

Elucidation of the mechanism of learning and memory deficits in adolescent offspring due to prenatal cannabinoid exposure

by

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Abstract

Cannabis is now one of the most commonly used illicit substances among pregnant and lactating women. This is particularly concerning given that developmental exposure to cannabinoids has been demonstrated to induce enduring neurofunctional and cognitive alterations in clinical studies, yet there is a relative paucity of preclinical literature investigating the underlying neurocognitive alterations resulting from prenatal exposure to cannabinoids. In the current study, we provide a mechanistic evidence on how learning and memory deficits result from prenatal cannabinoid exposure (PCE) in adolescent offspring. We have investigated the effect of PCE on hippocampal and cerebellar function in a pregnant rodent model. PCE induced deficits in hippocampal-dependent memory tasks in adolescent offspring, and these behavioral deficits were associated with decreased long-term potentiation (LTP) and enhanced long-term depression (LTD) at hippocampal Schaffer collateral-CA1 synapses, as well as an imbalance between GluN2A- and GluN2B- mediated signaling. Moreover, PCE reduced gene and protein expression of neural cell adhesion molecule (NCAM) and polysialylated-NCAM (PSA-NCAM), which is critical for GluN2A and GluN2B signaling balance. Restoration of PSA-NCAM activity restored the LTP deficits observed in PCE animals, suggesting PSA-NCAM mediated alterations in GluN2A- and GluN2B- signaling pathways are responsible for the altered synaptic plasticity in hippocampus resulting from PCE. Interestingly, PCE has shown to exert an anti-apoptotic effect on the cerebellum by decreasing oxidative stress markers and nitrite content. Our study indicates that effects of PCE on the cerebellum are unique compared to other brain regions by enhancing mitochondrial complex I and complex IV function to promote neuronal survival and reducing the level of pro-apoptotic factor caspase 3 and apoptosis associated signaling. These

findings enhance the current understanding of how PCE affects cognition and neuronal survival, and how this process can be manipulated for future therapeutic purposes.

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Chapter 1: Introduction to Dissertation

Marijuana is one of the most commonly used illicit drugs worldwide. According to the ‘National Center for Drug Abuse Statistics’ 188 million people worldwide used marijuana in 2019, 55 million of which are from the USA (“Marijuana Addiction: Rates & Usage Statistics – NCDAS,” 2019). Marijuana use during pregnancy is also rising because of the popular belief that morning sickness can be alleviated by marijuana during pregnancy. 1.5% of pregnant women used marijuana daily in 2018 while 4.7% of pregnant women used it occasionally (Hennessy, 2018). In addition, use of synthetic cannabinoids is increasing, especially among adolescents and young adults due to increased availability and because synthetic cannabinoids are undetectable in routine drug screenings. Synthetic cannabinoids bind to the same receptors in the brain as marijuana: cannabinoid receptor type 1 (CB1R) and cannabinoid receptor type 2 (CB2R) but they are more potent and harmful as compared to natural marijuana due to several differences in activity, chemical structure, and concentration in the commercially available products (Alipour, Patel, Shabbir, & Gabrielson, 2019; Cohen & Weinstein, 2018). Although it is evident from human studies that the use of marijuana during pregnancy have adverse behavioral effects on offspring including deficits in attention and executive function, still marijuana use rate among pregnant women is increasing worldwide. Various aspects of human behavior such as emotion, learning, and memory have been reported to be impaired due to prenatal cannabinoid exposure (PCE). Glutamate, being the major excitatory neurotransmitter in the central nervous system is implicated in the majority of these behavioral abnormalities. Especially, alteration in the glutamatergic system during developmental period can contribute to alteration in other neurotransmitter systems as well. Thus, the exploration of glutamatergic changes in response to PCE is crucial to develop an understanding of how homeostatic imbalance of neurotransmitter

systems occurs causing various long-term neurobehavioral deficits. Based on evidence from the current literature, most of these vast alterations to the neurotransmitter systems following PCE, are brain region-specific, time-dependent, and sexually dimorphic. In this project, we have investigated the effect of PCE on two of the vital brain regions: hippocampus and cerebellum. Located in the medial temporal lobes, the hippocampus is one of the most studied neuronal systems in the brain due to its importance in learning and memory formation (Bird & Burgess, 2008). Cerebellum located in the back of the skull, containing around 50 % of all neurons, control balance, motor activities, and coordination of voluntary movements (De Zeeuw & Ten Brinke, 2015). Using drugs of abuse during gestational period have a long-lasting effect both on hippocampal and cerebellar functions as psychoactive drugs can modulate various neurotransmitter system, and their components: receptors, transporters, and uptake (B. L. Thompson, Levitt, & Stanwood, 2009). PCE has shown to modulate several neurotransmitter systems: glutamate, GABA, serotonin, dopamine, and adrenergic (Pinky et al., 2019). Cannabinoid exerts its effect by binding with Cannabinoid type I (CB1R) and cannabinoid type II (CB2R) receptor. CB1R is present in abundance in both hippocampus and cerebellum and predominantly controls cognition, memory, emotion (Moreira & Lutz, 2008; Wotjak, 2005), and motor function (Chaouloff, Dubreucq, Bellocchio, & Marsicano, 2011). Following this, we examined how gestational cannabinoid exposure influences glutamate-mediated learning and memory via CB1R. The objective of this project was to dissect the molecular mechanisms by which PCE induces synaptic plasticity and cognitive deficits in the hippocampus as well as how cannabinoid affects the cerebellar function. We have used a multidisciplinary approach including behavioral, electrochemical, electrophysiological, cellular, and molecular methodologies to test our hypotheses. In the Chapter 2 literature review, we aim to provide a summary of observed

changes to glutamatergic neurotransmission related learning and memory following cannabinoid exposure during pregnancy and to draw possible correlations to reported behavioral and synaptic plasticity alterations in affected offspring. In the 3rd chapter, we identified that PCE causes hippocampus based spatial memory deficits during adolescent period along with synaptic plasticity deficits. Furthermore, we established a signaling mechanism that could be responsible for the observed cognitive deficits. Although the effect of PCE on hippocampus-based learning and memory has been somewhat well studied, how PCE affects the cerebellar function is still unknown. In the 4th chapter, we investigated the effect of PCE on the cerebellum and observed that PCE rather exerts a protective role on the cerebellum. PCE reduced the oxidative markers while increasing mitochondrial complex activity. PCE also reduced the level of various proteins that plays an important role in apoptosis while increasing the expression level of proteins that act as anti-apoptotic. Thus, we observed the effect of PCE could be brain region specific.

Currently, there are no therapeutic options to treat the hippocampus based cognitive deficits associated with PCE. We proposed the possible use of a molecule that has been shown to ameliorate memory deficits. Moreover, we also provided a unique perspective of PCE on the cerebellum. Thus, this study fills an important gap and provides more support to the existing body of evidence on the effects of PCE on glutamate-mediated neurotransmission.

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Chapter 2: Review on effect of prenatal cannabinoid exposure on adolescent offspring

1. Introduction

Marijuana is the most commonly abused illicit drug by women (Azofeifa, Mattson, Schauer, et al., 2016; Fantasia, 2017; Metz & Stickrath, 2015), and marijuana use is associated with higher rates of cigarette smoking, alcohol intake, and use of other illicit drugs (Grant, Petroff, Isoherranen, Stella, & Burbacher, 2018; Gunn et al., 2016; Noland et al., 2003; Schauer & Peters, 2018; Secades-Villa, Garcia-Rodríguez, Jin, Wang, & Blanco, 2015). Prenatal exposure to drugs, including nicotine and ethanol, have been associated with a number of negative consequences (Cross, Linker, & Leslie, 2017; Healy, Le Noury, & Mangin, 2016; Holbrook, 2016; M. M. Martin, Graham, McCarthy, Bhide, & Stanwood, 2016; Pennell, 2018). However, the rate of marijuana use by young, pregnant women has been steadily increasing in recent years (Young-Wolff et al., 2017). It is unclear why usage would increase despite the known ill-effects of fetal drug exposure, but studies show 1 in 25 women report marijuana use during pregnancy, and surprisingly 70% of pregnant women are of the opinion that there is little to no risk of harm associated with once or twice weekly marijuana use (Ko, Farr, Tong, Creanga, & Callaghan, 2015). This disconnect between the known untoward, long-term effects of marijuana use and the opinions of those likely to use it during pregnancy may be due to factors such as decreased social stigmatization coupled to increased availability of marijuana in the marketplace, increased prescribing of medical marijuana by physicians, self-medication during pregnancy due to gestational-induced nausea and vomiting, and overall decriminalization of marijuana use in many states (Azofeifa et al., 2016a, 2016b, Niemeijer et al., 2014, Grant et al., 2018; NIDA, 2017; Sobrian, 2016). Furthermore, those who smoke marijuana during pregnancy are also more likely

to be of lower socioeconomic status, have a lower level of education, and not seek out appropriate prenatal care (El-Mohandes et al., 2003; Ko et al., 2015).

Advising women to abstain from psychoactive drug use during pregnancy is the standard of care and should be considered best practice for prevention of negative pregnancy outcomes and reduction of post-natal complications and disease development in the offspring. Furthermore, cannabis use by the mother can impair the mother's attention and judgement while taking care of the baby, leading to unsafe conditions (Jansson, Jordan, & Velez, 2018). To date, clinical literature on the topic is dominated by data from two large longitudinal studies of PCE in humans, the Ottawa Prenatal Prospective Study (OPPS) and the Maternal Health Practices and Child Development Project (MHPCDP) (Campolongo et al., 2009; Fried, 2002b; Fried et al., 1998; Fried and Watkinson, 1990; Goldschmidt et al., 2012; Trezza et al., 2008). Additionally, recently published results from another large longitudinal study conducted in the Netherlands add more current insights to this body of literature from a different population (El Marroun et al., 2018). While attributing the findings of these studies specifically to prenatal marijuana exposure is complicated by concomitant cigarette and alcohol use, they nonetheless provide valuable insight on neurodevelopmental and behavioral disorders commonly observed in offspring of mothers who used marijuana during pregnancy. Findings from these studies indicate that PCE results in detectable neurobehavioral changes and executive functioning deficits in offspring that persist into later stages of brain development during childhood and adolescence. Additionally, affected offspring show variable, age-dependent deficits in attentiveness and cognition, as well as higher rates of hyperactivity and use of marijuana themselves during adolescence (Day, Goldschmidt, & Thomas, 2006; Lidush Goldschmidt et al., 2012). Although PCE has been

associated with neurobehavioral effects, it is not clear whether teratogenicity can also result in due to PCE (Orsolini et al., 2017). Hence, there remains a dire need for further exploration of the neurobiological mechanisms underlying behavioral and cognitive deficits caused by cannabinoid exposure during fetal development.

In this review, we provide a summary of literature reporting changes to glutamatergic systems following cannabinoid exposure during pregnancy and draw possible correlations as to how gestational alterations in neuroplasticity manifest as behavioral dysfunction in affected offspring. A comprehensive understanding of alteration in glutamatergic regulation in response to PCE also includes recognizing many of these alterations within the brain are region specific, time-dependent, and sexually dimorphic.

2. Human pharmacology and pathophysiology

Delta-9-tetrahydrocannabinol (THC) is the primary psychoactive substance associated with marijuana abuse (Wilkinson, Yarnell, Radhakrishnan, Ball, & D'Souza, 2016). There are several ways of THC consumption either by vaping, smoking or eating edibles and the absorption and metabolism of THC greatly depend on its route of delivery. The oral LD₅₀ of THC in rats is reported to be 800–1900 mg/kg, depending on formulation, strain and sex (G. R. Thompson, Rosenkrantz, Schaeppi, & Braude, 1973) and the intragastric LD₅₀ with the emulsion was 800 mg/kg and 1270 mg/kg while with the sesame oil. LD₅₀ for intravenous administration was 36–40 mg/kg, which is similar to the inhalation dose (Rosenkrantz, Heyman, & Braude, 1974). THC when administered orally undergoes first pass metabolism in the liver and metabolized into 11-hydroxy- Δ^9 -THC (THC-OH). It then further undergoes oxidation to the inactive 11-nor-9-

carboxy- Δ^9 -THC (THC-COOH) (Huestis, 2007). THC, being a lipophilic compound, can enter highly vascularized tissues like the liver, and tends to accumulate in body fat for days to weeks. THC metabolites enters enterohepatic recirculation and finally excretes through urine and feces and a small amount through sweat and saliva (Huestis, 2007). THC has regularly served as a basis for the development of synthetic cannabinoids used in both clinical and recreational settings. Additionally, selective breeding and cultivation has led to a four-fold increase in the average THC content of confiscated cannabis-family specimens over the past twenty-five years (ElSohly et al., 2000; ElSohly et al., 2016; Potter et al., 2008). Though information regarding human placental penetration of THC is relatively scarce (Marchetti, Di Masi, Cittadini, La Monaca, & De Giovanni, 2017), THC being a lipophilic psychoactive compound readily crosses the blood placenta barrier (Asch & Smith, 1986; Bailey, Cunny, Paule, & Slikker, 1987a; Freudenthal, Martin, & Wall, 1972; Vardaris, Weisz, Fazel, & Rawitch, 1976). Also, THC is only one of over one-hundred chemical compounds found within cultivated cannabis plants, which complicates the isolation of effects attributable to a single component (Wilkinson et al., 2016). Combining aforementioned medical, legal, and psychosocial factors, a steady increase in THC content of cannabis family plants, and a reasonable assumption of human placental pharmacokinetics, it is safe to say that the risk of PCE is growing through a combination of complex biologic and sociologic elements.

PCE results in low birth weight, preterm labor, intra uterine growth retardation and even still birth. PCE can also increase the admission rate to NICUs due to asphyxia or infection (Hayatbakhsh et al., 2012; Varner et al., 2014). However, some reports has shown no such effects of PCE on birth outcomes (Fergusson, Horwood, & Northstone, 2002; Huizink, 2014; Mark, Desai, & Terplan, 2016; van Gelder et al., 2010) which might be because often the

postnatal effect of PCE is often subtle based on the dose and duration of usage and only very high dose might result in an immediate overt neurodevelopmental phenotype (K. A. Richardson, Hester, & McLemore, 2016). Hence, PCE often exerts a long term effect as observed in the Ottawa Prenatal Prospective Study (OPPS) study, such as increased hyperactivity and impulsivity, decreased attention deficits during infancy and decrease concentration and verbal reasoning as well as decreased activity in various brain regions during adolescent period.

The endocannabinoid system, described in more detail below, remains the pharmacologic target for THC in both maternal and fetal nervous systems. However, due to variability in the function of this system in the mature versus developing brain, long-term implications of cannabinoid exposure to the fetus are presumably more impactful than long-term effects on the mother. Much of what is known regarding human-specific effects of PCE is limited to *ex vivo* studies comparing brain tissue from aborted fetuses with and without *in utero* cannabis exposure (Morris et al., 2011). Interestingly, the investigation of these samples elucidates an association between marijuana use in pregnancy and a quantifiable change in fetal neuronal physiology. For example, expression of dopamine receptor 2 (D2) mRNA in the amygdala and mesolimbic nucleus accumbens, but not the dorsal striatum, is inversely correlated with maternal marijuana use. This change is not attenuated after adjustment for simultaneous alcohol and nicotine exposure. Although it has been suggested that this change could be explained by a genetic predisposition for marijuana use in the mother, this finding has been replicated in a more controlled setting using prenatally exposed rat models (DiNieri et al., 2011; Xinyu Wang, Dow-Edwards, Anderson, Minkoff, & Hurd, 2004).

The clinical significance of these findings is multifactorial. D2 expression often seems to be altered having a potential role in substance abuse and addiction in humans. Altered D2 level is also correlated with changes in dopaminergic signaling within the striatum (Gorwood et al., 2012; J. D. Jones & Comer, n.d.; Kenny, Voren, & Johnson, 2013; Trifilieff & Martinez, 2014). Additionally, genetic alterations in the D2 receptor and dopaminergic signaling are established risk factors for various neurobehavioral pathologies including attention deficit hyperactive disorder, antisocial behavior, pathological gambling, and sex addiction (Farré et al., 2015; Gold, Blum, Oscar-Berman, & Braverman, 2014; Yau & Potenza, 2015). Alteration in the level of D2 receptor expression is also evident in prenatal alcohol and nicotine exposure, where the effects of marijuana use can simultaneously be masked and exacerbated by co-administration of these substances (Nutt, Lingford-Hughes, Erritzoe, & Stokes, 2015). However, since exposure to nicotine and alcohol were controlled for within the various groups of fetal brain samples, it appears that marijuana exposure independently alters dopaminergic receptors in certain brain areas. Thus, the behavioral phenotypes observed in the OPPS and MHPCDP studies, which also controlled for co-utilization of nicotine and alcohol, may be in part due to alterations in dopaminergic neurotransmission (DiNieri et al., 2011; Xinyu Wang et al., 2004). However, it is important to note that only male subjects exhibited these effects on the amygdala, which is consistent with other trends of sexual dimorphism observed in various human and animal studies (M. E. Carroll & Smethells, 2015; Jutras-Aswad, DiNieri, Harkany, & Hurd, 2009; Sanchis-Segura & Becker, 2016). Additionally, expression of dopamine receptor 1 (D1) in the striatum, a receptor strongly implicated in motor function, reward, and cognition (Nishi, Kuroiwa, & Shuto, 2011), remained unaltered in the tissue samples used for these studies. Regardless, it is

anticipated that early-life, region-dependent modification of dopaminergic signaling will have long-standing impacts on these and other areas of the brain.

PCE has not only been linked to alterations in dopaminergic neurophysiology, but also to effects on the endogenous opioid system in human fetuses. Exposure results in increased expression of mu opioid receptors in the amygdala and decreased expression of kappa opioid receptors in portions of the thalamus (X Wang, Dow-Edwards, Anderson, Minkoff, & Hurd, 2006). In terms of the impact of opioid and dopaminergic systems on the propensity for substance abuse, habit formation, and addiction, the involvement of both systems indicates a significant risk additive to the more immediate neurobehavioral changes observed throughout human development. Because changes to these two systems could potentially increase an offspring's risk for future drug abuse, the implications of PCE may introduce a generational effect where the offspring of mothers who smoke marijuana during pregnancy are more likely to expose themselves and their offspring to marijuana and other drugs of abuse during gestation. This is at least partially supported by the observation that prenatally exposed adolescents begin using marijuana earlier in life and with a higher frequency than their non-exposed counterparts (Day et al., 2006). MRI study of 10-14 year old children with prenatal exposure to drugs of abuse demonstrates reduced cortical gray matter was reduced in those who were exposed to cannabis with or without other drugs as well (Rivkin et al., 2008). While gestational exposure to both cocaine and marijuana caused severe impairment in frontal white matter development in 10 years old children which was worse than gestational exposure to cocaine alone (Warner et al., 2006). fMRI study on 18-22 years old revealed a significant increase in prefrontal cortex activity with decreased left cerebellar activity associated with increased errors of commission while performing Go/No-Go task (Smith, Fried,

Hogan, & Cameron, 2004a). In the same study group, with increasing amount of prenatal cannabis use, increased activity in the left inferior and middle frontal gyri, left para hippocampal gyrus, left middle occipital gyrus and left cerebellum has been observed resulting in visuo-spatial memory deficits (Smith, Fried, Hogan, & Cameron, 2006). Additionally, cannabis use as early adolescence can also result in a disproportionate ratio in the cortical white and gray matter. Interestingly, people in this group has also short body stature and less body weight (W. Wilson et al., 2000).

There have been few studies in prenatally exposed human fetal brains to elucidate changes to other neurotransmitter systems. Although alterations in learning and memory are exhibited by offspring prenatally exposed to cannabinoids, the glutamatergic system has not been studied in human fetal brains to our knowledge. This review will briefly discuss the role of glutamate in learning and memory and how it is controlled by endocannabinoid system as well as altered in case of exogenous cannabinoid exposure.

3. Overview of the endocannabinoid system

3.1 Endogenous cannabinoids

The endocannabinoid system is comprised of endogenous cannabinoids, enzymes that synthesize and degrade these cannabinoids, cannabinoid receptors, and their interacting proteins (Lu & Mackie, 2016). Endocannabinoids are endogenous lipids derived from polyunsaturated fatty acids, namely arachidonic acid. Of the various endocannabinoids, arachidonyl ethanolamine

(AEA) was first discovered in the porcine brain in 1992 (Devane et al., 1992), and the presence of 2-arachidonoylglycerol (2-AG) in the rat brain was first reported in 1995 (Sugiura et al., 1995). Unlike classical neurotransmitters and neuropeptides, endocannabinoids are not synthesized and stored into synaptic vesicles. Instead, these unique lipid derivatives are produced on demand in the plasma membrane by receptor-stimulated enzymatic cleavage of their associated lipid precursors. Following influx of calcium through voltage-gated calcium channels and depolarization of the postsynaptic cell, endocannabinoids are synthesized and released into the extracellular space. Anandamide and AEA are produced by hydrolysis of the phospholipid precursor *N*-arachidonoyl phosphatidylethanolamine (PE) (Di Marzo et al., 1994; Freund et al., 2003), whereas 2-AG is produced from 1,2-diacylglycerol (DAG), which is cleaved from phosphatidylinositol (PI) (Farooqui, Rammohan, & Horrocks, 1989; Piomelli, 2003). Endocannabinoids travel to the presynaptic nerve terminal and exhibit retrograde signaling upon binding to cannabinoid receptors (Basavarajappa, 2007). Through this retrograde signaling, endocannabinoids are responsible for depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) at GABAergic and glutamatergic synapses, respectively (Diana & Marty, 2004). Stated simply, depolarization of the post-synaptic neuron leads to calcium-dependent endocannabinoid release, and the resultant activation of pre-synaptic CB1Rs leads to reduced neurotransmitter release. Termination of cannabinoid signaling may occur via one of two mechanisms, reuptake or degradation. However, the existence of a specific endocannabinoid transporter for the reuptake of endocannabinoids is debated (Fowler, 2013; Jhaveri, Richardson, & Chapman, 2007), as it is not clear whether reuptake is mediated by a specific cannabinoid transporter or by carrier mediated facilitated diffusion (Giuffrida, Beltramo, & Piomelli, 2001; Hillard, Edgmond, Jarrahian, & Campbell, 1997). Both of the

endocannabinoids are degraded via enzymatic hydrolysis (M Bouaboula et al., 1995), and the enzymes responsible for endocannabinoid degradation are monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), which hydrolyze 2-AG and AEA, respectively (Castillo, Younts, Chávez, & Hashimoto, 2012).

3.2 Cannabinoid receptors

Endocannabinoid signaling is involved in various physiologic processes which include synaptic function modulation in the central nervous system, analgesia, vasoregulation, thermoregulation, inflammation, and peripheral lipid and glucose homeostasis (Rodríguez de Fonseca et al., 2005). To date, there are two cannabinoid receptors that are well established in the literature, though some studies suggest more receptors may exist (A. J. Brown, 2007; Kreitzer & Stella, 2009; Q. Shi et al., 2017). The discovery of the two first receptors occurred over two decades ago, with CB1Rs first discovered in the brain in 1990 (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990), and CB2Rs discovered in immune cells in 1993 (Munro, Thomas, & Abu-Shaar, 1993). These two cannabinoid receptors are the part of the canonical endocannabinoid system along with AEA and 2-AG (Iannotti, Di Marzo, & Petrosino, 2016). Both CB1R and CB2R are G-protein coupled receptors (GPCRs) that retain approximately 44% amino acid homology in humans (Onaivi et al., 2002). Additionally, the intrinsic efficacy of endogenous cannabinoids at these receptors varies. 2-AG is a high-efficacy agonist for both CB1R and CB2R, whereas AEA is a low-efficacy agonist at CB1R and a very low-efficacy agonist at CB2R (Luk et al., 2004).

In humans, CB1R consists of 472 amino acid and is encoded by the gene CNR1. Besides the typical structure of CB1R, In addition to the canonical long form of the CB1R, two additional isoforms with shorter N-terminus can be found created by alternative splicing known as hCB1a (Shire et al., 1995) and hCB1b (Erik Ryberg et al., 2005). While hCB1a can be predominantly found in the brain and skeletal muscle along with full length CB1R, the CB1Rb shows a higher expression level in the liver and pancreatic islet cells(Zou & Kumar, 2018). CB1Rs are highly expressed in both excitatory and inhibitory presynaptic terminals and are most abundant in the hippocampus, cortex, basal ganglia, and cerebellum (reviewed by Mackie, 2005; Svíženská et al., 2008).

CB1Rs has an important role in the modulation of activity-dependent neuronal function, plasticity, and neurogenesis (Castillo et al., 2012). They are also implicated in numerous other functions such as cognition, memory, emotion (Moreira & Lutz, 2008; Wotjak, 2005), and motor function (Chaouloff et al., 2011). CB1Rs can be considered essential in shaping emotional response as they are extensively involved in fear memory extinction processes (Akirav, 2011; Lutz, 2009). Thus, CB1R agonists can act as potential anxiolytics and anti-depressants (Marco et al., 2011; Trezza & Campolongo, 2013). Although previously CB2Rs are thought to mediate peripheral immune activity through regulation of immune cell migration and cytokine release (Cabral & Griffin-Thomas, 2009), but the role of CB2Rs in the brain is an emerging topic. In humans, CB2R is consist of 360 amino acid and is encoded by the gene CNR2. Like CB1R, two isoforms of CB2R have been identified in humans, with CB2a predominantly expressed in testis and brain, whereas the CB2b is mainly expressed in peripheral tissues such as spleen, muscle and leukocytes (Q.-R. Liu et al., 2009). In brain, CB2R has been implicated in the maintenance of

hippocampal contextual and spatial memory (Yong Li & Kim, 2016a), as well as GABA-dependent consolidation of short term and long term memory (Nasehi, Gerami-Majd, Khakpai, & Zarrindast, 2018). CB2R dependent mTOR signaling as shown to facilitate memory consolidation in hippocampus in presence of endocannabinoids (Ratano et al., 2018). Though CB2R expression is typically low in the healthy brain (Y. Li & Kim, 2015), certain neuroinflammatory diseases exhibit an increase in CB2R expression in both neuronal and glial cells (Aso & Ferrer, 2016; Kruk-Slomka, Dzik, Budzynska, & Biala, 2017). Some of the functions of CB2Rs in CNS include neuronal progenitor cell proliferation (Palazuelos, Ortega, Díaz-Alonso, Guzmán, & Galve-Roperh, 2012), axon guidance (Duff et al., 2013), and synaptic transmission (Yong Li & Kim, 2016b). CB2Rs may act as neuroprotective in Alzheimer's disease, Huntington's chorea, and amyotrophic lateral sclerosis by modulating central astrocytic and microglial proliferation and migration (Fernández-Ruiz, Pazos, García-Arencibia, Sagredo, & Ramos, 2008).

Recent emerging evidence suggests the existence of a third novel cannabinoid receptor which is known as The G protein-coupled receptor 55 or GPR55. In rat, GPR55 mRNA is predominantly expressed in found in the adrenals, parts of the gastrointestinal tract i.e. ileum and jejunam, and in the CNS. Although the levels of GPR55 in frontal cortex, striatum, hippocampus, cerebellum, hypothalamus and brain stem were much lower than CB1R level in those brain regions. CBGPR55 is activated by the Δ^9 -THC as well as by the endocannabinoids and certain cannabinoid agonists (Q.-R. Liu et al., 2009). Pharmacologic effects of GPR55 has been observed in case of pain, inflammation (Staton et al., 2008) anxiety and depression (Rahimi,

Hajizadeh Moghaddam, & Roohbakhsh, 2015; Q. X. Shi et al., 2017) which makes GPR55 an interesting drug target.

Cannabinoid receptor expression in brain is also sexually dimorphic (Rubino & Parolaro, 2011), an effect that is largely dependent on environmental factors. Female rats display higher baseline CB1R and CB2R mRNA expression levels in the hippocampus, amygdala, and prefrontal cortex than their male counterparts, and these receptors are downregulated after repeated tail shock stress (Xing et al., 2014). In contrast, prenatal stress reduces CB1R expression in the cortex, striatum, hippocampus, and amygdala of male rats, while increasing CB1R expression in the hippocampus of female rats (Dow-Edwards, Frank, Wade, Weedon, & Izenwasser, 2016). In response to early maternal deprivation, females offspring exhibits a higher CB1 immunoreactivity than males in CA3 region, and a lower CB2 immunoreactivity than males in dentate gyrus of hippocampus (J. Suárez et al., 2009). Similarly in response to early stress, GPR55 expression increases in the frontal cortex of male while it increases in the hippocampus of female rats (Marco et al., 2014). Therefore, it should be investigated how the endocannabinoid system is differentially regulated in males and females, and generalization of observed effects from one sex to the other should be avoided.

3.3 Cannabinoid receptor signaling in the CNS

CB1 receptors are predominantly present at presynaptic and axonal compartments (Straiker, Wager-Miller, Hutchens, & Mackie, 2012) as well as can be found intracellularly or on the cell surface (Leterrier et al., 2006).

Postsynaptic neuronal activation and calcium influx leads to retrograde signaling and endocannabinoid activation of presynaptic CB1Rs. Upon activation by binding with endocannabinoids or exo-cannabinoids such as Δ^9 -THC or synthetic cannabinoids, CB1Rs couple with pertussis toxin (PTX)-sensitive inhibitory $G_{i/o}$ type G proteins, leading to a rapid decreased formation of cyclic adenosine monophosphate (cAMP) by inhibiting adenylate cyclase enzyme activity (Kendall & Yudowski, 2017a). This results in inhibition of calcium channel activity, stimulation of inwardly rectifying potassium currents, and activation of mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase 1 (ERK1), ERK2, p38, and JUN N-terminal kinases (JNKs) (Turu & Hunyady, 2010). These numerous MAPKs are involved in downstream signal transduction and regulation of nuclear transcription factors for both CB1Rs and CB2Rs. CB1R activates the PI3K-Akt pathway (Gómez del Pulgar, Velasco, & Guzmán, 2000), which is likely important for cannabinoid-mediated effects of neurogenesis and neuroprotection (Molina-Holgado et al., 2002). Additionally, activation of CB1Rs inhibits N-type voltage-gated calcium currents in differentiated neuroblastoma cells (Caulfield & Brown, 1992), P/Q-type calcium currents in rat cortical and cerebellar neurons (A. J. Hampson et al., 2002), and L-type calcium currents in arterial smooth muscle cells in the brain (Gebremedhin, Lange, Campbell, Hillard, & Harder, 1999). CB2Rs are also $G_{i/o}$ GPCRs and follow the same downstream signaling as described for CB1Rs. CB2Rs are mainly located on microglial cells mediating microglial migration (Walter et al., 2003) and proliferation (Carrier et al., 2004). The modulatory effect of the CB2R on microglia function is important since aberrant microglial activation has been observed in some neurodegenerative disease (Ramírez, Blázquez, Gómez Del Pulgar, Guzmán, & De Ceballos, 2005; Sagredo et al., 2007). Since, microglia produce endocannabinoids, and thus, activation of CB2Rs on their

surface appears to be at least partly autocrine in nature (Carrier et al., 2004). Neuronal CB2 receptors also control synaptic function and synaptic plasticity (Kendall & Yudowski, 2017a; Stempel et al., 2016) Like CB1 receptors, upon activation CB2 receptors also activate the downstream signaling of adenylyl cyclase which then activates mitogen-activated protein kinase (MAPK) such as c-Jun N-terminal kinase, Akt and others (Monsif Bouaboula et al., 1996; Ehrhart et al., 2005; Herrera, Carracedo, Diez-Zaera, Guzmán, & Velasco, 2005a).

In contrast to CB1R and CB2R, GPR55, mainly couples with Ca^{2+} signaling (Baker, Pryce, Davies, & Hiley, 2006; Ross, 2009). However, GPR55 can also couple with GQ or G13 protein with or without activating Ca^{2+} signaling (Lauckner et al., 2008; E Ryberg et al., 2009).

3.4 Role of the endocannabinoid system in neurodevelopment

The endocannabinoid system controls a number of key processes throughout brain development, during which AEA and 2-AG predominate at different time points. AEA is necessary for embryonic implantation, and levels are higher during early embryonic time points and in the blastocyst (Fride et al., 2009). As tissue differentiation occurs, 2-AG levels increase substantially (Fernando Berrendero, Sepe, Ramos, Di Marzo, & Fernández-Ruiz, 1999), and 2-AG becomes critical in controlling the size of neural progenitor cell pools within the brain, as well as in the determination of neuronal versus astrocyte cell fate. Relatively higher levels of 2-AG compared to AEA are present in the early postnatal period (Trezza et al., 2008), but both AEA and 2-AG play an important role in interneuronal migration and axonal specification (Harkany, Keimpema, Barabás, & Mulder, 2008). Endocannabinoid synthesizing enzyme DAGL is increased in developing pyramidal axons, and inhibition of DAGL can lead to impaired glutamate transporter VGLUT1 expression (Mulder et al., 2008). AEA induces BDNF-dependent differentiation of

hippocampal and cortical interneurons via activation of CB1R present on cholecystokinin (CCK)-containing GABAergic interneurons (Berghuis et al., 2005). However, exogenous AEA supplementation can trigger the internalization of CB1R, leading to growth cone abnormalities in GABAergic interneurons (Berghuis et al., 2007).

CB1Rs are abundantly present in the subgranular zone of the hippocampal dentate gyrus, and they play an important role in adult neurogenesis (Morales & Bäckman, 2002). Conflicting findings exist in *in vivo* and *in vitro* studies that show CB1 can have both positive and negative effects on neurogenesis. (Prenderville, Kelly, & Downer, 2015). Acute exposure to CB1 agonists increases adult neurogenesis in hippocampal dentate gyrus (Jiang et al., 2005). However, chronic exposure to CB1 agonists has shown to reduce hippocampal neurogenesis in adolescent rats (Abboussi, Tazi, Paizanis, & El Ganouni, 2014). Endocannabinoids also inhibits neuronal proliferation and adult neurogenesis via activating the CB1R dependent ERK pathway (Rueda, Navarro, Martinez-Serrano, Guzman, & Galve-Roperh, 2002). Interestingly, endocannabinoid uptake inhibitor can mitigate the stress mediated decrease in hippocampal cell proliferation (Hill, Kambo, Sun, Gorzalka, & Galea, 2006), suggesting a role of endogenous endocannabinoids in promotion of neurogenesis.

CB2Rs, found in neural progenitor cells, promote cell proliferation via mTORC1 signaling contributing to the expansion of the cell pool (Palazuelos et al., 2012). In post-mitotic neurons, there is a switch to a predominant expression of CB1Rs. CB1R expression reaches particularly high density in the cerebral cortex, hippocampus, caudate nucleus, and putamen in the fetal brain (C.-S. Wu, Jew, & Lu, 2011). During development, CB1Rs are expressed on the axonal surfaces

of neurons destined for the cerebral cortex. Here, the receptors contribute to axonal guidance through growth cone collapse, reinstatement, and extension (Fride et al., 2009). This process of axonal guidance is assumedly crucial for complex organization of neurons within the cerebral cortex (Maccarrone, Guzmán, Mackie, Doherty, & Harkany, 2014). Additionally, endocannabinoid signaling is important for regulation of glial cells, as CB1R activation in neural progenitor cells increases astrocyte differentiation and enhances astrocyte survival (Aguado et al., 2006). Therefore, spanning embryonic implantation to synaptogenesis, the endocannabinoid system can be considered critical for normal physiologic processes in the fetal brain.

3.5 Exogenous cannabinoid exposure

Exogenous cannabinoids, including marijuana and synthetic cannabinoids, are commonly used psychoactive agents. As mentioned previously, the primary psychoactive component of cannabis is THC, which is rapidly absorbed and converted into the active metabolite 11-hydroxy- Δ^9 -THC in the lungs and liver (Abood & Martin, 1992). The psychoactive effects of cannabinoids are thought to be due primarily to CB1R signaling, while the role for CB2R signaling in the brain following cannabis administration is not well established (Dhopeshwarkar & Mackie, 2014). Acute administration of exogenous cannabinoids results in activation of CB1Rs, leading to decreased glutamate release and a corresponding decrease in excitatory postsynaptic currents with implications for learning and memory (Mereu et al., 2002). Chronic cannabis use can lead to downregulation of CB1Rs (Hirvonen et al., 2012) and impairment in hippocampal long-term potentiation (LTP) (Hoffman, Oz, Yang, Lichtman, & Lupica, 2007). In contrast, a recent study showed that chronic activation of CB2Rs can actually *increase* hippocampal excitatory synaptic transmission (Jimok Kim & Li, 2015a). This novel finding indicates the presence of CB2Rs in

the hippocampus and implies a difference in the downstream molecular mechanisms of CB1R and CB2R.

As mentioned earlier, due to the dimorphic feature of cannabinoid receptors, female and male can respond differently to cannabinoid exposure. Females in general are more sensitive to the behavioral effects of cannabinoids than their male counterparts. Women reports significantly more dizziness than men due to a greater drop in mean arterial pressure (Mathew et al., 2003). Female marijuana smokers also showed a greater visuospatial memory than male smoker (Pope, Jr. et al., 1997) (Pope et al., 1997). However, increasing number of women have been reported to use cannabinoid for chronic pain, rheumatoid arthritis, or for conditions other than pain such as anxiety, anorexia, weakness and irritable bowel syndrome (Aggarwal et al., 2018; Cuttler et al., 2016; Ste-Marie et al., 2016; Waissengrin et al., 2015).

Since cannabinoids cross the blood-placenta barrier and are secreted in breast milk (Perez-Reyes & Wall, 1982) similar to other psychoactive drugs, the likelihood that exogenous cannabinoids reach the fetal brain is high. Consequently, maternal cannabinoid intake can affect the expression of genes key for proper neural development, leading to disturbances in neurotransmitter systems and behavior (Fernández-ruiz, Gómez, Hernández, Miguel, & Ramos, 2004). Of note, administration of AEA to pregnant rats did not alter CB1R expression in the offspring despite increasing CB1Rs and decreasing endothelin receptors in pregnant dams (Amlani et al., 2017). As the endocannabinoid system mediates numerous developmental processes as well as neurogenesis, neuronal plasticity, and memory, prenatal exposure to exogenous cannabinoids is likely to have a profound influence on brain function.

4. Role of glutamate in learning and memory

Glutamate is the major excitatory neurotransmitter in CNS. Glutamate is synthesized from glutamine. Glutamine is released by glial cells in presynaptic terminals and metabolized to glutamate by the mitochondrial enzyme glutaminase. Glutamate is also synthesized in the tricarboxylic acid (TCA) cycle by transamination of 2-oxoglutarate. Upon synthesizing, glutamate is packaged into vesicles. Vesicular glutamate transporters or VGLUTs are responsible for glutamate uptake into synaptic vesicles via a Mg^{2+} /ATP-dependent transport process. There are 3 types of VGLUTs -VGLUT1, VGLUT2, VGLUT3. Both VGLUT1 and VGLUT2 are expressed in the adult brain but VGLUT2 expression predominates during the first two weeks of postnatal development. VGLUT1 is expressed by glutamatergic neurons of the cerebral and cerebellar cortices, the hippocampus and the thalamus; whereas VGLUT2 is found in excitatory neurons throughout the diencephalon and the brainstem (Herzog et al., 2001; Hioki et al., 2003; Sakata-Haga et al., 2001). Interestingly, VGLUT3 holds a different characteristic as it is found in cholinergic neurons in the caudate-putamen and in serotonergic neurons in the raphe nucleus (Gras et al., 2002) rather than glutamatergic synapses. However, VGLUT1 plays the most important role as glutamatergic neurotransmission is drastically reduced in VGLUT1-deficient mice and targeted deletion of VGLUT1 in mice reduce the life span significantly. However, overexpression of VGLUT1 can restore the reduction in glutamatergic neurotransmission (Gras et al., 2002). Glutamate-filled vesicles are docked and released from presynaptic sites and bind to post synaptic receptors to exert its effect. Excess glutamate is uptaken into neurons and surrounding glial cells via specific transporters. Glutamine released by glial cells and taken up by neurons from the nerve terminal to convert back to glutamate. Glutamate is removed from the

synaptic cleft by glutamate transporters named excitatory amino acid transporters (EAATs), present in both glial cells and presynaptic terminals. There are five types of EAATs have been characterized so far. EAAT1 and EAAT2 are primarily expressed in glial cells, EAAT3-5 are expressed in neurons, with EAAT4 specifically localized to Purkinje cells in the cerebellum and EAAT5 expressed in the retina. EAAT1 (glutamate/aspartate transporter/GLAST) and EAAT2 (glutamate transporter -1 /GLT-1) are abundant in the brain and responsible for majority of glutamate uptake preventing glutamate spillover and excitotoxicity. GLAST and GLT-1 deficiency has been implicated in many psychiatric and neurodegenerative disease including bipolar disorder, major depression, schizophrenia, Alzheimer's disease (Jacob et al., 2007; Mookherjee et al., 2011), Parkinson's disease (Chotibut et al., 2017; Rihua Wang et al., 2018; Zhang, Tan, Xu, & Qu, 2016), Huntington disease (Estrada-Sánchez, Montiel, Segovia, & Massieu, 2009; Liévens et al., 2001) and others. Upon uptake into the glial cells by the glutamate transporters, glutamate is converted into glutamine by the enzyme glutamine synthetase and transported out of the glial cells into the nerve terminals. Decrease in glutamine synthetase can also result in cognition and memory deficits (Kulijewicz-Nawrot, Syková, Chvátal, Verkhatsky, & Rodríguez, 2013; Olabarria, Noristani, Verkhatsky, & Rodríguez, 2011). Thus, the continuous synchronous work between synaptic terminals and glial cells maintain an adequate supply of the neurotransmitter which is referred as the glutamate-glutamine cycle. Glutamate exerts its effect by binding with its postsynaptic receptors. There are mainly two classes of receptors, ligand gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. There are three ionotropic glutamate receptors: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), Kainate. These receptors possess four hydrophobic transmembrane regions (TMI – IV) having an extracellular longer N-

terminal domain and intracellular shorter C-terminal domain. The ionotropic receptors are ligand gated ion channels. When glutamate binds to these receptors, the channels open and Na⁺ and Ca²⁺ pass through them. The flow of ions depolarizes the plasma membrane and generate an electrical current (also known as excitatory post synaptic potential/epsp). It is then propagated through dendrites and axons and pass to the next neuron. Metabotropic glutamate (mGlu) receptors are G-protein coupled receptors (GPCRs) and mainly subdivided into three groups: Group I mGlu receptors (mGlu1 and mGlu5), group II (mGlu2 and mGlu3) and group III receptors (mGlu4, mGlu6, mGlu7 and mGlu8). Group I coupled to Phospholipase C (PLC) and intracellular calcium signaling, while group II and group III receptors are negatively coupled to adenylyl cyclase. Activation of both ionotropic and metabotropic receptors is responsible synaptic plasticity. Long-term potentiation (LTP) and long-term depression (LTD), are two such parameters which are termed as cellular correlate of learning and memory (Lüscher & Malenka, 2012; Miller & Mayford, 1999). In the next portion, we will mainly discuss role of ionotropic receptor AMPA and NMDA in hippocampus dependent learning and memory since we have mainly explored alteration of these two receptors in case of prenatal cannabinoid exposure.

AMPA receptors composed of mediate fast synaptic transmission in the CNS and are composed of subunits GluA1-4, AMPAR channels are usually impermeable to calcium due to post-transcriptional editing (Q/R editing) of GluA2 subunit) in the TMIII region where glutamine (Q) changes to arginine (R) making the receptor calcium impermeable (Pachernegg, Münster, Muth-Köhne, Fuhrmann, & Hollmann, 2015). However, GluA2 lacking AMPAR has shown to be calcium permeable and contribute to homeostatic synaptic plasticity (Lee, 2012). AMPAR regulates fast excitatory synaptic transmission throughout the CNS. AMPAR regulates LTP by

either increase post synaptic insertion of the receptors or by increasing the single channel conductance of the receptors expressed (Benke, Luthi, Isaac, & Collingridge, 1998; Plant et al., 2006). There are 2 phase of LTP: early phase (E-LTP), which lasts 2–3 h, and long-lasting LTP (L-LTP), which lasts several hours in vitro and weeks in vivo (M. A. Lynch, 2004). While delivery of new AMPARs to the postsynaptic sites is responsible for early-LTP, the late-LTP requires gene transcription and new protein synthesis (Plant et al., 2006). Sustained activation of AMPA receptors eventually leads to NMDAR activation. The NMDAR has similar structure like AMPAR, with an extracellular N-terminus, intracellular C-terminus and a re-entrant transmembrane domain. There are 4 subunits of NMDA: GluN1, GluN2, GluN3, GluN4. Again, each of them has multiple subunits. At resting membrane potentials, NMDA receptors are inactive due to voltage dependent Mg^{2+} blockade of the channel pores. Activation of AMPA depolarizes the post synaptic cell and repel the Mg^{2+} ions from the pore, thereby allowing the flow of permeant ions. NMDA receptors are permeable to calcium ions as well as to other ions (Mayer, Westbrook, & Guthrie, 1984). Thus, NMDA receptor activation leads to a calcium influx into the post-synaptic cells causing activation of postsynaptic Ca^{2+} -dependent kinases including Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) mediated signaling cascades. Activation of these kinases promotes AMPAR insertion into the post synaptic membrane causing a long-lasting enhancement of excitatory synaptic transmission that is required for LTP. LTP in hippocampal CA1 area is mainly NMDA dependent while it can be NMDA independent in other brain regions such as in mossy fiber pathway (Kapur, Yeckel, Gray, & Johnston, 1998), visual cortex (Aroniadou & Teyler, 1992), somatosensory cortex (Feldman, Nicoll, & Malenka, 1999) and others. Induction of LTD is also NMDAR-dependent in the hippocampal CA1 region and, like LTP induction and requires Ca^{2+} influx through NMDARs (Bear & Malenka, 1994).

However, in aged animals LTD seems to be mGluR dependent rather than NMDA (Bortolotto, Fitzjohn, & Collingridge, 1999). For LTD, NMDA mediated dephosphorylation of AMPARs, leads to a reduction in the open probability and removal of AMPARs from the synaptic plasma membrane by endocytosis causing receptor internalization (Beattie et al., 2000; R. C. Carroll, Beattie, Von Zastrow, & Malenka, 2001). Since both LTP and LTD is elicited by NMDAR-dependent calcium influx, it is important to establish how they can be induced in a particular cell. Reports indicate that, modest activation of NMDARs causes a modest increases in postsynaptic calcium and results in LTD while a stronger NMDAR is required to cause a robust change in postsynaptic calcium to trigger LTP (Bear & Malenka, 1994). Hence, in acute slices of the hippocampus, we use a high frequency stimulus or theta burst stimulus which are usually comprised several trains of pulses at 50–100 Hz, to elicit LTP. A comparatively low frequency stimuli of 1–3 Hz for 5–15 min is usually used to elicit LTD. Therefore modest depolarization through low frequency stimuli protocol is sufficient for LTD induction, whereas stronger depolarization through high frequency stimuli protocol required for LTP induction (Lüscher & Malenka, 2012). Thus, LTP and LTD two cellular and molecular mechanism of synaptic plasticity contributes to learning and memory and the alterations/deficits in these two parameters are usually manifested in behavioral outcomes. Reports depict that, the deficits in synaptic plasticity can be partly alleviated by using AMPAR agonists. However, overactivation of AMPA receptors could damage the brain, inducing convulsions and/or cell death (P. K.-Y. Chang, Verbich, & McKinney, 2012). Positive allosteric AMPAR modulator or ampakines cause a slower AMPAR desensitization and ligand-induced deactivation without a direct agonist or antagonist activities. Thus, ampakines mediate a fast excitatory transmission by modestly enhancing glutamatergic synaptic responses (Gall, Lynch, & Lauterborn, 2009). Ampakines

facilitates LTP by upregulating brain-derived neurotrophic factor (Lauterborn et al., 2003; Rex et al., 2006) and thus can improve learning and memory. However, even though ampakines have initially shown to improve cognition in several preclinical trials (Bloss et al., 2008; Dicou, Rangon, Guimiot, Spedding, & Gressens, 2003; Ingvar et al., 1997; G. Lynch, Rex, Chen, & Gall, 2008), they did not show promising effect in the long run. There are also AMPA receptor antagonists which have shown efficacy in cerebral ischemia and neuronal injury where AMPAR get overactivated. Competitive AMPAR antagonists such as ZK200775 (Elting et al., 2002; Lechoslaw Turski, 1998) and noncompetitive AMPAR antagonist such as perampanel (Aida, Niedzielko, Szaflarski, & Floyd, 2020; Suda & Kimura, 2019), Talampanel (Belayev et al., 2001) and LY 300168 (X. H. Liu, Wang, & Barks, 1997) have shown to improve stroke, head trauma and convulsion treatment outcome. Working with NMDAR agonist/antagonist is not as straightforward as AMPAR modulators because of NMDA subtypes and their different activity based on their synaptic vs extrasynaptic localization. While activation of synaptic NMDAR is crucial for maintaining synaptic plasticity, overactivation of NMDARs, particularly extrasynaptic NMDARs, is implicated in many disease process such as Alzheimer's disease (J. Liu, Chang, Song, Li, & Wu, 2019), Parkinson's disease (Vanle et al., 2018), Huntington's disease (Milnerwood et al., 2010) etc. Hence, a fine balance is required between synaptic and extrasynaptic NMDAR function (Rui Wang & Reddy, 2017). Among all the NMDAR antagonists, memantine which is a noncompetitive antagonist has gained particular attention as it has shown to predominantly block overactivation of extrasynaptic NMDA and reverse memory deficits (Frankiewicz, Potier, Bashir, Collingridge, & Parsons, 1996; Martinez-Coria et al., 2010; Song, Rauw, Baker, & Kar, 2008; Zajackowski, Quack, & Danysz, 1996). An improved analog of memantine is nitromemantine, which selectively blocks extrasynaptic NMDARs has been

proven beneficial in Alzheimer's disease (Zheng, Fridkin, & Youdim, 2014) and stroke (H. Takahashi et al., 2015). However, there are also certain NMDAR agonists like D-cycloserine which has been effective in treating encephalitis (Guan et al., 2016), schizophrenia (Goff, 2016), major depression (Heresco-Levy et al., 2006) and phobia (Walker, Ressler, Lu, & Davis, 2002).

5. Endocannabinoid and glutamate interplay:

As evident from the previous section, glutamate is essential for neuronal plasticity and differentiation, brain development and synaptic plasticity, directly affecting learning and memory consolidation. Since endocannabinoid system also controls a number of key processes throughout brain development a cross talk between glutamate and endocannabinoid exists having GPCR unit in common. Recent reports suggest that, NMDAR is localized not only post-synaptically or extra-synaptically, they can also be found presynaptic (Brasier & Feldman, 2008; McGuinness et al., 2010). While presynaptic NMDARs has some similarity with postsynaptic NMDARs in terms of LTP and LTD regulation, presynaptic NMDARs indeed possess novel functions such as regulation of presynaptic release probability and short-term plasticity (Corlew, Brasier, Feldman, & Philpot, 2008). Since CB1R is also present both pre and post synaptic, it can interact with NMDAR at both sites influencing its activity. Pre-synaptic CB1Rs inhibits glutamate release leading to reduced activation of NMDAR (X. J. Liu & Salter, 2010) while post-synaptic CB1Rs can influence NMDAR mediated calcium influx exerting neuroprotection (Qing Liu, Bhat, Bowen, & Cheng, 2009; Zhuang et al., 2005). CB1R can be colocalized with NMDAR i.e. GluN1 in CA3 hippocampal neurons and can directly influence NMDAR activity (Marchalant, Cerbai, Brothers, & Wenk, 2008). CB1R couples with NMDAR-GluN1 via histidine triad nucleotide-binding protein 1 (HINT1) proteins and promotes co-internalization.

Thus, it can inhibit overactivation of NMDAR (Sánchez-Blázquez, Rodríguez-Muñoz, Vicente-Sánchez, & Garzón, 2013). NMDAR activity in turn stimulates endocannabinoid release causing CB1R stimulation, which then inhibits NMDAR activity and NMDAR mediated excitotoxicity (Marsicano et al., 2003). Interestingly, HINT1 deficiency unables CB1R-GluN1 coupling resulting in vulnerability to glutamate excitotoxicity (Vicente-Sánchez, Sánchez-Blázquez, Rodríguez-Muñoz, & Garzón, 2013). However, excess endocannabinoid can also cause overactivation of CB1R and excess coupling leading to NMDAR hypofunction. Thus, a well-balanced CB1R-NMDAR crucial for normal synaptic function and implicated in mood, pain and various neuropsychiatric disorders such as schizophrenia and epilepsy. Since, mGluRs are also GPCR, a crosstalk between mGluR and CB1R can be observed in various physiologic events, i.e. LTD and neurotransmitter release. mGluR1/5 activation cause increase endocannabinoid preferably 2AG synthesis and release which then travel retrogradely and binds with presynaptic CB1R and trigger LTD (Jung et al., 2007; Maejima, Hashimoto, Yoshida, Aiba, & Kano, 2001). mGluRs mediated CB1 activation can in turn decrease glutamate release rendering a neuroprotective effect (Marsicano et al., 2003). Stimulation of mGluRs causes AKT and ERK phosphorylation through PI3K (Phosphoinositide 3 kinase)-PIP3 complex formation (Derkinderen et al., 2003; Hou & Klann, 2004; Rong et al., 2003). Activation of these cell signaling pathways can lead to increase brain derived neurotrophic factor (BDNF) synthesis, which is essential for neuronal survival (Blázquez et al., 2015).

Cannabinoid use has shown to affect different brain regions differently based on acute or chronic, prenatal or postnatal exposure as they can affect a range of neurotransmitter system (Pinky et al., 2019). Acute consumption of cannabis is usually associated with a sense of well-

being and euphoria, easy laughter, talkativeness, sedation, distortion of time perception, increased perception of external stimuli, and memory lapses. Based on the dose consumed, users can also experience anxiety attack, dysphoria, panic reactions, paranoia, and even delusion and hallucination (Panlilio, Goldberg, & Justinova, 2015). However, the long-term effects of chronic cannabis use are more profound with significant psychosocial, epidemiological, neurobiological, and general health problems. Chronic cannabis use during adolescence can impair neural connectivity leading to lower IQ, poor school/college performance, cognitive impairment, with lower IQ and ultimately diminished life satisfaction and achievement. Moreover, cannabis smoking can result in chronic bronchitis and other respiratory disease. Regular marijuana use is also associated with an increased risk of anxiety and depression (Patton et al., 2002) and increased risk of psychosis and schizophrenia in a genetically predisposed population (Hall & Degenhardt, 2008). In animal studies, cannabinoid exposure has been associated with reduced performance in spatial memory task. When the reason behind the cognitive impairment and learning and memory deficits has been investigated alteration in glutamatergic systems has been found to be largely responsible. Acute cannabinoid exposure increases glutamate release via activation of astroglial CB1Rs further causing activation of postsynaptic glutamate receptors (Han et al., 2012). Cannabinoid use also impairs hippocampus dependent synaptic plasticity parameters i.e. LTP (Hoffman et al., 2007) and LTD (Han et al., 2012). Although most of the studies suggest cannabinoid exposure cause a possible reduction in presynaptic glutamate release probability leading to reduced extracellular glutamate level (Anwyl, 2006; Dale A. Fortin & Eric S. Levine, 2006; Heifets & Castillo, 2009; Hoffman et al., 2007; Lovinger, 2008; Sánchez-Blázquez, Rodríguez-Muñoz, & Garzón, 2014), certain studies report that cannabinoid may also

stimulate presynaptic glutamate release (Colizzi et al., 2019; Fan, Yang, Zhang, & Chen, 2010; Han et al., 2012).

However, each of these studies are unique in terms of dosage, route of administration and the age of the animal examined, so it is comprehensible that the effect of cannabinoid exposure might vary depending on those factors.

6. Prenatal cannabinoid exposure and the endocannabinoid system

Though the mechanism has not been widely studied, neurobehavioral deficits observed in prenatally exposed offspring are typically assumed to be regulated by activity of exogenous cannabinoids at CB1Rs. Evidence indicates that CB1Rs are responsible for motor deficits seen in offspring following prenatal cannabinoid exposure, as conditional CB1R knockout mice are resistant to prenatal THC-induced motor alterations and CB1R expression reverses deficits in adulthood (de Salas-Quiroga et al., 2015b). Few other studies have addressed mechanisms responsible for neurobehavioral deficits, but some have evaluated CB1R-related parameters in affected offspring. Prenatal cannabinoid exposure appears to alter expression or functionality of CB1Rs in specific brain regions.

In humans, CB1R downregulation occurs in adults who use cannabis chronically, but this can be at least partially alleviated during periods of abstinence where levels of CB1R increase over time (Cyril et al., 2015). Comparatively, CB1R protein is downregulated in the cerebral cortex at gestational day 17.5 (GD17.5) following prenatal exposure to THC. However, CB1R expression returns to normal at postnatal day 2 (PND2), which denotes that the decrease in CB1R levels could be transient, at least in the exposure paradigm used (3mg/kg/day intraperitoneal (i.p.)

GD12.5 to GD16.5) (de Salas-Quiroga et al., 2015b). Conflicting results are reported regarding CB1R expression in adult male offspring following prenatal cannabinoid exposure, with no available data on female offspring to our knowledge. A recent study reported reduced CB1R density (Bmax) in the hippocampus of PND90 male rats with no change in receptor affinity following oral gavage administration of THC (5mg/kg) from GD15 to PND9 (Beggiato et al., 2017). In contrast, a different study found no change in the density or affinity of CB1R in the cortex, hippocampus, striatum, limbic areas, and cerebellum of PND80 male offspring following subcutaneous (s.c.) exposure to WIN55,212-2 (0.5 mg/kg) from GD5 to GD20 (Castelli et al., 2007a). However, based on the EC50 value of WIN55,212-2 needed to stimulate G-protein coupling of CB1R, sensitivity of hippocampal CB1Rs was increased while sensitivity of striatal CB1Rs was decreased. Both of these studies suggest long-term alterations in CB1R following prenatal exposure. Another study with prenatal THC exposure (3mg/kg/day i.p. from GD5.5 to GD17.5) reveals reduced CB1R mRNA levels in the fetal brain accompanied by increased MAG lipase and reduced diacylglycerol (DAG) lipase enzyme levels (Tortoriello et al., 2014). It has recently been reported that prenatal exposure to cannabinoid can also modulate endocannabinoid levels in a sex-dependent manner, as there was a significant reduction of DAGL α mRNA level in the prefrontal cortex of female but not male offspring despite no changes CB1R expression in either male and female offspring (Bara et al., 2018a). As MAGL and DAGL are responsible for degradation and synthesis of endogenous cannabinoids respectively, changes in these enzymes indicate a possible disruption in endogenous cannabinoid production and maintenance.

Evidence of changes to endocannabinoid levels in adult male offspring following prenatal cannabinoid exposure accompanies alterations in receptor expression discussed above. In the

previously mentioned study, Castelli et al. reported increased AEA levels in the striatum and reduced AEA in the limbic areas. Along with this, AEA levels in these regions were inversely correlated with activity of the AEA degrading enzyme FAAH and positively correlated with activity of the synthesizing enzyme, NAPE-PLD. However, these changes were not found within the endocannabinoid systems of the cortex, hippocampus, or cerebellum. The mechanism by which endocannabinoid imbalance occurs is not yet well elucidated. However, some evidence suggests that alterations in posttranslational modification may affect 2-AG signaling during corticogenesis (Keimpema et al., 2013). Also, degradation of superior cervical ganglion (SCG10) microtubule protein, which is necessary for maintenance of normal neuronal growth, can play a role in this dysregulation. SCG10 is downstream of 2-AG-CB1R signaling and its loss can cause cytoskeletal instability in response to exogenous cannabinoid exposure (Cristino & Di Marzo, 2014) This response can be exacerbated by simultaneous effects on 2-AG levels following JNK phosphorylation and feedback inhibition (Calvigioni, Hurd, Harkany, & Keimpema, 2014).

Taken together, these studies indicate that prenatal cannabinoid exposure leads to long-lasting changes to the endocannabinoid system, with most evidence pointing to alterations in the hippocampus, striatum, and limbic areas. Additional studies are needed to assess alterations in the brains of female animals and to clarify factors leading to commonly contrasting results between different studies. Finally, as an increase in endocannabinoid production can further lead to CB1R internalization and affect the axonal growth cone and neuronal connectivity (Berghuis et al., 2007), so correlating endogenous cannabinoid levels with CB1R expression in prenatally cannabinoid exposed offspring would be an interesting approach for future studies.

7. Prenatal cannabinoid exposure and the glutamatergic neurotransmission

Apart from maintaining synaptic plasticity, glutamate also regulates neuronal differentiation, migration, and survival in the developing brain (Tapiero, Mathé, Couvreur, & Tew, 2002). As mentioned previously, endocannabinoids act as retrograde messengers through CB1R to decrease release of neurotransmitters from presynaptic neurons. Since CB1Rs are known to influence glutamate neurotransmission and glutamate is important for learning and memory processes, there exists an immense interest in the long-lasting effects of prenatal cannabinoid exposure on the glutamatergic system.

Currently, there is limited literature that explores modulation of glutamatergic neurotransmission in prenatally cannabinoid-exposed offspring. The evidence that does exist varies greatly in terms of cannabinoid exposure period, route of administration, and age of the offspring during experimentation. Nevertheless, these studies overwhelmingly demonstrate that prenatal cannabinoid exposure has long-term effects on the glutamatergic system. Specifically, release and uptake of glutamate is altered, synaptic plasticity is impaired, and behavioral abnormalities are present. Gestational exposure (GD5-GD20) to the synthetic cannabinoid WIN55,212-2 at a dose of 0.5mg/kg body weight reduces potassium-evoked glutamate release in the hippocampus at PND40 and 80 when measured using *in vivo* microdialysis (Mereu et al., 2003). LTP in the Schaffer collateral pathway is reduced in this exposure paradigm, an effect that may be due to disrupted glutamate release in this region. This WIN55, 212-2 exposure paradigm does not appear to cause significant changes in the long-term (PND80) motor activity of the offspring, but it does lead to disruption of retention memory in the passive avoidance task at both PND40 and PND80. Therefore, it can be hypothesized that alterations in glutamate release might be

responsible for impairments in retention memory as well as deficits in LTP, the cellular mechanism of synaptic plasticity. Prenatal cannabinoid exposure (THC, 3mg/kg i.p. GD5.5-GD17.5) diminishes LTD and increases paired pulse ratio (PPR) in CA1 stratum radiatum of the hippocampus. Generally, an increased PPR indicates decreased neurotransmitter release probability (Manabe, Wyllie, Perkel, & Nicoll, 1993). This further indicates alterations in synaptic plasticity and glutamate release in the hippocampi of offspring prenatally exposed to cannabis (Tortoriello et al., 2014).

There are multiple hypotheses addressing possible mechanisms for glutamate dysregulation in these prenatally cannabinoid-exposed offspring. One explanation is that overactivation of CB1R innately leads to decreased release of glutamate and associated internalization of its specific NMDA receptor subtypes, further decreasing calcium influx and presynaptic glutamate release (Q. Liu, Bhat, Bowen, & Cheng, 2009). Primary cell culture from prenatal WIN55,212-2 (0.5mg/kg/day s.c. from GD5-GD20) exposed rodents demonstrates reduced basal and potassium-evoked cortical glutamate release, as well as reduced cortical neuronal populations and abnormal neurite branching and outgrowth. Exogenous NMDA application to the cortical cell cultures from control rodents led to a concentration-dependent increase in glutamate levels, which is absent in cell cultures obtained from WIN55,212-2-exposed pups (Tiziana Antonelli et al., 2005). Again, the loss of NMDA receptor activity observed in this study could be due to interference with calcium release via the inositol phosphokinase 3- phospholipase C (IP3-PLC) pathway (Netzeband, Conroy, Parsons, & Gruol, 1999) or through post-junctional phosphorylation mechanisms (Ferraro et al., 2001), as both of these have been observed in adult animals following acute cannabinoid exposure. Another interesting possibility is that exogenous

cannabinoid exposure causes a transient increase in endocannabinoids that subsequently inhibit glutamate release via activation of presynaptic CB1Rs in the process termed DSE (Melis et al., 2004). The cannabinoid system controls a number of synaptic processes including DSE, DSI, and certain types of long-term depression (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009). Relevant to the glutamatergic system, cannabinoid-mediated DSE can lead to reductions in glutamate outflow. In DSE, a rise in post-synaptic intracellular calcium concentrations results in release of endocannabinoids, which act as retrograde messengers on CB1R to reduce glutamate release (Diana & Marty, 2004). In line with this, AEA application decreases cell firing and amplitudes of field excitatory postsynaptic currents (fEPSP) while increasing the magnitude of PPR in acute hippocampal slices of adult rats (Ameri, Wilhelm, & Simmet, 1999).

Prenatal cannabinoid exposure also reduces basal and potassium-evoked glutamate release in the cerebral cortex of adult anesthetized rats (PND90) (T Antonelli et al., 2004) and in cerebral cortical cell cultures obtained from PND1 offspring (T. Antonelli et al., 2006). Additionally, acute injection of WIN55,212-2 (0.5mg/kg/day s.c. from GD5-GD20) increases the dialysate glutamate level in the cerebral cortex in both prenatal vehicle and WIN55,212-2 treated offspring at PND90 (T Antonelli et al., 2004). In the control group, the WIN55,212-2-induced increase in glutamate levels was fully counteracted by pretreatment with a CB1R antagonist but not with a CB2R antagonist. In the prenatal cannabinoid-treated offspring, acute WIN55,212-2-induced an increase in glutamate that was not reversed by a CB1R or a CB2R antagonist. This suggests that WIN55,212-2-induced increases in glutamate levels in the cannabinoid exposed offspring may be independent of CB1R and CB2R.

While chronic cannabinoid use decreases levels of glutamate, short-term cannabinoid exposure is often accompanied by an acute increase in glutamate levels. Similarly, WIN55,212-2 promotes glutamate release in the hippocampus of control offspring, which was absent in the hippocampus of the THC (5mg/kg/day oral gavage from GD15-PND9) exposed offspring (Castaldo et al., 2010a). The absence of acute WIN55,212-2-induced glutamate release in cannabinoid exposed offspring could be due to alterations in CB1R. For example, either reduced expression of CB1R in the hippocampus (García-Gil, Romero, Ramos, & Fernández-Ruiz, 1999) or rapid loss of cannabinoid responsiveness due to desensitization of these receptors (Rodríguez de Fonseca, Gorriti, Fernández-Ruiz, Palomo, & Ramos, 1994) could be responsible for the lack of response to WIN55,212-2 in the prenatal cannabinoid exposed offspring. Another possible explanation for the failure of acute THC application to increase glutamate release in the hippocampus of perinatally THC exposed rats could be that basal endocannabinoid levels are increased, which could saturate receptors and render them unresponsive to exogenous cannabinoid application (Castaldo et al., 2010a). Since astrocytic features are markedly affected by prenatal drug abuse (Miguel-Hidalgo, 2009), this could be another reason for the absence of increased glutamate release in perinatally cannabinoid-exposed offspring.

Studies designed to evaluate alterations in glutamate uptake have also been performed in offspring following prenatal cannabinoid exposure. While Antonelli et al. failed to observe any effect of prenatal WIN55,212-2 exposure on glutamate uptake, an acute hippocampal slice study of PND40 offspring showed that perinatal (GD15-PND19) exposure to THC reduces glutamate uptake in the hippocampus accompanied by reduced potassium-evoked glutamate release

(Castaldo et al., 2010a). The decreased glutamate uptake was correlated with lower expression of glutamate transporter 1 (GLT1) and glutamate aspartate transporter (GLAST), with no change of excitatory amino acid carrier 1 (EAAC1), in the hippocampus of THC-exposed offspring. These transporters are important in maintaining synaptic glutamate concentrations (Perego et al., 2002). Similar to the hippocampal data, a reduction in GLAST and EAAC1 expression occurs in rat cerebellum following cannabinoid exposure (THC 5mg/kg/day orally from GD5- PND20). This effect is observed at PND20, PND30, and PND70, suggesting a persistent long-term outcome (I. Suárez et al., 2004a). A decrease in glutamate uptake increases synaptic glutamate concentration, a phenomenon that is compensated for via presynaptic metabotropic glutamate receptor activation and a subsequent decrease in glutamate release (T. M. Brown, Brotchie, & Fitzjohn, 2003). However, conflicting evidence shows an increase in glutamate uptake in the frontal cerebral cortices of male offspring following prenatal exposure (GD5 - GD20) to WIN55,212-2 or THC (Castaldo et al., 2007) at a dose of 0.5mg/kg or 5mg/kg, respectively, indicating a possible region-specific effect of differential glutamate uptake. This increased uptake correlates with higher expression of GLT1 and EAAC1 and no change in GLAST expression. Since glutamate transporters mediate the development of cannabinoid tolerance (Gunduz, Oltulu, & Ulugol, 2011), exploring the expression of these transporters in relation to prenatal cannabinoid use may provide an opportunity for development of a novel therapeutic approach to rescue cognitive deficits caused by impaired glutamate transmission (Roberts-Wolfe & Kalivas, 2015).

Interestingly, carbon monoxide (CO) exposure, which occurs when marijuana is smoked (Tilles et al., 1986), inhibits glutamate uptake in both male and female rat striatum in a time and temperature dependent manner. Female rats show higher inhibition of glutamate uptake than

males, which indicates possible sexual dimorphism in regard to CO's effects in the brain (Taskiran, Kutay, & Pogun, 2003). Prenatal CO exposure also reduces potassium-evoked extracellular glutamate levels in primary rat cerebral cortical neuronal cell cultures (T. Antonelli et al., 2006). Although this study did not observe a significant additive effect on potassium-induced glutamate release when prenatal CO and WIN55,212-2 exposure was combined, it could be hypothesized that CO contributes to alterations in the glutamatergic system in human offspring exposed to concomitant maternal smoking. In regard to CO-related alterations in glutamate synthesis and release, conflicting mechanisms have been proposed for increasing or decreasing levels of glutamate in the synapse. For example, CO can differentially modulate the process of synaptic transmission by either raising glutamate levels via increased synthesis of the glutamate precursor glutamic acid (Piantadosi, Zhang, Levin, Folz, & Schmechel, 1997) or by decreasing glutamate release through reduction of calcium current dependent depolarization (Shinomura, Nakao, & Mori, 1994). Future studies should further elucidate the role of CO in combination with cannabinoids and fully assess changes to glutamate production, release, reuptake, or a mix of these components. This is especially important to real-life application considering that not all cannabinoid use occurs through marijuana smoking, but also includes ingestion of THC-rich edibles that do not introduce a CO component to prenatal exposure.

Although literature discussing prenatal cannabinoid exposure indicates both short- and long-term alterations in the glutamatergic system, generalization of these findings remains difficult due to reports of conflicting results. Variability in experimental observations is likely due to inconsistent exposure paradigms and methodologies, as well as differences in the age of offspring tested. Additionally, despite the present availability of numerous synthetic

cannabinoids, the prenatal effects of only a few agents have been examined to date. Observable differences in terms of variable severity or persistence of negative outcomes may occur for different cannabinoid compounds. Thus, future studies should attempt to expand evidence surrounding these agents. Also, mapping time-dependent changes to the glutamatergic system from early post-natal days to adulthood in exposed offspring may be beneficial to gain a better understanding of possible age-dependent alterations.

8. Prenatal cannabinoid exposure and the GABAergic neurotransmission and locomotor activity

Compared to glutamate, less is known about the effects of prenatal cannabinoid exposure on other neurotransmitters systems in preclinical models. Evidence suggests that long-lasting changes to learning and memory in offspring prenatally exposed to cannabinoids could also be due to alterations in GABAergic neurotransmission. Basal and potassium-evoked GABA outflow in the hippocampus is significantly reduced in adult (PND90) offspring prenatally exposed to THC (5mg/kg/day orally) from GD15 to PND9 (Beggiato et al., 2017). Additionally, reduced uptake of GABA is also observed in these animals, which could be partially attributed to reduction in levels of GABA transporter 1 (GAT-1), a membrane protein responsible for removal of GABA from the synaptic cleft. Activation of CB1R is thought to alter phosphorylation of GAT-1 through cAMP dependent protein kinases A and C (PKA/PKC), thus possibly modifying the uptake process (Maneuf, Nash, Crossman, & Brotchie, 1996; Tian, Kapatos, Granneman, & Bannon, 1994). Acute application of THC to hippocampal slices of both vehicle and THC perinatally-treated animals reduces GABA outflow, an effect antagonized by a CB1R blocker (Beggiato et al., 2017). Interestingly, CB1R expression is also reduced in the hippocampus of

these animals, an observation opposite of what might be predicted given the observed reduction in GABA outflow. This suggests that reduction of GABA outflow in these offspring may not be exclusively CB1R mediated, since reduced GABA outflow is expected to be related to increased presynaptic CB1R expression. However, since the hippocampus contains many types of neurons, the possibility of increased CB1R in the GABAergic population of neurons cannot be ruled out.

In adult animals, presynaptic CB1Rs on hippocampal basket cells regulate GABA release by inhibiting hippocampal GABA outflow, as demonstrated by WIN55,212-2 application (Katona et al., 1999). Whole cell patch clamp recording on adult rat midbrain slices also reveals reduction in the evoked and spontaneous inhibitory post synaptic currents (IPSC) in presence of WIN55, 212-2. These currents are typically mediated by the GABA-A receptor (P. K. Chan, Chan, & Yung, 1998). However, the relationship between CB1R expression and effects on GABA release is complicated by recent evidence indicating that effects of endogenous cannabinoids on GABA release may be primarily determined by the concentration of endogenous cannabinoids rather than CB1R expression (Lenkey et al., 2015). The same effect could be considered as a possibility for release of other neurotransmitters with similar release and feedback mechanisms.

As CB1Rs are present on GABAergic neurons during prenatal development, alterations in cannabinoid signaling at these neurons may lead to neurodevelopmental deficits. CB1Rs are found on migrating GABAergic interneurons during late stages of corticogenesis, which is important for axonal cone growth and neuritogenesis (Berghuis et al., 2007). An increased number of GABAergic interneurons are also observed in the marginal zone of telencephalic coronal sections from WIN55,212-2 exposed (0.75mg/kg/day s.c. from GD5 to GD16) embryos

at GD16.5 (Saez, Aronne, Caltana, & Brusco, 2014a). Since GABAergic interneurons invade the hippocampus mainly through migratory zone streams (Manent, Jorquera, Ben-Ari, Aniksztejn, & Represa, 2006), increased GABAergic expression may cause a defect in the positioning of hippocampal interneurons. This positioning defect could lead to an imbalance in the glutamatergic and GABAergic neurotransmitter systems in cannabinoid-exposed offspring. However, perinatal exposure (GD5 – PND4) to THC failed to show any changes in the GABA content within the nucleus accumbens, globus pallidus, substantia nigra, caudate-putamen, and ventral tegmental area of adult offspring (Garcia-Gil et al., 1999a). A lack of observable changes in these areas could suggest either that these alterations do not occur in the brain areas tested or that alterations in these areas may not persist into adulthood. Determining whether hippocampal GABA concentrations are altered in adult offspring would be interesting, as this area of the brain is implicated in learning and memory deficits.

The role of the GABAergic system in controlling motor activity is well established. CB1Rs are found in the intrinsic striatal medium-spiny GABAergic neurons that project toward two major areas for movement control, the globus pallidum and substantia nigra (Rodríguez de Fonseca, Del Arco, Martín-Calderón, Gorriti, & Navarro, 1998). In prenatal cannabinoid literature, the observed effects of GABA-A activation on motor activity are somewhat dose-dependent, with low doses of GABA-A agonists leading to motor depression but high doses causing motor enhancement (Wong, Eshel, Dreher, Ong, & Jackson, 1991). However, the commonly used GABA-B agonist baclofen acutely reduces locomotor activity in adult rats at both low and higher doses (Wong et al., 1991). Following prenatal exposure to THC (5mg/kg/day orally from GD5-PND1), affected offspring are more susceptible to motor depression when administered a

GABA-B agonist, but not a GABA-A agonist, as compared to their non-exposed counterparts. (Garcia-Gil et al., 1999a). This finding suggests that prenatal cannabinoid exposure might lead to motor impairment involving GABA-B receptors, an effect in concordance with observations following acute cannabinoid administration in adult animals (Romero, García-Palomero, Fernández-Ruiz, & Ramos, 1996).

Observed increased GABA should theoretically accompany motor-behavioral deficits outflow, but no change in basal GABA levels have been observed to our knowledge. Since GABAergic and glutamatergic neurons are interrelated in the previously mentioned brain areas, it is possible that these observed effects are instead due to alterations in glutamatergic signaling rather than GABAergic signaling. NMDA receptor subtypes can cause an increase in surface expression of GABA-B receptors (Kantamneni et al., 2014), and since acute doses of cannabinoids may actually increase glutamate release, eventual increases in NMDA-dependent GABA-B expression may manifest a more inhibitory effect. Similar GABA-glutamate interplay has been observed in relation to the anxiogenic and anxiolytic properties of cannabinoids in adult literature (Rey, Purrio, Viveros, & Lutz, 2012), where cannabinoids exert anxiolytic effects via glutamatergic signaling and anxiogenic effects through GABAergic signaling. Therefore, future studies should target in depth exploration of dose and region-specific changes to GABAergic circuitry in response to prenatal cannabinoid exposure and should further address parameters of locomotion and anxiety in affected offspring.

9. Role of cannabinoid in oxidative stress and neuronal apoptosis:

CB1 receptors can be found on neuronal mitochondrial outer membranes (mtCB1R). mtCB1R activation inhibits mitochondrial cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/complex I pathway and thus can regulate mitochondrial bioenergetics (Bénard et al., 2012a). mtCB1R are also responsible for DSI type of short-term synaptic plasticity at GABAergic synapses. Acute administration of THC reduces mitochondrial respiratory chain complex I activity in hippocampal mitochondria and inhibition of complex I activity enhanced DSI by inhibiting presynaptic mtCB1R (Bénard et al., 2012a). THC administration also inhibits brain mitochondrial complexes II, III, and IV activities and increase H₂O₂ level resulting in oxidative stress (Singh, Hroudová, & Fišar, 2015a; Wolff V, Schlagowski AI, Rouyer O, 2015). The alteration in oxidative stress markers are also associated with increased neuronal DNA damage (Kopjar et al., 2019). Interestingly, opposing reports also exist where cannabinoid administration has shown to render a protective effect in traumatic brain injury and cerebral ischemia. mtCB1R can indeed play a dual role in neuronal apoptosis and cell death. Traumatic brain injury can upregulate mtCB1R activation and inhibit cAMP/PKA/complex I resulting in exacerbation of metabolic defects and neuronal apoptosis. Interestingly, activation of mtCB1R also upregulates mitochondrial AKT/complex V activity which then exert an anti-apoptosis effects and protect the cellular metabolism (Z. Xu, Lv, Dai, Ge, & Xu, 2016). Cannabinoid agonist administration significantly up-regulates mtCB1R level in hippocampus and thus reduce reactive oxygen species (ROS) and lactate dehydrogenase (LDH) restoring cell viability (Ma et al., 2015). Plant cannabinoid cannabidiol has shown to reduce cell viability with higher dose while it also showed to protect against H₂O₂ mediated neurotoxicity with a comparatively lower dose than LC₅₀ (Jungnam Kim, Choi, Seo, & Choi, 2020). The protection against H₂O₂-induced death is probably through decreasing several apoptotic effectors such as CHOP, Bax and caspase

12, while increasing anti-apoptotic Bcl-2 level (Mecha et al., 2012). THC administration also protects against NMDA-induced cell death by decreasing NMDA-induced ROS generation and inhibiting p38 MAPK phosphorylation (Chen, Errico, & Freed, 2005). However, although cannabinoid treatment has shown to improve the oxidative stress in certain brain areas it did not alter the immunohistochemical neurodegenerative changes in striatum and substantia nigra as a result of toxic insult (Abdel-Salam et al., 2015). Isoflurane also shows to activate mtCB1R leading to increased brain ischemic tolerance and rendering neuroprotection (Cai et al., 2017). CB1R activation upon oxidative injury inhibits PKA signaling and NO generation and protects against excitotoxicity (Sun, Seok, Xiao, Jin, & Greenberg, 2006) and cortical neuronal death (S. H. Kim, Won, Mao, Jin, & Greenberg, 2005). In *in vivo* model cannabinoid administration also protects hippocampus from global and focal ischemia in a CB1R dependent manner while *in vitro* model showed cannabinoid reduced excitotoxicity through a receptor-independent mechanism (Nagayama et al., 1999).

Thus, cannabinoid can be both neurotoxic and neuroprotective and the effects might be either receptor-mediated or non-receptor-mediated. These discrepancies are likely due to a variety of influences, i.e. the dose of cannabinoid, the type of the cannabinoid used (phenolic vs non phenolic), the brain area or cells that were used (hippocampal, cortical, cerebellar) and the nature of the toxic insult (acute vs chronic).

10. Prenatal cannabinoid exposure and oxidative stress:

Although cannabinoid mediated effect on oxidative stress and neuronal apoptosis is a continuous evolving research area, how prenatal/perinatal cannabinoid exposure mediates these effects are still relatively unknown. Perinatal exposure to THC can have long-term impact on the immune

system of the offspring by triggering fetal thymic atrophy. This is mediated by both CB1R and CB2R signaling leading to induction of apoptosis. Furthermore, this can lead to a developmental defect in thymus and spleen functionality resulting in diminished immune response to HIV-1 antigen (Lombard, Hegde, Nagarkatti, & Nagarkatti, 2011; Roth, Tashkin, Whittaker, Choi, & Baldwin, 2005). A single dose of THC during at PND 10 has shown to alter the gene transcription levels of that are involved in neurotrophic, endocannabinoid and oxidative stress signaling resulting in increased apoptosis in rat's cortex and hippocampus (Philippot, Forsberg, Tahan, Viberg, & Fredriksson, 2019b). A recent report suggested that prenatal exposure to cannabinoid can result in pathological and behavioral phenotypes similar to fetal alcohol spectrum disorder (Boa-Amponsem, Zhang, Mukhopadhyay, Ardrey, & Cole, 2019) which might be due to increased ROS-mediated signal transduction and oxidative DNA damage, leading to pathologic genetic mutations and epigenetic changes (Bhatia, Drake, Miller, & Wells, 2019). Furthermore, THC mediated increased production of ROS generation is often associated with DNA strand breaks (G. C. K. Chan, Hinds, Impey, & Storm, 1998) which is thought to be mediated by epoxidation of the 9, 10-alkene linkage (Narimatsu et al., 1992). Epoxide also itself mediates DNA alkylation and generate ROS (Kovacic & Jacintho, 2012; Kovacic & Osuna Jr., 2005). Thus, cannabinoid can influence the oxidative stress level in the brain both in positive and negative way which has also shown to be brain region specific.

11. Conclusion

It is becoming clear that prenatal exposure to cannabinoids leads to long-lasting deficits in central processes of behavior. Because human-specific research is limited to prospective or retrospective evaluations, emerging studies in animal models are essential for the continued

understanding of complex effects related to prenatal cannabinoid exposure. The endocannabinoid system plays an important role in early development, and changes to this system may lead to persisting, multifaceted effects in the offspring. Furthermore, cannabinoid receptors are important regulators of other neurotransmitter systems, so alterations in these receptors are likely to vastly impact numerous brain processes. However, the results from prenatal cannabinoid studies are difficult to generalize based on a number of factors. Sexual dimorphism exists for many of the effects associated with prenatal cannabinoid exposure, and investigators have consistently noted opposing effects in terms of downregulation or upregulation of specific proteins. It is also important to note that multiple studies have been performed using only male offspring. In addition, many observed alterations in neurotransmitter systems appear to be time dependent. Finally, different doses, variable times and durations of exposure, or even the type of cannabinoid used can all lead to individualized experimental results. Therefore, it remains difficult to generalize the conclusions from one experimental design to other studies. This highlights the importance of additional investigation into human-specific prenatal cannabinoid exposure to help further establish the clinical application of animal studies and identify a specific therapeutic target to ameliorate the observed cognitive deficits. Based on available evidence, it appears that prenatal cannabinoid exposure leads to widespread changes in glutamatergic, GABAergic, dopaminergic, serotonergic, and adrenergic neurotransmitter systems. These alterations are likely responsible for the behavioral deficits observed in both human offspring and in animal studies.

12. Tables

Table 2.1 Effect of PCE on glutamatergic neurotransmission

Treatment protocol	Age of Offspring	Effect	Reference
WIN55,212-2 (0.5 mg/kg) THC (5 mg/kg) Daily, Subcutaneous injection GD 5 to GD 20.	male and female offspring PND 90 – 130	PCE does not parameter of cognitive and anxious behaviors in both sexes. In males but not in female offspring, PCE reduced social interaction along with LTD alteration and heightened excitability of prefrontal cortex pyramidal neurons WIN55,212 treatment of dams decreased TRPV1, mGlu5, and DAGL α mRNA in female offspring but only mGlu5 mRNA in male offspring in prefrontal cortex.	Sex-dependent effects of in utero cannabinoid exposure on cortical function (Bara et al., 2018b)
THC (5 mg/kg) Daily oral dose THC from gestational day GD 5 to PND 20	PND20, PND30 and PND70	PCE causes reduced expression of Glutamate transporter GLAST in astroglial cells and EAAC1 in Purkinje neurons mainly in male offspring.	Prenatal Cannabinoid Exposure Down- Regulates Glutamate Transporter Expressions (GLAST and EAAC1) in the Rat Cerebellum (I. Suárez et al., 2004b)
WIN55,212-2 (0.5 mg/kg) THC (5 mg/kg) Daily subcutaneous injection GD 5 to GD20	PND 40	PCE reduced dialysate glutamate levels and enhanced glutamate uptake in frontal cerebral cortex. Increased expression of glutamate transporter 1 (GLT1) and excitatory amino acid carrier 1 (EAAC1) in frontal cerebral cortex.	Prenatal Exposure to the Cannabinoid Receptor Agonist WIN 55,212-2 Increases Glutamate Uptake Through Overexpression of GLT1 and EAAC1 Glutamate Transporter Subtypes in Rat Frontal Cerebral Cortex (Castaldo et al., 2007)
WIN 55,212-2	Cerebral	PCE reduced Basal ₅₁ and K(+)-evoked	Prenatal Exposure to the

(0.5 mg/kg) Daily subcutaneous injection GD 5 to GD 20	cortical prepared from 1- day-old	extracellular glutamate levels in cortical cultures	Cannabinoid Receptor Agonist WIN 55,212-2 and Carbon Monoxide Reduces Extracellular Glutamate Levels in Primary Rat Cerebral Cortex Cell Cultures (T. Antonelli et al., 2006)
WIN-55,212-2 (0.5mg/kg/day) Daily subcutaneous injection GD5 to GD20		PCE caused hyperactivity at PND 40 days but not at PND 80. PCE causes a reduction in LTP at PND 40 but normal basal synaptic transmission decreased K ⁺ evoke glutamate release on microdialysis at PND 40 and PND 80	Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long term potentiation and glutamate release (Mereu et al., 2003)
WIN55,212-2 (0.5 mg/kg) Daily subcutaneous injection GD5 to GD 20	PND 90	PCE reduced basal and K ⁺ -evoked extracellular glutamate levels in the prefrontal cortex of the awake rat offspring which was a CB1R mediated	Long-term Effects on Cortical Glutamate Release Induced by Prenatal Exposure to the Cannabinoid Receptor Agonist (R)-(+)-[2,3-dihydro-5-methyl-3- (4-morpholinyl- methyl)pyrrolo[1,2,3-de]-1,4- benzoxazin-6-yl]-1- naphthalenylmethanone: An in Vivo Microdialysis Study in the Awake Rat (T Antonelli et al., 2004)
WIN 55-212,2 (0.75 mg/kg) Daily subcutaneous injection GD 5 to GD12,	E12.5, E13.5, E14.5, E16.5, and E20.5	PCE impaired tangential and radial migration of post-mitotic neurons in the dorsal pallium. PCE increased GABAergic interneurons in the cortical marginal zone and the number of glutamatergic intermediate	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

GD14, GD16 or GD20, respectively		progenitors in the dorsal pallium of cerebral cortex. The number of glutamatergic post-mitotic neurons was decreased in the developing cerebral cortex at E12.5 and E14.5	Rat Cerebral Cortex (Saez, Aronne, Caltana, & Brusco, 2014b)
THC (5 mg/kg) Daily orally GD 15 to PND 9	PND 40	PCE reduced both basal and K ⁺ -evoked glutamate outflow and reduced glutamate uptake in hippocampus. A reduction in glutamate transporter 1 (GLT1) and glutamate/aspartate transporter (GLAST) protein were also observed	Altered Regulation of Glutamate Release and Decreased Functional Activity and Expression of GLT1 and GLAST Glutamate Transporters in the Hippocampus of Adolescent Rats Perinatally Exposed to Delta (9)-THC (Castaldo et al., 2010b)
WIN 55,212-2 (0.5 mg/kg) was Daily subcutaneous injection GD 5 to GD 20	PND 60	PCE reduced BDNF levels in hippocampus and frontal cortex and reduced phosphorylation of ERK1/2 in hippocampus, frontal and prefrontal cortex. PCE also reduced total and phospho-alpha-CaMKII level in the hippocampus	Long-term reduction of brain-derived neurotrophic factor levels and signaling impairment following prenatal treatment with the cannabinoid receptor 1 receptor agonist (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (Maj et al., 2007)
WIN 55,212-2 (0.5 mg/kg) Daily subcutaneous injection GD 5 to GD 20	Cortical Cell Cultures PND 10-12, PND 80	PCE reduced both basal and K ⁺ -evoked extracellular glutamate levels cortical cell cultures which was failed to increase in after NMDA application. This was also associated with reduced number of cortical neuronal population. PCE offspring preformed poorly in	Prenatal Exposure to the CB1 Receptor Agonist WIN 55,212-2 Causes Learning Disruption Associated With Impaired Cortical NMDA Receptor Function and Emotional Reactivity Changes in Rat

		homing (PND 10-12) and active avoidance tests (PND 80) as well as a decrease in the rate of separation-induced ultrasonic emission (PND 10)	Offspring (Tiziana Antonelli et al., 2005)
THC (5 mg/kg) Daily orally GD 5 to GD 20	PND 65 to PND 90	PCE increases extracellular basal Kynurenic acid levels and reduces basal glutamate levels. PCE offspring exhibited reduced spontaneous alternation but not locomotor activity (arm entries) in Y maze test	Prenatal THC Exposure Raises Kynurenic Acid Levels in the Prefrontal Cortex of Adult Rats (Beggiato, Ieraci, Tomasini, Schwarcz, & Ferraro, 2020)
WIN 55212-2 (0.5 mg/kg/day) Daily subcutaneous injection GD 5 to GD 20	PND 120 to PND 150	PCE causes an increase in glutamic acid decarboxylase (GAD) and GABA immunoreactivities in the molecular layer and axon terminals in the Purkinje neuron layer indicating selective up-regulation of GABA-mediated neurotransmission	Effects of Prenatal Exposure to the CB-1 Receptor Agonist WIN 55212-2 or CO on the GABAergic Neuronal Systems of Rat Cerebellar Cortex (Benagiano et al., 2007)
THC (5mg/kg) Daily orally GD5 to PND 20	PND 20, PND 30 and PND 70	PCE decreased GluR1 level in Bergmann glial cells, as well as levels of the GluR2/3 in Purkinje neurons WB expression reduced in Cerebellar cortex	Down-regulation of the AMPA Glutamate Receptor Subunits GluR1 and GluR2/3 in the Rat Cerebellum Following Pre- And Perinatal delta9-tetrahydrocannabinol Exposure (Isabel Suárez et al., 2004)
THC (5 mg/kg) Daily oral dose GD 5 to PND 20	PND 30, PND 70	PCE reduces GFAP immunoreactivity in cerebellar astrocytes and Bergmann glial cells. PCE reduced glutamine synthetase more prominently in male offspring than female offspring	Reduced Glial Fibrillary Acidic Protein and Glutamine Synthetase Expression in Astrocytes and Bergmann Glial Cells in the Rat Cerebellum Caused by delta(9)-tetrahydrocannabinol Administration During

			Development (Isabel Suárez et al., 2002)
THC (5 mg/kg weight) Daily oral dose GD 5 to PND 24	>PND70	PCE did not modify GAD activity or GABA content in the ventral-tegmental area, nucleus accumbens, substantia nigra, caudate-putamen, and globus pallidus. Open-field test revealed, GABA-B agonist baclofen facilitate PCE induced decreased ambulation and stereotypy and increased inactivity.	Perinatal delta9-tetrahydrocannabinol Exposure Augmented the Magnitude of Motor Inhibition Caused by GABA(B), but Not GABA(A), Receptor Agonists in Adult Rats (Garcia-Gil et al., 1999b)
THC (5mg/kg) Daily orally GD15 to PND 9	PND 90	PCE reduced basal and K+-evoked [3H]-GABA outflow in the hippocampus. These effects were associated with a reduction of hippocampal [3H]-GABA uptake. PCE also reduced CB1 receptor binding in the hippocampus. THC (0.1 µM) or WIN55,212-2 (2 µM) administration also reduced K+-evoked GABA outflow in the both group of offspring which were significantly blocked by CB1R antagonist	Long-lasting alterations of hippocampal GABAergic neurotransmission in adult rats following perinatal Δ9-THC exposure (Beggiato et al., 2017)

Table 2.2: Effect of PCE on spatial memory behavior and locomotor activity

Treatment protocol	Age of offspring tested	Findings	Reference
THC (3mg/kg) intraperitoneal injection GD 10.5 to GD17.5	PND60	THC did not alter NOR but exhibited significant alteration in NOL	Long-term Hippocampal Interneuronopathy Drives Sex-Dimorphic Spatial Memory Impairment Induced by Prenatal THC Exposure (de Salas-Quiroga et al., 2020)
CP (0.4 mg/kg/day) Intra gastric administration PND 4-PND 9 (corresponding to third semester in human)	OFT- PND 18–21, EPM- PND-25, and MWM (PND 40 - 46)	PCE increased locomotor activity and reduced habituation in the OFT task, increased thigmotaxis during acquisition without altering spatial memory during probe trial in the MWM, and increased time spent in the open arms on EPM task	The effects of alcohol and cannabinoid exposure during the brain growth spurt on behavioral development in rats (Breit, Zamudio, & Thomas, 2019)
WIN55,212-2 (0.5 mg/kg) Subcutaneous injection GD5 to GD20)	infancy (PND 10 and 13) prepubertal period (PND 28–35 for males and PND 22–28 for	PCE male but not female showed a decreased ultrasonic vocalization. Homing behavior- the frequency of crossing in the test arena was	Sex-specific behavioral deficits induced at early life by prenatal exposure to the cannabinoid receptor agonist

	females) puberty (PND 50–60 for males and PND 30–40 for females offspring)	increased only in PCE male offspring while females were spared. Temporal order memory test- PCE did not affect social, anxious and cognitive behaviours in prepubescent male and female offspring.	WIN55, 212-2 depend on mGlu5 receptor signalling (Manduca et al., 2020)
THC (0.15 mg/kg) Daily intravenous injections. GD1 to GD21.	passive avoidance- PND 22 active place avoidance - PND 45 attention task- PND 60	Passive avoidance- no effect on acquisition but impaired consolidation during retention testing. The active place avoidance- showed no effects on acquisition but PCE offspring tending to take more trials to complete the task in reversal performance. Attention task – smaller percentage of PCE rats completed the test, although the failure rate of both groups was quite high PCE male offspring showed more reduction in locomotor activity compared to female offspring in response to	Prenatal tetrahydrocannabinol (THC) alters cognitive function and amphetamine response from weaning to adulthood in the rat (Silva, Zhao, Popp, & Dow-Edwards, 2012)

		amphetamine	
			Delta-9-tetrahydrocannabinol during pregnancy in the rat: II. Effects on ontogeny of locomotor activity and nipple attachment in the offspring (Brake, Hutchings, Morgan, Lasalle, & Shi, 1987)
WIN 55,212-2 (0.5 mg/kg, Daily subcutaneous injection GD5-GD20	PND 80	PCE did not result in significant locomotor deficits	Dysregulation of the endogenous cannabinoid system in adult rats prenatally treated with the cannabinoid agonist WIN 55,212-2 (Castelli et al., 2007b)
THC (5 mg/kg) Daily oral dose GD15-PND9	PND 80	PCE reduced extracellular glutamate level in the PFC Inhibitory avoidance- PCE reduced avoidance latencies of adult offspring. Social discrimination-	Perinatal exposure to delta-9-tetrahydrocannabinol causes enduring cognitive deficits associated with alteration of cortical gene expression and

		PCE reduced discrimination abilities at adulthood	neurotransmission in rats (Campolongo et al., 2007)
			Sex-dimorphic Psychomotor Activation After Perinatal Exposure to (-)-Delta 9-tetrahydrocannabinol. An Ontogenic Study in Wistar Rats (Navarro, Rubio, & Rodríguez de Fonseca, 1994)
THC (2 mg/kg) daily subcutaneous injections GD 5 to GD 2	PND 25 onwards	OFT - PCE increased locomotor activity NOR- no significant effects. Emotional NOR- PCE rats showed a significant decrease in the avoidance of the fear-associated object instrumental learning- decreased alcohol seeking behavior, drinking, relapse- increased operant chamber-	In Utero Δ 9-tetrahydrocannabinol Exposure Confers Vulnerability Towards Cognitive Impairments and Alcohol Drinking in the Adolescent Offspring: Is There a Role for Neuropeptide Y? (Brancato, Castelli, Lavanco, Marino, & Cannizzaro, 2020)

		increased percentage of punished responses for alcohol	
<i>Paternal Cannabinoid exposure</i> WIN55,212-2 (1.2 mg/kg) daily intraperitoneal injections For 20 days	PND 60	OFT- In the absence of stress- no alterations in the parameters In presence of stress- significant decrease in the time spent in the center of the open field arena NOR- No change is objects discrimination	Behavioral and epigenetic effects of paternal exposure to cannabinoids during adolescence on offspring vulnerability to stress (Ibn Lahmar Andaloussi, Taghzouti, & Abboussi, 2019)
THC (2 mg per kg) Daily subcutaneous injection GD 5 -GD 20	PND15 - 28	Startle reflex and PPI and OFT- No differences were observed between progenies, unless they were acutely treated with THC, as revealed by increased startle, hyperlocomotion and reduced thigmotaxis in PCE offspring EPM- no alteration in the parameters Wire-beam bridge test – Acute THC administration does not modify the latency to crossing the bridge but	Prenatal THC exposure produces a hyperdopaminergic phenotype rescued by pregnenolone (Frau et al., 2019)

		reduces the number of stretched-attend postures (SAP) in PCE male offspring.	
THC (2.5-5 mg/kg) Daily orally GD 15 – PND 9	USV- PND 12 Social interaction - PND 35 EPM- PND 80	In 5mg/kg dose, USV- PCE increased the number of ultrasounds emission social interaction- PCE offspring was less engaged in pinning, Pouncing and boxing–wrestling while social behaviors unrelated to play were unaffected EPM- PCE decreased the % of time spent on the open arms and the % of entries in the open arms, decreased the number of exploratory head dippings and increased the number of SAP	Effects of Perinatal Exposure to delta-9-tetrahydrocannabinol on the Emotional Reactivity of the Offspring: A Longitudinal Behavioral Study in Wistar Rats (Trezza, Campolongo, et al., 2008)
THC (2 mg/kg) injections of natural twice daily subcutaneous injection GD 1- PND 10.	PND 90	OFT- PCE offspring travelled less distance and spent significantly less time in the center Active social interaction- PCE exhibited more investigative sniffing	Perinatal delta-9-tetrahydrocannabinol Exposure Disrupts Social and Open Field Behavior in Adult Male Rats (Newsom & Kelly,

		Forced swim test- No difference compared to control	2008)
THC (0.1, 0.5 or 2 mg/kg) daily oral dose GD 5 -PND 24	PND 70	OFT- PCE shows a robust reduction in locomotor activity compared to female	Perinatal exposure to Δ 9-tetrahydrocannabinol increases presynaptic dopamine D2 receptor sensitivity: a behavioral study in rats (Moreno, Trigo, Escuredo, Rodríguez de Fonseca, & Navarro, 2003)
THC (5 mg/kg) or WIN55,212-2 (0.75 mg/kg) daily intraperitoneal injections GD10.5–GD18.5	PND - 8 to 12 weeks	NOR- PCE spent significantly less time interacting with the novel object EPM- no alteration has been observed	Persistent Inhibitory Circuit Defects and Disrupted Social Behaviour Following in Utero Exogenous Cannabinoid Exposure (Vargish et al., 2017)

12. Figures

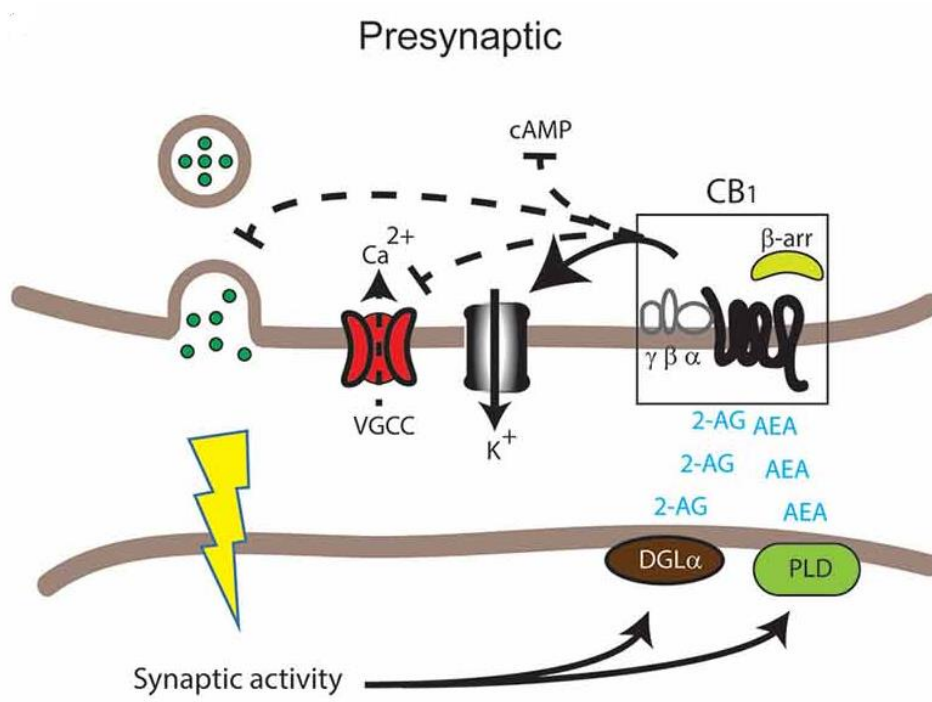


Figure 2.1. cannabinoid (CB) receptor signaling: Presynaptically arachidonylethanolamide (AEA) and 2-arachidonylglycerol (2-AG) is produced from arachidonic acid by the key enzymes: diacylglycerol lipase (DGL α) and phospholipase D (PLD). These activate the cannabinoid 1 receptor (CB1R). CB1R then inhibit cAMP accumulation, voltage-gated calcium channels (VGCC), K⁺ channels and neurotransmitter release in presynaptic excitatory and inhibitory synapses.

Postsynaptically, following activation of the CB1R by ligand binding, signaling via G protein and/or β -arrestin may occur at the plasma membrane. G proteins bind the unphosphorylated receptor while β -arrestin binds the receptor phosphorylated by G protein receptor kinases.

This figure is adapted from ‘Kendall, D.A. and Yudowski, G.A., 2017. Cannabinoid receptors in the central nervous system: their signaling and roles in disease. *Frontiers in cellular neuroscience*, 10, p.294.’ with appropriate permission

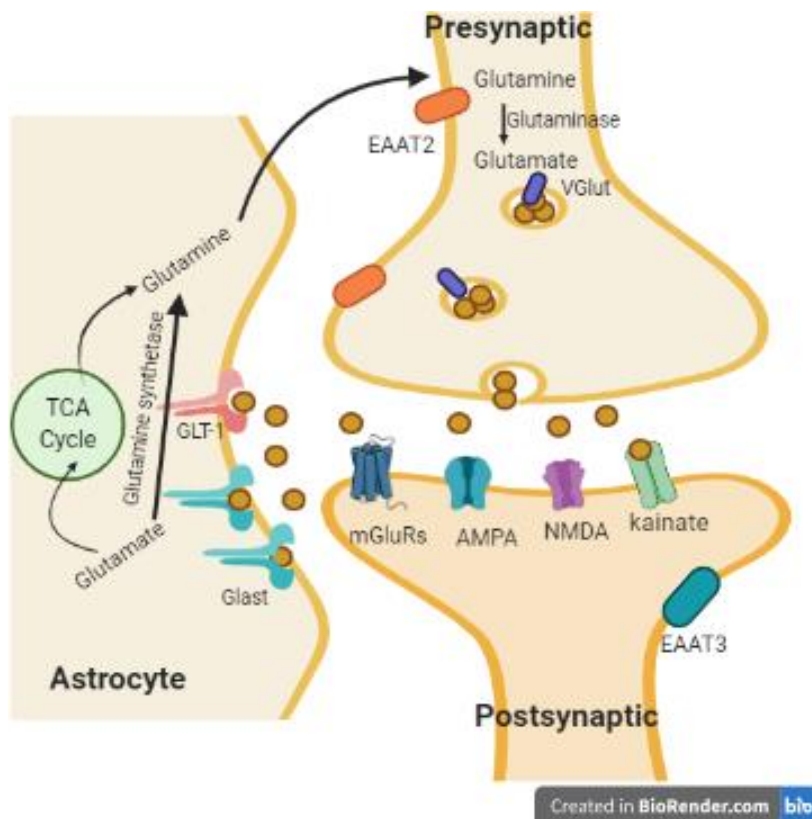


Figure 2.2: Glutamate synthesis and uptake: Glutamate is synthesized from the amino acid glutamine and is released from nerve terminals by an active process. In the synaptic space, glutamate acts on receptors of both ionotropic (AMPA, NMDA, Kainate) and metabotropic (mGluR) subtypes. The termination of glutamate action occurs by quick removal from synaptic space either by the neuronal EAAT3 transporter, primarily located on the postsynaptic membrane, or by astrocytic GLAST and GLT-1 transporters. Glutamate is subjected to transformation into glutamine either through TCA cycle or by the enzyme glutamine synthetase. The glutamine released by glial cells within the nerve terminal and taken up by neurons is converted back to glutamate. Glutamine is then transported back to neuronal cells by EAATs. This figure was “Created with BioRender.com” (<https://biorender.com/>)

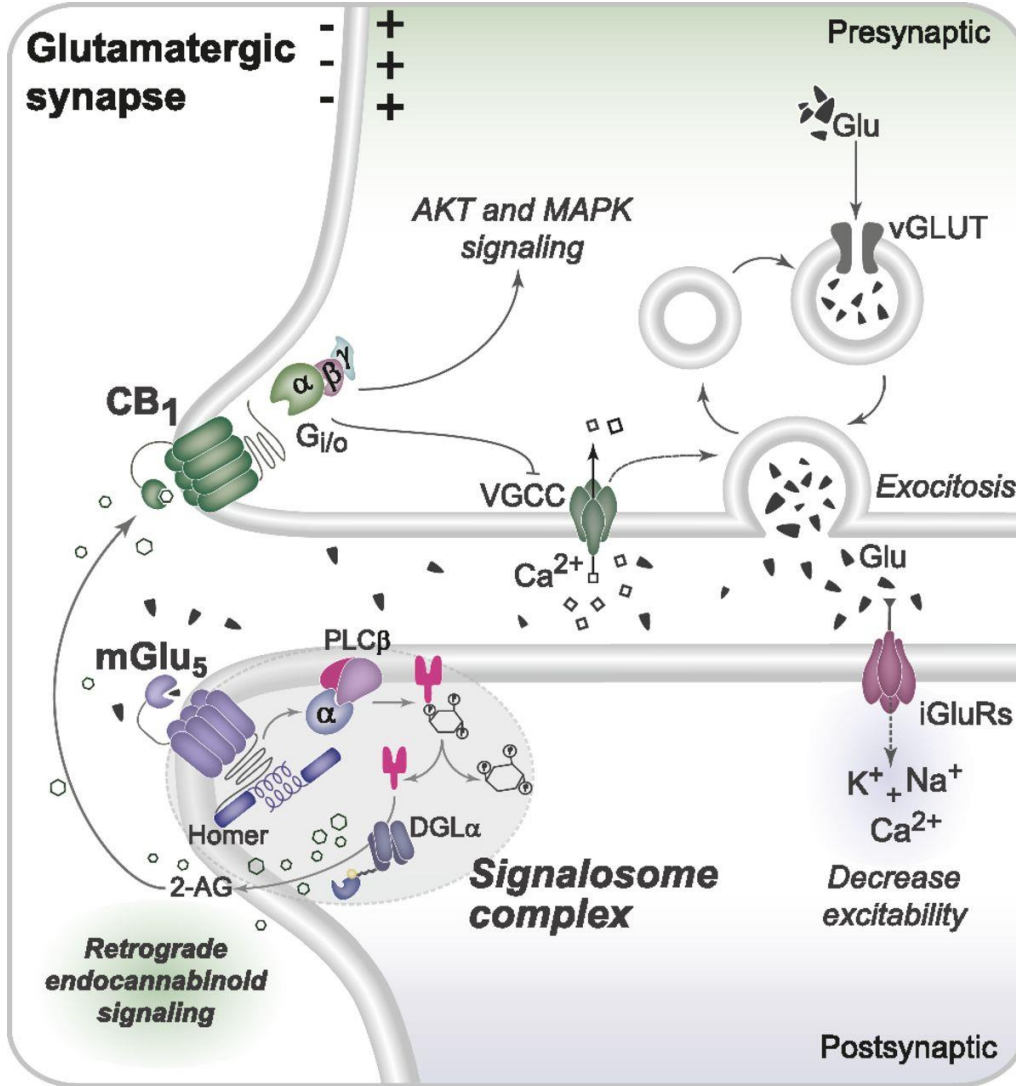


Figure 2.3 Cannabinoid and glutamate crosstalk: At glutamatergic synapses, mGlu5Rs interact with Homer protein, to form signalosome complex. mGlu5R stimulation leads to the activation of PLCβ and formation of IP3 and DAG, which is metabolized to 2-AG. 2-AG moves across the synaptic cleft and activates CB1R at the presynaptic terminal. Activation of CB1 inhibits the voltage-gated calcium channel, reducing the influx of Ca²⁺ and, consequently, regulates vesicle exocytosis and postsynaptic neurons excitability.

Activated elements are represented by continuous arrows, inhibition is represented by blocked arrows and reduced activity of the biologic process is represented by the dashed arrow. The protein complex depicted in the dashed area, named the signalosome complex, is located at the plasma membrane of the perisynaptic region. Glu, glutamate; iGluR, ionotropic glutamate receptor; VGCC, voltage-gated calcium channel; vGLUT, vesicular glutamate transporter 1.

This figure is adapted from 'Olmo, I.G., Ferreira-Vieira, T.H. and Ribeiro, F.M., 2016.

Dissecting the signaling pathways involved in the crosstalk between metabotropic glutamate 5 and cannabinoid type 1 receptors. *Molecular pharmacology*, 90(5), pp.609-619.' with appropriate permission

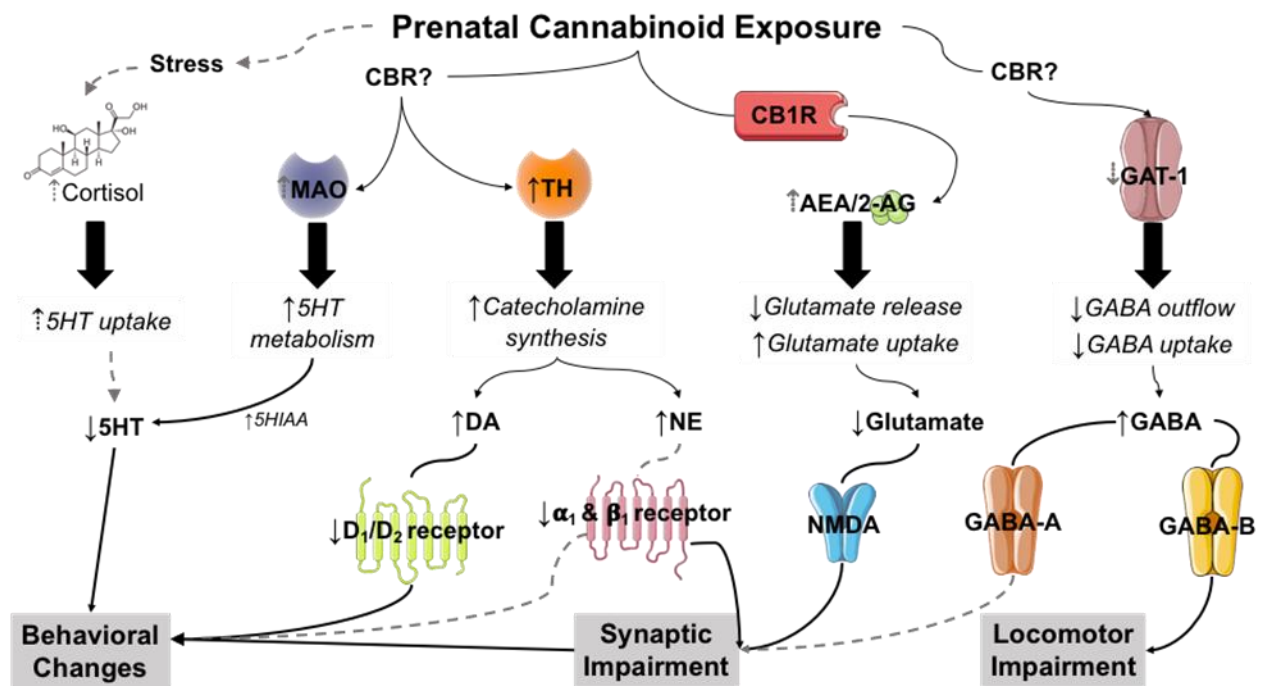


Figure 2.4: Proposed neurotransmitter system alterations following prenatal cannabinoid exposure (PCE). PCE can cause depression by decreasing serotonin levels while increasing dopamine levels. PCE can mediate cognitive impairment via an increase in norepinephrine and a decrease in glutamate level. Locomotor impairment is mediated by changes in the GABA and glutamatergic systems. Solid lines indicate an established mechanism from existing literature, while dotted lines indicate hypothetical mechanism based on the literature. CBR- Cannabinoid receptor; CB1R- Cannabinoid receptor type 1; MAO- Monoamine oxidase; TH- Tyrosine hydroxylase, AEA- Arachidonyl ethanol amine; 2-AG- Arachidonyl glycerol; GAT-1- GABA transporter type1; 5HT-serotonin; 5HIAA-5-hydroxyindoleacetic acid; D1- dopamine receptor type 1; D2- dopamine receptor type 2; NMDA- N methyl D aspartic acid; GABA-A- GABA receptor type A; GABA-B- GABA receptor type B. This figure was produced using Servier Medical Art (<https://smart.servier.com/>) and Library of science and medical Illustrations (<http://www.somersault1824.com/science-illustrations/>)

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Chapter 3 Prenatal cannabinoid exposure results in synaptic plasticity and memory deficits in adolescent offspring due to reduced PSA-NCAM expression and altered glutamate receptor mediated signaling

1. Abstract

Cannabis is now one of the most commonly used illicit substances among pregnant and lactating women. This is particularly concerning given that developmental exposure to cannabinoids has been demonstrated to induce enduring neurofunctional and cognitive alterations in clinical studies, yet there is a relative paucity of preclinical literature investigating the underlying neurocognitive alterations resulting from prenatal exposure to cannabinoids. The aim of our study is to investigate the mechanisms of learning and memory deficits resulting from prenatal cannabinoid exposure (PCE) in adolescent offspring. The synthetic cannabinoid agonist WIN55,212-2 was administered to pregnant rats during the gestational period and a series of behavioral, electrophysiological, and immunochemical studies were performed to identify potential mechanisms of memory deficits in the adolescent offspring. PCE induced deficits in hippocampal-dependent memory tasks in adolescent rats, and these behavioral deficits were associated with decreased long-term potentiation (LTP) and enhanced long-term depression (LTD) at hippocampal Schaffer collateral-CA1 synapses, as well as an imbalance between GluN2A- and GluN2B- mediated signaling. Moreover, PCE reduced gene and protein expression of neural cell adhesion molecule (NCAM) and polysialylated-NCAM (PSA-NCAM), which is critical for GluN2A and GluN2B signaling balance. Restoration of PSA-NCAM activity restored the LTP deficits observed in PCE animals, suggesting PSA-NCAM mediated alterations in GluN2A- and GluN2B- signaling pathways are responsible for the altered synaptic plasticity in

hippocampus resulting from PCE. These findings enhance the current understanding of how PCE affects cognition, and how this process can be manipulated for future therapeutic purposes.

2. Introduction

Cannabis is the most commonly used illicit drug with current users of approximately 192 million people worldwide (UNODC, 2018). Cannabis use has increased in the American continent in the past decade from 40.5 million people in 2006 (6.9% of the population aged 15–64 years) to 52.9 million in 2016 (8% of the population aged 15–64 years). Its use during pregnancy has also increased dramatically in the past 10 years (Q. L. Brown et al., 2017), and the recent relaxation of marijuana policies in several countries is expected to result in even greater maternal use (J. T. Jones, Baldwin, & Shu, 2015). The major psychoactive ingredient in marijuana and other cannabis-based preparations is Δ^9 -Tetrahydrocannabinol (THC), which can cross the placental barrier to expose the fetus (Asch & Smith, 1986; Grant et al., 2018). As the prenatal brain is particularly sensitive to maternal drug use during ongoing brain development, prenatal cannabinoid exposure (PCE) has been demonstrated to cause behavioral and cognitive alterations in the offspring. These deficits persist and include increased impulsivity and hyperactivity in young children (L Goldschmidt, Day, & Richardson, 2000; G. A. Richardson, Ryan, Willford, Day, & Goldschmidt, 2002) and memory and executive function impairment during adolescence and early adulthood (Noland et al., 2005; C.-S. Wu et al., 2011).

The cannabinoid receptor type 1 (CB1), which is more densely expressed in the brain than CB2 receptors (Kendall & Yudowski, 2017b), emerge early during prenatal brain development and are functionally coupled to signal transduction mechanisms from early prenatal stages in both

rodents (F Berrendero et al., 1998) and humans (Mato, Del Olmo, & Pazos, 2003). Given that CB1 receptors play an important role in CNS development, affecting synaptogenesis, proliferation and migration of neuronal cells, functional synaptic organization, and signal transduction (as reviewed in Harkany et al., 2007), persistent cognitive deficits after PCE are not surprising. Nevertheless, there is still a relative paucity of literature regarding the mechanisms mediating cognitive deficits resulting from PCE (de Salas-Quiroga et al., 2015a; Mereu et al., 2003). This is particularly concerning given the high density of CB1 receptors in brain regions devoted to higher cognitive function, including the hippocampus (Herkenham et al., 1991; Mackie, 2005), as well as the long-lasting functional changes in the glutamatergic system mediated by CB1 receptors in the hippocampus (Colizzi et al., 2019; Sánchez-Blázquez et al., 2014)

In the current study, we used a rodent model to determine the long-lasting consequences of PCE on hippocampal glutamatergic neurotransmission and hippocampal-dependent memory during adolescence, an age when observational human studies have demonstrated PCE results in memory deficits (P A Fried & Watkinson, 2001; Peter A. Fried, Watkinson, & Gray, 2003). We also, for the first time, identified a potential mechanism for these deficits, notably a reduction in polysialylated neural cell adhesion molecule (PSA-NCAM). NCAM is a transmembrane glycoprotein, essential for maintaining neurite outgrowth, cell migration, and synaptogenesis. PSA, a highly negatively charged homomeric polymer of sialic acid, is attached to the extracellular domain of NCAM and greatly affects NCAM function (Kochlamazashvili et al., 2010; O. Senkov et al., 2006; Oleg Senkov, Tikhobrazova, & Dityatev, 2012a). PSA-NCAM is a well-established mediator of hippocampal glutamatergic neurotransmission (Gascon, Vutskits, & Kiss, 2007), being required for both long-term potentiation (LTP) and long-term depression

(LTD) in the hippocampus (Becker et al., 1996; Eckhardt et al., 2000). PSA-NCAM is found at both synaptic and extrasynaptic sites, where it facilitates activity of AMPA receptors and restrains activity of extrasynaptic GluN2B-containing NMDA receptors, respectively (Kochlamazashvili et al., 2012, 2010; Oleg Senkov, Tikhobrazova, & Dityatev, 2012b; Vaithianathan et al., 2004). We have previously shown that perturbation in either polysialylation or NCAM expression causes deficits in hippocampal LTP and hippocampal-dependent memory tasks (O. Senkov et al., 2006), and these alterations can be rescued by blocking extrasynaptic GluN2B activity (Kochlamazashvili et al., 2010) Together with our current findings, we propose that PCE leads to alterations in glutamatergic synaptic plasticity through reductions in PSA-NCAM, ultimately leading to memory deficits.

3. Materials & Methods

Animals

Time pregnant Sprague Dawley rats were purchased from commercial vendor (Envigo laboratories). Pregnant dams were anaesthetized on the gestational day 3 (GD3) under isoflurane anesthesia to implant an osmotic mini pump (Alzet, 2004) subcutaneously. The pump, which dispensed the drug until the delivery of the pups, was filled with either vehicle or the cannabinoid agonist, WIN55,212-2 (Sigma-Aldrich). The dose of WIN55,212-2 (2mg/kg body weight/day) was based on previous studies that corresponds with moderate to heavy cannabinoid exposure (Campolongo & Trezza, 2012; French, Dillon, & Wu, 1997; R. E. Hampson & Deadwyler, 2000; Tortoriello et al., 2014). The pumps were removed from the mothers on post-

natal day 2 (PND2), so that the offspring were exposed to the drugs primarily during the gestational period. Offspring were weaned at post-natal day (PND) 21 and housed in groups of three per cage. Behavioral experiments were performed first at PND 40-50 with electrophysiological experiments later performed in the same animals at PND 50-60. A separate group of pups were sacrificed at PND 42 to collect the brain sample for PCR and immunoblotting.

All animals were housed in a vivarium maintained on a 12 h: 12 h light: dark cycle (lights on at 6:00 am) and at a temperature of 22–24 °C. All experimental procedures were approved by the Auburn University Animal Care and Use Committee (IACUC) and conducted in compliance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

Behavioral Testing

Pre-handling

Prior to behavioral testing, animals were acclimated to transport and handling by pre-handling for 5 days. Pre-handling consisted of daily weighing and 5 minutes of gentle handling while walking.

Open Field

The rat was placed in a transparent 60 x 60 cm Plexiglas arena and allowed to explore for 10 minutes, after which the rat was returned to its home cage. The trial was recorded via a camera placed above the open field and EthoVision software (Noldus Information Technologies) was used to track the rat. A virtual center square (measuring 45 x 45 cm) was defined as the ‘center zone’, while the remainder was defined as the ‘outer zone’. Anxiety parameters included the number of entries into, and time spent in (sec), the center zone of the arena. Activity measures included mean speed (cm/s) and distance traveled (cm) in the whole arena.

Contextual Fear Conditioning

Contextual fear conditioning training occurred in a MED-VFC-008 chamber (12"H × 9.5"W × 8.25"H; Med Associates Inc.) situated in sound-attenuating cabinets located in a brightly lit and isolated room. Through a connection from the PC to the chamber, the software (MED-SYST-VFC, Med Associates Inc.) controlled the test shock current. After a shock current was delivered, the program measured freezing behavior, defined as the absence of motion except that required for respiration, for 1 s. The software calculated the percent of freezing using data from the near infrared camera. Each chamber was scented by placing 1 mL of undiluted Pine-Sol in a small, open container in the sound-attenuating chamber. A 70% ethanol solution was used to clean the chamber after each trial.

The contextual fear conditioning procedure was conducted over 2 days. On day 1, animals were placed in the conditioning chamber, and 180s later, a 2s, 0.75mA foot-shock was delivered.

Animals were removed from the context 30s after foot-shock delivery. Freezing behavior prior to

the onset of the shock was used to compare baseline levels of freezing among the groups. On day 2, 24 hours later, the animals were returned to the training context and exposed to all contextual stimuli, but not the shock, for 180 seconds. Freezing behavior during the 180-second period was used to compare contextual fear memory among the groups.

Morris Water Maze

Water maze rapid acquisition was performed as previously described (Fakhfouri et al., 2012; Farahmandfar, Karimian, Naghdi, Zarrindast, & Kadivar, 2010). Briefly, the apparatus was an opaque black circular pool of 182.88 cm in diameter filled with water colored black with tempera non-toxic paint. The hidden platform was 10 cm in diameter and located approximately 2 cm beneath the surface of the water. Extramaze cues of varying color, dimensions, and shapes were placed about the room. During training, the water temperature was kept in a range of 19 to 22° C. The rats received eight training trials in which they were released into pool from four different start locations (North, South, East, and West). Each start location was repeated twice, and the start locations were randomized. Animals were allotted 60 seconds to find the submerged platform. The animals then had to remain on the platform for 15 seconds before being removed from the pool. In the event that the animals were unable to locate the platform, they were then gently guided to the platform by the experimenter. After each trial, the animals were gently dried during the intertrial interval of 30 seconds and allowed to sit in a clean cage on top of a heating pad before resuming the next trial. For the rapid acquisition training, pathlength (or distance to locate the platform in cm) was compared between groups. To assess hippocampal-dependent spatial reference memory, a probe trial was conducted twenty-four hours after the last training

session. For the probe trial, the platform was removed, and animals were placed in the pool for 60 seconds. Percent time spent in the target quadrant versus the average of the other three quadrants was compared within and between groups.

Electrophysiological Recording

Preparations of acute hippocampal slices

Pups were sacrificed at PND 50-60 using CO₂ and whole brain were obtained after decapitation. Transverse hippocampal slices (350 μ m) were prepared as described earlier (Parameshwaran et al., 2013). Briefly, hippocampal slices were sectioned using a Leica VT1200 S Vibratome (Leica Biosystems Inc., Buffalo Grove, IL, United States). During the slicing, the brain was submerged in ice cold cutting buffer containing 85 mM NaCl, 2.5 mM KCl, 4.0 mM MgSO₄, 0.5 mM CaCl₂, 1.25 mM NaH₂PO₄, 25mM NaHCO₃, 25 mM glucose, 75 mM sucrose, 0.5 mM ascorbate. The solution was bubbled with 95%O₂/5%CO₂, Slices were incubated for at least one hour in room temperature before electrophysiological recording in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.5 MgSO₄, 2.4CaCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃ and 10-D glucose. (for detailed methods see (Alhowail et al., 2019; Parameshwaran et al., 2013))

Extracellular field recordings

Electrophysiological field recordings were performed as described previously (Alhowail et al., 2019; S. Bhattacharya et al., 2017) After at least one hour of incubation slices were transferred to a recording chambers continuously perfused in ACSF bubbled with 95%O₂/5%CO₂ at 30°C. Field excitatory postsynaptic potentials (fEPSP) were recorded from Schaffer collateral pathway of the hippocampus. CA3 region was stimulated with a platinum bipolar electrodes and the recording glass electrode filled with ACSF (2-6 MΩ), was placed in CA1 stratum radiatum at approximately 200 μm away from the stimulating electrode. The basal synaptic transmission was recorded in response to increasing stimulus intensity between 0 to 0.2 mA every 20s. For paired pulse facilitation (PPF) and LTP experiments, current intensity was set at 50% of maximal fEPSP when a pop spike occurs. Interpulse intervals were set to 20, 50, 100, 150 and 200 ms in PPF experiments. In LTP experiments, after 10 min of stable baseline recording, induction was initiated by using theta burst stimulation (TBS) protocol. Five TBS sweeps were applied with an inter-TBS interval of 20 s. Each TBS consists of 10 bursts delivered at 5 Hz, each burst containing 4, 0.2 ms, pulses at 100 Hz. LTP was recorded for 60 min post TBS. For LTD recording, induction was given using two low frequency stimuli (LFS: 900 pulses at 1 Hz) delivered at an interval of 10 min and preceded by 10 min of stable baseline. Stimulation intensity was set at 60% (during LFS) or 40% (all other times excluding LFS) of the amplitude at which initial population spikes began to appear. LTD and LTP were measured as an average of fEPSP slopes from 50-60 minutes after the end of induction. Field potentials were recorded using LTP software with Axoclamp 2B (Axon Instruments, Foster City, CA) and analyzed using WinLTP software.

Preparation of synaptosomes and single channel electrophysiology

Synaptosomes were prepared by previously described methods (Johnson, Chotiner, & Watson, 1997; Suppiramaniam, Vaithianathan, & Parameshwaran, 2006) in which hippocampi were dissected out and homogenized in a buffer (mKRBS) consisting of (in mM): 118.5 NaCl, 4.7 KCl, 1.18 MgSO₄, 2.5 CaCl₂, 1.18 KH₂PO₄, 24.9 NaHCO₃, 10 dextrose and 10 mg/ml adenosine deaminase. The pH was adjusted to 7.4 by bubbling with 95% O₂/5% CO₂. The buffer was also supplemented with 0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin and 5 mM benzamide to minimize proteolysis. The homogenate was filtered twice and centrifuged at 1000g for 15 min at 4 °C. The supernatant was removed, and the pellets, which contained synaptosomes, were resuspended in mKRBS buffer (Bloemer et al., 2019).

Incorporation of NMDARs from synaptosomal fractions in artificial lipid bilayers was carried out using ‘tip-dip’ method (Suppiramaniam et al., 2006; Vaithianathan et al., 2005). Briefly, a thin phospholipid bilayer was formed at the tip of a borosilicate glass pipette (100 M) and filled with artificial intracellular (110 mM KCl, 4 mM NaCl, 2 mM NaHCO₃, 1 mM MgCl₂, 0.1 mM CaCl₂, and 2 mM 3-N-Morpholino propanesulfonic acid (MOPS); pH 7.4) fluid. The synthetic phospholipid was prepared by dissolving 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) in anhydrous hexane (Aldrich Chemical Co., Milwaukee, WI) at a concentration of 1 mg/ml. Approximately 3–5 µl of the synthetic phospholipid was delivered into 500 µl of extracellular bath solution (125 mM NaCl, 5 mM KCl, 1.25 mM NaH₂PO₄, and 5 mM Tris HCl). Bilayer formation was initiated by successive transfer of two monolayers onto the tip of the patch pipette in an asymmetric saline condition with “outside-out” configuration. After forming a stable membrane, 3-5 µl suspension of the synaptosomes was delivered to the bath solution. Single-channel currents were elicited by application 3 µM glutamate. In presence

of an AMPA blocker (DNQX), NMDA currents were recorded at holding voltages averaging ± 65 mV. Single channel currents were low-pass filtered (2 kHz), digitized (5 kHz) (DigiData 1440B, Molecular Devices), acquired with pClamp10 software (Molecular Devices), and saved on a computer for off-line analysis. Only the data exhibiting long stretches of single channel current transitions without major base line drifts were chosen for quantitative analysis. All points-current amplitude histograms were constructed and fitted with Gaussian method to identify individual conductance levels. Single channel open probability was computed from areas under the current-amplitude histograms.

Immunoblotting

Half of rat hippocampus tissue was homogenized in 200 μ l of RIPA buffer (Thermo Fisher Scientific) in an Eppendorf tube fit with a small homogenizer, followed by pipetting 10 times with a 27-g-needle syringe. Half of the lysate was saved for RNA isolation, and the remaining half was diluted with 500 μ l RIPA buffer and continuously lysed at 4°C for 20 min on a rotator, then centrifuged at 11,000g at 4°C for 10 min. The supernatant was collected and the pellet was discarded. Protein concentration was measured by BCA assay kit (Pierce BCA Protein Assay Kit, ThermoFischer Scientific). 15 μ g of protein underwent electrophoresis and was then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, pore size 0.45 μ m). Membranes were blocked in 5% non-fat dry milk in mM Tris-HCl, pH 7.4, 150 mM NaCl buffer with 0.1% Tween 20 (TBST) for 4 hrs at room temperature (RT) followed by overnight incubation with the primary antibody at 4°C. Primary antibodies (GluA1, GluN2A, GluN2B, p44/42 MAPK, p-p44/42 MAPK, p38MAPK, pP38 MAPK, CAMKII, pCAMKII) were brought

from cell signaling technology and was used at 1:1000 dilution in 5% BSA in TBST except these: Anti NCAM antibody, EMD Millipore, 1:500; Anti-Cannabinoid Receptor I antibody, abcam, 1:200, PSA-NCAM from Dr. Alexander Dityatev's lab, 1:200; GAPDH, Calbiochem, 1:10,000, Anti BDNF antibody, abcam, 1:500 dilution in 5% BSA in TBST) at 4°C. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:3000) for 1.5 hrs at room temperature. Immunoreactivity was visualized using enhanced chemiluminescence (Thermo Fisher) in a FluorChem Q imager system (Protein Simple). Protein band intensity was quantified by Image J software.

RNA isolation and Quantitative PCR

Half of hippocampus lysate (100 µl) was mixed well with 1 ml of Trizol reagent (Life Technologies) and incubated for 5 min at RT. After adding 200 µl of chloroform the mixture was shaken vigorously by hand for 15 s followed by 2-min incubation at RT. The homogenate was then centrifuged at 12,000g for 15 min at 4°C. The upper phase was carefully transferred to a new Eppendorf tube and mixed with an equal volume of 70% ethanol, then loaded in a spin cartridge from PureLink RNA mini kit (Ambion). RNA was purified and DNA-freed following the kit protocol. RNA concentration was measured using Nano drop, and 1 µg of RNA was used to make cDNA by Superscript III first strand synthesis system for RT-PCR (Invitrogen) according to manufacturer's guidelines. Then, 1.5 µl of cDNA was used to set up each 20-µl RT-PCR reaction mixture following SYBR Green Master Mix manual (Thermo Fisher). Primers for RT-PCR were as follows: GAPDH forward 5'-GGTGAAGGTCGGTGTGAAC G-3' and reverse 5'-CCTTGACTGTGCCGTTGAA -3'; NCAM forward 5'- CATCTGCACTGCCAGCAACA -

3' and reverse 5'- CTTGGGTAGGCAAAGACCTCACA -3'. Quantitative PCR was performed using the ABI 7500 Real Time PCR system. Each sample was performed in triplicates, and each experiment was repeated at least 3 times. GAPDH was used as an endogenous control for normalization. The method of $2^{-\Delta\Delta C_t}$ was used to analyze data.

In Vivo Glutamate Recordings

To examine changes in tonic extracellular glutamate and glutamate clearance, a ceramic-based microelectrode array with platinum recording sites (Quanteon, Nicholasville, KY) coated with glutamate oxidase with an attached glass micropipette for drug delivery was inserted into the CA1 region of the hippocampus (coordinates from the bregma were AP: 4.1 mm, ML: 3.5 mm, DV: 3.5) after calibration, as previously described (Hunsberger, Hickman, & Reed, 2016; Hunsberger, Konat, & Reed, 2017; Hunsberger, Rudy, Batten, Gerhardt, & Reed, 2015). All MEA recordings were performed using isoflurane to avoid anesthetic-induced changes in resting glutamate levels (Mattinson et al., 2011) and were performed at 10 Hz using constant-potential amperometry. To measure glutamate uptake, animals received 1–2 injections at 50 nL increments within a 50–250 nL range of 200 μ M glutamate (Sigma-Aldrich, St. Louis, MO) delivered every 2–3 minutes in one hemisphere. Temporal clearance of glutamate was monitored and expressed as the net area under the curve (AUC). The amperometric data were analyzed using a custom Microsoft Excel software program (MatLab).

Statistical Analysis

Data analysis was performed using JMP (SAS, Cary, NC, USA) and GraphPad PRISM. Statistical analysis consisted of two-way ANOVAs or repeated measures ANOVAs (RMANOVAs) in which the main effects of Treatment (Control vs. PCE) and Sex (male, female), as well as the interaction between the two (Treatment*Sex) were assessed. Outliers were eliminated from data using a Grubb's test, also called the ESD method (extreme studentized deviate) before analyses were conducted. Results were presented as mean \pm SEM, and differences between groups were considered statistically significant at $p \leq 0.05$. Unless otherwise noted, there were no differences between the sexes and no Treatment*Sex interactions, and thus, focus is given to the effect of Treatment.

4. Results

4.1 Bodyweights

Exposure to WIN55,212-2 (2 mg/kg/wt) did not result in smaller litter sizes, pup mortality differences, or produce gross abnormalities. Bodyweights were assessed as part of pre-handling prior to behavioral testing. For females, PCE did not alter bodyweights compared to controls (Day*Treatment: $F(4,44)=2.82$, $p=.1$ Figure 3.1A). In contrast, PCE males initially weighed more than control males, though this difference decreased across the days (Day*Treatment: $F(4,40)=7.43$, $p=.008$, Figure 3.2B).

4.2 PCE decreases anxiety without altering general locomotor activity

To assess general locomotor activity, animals were placed in an open arena and allowed to explore for 10 minutes. There were no differences in the total distance traveled ($F(1, 24)=0.62$, $p=.44$; Figure 2A) or mean speed ($F(1, 24)=0.66$, $p=.43$; Figure 3.2B), suggesting PCE did not produce overt alterations in locomotor activity. Because rodents will typically spend more time close to the wall, an anxiety-like response referred to as thigmotaxis, as opposed to the unprotected center area (Lamprea, Cardenas, Setem, & Morato, 2008), we next examined the number of entries into, and time spent in, the center portion of the arena during the open field task to provide an initial screen for anxiety-related behavior (Prut & Belzung, 2003). Though PCE only marginally increased the number of entries into the center zone ($F(1, 23)=3.76$, $p=.07$; Figure 3.2C), the time spent in the center zone was significantly increased in PCE animals compared to controls ($F(1, 23)=4.76$, $p=.04$; Figure 3.2D), suggesting a reduction in anxiety in cannabinoid-exposed animals.

4.3 PCE impairs hippocampal-dependent learning and memory

We next assessed whether PCE would impair hippocampal-dependent contextual fear memory. During training, no significant differences in percent time freezing between controls and PCE animals were observed before the first shock presentation ($F(1, 24)=0.01$, $p=.91$; Figure 3.3A), indicating similar levels of baseline freezing and activity for the two groups. The following day, animals were placed back in the training context, and retention of contextual CS:US memory was assessed. Both groups froze significantly more the second day compared to their respective baseline freezing levels ($ps<.001$), suggesting learning did occur in both groups. However, PCE animals exhibited a significant decrease in the percent time spent freezing during contextual fear

retention compared to control animals ($F(1, 24)=6.5, p=.018$; Figure 3.3A), suggesting an impairment in hippocampal-dependent contextual fear memory.

To determine if hippocampal-dependent spatial learning and memory were also impaired, the Morris water maze (MWM) was used. PCE animals did not differ from controls in their acquisition of hidden platform training across the eight training trials ($F(7,15)=0.85, p=.57$; Figure 3.3B). Twenty-four hours after the last training trial, a probe trial was conducted to assess hippocampal-dependent spatial reference memory. Whereas controls exhibited a preference for the target quadrant relative to the average of the other three quadrants ($F(1,14)=10, p=.007$; Figure 3.3C), PCE animals did not ($F(1,9)=0.1, p=.75$), indicating PCE induces deficits in hippocampal-dependent spatial reference memory (Figure 3.3C).

4.4 PCE alters basal hippocampal glutamatergic synaptic transmission

We next determined whether the observed memory deficits in PCE rats are related to alterations in glutamatergic neurotransmission by examining basal synaptic transmission in acute hippocampal slices. For basal synaptic transmission, the slope of fEPSP responses were measured at increasing stimulus intensities. fEPSP slopes were reduced in PCE animals at higher stimulus intensities compared to controls ($F(1,13)=16.5, p=.001$; Figure 3.4A), suggesting a deficit in baseline glutamatergic synaptic transmission. To determine whether the deficit in basal synaptic transmission in PCE group is due to alterations in presynaptic axonal recruitment, we analyzed the presynaptic fiber volley (FV) amplitude and slope of EPSPs from CA1 synapses at different stimulus intensities. Summation of action potentials arriving at the CA1 region

following Schaffer collateral stimulation are represented by FV amplitudes, which provides a measure of the number of presynaptic neurons recruited (Parameshwaran et al., 2012). FV amplitudes in PCE animals were higher than that of controls at increasing stimulus intensities ($F(1,10)=29.96, p=.0005$; Figure 3.4B), suggesting an increase in the number of active afferent axons in hippocampus in PCE animals. We next sought to determine whether this increase in active afferents, as observed by increased FV amplitude, leads to an increase in presynaptic release probability by measuring paired pulse facilitation (PPF) across a range of inter-stimulus intervals. PCE animals demonstrated a lower PPF value ($F(1,9)=7.63, p=.02$; Figure 3.4C), indicative of a higher release probability of glutamate. However, when fEPSP was compared to fiber volley amplitude (presynaptic action potential) across a range of increasing stimuli, PCE animals exhibited a significant reduction in basal synaptic transmission ($F(1,9)=19.49, p=.002$; $p=.04$; Figure 3.4D). Furthermore, we also observed a significant increase in vesicular glutamate transporter ($F(1,11)=9.77, p=.01$; Figure 3.4E,F) in PCE animals, which may have contributed to the increased release as observed by PPF. The increased glutamate release in PCE animals is contrary to what is expected based on the stimulus/response curves, it is reasonable to presume that saturation of synaptic glutamate receptors by excessive glutamate concentration could have resulted in receptor desensitization and thus decreased responses to stimuli (Yamashita, Kanda, Eguchi, & Takahashi, 2009).

4.5 PCE decreases LTP and enhances LTD in the hippocampus

To determine whether the change in basal and presynaptic transmission in PCE animals also alters synaptic plasticity, we utilized acute hippocampal slices to induce LTP or LTD in the

Schaffer collateral pathway. The average slope, as a percentage of baseline, showed approximately 50% diminished LTP maintenance in PCE rats compared to controls ($F(1,19)=46.46$ $p<.0001$; Figure 3.5A,B). Because we observed LTP deficits in the PCE group, we next investigated whether the LTP induction in these animals was also impaired. When amplitudes of the first EPSPs elicited within each sweep were normalized to the first train, each subsequent pulse showed significantly reduced potentiation ($F(1,9)=5.69$, $p=.04$; Figure 3.5C), indicating an impairment in LTP induction preferably through reduced GluN2A expression and associated signaling. In a separate set of experiments, LTD was compared between the groups, and a long-term reduction of the fEPSP slope by >20% from baseline in control slices as compared to a >40% reduction in PCE slices was observed ($F(1,11)=59.78$, $p<.0001$; Figure 3.5 D,E). Together, these results demonstrate that PCE results in a loss of LTP and enhancement of LTD in adolescence (PND 50-60).

4.6 Effects of PCE on single-channel properties of synaptosomal NMDARs

Alterations in single-channel activity could result in alterations in overall synaptic currents and LTP. Since our LTP induction protocol is mainly NMDA dependent and PSA-NCAM can alter NMDAR activity (Kochlamazashvili et al., 2012, 2010), the deficits in LTP induction suggest an alteration in either NMDA receptor functioning (Shipton & Paulsen, 2014). Analysis of synaptic NMDAR single-channel currents from synaptosomes reconstituted in lipid bilayers revealed an increase in the channel open probability (P_o) in PCE offspring compared to the controls ($F(1,19)=7.00$ $p=.01$; Figure 3.6) as represented by the upward transition of the current. This

suggests that alterations in single-channel properties of NMDARs may mediate the synaptic plasticity deficits observed in PCE animals.

4.7 PCE results in reduced NCAM and PSA-NCAM expression in the hippocampus

Studies have shown an impairment of LTP in the CA1 region after perturbation of PSA-NCAM (Muller et al., 1996). Hence, we investigated NCAM and PSA-NCAM expression in the hippocampus of PCE animals and observed a reduction in mRNA level of NCAM ($F(1,18)=10.99$, $p=.004$; Figure 7A) in the PCE animals. We also observed a significant reduction in NCAM and PSA-NCAM protein levels in the PCE rats ($F(1,12)=4.68$, $p=.05$; $F(1,5)=12.29$, $p=.02$, respectively; Figure 3.7B,C).

4.8 PCE alters PSA-NCAM mediated downstream glutamatergic signaling

LTP is dependent on NCAM and PSA-NCAM level in the hippocampus as we have previously observed that perturbation in either polysialylation of NCAM or NCAM expression causes alterations LTP/LTD by reducing GluA1 (Francija et al., 2019; Sytnyk, Leshchyns’Ka, Nikonenko, & Schachner, 2006) and GluN2A mediated signaling while enhancing extrasynaptic GluN2B signaling (Kochlamazashvili et al., 2010; O. Senkov et al., 2006). Hence, we next evaluated the levels of GluA1, GluN2A, and GluN2B, as well as their downstream signaling pathways in whole hippocampal extracts. We observed a significant reduction in the mRNA level of GluA1 and GluN2A but no significant changes in GluN2B mRNA level ($F(1,18)=4.70$,

$p=.04$; $11.02, p=.004$; $3.61, p=.07$, respectively; Figure 3.8A). We also observed a PCE-mediated decrease in both total GluA1 and phosphorylation of GluA1 Ser831 ($F(1,9)= 18.38, p=.002$; $24.77, p=.001$, respectively; Figure 8B,C). Phosphorylation of Ser831 increases the conductance of GluA1 in the presence of transmembrane AMPA receptor regulatory proteins, facilitating AMPAR insertion and thus implicated in the maintenance of LTP (Henley & Wilkinson, 2013). In congruence with the downregulation of GluA1 expression, the downstream phosphorylation of CAMKII was also decreased in PCE animals ($F(1,5)=11.07, p=.02$), as was BDNF ($F(1,9)= 5.62, p=.04$; Figure 3.8 B,C). GluN2A receptor levels, as well as the downstream signaling pathway that includes pERK and Ras-grf1, were also significantly decreased in the hippocampus of PCE animals ($F(1,7)=11.08, p=.01$, $F(1,9)=5.78, p=.04$; $F(1,11)=,5.99, p=.03$, respectively; Figure 3.8 D,E). Although we have observed no changes in the GluN2B receptor levels ($p>.05$), an increase in the phosphorylation of the p38 was observed ($F(1,7)=5.59 p=.04$; Figure 3.8F,G) in the hippocampus of PCE animals, suggesting an increase in GluN2B signaling possibly due to increased receptor activity.

Next, we examined whether the glutamate receptor mediated imbalance results in glutamate clearance from the extracellular space. A decrease in glutamate uptake in the CA1 region has been observed in PCE animals in response to rapid application of glutamate via a micropipette into the extracellular space ($F(1,16)=6.9, p=.0087$;Figure 3.8C). The reduced uptake in turn can increase the glutamate level in extracellular space potentially leading to an increase in extrasynaptic GluN2B activation and signaling.

4.9 Exogenous application of a bacterially derived PSA compound, colominic acid, ameliorates LTP deficits in PCE animals

Next, we investigated whether application of a PSA compound, colominic acid (CA) sodium salt derived from *E. Coli* (6 μ M), in acute hippocampal slices for 20 min before induction could restore LTP in the PCE group (O. Senkov et al., 2006). Application of CA led to an increase in LTP in the PCE group. The average EPSP slope in PCE animals 50–60 min after TBS was 147.1 ± 3.506 ($F(2,23)=23.9$, $p<.001$; Figure 3.9B). Within train data analysis revealed an increase in the amplitude of sweep EPSPs ($F(2,15)= 4.36$, $p=.03$; Figure 3.9 C) following CA application, denoting increased NMDAR activation. Based on our findings and previously published data (Kochlamazashvili et al., 2012, 2010; O. Senkov et al., 2006), we can hypothesize that PCE may alter synaptic plasticity via a reduction in PSA.

5. Discussion

The current study demonstrates that PCE results in hippocampus-based learning and memory deficits in a rodent model that can persist to the adolescent period of the offspring. These behavioral and synaptic plasticity deficits were associated with alterations in glutamate receptor levels in the hippocampus along with alterations in their downstream signaling. Furthermore, deficits in hippocampal PSA-NCAM levels were identified as a therapeutic target, that when increased, restored the observed synaptic deficits induced by PCE.

We have used a pregnant rodent model for investigating gestational drug exposure effects (D Bhattacharya et al., 2015; Parameshwaran et al., 2013). Based on existing reports, 5 mg/kg of Δ 9-THC in rats corresponds to a moderate exposure of the drug in humans (García-Gil et al.,

1997; García-Gil, Ramos, Rubino, Parolaro, & Fernández-Ruiz, 1998), and WIN55,212-2 is estimated to be 3-10 times more potent depending on the outcome measured (French et al., 1997; R. E. Hampson & Deadwyler, 2000). Hence, we estimate that a dose of 2mg/kg corresponds to a moderate exposure in humans (Campolongo & Trezza, 2012), especially given that the potency of marijuana available in the United States has been steadily increasing over the last few decades. In our study, WIN55,212-2-treated dams did not show any overt signs of toxicity or resulted in any gross malformations or altered reproductive and developmental parameters such as reduced weight or increased mortality rate in the offspring. We have opted to examine the consequences of PCE on the adolescence period (PND 40-65) because most studies examining the consequences of PCE in humans have focused on this period, allowing for comparison of our results to those obtained in humans. Observational human studies demonstrates prenatal cannabinoid exposure results in cognitive impairments, including impairments in memory, analysis, and attention, during the adolescent period (P A Fried & Watkinson, 2001; Peter A Fried, Watkinson, & Gray, 2003). Since, adolescent success is highly predictive of adulthood outcomes (Kansky, Allen, & Diener, 2016; Seiffge-Krenke, Luyckx, & Salmela-Aro, 2014), deficits during this period are likely to produce long-lasting consequences even if neurological alterations associated with PCE do not persist into adulthood.

Though PCE has previously been shown to induce motor hyperactivity during infancy and adolescence both in animal (Boulanger et al., 1995; Mereu et al., 2003) and human studies (P.A. Fried & Smith, 2001; Lidush Goldschmidt, Richardson, Cornelius, & Day, 2004), in the current study, we did not observe any gross abnormalities in motor activity. The discrepancy might be due to variation in the dose administered, route of administration or the age when the offspring

where examined (Pinky et al., 2019b). A reduction in the social interaction along with an anxiety like behavior was reported previously in perinatally cannabinoid exposed offspring (Trezza, Campolongo, et al., 2008). However, we observed a reduction in the anxiety parameters in our study similar to adult animals where an anxiolytic effects of cannabidiol has been observed (Campos et al., 2013). The anxiolytic and/or anxiogenic effects of cannabinoids is mainly dose dependent with lower doses tending to be anxiolytic and higher doses tending to be anxiogenic (Rubino et al., 2007, 2008) as well as environment-dependent and CB1R activation dependent (M. Martin, Ledent, Parmentier, Maldonado, & Valverde, 2002; Onaivi E S, 1990; Rey et al., 2012)

Cannabinoid exposure during adolescent and adult periods also negatively affects spatial memory (Abboussi et al., 2014; Abush & Akirav, 2012; Cha, White, Kuhn, Wilson, & Swartzwelder, 2006). Similarly, we have observed poor performance in PCE offspring in MWM and CFC task in our study. The observed spatial memory deficits may be due to an increase in endocannabinoid level as speculated in adolescent studies (Tomas-Roig et al., 2017; Zumbrun, Sido, Nagarkatti, & Nagarkatti, 2015). CB1R activation also decreases PSA-NCAM level resulting in impairment in memory consolidation (M. Maćkowiak, Chocyk, Dudys, & Wedzony, 2009; Marzena Maćkowiak, Chocyk, Markowicz-Kula, & Wedzony, 2007). Since we also observed a reduction in NCAM and PSA-NCAM level, we suggest that, the observed behavioral deficits in our study are due to reduced PSA-NCAM activity, possibly through changes in CB1R level (Pamplona & Takahashi, 2006).

To further investigate the reason behind behavioral deficits, we measured the changes in hippocampal synaptic plasticity parameters. The deficits in basal synaptic transmission in the PCE offspring indicates an alteration in glutamatergic receptor signaling in the PCE hippocampus. This can be either due to presynaptic alteration and changes in glutamate release, or uptake, resulting in altered activation of glutamatergic receptors (Sweatt, 2010). The increased glutamate release in our study can be due to increased availability of the glutamate filled vesicles in the presynaptic terminal as observed by increased VGLUT level in the PCE offspring (Herman, Ackermann, Trimbuch, & Rosenmund, 2014; N. R. Wilson et al., 2005). CB1R activation can also lead to a reduction in GABAergic outflow causing an increase in the glutamatergic outflow (Beggiato et al., 2017; Pistis et al., 2002) However, we did not measure the GABA level or activity in our study and should be explored in future studies. As we observed an increase in presynaptic mechanism, the reduction in the basal synaptic transmission in our study may be related to a reduction in the post synaptic receptor density, most probably AMPAR subunit, GluA1. MEA study revealed a decrease in the removal (or uptake) of glutamate from the extracellular space in the CA1 region. Hence, it is reasonable to presume that saturation of synaptic glutamate receptors by excessive glutamate concentration could have resulted in receptor desensitization and thus decreased responses to stimuli.

Altered basal synaptic transmission and presynaptic release probability in PCE offspring also contributed to synaptic plasticity deficits as observed by decreased LTP and enhanced LTD. LTP induction requires concurrent activation of the glutamatergic receptors AMPA and NMDA (M. A. Lynch, 2003; Park et al., 2014) while AMPAR expression and trafficking are important for the maintenance of LTP (Lüscher & Malenka, 2012). Larger membrane depolarization, leads

to increased activation of NMDARs during LTP induction (Bliss & Collingridge, 1993). Hence, a decreased LTP induction in PCE offspring, indicates reduced activation of NMDAR preferably GluN2A (Bellone & Nicoll, 2007; Franchini et al., 2019; Sakimura et al., 1995). Activation of NMDA receptors are also necessary for acquisition of spatial information and memory retention (Huerta, Sun, Wilson, & Tonegawa, 2000). We observed a significant reduction in GluN2A level in the hippocampus of PCE offspring along with alteration in its downstream signaling. LTD induction is mostly dependent on GluN2A than GluN2B signaling (Bartlett et al., 2007; Morishita et al., 2007) which further supports the notion that impaired LTD in PCE animals might be due to changes in GluN2A level in PCE offspring.

NCAM and PSA-NCAM are also implicated in the maintenance of synaptic plasticity (Becker et al., 1996; Muller et al., 1996). PSA-NCAM enhances synaptic GluN2A activity while suppressing the extrasynaptic GluN2B activity (Kochlamazashvili et al., 2012). We observed a reduction in hippocampal NCAM and PSA NCAM level in PCE offspring followed by NMDAR dependent impairment in LTP and LTD. Interestingly, exogenous application of a bacterially derived PSA compound, colominic acid (CA) restored LTP along with an increase in the LTP induction in the PCE offspring. The increase in the LTP induction further supports the notion that the PSA-NCAM mediated synaptic plasticity deficits is mostly NMDAR dependent. PSA application in NCAM deficient mice have been shown to ameliorate behavioral deficits (O. Senkov et al., 2006). We observed a reduction in hippocampal GluN2A level in PCE offspring along with a reduction in its downstream signaling molecules ras-grf1 and ERK. Phosphorylation of ERK is necessary for the LTP maintenance (Winder et al., 1999). A reduction in GluN2A can decrease surface insertion of GluA1 via reduced ras-grf1-ERK signaling (Myung, Dunah, Yu, &

Sheng, 2005). We observed a decrease in total GluA1 level as well as reduced phosphorylation of GluA1 at serine 831. The phosphorylation of GluA1 at serine 831 is implicated in both LTP and LTD (Crombag et al., 2008). Reduction of PSA-NCAM also reduces BDNF dependent signaling (Jong, Sergin, Purgert, & O'Malley, 2014; Muller et al., 2000) which can lead to reduced synaptic delivery of GluA1 resulting in deficits in LTP through reduced CAMKII phosphorylation and ERK activity. Moreover, the reduction in glutamate uptake that we observed in the MEA study can result in glutamate spill over causing activation of extrasynaptic GluN2B (Lozovaya et al., 2004; Potier et al., 2010). Although we did not observe a change in total hippocampal level of GluN2B in the PCE offspring we indeed observed an increase in p38 activation which is downstream of extrasynaptic GluN2B. Synaptic GluN2A deficiency rather than GluN2B is implicated in LTP (Bellone & Nicoll, 2007; Franchini et al., 2019; Sakimura et al., 1995) and LTD induction (Bartlett et al., 2007; Morishita et al., 2007). In congruence, we observed an increase in the probability of opening in synaptosomal NMDA channel in PCE offspring. We acknowledge the increase in the NMDA channel could be either due to GluN2A or GluN2B or both. Since, we observed a reduced activation of GluN2A in our LTP induction analysis and a reduction in the GluN2A level followed by reduced downstream protein level and an increase in downstream extrasynaptic GluN2B signaling, we speculate that the increased channel activity is probably due to increased GluN2B. This can be further validated by the holding voltage/ voltage clamp at which we recorded the NMDA currents was similar to the voltage clamp of extrasynaptic NMDAR channels' in our previous studies (Hammond et al., 2006; Kochlamazashvili et al., 2010). Thus, we postulate that, PCE causes alteration in glutamate mediated neurotransmission via reduced activity of GluA1 and GluN2A and increased

activity of extrasynaptic GluN2B, ultimately leading to altered synaptic plasticity and behavioral deficits.

6. Conclusion

Current study aims to elucidate the mechanisms by which exposure to cannabinoids during pregnancy causes learning and memory deficits in offspring. We have established a potential signaling mechanism by which gestational cannabinoid exposure may lead to learning and memory deficits. This provides a signaling pathway for additional mechanistic studies to investigate therapeutic targets to ameliorate learning and memory deficits in PCE. Future studies should look at how AMPAR channels are also regulated by PSA-NCAM deficiency as well as GABAergic neurotransmission is altered as glutamate and GABA neurotransmission mostly regulates learning and memory codependently.

7. Tables

Table 3.1. Summary of antibodies and working conditions used in the experiments

Antibodies	Host & Type	specificity	Source	Catalog#	Dilution
<i>Primary Antibodies</i>					
GAPDH	mouse monoclonal	H M Pg Rb R C Dg	EMD Millipore	1001	1:1,000
β -actin	Rabbit, monoclonal	H M R Mk Dm Z	Cell Signaling Technology	8457	1:1,000
GluA1	Rabbit, monoclonal	M R	Cell Signaling Technology	13185	1:1,000
GluA1 Ser 831	Rabbit, monoclonal	H M	Cell Signaling Technology	75574	1:1,000
CaMKII	Rabbit, monoclonal	H M R	Cell Signaling Technology	11945	1:1,000
Phospho- CaMKII	Rabbit, monoclonal	H M R Dr	EMD Millipore	3865	1:1,000
BDNF	rabbit, monoclonal	H M R	Abcam	108319	1:1,000
Synaptophysin	Rabbit, monoclonal	H M R	Cell Signaling Technology	36406	1:1,000
VGLUT1	Rabbit, monoclonal	M R	Cell Signaling Technology	12331	1:500
GluN2A	Rabbit, monoclonal	M R	Cell Signaling Technology	4205	1:1,000
ERK1/2	Rabbit, monoclonal	H M R Hm Mk Mi Dm Z	Cell Signaling Technology	4695	1:2,000

		B Dg Pg Ce			
Phospho-ERK1/2	Rabbit, monoclonal	H M R Hm Mk Mi Dm Z B Dg Pg Sc	Cell Signaling Technology	4370	1:1,000
Ras-grf1	Mouse, monoclonal		Santa Cruz	377234	1:700
Glun2B	Rabbit, monoclonal	H M R	Cell Signaling Technology	4207	1:1000
Anti p38 MAPK	Rabbit, monoclonal	H M R Hm Mk B Pg	Cell Signaling Technology	8690	1:1000
Anti-Phospho-p38 MAPK	Rabbit, monoclonal	H M R Mk Dm Pg Sc	Cell Signaling Technology	9211	1:1000
NCAM	Rabbit, polyclonal	Ch, H, M, R	EMD Millipore	5032	1:700
<i>Secondary Antibody</i>					
Anti-rabbit IgG	Goat	R	Cell Signaling Technology	7074	1:5,000
Anti-mouse IgG	N/A	M	Santa Cruz	516102	1:2,000

H-Human, M-Mouse, R-Rat, Hm-Hamster, Mk-Monkey, Mi-Mink, C-Chicken, Dm-D. melanogaster, X-Xenopus, Z-Zebrafish, B-Bovine Dg-Dog, Pg-Pig, Sc-S. cerevisiae, Ce-C. elegans, Hr-Horse, Dr-Drosophila, Rb- Rabbit

8. Figures

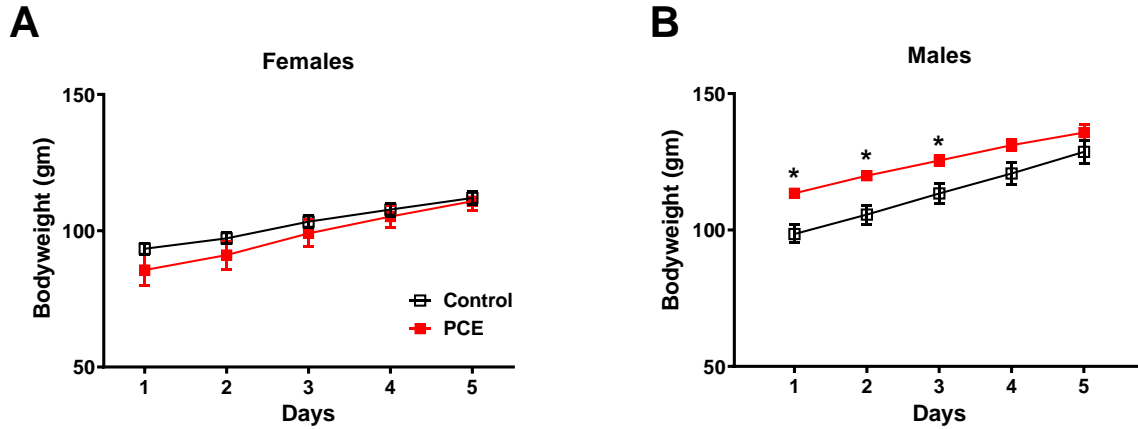


Figure 3.1 Effects of prenatal cannabinoid exposure (PCE) on bodyweight

Bodyweights were similar between PCE and control females (A), whereas PCE males weighed significantly more than control males. Symbols represent means \pm SEM from 12 to 13 rats per group. *indicates significant difference in control vs. PCE; $*p \leq .05$

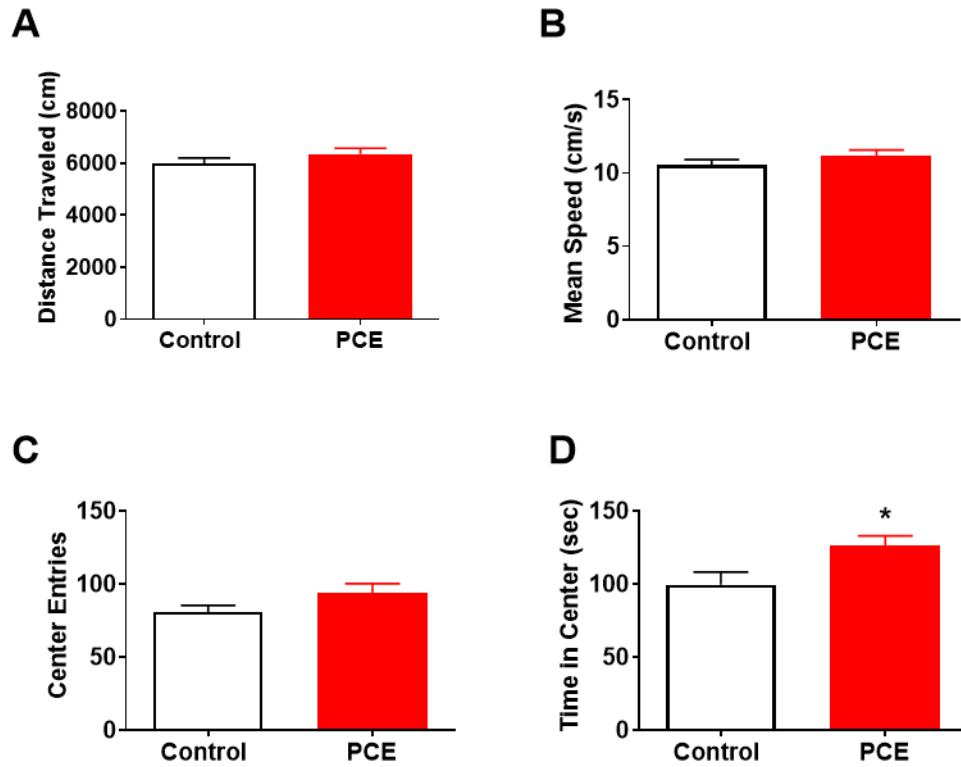


Figure 3.2 PCE decreases anxiety without altering general locomotor activity

Total distance travelled (A) and mean speed (B) were similar between control and PCE animals during the open field task. PCE animals made slightly more entries into the center zone (C) and spent significantly more time in the centre zone (D) than controls. Symbols represent means \pm SEM from 12 to 13 rats per group. *indicates significant difference in control vs. PCE; * $p \leq .05$

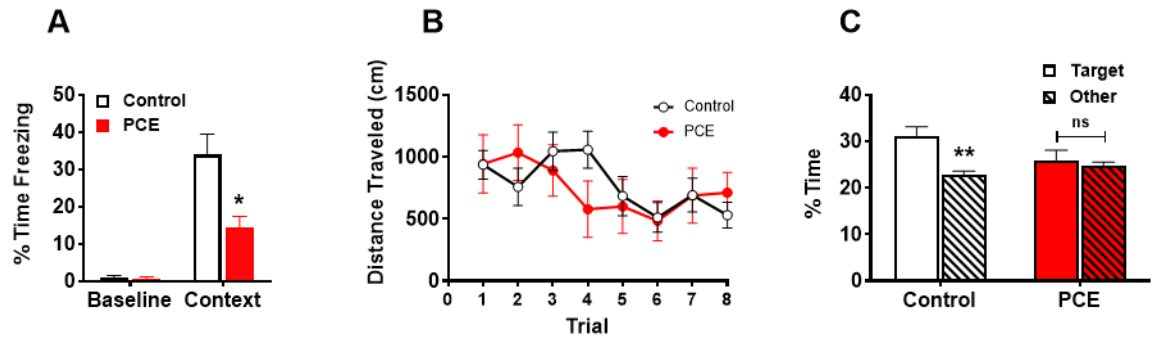


Figure 3.3 PCE impairs hippocampal-dependent memory

(A) While baseline freezing was similar between control and PCE animals during training, PCE animals froze significantly less than controls during the contextual fear test ($p = .018$). (B) PCE animals exhibited similar acquisition in the hidden platform training trials. (C) Controls exhibited a preference for the target quadrant relative to the average of the other 3 target quadrants (“Other”), whereas PCE animals did not. Dashed line represents chance performance. Symbols represent means \pm SEM from 10 to 15 rats per group. ns, nonsignificant; *indicates significant difference in control vs. PCE; $*p \leq .05$, $**p \leq .01$

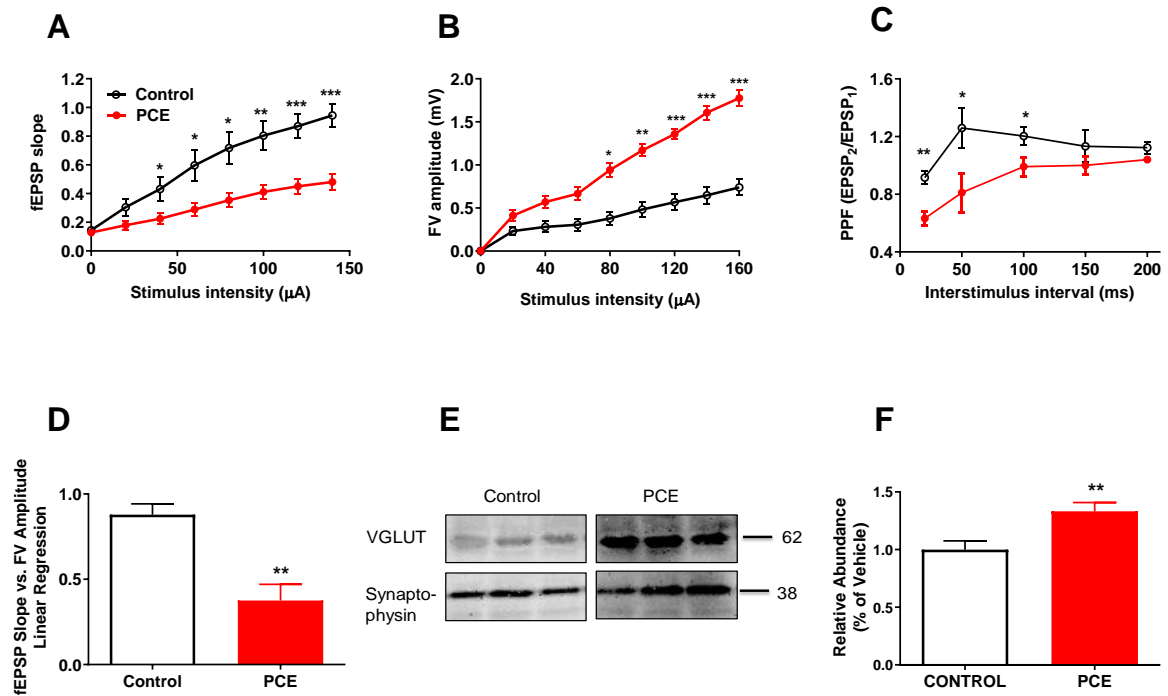


Figure 3.4 PCE alters presynaptic release probability and basal synaptic transmission

(A) Input-output curve of fEPSP slope measured at increasing stimulus intensities. (B) Input-output curve of FV amplitude measured at increasing stimulus intensities. (C) Paired-pulse facilitation expressed as the change in ratio of the second stimulus fEPSP to the first stimulus fEPSP slope plotted as a function of interstimulus interval. (D) Slope of the linear regression line of best fit from plotting fEPSP slope vs. FV amplitude. (E, F) Western blot data showed significant increase in VGLUT levels in PCE animals. Symbols represent means \pm SEM from 5-6 rats per group; *indicates significant difference in control vs. PCE; * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$

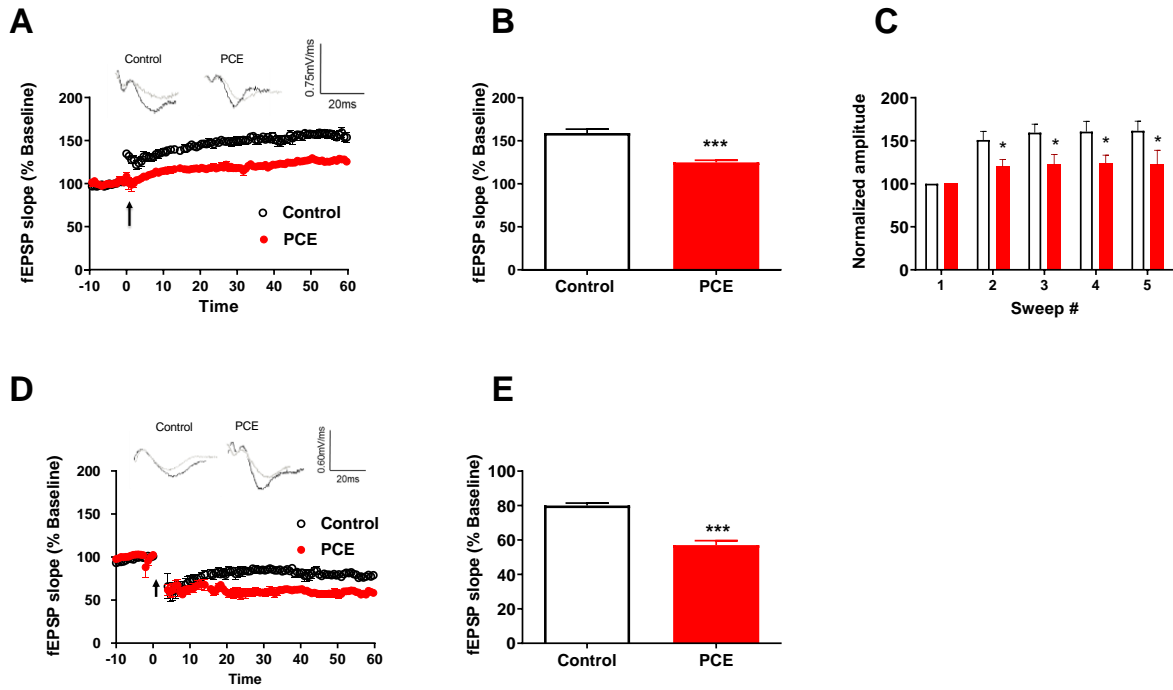


Figure 3.5 Prenatal cannabinoid exposure alters synaptic plasticity

(A) LTP graph represents fEPSP slope before and after induction by TBS. Mean slope of fEPSPs recorded 10 min prior to TBS was taken as 100% and arrow indicates delivery of TBS. (B) LTP bar graph shows fEPSPs recorded during the time period 50-60 min following TBS induction normalized to baselines levels. (C) Facilitation of the first fEPSP between trains computed by normalizing the amplitude of the first fEPSP for trains #2-5 with the first fEPSP of the first train. (D) LTD graph represents fEPSP slope before and after induction by LFS. Mean slope of fEPSPs recorded 10 min prior to TBS was taken as 100% and arrow indicates delivery of LFS. (E) LTD bar graph shows fEPSPs recorded during the time period 50-60 min following LFS induction normalized to baselines levels. Symbols represent means \pm SEM from 8 to 10 rats per group.

*indicates significant difference in control vs. PCE; * $p \leq .05$, ** $p \leq .01$; *** $p \leq .001$, **** $p \leq .0001$

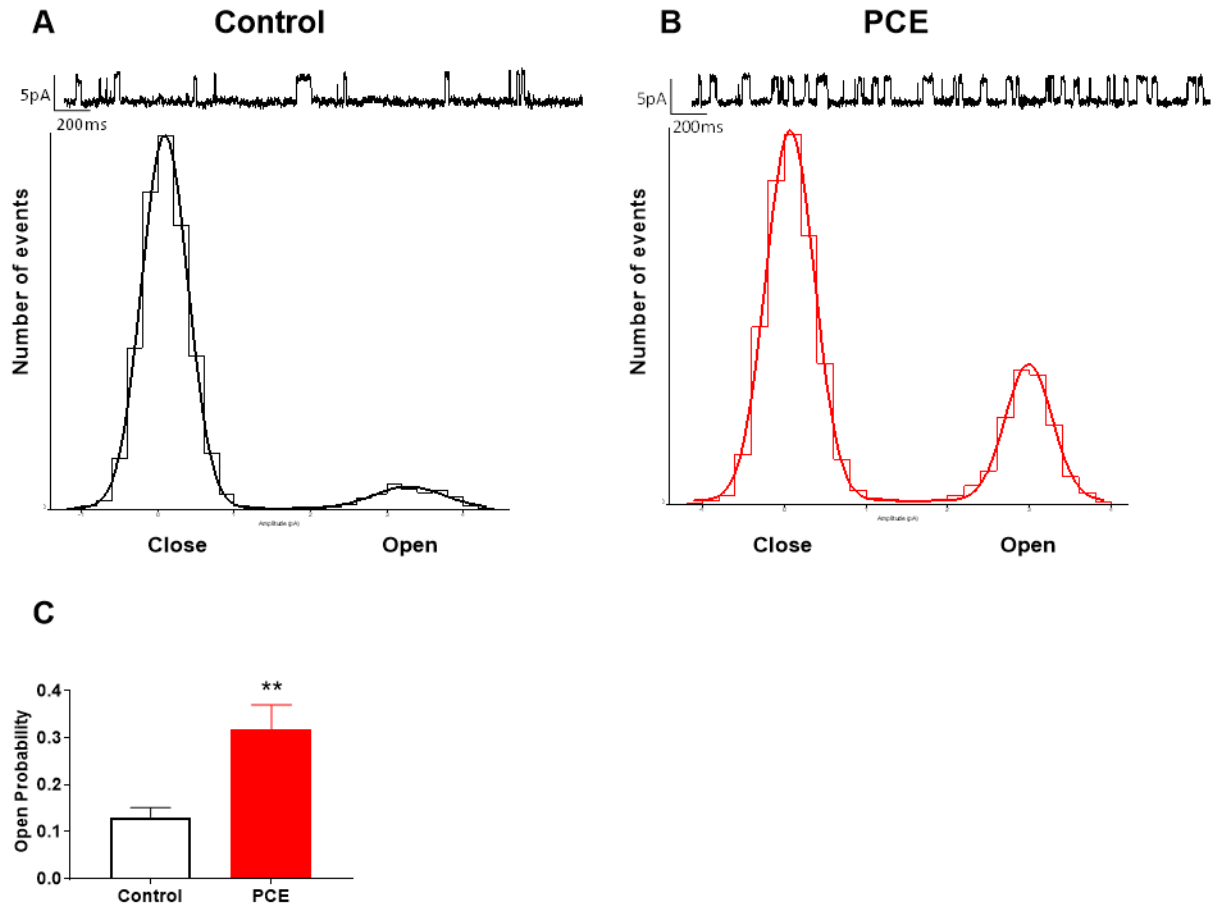


Figure 3. 6 PCE is associated with alterations in the single channel properties of hippocampal synaptic NMDARs

(A, B) Representative traces and amplitude histogram illustrating the increased channel activity (upward deflections) in the PCE rats. (C) Bar plot showing significantly increased channel open probability in PCE rats. Symbols represent means \pm SEM from 3 rats per group. *indicates significant difference in control vs. PCE; ** $p \leq 0.01$

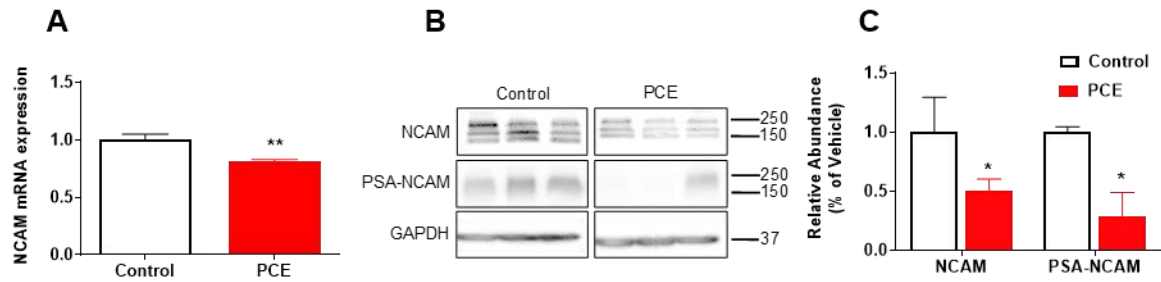


Figure 3. 7 PCE reduces hippocampal NCAM and PSA-NCAM mRNA expression and protein levels

A. PCR data shows significant reduction in mRNA level of NCAM ($p \leq 0.05$) in the PCE hippocampus. (B, C) Western blot data shows significant reduction in both NCAM and PSA-NCAM levels in PCE hippocampus. Symbols represent means \pm SEM from 3 to 5 rats per group. *indicates significant difference in control vs. PCE; * $p \leq .05$; ** $p \leq .01$

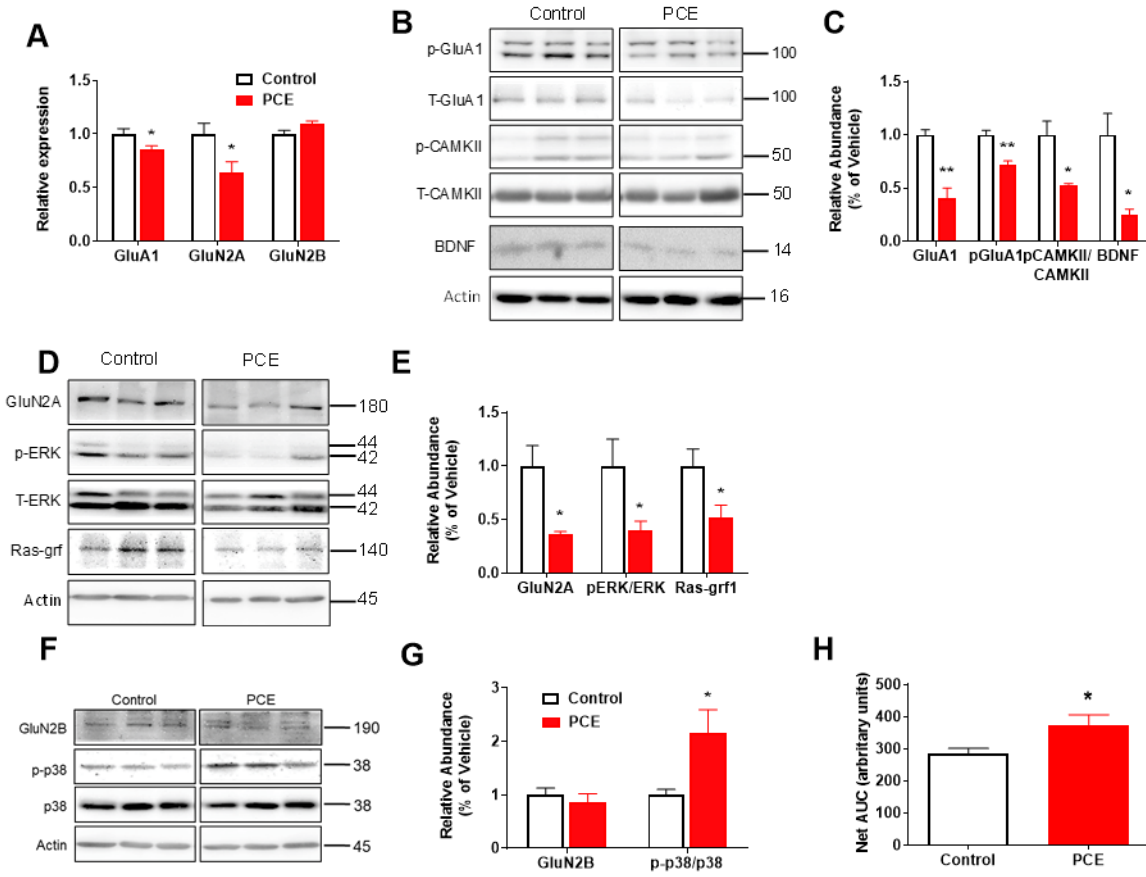


Figure 3.8 PCE results in increase GluN2B mediated signaling followed by reduced glutamate uptake in CA1 hippocampal subregion of PCE animals

(A) PCR data revealed increase in GluA1, GluN2A but no difference in GluN2B mRNA level, (C,D) Western blot analysis revealed significant reduction in total GluA1, phosphorylation of GluA1 and CAMKII level in PCE animals (E,F) significant reduction in GluN2A followed by significant reduction in phosphorylation of ERK and Ras-grf1. (F,G) Western blot data showed no difference in the GluN2B expression in the hippocampus of control and PCE animals. An increase in phosphorylation of p38 has been observed in PCE animals. (D) Glutamate uptake

expressed as the net area under the curve (AUC) was significantly reduced in PCE animals.

Symbols represent means \pm SEM from 3 to 5 rats per group. *indicates significant difference in control vs. PCE; * $p \leq .05$, ** $p \leq .01$

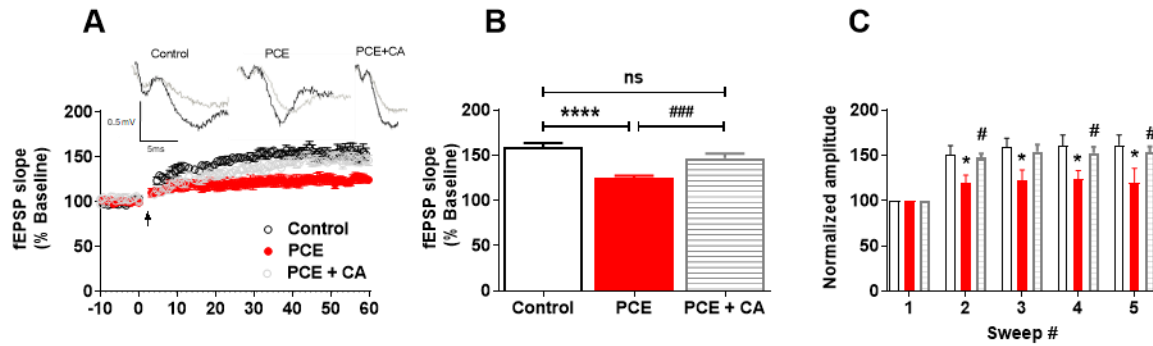


Figure 3. 9: Colominic acid application restores the LTP in PCE offspring

(A) LTP graph represents fEPSP slope before and after induction by TBS. Mean slope of fEPSPs recorded 10 min prior to TBS was taken as 100% and arrow indicates delivery of TBS (induction period not shown). (B) LTP bar graph shows fEPSPs recorded during the time period 50-60min following TBS induction normalized to baselines levels. Significant improvement in LTP after CA application as measured by fEPSP slope in PCE animals. (C) Within-train facilitation, Facilitation of the first fEPSP between trains, computed by normalizing the amplitude of the first fEPSP for trains #2-5 with the first fEPSP of the first train. Symbols represent means \pm SEM from 5 to 8 rats per group. *indicates significant difference in control vs. PCE; n= 5-8 rats per group #indicates significant difference in PCE vs. PCE+CA; */# $p \leq .05$, ### $p < .001$, **** $p \leq .0001$

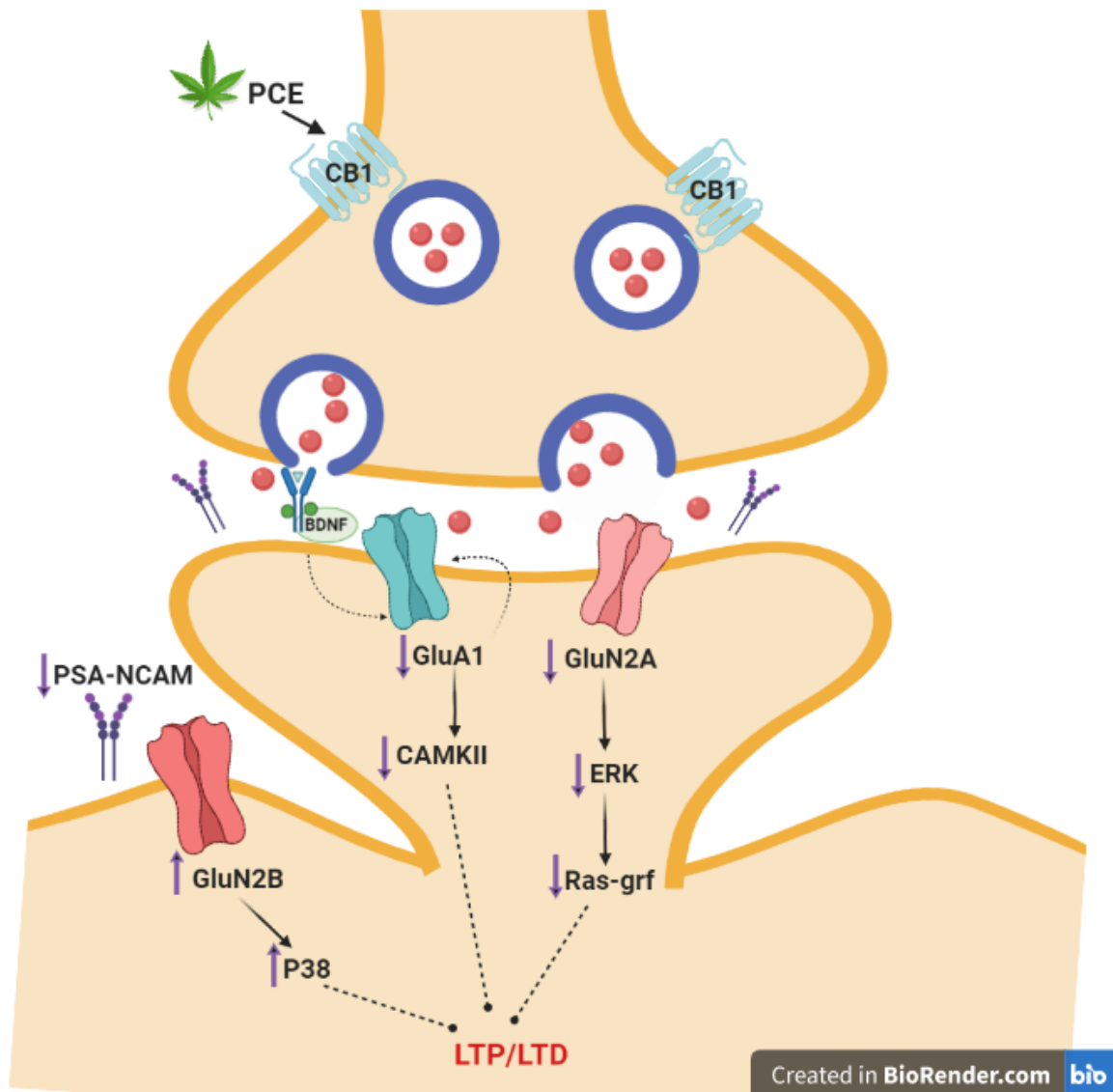


Figure 3.10 PCE impairs hippocampal LTP/LTD through alteration in glutamate mediated signaling

PCE causes activation of Cannabinoid receptor (CB1) which leads to reduction in the PSA-NCAM level. LTP is induced through activation of GluN2A, whereas LTD requires the activity of both GluN2A- and GluN2B-containing receptors. Decreased PSA-NCAM reduces synaptic

GluN2A mediated signaling and enhances extrasynaptic GluN2B-containing receptor mediated signaling, resulting in impaired LTP/LTD. LTP is induced through activation of GluN2A, whereas LTD requires the activity of both GluN2A- and GluN2B-containing receptors.

Decreased PSA-NCAM also causes a reduction in BDNF mediated signaling i.e. reduced trafficking and insertion of AMPA into the post synaptic membrane. PSA-NCAM can also inhibit GluA1 directly which in turn can negatively affect BDNF level and its signaling.

CB1- cannabinoid receptor type 1; PSA-NCAM- polysialylated neural cell adhesion molecule;

BDNF- Brain derived neurotrophic factor; LTP- Long term potentiation; LTD- Long term

depression. This figure was “Created with BioRender.com” (<https://biorender.com/>)

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Chapter 4 Effects of Prenatal Cannabinoid Exposure on the Cerebellum of Adolescent Rat Offspring

1. Abstract:

Cannabis is the most commonly used illicit drug worldwide. Recently, it is getting more popular among young pregnant women to help them with morning sickness. However, prenatal cannabinoid exposure (PCE) leads to long-lasting cognitive, motor, and behavioral deficits in the offspring and alterations in neural circuitry through various mechanisms. Although these effects have been studied in the hippocampus, the effects of PCE on the cerebellum are not well elucidated. The cerebellum plays an important role in balance and motor control, as well as cognitive function such as attention, language, and procedural memories. The aim of this study is to investigate the effects of PCE on the cerebellum of adolescent offspring. Pregnant rats were treated with synthetic cannabinoid agonist WIN55,212-2 and their adolescent offspring were evaluated for various cerebellar markers of oxidative stress, mitochondrial function, and apoptosis. Additionally, signaling proteins associated with glutamate dependent synaptic plasticity were examined. Administration of WIN55,212-2 during pregnancy altered markers of oxidative stress by significantly reducing oxidative stress and nitrite content. Mitochondrial Complex 1 and Complex 4 activities were also enhanced following PCE. With regard to apoptosis, pP38 levels were significantly increased and pro-apoptotic factor caspase 3 activity, pERK, and pJNK levels were significantly decreased. CB1R and GluA1 level remained unchanged; however, GluN2A level was significantly reduced. There was a significant decrease in MAO activity although tyrosine hydroxylase activity was unaltered. Our study indicates that effects of prenatal cannabinoid exposure on the cerebellum are unique compared to

other brain regions by enhancing mitochondrial function to promote neuronal survival. Further studies are required to evaluate the mechanisms by which prenatal cannabinoid exposure alters cerebellar processes and the impact of these alterations on behavior.

2. Introduction:

Historically, derivatives of the plant *Cannabis sativa* have been used for recreational, medical, and veterinary purposes (Mahmoud A ElSohly et al., 2016). Cannabis is currently one of the most commonly used recreational drugs in the world. In the United States of America, as more and more states are legalizing the use of marijuana, the rate of current marijuana abuse has increased (Center for Behavioral Health Statistics, 2018). The active ingredient, trans- Δ^9 -tetrahydrocannabinol (THC) can readily cross the blood placental barrier and affect fetal growth and development (Bailey, Cunny, Paule, & Slikker, 1987b). Studies have demonstrated a strong correlation between maternal use of cannabinoid and cognitive outcomes in the offspring, particularly in younger children. Animal studies have confirmed that PCE results in learning and memory deficits that are consistent with the cognitive deficits observed in children who were prenatally exposed to cannabinoids (Campolongo, Trezza, Ratano, Palmery, & Cuomo, 2011). Impaired short-term memory, verbal reasoning, and attention and increased impulsivity and hyperactivity in early childhood as well as impairment in visual reasoning, problem-solving, sustained attention, and visual-motor coordination is reported (Sobrian, 2016). Studies on children of various ages suggest that the prenatal exposure to high doses of marijuana can affect the learning of tasks as well as emotional outcomes (Day et al., 2006; L Goldschmidt et al., 2000; Gray, Day, Leech, & Richardson, 2005). These neurobehavioral changes are long lasting and might be associated with alterations in various neurotransmitter systems (Pinky et al., 2019b).

There are two types of cannabinoid receptors - cannabinoid receptor type 1 (CB1R) (Matsuda et al., 1990) and cannabinoid receptor type (CB2R) (Munro et al., 1993). The density of CB1R in

the central nervous system (CNS) is particularly high and its distribution is widespread in the basal ganglia, cerebellum, cerebral cortex, and hippocampus (Felder et al., 1995). The cerebellum is involved motor coordination, the implementation of associative learning, and the processing of temporal operations (Stella, 2013). In the cerebellum, the vast majority of CB1Rs are found at the presynaptic terminals received by Purkinje cells (K. A. Takahashi & Linden, 2000). CB1R is moderately high in the molecular layer and low in the granule cell layer of the cerebellum. Chronic cannabinoid exposure can cause motor incoordination and abnormal eyeblink reflex conditioning in mice, leading to impairment in cerebellar-associated learning (Cutando et al., 2013). Altered time perception and self-paced behaviors due to THC intoxication have also been reported, which were linked to an altered internal clock (Mathew, Wilson, Turkington, & Coleman, 1998; O'Leary et al., 2003).

Although there are several studies which examined effect of prenatal alcohol, nicotine, and other substance use in the offspring (Dwipayana Bhattacharya et al., 2018; Bookstein, Streissguth, Connor, & Sampson, 2006; Koning et al., 2017; Luo, 2015), to date, there is very limited data regarding the role of cannabinoid exposure on the cerebellum during the developmental period. Previously, substance abuse during pregnancy was shown to alter various behavioral parameters and motor development in the offspring (De Salas-Quiroga et al., 2015; Farah Naquiah et al., 2016; Kelly et al., 2000; Saberi Moghadam et al., 2013; Sobrain, 1977; Wu et al., 2011).

Therefore, we also determined whether the dose of cannabinoid that we used here induced any alteration in common behavioral parameters. Additionally, we have evaluated how cerebellar mitochondrial function is altered as a result of PCE, since CB1Rs are found in mitochondria where they modulate neuronal energy homeostasis (Bénard et al., 2012b). The generation of reactive oxygen species (ROS) triggers oxidative stress and induces irreversible oxidation of

lipids and proteins, resulting in cell death. We investigated ROS, nitrite, and lipid peroxidation content to see whether PCE alters these parameters in cerebellum. Since cannabinoid induce apoptosis in cerebellar granular cells (Pozzoli et al., 2006), we also wanted to investigate whether PCE can initiate any apoptotic response in cerebellum during developmental period via measuring caspase 1 and caspase 3 activity. The study further evaluated, the effect of PCE on the key signaling molecules, which are important for maintenance of synaptic plasticity, cerebellar mitochondrial function & apoptosis.

3. Materials and methods:

Animals

Time pregnant Sprague Dawley rats were purchased from commercial vendor (Envigo laboratories). Animals were housed in a vivarium maintained on a 12 h: 12 h light: dark cycle (lights on at 6:00 am) and at a temperature of 22–24 °C. All experimental procedures were approved by the Auburn University Animal Care and Use Committee (IACUC) and conducted in compliance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals. Briefly, rats were anaesthetized on gestational day 3 with isoflurane anesthesia for subcutaneous implantation of an osmotic mini-pump (Alzet 2004). The pump delivered either vehicle or the synthetic cannabinoid agonist (WIN55,212-2, 2mg/kg body weight/day) until the pups were born. The dose of cannabis was based on previous studies and corresponds to moderate to heavy cannabinoid exposure (Campilongo & Trezza, 2012; French et al., 1997; R. E. Hampson & Deadwyler, 2000; Tortoriello et al., 2014) . The pumps were removed from the dams after delivery. Pups were weaned at postnatal day 21. Animals were housed in a vivarium

maintained on a 12 h:12 h light: dark cycle (lights on at 6:00 am) and at a temperature of 22–24 °C. Pups were housed in groups of three per cage after weaning. At postnatal day 42, pups were euthanized via CO₂, and cerebellar tissue was extracted. There was no significant difference in the number of pups per litter, body weight, or weight of the cerebellum and whole brain in the PCE offspring compared to the control.

Chemicals

All the chemicals and antibodies used in the current study were purchased from Sigma (St. Louis, MO) and Cell Signaling Technology (Danvers, Massachusetts), respectively, unless otherwise specified.

Behavioral Studies:

Animals were observed for various physical and behavioral parameters including tremor, Straub tail, seizures, hyperactivity (excessive jumping), hind limb abduction, head twitching, hair coat erection, fighting, drooling, diarrhea, and ataxia. Observations were performed by blinded reviewers.

Biochemical studies

Animals were sacrificed in the morning to avoid diurnal variations of endogenous amines, enzymes, and other antioxidant molecules. The cerebellum was dissected, flash frozen in liquid

nitrogen, and stored at -80°C . The cerebellar homogenate for the biochemical tests was prepared by homogenizing the tissue in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer, followed by centrifugation at 10,000g for 60 min at 4°C , and the supernatant was collected (Ahuja et al., 2017).

Generation of reactive oxygen species (ROS)

Spectrofluorometric method was used to determine ROS in the cerebellar samples. ROS generates as result of conversion of the non-fluorescent chloromethyl-DCF-DA (2',7'-dichlorofluorescein diacetate, DCF-DA) dye into fluorescent DCF. The amount of ROS was measured at the excitation wavelength of 492 nm and emission wavelength of 527 nm ROS (fluorescence units) measured was normalized to total protein content as relative fluorescence intensity/mg protein. Results are expressed as (%) change as compared to the control (Alhowail et al., 2019; Katz et al., 2018; Majrashi et al., 2018).

Assessment of lipid peroxidation

Tissues were homogenized with PBS and lysis buffer. The samples were sonicated (Qsonica) for 2-3 min. Then samples were centrifuged at 12,000 x g for 20 minutes at 4°C . The supernatant was transferred into new centrifuge tubes. The proteins were quantified by Bradford Protein Assay before running the lipid peroxidation assay. A spectrophotometric method using thiobarbituric acid was used to assess lipid peroxidation. The index of lipid peroxidation was estimated by the formation of thiobarbituric acid-reactive substances (TBARS) at 532 nm.

TBARS was normalized to total protein content as TBARS formed/mg protein (Alhowail et al., 2019; Majrashi et al., 2018)

Monoamine oxidase (MAO) activity

Spectrofluorometric method using kynuramine as a substrate was used to measure MAO activity (315 nm-excitation and 380 nm emission in the cerebellar samples. MAO activity refers to 4-hydroxy quinolone (μM)/mg protein (Thrash-Williams et al., 2016).

Mitochondrial Complex I activity

Samples were homogenized with phosphate-buffered saline (PBS) and lysis buffer and centrifuged at $14000 \times g$ and $2-4^\circ\text{C}$ for 15 min. The supernatant was transferred into new, clean 1.5 mL microcentrifuge tubes and frozen at -80°C until use. The proteins from each sample were quantified by Bradford Protein Assay before starting assessment of mitochondrial Complex I activity. Spectrophotometric method using NADH as a substrate was used to measure Complex I activity (340 nm) (Thrash-Williams et al., 2016). The Complex I activity refers to NADH oxidized/mg protein.

Complex-IV activity

Spectrophotometric method using cytochrome c as a substrate was used to measure Complex-IV activity (550 nm) in the cerebellar sample of the control and WIN55,212-2 treated animals. The Complex-IV activity refers to cytochrome c oxidized/mg protein (Thrash-Williams et al., 2016).

Nitrite content

Nitrite content was measured spectrophotometrically using the Griess reagent which was developed by Griess in 1879. The Griess method relies on Nitrite reaction with sulfanilamide under acidic condition resulting in the production of diazonium ion. The diazonium ion then combine with N-(1-naphthyl) ethylenediamine to form chromophoric azo product which can be measured spectrophotometrically at 545 nm. A sodium nitrite standard curve was prepared from commercially acquired sodium nitrite. Results are expressed as (%) change as compared to the control (Giustarini, Dalle-Donne, Colombo, Milzani, & Rossi, 2008; Green et al., 1982; Majrashi et al., 2018).

Caspase-1 activity

Spectrofluorometric method using Ac-Tyr-Val-Ala-Asp-7-amino-4-Trifluoromethylcoumarin (Ac-YVAD-AMC) as a substrate was used to measure Caspase-1. Cleavage of AMC by Caspase-1 generates strongly fluorescent AMC that is fluorometrically monitored at excitation of 340-350nm and emission of 440-460nm. The Caspase-1 activity refers to free AMC/mg total protein. Results are expressed as (%) change as compared to the control (Majrashi et al., 2018; Usha et al., 2000).

Caspase-3 activity

Caspase-3 activity was measured spectrofluorometrically using the non-fluorescent Caspase-3 substrate (Ac-DEVD-AMC). Cleavage of AMC by Caspase-3 generates strongly fluorescent AMC that is fluorometrically monitored at excitation of 340-350nm and emission of 440-460nm. The Caspase-3 activity refers to free AMC/mg total protein. Results are expressed as (%) change as compared to the control (Dwipayana Bhattacharya et al., 2018; Usha et al., 2000).

Tyrosine Hydroxylase activity

The formation of L-Dopa was measured by using sodium periodate to oxidize L-Dopa to form the spectrophotometrically detectable chromophore dopaquinone at 475nm. A L-Dopa standard curve was prepared from commercially acquired L-Dopa. Results are expressed as (%) change as compared to the control (Vermeer, Higgins, Roman, & Doorn, 2013).

Western blot analysis

Total protein was isolated using cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) containing protease inhibitor cocktail (P8340, Sigma, St. Louis, MO) and phosphatase inhibitors (P 5726, Sigma, St. Louis, MO). Protein concentration was measured using the bicinchoninic acid assay (BCA assay). Western blot analysis was performed as previously described (Alhowail et al., 2019). Each sample was denatured at 95°C for 5minutes before loading onto freshly

prepared 10% SDS-PAGE gel for protein separation. Separated proteins on SDS-PAGE were transferred onto PVDF membranes (Immobilon-p Millipore, Germany). Non-specific binding sites on the membranes were blocked with 5% non-fat milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) at pH 7.4. The membranes were then incubated overnight at 4°C with specific antibody constituted in 5% BSA in TBST. Primary antibodies used in this study included: anti-PSD95, anti-GLuR1, anti-ILK, anti-AKT, anti-JNK, anti-GSK3B, anti-ERK, anti-p38 MAPK (Cell signaling, Denver) anti-CB1 (Abcam). All the primary antibodies were used at a 1:1000 dilution. Membranes were then washed with TBST (3X, each for 10 min) and incubated with Goat Anti-Rabbit conjugated secondary antibodies (CST, 1:3000) for 60-90 min at room temperature. Membranes were again washed three times for 10 minutes with TBST after incubation with the secondary antibody. After washing, membranes were analyzed in FluorChemQ® system Imaging. Band densities for each sample were normalized to their respective β -actin (1:1000, Cell Signaling) or GAPDH (1:10000, EMD Millipore) signal and reported as percentage change from control.

Statistical analysis

The results are presented as means \pm SEM. Statistical analysis was performed using the Prism-V software. The experimental data were analyzed using a two-tailed Student's t-test. Statistical differences were considered significant at $p \leq .05$.

4. Results:

4.1 PCE did not alter common behavioral parameters:

Previously, substance abuse during pregnancy has shown to alter various behavioral parameters and motor development in the offspring (Breit et al., n.d.; De Salas-Quiroga et al., 2015; Farah Naquiah et al., 2016; S. J. Kelly et al., 2000; Saberi Moghadam et al., 2013; Sobrain, 1977; C. S. Wu et al., 2011). Following this, we also determined whether the dose of cannabinoid that we used induced any alteration in common behavioral parameters as mentioned in the table 1. No changes in any of the behavioral parameters has been observed in the WIN exposed offspring at 2mg/kg/bodyweight dose as described in Table 4.1

4.2 PCE reduces ROS, lipid peroxidation content and nitrite in the cerebellum

The generation of reactive oxygen species (ROS) triggers oxidative stress and induces irreversible oxidation of lipids and proteins, resulting in cell death. We investigated the ROS content and the TBAR content to see whether PCE exerts a neurotoxic effect on cerebellum. With respect to the DCF based ROS assay, PCE reduced ROS generation although it was not statistically significant compared to the control ($F(1,9) = 3.96$, $p = .08$; Figure 4.1A). However, TBAR content and nitrite content estimation revealed a significant reduction of those in the cerebellum of PCE offspring. ($F(1,9) = 5.63$, $p = .04$; Figure 1B, $F(1,7) = 9.36$, $p = .02$; Figure 4.1C)

4.3 PCE increases mitochondrial complex I and complex IV activity

To investigate how PCE affects mitochondrial system, we measured Complex I and Complex-IV activity. In the PCE offspring, there was an increase in both complex I and complex IV activity. PCE notably improved mitochondrial bioenergetics, as demonstrated by significant increase in Complex-I ($F(1,7) = 7.28, p = .03$; Figure 4.2A) and in complex IV ($F(1,7) = 7.24, p = .03$; Figure 4.2B).

4.4 PCE does not alter caspase-1 and but reduces caspase-3 activity

Caspases are a family of endoproteases which is crucial for regulating inflammation and cell death (McIlwain, Berger, & Mak, 2013a). To determine whether cannabinoid exposure can initiate apoptotic cascade in cerebellum during developmental period, we investigated caspase 1 and caspase 3 activity (Fig. 4.3A, B). PCE did not result in any changes in caspase-1 ($F(1,7) = .31, p = .59$, Figure 3A). However, caspase 3 was significantly reduced in the WIN55,212-2 group ($F(1,7) = 6.99, p = .03$; Figure 4.3B) suggesting that PCE might play an anti-apoptotic role in cerebellum.

4.5 PCE decreases MAO activity but no change in tyrosine hydroxylase activity

Since MAO is known to play a ‘morphogenetic’ role during development and aging process, we investigated the total MAO activity in the cerebellum of these animals. (McIlwain et al., 2013a) MAO activity was significantly reduced in the cerebellum of WIN55,212-2 exposed animals ($F(1,7) = 8.21, p = .02$; Figure 4.4A). Tyrosine hydroxylase activity was also measured to explore whether PCE affects the cerebellar catecholamine content. There was no significant difference in the TH activity in the two groups ($F(1,9) = .83, p = .38$; Figure 4.4C).

4.6 PCE alters the key proteins important for mitochondrial function and synaptic plasticity

Next, we investigated the effect of PCE on the key signalling molecules, which are important for maintenance of synaptic plasticity and cerebellar mitochondrial function. Western blot analyses (Figure 4.5) revealed that WIN 55,212-2 use during pregnancy does not alter CB1R expression in the cerebellum

($p > .05$). Following this there was no alteration in GluA1 expression but a significant reduction in GluA2 ($p < .05$) has been observed. PCE also does not alter AKT, GSK3 β phosphorylation or ILK expression ($p > .05$). However, a significant reduction in the ERK and JNK phosphorylation has been observed which was accompanied with an increase in P38 phosphorylation ($F(1,7)=6.27$, $p=.04$, Figure 4.5C).

5. Discussion:

Neuronal cell death is characterized by oxidative stress, mitochondrial energy dysregulation and excitotoxicity mediated through various apoptotic signals (Zádori et al., 2012). In the current study, we report that PCE can reduce oxidative stress and increase mitochondrial function in the adolescent offspring accompanied by alterations in various key regulators of apoptosis and synaptic function. Chronic THC intake is associated with altered cerebellar dependent learning in adult rodents (Cutando et al., 2013). In adolescent humans, PCE is linked to reduced left cerebellar activity (Smith, Fried, Hogan, & Cameron, 2004b). Although there are studies which explored the effect of cannabinoids on oxidative stress (Booz, 2011), inflammation, and apoptosis (Pozzoli et al., 2006), to date, few studies have examined the effects of PCE on the

above mentioned parameters in the cerebellum. However, it is important to understand how PCE alters cerebellar functions since cerebellum plays an essential role in learning and motor function.

We measured oxidative stress and lipid peroxidation in response to PCE as an indicator of neurotoxicity. Oxidative stress and lipid peroxidation occur as a result of dysregulated redox homeostasis leading to accumulation of highly reactive molecules resulting in cellular and neuronal injury (Gallelli et al., 2018). Our finding is in congruent with finding in adult animals where cannabinoid exposure has shown to reduce ROS and lipid peroxidation content in cortical neuron (Rangel-López et al., 2015) and cerebellar granular cells and hippocampal neuronal cells, (Marsicano, Moosmann, Hermann, Lutz, & Behl, 2002). CB1R can be found in mitochondrial membranes (mtCB1), controlling cellular respiration and energy production (Bénard et al., 2012b). ROS and lipid peroxidation products are proapoptotic factors and can activate apoptosis via mitochondrial dependent pathways (Wójcik, Žarković, Gęgotek, & Skrzydlewska, 2020). Since we observed a reduction in the ROS and lipid content, we also expected an improvement in the mitochondrial function. Although previous studies utilizing acute exposure of cannabinoids in adult rodents demonstrated reduced complex I and complex IV activity (Singh et al., 2015a), we observed a significant increase in both Complex I and Complex IV activity. This suggests cannabinoids might regulate the mitochondrial respiratory chain function differentially with acute versus chronic exposure as well as in adult versus developmental exposure. We also observed a reduction of nitrite following PCE. The reduction of nitrite in our study could be due to increased utilization of NO or increased conversion of nitrite to NO (Rassaf, Ferdinandy, & Schulz, 2014), possibly indicating a neuroprotective effect

in response to PCE. Nitrite is an inhibitor of complex I (Shiva, 2010), so the increase in complex I activity observed in this study may be due to the reduction in nitrite following PCE.

Cannabinoids can also affect various brain monoamines levels i.e. dopamine, norepinephrine, and serotonin. A significant reduction in total MAO activity in the cerebellum of PCE offspring in our study aligns with previous findings in adult pig brain cortex (Fišar, 2010). An inhibition of MAO activity is an interesting finding since MAO inhibition has been linked to alcohol and nicotine addiction previously (Amsterdam, Talhout, Vleeming, & Opperhuizen, 2006) and PCE indeed can contribute to cannabis use in early adolescent age (Day et al., 2006).

PCE also alters tyrosine hydroxylase (TH) activity in the hippocampus (Castaño et al., 1995) and other brain regions (A. Bonnin, de Miguel, Hernández, Ramos, & Fernández-Ruiz, 1995; Ana Bonnin, de Miguel, Castro, Ramos, & Fernandez-Ruiz, 1996) along with changes in dopamine activity. In the current study, we did not observe any change in the TH activity in response to PCE, which is in contrast with previous reports in other brain regions (A. Bonnin et al., 1995; Ana Bonnin et al., 1996; Rodríguez De Fonseca et al., 1992). CB1R colocalizes with TH in fetal mesencephalic neurons and causes an increase in the enzyme levels via receptor activation (Hernández et al., 2000). We also did not observe any changes in CB1R level in cerebellum while CB1R activation is required for inducing an alteration in the TH level (Frau et al., 2019; Ginovart et al., 2012). However, it is possible that the dose that has been used here is unable to produce the changes in cerebellum, as other studies have found alterations in CB1R levels along with changes in TH level in other brain regions following PCE (A. Bonnin et al., 1995; Ana Bonnin et al., 1996; Castaño et al., 1995).

Since the cerebellum has a high density of CB1Rs, and glutamatergic neurotransmission is altered by CB1R activation, we wanted to examine whether PCE also alters glutamatergic receptor subunits in the cerebellum. We observed a significant reduction in GluN2A level with no changes in GluA1 in the cerebellum following in the cerebellum of PCE offspring. Overactivation of NMDA receptors such as GluN2A shuts off the pro-survival signaling while molecular knockdown of GluN2A attenuates NMDAR-mediated neuronal death as observed in cortical neurons (Zhou, Ding, Chen, Yun, & Wang, 2013b). This is probably due to sustained Ca²⁺ influx leading to cellular excitotoxicity (Deep, Mitra, Rajagopal, Paul, & Poddar, 2019). Because GluN2A can be considered as excitotoxic (Zhou, Ding, Chen, Yun, & Wang, 2013a) a decrease in GluN2A may be neuroprotective against calcium mediated cellular excitotoxicity. Although NMDA dysfunction is associated with motor discoordination, it is usually due to combined disruption of both GluN2A and GluN2C, but not from single disruption of the GluN2A in cerebellum (Kadotani et al., 1996). This might explain why we did not observe any changes in the common behavioral parameters.

To further investigate whether PCE enhances apoptosis, we examined caspase 1 and caspase 3 activity. Activation of inflammatory caspases like caspase 1 and caspase 3 can increase the production of various pro-inflammatory cytokines initiating cell death and apoptosis (McIlwain, Berger, & Mak, 2013b). We did not observe any change in caspase 1, which is an inflammatory marker, but we observed a significant decrease in caspase 3, which is an apoptotic marker. This indicates that PCE might lead to a reduction in apoptotic event in the cerebellum. Furthermore, we investigated the signaling proteins that are associated with apoptosis. Three major mitogen-

activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-JUN N-terminal kinase (JNK), and P38 are known regulators of apoptosis (Chuang, Wang, & Yang, 2000). In this study, we observed significant reduction in the phosphorylation of ERK and JNK accompanied with an increase in P38 phosphorylation. Although ERK activation is traditionally thought to promote cell viability, activation of ERK in response to DNA damage from insults can indeed result in proteosomal degradation and inhibition of apoptosis (Mebratu & Tesfaigzi, 2009). JNK induces brain-region specific apoptosis during early developmental period and help in proper brain development (Kuan et al., 1999), while regulates neuronal migration, dendrite formation, and axon maintenance during later brain development (Björkblom et al., 2005; L. Chang, Jones, Ellisman, Goldstein, & Karin, 2003). Here, we observed reduced JNK phosphorylation along with a reduction in ERK phosphorylation in the cerebellum of PCE offspring. Since, we also observed a reduction in oxidative stress in the PCE offspring, alterations in phosphorylation of ERK and JNK may indicate an anti-apoptotic role. An increase in the phosphorylation of ERK and JNK has been associated with cerebellar neuronal death (Zahir et al., 2012). Reduction in the phosphorylation of these proteins may indicate a possible protective effect of cannabinoid exposure on cerebellar neuronal growth and survival. The role of p38 in cell death/survival is a controversial issue for decades and evidence depict that P38 can act as a double edge sword. However, inhibition of P38 has shown to elicit an apoptotic event in certain tumor cells (Phong et al., 2010; Refaat, Abdelhamed, Saiki, & Sakurai, 2015). Since we found a decrease in anti-apoptotic marker caspase 3 activity (Chuang et al., 2000; Herrera, Carracedo, Diez-Zaera, Guzmán, & Velasco, 2005b), we hypothesize that the increase in pP38 indicates an anti-apoptotic role.

The serine/threonine kinase AKT (also known as protein kinase B) plays a critical role in mediating diverse cellular functions including metabolism, growth, proliferation, survival, transcription and protein synthesis. AKT dysregulation has been implicated in various diseases as well as learning and memory (Hers, Vincent, & Tavaré, 2011). Increased oxidative stress and mitochondrial dysfunction can lead to AKT activation resulting in premature cell cycle arrest and apoptosis (Nogueira et al., 2008). AKT also serves as a regulator of GSK3 β , which is a critical protein for synaptic plasticity. In our study, we did not observe any changes in AKT or GSK3 β phosphorylation. However, acute administration of a cannabinoid agonist increased both AKT and GSK3 β phosphorylation in the hippocampus of adult rats, which was CB1R dependent (Ozaita, Puighermanal, & Maldonado, 2007). Since we did not observe any changes in the cerebellar CB1R, the changes in AKT and GSK3 β might be observed in acute dosing regimens but not with developmental exposure (Trazzi, Steger, Mitrugno, Bartesaghi, & Ciani, 2010). We also did not observe any change in cerebellar ILK level following unaltered AKT/GSK3 β . ILK regulates various cellular processes, including migration, differentiation, survival as well as neurite outgrowth & dendritic morphogenesis (A. Kelly et al., 2003; X.-F. Xu et al., 2015). ILK also regulates GSK3 β activity in neurons, as it can phosphorylate GSK3 β directly, or indirectly through AKT (Naska et al., 2006)(Delcommenne et al., 1998). Although we did not observe changes in the ILK levels or phosphorylation of AKT and GSK3 β , more studies are needed to identify whether there are any alterations in neuronal morphology or synaptic plasticity in the cerebellum due to PCE (D. Bhattacharya et al., 2015).

In conclusion, our results demonstrate a unique effect of PCE in the cerebellum of adolescent offspring. Our results indicate that PCE may have a neuroprotective effect in the cerebellum

during early phases of brain development (Table 2). Future studies should evaluate the in depth mechanisms by which PCE alters cerebellar function and the impact of these alterations on cerebellum specific behavioral outcome.

Tables:

Table 4.1: Effect of PCE on various physical and behavioral parameters

Behavioral parameters	Control	PCE
Tremor	N	N
Straub tail	N	N
Seizure	N	N
Mortality observed	N	N
Hyperactivity (Excessive Jumping)	N	N
Hind limb abduction	N	N
Head twitching	N	N
Hair coat erection	N	N
Fighting (Aggressive Behavior)	N	N
Drooling	N	N
Diarrhea	N	N
Ataxic behaviors	N	N
Anaphylactic shock/ Death	N	N
Allergic reaction (redness of the Skin or Eye)	N	N

N= Not observed

Table 4.2: Normal physiological functions of various markers and the effect of PCE

Markers	Physiological Functions	Prenatal Cannabinoid Exposure
ROS	Oxidative stress, cellular and neuronal injury	No changes in ROS generation
Nitrite	Neuroprotective	Nitrite content reduced in prenatal cannabinoid exposure
Lipid Peroxidation	Maintain cellular assembly and cell dynamics	No change in lipid peroxidation
Complex I & complex IV	Regulates oxidative stress, neuronal cell death & degeneration	Increased Complex I and Complex IV activity in prenatal cannabinoid exposure
Caspase 1	Inflammation	No change in Caspase 1 activity
Caspase 3	Cell death	Reduced Caspase 3 activity in prenatal cannabinoid exposure
ERK, JNK, P38	Cell differentiation, proliferation, neuronal death	Reduced ERK and JNK phosphorylation and increase in P38 phosphorylation in prenatal cannabinoid exposure
GluA1	Synaptic plasticity, regulator of pain pathway	No change in GluA1 level
GluN2a	Synaptic plasticity, neuronal excitotoxicity	Decreased GluN2A level in prenatal cannabinoid exposure
MAO	Regulates monoamine neurotransmitters (Serotonin & norepinephrine) crucially	Reduced MAO activity in prenatal cannabinoid exposure
Tyrosine	Rate limiting enzyme for catecholamine synthesis, regulates motor behavior	No change in tyrosine hydroxylase activity
AKT	cell viability & neuronal proliferation,	No change in AKT or pAKT

	learning & memory process, premature cell cycle arrest& apoptosis	levels
GSK3 β	Synaptic plasticity	No change in GSK3B or pGSK3B levels
ILK	Cell migration, adhesion, differentiation & survival. Neurite outgrowth and dendritic morphogenesis	No change in ILK levels

7. Figures:

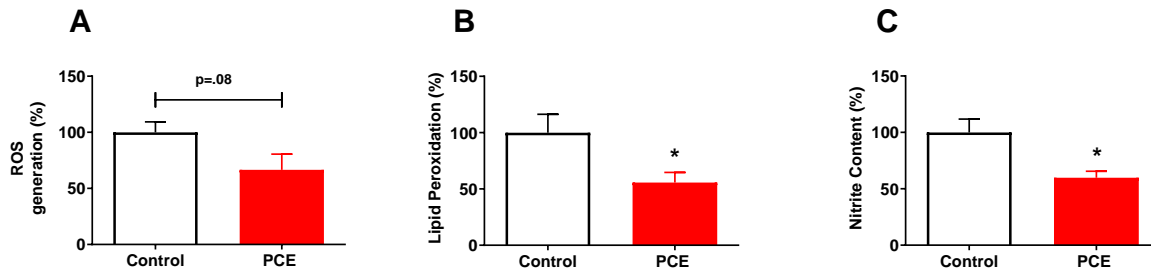


Figure 4. 1: Effect of PCE on ROS, lipid peroxide, and nitrite content in the cerebellum

(A) Reduction in the oxidative stress level measured by ROS generation ($F(1,9) = 3.96$, $p = .08$).

(B) significant reduction in cerebellar lipid peroxide content in PCE offspring ($F(1,9) = 5.63$, $p = .04$).

(C) Nitrite content was significantly reduced in prenatally cannabinoid exposed group ($F(1,7) = 9.36$, $p = .02$).

Results are expressed as Mean \pm SEM, $n=4-5$ rats per group. * indicates a significant difference when $p \leq .05$.

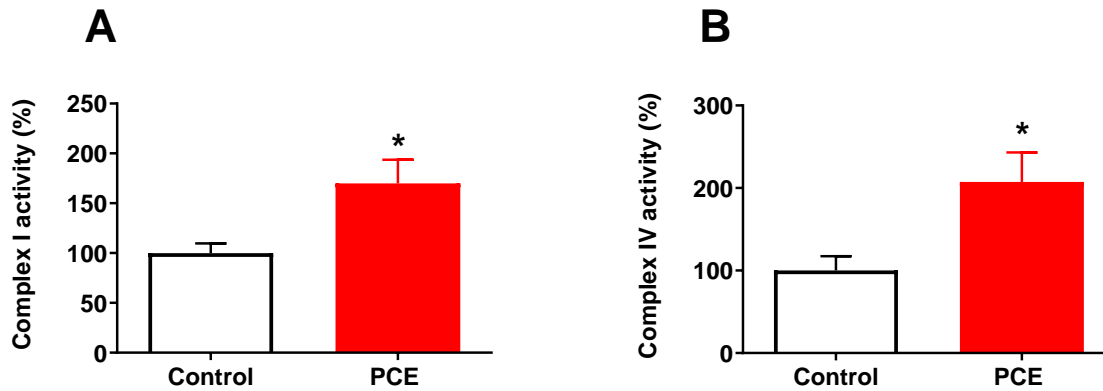


Figure 4.2: Effect of PCE on complex I and complex IV activity

(A) significant increase in complex –I activity in PCE offspring ($F(1,7) = 7.28, p = .03$). (B)

Complex IV activity has also increased in WIN55,212-2 exposed group ($F(1,7) = 7.24, p = .03$).

Results are expressed as Mean \pm SEM, n=4 rats per group. * indicates a significant difference when $p \leq .05$.

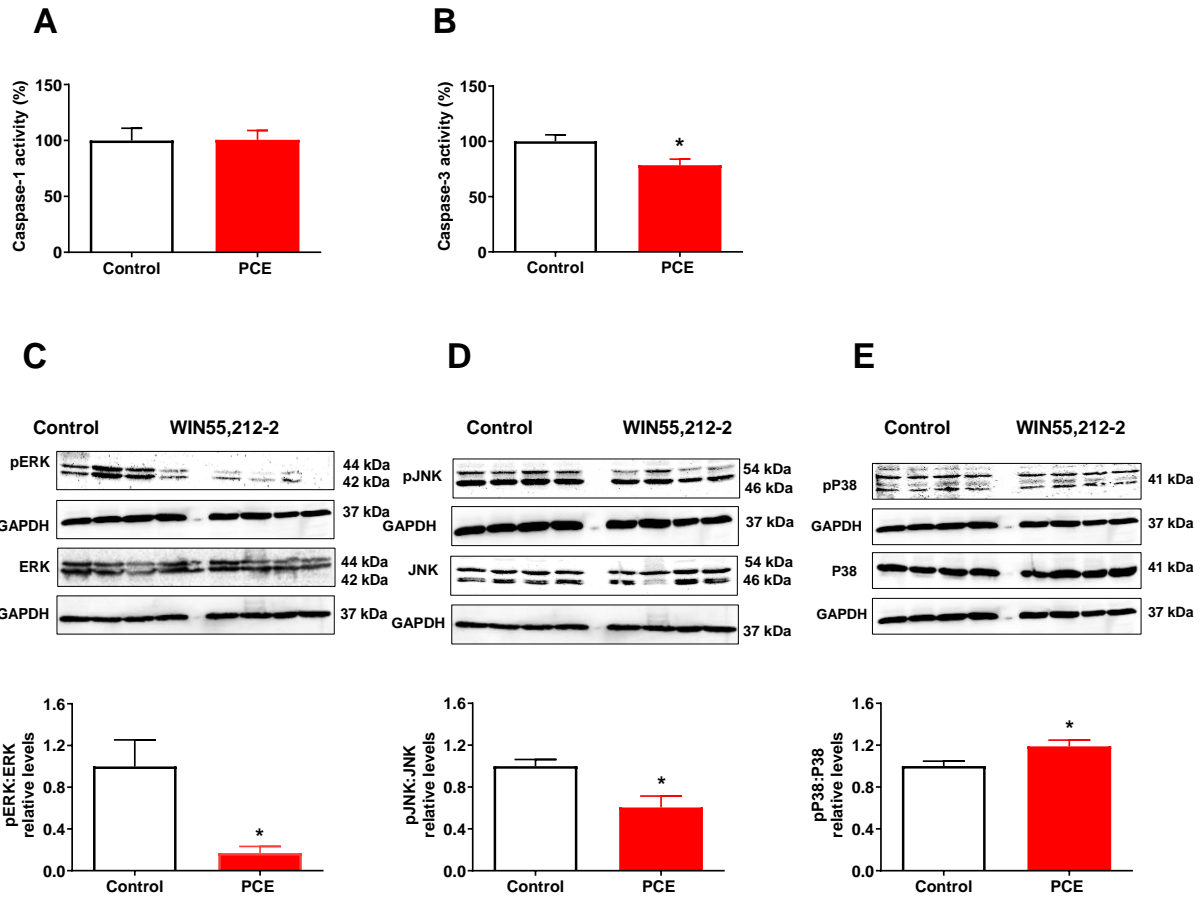


Figure 4.3: Effect of PCE on apoptotic markers

(A) PCE did not cause any alteration in the caspase 1 activity ($p > .05$). (B) Caspase-3 activity was significantly reduced in the PCE offspring ($F(1,7) = 6.99$, $p = 0.03$);). Representative immunoblots showing (C) pERK/ERK ($F(1,7)=10.09$, $p=.01$), (D) pJNK/JNK ($F(1,7)=9.70$, $p=.02$), (E) pP38/P38 ($F(1,7)=5.49$, $p=.05$).relative densities. Results are expressed as Mean \pm SEM, $n=3-4$ rats per group. * indicates a significant difference when $p \leq .05$.

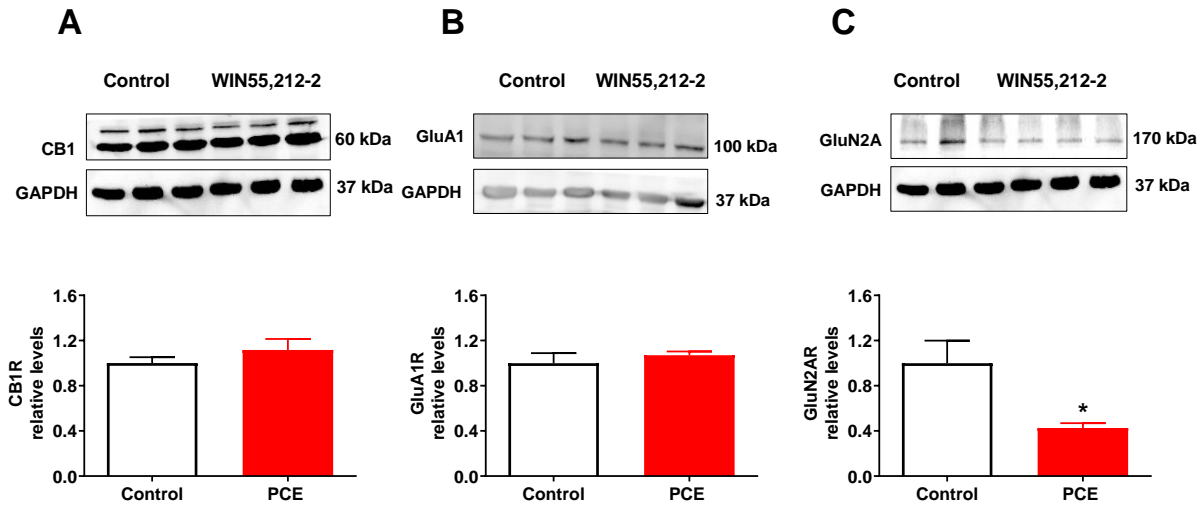


Figure 4.4: Effect of PCE on cerebellar signaling molecules associated with cannabinoid and glutamatergic neurotransmission

Representative immunoblots showing CB1/GAPDH, GluA1/GAPDH and GluN2A/GAPDH relative expression. (A) No change in CB1R expression in cerebellum ($p > .05$) (B) No change in GluA1 level in cerebellum ($p > .05$). (C) A significant reduction in GluN2A level in the prenatally cannabinoid exposed group ($F(1,4)=7.97$, $p=.04$) Results are expressed as Mean \pm SEM, $n=3-4$ rats per group. * indicates a significant difference when $p \leq .05$. * indicates a significant difference when $p \leq .05$

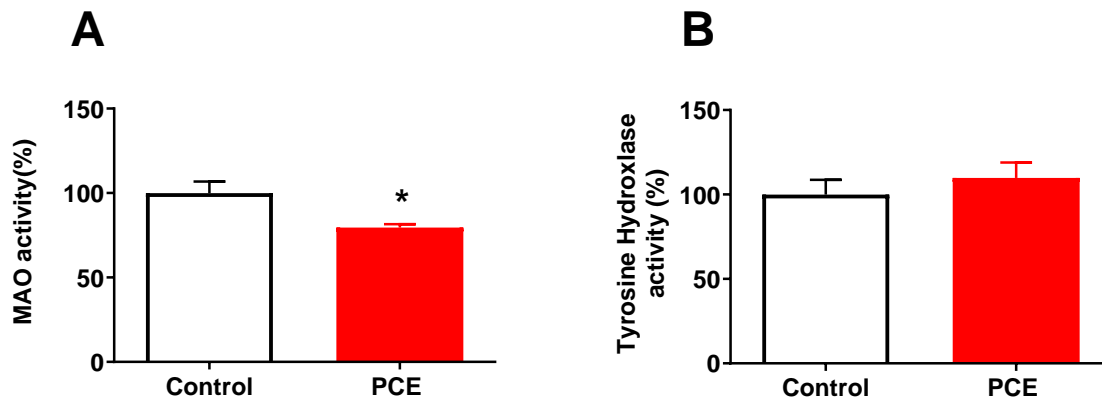


Figure 4.5: Effect of PCE on MAO and tyrosine hydroxylase activity:

(A) MAO activity was significantly reduced in PCE offspring ($F(1,7) = 8.21$, $p = 0.02$). (B) No significant change in the tyrosine hydroxylase content in between the groups ($p > .05$). Results are expressed as (%) change as Mean \pm SEM. $n = 4$ rats per group. * indicates a significant difference when $p \leq .05$

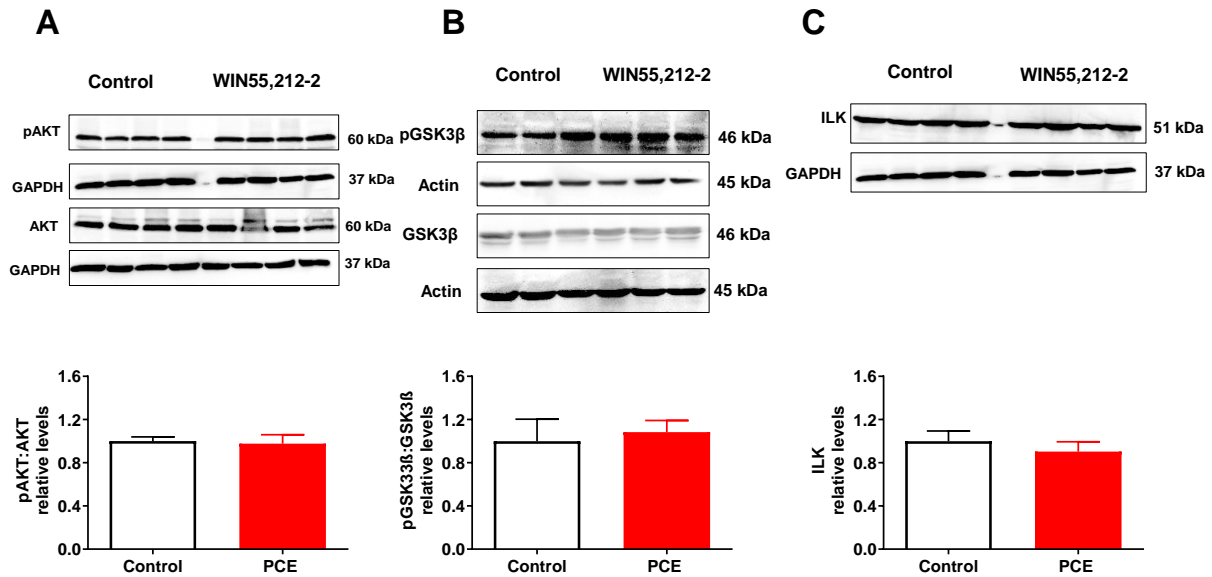


Figure 4.6: Effect of PCE cerebellar signaling molecules associated with markers of excitotoxicity and synaptic plasticity

Representative immunoblots showing pAKT/AKT, pGSK3β/GSK3β, ILK/Actin /GAPDH, relative expression. (A) No change in the phosphorylation of AKT in response to PCE ($p > .05$) (B) No change in the phosphorylation of GSK3β at Serine-9 between the two groups ($p > .05$). (C) No change in ILK expression in the cerebellum in response to PCE ($p > .05$). Results are expressed as Mean \pm SEM. $n = 3-4$ rats per group. * indicates a significant difference when $p \leq .05$

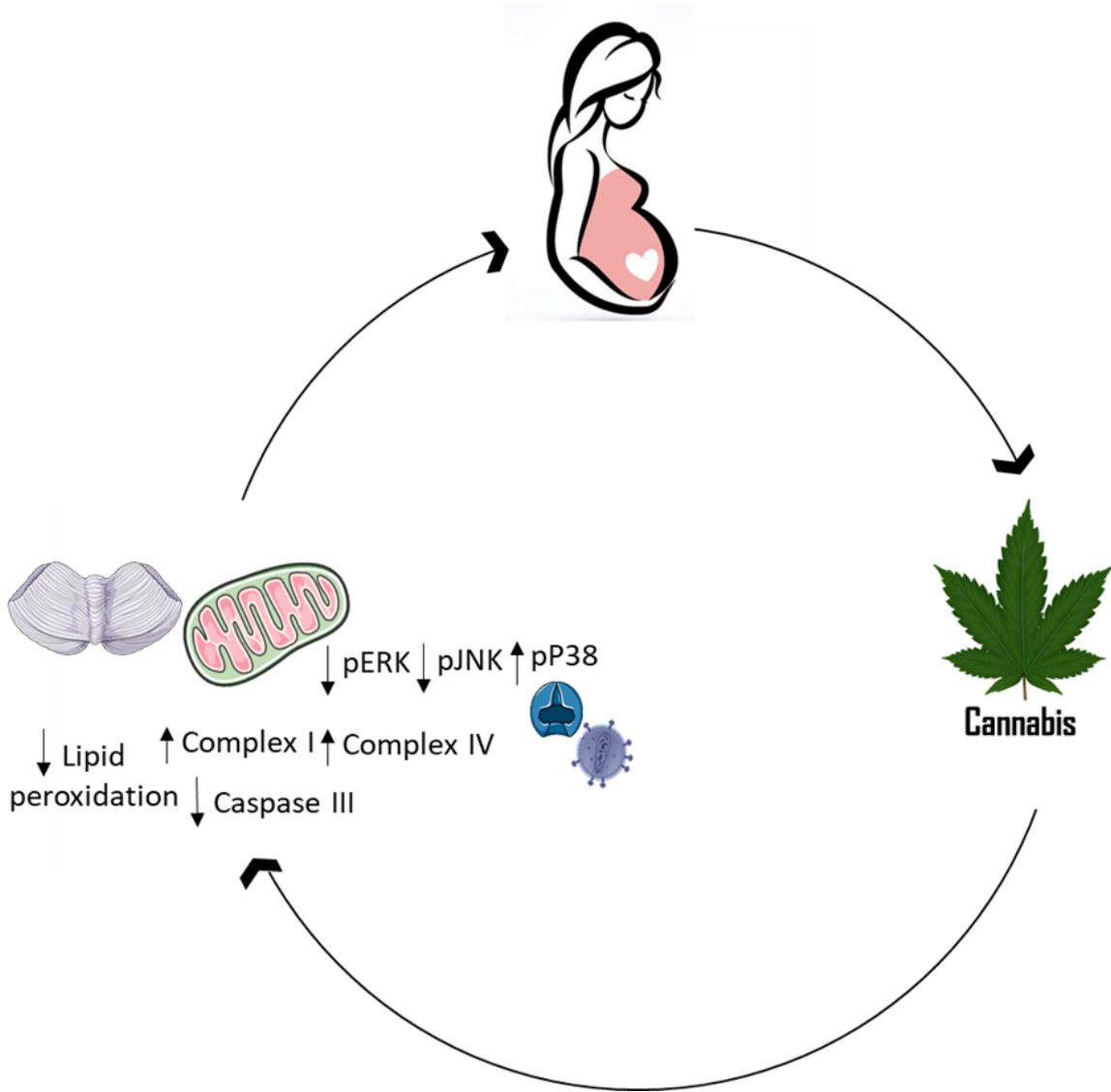


Figure 4.7: Effect of PCE on the developing cerebellum

PCE results in alteration of phosphorylation of several molecules i.e. ERK, JNK, P38. It can also increase complex I and complex IV activity accompanied with reduction in caspase 3 activity and lipid peroxidation content demonstrating altered mitochondrial function. This figure was produced using Servier Medical Art (<https://smart.servier.com/>) and Library of science and medical Illustrations (<http://www.somersault1824.com/science-illustrations/>).

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Chapter 5: Conclusion and Future Directions

The current work focused on elucidating the effect of prenatal cannabinoid exposure (PCE) on the hippocampus and cerebellum of adolescent offspring. We also investigated the possible mechanism through which the observed synaptic plasticity deficits in the hippocampus of PCE offspring could be ameliorated. To summarize key results, (1) PCE causes hippocampus dependent spatial learning deficits; (2) PCE leads to deficits in basal synaptic transmission, deficits in long-term potentiation (LTP), and an increase in presynaptic release probability accompanied with increased presynaptic axonal recruitment; (3) PCE causes a reduction in neural cell adhesion molecule (NCAM) and polysialylated NCAM (PSA-NCAM) leading to an imbalance between NMDAR subtype GluN2A and GluN2B mediated signaling accompanied with a reduction in AMPAR subtype GluA1 mediated downstream signaling alterations; (4) The deficits in LTP in the PCE offspring are ameliorated by acute application of a PSA-mimetic compound colominic acid on the hippocampal slices; (5) PCE exerted rather a protective effect on the cerebellum of the offspring by reducing oxidative stress marker: Reactive Oxygen Species (ROS), lipid peroxidation and nitrite content and improving mitochondrial bioenergetics complex I and complex IV activity; (6) A reduction in the level of pro-apoptotic proteins indicating an anti-apoptotic actions of PCE on cerebellum has also been observed. The data from the current study not only identified a specific mechanism responsible for PCE related memory deficits but will also comprehensively assess the different roles played by various synaptic proteins responsible for plasticity mechanisms closely associated with cognition.

There are a few limitations in the current study. According to the National Institutes of Health, sex should be considered as a biological variable for vertebrate studies. We utilized both male

and female rat offspring initially for the behavioral and electrophysiological experiments and did not see any sex-specific deficits. Hence, we did not continue the next set of experiments i.e. western blot and single-channel study on the hippocampus for both sexes and only utilized male pups. All the biochemical assays and western blot for the cerebellar study were performed on the male pups only. This can be assumed as a major caveat of the study since we discussed in the review section how sexual dimorphic exists for cannabinoid receptors in both animal and human neurotransmitter systems and physiological and pathological processes (M. E. Carroll & Smethells, 2015; Jutras-Aswad et al., 2009; Sanchis-Segura & Becker, 2016). Although we have observed the behavioral and synaptic plasticity deficits in response to PCE, we did not measure the amount of WIN55,212-2 in the fetal brain. We removed the osmotic pump after the delivery of the pups and compared its weight with its previous weight filled with the drug. Thus, the weight difference gave us the idea that the drug has been undergone systemic circulation into the pregnant dams and their offspring. Ideally, measurement of blood levels of the drug in dams and pups and brain levels of the drug in pups should be measured. Based on our previous literature discussion, the glutamatergic and GABAergic neurotransmitter systems seem to be co-dependent and cannabinoid exposure affects both of those neurotransmitter systems. CB1R is more in GABAergic interneurons which is at least 10-20 times higher than in glutamatergic neurons (Kawamura et al., 2006, Bellocchio et al., 2010). We have got an idea about increased presynaptic glutamate release from the electrophysiological paired pulse facilitation data and reduced glutamate uptake from the extracellular space by analyzing the multi-electrode array study data, altogether which indicate an increase in the basal glutamate level in the PCE offspring. The increase in glutamate level also indicates a possible reduction in the GABA level as GABA usually exerts an inhibitory effect on glutamate (Iversen, 2003; B. L. Thompson et al.,

2009). However, the absolute resting level of glutamate and GABA should be measured in those brain regions.

Another limitation is the use of *ex vivo* hippocampal slices instead of *in vivo* hippocampal recordings. In the current study, acute hippocampal slices in an artificially created *in vivo* environment, preserving synaptic circuitry and neuronal function, were utilized for electrophysiological recordings. While providing direct access to specific pathways for studying the effects of various drugs. However, the results of the current study, especially the rescue of LTP by PSA mimetic colominic acid, should be validated *in vivo* to confirm the physiological relevance of these findings. Furthermore, these should be evaluated with behavioral experiments. Previously, administration of PSA mimetic has shown to rescue cognitive deficits (O. Senkov et al., 2006). However, rescuing cognitive deficits by PSA-mimetic in a pregnant rodent model of PCE has not been investigated so far. As LTP in the hippocampus usually correlates with the hippocampus based memory tasks' performance (Lüscher & Malenka, 2012), it is expected that PSA could rescue behavioral deficits too, if sufficient brain concentrations are achieved.

Although we hypothesized PSA-NCAM deficits are responsible for the observed imbalance in the NMDA subtype mediated signaling, we did not validate it with examining the NMDA downstream signaling upon PSA-NCAM application. Future studies should also evaluate the pharmacokinetic parameters of PSA mimetic to establish oral or parenteral dosing regimens for easier administration and improved compliance. If this fails, the development of other therapeutic options could be considered that can inhibit the excess gluN2B mediated signaling or restore GluN2A mediated signaling deficits.

Another major limitation of this study is we did not examine the oxidative stress markers in the hippocampus. In the adult animals exposure to cannabinoid has shown to influence the oxidative stress and mitochondrial activity in both positive and negative ways (Ma et al., 2015; Philippot, Forsberg, Tahan, Viberg, & Fredriksson, 2019a; Singh, Hroudová, & Fišar, 2015b). So, it will be an interesting study to examine how PCE modulates the oxidative stress in the hippocampus and whether that could be correlated with observed behavioral deficits. Likewise, the animal also should have examined for cerebellar dependent motor activity deficits through specific behavioral paradigm to investigate whether reductions in oxidative stress and improvement of mitochondrial activity have any effects on motor learning.

Since WIN55-212-2 is a potent agonist at both type 1 and type 2 cannabinoid receptors (CBR), ideally CB2R level should have also examined. Recent evidence suggest that in adult animals' CB2R also plays an important role in the hippocampus dependent synaptic plasticity and LTP (Jimok Kim & Li, 2015b; Yong Li & Kim, 2016c). Hence, the change in LTP in this study could be also partially regulated by CB2R.

Taken together, the results from the current study indicate that PCE negatively influences hippocampal synaptic functions while exerts a possible neuroprotective effect on the cerebellar function. Thus, amelioration of hippocampal synaptic dysfunction can provide a scope of therapeutic use marijuana to attenuate the symptoms of morning sickness. The elucidation of underlying mechanisms has vertically advanced and expanded the understanding of the mechanisms by which PCE induces neurocognitive alterations. This work also contributes to the

growing body of evidence for the role of PSA-NCAM function in synaptic plasticity and memory, not only in PCE-exposed rodents but also in other neurocognitive disorders where PSA-NCAM expression or function is impaired. Knowledge from this study could be also utilized to identify the therapeutic potential for cannabinoid while reducing its specific adverse effect on certain synaptic circuitry.

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