Canon EOS Rebel digital camera. The videos were scored by two observers who were blind to treatment.

The observers scored the following behavioral parameters: (1) number of entries into the open arms, (2) number of entries into the closed arms, (3) time spent in the open arms, (4) time spent in the closed arms, and (5) time spent in the central square. From these data, the (6) percent open arm entries and (7) percent open arm time were calculated. In addition, the following ethological oriented parameters were scored: (8) number of stretch-attend postures (defined as exploration of the open arm with the front part of the body, while the hind region remains in the closed arm) and (9) number of head-dips (protruding the head over the ledge of an open arm and downwards towards the floor regardless of the location of the animals body). Entries into closed arm or open arm were counted only when all four paws including tail of rats were in respective arms.

Different anxiety levels have been observed under different levels of illumination following stimulation of the endocannabinoid system in rats (Haller et al., 2002). Thus, testing was conducted under two different illumination scenarios. The first was high illumination in which the illumination was ~600 LUX in the open arms and ~100 LUX in the closed arms. For this scenario, the controls (n=11 of each sex) were derived from 8 litters and the Δ9-THC treated (n=12 of each sex) were derived from 10 litters. The second was low illumination in which the illumination was ~10 LUX in the open arms and ~2 LUX in the closed arms. For this scenario, the controls (n=26-30 of each sex)

were derived from 22-23 litters and the Δ9-THC treated (n=20 of each sex) were derived from 15-16 litters. There were never more than two animals of the same treatment and sex from same litter used in any aspect of this study.

2.2.5   Social behavior (PND37-38)

Since previous behavioral test can affect the performance in subsequent behavioral test (Blokland et al., 2012; Mellwain et al., 2001), social behavior was conducted only on rats tested under low illumination in the elevated plus maze. The low illumination was selected over high illumination because, intuitively, it was considered to be less invasive and exerts less stressful effect. The social play test requires two rats of the same treatment in order to obtain a single replication and this is the reason for there being a greater numbers of litters and rats tested under low illumination in the elevated plus maze as compared to those tested under high illumination. The behavioral arena was a clear empty litter-cage with bright light (600 lux). Following a 24 hour isolation period, two rats of the same treatment, sex, age and size but from different litters were placed into different corners of the cage and allowed to interact for 10 min. Each test session was recorded using a remotely operated Canon EOS Rebel digital camera. After each test, the cage was emptied, cleaned with 70% ethanol, dried, and refilled with fresh litter. The videos were scored by two observers who were blind to treatment.

The observers scored the behavioral parameters as previously described (Ku et al., 2016; Himmler et al., 2013; Trezza et al., 2010; Varlinskaya & Spear 2002; Vanderschuren et al., 1997). These included: (1) time to first interaction, (2) frequency of self-grooming, (3) frequency of social exploration (body or anogenital sniffing or licking), (4) frequency of crawling over and under, (5) frequency of chasing, (6) frequency of pouncing/nape

attacks (playful attacks directed at the nape of the neck), (7) frequency of play fighting (boxing and wrestling), (8) frequency of pinning (one animal lying with its back on the floor with the other animal standing over it), and (9) the time spent play fighting. When scoring these parameters, the behavior of each individual rat was not recorded and the score obtained is a combined measure of the occurrence of the behavior in both rats regardless of which rat performed/initiated the behavior.

2.2.6 Statistical analysis

All data were subjected to testing by analysis of variance (ANOVA) using the SAS statistical package (SAS Institute Inc., Cary, NC). For body weights, the data were tested using the general linear model with a repeated measures paradigm and was found to violate the assumption of sphericity. Therefore, subsequent analysis using the Mixed procedure (Littell et al., 1996) was conducted with a repeat measures paradigm with a Huynh-Feldt covariance structure (Huyuh & Feldt 1970), followed by separation of means using least squares mean (LSM). The analysis identified significant differences in the main effects (sex, treatment, and day) and all possible interactions.

Behavioral data were analyzed by ANOVA using the Mixed procedure followed by mean separation by Least Square Means (LSM). Prior to analysis, the distribution of the conditional residuals for the behavioral data were evaluated for each parameter to determine the appropriateness of the statistical model. Any parameter not found to be sufficiently normally distributed were subjected to Blom's transformation in SAS using the PROC RANK procedure with the NORMAL option (Altman 1991). The analysis identified significant differences in the main variables (sex, treatment and day) and all possible interactions. If either main effect was significant or tending towards

significance, an additional lower level analysis (one-way ANOVA) was performed to determine differences between sexes within each treatment or treatment within each sex. The criterion for significance was set at $p < 0.05$.

2.3   Results

2.3.1   Weight gain

Oral exposure to 10 mg/kg Δ9-THC significantly reduced the amount of weight gained in both females and male pups (Figure 1) with no statistically significant difference between sexes. No signs of toxicity were observed in the pups. The pups were not lethargic and did not exhibit any abnormal movements or behavioral patterns during the exposure period. By the time of behavioral testing, there were no longer any statistically significant differences between treatment in either sex. However, male rats weighed significantly more than female rats (P=0.0001).

2.3.2   Elevated plus maze

For the elevated plus maze, the parameters measured under high illumination and the parameters measured under low illumination were analyzed separately. For both high and low illumination, the overall statistical analyses indicated that there was no significant overall effect of treatment and no significant treatment × sex interaction on any activity parameter associated with any open arm activity but some significant differences were detected in certain parameters. When detected, an additional lower level analysis was conducted to determine the presence of either sex differences within each treatment or treatment differences with each sex.

2.3.3   Open arm activity

There was no significant effect of treatment and no significant treatment × sex interaction in any parameter associated with open arm activity tested under either high or low illumination. However, statistically significant sex differences were detected in certain parameters.

There was a significant overall effect of sex in the number of entries into the open arm under both high illumination ($F_{(1,32)}$ = 7.8, p = 0.089) and low illumination ($F_{(1,46)}$ = 7.57, p = 0.0085). Under high illumination, males made significantly more open arm entries than females (Figure 2A). The effects appear to be more significant between males and females in the Δ9-THC treated group (post hoc analysis, p = 0.0145) with no significant difference between sexes in the control group. Under low illumination, the pattern was reversed with females making significantly more open arm entries than males (Figure 2B) but post hoc analysis did not reveal any treatment related effects. When calculated in terms of % of open arm entries, there was a significant effect of sex only under high illumination ($F_{(1,32)}$ = 14.56, p = 0.0006). Under high illumination, both Δ9-THC treated males (post hoc analysis, p = 0.0175) and control males (post hoc analysis, p = 0.0273) had a higher % of open arm entries (Figure 2C) than their respective female treatment group. Under low illumination, females had a slightly higher % of open arm entries than males but there was only a trend towards significance ($F_{(1,46)}$ = 3.53, p = 0.0668) (Figure 2D).

There was a significant effect of sex in the time spent in the open arms under both high ($F_{(1,32)}$ = 5.52, p = 0.0253) and low illumination ($F_{(1,46)}$ = 4.97, p = 0.0308). Under high illumination, males spent significantly more time in the open arms than females (Figure

1E). The effects appear to be more significant between males and females in the Δ9-THC treated group (post hoc analysis, p = 0.0386) with no significant difference between sexes in the control group. Under low illumination, the pattern was reversed with females making significantly more open arm entries than males (Figure 2F) but lower level analysis did not reveal any treatment related effects. When calculated in terms of % of time spent in the open arms, there was a significant effect of sex under both high ($F_{(1,32)}$ = 5.52, p = 0.0253) and low illumination ($F_{(1,46)}$ = 4.97, p = 0.0308). Under high illumination, males had a higher % of time spent in the open arm than females (Figure 2G). The effects appear to be more significant between males and females in the Δ9-THC treated group (post hoc analysis, p = 0.0386) with no significant difference between sexes in the control group. Under low illumination, females had a higher % of time spent in the open arm than males but lower level analysis did not reveal any treatment related effects (Figure 2H).

2.3.4 Ethological oriented parameters and overall activity

Under both high and low illumination, there was no significant effect of treatment and no significant treatment × sex interaction in any ethological oriented parameter. However, sex differences were detected in certain parameters.

With respect to the numbers of stretch attended postures made under high illumination, the Δ9-THC treated rats of both sexes made more as compared to their respective controls but was not statistically significant (Figure 3A). Under low illumination, there was a significant overall effect of sex ($F_{(1,46)}$ = 4.63, p = 0.0367) with males making more than females. However, this effect appears to be more significant in the Δ9-THC

treated groups (post hoc analysis, p = 0.0291) with no significant difference between males and females in the control group (Figure 3B).

With respect to the number of head dips made under high illumination, there was a trend toward significance in the overall effect of sex ($F_{(1,46)}$ = 3.77, p = 0.0593) with males making more than females. Although not statistically significant, the Δ9-THC treated males made more head dips than did the Δ9-THC treated females with similar levels made between male and female controls (Figure 3C). Under low illumination, there was a trend towards significance for the effect of treatment (p=0.0590). This effect appeared to be more significant in males with greater number of head dips in the Δ9-THC treated group than controls (post hoc analysis, p = 0.0227) with no significant difference observed between treatment groups in females (Figure 3D).

During elevated plus maze testing, entries into the closed arms can be considered an indicator of activity levels (Cruz et al., 1994). Overall analysis indicated that under both high and low illumination, there was no significant effect of sex and no significant treatment × sex interaction. However, there was a trend towards a significant effect of treatment under high illumination ($F_{(1,32)}$ = 3.35, p = 0.0771) and a significant effect of treatment under low illumination ($F_{(1,46)}$ = 6.02, p = 0.0180). However, the effect appeared to mainly occur in males where Δ9-THC treated males made more entries into the closed arms than did control males under both high (Figure 3E) and low (Figure 3F) illumination (post hoc analysis, p=0.0453 and p=0.0210, respectively). The number of entries was similar between the two treatment groups in females regardless of illumination level.

2.3.5   Social behavior

There were no significant overall effects of treatment or sex and no significant sex ×
treatment interactions in the time to first interaction, the frequency of self-grooming, the
frequency of crawling over and under, the frequency of chasing, the frequency of
pouncing, or the frequency of pinning (data not shown). However, treatment effects were
observed on the frequency of social exploration (body or anogenital sniffing or licking),
the frequency of nape attacks, the frequency of play fighting (boxing and wrestling), and
the time spent play fighting. In these four parameters, there was no significant overall
effect of sex and no significant sex × treatment interaction.

With respect to social exploration, there was a significant overall effect of treatment
$(F(1,30) = 7.65, p = 0.008)$. Episodes of social exploration were significantly decreased
in rats exposed to Δ9-THC as compared to that in control rats (Figure 4A). It appeared
that the reduction in social exploration occurred equally in both males and females
exposed to Δ9-THC (Figure 4A inset). With respect to the frequency of nape attacks,
there was a significant overall effect of treatment $(F(1,30) = 16.93, p = 0.0004)$. Rats
exposed to Δ9-THC made significantly higher number of nape attacks than did control
rats (Figure 4B). Although both Δ9-THC-treated females and males exhibited increased
episodes of nape attacks, there appeared to be a greater numbers in males (Figure 4B
inset). With respect to the frequency of play fighting, there was a significant overall
effect of treatment $(F(1,30) = 6.35, p = 0.0188)$. Rats exposed to Δ9-THC engaged in
more episodes of play fighting than did the control rats (Figure 4C). This increased level
of play fighting was present in both Δ9-THC exposed males and females (Figure 4C
inset). With respect to the time spent playing, there was an overall significant difference

of treatment ($F_{(1,30)}$ =6.57, p=0.0171). Rats exposed to Δ9-THC spent a significantly

greater amount of time engaged in play fighting that did control rats (Figure 4D). This

increased amount of time spent play fighting was observed in both Δ9-THC exposed

males and females (Figure 4D inset).
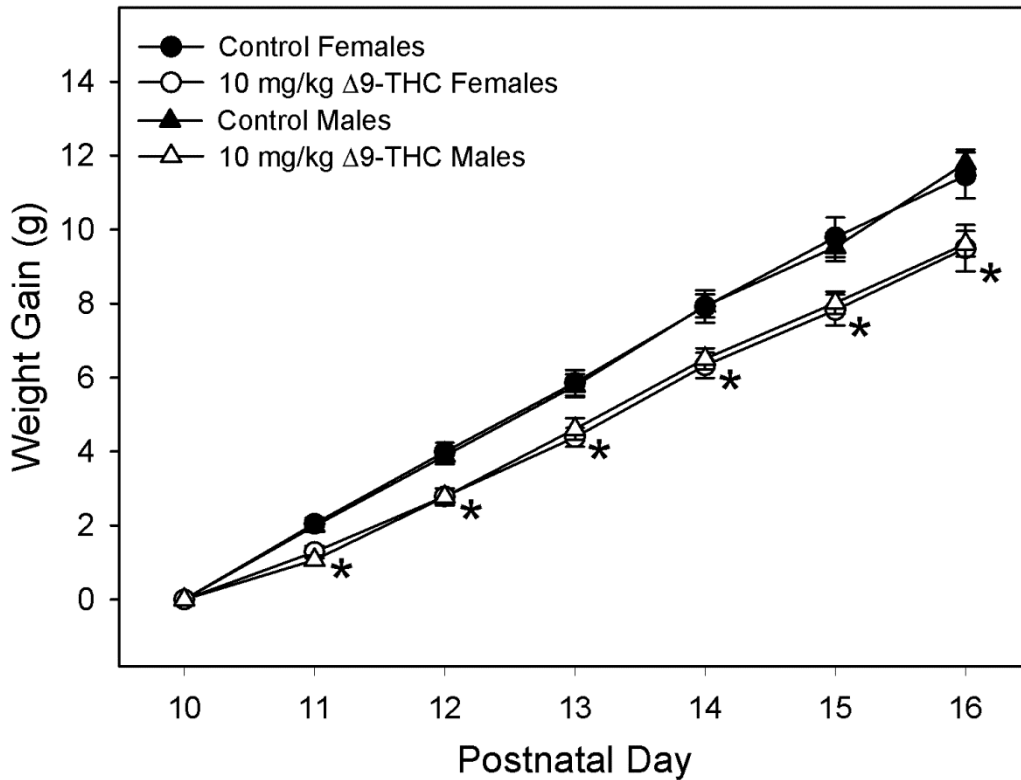
Figure 2.1   Plot of weight gain of male and female rat pups exposed daily to either
               corn oil (control) or 10 mg/kg of Δ9-THC as juveniles from postnatal days
               10-16. Values are expressed as weight ± SE (n = 31-37). For both sexes,
               weight gain in treated rats was significantly different from that in the
               controls (p≤ 0.05) at all time points.
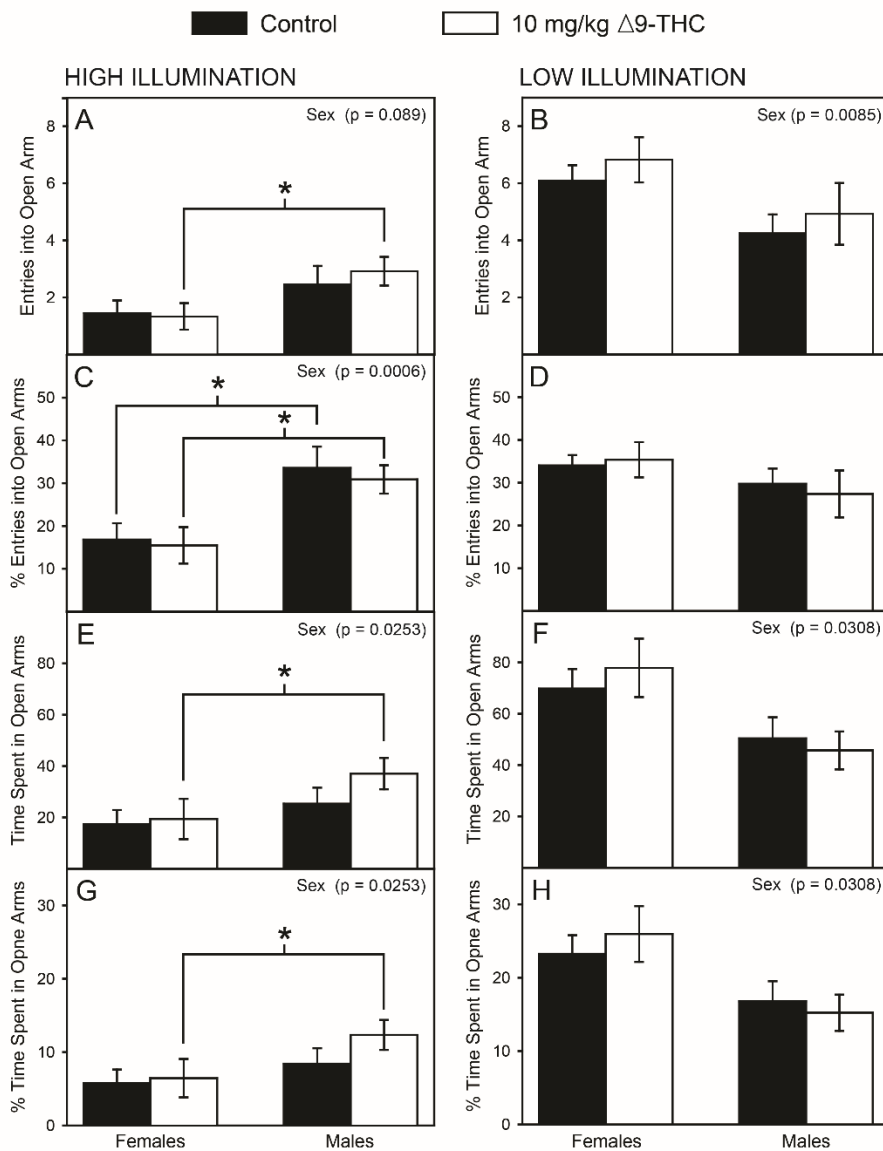
Figure 2.2   Open arm activity in the elevated plus maze under different levels of illumination as described in the Materials and Methods. Number of entries into open arms under high (A) and low (B) illumination, percent entries into open arms under high (C) and low (D) illumination, time spent in the open arms under high (E) and low (F) illumination, and percent time spent in the open arms under high (G) and low (H) illumination, in male and female adolescent rats (PND29) exposed to either corn oil or 10 mg/kg of Δ9-THC as juveniles from postnatal days 10-16. Values are expressed as means ± SEM or percent ± SEM (n=11-30). Brackets with asterisks (*) are statistically significant (p≤0.05) from one another

Figure 2.3   Open arm activity in the elevated plus maze under different levels of illumination as described in the Materials and Methods. Number of entries into open arms under high (A) and low (B) illumination, percent entries into open arms under high (C) and low (D) illumination, time spent in the open arms under high (E) and low (F) illumination, and percent time spent in the open arms under high (G) and low (H) illumination, in male and female adolescent rats (PND29) exposed to either corn oil or 10 mg/kg of Δ9-THC as juveniles from postnatal days 10-16. Values are expressed as means ± SEM or percent ± SEM (n=11-30). Brackets with asterisks (*) are statistically significant (p≤0.05) from one another

Figure 2.4   Social behavior interactions as described in the Materials and Methods. Frequency of social exploration (A), frequency of nape attacks (B), frequency of play fighting (C), and time spent play fighting (D) in adolescent rats (PND38) exposed to either corn oil or 10 mg/kg of Δ9-THC as juveniles from postnatal days 10-16. Insets are values separated by sex. Values are expressed as means ± SEM (n=10-12). Bars with asterisks (*) are statistically significant (p≤0.05) from controls.

59

2.4   Discussion

The results of this study suggest that Δ9-THC administered during the postnatal period altered anxiety and social behaviors in adolescent rats. The first interesting observation was that Δ9-THC decreased the expected weight gain in postnatal rats. Importantly, the decreased weight gain was not accompanied by any signs of toxicity. Previous studies have also observed decreased weight gain in rat pups exposed postnatally to Δ9-THC (O'Shea et al., 2004). It has been demonstrated that short term exposure of Δ9-THC to naïve humans and animals induces increased sleep (Babson et al., 2017; Angarita et al., 2016). It is possible that the short term exposure to Δ9-THC induced increased sleep resulting in decreased feeding behavior. Another hypothesis may the dose and route of exposure used in present study may have significantly contributed to reduced weight. The fact that Δ9-THC being an appetite stimulant as reported by other studies holds true only at lower doses. Higher doses on the other hand are responsible for decrease in the food intake and body weight (Cota et al., 2003).

The elevated plus maze (EPM) is a commonly used model to measure the level of anxiety in rats and mice and the illumination of the maze plays an important factor. Combined increases in the percentage of open arm entries and percentage of time spent in the open arms entries are indications of anxiolytic behavior (Bradley et al., 2007; Pellow et al., 1985), and increased ethological risk assessment behaviors, such as head dipping and stretch attended postures, have been proposed to be sensitive measures related to anxiety in the elevated plus maze (Cruz et al., 1994; Rodgers et al., 1999). However, we did not

observe effects on any parameter that would suggest increased or decreased anxiety levels in adolescent rats exposed to Δ9-THC as juveniles. While not directly comparable, O'Shea et al. (2006) reported no effect on anxiety in pubertal rats following early postnatal exposure to the cannabinoid receptor agonist CP 55,940. In other types of developmental exposures, increased anxiety has been reported in adults exposed either during the gestational/lactational period or the pubertal period to Δ9-THC (Keeley et al., 2015; Campolongo et al., 2008) while decreased anxiety has been reported in adults exposed as adolescents (Rubino et al., 2008; Wakeford et al., 2016). Thus, a direct link between developmental activation of the endocannabinoid system and long term effects on anxiety levels is not straightforward.

In the EPM, multiple studies have reported that increasing the level of illumination decreases activity in the open arms (Garcia et al., 2005; Pereira et al., 2005). Although not directly compared, the pattern of our data are in agreement with this line of thought in that regardless of treatment, the rats tested under low illumination had higher numbers of entries into the open arms and spent more time in the open arms than did the rats tested under high illumination. It has also been suggested that anxiogenic effects of drug exposures are more likely to be detected under low-intensity lighting, whereas anxiolytic effects of drug exposures are more likely to be detected under higher intensity lighting (200-400 Lux or more; Leo & Pamplona 2014). Exposure of juvenile rats to Δ9-THC appears to induce sex specific effects in adolescents. The sex specific differences in open arm activity were observed under high illumination, but totally disappeared when the testing was performed under low illumination suggesting that the effects of treatment only emerge during aversive conditions. Under these conditions, control males did

exhibit a slightly higher open arm activity than did control females but not enough to be statistically significant. Treatment with Δ9-THC did not alter female behavior but exacerbated the open arm activity in males such that sex differences were detected between Δ9-THC males and females. The basis for these sex differences could be due to the differential effect of Δ9-THC exposure on CB1 receptor function between in males and females. Differences exist in CB1 receptor density between males and females in the early postnatal period (Rodriguez de Fonseca et al., 1993). Adolescent exposure to Δ9-THC has been reported to induce sex-differences in the intensity of CB1 receptor-G protein interactions and increased altered CREB phosphorylation (Rubino et al., 2008). It is possible that Δ9-THC exposure during the postnatal period could induce sex differences in CB1 signaling levels as well.

An increase in the number of closed arm entries is an index of locomotor activity (Hogg 1996) and regulating locomotor activity is one of the physiological functions of CB1 receptors in adolescents (Fride 2002). Increased closed arm entries were observed in Δ9-THC treated males, but not Δ9-THC treated females, regardless of the level of illumination. We also observed increased head dipping under both high (increased but not statistically significant) and low illumination. While increased head dipping has been associated with anxiety levels, the increase observed in this study is more than likely merely an artifact of the overall level of increased activity. Increased spontaneous behavior and decreased habituation has also been reported in adult mice exposed to 10 mg/kg Δ9-THC as neonates (Philoppot et al., 2016). However, other studies have reported no effects on activity in adults exposed to Δ9-THC either prenatally or as

adolescents (Newsome & Kelly 2008; Rubino et al., 2008) indicating that, the exposure period and age of testing plays a role in the long term behavioral effects.

Social play is the earliest form of non-mother directed behavioral contact and is crucially important for the normal development of social and cognitive function (Vanderschuren et al., 1997). This behavior is observed to the greatest extent in adolescent rats as compared to juveniles and adults and represents the characteristic social interaction between adolescent rats (Trezza & Vanderschuren 2008). While the actual neural mechanisms involved in social play remain to be elucidated, it has been demonstrated that social play is strongly influenced by the endocannabinoid system in the brain and can be modulated by drugs that directly or indirectly affect activation of the CB1 receptors (Trezza et al., 2012; Trezza & Vanderschuren 2008). In fact, developmental exposure to CB1 receptor agonists can have long term effects on social behavior but the results vary based on the age of treatment, the duration of treatment, and the age of testing. For example, gestational/lactational Δ9-THC exposure did not alter social exploration but decreased social play in adolescents (Trezza et al., 2008) while gestational Δ9-THC exposure increased total social interactions (all social parameters combined) in adults (Newsome & Kelly 2008). Adolescent exposure to Δ9-THC has been reported to either decrease total social investigation (Realini et al., 2011; Zamberletti et al., 2012) or have no effect (Quinn et al., 2008) in adults. In the present study, juvenile exposure to Δ9-THC resulted in a lower level of social investigation in adolescent rats but an increase in social play. At face value, it seems unlikely that rats that exhibit lower levels of investigative interactions would actually have increased levels of playful interactions. One possible explanation could be that since the Δ9-THC treated rats exhibited a higher propensity for

63

pouncing/nape attack which led to a higher propensity for rough and tumble play, the level of social contact required prior to initiation of play behavior was reduced as compared to controls. However, many consider social exploration behavior to be not related to social play behavior and have suggested that these two parameters differ with respect to both their ontogeny and their regulation by neurological systems (Trezza et al., 2010; Vanderschuren et al., 1997; Spears 1999). The decreased social exploration could be the result of the fact that the Δ9-THC treated rats spent more time engaged in play fighting which reduced the opportunities for social exploration to occur.

Since social play can become very rough and tumble, it could be construed as a sign of adult-like aggressive behavior especially during the late stages of adolescence. However, it has been suggested that the rough and tumble play in developing rats is controlled by a separate neural system than the aggressive interactions observed in adult rats (Panksepp & Burgdorf 2003; Peills & Peills 2007). While there are conflicting reports about the Δ9-THC exposure leads to aggression, it does have a profound effect on the brain reward circuit (Katsidoni et al., 2013; Lupica et al., 2004). Social play induces a rewarding effect and is modulated by the dopaminergic, noradrenergic, opioid, and endocannabinoid systems, all of which have been implicated in reward (Achterberg et al., 2016; Manduca et al., 2015; Trezza et al., 2010; Trezza & Vanderschuren 2008; Vanderschuren et al., 2016). Thus, the modulation in social play observed in this study may involve discoordination between these different signaling systems., The disruption of the function of a single system during development could affect the appropriate functioning of the other systems at the time of behavioral testing. Regardless of the system responsible for

the altered behavior, this observed dysfunction is the result of the action of Δ9-THC on its primary target, the CB1 receptors, during the exposure period.

The CB1 receptors are responsible for maintaining the excitatory inhibitory balance in the brain by controlling the release of glutamate and γ-aminobutyric acid (GABA) at their respective synapses (Castillo et al., 2012; Kano et al., 2009). This balance is crucial for regulating and maintaining proper synaptic activity at most of the neurotransmitter systems involved in mediating social behavior. Of interest, van Kerkhof et al. (2013) reported both the activation of the GABAergic signaling in the nucleus accumbens core or the inhibition of the glutamatergic signaling in the dorsomedial striatum increased social play duration similar to that observed in this study. Therefore, a possible hypothesis is that exposure to Δ9-THC altered the appropriate developmental events necessary for maintaining the excitation-inhibition balance between glutamatergic and GABAergic signaling in specific regions important in social behavior. Development of the GABAergic inhibitory synapses occurs postnatally prior to adolescence in rodents (De Felipe et al., 1997) suggesting that this development might be a target.

In conclusion, given the packaging used in many cannabis edibles (MacCoun et al., 2015), it is not surprising that children find them very enticing. However, the dosages found in these products are highly variable and unregulated but the psychotropic effects are significantly more persistent than other routes of intake (Grotenhermen 2003) suggesting prolonged activation of the neurological target. This is of significant concern with respect to developmental exposures. This research provides an initial investigation into childhood oral exposure to Δ9-THC, an exposure scenario that has recently dramatically increased in occurrence. The results presented here suggest that repeated

oral exposure to Δ9-THC during postnatal development has no effect on anxiety, but can potentially affect the locomotor activity, and social behaviors during adolescence. However, it is not clear if these are accompanied by increased levels of other behaviors (i.e., risk-taking and novelty-seeking) that are characteristic of adolescent ages (Spears 1999). It is also not clear if exposure to lower dosages will also exert similar effects. This study was limited to a single dosage due to the amount of Δ9-THC available for study. Additional studies are required to investigate these issues as well as determine the mechanisms responsible for the altered behavior observed in the present study.

2.5   Reference:

Achterberg, E.J.M., van Kerkhof, L.W.M., Servadio, M., van Swieten, M.M.H., Houwing, D.J., Aalderink, M., Driel, N. V, Trezza, V., Vanderschuren, L.J.M.J., 2016. Contrasting roles of dopamine and noradrenaline in the motivational properties of social play behavior in rats. Neuropsychopharmacology 41, 858–868.

Altman, D.G., 2006. Practical Statistics for Medical Research. Chapman & Hall/CRC.

Angarita, G.A., Emadi, N., Hodges, S., Morgan, P.T., 2016. Sleep abnormalities associated with alcohol, cannabis, cocaine, and opiate use: a comprehensive review. Addict. Sci. Clin. Pract. 11, 9.

Babson, K.A., Sottile, J., Morabito, D., 2017. Cannabis, cannabinoids, and sleep: A review of the literature. Current Psychiatry Reports, 19, 23.

Basavarajappa, B.S., Nixon, R.A., Arancio, O., 2009. Endocannabinoid system: emerging role from neurodevelopment to neurodegeneration. Mini Rev. Med. Chem. 9, 448–462.

Beck, M.J., Padgett, E.L., Parker, G.A., Maginnis, G.M., Toot, J.D., Varsho, B.J., Varsho, J.S., 2011. Nonclinical juvenile toxicity testing, in: Hood, R.D. (Ed.), Developmental and Reproductive Toxicology. CRC Press, Boca Raton, FL, pp. 302–345.

Belue, R.C., Howlett, A.C., Westlake, T.M., Hutchings, D.E., 1995. The ontogeny of cannabinoid receptors in the brain of postnatal and aging rats. Neurotoxicol. Teratol. 17, 25–30.

Berrendero, F., García-Gil, L., Hernández, M.L., Romero, J., Cebeira, M., de Miguel, R., Ramos, J.A., Fernández-Ruiz, J.J., 1998. Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development. Development 125, 3179–3188.

Blokland, A., Ten Oever, S., van Gorp, D., van Draanen, M., Schmidt, T., Nguyen, E., Krugliak, A., Napoletano, A., Keuter, S., Klinkenberg, I., 2012. The use of a test battery assessing affective behavior in rats: order effects. Behavioral Brain Research 228, 16–21.

Blom, G., 1958. Statistical Estimates and Transformed Beta Variables. John Wiley and Sons, Inc., New York.

Bradley, B.F., Starkey, N.J., Brown, S.L., Lea, R.W., 2007. Anxiolytic effects of Lavandula angustifolia odour on the Mongolian gerbil elevated plus maze. J. Ethnopharmacology 111, 517–525.

Burston, J.J., Wiley, J.L., Craig, A.A., Selley, D.E., Sim-Selley, L.J., 2010. Regional enhancement of cannabinoid CB1 receptor desensitization in female adolescent rats following repeated Δ9-tetrahydrocannabinol exposure. Br. J. Pharmacol. 161, 103–112.

Castillo, P.E., Younts, T.J., Chávez, A.E., Hashimotodani, Y., 2012. Endocannabinoid signaling and synaptic function. Neuron 76, 70–81.

Cha, Y.M., White, A.M., Kuhn, C.M., Wilson, W.A., Swartzwelder, H.S., 2006. Differential effects of delta9-THC on learning in adolescent and adult rats. Pharmacol. Biochem. Behav. 83, 448–455.

Chen, J., Paredes, W., Li, J., Smith, D., Lowinson, J., Gardner, E.L., 1990. Δ9-tetrahydrocannabinol produces naloxone-blockable enhancement of presynaptic basal dopamine efflux in nucleus accumbens of conscious, freely-moving rats as measured by intracerebral microdialysis. Psychopharmacology (Berl). 102, 156–162.

Clancy, B., Finlay, B.L., Darlington, R.B., Anand, K.J.S., 2007. Extrapolating brain development from experimental species to humans. Neurotoxicology 28, 931–937.

Cosquer, B., Galani, R., Kuster, N., Cassel, J.-C., 2005. Whole-body exposure to 2.45GHz electromagnetic fields does not alter anxiety responses in rats: a plus-maze study including test validation. Behav. Brain Res. 156, 65–74.

Cota, D., Marsicano, G., Lutz, B., Vicennati, V., Stalla, G.K., Pasquali, R., Pagotto, U., 2003. Endogenous cannabinoid system as a modulator of food intake. Int. J. Obes. 27, 289–301.

Cruz, A.P., Frei, F., Graeff, F.G., 1994. Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacol. Biochem. Behav. 49, 171–176.

De Felipe, J., Marco, P., Fairén, A., Jones, E.G., 1997. Inhibitory synaptogenesis in mouse somatosensory cortex. Cereb. Cortex 7, 619–634.

den Boon, F.S., Werkman, T.R., Schaafsma-Zhao, Q., Houthuijs, K., Vitalis, T., Kruse, C.G., Wadman, W.J., Chameau, P., 2015. Activation of type-1 cannabinoid receptor shifts the balance between excitation and inhibition towards excitation in layer II/III pyramidal neurons of the rat prelimbic cortex. Pflügers Arch. 467, 1551–1564.

Devane, W.A., Dysarz, F.A., Johnson, M.R., Melvin, L.S., Howlett, A.C., 1988. Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. 34, 605–613.

Dobbing, J., Sands, J., 1979. Comparative aspects of the brain growth spurt. Early Hum. Dev. 3, 79–83.

Fellermeier, M., Eisenreich, W., Bacher, A., Zenk, M.H., 2001. Biosynthesis of cannabinoids. Incorporation experiments with (13)C-labeled glucoses. Eur. J. Biochem. 268, 1596–1604.

Fleming, C.B., Guttmannova, K., Cambron, C., Rhew, I.C., Oesterle, S., 2016. Examination of the divergence in trends for adolescent marijuana use and marijuana-specific risk factors in Washington state. J. Adolesc. Heal. 59, 269–275.

Fontes, M.A., Bolla, K.I., Cunha, P.J., Almeida, P.P., Jungerman, F., Laranjeira, R.R., Bressan, R.A., Lacerda, A.L.T., 2011. Cannabis use before age 15 and subsequent executive functioning. Br. J. Psychiatry 198, 442–447.

Fride, E., 2002. Endocannabinoids in the central nervous system-an overview. Prostaglandins, Leukot. Essent. Fat. Acids 66, 221–233.

Fried, P., Watkinson, B., James, D., Gray, R., 2002. Current and former marijuana use: preliminary findings of a longitudinal study of effects on IQ in young adults. CMAJ 166, 887–891.

Gaoni, Y., Mechoulam, R., 1964. Isolation, structure, and partial synthesis of an active constituent of hashish. J. Am. Chem. Soc. 86, 1646–1647.

Garcia, A.M.B., Cardenas, F.P., Morato, S., 2005. Effect of different illumination levels on rat behavior in the elevated plus-maze. Physiol. Behav. 85, 265–270.

Goonawardena, A. V, Plano, A., Robinson, L., Ross, R., Greig, I., Pertwee, R.G., Hampson, R.E., Platt, B., Riedel, G., 2015. Modulation of food consumption and sleep–wake cycle in mice by the neutral CB1 antagonist ABD459. Behav. Pharmacol. 26, 289–303.

Gourdet, C., Giombi, K.C., Kosa, K., Wiley, J., Cates, S., 2017. How four U.S. states are regulating recreational marijuana edibles. Int. J. Drug Policy 43, 83–90.

Grotenhermen, F., 2003. Pharmacokinetics and pharmacodynamics of cannabinoids. Clin. Pharmacokinet. 42, 327–360.

Haller, J., Bakos, N., Szirmay, M., Ledent, C., Freund, T.F., 2002. The effects of genetic and pharmacological blockade of the CB1 cannabinoid receptor on anxiety. Eur. J. Neurosci. 16, 1395–1398.

Higuera-Matas, A., Ucha, M., Ambrosio, E., 2015. Long-term consequences of perinatal and adolescent cannabinoid exposure on neural and psychological processes. Neurosci. Biobehav. Rev. 55, 119–146.

Himmler, B.T., Pellis, V.C., Pellis, S.M., 2013. Peering into the dynamics of social interactions: measuring play fighting in rats. Journal of visual experiments, 71, 4288.

Hogg, S., 1996. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol. Biochem. Behav. 54, 21–30.

Hutchings, D.E., Fico, T.A., Banks, A.N., Dick, L.S., Brake, S.C., 1991. Prenatal delta-9-tetrahydrocannabinol in the rat: effects on postweaning growth. Neurotoxicol. Teratol. 13, 245–248.

Huynh, H., Feldt, L.S., 1970. Conditions under which mean square ratios in repeated measures designs have exact F-distributions. J. Am. Stat. Assoc. 65, 1582–1589.

Järbe, T.U., Sheppard, R., Lamb, R.J., Makriyannis, A., Lin, S., Goutopoulos, A., 1998. Effects of Δ9-tetrahydrocannabinol and (R)-methanandamide on open-field behavior in rats. Behav. Pharmacol. 9, 169–174.

Katsidoni, V., Kastellakis, A., Panagis, G., 2013. Biphasic effects of Δ9-tetrahydrocannabinol on brain stimulation reward and motor activity. Int. J. Neuropsychopharmacol. 16, 2273–2284.

Keeley, R.J., Trow, J., Bye, C., McDonald, R.J., 2015. Part II: Strain- and sex-specific effects of adolescent exposure to THC on adult brain and behaviour: Variants of learning, anxiety and volumetric estimates. Behav. Brain Res. 288, 132–152.

Ku, K.M., Weir, R.K., Silverman, J.L., Berman, R.F., Bauman, M.D., 2016. Behavioral Phenotyping of Juvenile Long-Evans and Sprague-Dawley Rats: Implications for Preclinical Models of Autism Spectrum Disorders. PLoS One 11,58150.

Leo, L.M., Pamplona, F.A., 2014. Elevated plus maze test to assess anxiety-like behavior in the mouse. Bio-protocol 4, 1211.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D., 1996. Sas System for Mixed Models. SAS Institute, Inc, Cary, NC.

Lupica, C.R., Riegel, A.C., Hoffman, A.F., 2004. Marijuana and cannabinoid regulation of brain reward circuits. Br. J. Pharmacol. 143, 227–234.

MacCoun, R.J., Mello, M.M., 2015. Half-baked - The retail promotion of marijuana edibles. N. Engl. J. Med. 372, 989–991.

Manduca, A., Servadio, M., Damsteegt, R., Campolongo, P., Vanderschuren, L.J., Trezza, V., 2016. Dopaminergic neurotransmission in the nucleus accumbens modulates social play behavior in rats. Neuropsychopharmacology 41, 2215–2223.

McIlwain, K.L., Merriweather, M.Y., Yuva-Paylor, L.A., Paylor, R., 2001. The use of behavioral test batteries: effects of training history. Physiol. Behav. 73, 705–717.

Meier, M.H., Caspi, A., Ambler, A., Harrington, H., Houts, R., Keefe, R.S.E., McDonald, K., Ward, A., Poulton, R., Moffitt, T.E., 2012. Persistent cannabis users show neuropsychological decline from childhood to midlife. Proc. Natl. Acad. Sci. U. S. A. 109, 2657-2664.

Micheva, K.D., Beaulieu, C., 1996. Quantitative aspects of synaptogenesis in the rat barrel field cortex with special reference to GABA circuitry. J. Comp. Neurol. 373, 340–354.

Miczek, K.A., Barry, H., 1974. Δ9-tetrahydrocannabinol and aggressive behavior in rats. Behav. Biol. 11, 261–267.

Moore, N.L.T., Greenleaf, A.L.R., Acheson, S.K., Wilson, W.A., Swartzwelder, H.S., Kuhn, C.M., 2010. Role of cannabinoid receptor type 1 desensitization in greater tetrahydrocannabinol impairment of memory in adolescent rats. J. Pharmacol. Exp. Ther. 335, 294–301.

Newsom, R.J., Kelly, S.J., 2008. Perinatal delta-9-tetrahydrocannabinol exposure disrupts social and open field behavior in adult male rats. Neurotoxicol. Teratol. 30, 213–219.

O'Shea, M., Mallet, P.E., 2005. Impaired learning in adulthood following neonatal Δ9-THC exposure. Behav. Pharmacol. 16, 455–461.

O'Shea, M., McGregor, I.S., Mallet, P.E., 2006. Repeated cannabinoid exposure during perinatal, adolescent or early adult ages produces similar longlasting deficits in object recognition and reduced social interaction in rats. J. Psychopharmacol. 20, 611–621.

Onders, B., Casavant, M.J., Spiller, H.A., Chounthirath, T., Smith, G.A., 2016. Marijuana exposure among children younger than six years in the United States. Clin. Pediatr. (Phila). 55, 428–436.

Panksepp, J., Burgdorf, J., 2003. "Laughing" rats and the evolutionary antecedents of human joy? Physiol. Behav. 79, 533–547.

Pellis, S.M., 2002. Sex differences in play fighting revisited: traditional and nontraditional mechanisms of sexual differentiation in rats. Arch. Sex. Behav. 31, 17–26.

Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods 14, 149–167.

Pereira, L.O., da Cunha, I.C., Neto, J.M., Paschoalini, M.A., Faria, M.S., 2005. The gradient of luminosity between open/enclosed arms, and not the absolute level of Lux, predicts the behaviour of rats in the plus maze. Behav. Brain Res. 159, 55–61.

Philippot, G., Nyberg, F., Gordh, T., Fredriksson, A., Viberg, H., 2016. Short-term exposure and long-term consequences of neonatal exposure to Δ9-tetrahydrocannabinol (THC) and ibuprofen in mice. Behav. Brain Res. 307, 137–144.

Pope, H.G., Gruber, A.J., Hudson, J.I., Huestis, M.A., Yurgelun-Todd, D., 2001. Neuropsychological performance in long-term cannabis users. Arch. Gen. Psychiatry 58, 909–915.

Quinn, H.R., Matsumoto, I., Callaghan, P.D., Long, L.E., Arnold, J.C., Gunasekaran, N., Thompson, M.R., Dawson, B., Mallet, P.E., Kashem, M.A., Matsuda-Matsumoto, H., Iwazaki, T., McGregor, I.S., 2008. Adolescent rats find repeated Δ9-THC less aversive than adult rats but display greater residual cognitive deficits and changes in hippocampal protein expression following exposure. Neuropsychopharmacology 33, 1113–1126.

Realini, N., Viganò, D., Guidali, C., Zamberletti, E., Rubino, T., Parolaro, D., 2011. Chronic URB597 treatment at adulthood reverted most depressive-like symptoms induced by adolescent exposure to THC in female rats. Neuropharmacology 60, 235–243.

Richards, J.R., Smith, N.E., Moulin, A.K., 2017. Unintentional cannabis ingestion in children: A systematic review. J. Pediatr. 190, 142–152.

Rodgers, R.J., Cole, J.C., 1993. Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. Physiol. Behav. 54, 729–736.

Rodgers, R.J., Dalvi, A., 1997. Anxiety, defence and the elevated plus-maze. Neurosci. Biobehav. Rev. 21, 801–810.

Rodgers, R.J., Haller, J., Holmes, A., Halasz, J., Walton, T.J., Brain, P.F., 1999. Corticosterone response to the plus-maze: high correlation with risk assessment in rats and mice. Physiol. Behav. 68, 47–53.

Rodríguez de Fonseca, F., Ramos, J.A., Bonnin, A., Fernández-Ruiz, J.J., 1993. Presence of cannabinoid binding sites in the brain from early postnatal ages. Neuroreport 4, 135–138.

Rubino, T., Parolaro, D., 2011. Sexually dimorphic effects of cannabinoid compounds on emotion and cognition. Front. Behav. Neurosci. 5, 64.

Rubino, T., Viganò, D., Realini, N., Guidali, C., Braida, D., Capurro, V., Castiglioni, C., Cherubino, F., Romualdi, P., Candeletti, S., Sala, M., Parolaro, D., 2008. Chronic Δ9-tetrahydrocannabinol during adolescence provokes sex-dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. Neuropsychopharmacology 33, 2760–2771.

Rubio, P., Rodríguez de Fonseca, F., Martín-Calderón, J.L., Del Arco, I., Bartolomé, S., Villanúa, M.A., Navarro, M., 1998. Maternal exposure to low doses of Δ9-tetrahydrocannabinol facilitates morphine-induced place conditioning in adult male offspring. Pharmacol. Biochem. Behav. 61, 229–238.

Rubio, P., Rodríguez de Fonseca, F., Muñoz, R.M., Ariznavarreta, C., Martín-Calderón, J.L., Navarro, M., 1995. Long-term behavioral effects of perinatal exposure to Δ9-tetrahydrocannabinol in rats: possible role of pituitary-adrenal axis. Life Sci. 56, 2169–2176.

Spear, L.P., 2000. The adolescent brain and age-related behavioral manifestations. Neurosci. Biobehav. Rev. 24, 417–463.

Substance Abuse and Mental Health Services Administration, 2014. Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings. Rockville, MD.

Tanda, G., Pontieri, F.E., Di Chiara, G., 1997. Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism. Science 276, 2048–2050.

Trezza, V., Baarendse, P.J.J., Vanderschuren, L.J.M.J., 2010. The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol. Sci. 31, 463–469.

Trezza, V., Campolongo, P., Cassano, T., Macheda, T., Dipasquale, P., Carratù, M.R., Gaetani, S., Cuomo, V., 2008a. Effects of perinatal exposure to delta-9-tetrahydrocannabinol on the emotional reactivity of the offspring: a longitudinal behavioral study in Wistar rats. Psychopharmacology (Berl). 198, 529–537.

Trezza, V., Cuomo, V., Vanderschuren, L.J.M.J., 2008b. Cannabis and the developing brain: Insights from behavior. Eur. J. Pharmacol. 585, 441–452.

Trezza, V., Damsteegt, R., Manduca, A., Petrosino, S., van Kerkhof, L.W.M., Pasterkamp, R.J., Zhou, Y., Campolongo, P., Cuomo, V., Di Marzo, V.,

Vanderschuren, L.J.M.J., 2012. Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. J. Neurosci. 32, 14899–14908.

Trezza, V., Vanderschuren, L.J.M.J., 2009. Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. J. Pharmacol. Exp. Ther. 328, 343–350.

Trezza, V., Vanderschuren, L.J.M.J., 2008. Cannabinoid and opioid modulation of social play behavior in adolescent rats: Differential behavioral mechanisms. Eur. Neuropsychopharmacol. 18, 519–530.

Ueki, S., Fujiwara, M., Ogawa, N., 1972. Mouse-killing behavior (muricide) induced by Δ9-tetrahydrocannabinol in the rat. Physiol. Behav. 9, 585–587.

Ullrich, S., Valdez, A.M., 2017. Rocky Mountain high: Preventing cannabis-related injuries. J. Emerg. Nurs. 43, 78–80.

van Kerkhof, L.W.M., Damsteegt, R., Trezza, V., Voorn, P., Vanderschuren, L.J.M.J., 2013. Social play behavior in adolescent rats is mediated by functional activity in medial prefrontal cortex and striatum. Neuropsychopharmacology 38, 1899–1909.

Vanderschuren, L.J.M.J., Achterberg, E.J.M., Trezza, V., 2016. The neurobiology of social play and its rewarding value in rats. Neurosci. Biobehav. Rev. 70, 86–105.

Vanderschuren, L.J.M.J., Niesink, R.J.M., van Ree, J.M., 1997. The neurobiology of social play behavior in rats. Neurosci. Biobehav. Rev. 21, 309–326.

Varlinskaya, E.I., Spear, L.P., 2008. Social interactions in adolescent and adult Sprague–Dawley rats: Impact of social deprivation and test context familiarity. Behav. Brain Res. 188, 398–405.

Varlinskaya, E.I., Spear, L.P., 2002. Acute effects of ethanol on social behavior of adolescent and adult rats: role of familiarity of the test situation. Alcohol. Clin. Exp. Res. 26, 1502–1511.

Varlinskaya, E.I., Spear, L.P., Spear, N.E., 1999. Social behavior and social motivation in adolescent rats: role of housing conditions and partner's activity. Physiol. Behav. 67, 475–482.

Wakeford, A.G.P., Flax, S.M., Pomfrey, R.L., Riley, A.L., 2016. Adolescent delta-9-tetrahydrocannabinol (THC) exposure fails to affect THC-induced place and taste conditioning in adult male rats. Pharmacol. Biochem. Behav. 140, 75–81.

Wang, G.S., 2017. Pediatric concerns due to expanded cannabis use: Unintended consequences of legalization. J. Med. Toxicol. 13, 99–105.

Wang, G.S., Roosevelt, G., Le Lait, M.-C., Martinez, E.M., Bucher-Bartelson, B., Bronstein, A.C., Heard, K., 2014. Association of unintentional pediatric exposures with decriminalization of marijuana in the United States. Ann. Emerg. Med. 63, 684–689.

Zamberletti, E., Prini, P., Speziali, S., Gabaglio, M., Solinas, M., Parolaro, D., Rubino, T., 2012. Gender-dependent behavioral and biochemical effects of adolescent delta-9-tetrahydrocannabinol in adult maternally deprived rats. Neuroscience 204, 245–257.

Zeese, K.B., 1999. History of medical marijuana policy in US. Int. J. Drug Policy 10, 319–328.

CHAPTER III

ALTERATIONS IN THE PROTEOMIC PROFILE AND CANONICAL PATHWAYS IN ADOLESCENT RATS EXPOSED TO Δ9-TETRAHYDROCANNABINOL AS JUVENILES.

3.1 Introduction

With the legalization of marijuana and related products in several states of USA, an increase in the marijuana intoxication in children has been reported (Onders et al., 2016; Wang et al., 2014). The psychoactive component of marijuana is Δ9-tetrahydrocannabinol (Δ9-THC ; Gaoni and Mechoulam 1964) which targets the type I cannabinoid receptors (CB1) in brain. The binding of Δ9-THC to CB1 receptors is responsible for producing various behavioral effects associated with Δ9-THC exposure. CB1 receptors are involved in a variety of functions in adult brains which are different from the functions of CB1 receptors in the developing brains of adolescents and juveniles. This fact is supported by the atypical distribution of CB1 receptors in different brain areas (Berrendero et al., 1998). During the gestational (Berrendero et al., 1998) or postnatal period (Romero et al., 1997), CB1 receptors are transiently detected in brain regions that are not normally associated with CB1 signaling in the adult brain. In addition, CB1 mRNA and protein decrease gradually and progressively in juvenile and adolescent brains to finally reach adult levels (Heng et al., 2011). Considering that juvenile and adolescence are two important stages in terms of brain development,

76

exposure to Δ9-THC during any of these periods could have a long-lasting impact on the functioning of the adult brain and may increase the likelihood of developing psychiatric disorders (Renard et al., 2014).

Previously, adult studies have addressed the effect of Δ9-THC exposure on the expression of different parameters such as tyrosine hydroxylase (Bosier et al., 2012), dopamine receptors (Ginovart et al., 2012), oxytocin receptors (Butovsky et al., 2006), brain derived neurotropic factor (BDNF; ElBatsh et al., 2012. Butovsky et al., 2005), glutamate receptor subunits (Fan et al., 2010), the transcription factor cFOS (Marchese et al., 2008, McCgregor et al., 1998, Miyamoto et al., 1997), orexin receptors (Flores et al., 2016), the synaptic vesicle protein VAMP2, and the postsynaptic protein PSD95 (Rubino et al., 2009). However, these studies reported the effects on these parameters in various brain regions immediately following Δ9-THC exposure. Other studies have addressed the issue of persistent behavioral and biochemical effects following Δ9-THC exposure in adolescents or adults (Kasten et al., 2017, Murphy et al., 2017, Weed et al., 2016, Zamberletti et al., 2016;2015;2014, Fishbein et al., 2012). However, the literature is limited with respect to addressing the adverse outcome of early onset of cannabis use on adolescents. We have previously reported that social behavior is altered in adolescents following repeated juvenile exposure to Δ9-THC (Mohammed et al. 2018). The present study focused on determining the changes induced by this repeated juvenile exposure to Δ9-THC on the proteomic profile in the amygdala of adolescent rats. The amygdala was selected because it is one of the brain regions that regulates social behavior (Trezza et al.,

2013) and because it contains a high density of CB1 receptors (Herkenham et al., 1990), making it one of the brain regions that is a likely target for Δ9-THC-mediated effects (Bindukumar et al., 2008, Renard et al., 2014). In this study, label free proteomic analysis was conducted to determine changes in protein expression followed by further analysis to determine the pathways and functions most strongly affected by juvenile Δ9-THC exposure.

3.2   Materials and Method

3.2.1   Chemicals

Δ9-tetrahydrocannabinol was provided by the National Institute on Drug Abuse. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

3.2.2   Animals

A breeding colony of adult male and female Sprague Dawley rats (CD IGS; Harlan Laboratories, Indianapolis, IN) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility with temperature control (22 ± 2ºC) and with lights on between 0700 and 1900 for a 12-h dark-light cycle. Tap water and LabDiet rodent chow were freely available during the experimentation. The procedures used in this project were approved by the Mississippi State University Institutional Animal Care and Use Committee. From this colony, male and female rat pups were obtained and the day of birth was considered as PND0. On PND10, rat pups within the same litter were assigned to different treatment groups. There was always a representative control animal of the same sex present in each litter to match the Δ9-THC-treated animal.

3.2.3   Exposure

Beginning on PND10, rats were exposed once a day for 7 days to either corn oil (vehicle) or 10 mg/kg Δ9-THC by oral gavage at a volume of 0.5 ml/kg. Solutions were delivered to the back of the throat using a 50-ul tuberculin syringe equipped with a 1-inch 24-gauge straight intubation needle (Popper and Sons, Inc., New Hyde Park, NY). Rats were weaned on PND21 and remained in cages undisturbed until PND38.

3.2.4   Proteomic analysis

On the PND38, the whole brain was collected, frozen on a stainless steel plate on top of dry ice, and stored at -80º C. Three biological replicates of control and Δ9-THC-treated male rats from different litters were used. Each brain was subjected to coronal sectioning to obtain 500-micron thick brain slices. Using the rat brain atlas as a reference (Pixons and Watson 1998), the amygdala was isolated using 1 mm punch and lysed in NP-40 lysis buffer. The protein concentration was measured using a PierceTM BCA protein assay kit (Thermo Scientific). From each sample, 100 μg of protein was precipitated by chloroform/methanol extraction. After precipitation, protein digestion was performed by suspending the pellet in 33 μl of 100 mM Tris-HCl (pH 7.8) containing 6 M urea. The samples were reduced with 1.6 μl of 200 mM dithiothreitol (DTT) followed by alkylation with 6.6 μl of 200 mM iodoacetamide (IAA) each for 45 min at room temperature. The alkylation reaction was quenched by adding 20 μl of 200 mM DTT for 45 min at room temperature. The urea concentration was reduced by adding 258 μl of milliQ-H_2O. Finally, the proteins were digested with trypsin at 1:50 ratio for 18 hr at 37˚C. Protein digestion was terminated by lowering the pH of each sample to <6 by adding concentrated acetic acid. The samples were desalted using C18 SepPak columns (Waters,

USA). These samples were submitted to the University of Arizona Proteomics

Consortium for analysis of samples by using Tandem mass spectrometry coupled to

liquid chromatography (LC-MS/MS).

The LC-MS/MS analysis of trypsin digested protein samples was performed by using

LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA)

equipped with an Advion nanomate ESI source (Advion, Ithaca, NY). Peptides were

eluted from a C18 pre-column (100-μm id × 2 cm, Thermo Fisher Scientific) onto an

analytical column (75-μm ID × 10 cm, C18, Thermo Fisher Scientific) using a beginning

concentration of 2% solvent B (acetonitrile, 0.1% formic acid) for 5 minutes, then a 2–

7% gradient of solvent B over 5 minutes, followed by a 7-15 % gradient of solvent B

over 50 minutes, a 15-35% gradient of solvent B over 60 minutes, a 35-40% gradient of

solvent B over 28 minutes, a 40-85% gradient of solvent B over 5 minutes, held at 85%

solvent B for 10 minutes, 85-2% gradient of solvent B for 1 minute then held at 2%

solvent B for 16 min. All flow rates were at 400 nl/min. Solvent A consisted of water and

0.1% formic acid. Data dependent scanning was performed by the Xcalibur v 2.1.0

software (Andon et al., 2002) using a survey mass scan at 60,000 resolution in the

Orbitrap analyzer scanning m/z 400–1600, followed by collision-induced dissociation

(CID) tandem mass spectrometry (MS/MS) of the fourteen most intense ions in the linear

ion trap analyzer. Precursor ions were selected by the monoisotopic precursor selection

(MIPS) setting with selection or rejection of ions held to a +/− 10 ppm window. Dynamic

exclusion was set to place any selected m/z on an exclusion list for 45 seconds after a

single MS/MS. All MS/MS spectra were searched against a protein database using

Thermo Proteome Discoverer 1.3 (Thermo Fisher Scientific, San Jose, CA) using a

Rattus Norveigicus protein database downloaded from Uniprot (http://www.uniprot.org).

Fully tryptic peptides with up to 2 missed cleavage sites were selected. Variable

modifications considered during the search included methionine oxidation (15.995 Da),

and cysteine carbamidomethylation (57.021 Da). Proteins were identified at 99%

confidence with XCorr score cut-offs (Qian et al., 2005) as determined by a reversed

database search.

3.2.5   Data analysis

The proteins and peptide identification results were visualized by using scaffold v

3.6.1 (Proteome software Inc., Portland, OR, USA). Protein identification with a

minimum of two peptides identified with a 0.1% false discovery rate (FDR) were deemed

correct. Significant changes in the protein expression was calculated by using Fisher's

exact test. Fold changes in the protein expression were calculated using weighted

normalized spectra with 0.2 imputation value.

3.2.6   IPA and gene ontology

For pathway and ontology analysis, the protein list was filtered by applying the LOG2FC

threshold of 0.6 and the Fisher's exact P-value of 0.08 to ensure the presence of closely

related and biologically significant proteins in the list. Biological interpretation of

identified proteins was performed by DAVID (Database of Annotation, Visualization

and Integrated Discovery). The functional annotation clustering categorizes the

differentially expressed proteins among the controlled vocabulary of Gene Ontology

(GO) in three main categories: Biological process, which describes broad biological goal;

Molecular function, which describes the task performed by individual gene products; and Cellular component, which describes the location of molecules inside the cell (Dennis et al., 2003). In current study, GO terms enriched with differentially expressed protein with a P-value ≤ 0.05 were selected.

Differentially expressed proteins were also analyzed in Ingenuity Pathway Analysis (IPA; Qiagen Inc., https://www.qiagenbioinformatics.com) to obtain the canonical pathways, physiological functions, and networks associated with these proteins. In the initial analysis for pathways in IPA, only differentially expressed peptides were included that passed the LOG2FC cutoff 0.6, and Fisher's exact P-value of 0.05 were included which identified some biologically relevant pathways. A subsequent analysis was performed using a Fisher's exact P-value of 0.08 to include more differentially expressed proteins. Addition of proteins did not change the enrichment of pathways but improved the z-scores of pathways that were enriched at P-value of 0.05 (Figure 1).

3.2.7 Quantitative PCR (QPCR)

Total RNA from the amygdala was extracted using the Qiagen RNeasy mini kit (Germantown, MD), and converted to cDNA by using the Qiagen RT2 cDNA synthesis kit provided with genomic DNA elimination buffer (Germantown, MD). Pre-designed gene specific primers, probes (Table 3.1), and Primetime Gene Expression master mix (Integrated DNA Technology, Coraville, IA) were used to preform real time QPCR. Each sample was run in triplicate in a final reaction volume of 20 ul according to manufacturer's instructions. The thermal cycler conditions were as follows: polymerase activation- 95º C for 3 min, amplification- 40 cycles of 95º C for 15 sec and 60º C for 1 min. In each experiment, standard curves for the gene of interest and the reference gene

were made by using a range of cDNA dilutions (1:5-1:1000) and the PCR efficiency factor was calculated. The threshold Ct value of the gene of interest were normalized with Ct values of the reference gene and PCR efficiency factor. Normalized Ct values were used to calculate the fold change from control by using method $2^{(Ct\,(control\,mean)\,-\,Ct\,(Treatment))}$ as described by Betancourt et al. (2007). Statistical analysis was conducted on fold change values by using non-parametric Kruskal-Wallis one-way analysis of variance to determine the significant treatment effect. The level of statistical significance was set at $P \leq 0.05$.

Table 3.1   Primers, probes, and assay names for reference and target genes

Gene name	Assay name	Sequence 5'-3'
Actb	Rn.PT. 39a.222214 838.g	Primer 1 GGCATAGAGGTCTTTACGGATG Primer 2 TCACTATCGGCAATGAGCG Probe /5HEX/TCCTGGGTA/ZEN/TGGAATCCTGTGGC/3IAkFQ
Gnai	Rn.PT.58.5 104082	Primer 1 GTCGTTTTCACTCTAGTTCTGAGA Primer 2 GAGTACCAGCTGAACGATTCG Probe /56FAM/CAACCAAAT/ZEN/TACATCCCAACCCAGCA3IABkFQ

Table 3.1 (continued)

Gnas   Rn.PT.58.6   Primer 1 CCTTCTCACCATCGCTGTT

088247   Primer 2 CACCATTGTGAAGCAGATGAG

Probe

/56FAM/CCGCCCTCT/ZEN/CCGTTAAACCCATTAA/3IABkF

Q

Gnaq   Rn.PT.58.3   Primer 1 CACAAGCTCTTTATTGCGTCTA

8203772   Primer 2 CACTCTCAAGATCCCATACAAGT

Probe

56FAM/ACACCTTCT/ZEN/CCACATCAACCTCTCGA/3IABkF

Q

Gna13   Rn.PT.58.3   Primer 1 GCCATCAACTTGTCTCCGT

4054555   Primer 2 CACCATCTACAGCAACGTGA

Probe

56FAM/CTTCTCTCG/ZEN/GGCATCTACCAGCAC/3IABkF

3.3   Results and Discussion

This study was designed to determine the persistent changes in amygdaloid proteomic

expression in adolescent rats that occur as a result of repeated exposure to Δ9-THC at

juvenile stage. The differential expression in proteins due to Δ9-THC exposure was determined by applying Fisher's exact P-value threshold (P<=0.08), and LOG2FC threshold of 0.6. Out of a total of 1169 proteins detected, there were 38 proteins that were up regulated and 52 proteins that were down regulated due to juvenile Δ9-THC exposure. Functional clustering of differentially expressed proteins in DAVID revealed that most of the differentially expressed proteins were part of the cellular components with GO terms such as synapse, presynaptic membrane, synaptic vesicle and post-synaptic density significantly enriched (Figure 2) by differentially expressed proteins.

In juveniles, atypical distribution of the CB1 receptors in different brain regions is responsible for guiding the elongating axons to their targets in order to form a synapse with the postsynaptic neurons in the target region (Harkany et al., 2008). Large numbers of differentially expressed synaptic proteins in our study suggest that the improper CB1 activation during juvenile Δ9-THC exposure affected the axonal guidance cue which disrupted appropriate synapse formation. Altered synapse formation could be the molecular basis for the social play deficits that we observed in our previous study (Mohammed et al., 2018).

Table 3.2 lists the total canonical pathways enriched in IPA analysis of differentially expressed proteins in this study. There are some relevant pathways with positive or negative activity score are opioid signaling, Gaq signaling, Rho family GTPase, RhoGDI signaing, and thrombin signaling (Figure 3). The differentially expressed proteins associated with these pathways are listed in Table 3.3.

Table 3.2   Canonical pathways predicted using differentially expressed proteins in the amygdala of adolescent rats (PND38) exposed to either corn oil or 10 mg/kg Δ9-THC during juvenile period (PND10-16).

Canonical pathways	Activity z-score
RhoGDI signaling	Negative
Signaling by Rho family GTPase	Positive
Protein ubiquitination pathway	No pattern
Thrombin signaling	Positive
CXCR4 signaling	Zero
CREB signaling in neurons	Zero
Ephrin signaling	Zero
Ethanol degradation II	No pattern
Noradrenaline and adrenalin degradation	No pattern
Gaq pathway	Zero
Tec kinase signaling	Zero
G beta-gamma signaling	Zero
IL-B signaling	Zero
Semaphorin signaling in neuron	No pattern
Breast cancer regulation by stathmin	No pattern

Table 3.2 (continued)

Actin nucleation by ASP-WASP complex	Zero
Serotonin degradation	No pattern
CCR3 signaling in eosinophile	Zero
Androgen signaling	Zero
Histamine degradation	No pattern
Cardiac hypertrophy signaling	Zero
Oxidative ethanol degradation III	No pattern
Chemokine signaling	Zero
Huntington disease signaling	No pattern
Fatty acid oxidation	No pattern
GM-CSF signaling	Zero
Putriscine degradation	No pattern
Ethanol degradation IV	No pattern
Choline degradation	No pattern
D-Mannose degradation	No pattern
Tryptophan degradation	No pattern
Alpha-adrenergic signaling	Zero
CCR5 signaling in macrophages	Zero
Ephrin receptor signaling	Zero
IL-1 signaling	Zero

Table 3.2 (continued)

Dopamine degradation	No pattern
G protein signaling mediated by Tubby	No pattern
Cholestokonin/Gestin mediated signaling	Zero
Farmaldehyde oxidation II	No pattern
Glutamate dependent acid resistance	No pattern
Oxidative phosphorylation	No pattern
Axonal guidance signaling	No pattern
Neuropathic pain signaling in dorsal horn neurons	Zero
Glioma signaling	Zero
Role of NFAT in cardiac hypertrophy	Zero
Mechanisms of viral exit from host cell	No pattern
Rho A signaling	Zero
EIF2 signaling	Negative
Synaptic long term potentiation	Zero
Phospholipase C	Zero
fMLP signaling	Zero
nNOS signaling in neurons	Zero

Table 3.2 (continued)

Pentose phosphate pathway	No pattern
Opioid signaling	Positive
Phagosome mutation	No pattern
Protein Kinase A signaling	Zero
Lysine degradation II	No pattern
Glutamate degradation III	No pattern
Xenobiotic metabolism	No pattern
Molecular mechanism of cancer	No pattern
Relaxin signaling	Zero
GDP mannose biosynthesis	No pattern
Synaptic long term depression	Zero
Mitochondrial dysfunction	No pattern
GDP-glucose biosynthesis	No pattern
Melatonin signaling	Zero
Role of NFAT regulation in Immune response	Zero
Glucose and glucose 1 phosphate degradation	No pattern
Antiproliferative role of somatostatin receptor 2	Zero
Pentose phosphate pathway	No pattern

Table 3.2 (continued)

UDP-N-acetyl D-galactosamine biosynthesis	No pattern
Purine nucleotide de novo biosynthesis	No pattern
Glycogen degradation	No pattern

3.3.2   Gaq signaling

The CB1 receptors are G-protein coupled receptors and their functioning receptors occurs

by binding to the Gai subunit leading to the inhibition of adenylyl cyclase (See review:

Howlett 2005). This inhibitory action induced by the CB1 receptors is reversed in the

presence of pertussis toxin which blocks the binding of the Gai subunit to the CB1

receptor allowing the synthesis and accumulation of cAMP (Felder et al., 1998). Several

studies have reported that a stimulatory action can be induced by the CB1 receptors and

this occurs through the binding of alternate subunits such as Gas or Gaq depending upon

the relative abundance of each subunit (Bonhaus et al., 1996; Glass and Felder 1997;

Felder et al., 1998; Lauckner et al., 2005). In the current study, we observed an

enrichment of the Gaq signaling pathway which could indicate that the repeated exposure

to Δ9-THC persistently altered the abundance of Gai relative to other subunits. This

hypothesis was tested by measuring the level mRNA expression of the Gai, Gas, Gaq,

and Ga13 subunits using QPCR. The results indicated that there was a 15% fold increase

in the mRNA levels of Gai and Gaq whereas the levels of Gas and Ga13 were unaffected

(Figure 4). Previously, it has been reported that down regulation of the mRNA expression

for the G protein subunits occurs immediately following repeated exposure to a CB1 agonist (Rubino et al., 1997). Our gene expression data suggest that a reversal of the effect on the gene expression of G protein subunits occurs after cessation of Δ9-THC exposure. However, this data may not explain the enrichment of Gαq signaling as seen in our IPA data because the binding of subunit to receptor may not be correlated to the amount of mRNA present in tissue (Rubino et al., 1997).

3.3.3   Opioid signaling

In previous studies, it was reported that behavioral and pharmacological effects induced by Δ9-THC involves co-activation of cannabinoid and opioid receptors (Manharaz et al., 1999; Ambrosio et al., 1999). This synergistic action of cannabinoid and opioid receptors is majorly seen with the reward behaviors and antinociception. Absence of either CB1 (Martin et al., 2000) or opioid receptor (Ghozland et al., 2002) abolished the reward behavior induced by the respective receptor agonist. Chronic exposure to Δ9-THC can enhance the antinociceptive potency of morphine which is an opioid receptor agonist (Cichewicz et al., 1999; Welch and Eads, 1999; Smith et al., 1998). Δ9-THC is also known to produce analgesic effects and one of the major hypotheses behind Δ9-THC-induced analgesia is that it involves synergistic actions of both cannabinoid and opioid signaling (Welch and Eads, 1999). In addition, it has been reported that CB1 and opioid receptors are co-localized in amygdala (Novarro et al., 2001) where activation of CB1 receptor is responsible for producing Δ9-THC-induced analgesia (Lee et al., 2011). Thus, the activation of the CB1 receptor by Δ9-THC can in turn activate opioid signaling. Based on our data, it appears that this activation of the CB1 receptors during juvenile brain development leads to a persistent enhancement of opioid signaling which lasts

through adolescence. One hypothesis behind this Δ9-THC-induced long term alteration in opioid signaling may be that repeated Δ9-THC exposure in juvenile rats has increased the opioid receptor density in amygdala as seen in previous study (Cochero et al., 2004). This may lead to persistent alterations in opioid signaling as seen in our data.

3.3.4   Rho GTPase and thrombin signaling

The Rho family GTPase belong to the Ras superfamily of small peptides. Typically, Rho peptides are cytoskeleton proteins and involved in the organization of microtubules (Gunderson et al., 2004) and formation of stress fibers at the cell terminal (Nobes and Hall 1995) which in turn governs the cell migration and differentiation. In terms of neuronal development, the members of the Rho family are associated with various aspects of neural development such as axon formation, neuronal migration, and neurite outgrowth (Watable-Uchida et al., 2006). Neurite formation and inhibition are two important phenomena during brain development in order to achieve proper connectivity between different neurons (Skaper 2005). Several studies reported that activation of Rho GTPase negatively regulates in the axonal outgrowth (Gu et al., 2013; Harkany et al., 2008; Ng and Luo 2004; Lehman et al., 1999). Thrombin signaling is also associated axonal growth. Thrombin signaling occurs through G protein coupled receptors and is directly related to neurite retraction both during injury and during normal brain functioning. The addition of *Clostridium botulinum C3 exoenzyme*, which inhibits Rho, blocks the thrombin-induced neurite retraction (Jalink et al., 1994) indicating collaborative action of thrombin signaling and Rho GTPase to control the axonal growth. As Rho GTPases cycle from the cytoplasmic inactive form to the membrane bound form where they are active to interact with their targets. RhoGDP-dissociation inhibitors

(RhoGDI) are regulatory peptides that control Rho signaling by binding to the Rho proteins in the inactive form (Rho-GDP complex) in cytoplasm thus restricting the translocation to the membrane where they are activated (Rho-GTP; Robbe et al., 2003). In our data, there was an inhibition of RhoGDI signaling and activation of Rho GTPase, and thrombin. Thus, these data are suggesting that the juvenile exposure to Δ9-THC has persistent effects on neuronal functioning such as axonal formation, and neuronal migration which are governed by Rho GTPase. Since the juvenile period is a period where synaptogenesis peaks (Casey et al., 2000) and CB1 receptors were detected in the elongating axons that would reach the target to form a synapse (Fernandez-Ruiz et al., 2000), Δ9-THC -mediated activation of CB1 receptor during juvenile period may alter the synaptogenesis by activating Rho GTPase and thrombin signaling.

3.4   Conclusion

It is well established that the endocannabinoids (EC) and the CB1 receptors are involved in antinociception and neuronal development. Activation of opioid signaling in our data may validates a previous hypothesis that antinociceptive action of THC is mediated through amygdala. Initially, studies only predicted their functions in neural development based on the presence of CB1 receptors on developing neurons in specific regions (Romero et al., 1997). Later, involvement of the CB1 receptors in synaptogenesis was proposed (Fernandez-Ruiz et al., 2000). Our data has provided insight about how the Δ9-THC exposure during postnatal ages could persistently affect the neuronal pathways that are negatively correlated with in axonal migration, or neuritogenesis. As these processes are eventually involved in synaptogenesis, it is possible that juvenile Δ9-THC exposure may affect the synaptogenesis in postnatal brain. This is also supported by the functional

clustering of differentially expressed proteins on DAVID which identified GO terms related to synapse.

Figure 3.1   Network overlap generated by IPA by list of differentially expressed proteins at fishers exact P-value of 0.05 (A) and 0.08 (B)
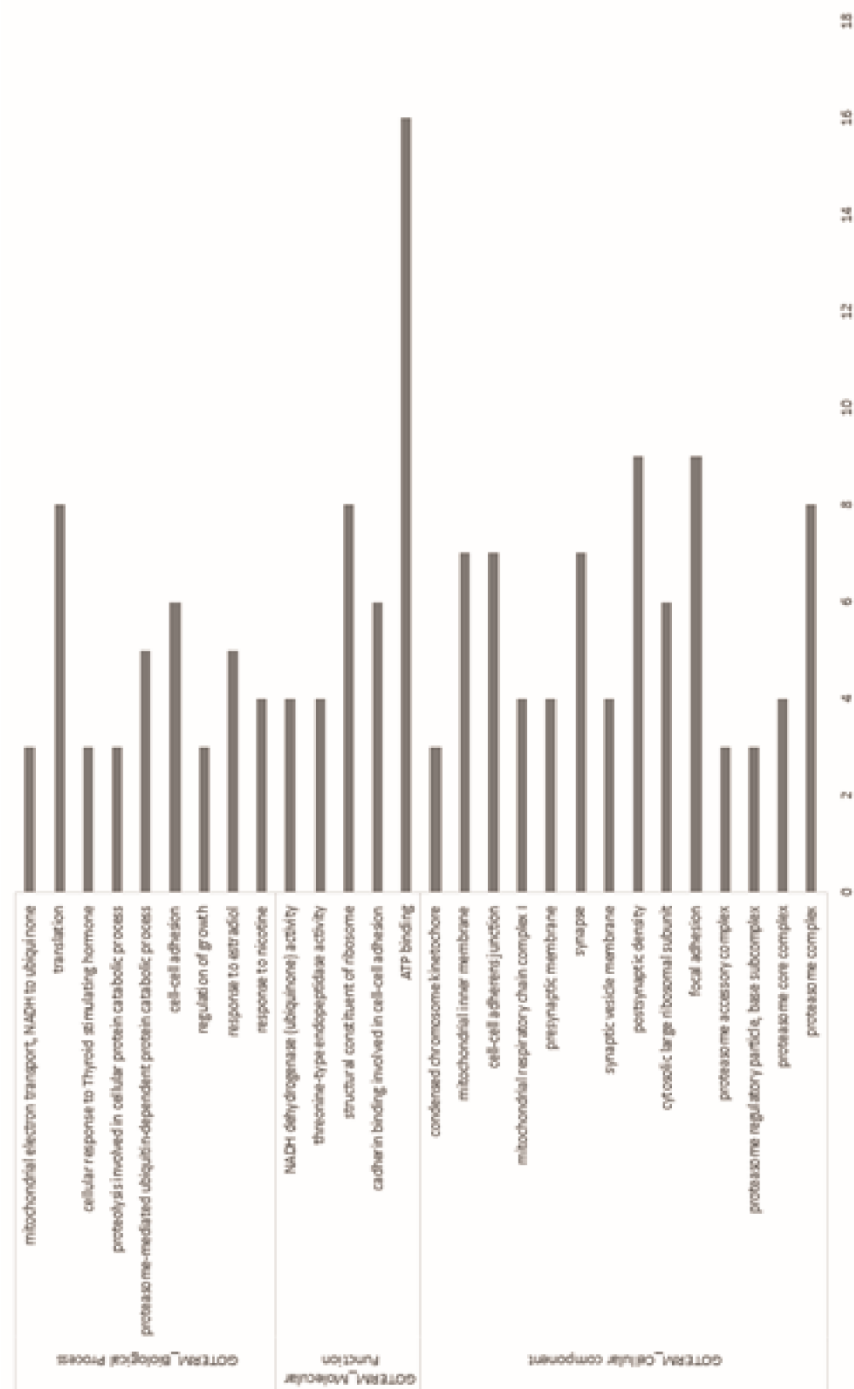
Figure 3.2   GO term analysis of differentially expressed proteins obtained from
amygdala of adolescent rats (PND38) repeatedly exposed to either corn oil
or 10 mg/kg Δ9-THC during juvenile ages (PND10-16).

95
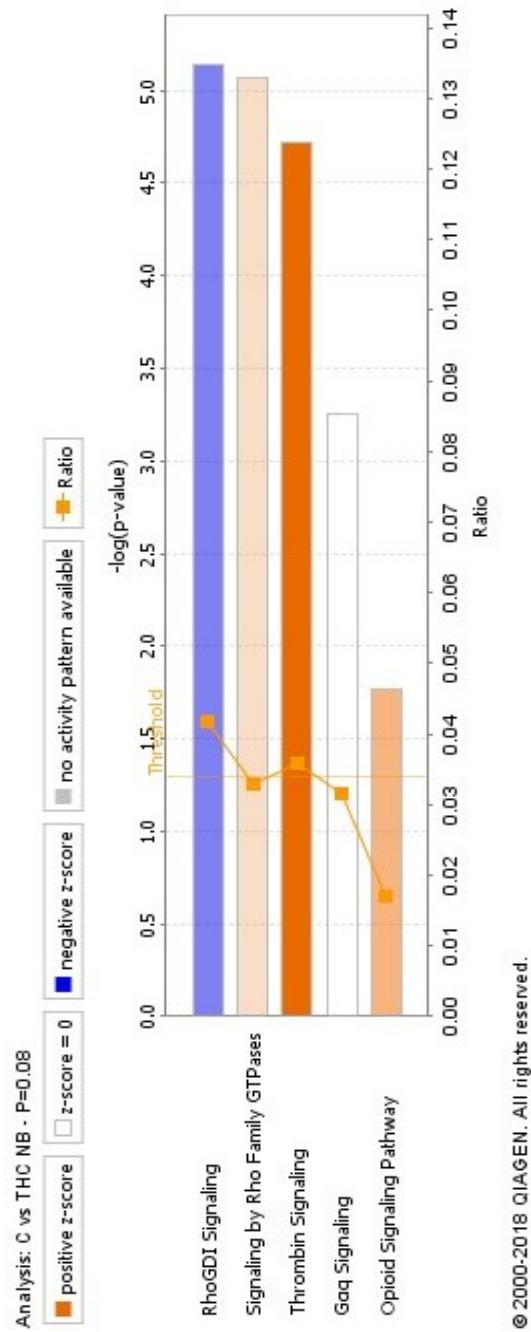
Figure 3.3   Canonical pathways associated with differentially expressed proteins obtained from amygdala (PND38) of rats exposed repeatedly to either corn oil or 10 mg/kg Δ9-THC at juvenile ages (PND10-16).

Table 3.3   List of differentially expressed protein involved in the relevant canonical
pathways shown in figure 3.3

Uniprot Accession	Gene Symbol	Entrez Gene name	Regulation
KCC2A_RAT	CAMK2A	Calcium/calmodulin dependent protein	Up
F1M7X3_RAT	CDH3	Cadherine 13	Down
A0A0G2K526_RAT	GNAL	G protein subunit alpha L	Up
G3V8K2_RAT	GNG3	G protein subunit gamma 3	Down
RACK1_RAT	RACK1	Receptor for activated C kinase 1	Down
A0A0G2K5N6_RAT	ROCK2	Rho associated coiled coil containing protein	Up
RHOB_RAT	RHOB	Ras containing family member B	Up
WASF1_RAT	WASF1	Was containing family member 1	Up

Table 3.3 (continued)

FILMH0_RAT	*SEPT 3*	Septin 3	Down
A0A0G2K526_RAT	*PRKB*	Protein kinase C Beta	Up
PENK_RAT	*PENK*	Proenkaphalin	Down
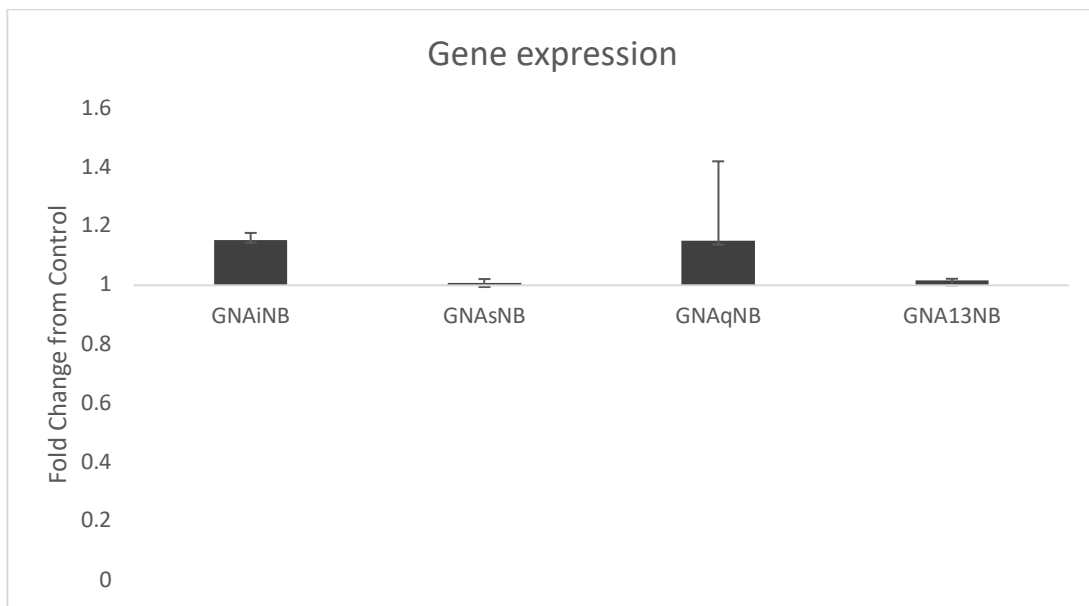
Figure 3.4   Change in mRNA expression in amygdala of adolescent rats (PND38) repeatedly exposed to either corn oil or 10 mg/kg of Δ9-THC during juvenile ages (PND10-16).

3.5   Reference:

Aguado, T., Monory, K., Palazuelos, J., Stella, N., Cravatt, B., Lutz, B., Marsicano, G., Kokaia, Z., Guzman, M., Galve-Roperh, I., 2005. The endocannabinoid system drives neural progenitor proliferation. The FASEB journal, 19, 1704-1706.

Ambrosio, E., Martin, S., Garcia-Lecumberri, C., Crespo, J, A., 1999. The neurobiology of sex dependence: Sex differences and potential interaction between cannabinoid and opioid system. Life sciences, 65, 687-694.

Berrendero, F., Garcia-Gil, L., Hernandez, M.L., Romero, J., Cebeira, M., de Miguel, R., Ramos, J.A., Fernandez-Ruiz, J.J., 1998. Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development. Development 125, 3179–3188.

Betancourt, A,M., Filipov, N,M., Carr, R,L., 2007. Alterations in the neurotrophins in the hippocampus and cerebral cortex of young rats exposed to chlorpyrifos and methyl parathion. Toxicological sciences, 100, 455-455.

Berghuis P., Rajnicek, AM., Morozov, YM., Ross, RA., Mulder, J., Urban GM., Monory, K., Marcicano, G., Matteoli, M., Canty, A., Irvin, AJ., Katona, I., Yanagawa, Y., Rakic, P., Lutz, B., Mackie, K., Harkany, T., 2007. Hardwiring the brain: Endocanabinoids shape neural connectivity. Science, 316, 1212-1216.

Bindukumar, SD., Mahajan, JLR. 2008. Genomic and proteomic analysis of the effects of cannabinoids on normal human astrocytes. Brain Re.2008 1–21.

Bonhaus, D.W., Chang, L.K., Kwan, J., Martin, G.R., 1998. Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. Journal of pharmacology and experimental therapeutics 287, 884–888.

Bosier, B., Muccioli, G.G., Mertens, B., Sarre, S., Michotte, Y., Lambert, D.M., Hermans, E., 2012. Differential modulations of striatal tyrosine hydroxylase and dopamine metabolism by cannabinoid agonists as evidence for functional selectivity in vivo. Neuropharmacology 62, 2327–2335.

Butovsky, E., Juknat, A., Elbaz, J., Shabat-Simon, M., Eilam, R., Zangen, A., Altstein, M., Vogel, Z., 2006. Chronic exposure to Δ9-tetrahydrocannabinol downregulates oxytocin and oxytocin-associated neurophysin in specific brain areas. Molecular and cellular neuroscience 31, 795–804.

Butovsky, E., Juknat, A., Goncharov, I., Elbaz, J., Eilam, R., Zangen, A., Vogel, Z., 2005. In vivo up-regulation of brain-derived neurotrophic factor in specific brain

areas by chronic exposure to Delta9-tetrahydrocannabinol. Journal of neurochemistry 93, 802–811.

Cadoni, C., Pisanu, A., Solinas, M., Acquas, E., Di Chiara, G, 2001. Behavioral sensitization after repeated exposure to Δ9-tetrahydrocannabinol and cross-sensitization with morphine. J. Neurochem. 106, 1586–1593.

Cichewics, D, L., Martin, Z, L., Smith, F, L., Welch, S, P., 1999. Enhancement of mu opioid antinociception by oral delta9-tetrahydrocannabinol: Dose response analysis and receptor identification. Journal of pharmacology and experimental therapeutics. 289, 859-867.

Chang, R.C.C., Yu, M.S., Lai, C.S.W., 2007. Significance of molecular signaling for protein translation control in neurodegenerative diseases. Neurosignals 15, 249–258.

Choi, H., Ghosh, D., Nesvizhskii, A.I., 2007. Statistical Validation of Peptide Identification cations in Large-Scale Proteomics Using the Target-Decoy Database Search Strategy and Flexible Mixture Modeling. Journal of proteome research 286–292.

Diaz-Alonso, J., Guzman, M., Galve-Roperh, I., 2012. Endocannabinoids via CB1 receptors act as neurogenic niche cues during cortical development. Philosophical transactions of the royal society B, 367, 3229-3241

Dennis, G. Jr., Sherman, B, T., Hosak, D, A., Yang, J., Gao, W., Lane, C., Lempiki, R, A.2003. DAVID: Database for annotation, visualization, and integrated discovery. Genome biology, 4, 9-60.

Elbatsh, M.M., Moklas, M.A.A., Marsden, C.A., Kendall, D.A., 2012. Antidepressant-like effects of Δ9-tetrahydrocannabinol and rimonabant in the olfactory bulbectomised rat model of depression. Pharmacology biochemistry and behavior 102, 357–365.

Fan, N., Yang, H., Zhang, J., Chen, C., 2010. Reduced expression of glutamate receptors and phosphorylation of CREB are responsible for in vivo Delta9-Δ9-THC exposure-impaired hippocampal synaptic plasticity. Journal of neurochemistry 112, 691–702.

Fernandez-Ruiz, J., Berrendero, F., Hernandez, M.L., Ramos, J.A., 2000. The endogenous cannabinoid system and brain development. Trends in neuroscience 23, 14–20.

Fishbein, M., Gov, S., Assaf, F., Gafni, M., Keren, O., Sarne, Y., 2012. Long-term behavioral and biochemical effects of an ultra-low dose of Δ9-tetrahydrocannabinol (Δ9-THC): Neuroprotection and ERK signaling. Experimental brain research 221, 437–448.

Felder, C.C., Joyce, K.E., Briley, E.M., Glass, M., Mackie, K.P., Fahey, K.J., Cullinan, G.J., Hunden, D.C., Johnson, D.W., Chaney, M.O., Koppel, G. a, Brownstein, M., 1998. LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. Journal of pharmacology and experimental therapeutics 284, 291–297.

Flores, Á., Julià-Hernández, M., Maldonado, R., Berrendero, F., 2016. Involvement of the orexin/hypocretin system in the pharmacological effects induced by Δ^9-tetrahydrocannabinol. British journal of pharmacology 173, 1381–1392.

Ghozland, S., Mathes, H, W D., Simonin, F., Fillici, D., Kieffer, B, L., Maldonado, R., 2002. Motivational effects of cannabinoids are mediated by mu and kappa-opioid receptors. The journal of neuroscience, 22, 1146-1154.

Ginovart, N., Tournier, B.B., Moulin-Sallanon, M., Steimer, T., Ibanez, V., Millet, P., 2012. Chronic Δ9-tetrahydrocannabinol exposure induces a sensitization of dopamine D2/3receptors in the mesoaccumbens and nigrostriatal systems. Neuropsychopharmacology 37, 2355–2367.

Glass, M., Felder, C.C., 1997. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. Journal of neuroscience 17, 5327–5333.

Gu, H., Yu, S, P., Gutekunst, C-A., Gross, R, E., Wei, L., 2013. Inhibition of Rho signaling pathway improves neurite outgrowth and neuronal differentiation of mouse neural stem cells. International journal of physiology pathophysiological pharmacology, 5, 11-20.

Gundersen, G.G., Gomes, E.R., Wen, Y., 2004. Cortical control of microtubule stability and polarization. Current opinion in cell biology 16, 106–112.

Heng, L., Beverley, J.A., Steiner, H., Tseng, K.Y., 2011. Differential developmental trajectories for CB1 cannabinoid receptor expression in limbic/associative and sensorimotor cortical areas. Synapse 65, 278–286.

Herkenham, M., Lynn, A.B., Litrle, M.D., Johnsont, M.R., Melvin, L.S., De Costa, B.R., Riceo, K.C., 1990. Cannabinoid receptor localization in brain. Neurobiology 87, 1932–1936.

Howlett A. C., 2005. Cannabinoid receptor signaling. Cannabinoids, 168, 53-79.

Jalink, K., Van Corven, E.J., Hengeveld, T., Morii, N., Narumiya, S., Moolenaar, W.H., 1994. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. Journal of cell biology 126, 801–810.

Kasten, C.R., Zhang, Y., Boehm, S.L., 2017. Acute and long-term effects of Δ9-tetrahydrocannabinol on object recognition and anxiety-like activity are age- and strain-dependent in mice. Pharmacology biochemistry and behavior, 163, 9–19.

Keller, A., Nesvizhkii, A, I., Aesbersold, R., 2002. Empirical statistical model to estimate the accuracy of peptide identification made by MS/MS and database search. Analytical chemistry, 74, 5383-5392.

Kramer, A., Green, J., Pollard Jr, J., Tugendeich, S. 2013. Causal analysis approaches in ingenuity pathway analysis. Bioinformatics, 30, 523-530.

Lauckner, J.E., Hille, B., Mackie, K., 2005. The cannabinoid agonist WIN55,212-2 increases the intracellular calcium via CB1 receptor coupling to Gq/11 G protein. PNAS, 102, 19144-19149.

Lehman, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebok, A., Leclerc, N., Tigyi, A., McKerracher, L., 1999. Inactivation of Rho signaling pathway promotes CNS axon regeneration. Journal of neuroscience, 19, 7537-7547.

Lee, M.C., Smith, F.L., Stevens, D.L., Welch, S.P., 2003. The role of several kinases in mice tolerant to delta 9-tetrahydrocannabinol. Journal of pharmacology and experimental therapeutics 305, 593–9.

Martin, M., Ledent, C., Parmentier, M., Maldonado, R., Valverde, O., Cocaine but not morphine induces conditioned place preference and sensitization to locomotor response in CB1 knock out mice. European journal of neuroscience, 12,4038-4046.

Manharaz, J., Corchero, J., Romero, J., Fernandez-Ruiz, J, J., Ramos, J, A., Fuentes, J, A., 1999. Pharmacological and Biochemical interaction between cannabinoid and opioids and cannabinoids. Trends in pharmacological sciences, 20. 287-294.

Marchese, G., Sanna, A., Casu, G., Casti, P., Spada, G.P., Ruiu, S., Pani, L., 2008. Delta-9-tetrahydrocannabinol differently affects striatal c-Fos expression following haloperidol or clozapine administration. European journal of pharmacology 598, 16–20.

McGregor, I.S., Arnold, J.C., Weber, M.F., Topple, a N., Hunt, G.E., 1998. A comparison of delta 9-Δ9-THC and anandamide induced c-fos expression in the rat forebrain. Brain research 802, 19–26.

Miyamoto, A., Yamamoto, T., Ohno, M., Watanabe, S., 1997. Desensitization of Fos protein induction in rat striatum and nucleus accumbens following repeated administration of delta9-tetrahydrocannabinol. Brain research 763, 137–140.

Murphy, M., Mills, S., Winstone, J., Leishman, E., Wager-Miller, J., Bradshaw, H., Mackie, K., 2017. Chronic Adolescent Δ^9-Tetrahydrocannabinol Treatment of Male Mice Leads to Long-Term Cognitive and Behavioral Dysfunction, Which Are Prevented by Concurrent Cannabidiol Treatment. Cannabis and cannabinoid research 2, 235–246.

Nesvizhskii, A., and Aebersold, R., 2004. Analysis, statistical validation and dissemination of large-scale proteomics data sets generated by tandem M/S. 6446, 173–181.

Nesvizhskii, A.I., Keller, A., Kolker, E., Aebersold, R., 2003. A statistical model for identifying proteins by tandem mass spectrometry. Analytical chemistry 75, 4646–4658.

Ng, J., Luo, L., 2004. Rho GTPases regulates axon growth through convergant and divergent signaling pathways. Neuron, 4, 779-793.

Nobes, C.D., Hall, A., 1995. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81, 53–62.

Qian, W, J., Liu, T., Monroe, M, E., Strittmatter, E, F., Jacobes, J, M., Kangas, L, J., Petritis, K., Camp, D, J., Smith, R, D. 2005. Probability based evaluation of peptides and protein identification from tandem mass spectrometry and SEQUEST analysis: the human proteome. Journal of proteome research, 4, 53-62.

Renard, J., Krebs, M.O., Le Pen, G., Jay, T.M., 2014. Long-term consequences of adolescent cannabinoid exposure in adult psychopathology. Frontiers in neuroscience 8, 1–14.

Robbe, K., Otto-Bruc, A., Chardin, P., Antonny, B., 2003. Dissociation of GDP dissociation inhibitor and membrane translocation are required for efficient activation of Rac by the Dbl homology-pleckstrin homology region of Tiam. Journal of biological chemistry 278, 4756–4762.

Rubino, T., Patrini, G., Parenti, M., Massi, P., Parolaro, D., 1997. Chronic treatment with a synthetic cannabinoid CP-55,940 alters G-protein expression in the rat central nervous system. Molecular brain research 44, 191–197.

Rubino, T., Realini, N., Braida, D., Guidi, S., Capurro, V., Viganò, D., Guidali, C., Pinter, M., Sala, M., Bartesaghi, R., Parolaro, D., 2009. Changes in hippocampal morphology and neuroplasticity induced by adolescent Δ9-THC treatment are associated with cognitive impairment in adulthood. Hippocampus 19, 763–772.

Searle, B.C., 2010. Scaffold: A bioinformatic tool for validating MS/MS-based proteomic studies. Proteomics 10, 1265–1269.

Searle, B.C., Turner, M., Nesvizhskii, A.I., 2008. Improving Sensitivity by Probabilistically Combining Results from Multiple MS / MS Search Methodologies 7, 245–253.

Smith, F.L., Cichewicz, D., Martin, Z.L., Welch, S.P., 1998. The enhancement of morphine antinociception in mice by Δ9- tetrahydrocannabinol. Pharmacol. Biochem. Behav. 60, 559–566.

Choi, H., Fermin, D., Nesvizhskii, A.I., 2008. Significance Analysis of Spectral Count Data in Label-free Shotgun Proteomics. Mol. Cell. Proteomics 7, 2373–2385.

Harkany, T., Keimpema, E., Barabás, K., Mulder, J., 2008. Endocannabinoid functions controlling neuronal specification during brain development. Mol. Cell. Endocrinol. 286, 84–90.

Skaper, S, D., 2005. Neuronal growth promoting and inhibitory cues in neuroprotection and neuroregeneration. Annuals in NY academy of science, 1053, 376-385.

Trezza, V., Damsteegt, R., Manduca, A., Petrosino, S., Van Kerkhof, L.W.M., Pasterkamp, R.J., Zhou, Y., Campolongo, P., Cuomo, V., Di Marzo, V., Vanderschuren, L.J.M.J., 2012. Endocannabinoids in Amygdala and Nucleus Accumbens Mediate Social Play Reward in Adolescent Rats. J. Neurosci. 32, 14899–14908.

Watabe-Uchida, M., Govek, E.-E., Van Aelst, L., 2006. Regulators of Rho GTPases in Neuronal Development. J. Neurosci. 26, 10633–10635.

Weed, P.F., Filipeanu, C.M., Ketchum, M.J., Winsauer, P.J., 2016. Chronic Delta(9)-Tetrahydrocannabinol during Adolescence Differentially Modulates Striatal CB1 Receptor Expression and the Acute and Chronic Effects on Learning in Adult Rats. J. Pharmacol. Exp. Ther. 356, 20–31.

Welch, S.P., Eads, M., 1999. Synergistic interactions of endogenous opioids and cannabinoid systems. Brain Res. 848, 183–190.

Zamberletti, E., Gabaglio, M., Grilli, M., Prini, P., Catanese, A., Pittaluga, A., Marchi, M., Rubino, T., Parolaro, D., 2016. Long-term hippocampal glutamate synapse and astrocyte dysfunctions underlying the altered phenotype induced by adolescent Δ9-THC treatment in male rats. Pharmacol. Res. 111, 459–470.

Zamberletti, E., Gabaglio, M., Prini, P., Rubino, T., Parolaro, D., 2015. Cortical neuroinflammation contributes to long-term cognitive dysfunctions following

adolescent delta-9-tetrahydrocannabinol treatment in female rats. Eur. Neuropsychopharmacol. 25, 2404–2415.

CHAPTER IV

ALTERATIONS IN CB1-RELATED CANONICAL PATHWAYS DURING SOCIAL

PLAY IN ADOLESCENT RATS REPEATEDLY EXPOSED TO Δ9-

TETRAHYDROCANNABINOL AS JUVENILES

4.1   Introduction

Social play is one of the earliest form of interactions with peers that occurs without any involvement of the mother. Because of this feature, adolescent social play is included among several important phenomena that are essential for developing normal adult social behaviors (Pellis and Pellis 2009; van den Berg et al., 1998). The occurrence of social play is one of the useful biomarkers for neurodevelopment (Blake and McCoy 2015) and deficits in play performance have been connected to certain neuropsychiatric disorders (Trezza et al., 2010; Meany and Stewart 1981). Play is a characteristic behavior that starts during the early stage of life, peaks during the adolescence, and declines at the time of puberty (Trezza et al., 2010; Martin and Caro et al., 1985). Despite its strong influence in terms of brain development, our knowledge towards understanding the physiological mechanism and neural substrates underlying social play is limited (Blake and McCoy 2015; Trezza and Vanderschuren 2008a). Currently, studies are focusing on elucidating the involvement of the brain endocannabinoid system in mediating social play behavior through type I Cannabinoid receptors (CB1; Trezza and Vanderschuren 2008b).

The modulation of social play through endocannabinoid signaling mainly occurs in the amygdala as demonstrated by Trezza and colleagues (2013). They investigated the effects of activation of endocannabinoid signaling on social play by administrating the FAAH inhibitor URB597 that acts as an indirect CB1 agonist and observed that the level of accumulation of endogenous CB1 ligand (anandamide or AEA) was highest in the amygdala as compared to other regions such as the hippocampus and the nucleus acceumbens. Another study focused on the role of CB1 ligands AEA and 2-AG (2-arachidonoyl glycerol) in the play behavior. They reported an increase in the social play activity in rats treated with a dual inhibitor of AEA and 2-AG hydrolysis. This increase in the social play activities was antagonized by CB1 receptor antagonist (Manduca et al., 2015).

The CB1 receptors are the primary target of Δ9-tetrahydrocannabinol (Δ9-THC; Devane et al., 1992) which is the main psychoactive component of marijuana (Gaoni and Mechoulam 1964). The CB1 receptors are involved in a variety of functions related to neurogenesis (Berrendoro et al., 1998; Romero et al., 1997; Fernandez-Ruiz et al., 2000; Heng et al., 2011) and some of the forms of neurogenesis, such as neuronal migration and synaptogenesis, peak during the juvenile period (Markus and Petit 1987). Thus, juvenile exposure to Δ9-THC could be catastrophic in terms of establishing normal neurological function which mainly depends on synaptogenesis during the early stages of life (Diaz-Alanso et al., 2012). When investigating the Δ9-THC-mediated alterations in brain development, determining the altered canonical pathways in the proteome as it relates to behavioral function could provide very insightful information. Several studies have reported immediate or long-term effects of Δ9-THC exposure on social interactions at

various life stages (Manduce et al., 2015; Trezza et al., 2013; Trezza and Vanderschuren 2008a,b Quinn et al., 2008; Newsome and Kelly 2008; Novarro et al., 1995; Cutler and Mackintosh 1984; van Ree et al., 1984; Seiber 198; Miczek 1974). However, none of these studies have investigated the Δ9-THC-mediated changes in the biochemical pathways related to social play. In our previous study, we reported that adolescent rats receiving Δ9-THC as juveniles engaged in playful activities for longer duration as compared to control adolescent rats (Mohammed et al., 2018). We have also determined the canonical pathways in the proteome altered in adolescents exposed to Δ9-THC as juveniles in the absence of any behavioral activity. In this study, we determined the alterations in the canonical pathways in the proteome related directly to Δ9-THC-mediated changes in social play.

4.2   Materials and Method

4.2.1   Chemical

Δ9-tetrahydrocannabinol was provided by the National Institute on Drug Abuse. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

4.2.2   Animals

A breeding colony of adult male and female Sprague Dawley rats (CD IGS; Harlan Laboratories, Indianapolis, IN) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility with temperature control (22 $\pm$ 2°C) and with lights on between 0700 and 1900 for a 12-h dark-light cycle. Tap water and LabDiet rodent chow were freely available during the experimentation. The procedures used in this project were approved by the Mississippi State University

Institutional Animal Care and Use Committee. From this colony, male and female rat

pups were obtained and the day of birth was considered as PND0. On PND10, rat pups

within the same litter were assigned to different treatment groups. There was always a

representative control animal of the same sex present in each litter to match the Δ9-THC-

treated animal.

4.2.3 Exposure

Beginning on PND10, rats were exposed once a day for 7 days to either corn oil (vehicle)

or 10 mg/kg Δ9-THC by oral gavage at a volume of 0.5 ml/kg. Solutions were delivered

to the back of the throat using a 50-ul tuberculin syringe equipped with a 1-inch 24-gauge

straight intubation needle (Popper and Sons, Inc., New Hyde Park, NY). Rats were

weaned on PND21 and categorized into either a behavioral cohort or a non-behavior

cohort. Each cohort consisted of set of rats receiving either vehicle or Δ9-THC.

4.2.4 Animal handling

4.2.4.1 Behavior rats

After weaning on PND21, rats belonging to the behavioral cohort were subjected to

testing in the elevated plus maze (EPM) on PND29 under low illumination followed by

testing for social interactions on PND38 (data presented in Chapter 2). On PND37, these

rats were individually housed for a 24-hour isolation period. A pair of rats of the same

sex and size and belonging to the same treatment group but from different litters were

placed into a novel cage under high illumination (600 lux) and allowed to interact for 10-

min interval. After social play, the rats were returned to the isolation cage and sacrificed

3-hours later.

4.2.4.2   Non-behavior rats

Rats from the non-behavior cohort were handled in the same way as behavior rats with the exception that they were not subjected to any type of behavior testing. After weaning on PND21, these rats were individually housed on PND37 for a 24-hour isolation period and then sacrificed 24-hours following isolation.

4.2.5   Proteomic analysis

On PND38, each rat in both the behavioral and non-behavioral cohorts were sacrificed to collect the whole brain which was frozen on a stainless steel plate on top of dry ice and stored at -80°C. Three biological replicates, following exposure conditions, were used.

1) Behavioral Control Rats, which received corn oil and subjected to social play.

2) Behavioral Δ9-THC Rats, which received 10 mg/kg of Δ9-THC and subjected to social play.

3) Non-behavioral Control Rats, which received corn oil but not subjected to social play.

4) Non-behavioral Δ9-THC Rats, which received Δ9-THC but not subjected to social play.

Each brain was subjected to coronal sectioning to obtain 500-micron thick brain slices. Using the rat brain atlas as a reference (Pixons and Watson 1998), the amydgala was isolated using 1 mm punch and lysed in NP-40 lysis buffer. The protein concentration was measured using a PierceTM BCA protein assay kit (Thermo Scientific). From each sample, 100 μg of protein was precipitated by chloroform/methanol extraction. After precipitation, protein digestion was performed by suspending the pellet in 33 μl of 100 mM Tris-HCl (pH 7.8) containing 6 M urea. The samples were reduced with 1.6 μl of 200 mM dithiothreitol (DTT) followed by alkylation with 6.6 μl of 200 mM

iodoacetamide (IAA) each for 45 min at room temperature. The alkylation reaction was quenched by adding 20 μl of 200 mM DTT for 45 min at room temperature. The urea concentration was reduced by adding 258 μl of milliQ-H$_2$0. Finally, the proteins were digested with trypsin at 1:50 ratio for 18 hr at 37˚C. Protein digestion was terminated by lowering the pH of each sample to <6 by adding concentrated acetic acid. The samples were desalted using C18 SepPak columns (Waters, USA). These samples were submitted to the University of Arizona Proteomics Consortium for Orbitrap LC-MS/MS analysis. The LC-MS/MS analysis of trypsin digested protein samples was performed by using LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Advion nanomate ESI source (Advion, Ithaca, NY). Peptides were eluted from a C18 pre-column (100-μm id × 2 cm, Thermo Fisher Scientific) onto an analytical column (75-μm ID × 10 cm, C18, Thermo Fisher Scientific) using a beginning concentration of 2% solvent B (acetonitrile, 0.1% formic acid) for 5 minutes, then a 2–7% gradient of solvent B over 5 minutes, followed by a 7-15 % gradient of solvent B over 50 minutes, a 15-35% gradient of solvent B over 60 minutes, a 35-40% gradient of solvent B over 28 minutes, a 40-85% gradient of solvent B over 5 minutes, held at 85% solvent B for 10 minutes, 85-2% gradient of solvent B for 1 minute then held at 2% solvent B for 16 min. All flow rates were at 400 nl/min. Solvent A consisted of water and 0.1% formic acid. Data dependent scanning was performed by the Xcalibur v 2.1.0 software (Andon et al., 2002) using a survey mass scan at 60,000 resolution in the Orbitrap analyzer scanning m/z 400–1600, followed by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) of the fourteen most intense ions in the linear ion trap analyzer. Precursor ions were selected by the monoisotopic precursor selection

(MIPS) setting with selection or rejection of ions held to a +/− 10 ppm window. Dynamic

exclusion was set to place any selected m/z on an exclusion list for 45 seconds after a

single MS/MS. All MS/MS spectra were searched against a protein database using

Thermo Proteome Discoverer 1.3 (Thermo Fisher Scientific, San Jose, CA) using a

Rattus Norveigicus protein database downloaded from Uniprot (http://www.uniprot.org).

Fully tryptic peptides with up to 2 missed cleavage sites were selected. Variable

modifications considered during the search included methionine oxidation (15.995 Da),

and cysteine carbamidomethylation (57.021 Da). Proteins were identified at 99%

confidence with XCorr score cut-offs (Qian et al., 2005) as determined by a reversed

database search.

4.2.6   Data analysis

The protein and peptide identification results were visualized with Scaffold v 3.6.1

(Proteome Software Inc., Portland, OR, USA). Protein identifications with a minimum of

two peptides identified at 0.1% peptide false discovery rate (FDR) were deemed correct.

Significant changes in protein expression were identified by Fisher's exact test. Fold

changes in protein expression were calculated using weighted normalized spectra with

0.5 imputation value.

The different comparisons were as follows:

   A) Control behavior verses Δ9-THC behavior

   B) Δ9-THC non-behavior verses Δ9-THC behavior

   C) Control non-behavior verses Control behavior

4.2.7 IPA and gene ontology

For pathway and ontology analysis, the protein list was filtered by applying the log2fc threshold of 0.6 and the Fisher's exact P-value of 0.05. Biological interpretation of identified proteins was performed by DAVID (Database of Annotation, Visualization and Integrated Discovery). The functional annotation clustering categorizes the differentially expressed proteins among the controlled vocabulary of Gene Ontology (GO) in three main categories: Biological process, which describes broad biological goal; Molecular function, which describes the task performed by individual gene products; and Cellular component, which describes the location of molecules inside the cell (Dennis et al., 2003). In current study, GO terms enriched with differentially expressed protein with a P-value ≤ 0.05 were selected.

Differentially expressed proteins were also analyzed in Ingenuity Pathway Analysis (IPA; Qiagen Inc., https://www.qiagenbioinformatics.com) to obtain canonical pathways, physiological functions, and networks associated with these proteins.

4.2.8 Quantitative PCR (QPCR)

Total RNA from the amygdala was extracted using the Qiagen RNeasy mini kit (Germantown, MD) and converted to cDNA by using the Qiagen RT2 cDNA synthesis kit provided with genomic DNA elimination buffer (Germantown, MD). Pre-designed gene specific primers, probes (Table 4.1) and Primetime Gene Expression Mastermix (Integrated DNA Technology, Coraville, IA) were used to perform real time QPCR. Each sample was run in triplicate in a final reaction volume of 20 ul according to manufacturer's instructions. The thermal cycler conditions were as follows: polymerase activation- 95C for 3 min, amplification- 40 cycles of 95C for 15 sec and 60C for 1 min.

In each experiment, standard curves for the gene of interest and the reference gene were made by using a range of cDNA dilutions (1:5-1:1000) and the PCR efficiency factor was calculated. The threshold Ct value of the gene of interest were normalized with Ct values of the reference gene, and PCR efficiency factor. Normalized Ct values were used to calculate the fold change from control by using method $2^{(Ct\,(Control\,mean)\,-\,Ct\,(Treatment))}$ as described by Betancourt et al. (2007). Statistical analysis was conducted on the fold change values by using a non-parametric Kruskal-Wallis one way analysis of variance test to determine the significant treatment effect. The level of significance was set at p ≤ 0.05.

Table 4.1   Primers, probes, and assay names for reference and target genes

Gene name	Assay name	Sequence 5'-3'
Actb	Rn.PT. 39a.222214838. g	Primer 1 GGCATAGAGGTCTTTACGGATG Primer 2 TCACTATCGGCAATGAGCG Probe /5HEX/TCCTGGGTA/ZEN/TGGAATCCTGTGGC/3IAkFQ
Gnai	Rn.PT.58.5104 082	Primer 1 GTCGTTTTCACTCTAGTTCTGAGA Primer 2 GAGTACCAGCTGAACGATTCG Probe /56FAM/CAACCAAAT/ZEN/TACATCCCAACCCAGCA3IABk FQ

Table 4.1 (continued)

Gnas	Rn.PT.58.6088 247	Primer 1 CCTTCTCACCATCGCTGTT Primer 2 CACCATTGTGAAGCAGATGAG Probe /56FAM/CCGCCCTCT/ZEN/CCGTTAAACCCATTAA/3IABkFQ
Gnaq	Rn.PT.58.3820 3772	Primer 1 CACAAGCTCTTTATTGCGTCTA Primer 2 CACTCTCAAGATCCCATACAAGT Probe 56FAM/ACACCTTCT/ZEN/CCACATCAACCTCTCGA/3IABkFQ
Gna1 3	Rn.PT.58.3405 4555	Primer 1 GCCATCAACTTGTCTCCGT Primer 2 CACCATCTACAGCAACGTGA Probe 56FAM/CTTCTCTCG/ZEN/GGCATCTACCAGCAC/3IABkF

4.3   Results and Discussion

In Chapter 2, we determined the effects of juvenile treatment with Δ9-THC on social

behavior in adolescent rats. In Chapter 3, we determined the changes in amygdaloid

proteomic expression and the associated canonical pathways in non-behavioral

adolescent rats treated with Δ9-THC as juveniles. Our lab has previously compared the changes in amygdaloid proteomic expression and the associated canonical pathways in control rats undergoing behavioral testing with non-behavioral control rats (Alugubelly et al., 2018). In this chapter, we extended those studies to determine the relationship between the changes in proteomic expression and the altered social behavior. This was done by comparing the differentially expressed proteins in the amygdala and the associated canonical pathways in (1) Control behavioral rats and Δ9-THC-treated behavioral rats and (2) Δ9-THC-treated behavioral rats and Δ9-THC-treated non-behavioral rats.

4.3.1   Control behavioral verses Δ9-THC-treated behavioral comparison

In this analysis, protein validation using Scaffold identified 51 proteins that were differentially expressed. DAVID analysis revealed that GO term related to the biological process such as microtubule cytoskeleton organization and cell adhesion, and the cellular component such as cell leading edge (Figure 4.1). These GO terms are involved in axonal guidance (Dent and Gertler 2003), contact between axonal growth cone and target cell (Brose 1999) or neurite growth (Rehaine and Madox 2015).

Further analysis of these differentially expressed proteins using IPA revealed the enrichment of canonical pathways listed in table 1. Figure 4.2 indicates several important pathways based on the number of differentially expressed proteins involved, and the relevance of these pathways in terms of brain development. Some of these pathways are possibly related to CB1 signaling such as protein kinase A, CREB signaling, and cAMP signaling, phospholipase C and calcium signaling. Other pathways such as thrombin signaling and opioid signaling are important in terms of brain development or social play

(Figure 1B). Thrombin signaling, a key pathway involved in the neurite retraction in developing brains (Jalink 1994), and opioid signaling were inhibited in the Δ9-THC-treated behavioral rats as compared to the vehicle treated behavioral rats. Interestingly, opioid signaling was activated in Δ9-THC-treated non-behavioral rats compared to vehicle-treated behavioral rats, whereas it was inhibited in Δ9-THC-treated behavioral rats. This suggests that juvenile Δ9-THC exposure may have affected the opioids (receptors or peptides) that would cause altered binding and/or activation of opioid signaling during adolescence.

Thrombin signaling in neurons is activated when cells are going through several biological processes such as neurite retraction (Jalink et al., 1994), or apoptosis (Denovan et al., 1997). Other studies reported that thrombin is produced in the brain following the brain injury which may lead to break down of blood brain barrier (Xi et al., 2003). In our study, thrombin signaling was enriched in Δ9-THC-treated behavior rats compared to Δ9-THC-treated non-behavior rats but was not enriched in vehicle-treated behavior rats compared to vehicle-treated non-behavioral rats. This suggests that the juvenile Δ9-THC exposure caused some damage to the brain, including neurons in the amygdala causing them to synthesize thrombin. However, inhibition of thrombin signaling in Δ9-THC-treated behavior rats, may indicate an adaptive response elicited by amygdala neurons following social play.

In Chapter 3 we reported the mRNA expression of G protein subunits in non-behavior adolescent rats receiving Δ9-THC as juveniles. Because social play activates the CB1 and opioid signaling which share the common G protein mediated mechanism of activation (Trezza and Vanderschuren 2008), we were interested to measure the abundance of G

protein subunit mRNA in Δ9-THC-treated behavior rats. There was a 6% increase in the Gnai subunit, but Gaq mRNA was reduced by 13% relative to control (Figure 1C). Down regulation of Gaq may indicate the reversal effect of social play on signaling because in Chapter 3, we reported the up regulation of Gaq in Δ9-THC-treated non-behavior rats. Down regulation of Gaq mRNA may be associated with inhibition of calcium signaling and phospholipase C (PLC) signaling (Figure 4.2). In previous study, it was reported that CB1 agonist-mediated activation of PLC and Ca^{++} signaling occurs through binding of Gaq subunit to CB1 receptor (Lauckner et al., 2005). Since PLC is an enzyme involved in the biosynthesis of the CB1 ligand 2-arachidonoyl glycerol (2-AG; Kano et al., 2009), it can be hypothesized that developmental Δ9-THC exposure may cause long term alterations in 2-AG synthesis.

In previous studies, it has been demonstrated that CB1 signaling can have an enhancing effect on social play behavior (Trezza et al., 2008 a,b; Trezza et al., 2013; Manduce et al., 2015). However, there are also studies that report the opposite effect of CB1 activation on social play behavior (O'Shea et al., 2006; Schneider and Koch 2005). This disparity was addressed by Trezza and Vanderscuren (2008a,b) who, considering the high reward value of social play in rats, demonstrated that a cross-talk between cannabinoid and opioid receptors exists and this is responsible for the Δ9-THC-induced and/or CB1 activation-mediated enhancement of social play behavior. In the present study, we observed inhibition of opioid signaling in the Δ9-THC-treated rats when they were subjected to social play. A possible explanation may be the inhibition of related pathways such as CREB signaling and PKA signaling. Both of these pathways are inhibited due to down regulation of cAMP (Koh et al., 1997). CREB is a transcriptional

118

factor which is phosphorylated by PKA to its active form. The activation or inhibition of

CREB in different brain regions can control different types of behaviors including

anxiety and reward behaviors (Review: Carlezon et al., 2005). It has been demonstrated

that microinjection of Δ9-THC into the amygdala inhibits CREB activation leading to an

anxiogenic response in rats (Rubino et al., 2008). It was also reported that CREB activity

is inhibited in the presence of opioid agonist, mainly because of the inhibitory action of

opioid receptor on cAMP (Guitart et al., 1992). On the other hand, opioid antagonist

activates CREB by releasing the tonic inhibition of opioid receptor on cAMP (Shaw-

Lutchman et al., 2002). In present study, inhibition of cAMP, CREB, and PKA signaling

in Δ9-THC treated rats is in agreement with previous studies.

Table 4.2   List of canonical pathways obtained from differentially expressed proteins
in amygdala of adolescent rats receiving either corn oil or 10 mg/kg Δ9-
THC during juvenile ages (PND10-16). Tissue was extracted on PND38,
either 3-hours following play

Canonical pathways	Activity z-score
Protein Kinase A	Negative
Sirtuin signaling pathway	Negative
Thrombin signaling	Negative
CREB signaling in neurons	Negative
Calcium signaling	Negative
GM-SCF signaling	Negative
Ephrine receptor signaling	Negative
B cell receptor signaling	Negative
Integrin signaling	Positive
Phospholipase C	Negative
cAMP mediated signaling	Negative

Table 4.3 (continued)

iCOS-iCOSL signaling in T helper cells	Negative
Neuropathic pain signaling in dorsal horn neuron	Negative
Chemokine signaling	Negative
Synaptic long term potentiation	Negative
Glioma signaling	Negative
Melanin signaling	Negative
GNRH signaling	Negative
PKCB in T lymphocytes	Negative
PI3K in B lymphocytes	Negative

Figure 4.1   GO term analysis of differentially expressed proteins obtained from amygdala tissue of behavioral rats repeatedly exposed to either corn oil or 10 mg/kg Δ9-THCduring juvenile ages (PND10-16). Tissue was extracted 3-hours following social play on PND38.
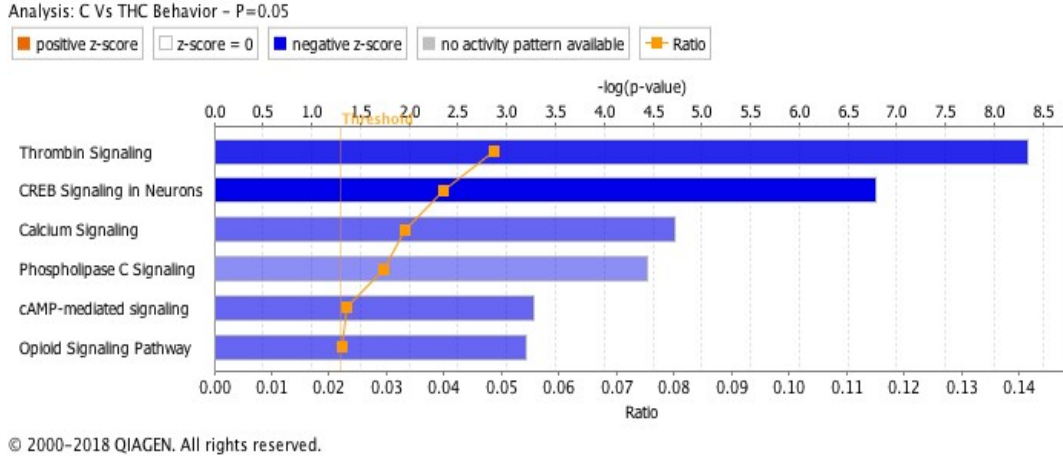
Figure 4.2   Relevant canonical pathways obtained from IPA analysis of differentially expressed proteins obtained from amygdala tissue of behavioral rats repeatedly exposed to either corn oil of 10 mg/kg Δ9-THC during juvenile ages (PND10-16). Tissue was extracted 3-hours following social play on PND 38.

Table 4.3   Differentially expressed proteins involved in the relevant canonical pathways shown in Figure 4.2.

Uniport Accession	Gene Symbol	Entrez Gene Name	Regulation
KCC4_RAT	CAMK4	Calcium/calmodulin dependent protein kinase IV	Down
KCC2A_RAT	CAMK2A	Calcium/calmodulin dependent protein kinase II	Down
FILUE2_RAT	CAMK2B	Calcium/calmodulin dependent protein kinase II beta	Down

121

Table 4.3 (continued)

KCC2D_RAT	CAMK2D	Calcium/calmodulin dependent protein kinase II	Down
A0A0G2K319_RAT	DUSP3	Dual specificity phosphatase	Down
GNA13_RAT	GNA13	G protein subunit alpha13	Down
M0R809_RAT	GNG4	G protein subunit gamma 4	Up
GRB2_RAT	GRB2	Growth factor receptor	Down
G3V9Y1_RAT	MYH10	Myosine heavy chain 10	Up
A0A0G2JSW0_RAT	MYL2A	Myosine light chain 12A	Up
PDE18_RAT	PDE18	Phosphodiesterase 18	Up
PPR18_RAT	PPP1R1B	Protein phosphatase 1 regulatory inhibition subunit	Up
RACK1_RAT	RACK1	Receptor for activated C kinase 1	Up

Figure 4.3   Relative change in mRNA expression of G protein subunits in amygdala of behavioral rats repeatedly exposed to either corn oil or 10 mg/kg of Δ9-THC during juvenile ages (PND10-16). Tissue was extracted 3-hours following social play on PND38.

4.3.2   Δ9-THC-treated behavioral versus Δ9-THC-treated non-behavioral comparison

In this analysis, protein validation using Scaffold identified 64 proteins that were up regulated and 67 proteins that were down regulated. Functional clustering of these differentially expressed proteins was performed by using DAVID which identified GO term related to cellular component such as cell junction, cell-cell adherence and synapse (Figure 4.4).

Further analysis of these differentially expressed proteins using IPA revealed enrichment of canonical pathways listed in Table 4. The canonical pathways such as thrombin signaling and opioid signaling PKA, cAMP and CREB signaling are the same pathways that we observed to be altered in our previous experimental comparison discussed above. The differentially expressed proteins associated with these pathways are listed in table 5.

Additional pathways significantly enriched included dopamine degradation and dopamine

DARPP32 feedback in cAMP signaling (Figure 4.5). The differentially expressed

proteins involved in these pathways are listed in Table 5.

The dopamine degradation pathway involved only monoamine oxidase (MAO) A and B

from our list of differentially expressed proteins. Down regulation of MAO may indicate

the decrease in dopamine degradation as this enzyme is responsible for dopamine

degradation. This finding is in agreement with previous studies that reported an increase

in the neuronal firing in dopamine producing brain regions such as the ventral tegmental

area and the substantia nigra (Renard et al., 2017; Wu et al., 2000) and an increase in the

dopamine release (Oleson et al., 2012; Chen et al., 1990) following Δ9-THC exposure.

However, an increase in the dopamine metabolites has also been observed following oral

exposure to Δ9-THC (Novorro et al., 1993). This contradicts our findings in that we

observed a down regulation of MAO which may not be correlated to increase in the

dopamine metabolites. Dopaminergic neurotransmission is modulated by the retrograde

action of CB1 receptors on GABA neurons which inhibits dopamine synthesis (Solinas et

al., 2006). Δ9-THC-mediated activation of the CB1 receptors may have been responsible

for Δ9-THC-mediated increase in dopamine levels observed by Novorro et al. (1993). It

is also possible that the Δ9-THC-mediated increase in dopamine was due to the inhibitory

action of Δ9-THC on MAO levels (Fisar 2010).

DARPP-32 is a protein phosphatase inhibitor mainly present in the dopaminergic neurons

(Fernandez et al., 2006) and present in the brain regions which are enriched with

dopamine innervations including the amygdala (Yger and Girault 2011). DARPP-32 is

activated due to the stimulatory action of dopamine receptor (D1) on cAMP and PKA,

which phosphorylates DARPP-32 on Thr34 site. Thr34 phosphorylation eventually

causes inhibition of CREB (Fernandez et al., 2006). Another study reported cyclin

dependent kinase (cdk5) can phosphorylate DARPP-32 on Thr75 site which is

responsible for inhibition of PKA (Bibb et al., 1999). Another study reported that

DARPP-32 feedback in cAMP signaling pathway is activated when there is an interaction

between opioid and dopaminergic neurotransmission (Mahajan et al., 2009). Also, the

dual activation of opioid and dopamine receptor is associated with increase in the social

play activity (Niesink and van Ree 1989). Therefore, inhibition of cAMP, CREB, PKA,

opioid, dopamine degradation and DARPP-32 feedback signaling pathways found in our

study may indicate that these pathways are inter related and play a role in mediating
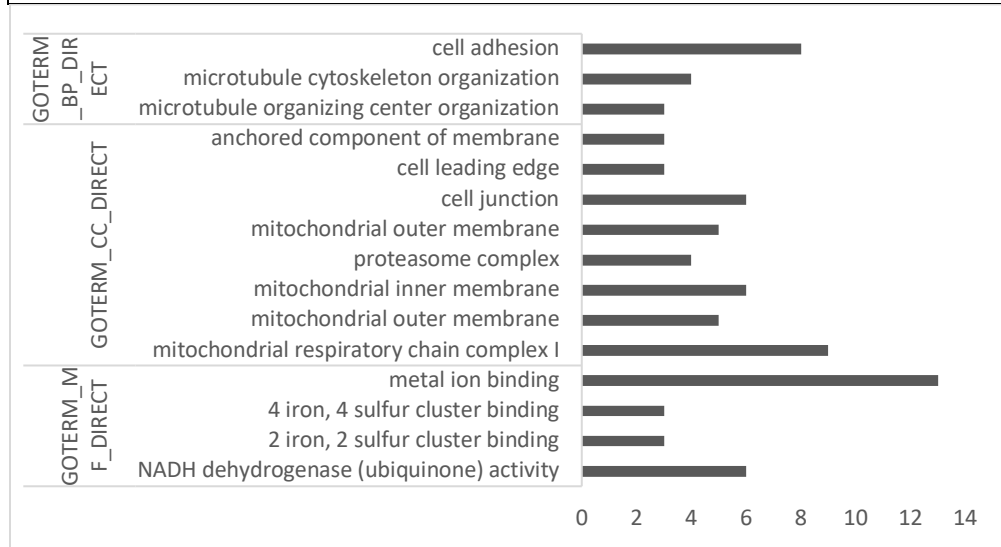
behaviors controlled by amygdala.
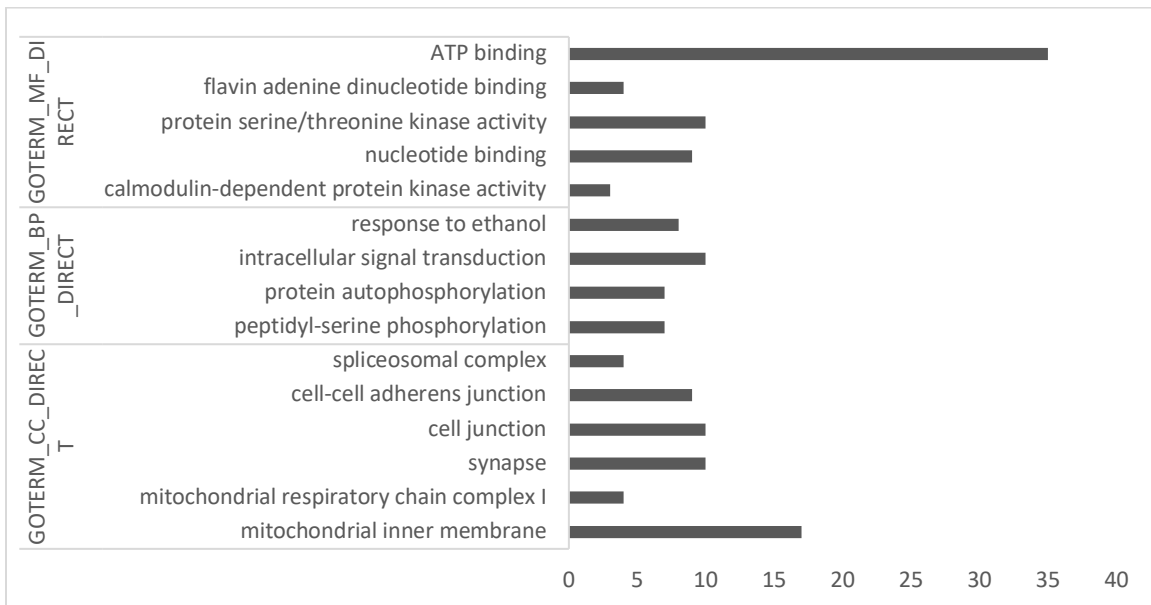
Figure 4.4   GO term analysis of differentially expressed proteins obtained from amygdala of adolescent rats repeatedly exposed to only Δ9-THC during juvenile ages (PND10-16). Tissue was extracted on PND38, either 3-hours following play (behavior Δ9-THC) or without being subjected to play (non-behavior Δ9-THC)

125

Table 4.4   List of canonical pathways obtained from differentially expressed proteins in amygdala of adolescent rats receiving only 10 mg/kg Δ9-THC during juvenile ages. Tissue was extracted on PND38, either 3-hours following play (behavior Δ9-THC) or without being subjected to play (non-behavior Δ9-THC).

Canonical pathways	Activity z-score
CREB signaling in neurons	Negative
Melatonin signaling	Negative
GNRH signaling	Negative
Thrombin signaling	Negative
Opioid signaling	Negative
Synaptic long term potentiation	Negative
CXCR4 signaling	Negative
CCR3 signaling in eosinophils	Negative
fMLP signaling in neutrophils	Negative
Androgen signaling	Negative
Chemokine signaling	Negative
Corticotrophin releasing hormone signaling	Negative
Protein kinase A signaling	Negative
GPCR signaling	Negative
Xenobiotic metabolism signaling	Negative
GPCR mediated nutrient sensing in enteroendocrine cells	Negative
IL-B signaling	Negative
UVC-induced MAPK signaling	Negative
Neuropathic pain signaling in dorsal horn neurons	Negative
Gaq signaling	Negative
G beta gamma signaling	Negative

Table 4.4 (continued)

Phospholipase C signaling	Negative
P2Y purigenic receptor signaling	Negative
UVB induced MAPK signaling	Negative
FC epsin RI signaling	Negative
Dopamine DRPP-32 feedback in cAMP signaling	Negative
Synaptic long term depression	Negative
GM-CSF signaling	Negative
Tryptophan degradation	Negative
Renin Angiotensin signaling	Negative
cAMP mediated signaling	Negative
NRF2-mediated oxidative stress	Negative
Neuroglin signaling	Negative
LPS-mediated MAPK	Negative
Ca induced T lymphocyte apoptosis	Negative
ErbB signaling	Negative
Cholestokonin mediated signaling	Negative
Thrombopiotin signaling	Negative
eNOS signaling	Negative
PPAR/RXR-alpha activation	Negative
PI3K signaling in B lymphocytes	Negative

Table 4.4 (continued)

Huntington disease signaling	Negative
CDK5 signaling	Negative
Endothein signaling	Negative
GP6 signaling	Negative
Adrenomeduline signaling	Negative
P7056K signaling	Negative
Ca signaling	Negative
Aldosterone signaling in epithelial cells	Negative
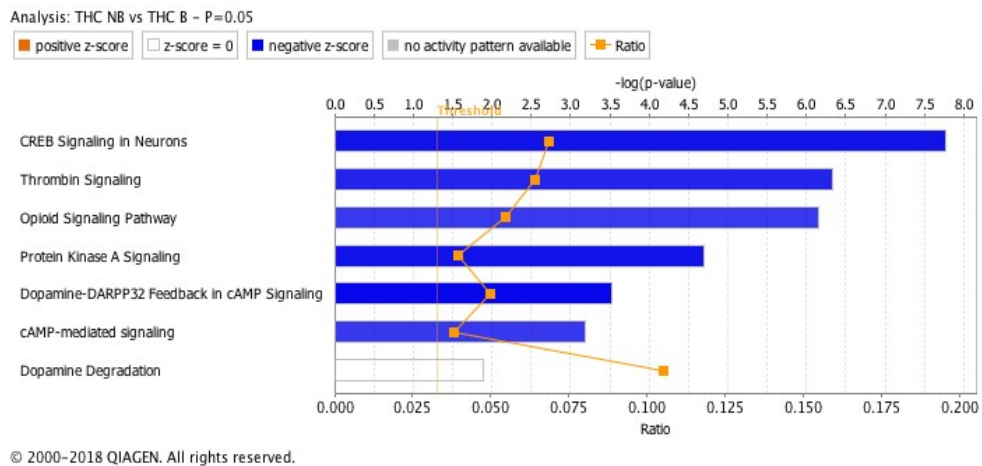
Figure 4.5   Relevant canonical pathways obtained from differentially expressed proteins in amygdala of adolescent rats receiving oly 10 mg/kg Δ9-THCduring juvenile ages (PND10-16). Tissue was extracted on PND38, either 3-hours following play (behavior Δ9-THC) or without being subjected to play (non-behavior Δ9-THC).

Table 4.5   Differentially expressed proteins involved in relevant canonical pathways shown in figure 4.5

Uniprot Accession	Gene Symbol	Entrez Gene Name	Regulation
FILN88_RAT	ALDH2	Aldehyde dehydrogenase	Down
G3V9G1_RAT	ADCYC	Adenylyl cyclase 5	Down
A0A0G2K7E5_RAT	CACNA2D1	Calcium voltage-gated channel auxiliary subunit alpha2delta 1	Down
KCC4_RAT	CAMK4	Calcium/calmoduline dependent protein kinase IV	Down
FILUE2_RAT	CAMk2B	Calcium/calmodulin dependent protein kinase II beta	Down
KCC2D_RAT	CAMK2D	Calcium/calmodulin dependent protein kinase II delta	Down
GNAS2_RAT	GNAS	GNAS complex locus	Up

Table 4.5 (continued)

M0R809_RAT	GNG4	G protein gamma subunit	Down
FILNE4_RAT	GRIA2	Glutamate ionotrophic receptor AMPA subunit 2	Up
MAP2K1_RAT	MAP2K1	Mitogen activated protein kinase kinase 1	Down
FILP57_RAT	MAP2K4	Mitogen actiavated protein kinase kinase II	Up
KPCA_RAT	PRKCA	Protein kinase C alpha	Down
A0A0G2KSQ0_RAT	PRKCB	Protein kinase C beta	Down
KPCG_RAT	PRKCG	Protein kinase C gamma	Down
RACK1_RAT	RACK1	Receptor for activated C kinase	Up
S12AS_RAT	SLC12AS	Solute carrier family 12 member 5	Down

Table 4.5 (continued)

FILX13_RAT	PDE10A	Phosphodiesterase 10A	Down
CDK5_RAT	CDKS	Cyclin dependent kinase 5	Down
FILV89_RAT	RAPGAP	Rap1 GTPase	Down
AOFA_RAT	MAOA	Monoamine oxidase A	Down

4.3.3 Control behavior and control non-behavior comparison

In this analysis, protein validation using Scaffold identified 38 proteins that were up

regulated and 64 proteins that were down regulated. Functional clustering of these

differentially expressed proteins by using DAVID revealed that there was enrichment of

molecular functions such as axonogenesis and positive regulation of axon elongation and

enrichment of cellular components related to the synapse such as postsynaptic density

and synaptic vesicles (Figure 4.6).

Further analysis of these differentially expressed proteins using IPA revealed enrichment

of canonical pathways listed in table 6. Figure 3B indicates the most relevant pathways

which are discussed in details. These pathways include opioid signaling, PKA signaling,

cAMP signaling and dopamine degradation. Several studies reported the enhancing effect

of opioid signaling on social play behavior (Trezza and Vanderschuren 2008 a,b;

Vanderschuren et al., 1995 a,b; Beatty et al., 1982). As activation of opioid signaling in

amygdala is mainly responsible for translating learned reward into motivation This

results in increased appetitive and/or consummatory behaviors towards that reward (Mahler and Berridge 2009). As play is a natural reward in rats, activation of opioid signaling in amygdala of behavioral rats was not surprising. The opioid signaling mainly occurs by binding of Gai subunits to opioid receptor causing inhibition of adenylyl cyclase enzyme. This causes decrease in cAMP and PKA signaling (Davis et al., 2003). Both cAMP and PKA signaling are decreased in our data.

The activation of opioid signaling was reversed in Δ9-THC-treated behavioral rats as discussed in the previous sections. This may indicate that juvenile Δ9-THC exposure altered opioid signaling in the brain that would alter the motivation for reward. However, despite the inhibition of opioid signaling, Δ9-THC-treated rats engaged in social play for more amount of time compared to controls as reported in chapter 2. Therefore, it can be hypothesized that juvenile Δ9-THC exposure causes functional changes in opioid signaling that would alter its activation during reward behaviors. Same pattern was observed for dopamine degradation pathway in both vehicle-treated and Δ9-THC-treated behavioral rats. In both comparisons, dopamine degradation pathway was enriched because of downregulation of MAO enzyme. This indicates inhibitory action of social play on dopamine degradation in amygdala. Previous studies have reported the enhancing effect of dopamine release on social play (Trezza et al., 2008; Peills et al., 1993; Neisink and van Ree 1989; Beatty et al., 1984). However, it is still need to be confirmed if the reduction in the MAO activity following social play can cause the accumulation of dopamine in amygdala and dopamine producing regions such as ventral tegmental area and striatum.

132
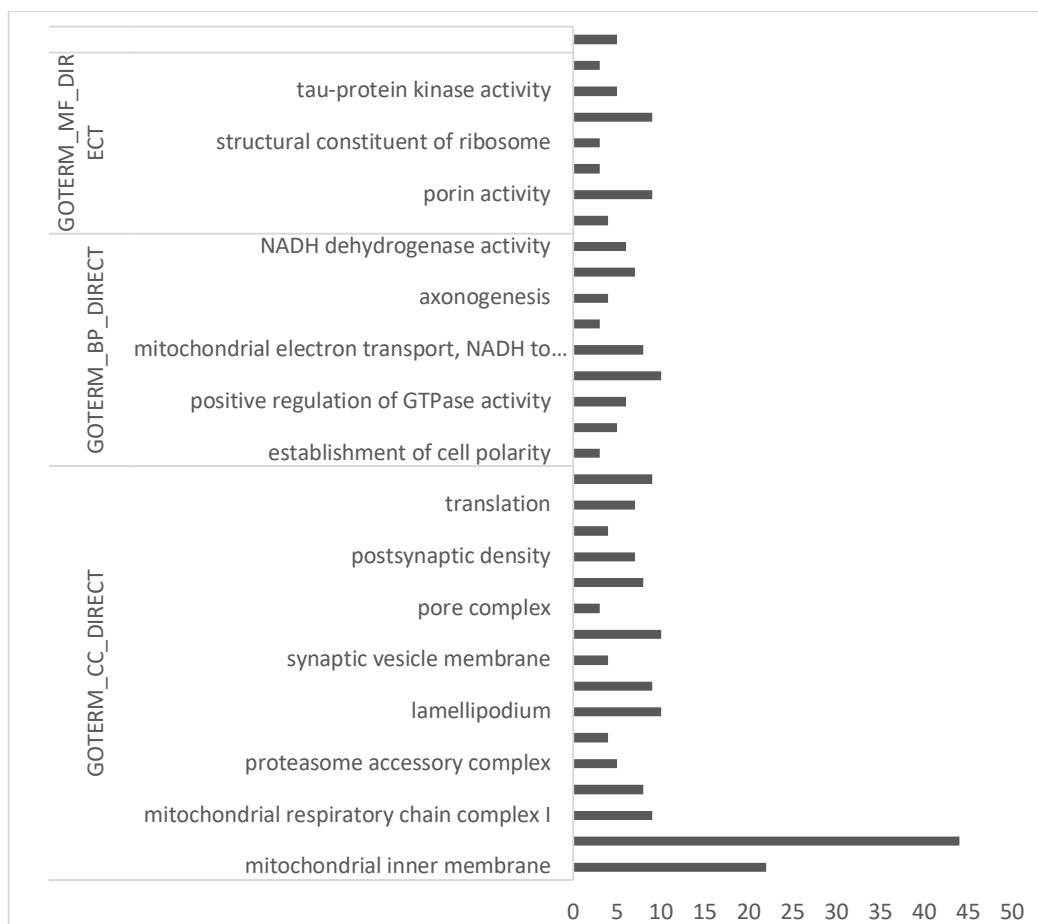
Figure 4.6   GO term analysis of differentially expressed proteins obtained from amygdala tissue of rats repeatedly exposed to corn oil during juvenile ages (PND10-16). Tissue was extracted on PND38, either 3-hours following play (behavior controls) or without being subjected to play (non-behavior controls).

Table 4.6   List of canonical pathways obtained from differentially expressed proteins in amygdala of adolescent rats exposed to corn oil during juvenile ages (PND10-16). Tissue was extracted on PND38, either 3-hours following play (behavior control) or without being subjected to play (non-behavior control).

Canonical pathways	Activity z-score
Mitochondrial dysfunction	No pattern
Sirbutin signaling	Positive

Table 4.6 (continued)

Glycogen degradation	Zero
Oxidative phosphorylation	Negative
Dopamine receptor signaling	Zero
Melatonin degradation	Zero
GDP-glucose biosynthesis	Zero
Glucose phosphatase degradation	Zero
Glycogen degradation	Zero
Protein kinase A signaling	Negative
Cardiac beta adrenergic signaling	Negative
Phenylalanine degradation	Zero
Serotonin receptor signaling	No pattern
Tryptophan degradation	Zero
Leukocyte extravasation	Zero
Dopamine degradation	Zero
Gai signaling	Zero
Noradrenaline degradation	Zero
Protein ubiquitnation	Zero
cAMP signaling	Negative
Relaxin signaling	Zero
Gaq signaling	Zero
Opioid signaling	Positive

Table 4.6 (continued)

NADH repair	Zero
tRNA splicing	Zero
Serotonin degradation	Zero
Melatonin degradation	Zero
GPCR signaling	Zero
EIF2 signaling	Zero
Gustation pathway	Zero
Semaphorin signaling	No pattern
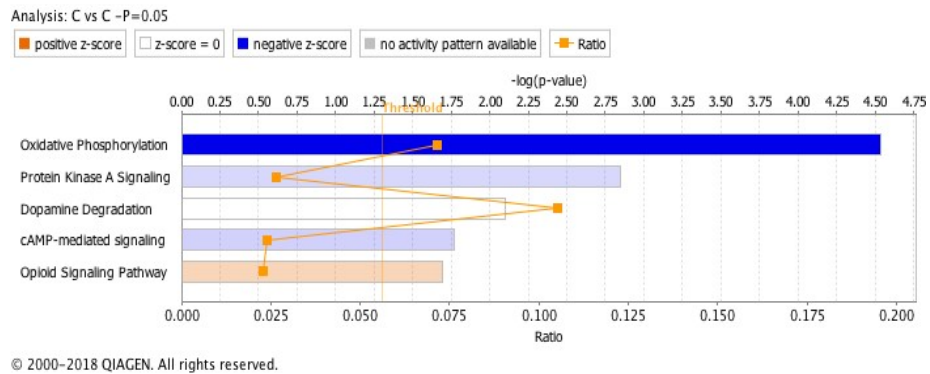
Figure 4.7   Relevant canonical pathways obtained from differentially expressed proteins obtained from amygdala of adolescent rats repeatedly exposed to only corn oil during juvenile ages (PND10-16). Tissue was extracted on PND38, either 3-hours following play (behavior controls) or without being subjected to play (non-behavior controls).

Table 4.7   List of differentially expressed proteins involved in canonical pathways listed in figure 4.

Uniprot Accession	Gene Symbol	Entrez Gene Name	Regulation
D3ZFQ8_RAT	CYC 1	Cytochrome c1	Down
NDUA9_RAT	NDUFA9	NADH Ubiquinone	Down
NDUAA_RAT	NDUAF10	NADH Ubiquinone	down
D4A0T0_RAT	NDUFB10	NADH Ubiquinone	Down
NDUS2_RAT	NDUSF2	NADH Ubiquinone	Down
UCR1_RAT	UQCRFS1	Ubiquinol cytochrome c	Down
G3V9G1_RAT	ADCYC5	Adenylyl cyclase 5	Down
AOFA_RAT	MAOA	Monoamine oxidase A	Down
AOFB_RAT	MAOB	Monoamine oxidase B	Down

Table 4.7 (continued)

A0A0G2KKB98_RAT	GSK3B	Glycogen synthetase kinase 3 beta	Up
FILX13_RAT	PDE10A	Phosphodiesterase 10A	Down
PDE1B_RAT	PDE1B	Phosphodiesterase 1B	Down
PP1R7_RAT	PPP1R7	Protein phosphatase 1 regulatory subnit 7	Down
RACK1_RAT	RACK1	Receptor for activated C kinase 1	Down
A0A0G2K5N6_RAT	ROCK2	Rho associated coiled coil containing protein	Up
FILV89_RAT	RAP1GAP	Rap1GTPase activating proteins	Down

Table 4.7 (continued)

D3ZWGZ_RAT	RGS7	Receptor for G protein signaling 7	Down
A0A0G2KB98_RAT	CLTA	Clathrin light chain A	Down

4.4 Conclusion

This study evaluated the social play-mediated enrichment of canonical pathways in the amygdala and how these pathways are altered by developmental Δ9-THC exposure. We report that developmental Δ9-THC exposure alters normal opioid signaling during social play behavior as evidenced by the fact that engaging in social play by control rats activates opioid signaling, whereas prior exposure to Δ9-THC inhibits the social play-induced opioid signaling in rats. This is the most significant finding in this study because opioid signaling is mainly activated during the reward behaviors. We also found inhibition of thrombin signaling in Δ9-THC-treated behavioral rats. As thrombin signaling is activated in response to neuronal damage, enrichment of thrombin signaling in Δ9-THC treated rats may indicate a response of the neurons to the damage induced by developmental Δ9-THC exposure.

4.5   Reference:

Aguedo, T., Monory, K., Palazuelos, J., Stella, N., Cravatt, B., Lutz, B., Marsicano, G., Kokaia, Z., Guzman, M., Galve-Roperh, I., 2005. The endocannabinoid system drives neural progenitor proliferation. The FASEB journal, 19, 1704-1706.

Beatty, W, W., Costello, K, B., 1982. Naloxon and play fighting in juvenile rats. Pharmacology biochemistry and behavior. 17, 905-907.

Beatty, W, W., Costello, K, B., Berry, S, L. 1984. Suppression of play fighting by amphetamine: effects of catecholamine antagonist agonist and synthesis inhibitors. Pharmacology biochemistry and behavior, 20, 747-754.

Bibb, J, A., Synder, G, L., Nishi, A., Yan, Z., Meijer, L., Feinberg, A, A., Tsai, L-H., Kwon, Y, T., Girault, J-A., Czernik, A, J., Huganir, R, L., Hemming Jr, H, C., Nairn, A, C., Greendarg, P. Phosphorylation of DARPP-32 by cdk5 modulates dopamine signaling in neurons. Letters to nature, 402, 669-671.

Brose, N., 1999. Synaptic cell adhesion proteins and synaptogenesis in mammalian central nervous system. Naturwiessenschaftern, 86, 516-524.

Blake, B.E., McCoy, K.A., 2015. Hormonal programming of rat social play behavior: Standardized techniques will aid synthesis and translation to human health. Neuroscience and biobehavioral reviews, 55, 184–197.

Bloomfield, M.A.P., Ashok, A.H., Volkow, N.D., Oliver, D., 2017. The effects of Δ9-tetrahydrocannabinol on the dopamine system. Europe PMC funders groups, 539, 369–377.

Calcagnetti, D.J., Schechter, M.D., 1992. Place conditioning reveals the rewarding aspect of social interaction in juvenile rats. Physiology of behavior 51, 667–672.

Carlezon, W.A., Duman, R.S., Nestler, E.J., 2005. The many faces of CREB. Trends in neuroscience, 28, 436–445

Castaneda, E., Moss, D.E., Oddie, S.D., Whishaw, I.Q., 1991. THC does not affect striatal dopamine release: microdialysis in freely moving rats. Pharmacology and biochemistry of behavior 40, 587–91.

Davis, M, I., Ronesi, J., Lovinger, D, M., 2003. A predominant role for inhibition of adenylyl cyclase/protein kinase A in ERK activation by cannabinoid receptor 1 in N1E-115 neuroblastoma cells. The journal of biological chemistry, 278, 48973-48980.

139

Denovan, F, M., Pike, C, J., Cotman, C, W., Cunningham, D, D. 1997. Thrombin induces apoptosis in cultured neurons and astrocytes via a pathway requiring tyrosine kinase and Rho A activities. Journal of neuroscience, 17, 5316-5326.

Dent, E, W., Gertler, F, B., 2003. Cytoskeletal dynamics and transport in growth cone motility and axon guidance. Neuron, 40, 209-227.

Fattore, L., Viganò, D., Fadda, P., Rubino, T., Fratta, W., Parolaro, D., 2007. Bidirectional regulation of mu-opioid and CB1-cannabinoid receptor in rats self-administering heroin or WIN 55,212-2. European journal of neuroscience, 25, 2191–2200.

Fernandez, E., Schiappa, R., Girault, J-A., Novere, N, L., 2006. DARPP-32 is a robust integrator of dopamine and glutamate signaling. PLOS computational biology, 2, 1619-1633.

Fišar, Z., 2010. Inhibition of monoamine oxidase activity by cannabinoids. Archives in pharmacology, 381, 563–572.

Hoffman, A.F., Oz, M., Caulder, T., Lupica, C.R., 2003. Functional tolerance and blockade of long-term depression at synapses in the nucleus accumbens after chronic cannabinoid exposure. Journal of neuroscience, 23, 4815–20.

Humphreys, A.P., Einon, D.F., 1981. Play as a reinforcer for maze-learning in juvenile rats. Animal behavior, 29, 259–270.

Jin, K., Xie, L., Kim, S.H., Parmentier-Batteur, S., Sun, Y., Mao, X.O., Childs, J., Greenberg, D. a, 2004. Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. Molecular pharmacology, 66, 204–208.

Kano, M., Ohno-Shousaku, T., Hashimotodani, Y., Uchigashima, M., Watanabe, M. 2009. Endocannabinoid-mediated control of synaptic neurotransmission. Psychological reviews, 89, 309-380.

Koh, W.S., Crawford, R.B., Kaminski, N.E., 1997. Inhibition of protein kinase A and cyclic AMP response element (CRE)-specific transcription factor binding by delta9-tetrahydrocannabinol (delta9-THC): a putative mechanism of cannabinoid-induced immune modulation. Biochemical pharmacology, 53, 1477–1484.

Koob, G.F., 2008. A role for brain stress systems in addiction. Neuron, 59, 11–34.

Lauckner, J.E., Hille, B., Mackie, K., 2005. The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. PNAS, 102, 19144–19149.

Lupica, C.R., Riegel, A.C., Hoffman, A.F., 2004. Marijuana and cannabinoid regulation of brain reward circuits. British journal of pharmacology, 143, 227–234

Lutz, B., Marsicano, G., Kokaia, Z., Guzmán, M., Galve-roperh, I., 2005. proliferation 19, 1704–1706.

Mahajan, S.D., Aalinkeel, R., Reynolds, J.L., Nair, B.B., Sykes, D.E., Hu, Z., Bonoiu, A., Ding, H., Prasad, P.N., Stanley, A., 2009. Therapeutic targeting of DARPP-32: A key signaling molecule in dopaminergic pathway for the treatment of opiate addiction. International reviews of neurobiology, 88, 199-222.

Mahler, V, S., and Berridge, K, C., 2009. Which cue to want? Central amygdala opioid activation enhances and focuses incentive salience on a proponent reward cue. The journal of neuroscience, 29, 6500-6513.

Manduca, A., Morena, M., Campolongo, P., Servadio, M., Palmery, M., Trabace, L., Hill, M.N., Vanderschuren, L.J.M.J., Cuomo, V., Trezza, V., 2015. Distinct roles of the endocannabinoids anandamide and 2-arachidonoylglycerol in social behavior and emotionality at different developmental ages in rats. European neuropsychopharmacology, 25, 1362–1374.

Markus, E.J., Petit, T.L., 1987. Neocortical synaptogenesis, aging, and behavior: Lifespan development in the motor-sensory system of the rat. Experimental neurology, 96, 262–278.

Martín-García, E., Fernández-Castillo, N., Burokas, A., Gutiérrez-Cuesta, J., Sánchez-Mora, C., Casas, M., Ribasés, M., Cormand, B., Maldonado, R., 2015. Frustrated expected reward induces differential transcriptional changes in the mouse brain. Addiction biology, 20, 22–27.

Martin, P., and Caro, T,M., 1985. On the functions of the play and its role in behavioral development. Advances in the study of behavior, 15, 59-99.

Meaney, M, J., and Stewart, J., 1981. The descriptive study of social development in rats (Rattus Norvegicus). Animal behavior, 29, 34-45.

Navarro, M., Carrera, M.R., Fratta, W., Valverde, O., Cossu, G., Fattore, L., Chowen, J.A., Gomez, R., del Arco, I., Villanua, M.A., Maldonado, R., Koob, G.F., de Fonseca, F.R., 2001. Functional interaction between opioid and cannabinoid receptors in drug self-administration. Journal of neuroscience, 21, 5344–5350.

Navarro, M., Fernández-Ruiz, J.J., de Miguel, R., Hernández, M.L., Cebeira, M., Ramos, J.A., 1993. An acute dose of δ9-tetrahydrocannabinol affects behavioral and neurochemical indices of mesolimbic dopaminergic activity. Behavioral brain research, 57, 37–46.

Neisink, R, J., van Ree, J, M., 1989. Involvement of opioid and dopaminergic systems in isolation induced pinning and social grooming of young rats. Neuropharmacology, 4, 411-418.

Normshell, L., and Panksepp, J., 1989. Effects of morphine and naloxone on play-rewarded spatial discrimination in juvenile rats. Developmental psychobiology, 23, 75-83.

Oleson, E.B., Cheer, J.F., 2012. A brain on cannabinoids: The role of dopamine release in reward seeking. Cold spring harbor perspectives in medicine, 2, 1–13.

Olioff, M., Stewart, J., 1978. Sex differences in the play behavior of prepubescent rats. Physiology of behavior, 20, 113–115.

O'Shea, M., McGregor, I, S., Mallet, P, E., 2006. Repeated cannabinoid exposure during perinatal, adolescent or early ages produces similar long-lasting deficits in object recognition and reduced social interaction in rats. Journal of psychopharmacology, 20, 611-621.

Panksepp, J., 1980. Social deprivation and play in rats. Behavioral and neural biology, 30, 197-206.

Panksepp, J., Sivity, S., Normshell, L., 1984. The psychology of play: Theoretical and methodological perspective. Neuroscience and behavioral reviews, 8, 465-492.

Pellis, S, M., Castaneda, E., McKenna, M, M., Tran-Nguyen, T, L, Ly., Whishaw, I, Q. 1993. The role of striatum in organizing the sequences of play fighting in neonatally dopamine depleted rats. Neuroscience letter, 158, 13-15.

Pellis, S., and Pellis, V., 2009. The playful brain: Venturing to the limits of neuroscience. Oneworld publication.

Pellis, S.M., Castañeda, E., McKenna, M.M., Tran-Nguyen, L.T.L., Whishaw, I.Q., 1993. The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats. Neuroscience letters, 158, 13–15.

Pellis, S.M., McKenna, M., 1995. What do rats find rewarding in play fighting? - an analysis using drug-induced non-playful partners. Behavioral brain research, 68, 65–73.

Pellis, S.M., Pellis, V.C., 1998. Play fighting of rats in comparative perspective: A schema for neurobehavioral analyses. Neuroscience and biobehavioral reviews, 23, 87–101.

142

Renard, J., Szkudlarek, H.J., Kramar, C.P., Jobson, C.E.L., Moura, K., Rushlow, W.J., Laviolette, S.R., 2017. Adolescent THC Exposure Causes Enduring Prefrontal Cortical Disruption of GABAergic Inhibition and Dysregulation of Sub-Cortical Dopamine Function Scientific reports, 7, 1–14.

Rezayof, A., Sardari, M., Zarrindast, M.R., Nayer-Nouri, T., 2011. Functional interaction between morphine and central amygdala cannabinoid CB1 receptors in the acquisition and expression of conditioned place preference. Behavioral brain research, 220, 1–8.

Robinson, T.E., Berridge, K.C., 1993. The neural basis of drug craving: An incentive-sensitization theory of addiction. Brain research revies, 18, 247–291.

Rubino, T., Guidali, C., Vigano, D., Realini, N., Valenti, M., Massi, P., Parolaro, D., 2008. CB1 receptor stimulation in specific brain areas differently modulate anxiety-related behaviour. Neuropharmacology, 54, 151–160.

Schneider, M., Koch, M., 2005. Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: Effects of chronic pubertal cannabinoid treatment. Neuropsychopharmacology, 30, 944–957.

Shaw-Lutchman, T.Z., Barrot, M., Wallace, T., Gilden, L., Zachariou, V., Impey, S., Duman, R.S., Storm, D., Nestler, E.J., 2002. Regional and cellular mapping of cAMP response element-mediated transcription during naltrexone-precipitated morphine withdrawal. Journal of neuroscience, 22, 3663–72.

Solinas, M., Justinova, Z., Goldberg, S.R., Tanda, G., 2006. Anandamide administration alone and after inhibition of fatty acid amide hydrolase (FAAH) increases dopamine levels in the nucleus accumbens shell in rats. Journal of neurochemistry, 98, 408–419.

Thor, D.H., Holloway, W.R., 1984. Social play in juvenile rats: A decade of methodological and experimental research. Neuroscience and biobehavioral reviews, 8, 455–464.

Tournier, B.B., Tsartsalis, S., Dimiziani, A., Millet, P., Ginovart, N., 2016. Time-dependent effects of repeated THC treatment on dopamine D2/3 receptor-mediated signalling in midbrain and striatum. Behavioral brain research, 311, 322–329.

Trezza, V., Baarendse, P.J.J., Vanderschuren, L.J.M.J., 2010. The pleasures of play: Pharmacological insights into social reward mechanisms. Trends in pharmacological sciences, 31, 463–469.

Trezza, V., Cuomo, V., Vanderschuren, L.J.M.J., 2008. Cannabis and the developing brain: Insights from behavior. European journal of pharmacology, 585, 441–452.

Trezza, V., Damsteegt, R., Manduca, A., Petrosino, S., Van Kerkhof, L.W.M., Pasterkamp, R.J., Zhou, Y., Campolongo, P., Cuomo, V., Di Marzo, V., Vanderschuren, L.J.M.J., 2012. Endocannabinoids in Amygdala and Nucleus Accumbens Mediate Social Play Reward in Adolescent Rats. Journal of neuroscience, 32, 14899–14908.

Trezza, V., Vanderschuren, L.J.M.J., 2008a. Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology, 197, 217–227.

Trezza, V., Vanderschuren, L.J.M.J., 2008b. Cannabinoid and opioid modulation of social play behavior in adolescent rats: Differential behavioral mechanisms. Eur. Neuropsychopharmacology,18, 519–530.

Trezza, V., Vanderschuren, L.J.M.J., 2009. Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. J. Pharmacology and experimental therapeutics, 328, 343–350.

Van Den Berg, C.L., Hol, T., Van Ree, J.M., Spruijt, B.M., Everts, H., Koolhaas, J.M., 1999. Play is indispensable for an adequate development of coping with social challenges in the rat. Developmental psychobiology, 34, 129–138.

Vanderschuren, L.J.M., Niesink, R.J.M., Spruijt, B.M., Van Ree, J.M., 1995a. Influence of environmental factors on social play behavior of juvenile rats. Physiology of behavior, 58, 119–123.

Vanderschuren, L.J.M.J., Stein, E.A., Wiegant, V.M., Van Ree, J.M., 1995b. Social play alters regional brain opioid receptor binding in juvenile rats. Brain research, 680, 148–156.

Viganò, D., Rubino, T., Vaccani, A., Bianchessi, S., Marmorato, P., Castiglioni, C., Parolaro, D., 2005. Molecular mechanisms involved in the asymmetric interaction between cannabinoid and opioid systems. Psychopharmacology, 182, 527–536.

Von Frijtag, J.C., Schot, M., Van Den Bos, R., Spruijt, B.M., 2002. Individual housing during the play period results in changed responses to and consequences of a psychosocial stress situation in rats. Developmental psychobiology, 41, 58–69.

Wu, X., French, E.D., 2000. Effects of chronic delta9-tetrahydrocannabinol on rat midbrain dopamine neurons: an electrophysiological assessment. Neuropharmacology, 39, 391–8.

Xi, G., Reiser, G., Keep, R, F., 2003. The role of thrombin and thrombin receptors in ischemic hemorrhagic brain injury: deleterious or protective? Journal of neuroscience, 84, 3-9.

Yger, M., Girault, J.-A., 2011. DARPP-32, Jack of All Trades? Master of Which? Frontiers in behavioral neuroscience, 5, 1–14.

CHAPTER V

CONCLUSION

There are numerous studies addressing the cognitive deficits in adults resulting from

marijuana use during adolescence (Vadhan et al., 2017; Forti et al., 2014; Cass et al.,

2014; Saez et al., 2014; Hajos et al., 2007) or gestation (Huizink 2014; Berghuis et al,

2007; Huizink et al., 2006). Given the increased popularity of ingesting marijuana edibles

that has recently developed, this research investigated the persistent behavioral deficits

and relevant signaling pathways that are altered following juvenile exposure to the

psychoactive component of marijuana Δ9-THC. Δ9-THC is considered to be the main

component responsible for producing the cognitive deficits associated with marijuana

(D'Souza et al., 2005). The primary target of Δ9-THC in the brain is stimulation of the

type I cannabinoid receptor (CB1) which is also involved in various aspects of brain

development (Watson et al., 2008). Thus, the persistent neurological deficits associated

with developmental marijuana exposure may be due to overstimulation of the CB1

receptor (Wilkinson et al., 2015) such that the normal progression of the brain

development is disrupted.

Neurite outgrowth and axonal migration are two important steps in brain development

that shape the connectivity of the brain by establishing proper synaptic connections

across the regions (Fernandez-Ruiz et al., 2000). The CB1 receptor is consistently present

in neurons undergoing these processes and is crucially important in the establishment of

the appropriate connections (Gaffari et al., 2012; Fernandez-Ruiz et al., 2000). During development, the acquisition of new behavioral skills depends upon the neuronal processes such as dendritic growth, the number of synapses and size of synapses in the brain (Dagwood et al., 1985). As juvenile period is associated with sharp increase in the number of synapses (Markaus and Petit 1987), over activation of CB1 receptor during this period can profoundly affect the acquisition of behavioral skills.

In our behavior data, we did not observe any direct effect of juvenile Δ9-THC exposure on anxiety in adolescent rats in the elevated plus maze (EPM). One possible reason have could been the age at which the testing was conducted. Adolescence is a period characteristically linked with increased risk taking (Steinbern 2007). This natural tendency to take risks during adolescence may have simply increased the overall level of exploration and this decreased the ability to measure any increases or decreases in the level of anxiety during testing. We did observe significant differences in the social interaction test in adolescent rats exposed to Δ9-THC as juveniles. The most significant parameters affected involved increased levels of play behavior by the Δ9-THC-treated rats. A possible neurobiological explanation for this increased play behavior could be that the Δ9-THC exposure induced persistent changes that disrupted the inhibitory and excitatory balance in the brain. Activation of the CB1 receptor on the gamma-aminobutyric acid (GABA) neurons inhibits the release of GABA which reduces the inhibitory action of GABA in the brain. This 'dis-inhibition' has been reported to be enhanced by Δ9-THC treatment in rats (Radhakrishnan et al., 2015).

Our proteomic data identified the cellular pathways associated with Δ9-THC exposure and/or social play behavior in amygdala neurons. Thrombin signaling was the most

relevant pathway affected in Δ9-THC rats. Thrombin signaling in neurons is mainly responsible for growth cone collapse and neurite retraction (Jalink et al., 1994) which impairs the navigation of the axon and prevents it from reaching its target synapse (Harkany et al., 2008). In our data, enrichment of thrombin signaling was detected in Δ9-THC-treated behavioral rats and non-behavioral rats but was not detected in vehicle-treated rats which indicates that a long-term effect of repeated Δ9-THC ingestion during the juvenile period is altered thrombin signaling in neurons of the amygdala. As thrombin signaling in neurons is involved in neurite retraction (Jalink et al., 1994), is activated following brain injury (Vaughan et al., 1995) and is associated with neuronal death (Choi et al., 2003), the enrichment of thrombin signaling in Δ9-THC-treated rats in the current study may indicate that juvenile Δ9-THC exposure has altered one or more of these processes that thrombin signaling is linked to.

In addition, opioid signaling was enhanced in Δ9-THC-treated non-behavioral rats as compared to control non-behavioral rats but was inhibited in Δ9-THC-treated behavioral rats as compared to control behavioral rats. The different effects of Δ9-THC exposure on opioid signaling induced by the presence or absence of social behavior may suggest altered competitive binding of the cannabinoid and/or opioid receptors to G proteins subunits. The cannabinoid and opioid receptors share a common G protein subunit and dual activation of both cannabinoid and opioid receptors during social play (Trezza and Vanderschuren 2008) in Δ9-THC-treated rats may have attenuated the binding of Gαi subunit to opioid receptor as previously reported (Rios et al., 2006). Since opioid signaling is mainly associated with nociception and repeated Δ9-THC treatment reduces the response to painful stimuli even long after treatment ceases (Martin et al., 2004;

148

Caroline et al., 2000), this suggests that repeated Δ9-THC dampens the response of opioid signaling to stimuli. Our results agree with this premise in that introduction of a behavioral challenge resulted in the inhibition of opioid signaling in the amygdala of rats treated with Δ9-THC.

Overall, this study investigated the persistent effects of oral Δ9-THC exposure during the juvenile period and included the behavioral alterations observed and the cellular pathways that were either activated or inhibited in the amygdala of those exposed rats once they reached adolescence. This research has limitations in that it only tested the persistent alterations in behaviors related to anxiety and social interactions in response to exposure to Δ9-THC. First, our EPM data suggest an increase in locomotor activity in Δ9-THC-treated rats. Future studies should focus on validating the effects on Δ9-THC on locomotor activity using a more appropriate behavioral test specific for measuring this type of behavior. Secondly, we observed Δ9-THC-mediated effects on thrombin signaling in the amygdala neurons. Both thrombin signaling (Harkany et al., 2008) and the CB1 receptor (Fernandez-Ruiz et al., 2000) are involved in the migration of axons to target neurons in order to form a synapse. Therefore, future studies should also include investigation of the potential alterations in synaptic density throughout the entire brain following oral juvenile exposure to Δ9-THC. Thirdly, we observed that in Δ9-THC-treated behavior rats, there is an alteration in opioid signaling. This may suggest alterations in the binding affinity of G protein subunits to the opioid receptor in the amygdala. Future studies should investigate the binding affinity of G protein subunits to the opioid receptor in animals previously treated with Δ9-THC by administering a challenge that would activate opioid receptors.

While the popularity of edible marijuana products is increasing in the United States, the data obtained in our study may provide an insight to the increasing popularity of marijuana and related products in the United States. Our data has clearly established a link between repeated juvenile Δ9-THC exposure and deficits in social behaviors during adolescence. This effect of Δ9-THC on the developing brain in juveniles may challenge to the public's attitude to support the popularity of marijuana products. As addressed by previous studies, the child friendly packaging of marijuana edibles and the lack of limitations on the maximum content of Δ9-THC in a single edible are major threats to the children. Our data suggest that consumption of edibles by juveniles may be catastrophic in terms of the acquisition of skills which are necessary for meeting the challenges in future life. The results of this study also strongly suggest that care should be taken when popularizing marijuana edible products in the media so that children will not misinterpret marijuana as being safe for them to ingest.

5.1   Reference:

Choi, S-H., Lee, D, Y., Ryu, J, K., Kim, J., Joe, E, H., Jin, B, K. 2003. Thrombin induces niagral   dopaminergic neurodegeneration in vivo by altering the expression of cell death related proteins. Neurobiology of disease, 14, 181-193

Di Forti, M., Sallis, H., Allegri, F., Trotta, A., Ferraro, L., Stilo, S.A., Marconi, A., La Cascia, C., Marques, T.R., Pariante, C., Dazzan, P., Mondelli, V., Paparelli, A., Kolliakou, A., Prata, D., Gaughran, F., David, A.S., Morgan, C., Stahl, D., Khondoker, M., MacCabe, J.H., Murray, R.M., 2014. Daily use, especially of high-potency cannabis, drives the earlier onset of psychosis in cannabis users. Schizophrenia bulletin 40, 1509–1517.

Fride, E., 2004. The endocannabinoid-CB 1 receptor system in pre- and postnatal life. European journal of pharmacology 500, 289–297.

Gaffuri, A.-L., Ladarre, D., Lenkei, Z., 2012. Type-1 Cannabinoid Receptor Signaling in Neuronal Development. Pharmacology 90, 19–39.

Hajós, M., Hoffmann, W.E., Kocsis, B., 2008. Activation of Cannabinoid-1 Receptors Disrupts Sensory Gating and Neuronal Oscillation: Relevance to Schizophrenia. Biological psychiatry 63, 1075–1083.

Harkany, T., Mackie, K., Doherty, P., 2008. Wiring and firing neuronal networks: endocannabinoids take center stage. Current opinion in neurobiology 18, 338–345.

Huizink, A.C., 2014. Prenatal cannabis exposure and infant outcomes: Overview of studies. Progress in neuro-psychopharmacology and biological psychiatry 52, 45–52.

Huizink, A.C., Mulder, E.J.H., 2006. Maternal smoking, drinking or cannabis use during pregnancy and neurobehavioral and cognitive functioning in human offspring. Neuroscience and biobehavioral review 30, 24–41.

Martin, B.R., Sim-Selley, L.J., Selley, D.E., 2004. Signaling pathways involved in the development of cannabinoid tolerance. Trends in pharmacological sciences 25, 325–330.

Moore, T.H.M., Zammit, S., Lingford-Hughes, A., Barnes, T.R.E., Jones, P.B., Burke, M., Lewis, G., 2007. Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. Lancet 370, 319–328.

Prini, P., Rusconi, F., Zamberletti, E., Gabaglio, M., Penna, F., Fasano, M., Battaglioli, E., Parolaro, D., Rubino, T., 2018. Adolescent △9-THC exposure in female rats

leads to cognitive deficits through a mechanism involving chromatin modifications in the prefrontal cortex. Journal of psychiatry and neuroscience 43, 87–101.

Radhakrishnan, R., Skosnik, P.D., Cortes-Briones, J., Sewell, R.A., Carbuto, M., Schnakenberg, A., Cahill, J., Bois, F., Gunduz-Bruce, H., Pittman, B., Ranganathan, M., D'Souza, D.C., 2015. GABA deficits enhance the psychotomimetic effects of Δ 9-△9-THC. Neuropsychopharmacology 40, 2047–2056.

Rios, C., Gomes, I., Devi, L.A., 2006. : opioid and CB1 cannabinoid receptor interactions: Reciprocal inhibition of receptor signaling and neuritogenesis. British journal of pharmacology 148, 387–395.

Saez, T.M.M., Aronne, M.P., Caltana, L., Brusco, A.H., 2014. Prenatal exposure to the CB1 and CB2 cannabinoid receptor agonist WIN 55,212-2 alters migration of early-born glutamatergic neurons and GABAergic interneurons in the rat cerebral cortex. Journa of neurochemistry 129, 637–648.

Steinberg, L., 2007. Risk taking in adolescence new perspectives from brain and behavioral science. Psychological sciences 16, 55–59.

Vaughan, P, J., Pike, C, J., Cotman, C, W., Cunningham, D, D. 1995. Thrombin receptor activation protects neurons and astrocytes from cell death produced by environmental insults. The journal of neuroscience, 15, 5389-5401.

Galloway, D.A., Laimins, L.A., Division, B., Hutchinson, F., 2016. Acute effects of smoked marijuana in marijuana smokers at clinical high risk of psychosis: A preliminary study. Psychiatric research, 257, 372-374.

Watson, S., Chambers, D., Hobbs, C., Doherty, P., Graham, A., 2008. The endocannabinoid receptor, CB1, is required for normal axonal growth and fasciculation. Molecular and cellular neuroscience 38, 89–97.

Wilkinson, S.T., Radhakrishnan, R., Haven, N., Mental, C., Haven, N., Haven, W., 2015. Impact of cannabis use on the development of psychotic disorders. Current addictive reports, 2, 115-128.

Zhou, D., Song, Z.H., 2001. CB1 cannabinoid receptor-mediated neurite remodeling in mouse neuroblastoma N1E-115 cells. Journal of neuroscience research.

APPENDIX A

Lists of Proteins Obtained from Proteomic Analysis Under Different Experimental

Conditions

Table A.1   List of differentially expressed proteins obtained from vehicle or Δ9-THC
treated non-behavior rats

Uniprot Accession	Fishers exact P-value	LOG2FC
TBA3_RAT	0.015	0.378511623
G3P_RAT	0.022	0.584962501
MBP_RAT	0.029	0.584962501
ATPB_RAT	0.035	0.485426827
ACON_RAT	0.072	0.485426827
ALBU_RAT	0.019	-0.152003093
DYN1_RAT	0.048	0.485426827
1433Z_RAT	0.065	0.485426827
F1MA36_RAT	0.0019	-0.321928095
VATB2_RAT	0.047	0.485426827
Q6T487_RAT	0.0015	-0.514573173
KCC2A_RAT	0.0035	0.765534746
DPYL4_RAT	0.04	0.678071905
CALR_RAT	0.059	-0.321928095
G3V741_RAT	0.039	-0.321928095
D4A0F5_RAT	0.059	-0.321928095
A0A0G2K5Q0_RAT	0.047	0.765534746
G3V9Y1_RAT	0.022	-0.514573173
Q6IMZ3_RAT	0.0055	1.201633861
DCE2_RAT	0.026	1
D3ZFQ8_RAT	0.039	-0.514573173
PP1R7_RAT	0.064	-0.514573173
F1LX07_RAT	0.061	-0.514573173
ROA1_RAT	0.056	-0.514573173
F1LMH0_RAT	0.057	-0.736965594
F1LN88_RAT	0.072	1.070389328
HPCL4_RAT	0.017	-0.736965594
A0A0G2K079_RAT	0.032	1.485426827
NDUAA_RAT	0.023	-1
A0A0A0MY13_RAT	0.032	1.378511623
RHOB_RAT	0.043	1
F1LPG3_RAT	0.0018	2.744161096
Q5RK08_RAT	0.061	1.263034406
WASF1_RAT	0.061	1.263034406

Table A.1 (continued)

A0A0G2JUT1_RAT	0.066	-0.736965594
RACK1_RAT	0.0039	-1.321928095
D3ZFX4_RAT	0.011	4.087462841
ADHX_RAT	0.038	-1
LIN7A_RAT	0.035	1.925999419
DC1I1_RAT	0.071	1.632268215
B0BNL2_RAT	0.038	-1.321928095
PRS6B_RAT	0.022	2.201633861
REEP5_RAT	0.0046	-1.736965594
D4AB17_RAT	0.0092	2.137503524
PENK_RAT	0.032	-1
DC1L1_RAT	0.0099	-2.321928095
F1M1Y0_RAT	0.035	2.070389328
USO1_RAT	0.0014	-4.321928095
A0A0G2K459_RAT	0.078	-3.321928095
F1M7X3_RAT	0.0099	-2.321928095
G3V8K2_RAT	0.066	-1
G3V8R2_RAT	0.0053	-2.321928095
YKT6_RAT	0.033	3.807354922
B4F7C7_RAT	0.0056	-3.836501268
G3V940_RAT	0.064	1.432959407
Q5EBD0_RAT	0.019	-1.321928095
6PGL_RAT	0.022	2.201633861
PSB3_RAT	0.021	-3.473931188
A0A0G2K3V4_RAT	0.071	-1
PSB1_RAT	0.0083	2.432959407
A0A0G2KA27_RAT	0.035	2.070389328
F1M013_RAT	0.041	-3.321928095
F1LM19_RAT	0.038	-1.321928095
TMM35_RAT	0.078	-3.321928095
A0A0G2K5N6_RAT	0.019	4
A0A0G2K6T9_RAT	0.018	-1.736965594
Q6P3V9_RAT	0.0099	-1.736965594
SFXN5_RAT	0.025	-1.321928095
PPM1E_RAT	0.021	-3.64385619
UBA5_RAT	0.058	-1.736965594

Table A.1 (continued)

AGAP2_RAT	0.033	3.700439718
A0A0G2JY03_RAT	0.033	-1.736965594
RCN2_RAT	0.0051	2.5360529
Q5RJN0_RAT	0.043	-1.321928095
RD23B_RAT	0.033	-1.736965594
MPI_RAT	0.078	-3.321928095
NTF2_RAT	0.033	-1.736965594
PRIO_RAT	0.041	-3.321928095
PSMD1_RAT	0.056	1.925999419
D4A5X7_RAT	0.058	-1.321928095
PHYIP_RAT	0.023	-1.321928095
D3ZT36_RAT	0.033	3.700439718
ZWINT_RAT	0.078	-3.321928095
CHRD1_RAT	0.033	3.700439718
AL7A1_RAT	0.055	3.584962501
TXTP_RAT	0.033	-1.736965594
G3V7Q6_RAT	0.055	3.584962501
G3V7U4_RAT	0.071	-1.321928095
G3V7I8_RAT	0.055	3.584962501
O88321_RAT	0.055	3.584962501
PSB2_RAT	0.078	-3.321928095
D3ZRM9_RAT	0.041	-3.473931188
M0RC57_RAT	0.011	4.087462841
A0A0G2JT00_RAT	0.055	3.584962501
sp|P62243|RS8_RAT	0.041	-3.321928095
G3V914_RAT	0.043	-1.736965594
G3V912_RAT	0.033	3.700439718
PA2G4_RAT	0.011	-3.836501268
A0A0G2K724_RAT	0.021	-3.473931188
B2RYS8_RAT	0.078	-3.321928095
RLA1_RAT	0.078	-3.321928095
RUVB1_RAT	0.055	3.584962501
B1H2A2_RAT	0.078	-3.321928095
RLA0_RAT	0.021	-3.473931188
D3Z9G9_RAT	0.041	-3.321928095
D3ZM33_RAT	0.078	-3.321928095

Table A.1 (continued)

A0A0G2K526_RAT	0.056	2.201633861
M0R7P0_RAT	0.078	-3.321928095
Q505I9_RAT	0.078	-3.321928095

Table A.2   List of differentially expressed proteins obtained from vehicle or Δ9-THC treated behavior rats

Uniprot Accession	Fishers Exact P-Value	LOG2FC
KCC2A_RAT	0.001	0.736965594
D4A8U7_RAT	0.00074	-1
F1LUE2_RAT [5]	0.0074	0.736965594
NDUS1_RAT	0.034	0.584962501
Q5XIH3_RAT	0.0082	1
D3ZA84_RAT	0.014	-1
CALX_RAT	0.042	0.678071905
A0A0G2K9J2_RAT	0.018	-1
KCC2D_RAT	0.013	0.736965594
G3V9Y1_RAT	0.0091	0.925999419
VDAC2_RAT	0.0032	1.070389328
NFM_RAT	0.0076	1.201633861
LSAMP_RAT	0.039	-1
A0A0G2JZM8_RAT	0.025	-1
UCRI_RAT	0.023	1.137503524
Q5M7T6_RAT	0.00042	1.736965594
PALM_RAT	0.0093	1.378511623
RACK1_RAT	0.023	1.137503524
PPR1B_RAT	0.02	1.5360529
GRB2_RAT	0.04	1.736965594
A0A0G2K8V2_RAT (+2)	0.046	1.321928095
PDE1B_RAT	0.011	2.201633861
CALB1_RAT	0.0068	1.632268215
A0A0G2JSW0_RAT [2]	0.044	1.137503524
A0A0G2K261_RAT	0.034	3.584962501
ACYP2_RAT	0.034	3.584962501
A0A0G2K3I9_RAT	0.012	2.321928095
RAP2B_RAT [2]	0.02	1.5360529
G3V7L6_RAT (+1)	0.03	1.736965594

Table A.2 (continued)

FUBP1_RAT	0.01	3.906890596
NDUV2_RAT	0.025	1.378511623
ERR1_RAT	0.0018	4.321928095
I6L9G6_RAT	0.033	1.321928095
AGAP2_RAT	0.036	3.473931188
G3V7Q6_RAT	0.019	3.700439718
NDUS2_RAT	0.0056	4.087462841
KCC4_RAT	0.02	1.736965594
ACBP_RAT [2]	0.02	3.836501268
A0A0H2UI10_RAT	0.036	-3.64385619
B2GUY4_RAT (+1)	0.034	3.584962501
A0A0G2K0P8_RAT	0.034	3.584962501
Q6PDW1_RAT	0.036	3.473931188
D3Z955_RAT	0.02	3.836501268
TOM34_RAT	0.036	-3.64385619
sp|K22E_HUMAN|	0.0014	2.321928095
M0R809_RAT	0.034	3.584962501
G3V964_RAT	0.0061	4.058893689
M0R4B6_RAT (+1)	0.018	2.070389328
A0A0G2KAZ7_RAT	0.019	3.700439718
GNA13_RAT	0.03	1.736965594
G3V8H3_RAT	0.036	-3.64385619

Table A.3   List of differentially expressed proteins obtained from Δ9-THC treated behavior or non-behavior rats

Uniprot Accession	Fishers Exact P-Values	LOG2FC
D4ABN3_RAT	0.0043	0.678071905
GPM6A_RAT	0.0017	0.925999419
D3ZDU5_RAT	0.03	0.765534746
PCLO_RAT	0.038	1
PGRC1_RAT	0.016	0.925999419
NFM_RAT	0.0082	1.263034406
A0A0G2JZH8_RAT	0.0087	1.321928095
A0A0G2JUT1_RAT	0.049	0.925999419
PCYOX_RAT	0.0052	1.5360529
F1LRZ7_RAT	0.041	2.03562391
D4A435_RAT	0.021	1.722466024
CX6C2_RAT	0.024	3.700439718

THIC_RAT	0.002	2.560714954
A0A0G2KAW4_RAT	0.0052	2.232660757
RACK1_RAT	0.013	1.321928095
B4F774_RAT	0.021	1.632268215
CSKI1_RAT	0.025	1.378511623
F1LV13_RAT	0.041	1.5360529
B0BNL2_RAT	0.014	1.5360529
ELOB_RAT	0.014	3.906890596
RTCB_RAT	0.014	3.906890596
A0A0G2K926_RAT	0.0004	4.584962501
sp|P62909|RS3_RAT	0.014	3.906890596
B1WC34_RAT	0.0055	2
F1LP57_RAT	0.015	1.847996907
PSA2_RAT	0.024	1.584962501
Q5EBD0_RAT	0.014	1.5360529
CDS2_RAT	0.0089	2.10433666
D3ZWA8_RAT	0.0052	2.201633861
SCAM1_RAT	0.025	2.292781749
D4A781_RAT	0.0044	4.169925001
PPM1E_RAT	0.0013	4.392317423
LASP1_RAT	0.041	1.722466024
B4F7C7_RAT	0.0078	4
A0A0G2K6T9_RAT	0.0052	2.232660757
G3V6L9_RAT	0.014	3.906890596
DNJA2_RAT	0.0089	2.10433666
M0R735_RAT	0.0024	4.247927513
A0A0G2K0V8_RAT	0.024	3.700439718
A0A0H2UHF6_RAT	0.025	1.847996907
D3ZDC0_RAT	0.0044	4.169925001
CSRP1_RAT	0.014	3.906890596
ZWINT_RAT	0.043	3.584962501
ACYP2_RAT	0.043	3.584962501
A0A0G2JZB8_RAT	0.014	3.906890596
A0A0G2K7W7_RAT	0.024	3.700439718
FUBP1_RAT	0.014	3.807354922
Q6P3V9_RAT	0.025	1.722466024
SCN2B_RAT	0.024	3.700439718
MPI_RAT	0.024	3.700439718
A0A0G2K0P8_RAT	0.043	3.584962501
PRRT2_RAT	0.014	3.906890596
UK114_RAT	0.041	2.137503524
LYPA2_RAT	0.043	3.459431619

Table A.3 (continued)

SGT1_RAT	0.043	3.459431619
PRIO_RAT	0.014	3.906890596
F1LNE4_RAT	0.037	1.378511623
NPTX1_RAT	0.024	3.700439718
AP1M1_RAT	0.043	3.459431619
M0R907_RAT	0.043	3.459431619
sp|P21531|RL3_RAT	0.0078	4
A0A0G2JVS2_RAT	0.043	3.459431619
M0R809_RAT	0.043	3.459431619
RAB4B_RAT	0.00022	4.700439718

Table A.4   List of differentially expressed proteins obtained from Δ9-THC treated behavior or non-behavior rats

Uniprot Accession	Fishers Exact P-Values	LOG2FC
F1LNK0_RAT	0.033	0.584962501
M0R8M9_RAT	0.022	0.584962501
D4A8U7_RAT	0.0016	1.263034406
Q499Q4_RAT	0.036	1.201633861
RUFY3_RAT	0.048	1.070389328
ERR1_RAT	0.0056	4.321928095
G3V6L4_RAT	0.029	1.5360529
D3ZHY9_RAT	0.0051	1.765534746
A0A0G2K4X8_RAT	0.02	1.584962501
A0A0G2JSZ5_RAT	0.024	1.432959407
PACS1_RAT	0.0077	2.5360529
Q5U2P5_RAT	0.048	1.807354922
I6L9G6_RAT	0.047	1.722466024
Q5M9H7_RAT	0.032	1.847996907
G3V6T1_RAT	0.047	2.070389328
LASP1_RAT	0.0094	4.247927513
PCYOX_RAT	0.0038	2.40599236
Q5U2S7_RAT	0.047	1.925999419
PRS7_RAT	0.03	2.201633861
A0A0G2KB98_RAT	0.016	4.087462841
ELOB_RAT	0.047	1.925999419
RTCB_RAT	0.042	3.700439718
LYAG_RAT	0.016	4.087462841

Table A.4 (continued)

M0R735_RAT	0.047	2.070389328
A0A0G2K5N6_RAT	0.0056	4.321928095
A0A0G2K7W7_RAT	0.032	1.925999419
CSRP1_RAT	0.042	3.700439718
PSMD1_RAT	0.047	2.070389328
O88321_RAT	0.042	3.700439718
PSB1_RAT	0.047	2.070389328
B5DFN4_RAT	0.042	3.807354922
D3ZWA8_RAT	0.026	3.906890596
PPM1H_RAT	0.042	3.807354922
RCN2_RAT	0.03	2.201633861
D3ZPR0_RAT	0.042	3.700439718
D3Z955_RAT	0.042	3.807354922
A0A0G2JT00_RAT	0.026	3.906890596
D3Z981_RAT	0.016	4.087462841