Characterizing the endocannabinoid system in the mouse ovary

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ABSTRACT

Since the legalization of recreational cannabis in 2018, its use among Canadian women has increased from 18% pre-legalization to 22% in 2021. The components of cannabis are called phytocannabinoids, of which tetrahydrocannabinol (THC) binds to the cannabinoid receptor isoforms, CNR1 and CNR2. Given these two receptors have been found in reproductive organs, the continued increase in cannabis usage raises the concern of its potential effects on the female reproductive system. However, not much has been explored in terms of their roles in ovarian functions. The objectives of this study were to use mice to:1) profile the expression pattern of the endocannabinoid receptors (*Cnr1* and *Cnr2*) and the endocannabinoid metabolizing enzymes (*Faah* and *Mgll*) in ovarian granulosa cells during follicular development and ovulation; 2) to determine the effect of the Cnr1 antagonist (AM251) on ovulation, and 3) to evaluate the effect of THC on estrous cycles and ovarian steroidogenesis.

For granulosa cell collection, we superovulated immature mice (N=3-4/time-point) with equine chorionic gonadotropin (eCG; 5IU, i.p.) to induce follicular growth and 48h later with human chorionic gonadotropin (hCG; 5IU, i.p.) to induce ovulation. To pharmacologically inhibit Cnr1, we treated immature mice (N=3-5/group) with AM251 (5mg/kg or 10mg/kg; i.p.) 30 mins before hCG stimulation and collected oviducts 18h later for oocyte count. The control mice received the vehicle at 30 mins before hCG stimulation. To assess the effect of THC inhalation on the estrous cycle and steroidogenesis, we treated adult mice (N=5/group) with smoke of cannabis dried flower (1g/day) of high THC concentration for 28 days and recorded daily estrous cycle stages using vaginal cytology in each mouse. We collected blood samples at euthanasia on d28 for steroid measurement.

The transcript abundance of *Cnr1* was higher ($p<0.05$) at 4h hCG suggesting that the preovulatory LH surge induces its expression. The *Cnr2* mRNA levels were lower ($p<0.05$) at all timepoints of follicular development compared to 0h eCG, suggesting that it is downregulated by the gonadotropin treatment. Interestingly, the mRNA levels of *Faah* and *Mgll* were higher($p<0.05$) at 14h hCG compared to all other time points, suggesting their expression is upregulated during corpus luteum formation after ovulation. The mean number of ovulations was lower ($p<0.05$) in AM251-treated than vehicle-treated mice. We found that the number of estrous cycles in the THC-treated mice was lower and the length of estrous cycles was longer than in control mice

($p<0.05$). Among estrous cycle stages, estrus and diestrus phases were longer in the THC treated group as compared to the control group ($p<0.05$). Further, we found that estradiol and progesterone levels were not different among the treated and control groups ($p>0.05$).

In conclusion, these results indicate that Cnr1 appears to be involved in LH pathway during ovulation and that THC has negative impact on the ovarian functions. This study lays the foundation for further research on the mechanisms by which CNR1 impacts ovarian functions, which may help in the development of pre-conception guidelines as well as treatment protocols to manage reproductive health in women.

RESUME

Depuis la légalisation du cannabis récréatif en 2018, son utilisation chez les Canadiennes est passée de 18 % avant la légalisation à 22 % en 2021. Les composants du cannabis sont appelés phytocannabinoïdes, dont le tétrahydrocannabinol (THC) se lie aux isoformes des récepteurs cannabinoïdes, CNR1 et CNR2. Étant donné que ces deux récepteurs ont été trouvés dans les organes reproducteurs, l'augmentation continue de la consommation de cannabis soulève des inquiétudes quant à ses effets potentiels sur le système reproducteur féminin. Cependant, peu de choses ont été explorées en termes de leurs rôles dans les fonctions ovariennes. Les objectifs de cette étude étaient d'utiliser des souris pour : 1) profiler le profil d'expression des récepteurs endocannabinoïdes (Cnr1 et Cnr2) et des enzymes métabolisant les endocannabinoïdes (Faah et Mgll) dans les cellules de la granulosa ovarienne pendant le développement folliculaire et l'ovulation ; 2) pour déterminer l'effet de l'antagoniste Cnr1 (AM251) sur l'ovulation, et 3) pour évaluer l'effet du THC sur les cycles oestraux et la stéroïdogenèse ovarienne.

Pour la collecte de cellules de la granulosa, nous avons superovulé des souris immatures (N = 3-4/temps) avec de la gonadotrophine chorionique équine (eCG ; 5 UI, i.p.) pour induire la croissance folliculaire et 48 h plus tard avec de la gonadotrophine chorionique humaine (hCG ; 5 UI, i.p.) pour provoquer l'ovulation. Pour inhiber pharmacologiquement Cnr1, nous avons traité des souris immatures (N = 3-5/groupe) avec AM251 (5mg/kg ou 10mg/kg; i.p.) 30 minutes avant la stimulation hCG et collecté les oviductes 18h plus tard pour le comptage des ovocytes. Les souris témoins ont reçu le véhicule 30 minutes avant la stimulation hCG. Pour évaluer l'effet de l'inhalation de THC sur le cycle œstral et la stéroïdogenèse, nous avons traité des souris adultes (N = 5/groupe) avec de la fumée de fleur séchée de cannabis (1 g/jour) à forte concentration de THC pendant 28 jours et enregistré les étapes quotidiennes du cycle œstral à l'aide de cytologie vaginale chez chaque souris. Nous avons prélevé des échantillons de sang à l'euthanasie à j28 pour la mesure des stéroïdes.

L'abondance du transcrit de Cnr1 était plus élevée ($p < 0,05$) à 4 h d'hCG suggérant que le pic de LH préovulatoire induit son expression. Les niveaux d'ARNm Cnr2 étaient inférieurs ($p <0,05$) à tous les moments du développement folliculaire par rapport à 0h eCG, ce qui suggère qu'il est régulé à la baisse par le traitement à la gonadotrophine. Fait intéressant, les niveaux d'ARNm de Faah et Mgll étaient plus élevés ($p <0, 05$) à 14h

hCG par rapport à tous les autres moments, ce qui suggère que leur expression est régulée à la hausse pendant la formation du corps jaune après l'ovulation. Le nombre moyen d'ovulations était inférieur ($p < 0,05$) chez les souris traitées avec AM251 par rapport aux souris traitées avec le véhicule. Nous avons constaté que le nombre de cycles oestraux chez les souris traitées au THC était plus faible et que la durée des cycles oestraux était plus longue que chez les souris témoins ($p<0,05$). Parmi les stades du cycle oestral, les phases d'oestrus et de diestrus étaient plus longues dans le groupe traité au THC par rapport au groupe témoin ($p<0,05$). De plus, nous avons constaté que les niveaux d'œstradiol et de progestérone n'étaient pas différents entre les groupes traités et témoins ($p> 0,05$).

En conclusion, ces résultats indiquent que Cnr1 semble être impliqué dans la voie LH pendant l'ovulation et que le THC a un impact négatif sur les fonctions ovariennes. Cette étude jette les bases de recherches supplémentaires sur les mécanismes par lesquels le CNR1 a un impact sur les fonctions ovariennes, ce qui pourrait aider à l'élaboration de directives préconceptionnelles ainsi que de protocoles de traitement pour gérer la santé reproductive chez les femmes.

CONTRIBUTION OF AUTHORS

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Jasmine Randhawa designed and conducted all the experiments, analyzed the data and wrote the manuscript. Ejimedo Madogwe trained me for the techniques used in the experiments and assisted in data analysis. Emily Wilson and Hussein Traboulsi assisted in doing the cannabis smoke exposures. Carolyn Baglole made all the resources available for the cannabis related experiments and Raj Duggavathi designed the experiments, supervised the primary author, reviewed the data and the manuscript.

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LIST OF ABBREVIATIONS

3β-HSD: 3β-hydrosteroid dehydrogenase

17β-HSD: 17β-hydrosteroid dehydrogenase

AEA: N-arachinodoylethanolamine

2-AG: 2-arachidonoylglycerol

AM251 : 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidinyl)-1H-pyrazole-3-carboxamide

ANOVA: Analysis of Variance

AREG: Amphiregulin

B2m: Beta 2 microglobulin

BMP: Bone morphogenetic protein

BTC: Betacellulin

cAMP: Cyclic adenosine monophosphate

CBD: Cannabidiol

CBN: Cannabinol

cDNA: complementary deoxyribonucleic acid

cGMP: Cyclic guanosine monophosphate

CNR1: Cannabinoid Receptor 1

CNR2: Cannabinoid Receptor 2

Cyp11A1: Cytochrome P450 family 11 subfamily A member 1

Cyp17A1: Cytochrome P450 family 17 subfamily A member 1

Cyp19A1: Cytochrome P450 family 19 subfamily A member 1

DMSO: Dimethyl sulfoxide

DHEA: Dehydroepiandrosterone

DHH: Desert hedgehog

eCG: equine chorionic gonadotropin

ECS: Endocannabinoid system

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EREG: Epiregulin

ERK1/2: Extracellular regulated kinases

FAAH: Fatty acid amide hydrolase

FSH: Follicle-stimulating hormone

FSHR: Follicle-stimulating hormone receptor

FOXL2: Forkhead box L2

GABA: Gamma amino butyric acid

GDF9: Growth differentiation factor 9

GPCR: G protein coupled receptors family

GnRH: Gonadotropin-releasing hormone

hCG: human chorionic gonadotropin

HPO: Hypothalamus-pituitary-ovarian

IHH: Indian Hedgehog

L19: Ribosomal protein

LH: Luteinizing hormone

LHCGR: Luteinizing hormone/chorionic gonadotropin receptor

LLC: Large luteal cells

MAPK: Mitogen activated protein kinases

MGLL: Monoglyceride lipase

MPF: Maturation promoting factor

mRNA: messenger ribonucleic acid

NOBOX: Newborn ovary homeobox

P450scc: Cytochrome P450 steroid sidechain cleavage

P450c17: Cytochrome P450 17-hydroxylase/17,20-lyase

P450aro: Cytochrome P450 aromatase

P450c11: Cytochrome P450 11-hydroxylase

PD: postnatal day

PGR: Progesterone receptor

PBS: Phosphate buffered saline

PDE3A: Phosphodiesterase enzyme

PKA: Protein kinase A

Ptgs2: Prostaglandin-endoperoxide synthase 2

PPIB: Peptidyl-prolyl cis-trans isomerase B

WNT4: Wnt family member 4

CHAPTER 1: INTRODUCTION

In Canada, the use of cannabis for recreational purposes was legalized in 2018. Since then, its use has been persistently increasing in the total population by up to 2% to 3% every year. Among women, the prevalence has increased from 18% pre-legalization to 22% in 2021 (Canadian Cannabis Survey). Such a persistent increase in cannabis usage raises the concern of its impact on women's reproductive health because the endocannabinoid receptors are expressed in female reproductive organs including ovaries (Cecconi et al., 2019).

The endogenous ligand that binds to CNR1 with high affinity is N-arachidonoylethanolamine (AEA, a.k.a. Anandamide) and the one that binds to CNR2 is 2-arachidonoylglycerol (2-AG) (Mechoulam et al.,1995). The endocannabinoids, AEA and 2-AG, are degraded by enzymes fatty acid amide hydrolase (Faah) and monoglyceride lipase (Mgll), respectively. The endocannabinoid receptors are expressed in female reproductive organs such as the fallopian tube and uterus, suggestive of their potential role in fertilization and implantation (Gervasi et al., 2009). A *Cnr1* and *Cnr2* knockout study in mice reported embryo retention in the oviduct indicating their importance in embryo transfer to the uterus for implantation (Wang et al., 2004). A human study revealed that plasma AEA levels increase during the follicular phase of the menstrual cycle peaking around ovulation, signifying its potential role in follicular development and ovulation. (Cui et al., 2017). But not much has been explored in terms of the regulation of these receptors and the pathways they are involved in with respect to folliculogenesis and steroidogenesis in the ovary. As the major cannabis component, tetrahydrocannabinol (THC), is an exogenous ligand for CNR1, there are multiple studies suggestive of the negative effects of THC on reproductive processes. One study demonstrated that THC treatment *in vitro* resulted in lower FSH-induced steroidogenesis and LH receptor (Lhcgr) expression in rat granulosa cells (Adashi et al., 1983). A mouse study showed that THC administered orally resulted in reduced litter size (Kostellow et al., 1980). However, further investigation is required to determine if THC has adverse effect on specific ovarian functions such as ovulation, steroidogenesis, and regulation of reproductive cyclicity.

In this study, using the mouse model, I examined the expression pattern of

endocannabinoid receptors and endocannabinoid degrading enzymes in ovarian granulosa cells, the effect of Cnr1 inhibition on ovulation, and the effect of cannabis smoke on estrous cyclicity and steroidogenesis. This article-based thesis is divided into chapters describing the pertinent literature review and an article describing my experimental data.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Cannabis and phytocannabinoids:

Cannabis has been used for medicinal purposes from ancient times especially in some countries like Greece (Butrica, 2002), China and India (Li, 1974). It has been used for treating ailments like asthma, gout, and malaria in Chinese treatment and for migraines, convulsions in India (Touw, 1981). In European countries and the USA, it was accepted for its medicinal purpose in the nineteenth century. Cannabis extract called as 'cannabis tincture' was used for curing rabies, cholera, tetanus, and other similar illnesses in those countries (Pisanti & Bifulco, 2017). *Cannabis indica* and *Cannabis sativa* are the two main species of cannabis. At least sixty-six components which are referred to as phytocannabinoids have been identified so far, of which the four most familiar ones are cannabinol (CBN), cannabidiol (CBD), and two forms of tetrahydrocannabinol (delta 9-THC and delta 8-THC). Of those, delta 9-THC is the most active component having psychotropic effect (Dewey, 1986). These phytocannabinoids have therapeutic potential for neurological conditions. As a potential cure against seizures, delta 9- THC was administered in rat models as a result it reduced seizures induced by kainic acid (Wallace, 2003)). Apart from this, a mixture of THC and CBD in 20:1 ratio was effective as an anticonvulsant (Tzadok, 2016). The oral solution of CBD known as Epidiolex reduces the frequency and duration of seizures in epilepsy patients (Gofshteyn et al., 2017) and it has been approved in USA for epileptic treatment.

Apart from its use in medicine, cannabis is used for recreational purposes including in Canada (Cannabis Act). This has led to an increase in its usage.

2.2 Prevalence of cannabis use:

In Canada, cannabis was legalized in 2001 for medicinal purposes, and for recreational use in October 2018 under the 'Cannabis Act' (Cannabis laws and regulations-Canada). Since 2018, prevalence of use has increased steadily among different age groups. The Canadian Tobacco, Alcohol and Drugs Survey (CTADS), which was done in 2017 before the 'Cannabis Act' came into effect, reported that there were 15% of those people who have used cannabis in the past one year were aged above 15

years; of which 19% were 15-19 years old, 33% were between 20-24 years and 13% were older than 25 years (Canadian Tobacco, Alcohol and Drugs Survey).

In 2018, Canadian Cannabis Survey showed that there was an increase in the percentage of cannabis users among the people aged 15 years and older. The prevalence of cannabis use for recreational purposes among the Canadian population increased from 22% pre legalization to 25% in 2021 according to 'past 12 months survey'. The use is more prevalent among 20-24 years of age group of up to 49%. Among women, there has been a rise in usage by 4 % (Table 1).

Years	**2018**	**2019**	**2020**	**2021**
Total Population	22%	25%	27%	25%
Gender				
Male	26%	29%	31%	29%
Female	18%	21%	23%	22%
Age Groups				
16-19 years	36%	44%	44%	37%
20-24 years	44%	51%	52%	49%
25 older	19%	21%	24%	22%

Table 1: **Past 12 months cannabis use** (Modified from Canadian Cannabis Survey)

Among individuals who used cannabis in the past 12 months in 2021, there were 19% of people who reported daily use and 11% who reported one to two days a week, which is an increase from 10% in 2018 (Canadian Cannabis Survey 2021). Apart from recreational purposes there were 14% respondents aged 16 years and above who reported to use cannabis for medical purposes of which only 3% used it on prescription. Most of them (32%) consumed it on daily basis (Canadian Cannabis Survey). There are various methods through which cannabis can be consumed, the most common being smoking, as reported by 89% of people in 2018. Although the percentage of people who use smoking as a method of consumption has reduced to 74% in 2021 yet, it remains as the most common method (Canadian Cannabis Survey 2021). Overall, there has been increase in the use of cannabis among Canadians including women of reproductive age.

2.3 Discovery of phytocannabinoids:

The first isolated phytocannabinoid was CBN in late 19th century. It was first misunderstood as the psychoactive component of Cannabis. In 1940, Adams and colleagues identified another phytocannabinoid, CBD (Adams et al., 1940) and in 1942, a mixture of tetrahydrocannabinol, delta 8-THC and delta 9-THC, was isolated by another group (Wollner et al., 1942). Almost twenty years after their discoveries, the structures for both CBD and delta 9-THC were elucidated by Raphael Mechoulam and colleagues in 1963 and 1964, respectively (Gaoni & Mechoulam, 1964).

2.4 Metabolism of phytocannabinoids:

There are various ways in which phytocannabinoids, in particular THC, are consumed for recreational purposes. Those include inhalation, oral, and transdermal, but the most common route is inhalation (Canadian Cannabis survey). The metabolites formed are the same irrespective of the route of intake, although time taken for the formation of these metabolites varies depending upon the route (Huestis, Henningfield, & Cone, 1992,Ohlsson et al., 1980). Of the two major phytocannabinoids, metabolism of delta 9-THC has been studied the most as it is the most active psychotropic compound. Irrespective of the route, delta 9-THC is metabolized through hydroxylation and oxidation processes catalyzed by the CYP2C9 enzyme in the liver. As a result of hydroxylation, it is converted into 11-hydroxy-delta 9-tetra hydroxycannabinol (11-OH-delta 9-THC), the active metabolite. Then it is converted via oxidation to 11-nor-9-carboxy-delta 9-tetrahydrocannbinol (THCCOOH), the inactive metabolite, which is conjugated to glucuronic acid and excreted through urine (Musshoff & Madea, 2006).

After inhalation, delta 9-THC, is very rapidly absorbed through lungs such that within seconds of the first puff delta 9-THC can be detected in the plasma and within 8 mins it reaches the peak concentration (Huestis et al., 1992). In case of oral ingestion, it takes 1-2 hours for delta 9-THC and 11-OH-THC to reach their maximum plasma concentrations (Ohlsson et al., 1980).

2.5 Endocannabinoids:

Endocannabinoids are lipophilic molecules synthesized by various cells including neurons. There are various endocannabinoids identified so far, including N-arachinodoylethanolamine (AEA), arachinodoylglycerol (2-AG), noladin,

virodhamine and N-arachinodoyldopamaine (Cacciola et al., 2010). But AEA and 2-AG are the most widely studied.

2.5.1 Synthesis of Endocannabinoids:

2.5.1.1 AEA (N-arachinodoylethanolamine):

AEA was first isolated by Mechoulam and co-workers from the porcine brain (Devane et al., 1992). There are two steps in its synthesis; the first step involves the calcium dependent-*N*-acyltransacylase (NAT) enzyme that catalyzes the transfer of arachidonic acid to phosphotidyl-ethanolamine (PE) from sn-1 position of 1,2-*sn*-diarachidonylphosphatidylcholine (PC) (Di Marzo et al., 1994). As a result, *N*-arachidonylphosphatidylethanolamine (NAPE) is formed. In the second step, NAPE is hydrolysed by *N*-acylphosphatidylethanolamine (NAPE)- specific phospholipase D (PLD) (NAPE-PLD) (Okamoto at al., 2004). Consequently, AEA and phosphatidic acid are formed. Instead of NAPE-PLD an another enzyme, secretory phospholipase 2 (sPLA2), can also hydrolyse NAPE to form *N*-arachidonoyl-lysophosphatidylethanolamine (lyso-NAPE), which is then converted into AEA by lyso phospholipase D (lyso-PLD) (Okamoto et al., 2007). Alternatively, NAPE can be cleaved by phospholipase C resulting in phospho-AEA is formed which is dephosphorylated by the enzyme protein tyrosine phosphatase to form AEA (Liu et al., 2006). (Figure 1). In the brain, it is thought to be synthesized pre-synaptically in axon terminals as its biosynthetic enzymes are highly concentrated in that area (Nyilas et al., 2008), but is degraded post-synaptically (Piomelli et al., 1998, Bracey et al., 2002).

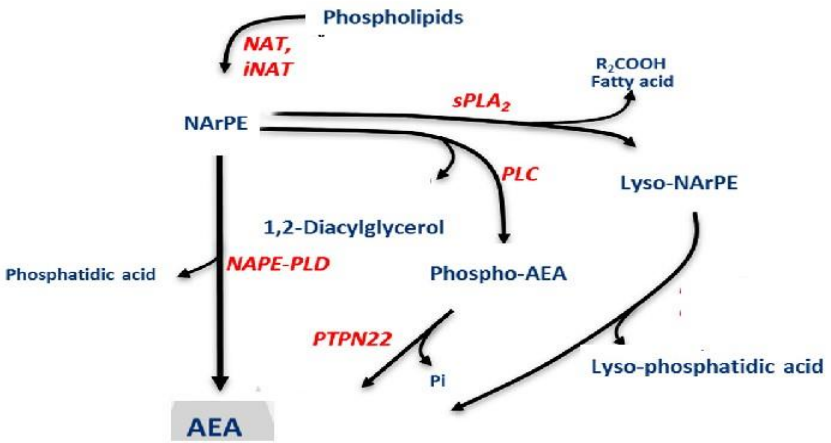

Figure 1: Synthesis of AEA modified from (Maccarrone, 2017)

2.5.1.2 2-AG (arachinodoylglycerol):

This endocannabinoid was isolated from canine intestinal tissue by Mechoulam and co-workers in 1995 (Mechoulam et al., 1995). In the first step of its synthesis initially 1,2-diacylglycerol (1,2-DAG) is formed from phosphatidylinositol (PI) by phospholipase C (Farooqui, Rammohan, & Horrocks, 1989). In the second step, DAG is converted into 2-AG by diacylglycerol lipase (DAGL) (Stella et al., 1997). In an alternative pathway, PI is hydrolyzed by phospholipase A thus producing lyso-phosphatidylinositol (LPI), which is then converted by lyso-phospholipase C to 2-AG (Sugiura et al., 1995). In the brain, it is synthesized post-synaptically in dendritic spines, but acts on the CNR1 receptors expressed pre-synaptically (Di Marzo, 2009), where it is also degraded as well (Dinh et al., 2002).

2.5.2 Degradation of endocannabinoids:

AEA is degraded by the enzyme fatty acid amide hydrolase (FAAH) to arachidonic acid and ethanolamine (Cravatt et al., 1996). This enzyme is present in the brain, testis, ovaries, liver and various other mammalian tissues (Cravatt & Lichtman, 2002). FAAH is an enzyme belonging to the amidase family and is an intracellular membrane bound protein (McKinney & Cravatt, 2005). This enzyme does not have much degrading effect on 2-AG, which is hydrolyzed by another enzyme called monoacylglycerol lipase (MGLL) to arachidonic acid. It is located in the cytosol and is present in mouse hippocampus, ovary, adipose tissue and brain of rat and human (Dinh et al., 2002).

2.6 Endocannabinoid Receptors:

These endocannabinoids bind to the receptors in the body known as endocannabinoid receptors. The endocannabinoid receptors, CNR1 and CNR2, are members of G protein coupled receptor (GPCR) family (Matsuda et al., 1990). These receptors were discovered in 1988 in Allyn Howlett's lab by using a radiolabeled cannabinoid receptor ligand [^{3}H]-CP55940 in the rat brain (Devane et al, 1988).

2.6.1 CNR1 Receptor:

Rat and human CNR1 receptors were cloned from a rat cerebral cortex cDNA library by Tom Bonner and colleagues (Matsuda et al., 1990). This receptor is highly conserved across mouse, rat, and human. Among mouse and human orthologs there is

90% identity in nucleic acid sequence and 97% identity in amino acid sequence (Abood at al., 1997). AEA has high affinity for this receptor and low affinity for CNR2 (Mechoulam et al., 1995). CNR1 is highly expressed in the brain, but its expression is also detected in other organs such as ovaries, uterus, gastrointestinal tract, urinary tract, and even human placenta (Pagotto et al., 2006). In the brain, it is present at the terminals of the neurons thereby having an inhibitory action on the secretion of various neurotransmitters including GABA and glutamate because of less calcium influx (Howlett et al., 2002). If activation is for a short time, it results in Ca2+ influx inhibition at presynaptic level (Kreitzer & Regehr, 2001). In case of long term activation it has inhibitory effect on adenylyl cyclase resulting in cAMP/PKA pathway downregulation (Heifets & Castillo, 2009).

2.6.2 CNR2 Receptor:

CNR2 was cloned from the human promyelocytic leukemia cell line, HL60, in 1993 by Sean Munro and coworkers (Munro et al., 1993). In contrast to CNR1, there is 82% identity in amino acid sequence between mouse and human (Shire et al., 1996). Human CNR1 and CNR2 receptors, display 44% identity in protein structure with 68% identity in the transmembrane region (Munro et al., 1993). Among endocannabinoids, 2-AG binds to CNR2 with higher affinity as compared to CNR1 (Sugiura et al., 1995). Similar to CNR1, CNR2 is also involved in the inhibition of adenylyl cyclase activity thereby reducing cAMP levels (Bouaboula et al., 1996). According to one of the mouse studies, cAMP levels were higher in the oocytes treated with cannabinoid receptor antagonists than in the vehicle treated controls, thus suggesting that CNR1 and CNR2 are involved in decreasing cAMP levels (Cecconi et al., 2019). CNR2 is primarily found in immune cells (Bouaboula et al., 1993), but it is also present in other organs such as testis (Gerard et al., 1991), brain (Gong et al., 2006) and ovary (El-Talatini et al., 2009).

2.7 Binding of phytocannabinoids to endocannabinoid receptors:

The phytocannabinoids bind to the endocannabinoid receptors but vary in their affinities for these receptors. THC in particular has more affinity for CNR1 than CNR2, but CBD does not bind to either of the main ligand binding sites of these receptors (Reggio et al., 2010). CBD has the potential to bind to an allosteric site of

CNR1 therefore negatively affecting the potency of its agonists (Laprairie et al., 2015).

2.8 Ovary:

Endocannabinoid receptors and ligands have been studied in female reproductive organs including ovaries (Di Blasio et al., 2013). Ovaries include the process of folliculogenesis which is development of follicle and oocyte, release of an oocyte which is called as ovulation, and development of corpus luteum which is termed as luteinization (Figure 2).

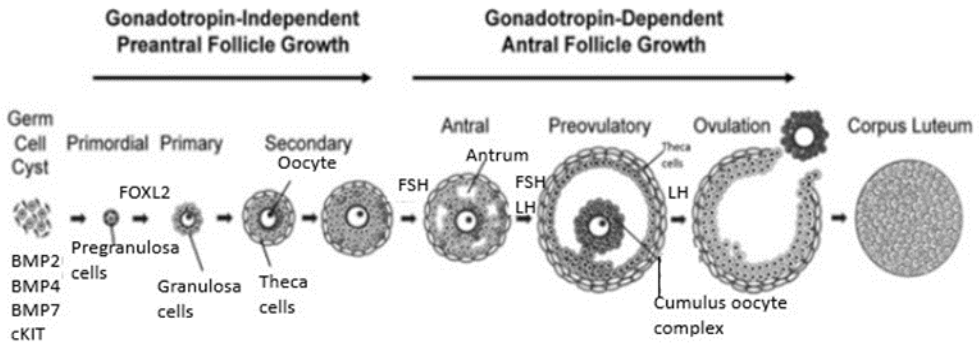

Figure 2: Preantral (early) and antral follicle development (Modified from (Edson et al., 2009))

2.8.1 Early follicle development:

During embryonic development the primordial germ cells (dorsal endoderm cells) migrate to the genital ridge. The bone morphogenetic proteins (BMP2, BMP4 and BMP7) (Pangas & Rajkovic, 2015) and cKIT- cKIT ligand (Driancourt et al., 2000) signaling pathways are responsible for the proliferation and survival of these germ cells. *FOXL2* is one of the ovary determining genes and is highly expressed in the somatic cells surrounding the germ cell nests and is involved in the process of ovarian somatic cell differentiation (Uhlenhaut et al., 2006). As a result of proliferation, syncytia, otherwise known as “nests”, are formed in the embryonic gonad. Nests are broken down by migration of the progenitor somatic cells (pregranulosa cells), thus forming primordial follicles leading to the formation of the ovarian reserve (Pangas & Rajkovic, 2015). There are two classes (waves) of primordial follicles in mice; the first wave involves activation of primordial follicles in the medulla region of the ovary

immediately after birth and the granulosa cells of these primordial follicle are FOXL2 positive cells. Whereas the second wave involves the activation of primordial follicles in the cortical region of the ovary and the granulosa cells of these follicles are derived from proliferative precursors which are activated in adulthood (Mork et al., 2012). These precursor cells undergo mitotic arrest prenatally and are activated postnatally. This leads to the formation of granulosa cells around the follicle, thus forming primary follicles and further division of granulosa cells leads to secondary follicle. In mice by postnatal day, (PD23), the first wave follicles mature into antral follicles, which are marked by formation of the fluid-filled antral cavity (Zheng et al., 2013) and gradually by PD90 almost all the first wave follicles are replaced by the second wave follicles, which will develop into ovulatory follicles (Zheng et al., 2013). Other than granulosa cells, there is a layer of supporting cells surrounding granulosa cells known as theca cells (Young & McNeilly, 2010). Growth differentiation factor (GDF9) expression in oocyte leads to the differentiation of theca cells at a secondary follicle stage (Solovyeva et al., 2000).

2.8.2 Antral follicle development:

With the formation of the antrum, the follicle development involves gonadotropins, FSH and LH, released from the pituitary in response to gonadotropin releasing hormone (GnRH) secreted from hypothalamus (Dalkin et al., 1989). The granulosa cells of antral follicle become responsive to FSH because of the expression of FSHR (George et al., 2011). As a result, the granulosa cells proliferate and differentiate into cumulus cells (the cells surrounding the oocyte) and mural cells (the cells surrounding the antrum) (Khamsi & Roberge, 2001). The gonadotropins regulate the processes of steroidogenesis and ovulation (Leung & Armstrong, 1980).

2.8.2.1 Steroidogenesis:

The process of steroidogenesis (Figure 3) begins at the antral follicle stages which are known as the secondary and tertiary follicle stages, where FSH and LH activate FSH receptor (FSHR) in granulosa cells and LHCGR in theca cells, respectively (Raju, 2013). Upon activation of FSHR, the expression of the enzyme CYP19A1 is induced along with production of estradiol resulting in the proliferation of granulosa cells. In theca cells the LHCGR is involved in the production of androgen (Richards et al., 1994). Initially in steroidogenesis, the steroidogenic acute regulatory protein encoded by the *STAR* gene transports cholesterol into mitochondria. Cholesterol is

converted to pregnenolone by the enzyme CYP11A1 and pregnenolone is converted to progesterone by 3β hydoxysteroid dehydroxygenase (3β-HSD). In the theca cells, CYP17A1, is highly expressed and it converts pregnenolone and progesterone to 17-OH pregnenolone and 17-OH progesterone, respectively. It also converts 17-OH pregnenolone and 17-OH progesterone to dehydroepiandrosterone (DHEA) and androstenedione, respectively. Androstenedione is then converted to testosterone by 17β hydroxysteroid dehydrogenase (17β-HSD). The androgens are then converted into estrogen by the enzyme aromatase (CYP19A1) expressed in granulosa cells of the follicle (Rey & Grinspon, 2011). There is a shift from estrogen to progesterone synthesis after the preovulatory LH surge. As a result of which, luteal cells (originating from both granulosa and theca cells), of the corpus luteum convert pregnenolone to progesterone using 3β HSD (Ohara et al., 1987). The LH surge reduces CYP19A1 expression in granulosa cells (Nimz et al., 2010).

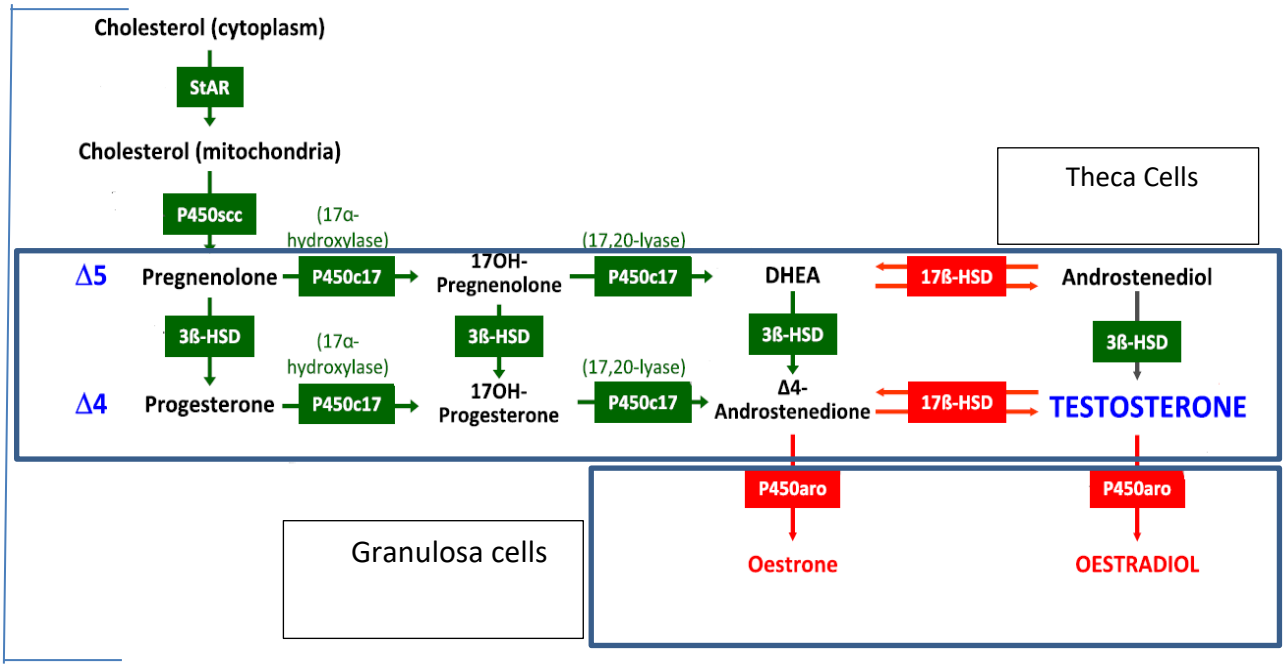

Figure 3. **Process of steroidogenesis in ovarian cells** (Modified from (Rey et al., 2011))

2.8.2.2 Ovulation:

Ovulation involves the rupture of preovulatory follicle hence releasing the expanded cumulus cell oocyte complex (COC) (Zhang et al., 2014). As follicles develop to preovulatory size, FSH and estradiol induce the expression of the LHCGR in granulosa cells thereby making the cells responsive to LH (Hunzicker-Dunn & Maizels, 2006). The LHCGR expression is however restricted to mural granulosa cells

(Peng et al., 1991). It is well established now that the LH surge through LHCGR induces the expression of EGF like factors, amphiregulin, epiregulin and betacellulin, in mural cells resulting in cumulus expansion and oocyte maturation (Park et al., 2004). In addition to cAMP, LH along with EGF-like factors activates ERK1/2 pathway, which appears to be the most critical signaling pathway regulating the gene expression program of granulosa cells during ovulation (Madogwe et al., 2021). One of the studies showed that ERK1/2$^{gc-/-}$ knockout female mice lead to anovulation and complete infertility (Fan et al., 2009).

Expression of specific genes in response to the LH surge include progesterone receptor (PGR) and prostaglandin synthase 2 (PTGS 2) among others (Kim & Duffy, 2016) (Richards & Pangas, 2010).-One of the events during follicle rupture involves the degradation of the proteoglycan versican by the enzyme ADAMTS1, a protease induced by PGR (Robker et al., 2000). At this preovulatory phase, oocyte is at germinal vesicle stage arrested at prophase 1 of meiosis, which is due to high levels of cAMP within oocyte and is maintained by constitutively active GPCRs (GPR3 and GPR12) within the oocyte. High levels of cAMP are also maintained because of inactive phosphodiesterase enzyme (PDE3A) in the oocyte. PDE3A is activated in response to the LH surge leading to reduced levels of cAMP and resumption of meiosis. This process involves cGMP, which is also elevated along with cAMP during meiotic arrest but is present in oocyte, granulosa cells and cumulus cells. Guanylate cyclase enzyme is responsible for maintaining cGMP levels and it has inhibitory action on PDE3A in oocytes as its transferred through cumulus- oocyte gap junctions upon secretion in granulosa and cumulus cells. Decrease in cGMP levels activate PDE3A resulting in decline in cAMP levels and hence resume meiosis (Gilchrist et al., 2011). As a result of meiotic resumption oocyte development takes place.

2.8.2.3 Luteinization:

Luteinization is the formation, in response to the LH surge, of the corpus luteum through differentiation of granulosa cells and theca cells into luteal cells of the ovulated follicle. During the transformation of a follicle into the corpus luteum, angiogenesis takes place through the action of vascular endothelial growth factor C and D (Kim et al., 2016). The granulosa luteal cells are referred to as large luteal cells (LLC) and thecal luteal cells are referred to as small luteal cells (SLC). Luteotrophic hormones such as LH, prolactin, human chorionic gonadotropin (hCG) and estrogen

are required for the maintenance and functioning of the corpus luteum (Stocco et al., 2007). Progesterone that is synthesized by corpus luteum through LLC is essential for implantation and maintenance of pregnancy (Stocco et al., 2007).

2.9 Endocannabinoid system in the human ovary:

One study investigated CNR1 and CNR2 expression in the ovary at different stages of folliculogenesis using immunohistochemistry (El-Talatini et al., 2009). In the primordial follicle, CNR2 is more strongly expressed in granulosa cells as compared to CNR1. Whereas in granulosa cells of primary and secondary follicles, CNR1 and CNR2 show similar levels of expression. But in granulosa cells of tertiary (antral) follicles the CNR1 expression is higher compared to CNR2. FAAH, the degrading enzyme of AEA, is present in theca cells of secondary and tertiary follicles. CNR1 and FAAH are also expressed in corpus luteum (El-Talatini et al., 2009). This shows that CNR2 expression in more prominent during early follicular development stages whereas CNR1 is expressed more at later stage.

In terms of endocannabinoids, one study examined the plasma AEA levels during menstrual cycle in women. Its levels peaked at the time of ovulation (12.24 ng/ml approximately) in comparison to early and late follicular phase. Its levels were lowest at the luteal phase (7.46 ng/ml approximately). There was a positive correlation between AEA levels and LH, FSH and estrogen levels (Cui et al., 2017). Estrogen has inhibitory effect on FAAH thus it increases the release of AEA from the endothelial cells (Maccarrone et al., 2002). Progesterone activates FAAH by upregulation of its gene expression in human T lymphocytes (Maccarrone et al., 2001). Both these studies suggests that AEA could be a part of ovulation process and post ovulation when progesterone levels increase during corpus luteum formation. The AEA levels could possibly decrease during luteinization because of an increase in the levels of the degrading enzyme (FAAH).

Following ovulation, fertilization takes place in the ampulla which is a part of fallopian tube, and the subsequent blastocyst development occurs in the isthmus region of the fallopian tube (oviduct). Significant concentrations of AEA are found in the fallopian tube fluid in humans (Schuel et al., 2002). In vitro treatment with metabolic substitute of AEA (AM 356) resulted in increased sperm motility at lower concentration and decreased motility at 10-fold higher concentration (Schuel et al.,

2002). This study indicates that an appropriate concentration of AEA may be required for fertilization.

2.10 Endocannabinoid System in the mouse reproductive system:

During in vivo meiotic maturation there is decrease in mRNA levels of *Cnr1* and *Cnr2* from geminal vesicle (GV) to meiosis I (MI) and meiosis II (MII) stages in oocytes. CNR1 and CNR2 had similar levels at GV stage whereas at MI stage CNR2 showed increase in its levels as compared to CNR1 which showed a significant decrease. Further at MII stage neither receptors showed a significant increase compared to MI (Cecconi et al., 2019).

In the uterus, AEA levels have been associated with uterine receptivity and refractoriness during implantation of the embryo in mouse. At the implantation sites AEA levels are lower as compared to the inter implantation sites in mice, indicating that high AEA levels may adversely affect implantation (Schmid et al., 1997).

2.11 Effect of THC on female reproduction:

Like other drugs such as tobacco, cannabis has been reported to have adverse effects on female reproduction. One study on rhesus monkeys indicated that delta 9-THC administered during follicular phase suppressed the LH surge, pattern of circulating estrogen was also altered and progesterone during the luteal phase was suppressed as well (Asch et al., 1981). However, another study showed no effect when delta 9 THC was administered during luteal phase (Asch et al., 1979). Another study in rhesus monkeys showed that a single dose of delta 9 THC administration at mid luteal phase caused a decline in the progesterone level, but this effect was reversed with hCG treatment (Almirez et al., 1983). Similar results of decreased plasma LH levels were also observed in a study conducted on women who used marijuana (Mendelson et al., 1986). A study in female mice showed that the litter size was smaller in THC administered group which was done through intubation (Kostellow et al., 1980).

All these studies indicate that THC may have suppressive effect on gonadotropins which can eventually lead to disrupted ovarian function and have effects on fertility.

CHAPTER 3: RATIONALE, HYPOTHESIS AND OBJECTIVES

The discovery of cannabinoid receptors (CNR1 and CNR2) in the 1980's led to further investigation about the role of these receptors in various physiological systems especially nervous system and immune system (Zou & Kumar, 2018). Later these receptors were also found to be expressed in the female reproductive system in the ovary, uterus and fallopian tube (Di Blasio et al., 2013). These receptors bind to endogenous ligands AEA and 2-AG with the former having high affinity towards CNR1 and the later towards CNR2 (Mechoulam et al.,1995). Besides these ligands, an exogenous compound THC also binds to CNR1 indicating that it might have effects on ovarian function (Reggio, 2010). Taking into consideration the increase in cannabis use among females from 18% in 2018 to 22% in 2021 (Canadian Cannabis Survey), it raises the concern of the effects of THC on female reproductive health because THC is known to bind to CNR1 (Reggio et al., 2010), which is found to be expressed in the ovary (El-Talatini et al., 2009).

I hypothesized that endocannabinoids and THC have direct effect on ovarian granulosa cells and thus female fertility. The following were the objectives of this study:

1. To profile the expression pattern of the *Cnr1* and *Cnr2* and the endocannabinoid metabolizing enzymes (*Faah* and *Mgll*) in granulosa cells during follicular development and ovulation
2. To determine the effect of the Cnr1 antagonist, AM251, on ovulation
3. To evaluate the effect of THC inhalation for 4 weeks on estrous cycles and steroidogenesis.

The results lay foundation for further studies to unveil underlying mechanisms. The effect of THC on reproductive parameters will expand the knowledge of the effects of cannabis use on female reproductive health.

CHAPTER 4

Characterizing the endocannabinoid system in the mouse ovary

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4.1 ABSTRACT:

The major component of Cannabis, tetrahydrocannabinol (THC) signals through cannabinoid receptors, CNR1 and CNR2. As these two receptors are found in ovaries, cannabis usage raises concerns about the potential effects of THC on ovarian functions. The objectives of this study were to use the mouse model to: 1) profile the expression pattern of the *Cnr1* and *Cnr2* and the endocannabinoid metabolizing enzymes (*Faah* and *Mgll*) in granulosa cells during follicle development and ovulation; 2) to determine the effect of the Cnr1 antagonist, AM251, on ovulation; and 3) to evaluate the effect of THC inhalation for 4 weeks on estrous cycles and steroidogenesis. We found that *Cnr1* transcript abundance was higher ($p<0.05$) at 4h hCG than 48h eCG time point, whereas *Cnr2* transcript was lower in ovulating follicles. Conversely, *Faah* and *Mgll* transcripts were higher at 14h hCG ($p<0.05$) suggesting their upregulation after ovulation. The ovulation rate was lower in AM251 than vehicle treated mice ($p<0.05$), indicating that Cnr1 signaling is pivotal for ovulation. The number of estrous cycles was lower, and their length was greater with longer estrus and diestrus phases in THC-treated mice ($p<0.05$). Further, we found that estradiol and progesterone levels were not different among THC and control groups ($p>0.05$). In conclusion, these results indicate that the endocannabinoid system appears to play a role in ovarian functions and that THC has negative impact on estrous cyclicity.

4.2 INTRODUCTION:

In Canada, Cannabis was legalized in October 2018 for recreational purposes and since then its use has increased among the total population from 22% in 2018 to 25% in 2021. The proportion of women users went up from 18% in 2018 to 22% in 2021 and the proportion of adult users in the total population aged 20 to 24 years went up from 44% in 2018 to 49% in 2021 (Canadian Cannabis Survey). Cannabis contains two main phytocannabinoids, a psychoactive THC (Wollner et al., 1942) and non-psychoactive CBD (Adams et al., 1940). The data from the Canadian Cannabis Survey raises concerns about the potential effects of THC on female reproductive health because THC is known to suppress the levels of gonadotropins (Walker et al., 2019) and result in small litter size in mice (Kostellow et al., 1980). As gonadotropins are required for normal functioning of ovaries (Richards et al., 1978), it is important to investigate if THC impacts ovarian functions.

THC acts by binding an endocannabinoid receptor, CNR1 (Devane et al., 1988), which is a member of the Gi/o class of GPCR (Felder et al, 1995). The endogenous ligands AEA, which preferentially binds to CNR1, and 2-AG, which preferentially binds to CNR2, are degraded by the enzymes FAAH and MGLL, respectively (Mechoulam et al., 1995). Considering the increase in the prevalence of cannabis use among Canadian women and the expression of endocannabinoid receptors in female reproductive system, it is critical to examine the role of these receptors and the effect of THC on normal functioning of the ovary.

4.3 MATERIALS AND METHODS:

4.3.1 Animals:

We used 3 weeks old C57BL/6 immature mice bought from Charles River laboratories and mature 7 to 9 weeks old bred in house mice weighing 10-12 g and 18-20g respectively. They were kept in standard cages under 12 hours light and dark cycles from 7:00 am to 7:00 pm and were given feed ad libitum. The experiments were designed in accordance with the Canadian Council of Animal Care guidelines and were approved by the Facility Animal Care Committee, McGill University (protocol number 2019-7550).

4.3.2 Superovulation:

Immature mice were super ovulated with equine chorionic gonadotropin (eCG; Folligon, Cat. 00806285, Intervet Canada; 5 IU/200µl, i.p.) and human chorionic gonadotropic (hCG; Chorulon, CH-475-1, Intervet Canada; 5 IU/200µl, i.p.) administered 48h apart. In the first study, ovaries were collected, for granulosa cell isolation, at different time points (N= 3-4 per time point) corresponding to different stages of follicle development, ovulation and corpus luteum formation (Table 4.8.1). In the second study, oviducts were collected at 18h post-hCG to count the number of oocytes ovulated.

4.3.3 Granulosa cell collection:

Ovaries were collected in Eppendorf tubes containing 400µl of 1X PBS on ice. These were then transferred to 35 x 10mm petri dishes having 200µl of 1xPBS and all the fat surrounding them were removed. The ovaries were then punctured with 27-gauge needles in new petri dishes with 200µl 1X PBS. The exudates from follicles of both ovaries of each mouse were pooled and filtered through 40µm sterile cell strainers

(Cat. 352340, Fisher Scientific, Canada) separating cumulus oocyte complexes from granulosa cells. The filtrate was then centrifuged at 7000rpm for 10 mins and the supernatant was discarded followed by RNA extraction from granulosa cells.

4.3.4 RNA extraction and cDNA synthesis:

RNA from granulosa cells was extracted using Direct-zol RNA Miniprep Kit (Cat. R2053, Zymo Research, VWR, Canada) as per manufacture's protocol. The quality and quantity of RNA extracted from each sample was measured using Nanodrop 2000 (Thermoscientific). The cDNA was synthesized using iScript Advance cDNA Kit (Cat. 1725038, Bio-Rad, Canada) from 250ng of RNA according to the manufacturer's protocol.

4.3.5 Real time PCR (qPCR):

All the primers used were ordered from Integrated DNA Technologies (Table 4.8.2) and they were validated for the efficiency between 90% and 110%, and R^2 from 0.98 to 1. The qPCR assays were performed according to MIQE guidelines (Bustin et al., 2009). For each qPCR reaction, Advanced qPCR Mastermix was used (Cat. 800-435-UL, Wisent, Canada) and the assay was performed according to the Wisent protocol. The following conditions were applied for qPCR assay: initial denaturation at 95°C for 2 mins followed by 39 cycles of denaturation at 95°C for 5 seconds, annealing and extension at 60°C for 30 seconds and final step at 95°C for 10 seconds. The readings were displayed on 384 CFX manager TM software (BioRad) and for the analysis of relative transcript abundance Starting Quantity values were taken and normalized against the three reference genes (*Sdha, Rpl19 and B2m*).

4.3.6 Inhibition of Cnr1 using AM251:

The 3-week immature female mice (N=3-5) were superovulated as mentioned above. Three groups of mice were treated with one of the two doses of AM251 (5µg/g or10µg/g, respectively; Cat. S2819, Selleckchem) or DMSO (vehicle group) administered 30 minutes prior to hCG treatment. Ovulation rate was determined by counting oocytes in the oviducts collected at 18h post-hCG treatment.

4.3.7 Cannabis smoke exposure and vaginal cytology:

Cannabis smoke exposure was performed on mature female mice of 7 to 9 weeks of age. Mice in the treated group were exposed to cannabis cigarette smoke using the inExpose inhalation exposure system (SCIREQ, Canada) and control group mice were

exposed to air through the same exposure system. Only the nose was exposed to the smoke. The exposures were given two times a day (morning and afternoon). The amount of cannabis given per exposure was 0.5g with 3 puffs per minute. The cannabis product used was an Indica strain (Blend) with high THC:CBD ratio (16% to 22% THC) (TWD, Canada). The control and treated mice (N=5/group) were subjected to daily vaginal cytology to monitor their estrous cycles for 28 days. The exposures and vaginal sample collection were started the same day and the exposures were ended on the 27th day whereas the sample collections were ended on 28th day. The exposures were ended a day before to include the effects of THC on the estrous cycle stage analyzed on the 28th day. The estrous cycles were analyzed by daily vaginal cytology. The vaginal samples were taken at 10 am everyday using a dropper and 1X PBS solution. The dropper with 1X PBS was inserted into the vagina to collect the sample and then it was smeared onto a 25mm x 75mm superfrost microscopic slide. The slide was dried for 30 mins and then stained with 10% May-Grunnwald stain (Cat. 89027, Fisher Scientific, Canada) and 5% Giemsa stain (Cat. 3250-16 (R3250000-500A, Ricca Chemicals, Canada). The slides were viewed using upright microscope under brightfield setting (Nikon Eclipse 80) using NIS- Elements software for analyzing the estrous cycle stage. On d28 of the experiment mice were euthanized and blood samples were collected regardless of the cycle stage.

4.3.8 Enzyme Linked Immunosorbent Assay (ELISA):

The sample size for measuring steroid levels among groups was, control (N=5) and THC treated group (N=4; one mouse died before the day of blood collection). The plasma was isolated from the blood samples using EDTA for ELISA assays to measure 17-β Estradiol with the ELISA Kit (Cat. ab10866, Abcam, Canada) and Progesterone with the ELISA Kit (Cat. 80559, Crystal Chem, USA) following the manufacturer's protocols. Following the incubation of plasma samples along with the assay reagents at 37°C for 2h for estradiol assay and at room temperature for 1h for progesterone assay, the plates were read using Infinite 200pro plate reader at 450 nm absorbance for estradiol, and at 450 nm and 630 nm absorbance for progesterone. The final measurement for progesterone was calculated by subtracting readings at 630 nm from those at 450nm absorbance. For both the assays the correlation coefficient was 0.99 and standard curve was four parametric logistic curve. The concentrations and standard curve were obtained using MyAssays software. The samples were run in

duplicates. The intraassay CV for 17-β Estradiol ELISA assay was 16.05% and for progesterone ELISA was 8.03%.

4.3.9 Statistical analysis:

All data were analyzed using R x64 4.0.3 version and the level of significance was set to $P < 0.05$. The transcript abundance data and ovulation rates were analyzed by one-way ANOVA followed by the multiple comparison Tukey's test. The parameters of estrous cyclicity and steroid concentrations were analyzed by the Student's t test. All data are expressed as Mean ±SEM.

4.4 RESULTS:

4.4.1 Transcript abundance of endocannabinoid receptors and degrading enzymes in granulosa cells during follicular development ovulation and CL formation:

The expression of the endocannabinoid receptors, *Cnr1* and *Cnr2*, and the endocannabinoid degrading enzymes, *Faah* and *Mgll*, was analyzed in granulosa cells at five specific time points (Table 4.8.1) using qPCR analysis. There was a significant difference in mRNA abundance of *Cnr1*, *Cnr2*, *Faah* and *Mgll* transcripts at different time-points analyzed. The *Cnr1* transcript abundance was suppressed by eCG which was later overcome by hCG. As a result, *Cnr1* transcript abundance was higher at 4h hCG than 24h and 48h eCG ($p<0.05$, Fig 4.7.1. A). In contrast, *Cnr2* mRNA levels were higher at 0h eCG than all other time points ($p<0.05$, Fig 4.7.1. B). The transcript abundance of *Faah* (Fig 4.7.1.C) and *Mgll* (Fig 4.7.1. D) were higher at 14h hCG compared to all other time points ($p<0.05$).

4.4.2 Effect of Cnr1 antagonist on ovulation:

The Cnr1 antagonist AM251 had a significant effect on the ovulation among treated groups (5μg/g and 10μg/g). The mean number of oocytes were lower in both AM251 treated than vehicle treated mice ($p<0.05$, Fig 4.7.2). There was no significant difference between the treated groups.

4.4.3 Estrous cyclicity in THC treated mice:

The mice of both control and THC treated groups were analyzed to ascertain the estrous cyclicity using daily vaginal cytology for 28 days. The four stages of the estrous cycle were examined based on types of cells viewed under the microscope. Three parameters were studied: number of estrous cycles, length of estrous cycle and

length of each stage of estrous cycle. The mean number of cycles was lower in THC treated mice than control mice ($p<0.05$, 4.7.3. A). In contrast, the mean length of estrous cycle was greater in treated mice ($p<0.05$, Fig 4.7.3. B). Among four stages, proestrus (Fig 4.7.4 A) and metestrus (Fig 4.7.4 C) were not significantly different between the groups ($p>0.05$) but estrus (Fig. 4.7.4 B) and diestrus (Fig. 4.7.4 D) were longer in THC treated mice as compared to control mice ($p<0.05$).

4.4.4 Steroid hormone analysis:

Although the THC treated mice had numerically higher plasma levels of estradiol and lower plasma levels of progesterone, the differences between the groups were not significant. ($p>0.05$, Fig 4.7.5 A and B respectively).

4.5 DISCUSSION:

The expression pattern of endocannabinoid receptors and degrading enzymes during follicle development provided the first hint of the potential mechanism by which endocannabinoid signaling regulates ovarian functions. Therefore, we first profiled the expression pattern of cannabinoid receptors (Cnr1 and Cnr2) and degrading enzymes (Faah and Mgll) in mouse ovarian granulosa cells. We found that *Cnr1* expression was higher at 4h hCG as compared to 24h eCG and 48h eCG indicating that hCG reverses the *Cnr1* expression that was downregulated by eCG in granulosa cells. This LH-driven expression pattern is similar to other genes such as *Star*, *Ptgs2* and *Pgr*, which are all known to play critical roles during ovulation (Richards, 1994, Lydon et al., 1995). The observation that *Cnr2* transcript abundance decreased through follicle development and ovulation indicated that this receptor may not mediate the endocannabinoid signaling at later stages of follicle development and ovulation. We observed that the expression of *Faah* and *Mgll* increased after ovulation suggesting that endocannabinoid signaling may be actively reduced during the formation of the corpus luteum. These results are in accordance with a study conducted on human ovary using the immunohistochemistry technique. That study also revealed that Cnr2 was more prominently expressed in early follicles and Cnr1 during late follicle stage along with FAAH expression (MGLL expression was not analyzed) in luteal cells (El-Talatini et al., 2009). Our results suggest that Cnr1 may be involved during preovulatory stage of follicle development. This inference is supported by a study that showed that plasma AEA levels were high at ovulation and were positively correlated with LH, FSH and estradiol but not with progesterone in women (Cui et al., 2017).

These observations provided justification for the experiment testing the role of Cnr1 signaling during ovulation.

We observed that treatment with two doses of the Cnr1-specific antagonist AM251 resulted in significant reduction in the ovulation rate. Taken together, this and the previous results demonstrate that Cnr1 signaling appears to be important for ovulation. These results also reinforce a previous study done in mice geminal vesicle staged oocytes surrounded by cumulus cells which showed that oocyte resumption of meiosis was slower in response to the treatment with Cnr1 and Cnr2 antagonists, SR1 and SR2, respectively than vehicle treated controls (Cecconi et al., 2019).

Although we did not further explore the mechanisms by which Cnr1 signaling regulates ovulation, it is possible to speculate the potential mechanism based on what is known about endocannabinoid signaling. The endocannabinoid receptors belong to Gi class of GPCR thus, regulating the cAMP levels upon activation by their ligands (Matsuda et al., 1990). It is therefore plausible that Cnr1 plays a modulatory role in regulating cAMP concentration by exerting negative effect on the adenylyl cyclase. This speculation is supported by the observation that treatment with forskolin, an adenylyl cyclase activator, results in lower number of ovulations in a dose dependent manner compared to hCG treatment in mice (Rodriguez et al., 2010). But further studies would be required to thoroughly understand the role of the endocannabinoid signaling in LH regulated pathways leading to ovulation. It would be very interesting to see if granulosa specific deletion of Cnr1 results in reduced ovulation rate similar to our observation of lower ovulation rate in response to AM251 treatment.

The other part of this study involved the use of cannabis smoke to assess the effect of THC on estrous cycle and steroidogenesis as THC signals through Cnr1 (Reggio, 2010). There are various studies that have depicted negative effects of cannabis use on female reproductive system. THC has a negative effect on implantation in mice and the THC effect was reversed through CNR1 antagonist indicating that THC acts via this receptor (Paria et al., 1998). Other studies in women have found that THC decreases the gestation length (Fried et al., 1984) and fetal birth weight (Hatch & Bracken, 1986). The gonadotropin levels were reduced in response to THC treatment in ovariectomized rhesus monkeys (Smith et al., 1979) mice (Dalterio, et al, 1983). The lower level of gonadotropins can inhibit follicular development, ovulation and corpus lutea formation. These various stages in the ovary are reflected through different

phases of estrous cycle. Based on these previous studies, we analyzed the effect of THC on estrous cyclicity and ovarian steroidogenesis for 4 weeks. Our results demonstrate that the average number of cycles was lower in Cannabis smoke treated mice by 42% than the control, thereby resulting a 40% longer cycle length in treated mice. In terms of different stages of estrous cycle, estrus and diestrus phases were prolonged in THC treated mice. These observations suggest that THC might have negative impact on ovulation during estrus phase and negative effect on corpus luteum function at diestrus stage.

We expected that THC may impact steroid levels, but our results depicted that the plasma estradiol and progesterone levels between control and treated groups were not different. However, we did observe numerical differences warranting further study with greater number of mice. This could be because all the mice within and among the control and treated group were not in the same estrous cycle stage at the day of their blood collection. The results of our study are in compliance with another study on female mice in terms of estrous cycle length which showed the increase in length of cycle around 8 days for first cycle after THC treatment through oral intubation (Kostellow et al., 1980). But, in the same study, there was no significant difference in cycle length between treated and control groups after another 60 days of treatment. This suggests that the negative impact of THC may wane after prolonged exposure. The difference in our results and this study (Kostellow et al., 1980) might be attributed to the method of administering THC, considering that the metabolism of THC greatly varies according to its consumption method (Ohlsson et al., 1980).

Overall, the results of the present study show that Cnr1 expression is upregulated by hCG, and it appears to have a role from preovulatory follicle stage until ovulation. As THC has adverse effect on estrous cyclicity, further studies will be required to understand Cnr1 pathways and the effect of THC on female reproductive health.

4.6 ACKNOWLEDGEMENTS:

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4.7 FIGURES AND FIGURE LEGENDS:

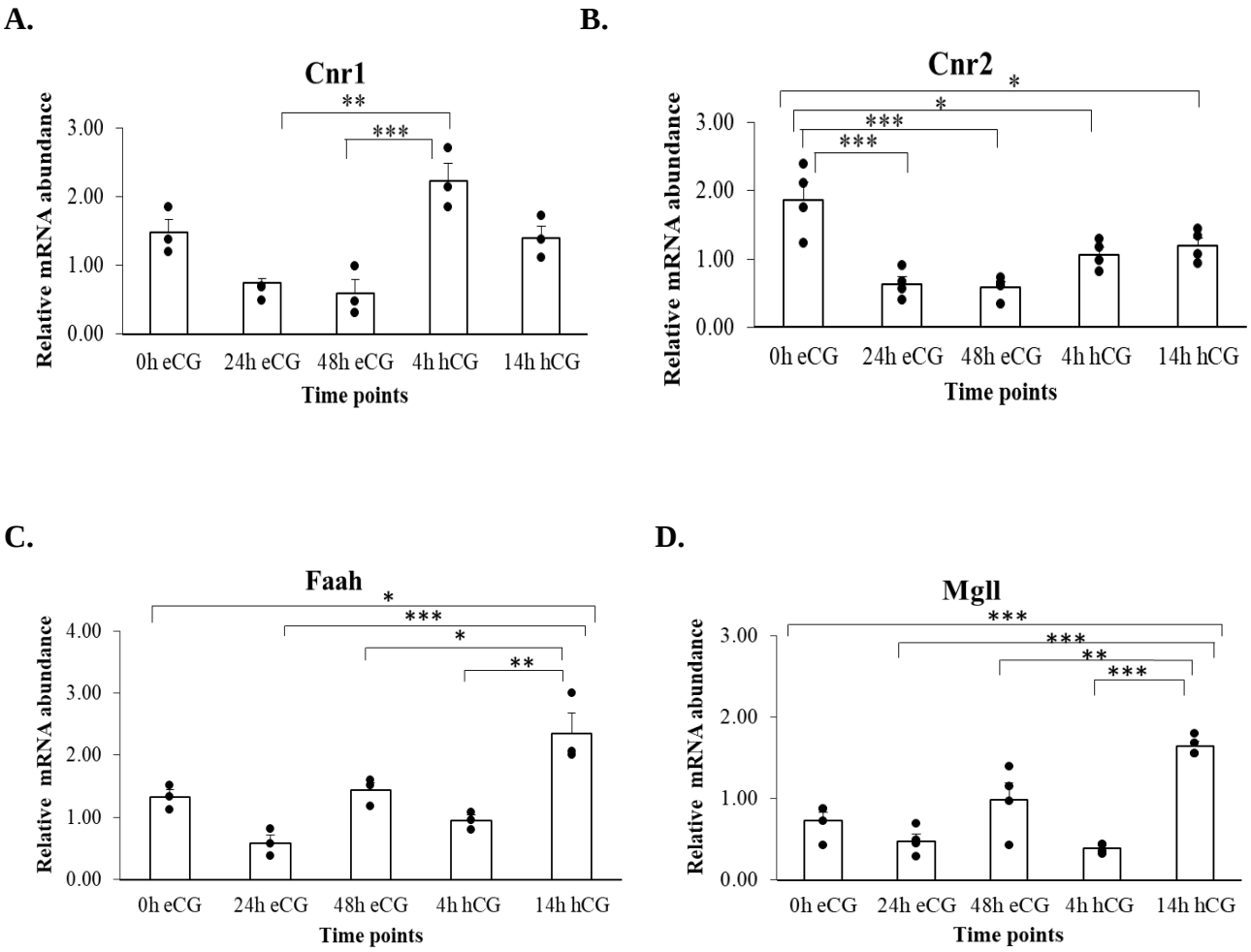


Figure 4.7.1. mRNA abundance of endocannabinoid receptors and degrading enzymes in granulosa cells. Quantitative-PCR was performed to profile the expression pattern of *Cnr1*, *Cnr2*, *Faah* and *Mgll* in granulosa cells collected at 0h eCG, 24h eCG, 48h eCG, 4h hCG and 14h hCG (N= 3-4/ time point). Data was normalized to reference genes *B2m, Rpl19* and *Sdha.* Data are represented as Mean ± SEM. The bars are means and the points are individual data points (•). * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$.

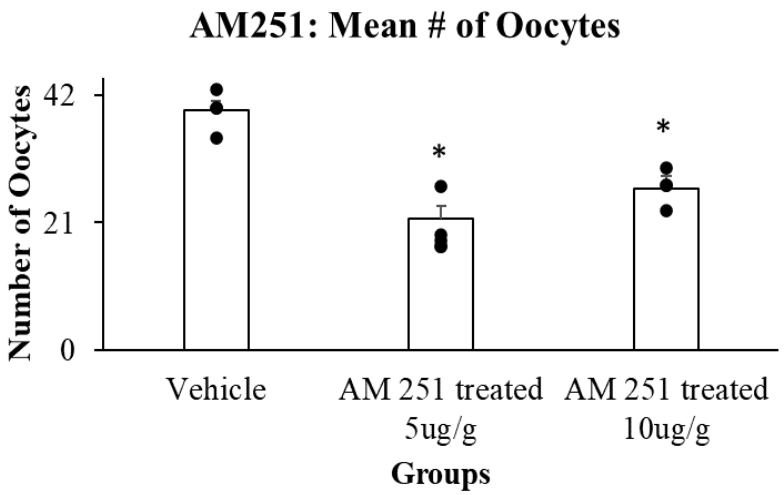
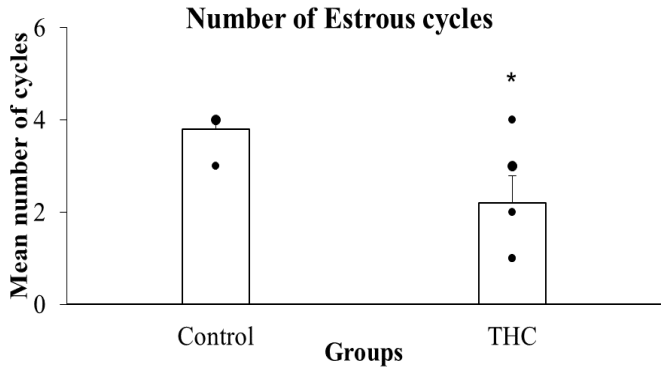


Figure 4.7.2. Effect of Cnr1 antagonist (AM 251) on ovulation in mice.
Immature female mice (N= 3-5) were superstimulated with exogenous gonadotropins (eCG and hCG). The vehicle group was treated with DMSO and the other two groups with Cnr1 antagonist AM251, each with different dose at 30 mins before hCG administration. Ovulation rate for each mouse was determined by counting the number of oocytes in both oviducts post 18h hCG. Data are represented as Mean ± SEM. The bars are means and the points are individual data points (●).

* denotes $p<0.05$.

A.



B.

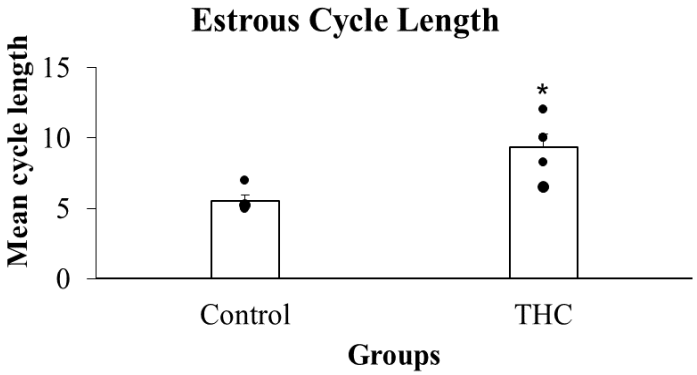


Figure 4.7.3 Effect of THC on estrous cycle number and length. Mature female mice (N=5/group) were exposed to high THC cannabis smoke for 4 weeks. Vaginal cytology was used to assess the estrous cycle stage. Estrous cycle number (A) and estrous cycle length (B) were analysed. Data are represented as Mean ± SEM. The bars are means and the points are individual data points (●). * denotes $p<0.05$.

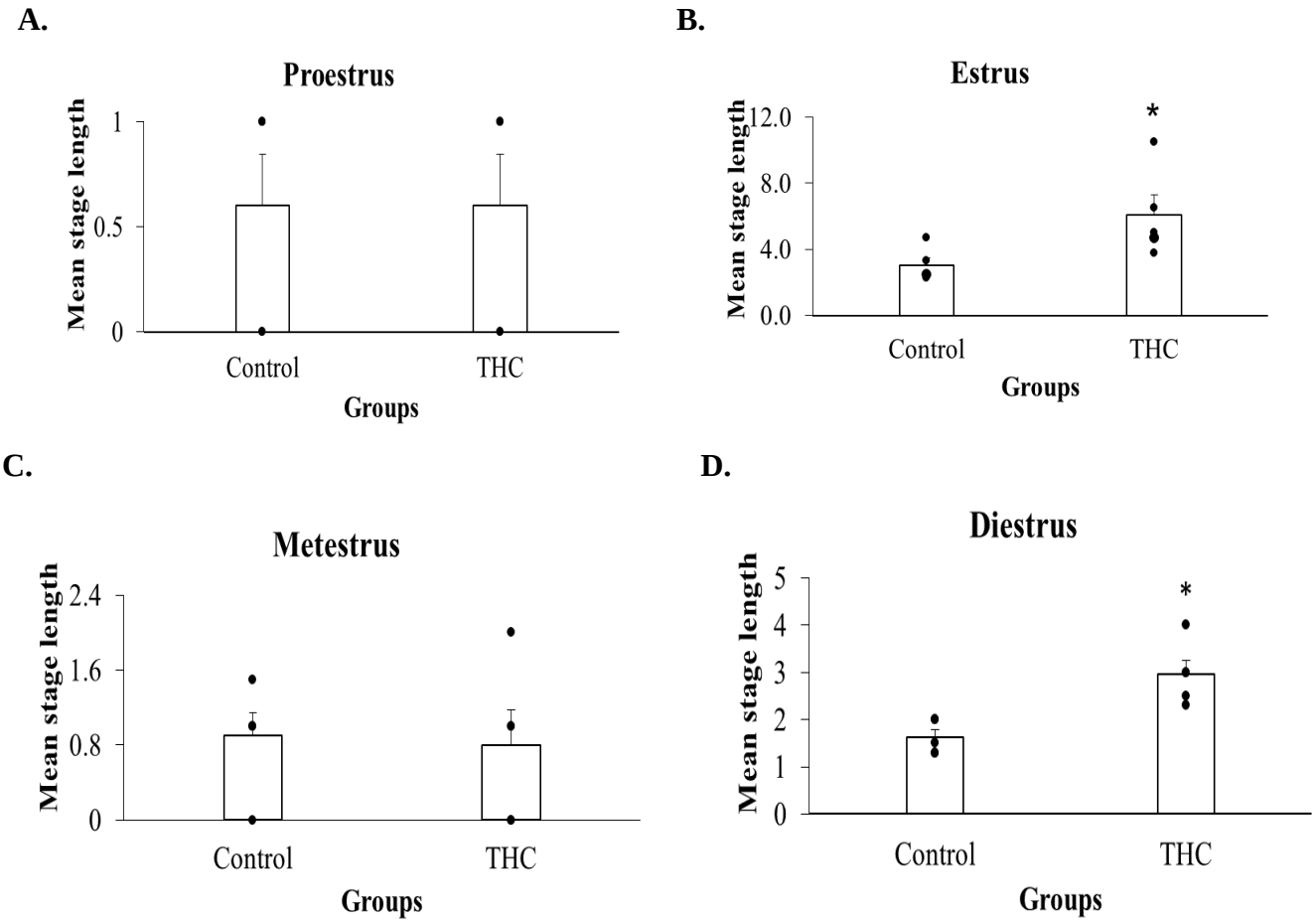
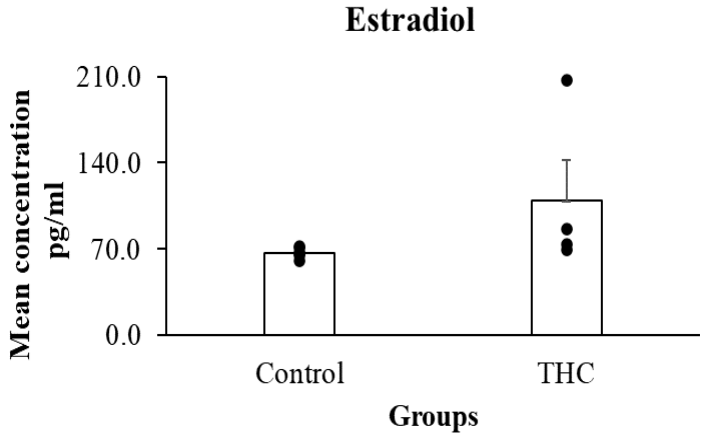


Figure 4.7.4. Effect of THC on estrous cycle stages. Mature female mice (N=5/ group) were exposed to high THC cannabis smoke for 4 weeks. Vaginal cytology was used to assess the estrous cycle stage. Estrous cycle stage length; Proestrus (A), Estrus (B), Metestrus (C) and Diestrus (D) was analysed. Data are represented as Mean ± SEM. The bars are means and the points are individual data points (●). * denotes $p<0.05$.

A.



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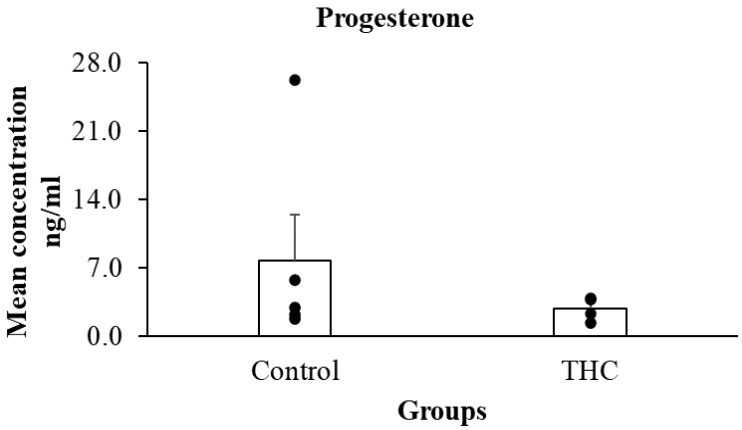

Figure 4.7.5 Effect of THC steroidogenesis. Mature female mice (N=5/group) were exposed to high THC cannabis smoke for 4 weeks. Post 4 weeks 17 β- Estradiol (A) and Progesterone (B) were analysed in blood plasma using ELISA. Data are represented as Mean ± SEM. The bars are means and the points are individual data points (●).

4.8. TABLES:

Table 4.8.1: Specific time points for granulosa cell collection (N=3-4/timepoint)

Time points	Follicular development stages
0h eCG	Small antral follicle
24h eCG	Growing antral follicle
48h eCG	Preovulatory follicle
4h hCG	Ovulating follicle
14h hCG	Corpus (hemorrhagicum) Luteum

Table 4.8.2: Primers used: Real time qPCR

Gene	Forward Primer 5'- 3'	Reverse Primer 5'- 3'
Cnr1	GTACATTCTCTGGAAGGCTCAC	CTGCACCTTGCCATCTTCT
Cnr2	GTTACCCGCCTACCTACAAAG	GAGCGGCAGGTAAGAAATCA
Faah	TGAACGAGGGTGTGACATCG	TTCCACGGGTTCATGGTCTG
Mgll	AGGCGAACTCCACAGAATGTT	ACAAAAGAGGTACTGTCCGTCT
Sdha	GGAACACTCCAAAAACAGACCT	CCACCACTGGGTATTGAGTAGAA
Rp119	ATGAGTATGCTCAGGCTACAGA	GCATTGGCGATTTCATTGGTC
B2m	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC

CHAPTER 5: CONCLUSIONS AND FUTURE PERSPECTIVES

Overall, the results of this study show that Cnr1 appears to part of the LH regulatory pathway, although hCG was used as LH analog, during ovulation in mice. The results also indicate that THC has adverse effects on the estrous cycle but not necessarily on estrogen and progesterone levels. However, further studies are warranted to clarify its effects on steroidogenesis using stage-specific steroid analyses. More importantly, further studies are needed to understand Cnr1 pathways and the effect of THC on women's reproductive health.

Future perspectives:

Since the CNR1 appears to be induced by hCG, it would be interesting to study, using SVOG cells (hCG responsive human granulosa cell line) (Lie et al., 1996), the molecular mechanisms by which it regulates LH-driven granulosa cell functions. Granulosa-specific *Cnr1* knockout mice will be useful to provide the *in vivo* evidence for the role of this receptor in ovulation. Such model will also enable testing the mode of action of THC, if any, on the ovary. Further, the effect of THC on both synthesizing and degrading enzymes of AEA, and the resulting impact on the endocannabinoid system of the ovary needs to be studied. It can be addressed by exposing the SVOG cells and mice to high THC cannabis smoke followed by measuring the expression pattern of the enzymes within granulosa cells at specific stages of follicular development and ovulation. Such studies examining the effects of THC on endocannabinoids are important as the normal Cnr1 signaling appears to be important for ovulation. As our results show that THC adversely affects the estrous cycle this follow up study will elucidate if THC suppresses the Cnr1 endogenous ligand activity (e.g., modulation of CNR1-driven cAMP concentration in granulosa cells) and thereby disrupts the CNR1 pathway explaining the potential cause of its adverse effects.

CHAPTER 6: REFERENCES

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