NEURODEVELOPMENTAL EFFECTS OF PRENATAL CANNABIDIOL EXPOSURE ON THE OFFSPRING OF RATS IN THE POSTNATAL PERIOD

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ABSTRACT

Cannabis use during pregnancy has recently increased worldwide. Cannabidiol (CBD), the main non-intoxicating compound in *Cannabis*, is often seen as a natural substance and has been used for the treatment of several health conditions. During pregnancy, women might choose to use CBD to treat very common pregnancy-related symptoms, such as nausea and vomiting. However, there is very little evidence regarding the safety of CBD use during pregnancy and the possible outcomes to maternal and fetal health. In this context, we tested the effects of prenatal CBD exposure on pregnancy outcomes, offspring physical health and neurodevelopment. Pregnant rats were treated by intraperitoneal injection with either a drug vehicle solution (1:1:18 ethanol:kolliphor:PBS), 5 mg/kg CBD or 10 mg/kg CBD during gestational days 6 to 20. Offspring physical health was assessed until weaning on post-natal day (PND) 21. Different neurodevelopmental tests were conducted from PND3 to PND21 to measure the development of neurological reflexes and postural mechanisms. Prenatal CBD exposure was associated with a lower body weight in offspring and a delay in the development of reflexes in early stages after birth. These findings contribute to the current evidence available on the consequences of *in utero* CBD exposure and brings light to the need for further research in the area.

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DEDICATIONS

My family. My rock in this world. My parents and sisters have molded me into what I am today and continue to encourage me to chase my dreams. The first people that I run to in times of despair and times of celebration, and who will always believe in me no matter how much I doubt myself. Thank you for the immense love and support. I wouldn't be where I am without it, and I am grateful for their willingness to hear my stories and my complaints, and their genuine wishes to see me succeed. To the friends that are also considered family, thank you.

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2-AG	2-arachdonoylglycerol
5-HT1A	Serotonin 1a receptor
ABHD12	α/β-hydrolase domain containing 12
ABHD6	α/β-hydrolase domain containing 6
ACOG	American College of Obstetricians and Gynecologists
AEA	Anandamide
BCRP	ABCG2/Breast cancer resistance protein
C_{max}	Maximum plasma concentration
CB1R	Cannabinoid type 1 receptor
CB2R	Cannabinoid type 2 receptor
CBD	Cannabidiol
CBDa	Cannabidiol acid
CNS	Central nervous system
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
eCB	Endocannabinoid
ECS	Endocannabinoid system
FAAH	Fatty acid amide hydrolase
FAS	Fetal alcohol syndrome
FDA	Food and Drug Administration
GD	Gestational day
GPCR	G protein-coupled receptors
GPR55	G-protein coupled receptor 55
i.p.	Intraperitoneal injection
KPNC	Kaiser Permanente Northern California
mRNA	Messenger RNA
NAPE-PLD	N-acyl-phosphatidylethanolamine specific phospholipase D
NSDUH	National Survey on Drug Use and Health
NVP	Nausea and vomiting in pregnancy

P-gp	P-glycoprotein
PAE	Prenatal alcohol exposure
PBS	Phosphate-buffered saline
PCE	Prenatal *Cannabis* exposure
PND	Post-natal day
RANZCOG	Royal Australian and New Zealand College of Obstetricians and Gynecologists
SGA	Small for gestational age
Shh	Sonic hedgehog signaling pathway
SOCG	Society of Obstetricians and Gynecologists of Canada
THC	Δ9-tetrahydrocannabinol
THCa	Δ9-tetrahydrocannabinol acid
TRPV1	Transient receptor potential cation channel subfamily V member 1
USA	United States of America
WHO	World Health Organization

1.0 GENERAL INTRODUCTION

1.1. *Cannabis* and the endocannabinoid system

Cannabis cultivation and use have been traced back to 12,000 years ago in Central Asia, its seeds being dispersed through the world following human migration patterns (reviewed in Crocq, 2020). The medicinal use of the plant also dates back thousands of years due to its analgesic, anti-inflammatory and anxiolytic properties, as well as its effectiveness in improving appetite and sleep (Crocq, 2020; Pertwee, 2006). Its euphoric properties also led to its use for religious ceremonies and recreationally (Crocq, 2020; Pertwee, 2006; Russo et al., 2008).

The *Cannabis* plant contains over 100 cannabinoids (phytocannabinoids), the two most-abundant ones being Δ9-tetrahydrocannabinol acid (THCa) and cannabidiol acid (CBDa), which are decarboxylated upon heating to yield THC (Δ9-tetrahydrocannabinol) and CBD (cannabidiol) (Crocq, 2020). CBD was first isolated in 1940 and its structure reported in 1963, followed by elucidation of the structure of THC in 1964 (Crocq, 2020; Pertwee, 2006). Both phytocannabinoids are present in the plant in their acidic form that are decarboxylated when heated (Pertwee, 2006).

Phytocannabinoids elicit many of their effects in humans and other mammals by acting on the endogenous endocannabinoid system (ECS). The ECS is comprised of G protein-coupled receptors (GPCR): cannabinoid type 1 receptor (CB1R) and cannabinoid type 2 receptor (CB2R); endogenous ligands, called endocannabinoids; and the enzymes responsible for their production and hydrolysis. The cannabinoid receptors are differentially distributed throughout the body, with CB1R found mostly in the central nervous system (CNS) and CB2R in immune cells, although they have also been detected in the CNS (e.g., in microglia) (reviewed in Howlett et al., 2002; Pertwee, 2006). The discovery of these receptors allowed the development of various antagonists, agonist and genetically modified animals that are valuable tools when investigating the ECS (reviewed in Howlett et al., 2002; Pertwee, 2006)

In the 1990s, after the discovery of cannabinoid receptors, researchers started to investigate the existence of endocannabinoids, leading to the discovery of the 2 major ones: 2-arachdonoylglycerol (2-AG) and anandamide (AEA) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Anandamide is produced via hydrolysis of membrane phospholipids, a reaction catalyzed by the enzyme *N*-acyl-phosphatidylethanolamine specific phospholipase D (NAPE-PLD) (Okamoto et al., 2004). On the other hand, the enzyme diacylglycerol lipase

(DAGL) hydrolyzes diacylglycerol (DAG), forming 2-AG (Bisogno et al., 2003). The 2 endocannabinoids are also hydrolyzed via different intracellular enzymes: fatty acid amide hydrolase (FAAH) degrades AEA (Cravatt et al., 1996) while 85% of 2-AG is broken down by monoacylglycerol lipase (MAGL) and the rest by 2 more recently discovered enzymes, ABHD6 and ABHD12 (α/β-hydrolase domain containing 6 and 12, respectively) (Blankman et al., 2007; Dinh et al., 2002; Savinainen et al., 2012). Both endocannabinoids are produced on demand in the postsynaptic neuron and travel to the presynaptic neuron to act retrogradely on CB1R, leading to a decrease in neurotransmitter release (Jarvis et al., 2017). Consequently, endocannabinoids are able to control synapses in different brain regions, regulating a wide range of behaviors (Bara et al., 2021)

The ECS also participates in several physiological functions and has an important role in maintaining homeostasis (Jarvis et al., 2017), as well as being involved in different aspects of reproduction (Meccariello et al., 2014) and being present from early stages of development (Fride, 2008). Importantly, the CBRs and endocannabinoids have fundamental roles in neurogenesis, neuronal migration and differentiation and synaptogenesis (reviewed in Bara et al., 2021). During brain development, CB1R and CB2R mRNA (messenger RNA) have been identified in embryonic rat brain as early as gestational day (GD) 11, with a higher expression of CB1R, particularly in regions associated with motor functions (Buckley et al., 1997). In humans, CB1R was detected in fetal brains at 9 weeks of gestation and its receptor density increased with gestational age, but the distribution pattern is distinct from that seen in adults (Biegon & Kerman, 2001; Zurolo et al., 2010). Endocannabinoids are also present in early development and show different developmental patterns. In rats, Berrendero and colleagues demonstrated that 2-AG is present in the fetal brain at GD21 with levels peaking and PND 1, whereas AEA was present at lower levels at GD21 and reached its maximum levels in adulthood (Berrendero et al., 1999).

1.2 *Cannabis* use during pregnancy

An increasing number of jurisdictions have legalized *Cannabis* in the past years. In October 2018, Canada became the second country to legalize recreational *Cannabis*, after Uruguay in 2013 (National Conference of State Legislatures, 2022). Various states in the United States have also legalized the non-medicinal use of *Cannabis* since 2012, including Colorado, Alaska, California, Michigan, and others (National Conference of State Legislatures, 2022)

Several reports point to a global increase in *Cannabis* consumption among pregnant women in the last decade. Data from the National Survey on Drug Use and Health (NSDUH) reported an increase in the prevalence of *Cannabis* use among pregnant women in the United States from 2002 to 2017 (Volkow et al., 2019). The NSDUH is a cross-sectional survey that uses a multistage, stratified design to recruit noninstitutionalized civilians in the USA, according to geographic location and age, to estimate prevalence and correlates of substance use (Center for Behavioral Health Statistics and Quality & Substance Abuse and Mental Health Services Administration, 2018). Volkow et al. (2019) analyzed data from women aged 12 to 44 years old, totaling in 467,100 respondents between 2002 and 2017 (Volkow et al., 2019). The prevalence of past-month *Cannabis* use among pregnant women increased from 3.4% to 7.0% and from 5.7% to 12.1% in the first trimester, which had higher rates than the second and third trimesters (Volkow et al., 2019). Another study that analyzed data from the 2007-2012 NSDUH reported that almost 70% of pregnant women (aged 18 to 44 years old) who had used *Cannabis* in the past year reported believing there was little to no harm associated with using *Cannabis* once or twice a week (Ko et al., 2015).

Following the same trends, data obtained from the Kaiser Permanente Northern California (KPNC) healthcare system showed an increase in overall prenatal *Cannabis* use from 2009 to 2016 in California (Young-Wolff et al., 2019). Among those, women with nausea and vomiting in pregnancy (NVP) had the highest prevalence of *Cannabis* use, with rates increasing from 6.5% in 2009 to 11.1% in 2016, although the prevalence increased in a similar rate whether women had NVP or not (Young-Wolff et al., 2019). Another study investigated the association between the legalization of recreational *Cannabis* in states in the US and maternal *Cannabis* use and reported that use increased in states that had legalized recreational *Cannabis* (i.e. Alaska and Maine) in the preconception and postpartum periods, but not during the prenatal period (Skelton et al., 2021).

The recent increase in *Cannabis* use by pregnant women is also seen in Canada. In Ontario, data from the BORN birth registry showed that the overall prevalence of prenatal *Cannabis* reached 1.8% in 2017, a relative increase of 61% from 2012 (Corsi et al., 2019). In British Columbia, rates of *Cannabis* use among women older than 19 years old increased from 3.64% before legalization (May 2018 – October 2018) to 4.62% in the post-legalization period (July 2019 – May 2020), according to data from the Pregnancy Specific Anxiety Scale study (Bayrampour &

Asim, 2021). Although not statistically significant, these rates are in line with the widespread increase seen in other locations.

It is important to note that longitudinal studies are subject to errors that might not reflect the reality: underreporting of *Cannabis* use is very likely as women may fear punitive action even in places where recreational use is legalized. To avoid that, studies that rely on both self-reporting and biochemical confirmation of *Cannabis* consumption can provide a more accurate view on the rates of consumption. In a recent study by Young-Wolff and colleagues, among pregnant women that reside in Northern California and who screened positive for prenatal *Cannabis* use, 13.7% were positive on self-report only *versus* 58.5% who were positive on toxicology only and 27.7% who were positive on both self-reporting and toxicology (Young-Wolff et al., 2019). Additionally, most studies are restricted to specific geographic locations and it is difficult to generalize their findings. However, there is a global trend towards an increase in overall *Cannabis* use, including among pregnant women (Volkow et al., 2019; Young-Wolff et al., 2019; Bayrampour & Asim, 2021)

Given this recent increase in *Cannabis* consumption, it is important to investigate what factors can influence mother's decisions of consuming *Cannabis* while pregnant. Location of *Cannabis* retailers is one of those factors, as rates of use are higher among women who live closer (within a 10-minute drive) to a retailer (Young-Wolff et al., 2022). Moreover, with the onset of the COVID-19 pandemic in 2020, prenatal *Cannabis* use increased by 25% from January 2019 to December 2020 in California, according to data obtained from the KPNC (Young-Wolff et al., 2021).

Another key factor into why women consume *Cannabis* and *Cannabis* products while pregnant is a popular belief that the plant and its derived products are not harmful due to their being "natural" (Chang et al., 2019). A New York Times article from 2017 highlights mothers who smoked during pregnancy and who relate believing there is no harm in consuming *Cannabis* while pregnant having observed no effects on their own or other's children (Louis, 2017). Similarly, a 2017 study from Baltimore, MD, surveyed pregnant women attending a prenatal visit and reported that 34% of the women who smoked *Cannabis* before pregnancy continued to do so after becoming pregnant (Mark et al., 2017). Additionally, 30% of the women included in the study believed *Cannabis* to not be harmful to the baby (Mark et al., 2017). Most women, however, do report

decreasing the frequency of use while pregnant, although the studies available do not have a validated method of quantifying consumption levels (reviewed in Weisbeck et al., 2021).

There is currently a lot of confusion around *Cannabis* consumption during pregnancy. Jarlenski et al. (2016) interviewed 26 women in Pittsburgh, PA, who had used *Cannabis* during pregnancy and reported that the most common source of information prenatal *Cannabis* use was the internet, followed by personal experiences or those of family and friends. Women also stated that the information available is inconsistent, leading to a generalizing thought that perinatal use is probably harmful, but there is no specification on how that affects the fetus (Jarlenski et al., 2016). Importantly, women reported not receiving adequate information from health care and social workers, receiving mostly punitive messages rather than information on the effects on the fetus and support/resources on how to stop *Cannabis* consumption (Jarlenski et al., 2016).

In regard to the consequences of maternal *Cannabis* consumption during pregnancy, the literature has been consistently growing over the last few years; however, there is still a gap between the scientific literature that is being produced, the information health care providers have and pass on to their patients, and the information available through other sources (e.g., internet, *Cannabis* dispensaries). Furthermore, and perhaps more relevant to this study, there is a critical gap between controlled scientific studies that are investigating the consequences of prenatal *Cannabis* and cannabinoids exposure, and the information that is being provided to health care professionals and the general population.

In a recent study of *Cannabis* dispensaries in the state of Colorado, when asked if any products were recommended for nausea at 8 weeks pregnant, 69% of dispensaries' staff recommended *Cannabis* products for "morning sickness" and 35.7% endorsed the safety of *Cannabis* use during pregnancy (Dickson et al., 2018). Most of the recommendations were based on personal opinions and only a small percentage reported potential risks for the fetus and/or the mother (Dickson et al., 2018). Of the dispensaries that recommended *Cannabis* products, 56% recommended products containing both THC and CBD, followed by 26% that recommended CBD-only products and 17% that recommended THC-only products. Lastly, the majority (81.5%) of dispensaries recommended talking to a health care professional, but only 31.8% of all dispensaries made that recommendation without being prompted by the caller/inquirer (Dickson et al., 2018).

There is still confusion when investigating the association of prenatal *Cannabis* use with adverse neonatal outcomes. Several epidemiological studies have associated prenatal *Cannabis* exposure (PCE) with preterm birth (Hatch & Bracken, 1986; Hayatbakhsh et al., 2012; Bailey et al., 2020), low birth weight (Bailey et al., 2020; T. L. Crume et al., 2018; Hatch & Bracken, 1986; Hayatbakhsh et al., 2012; Zuckerman et al., 1989), decreased birth length (Day et al., 1992) and admission to the neonatal intensive care unit (Hayatbakhsh et al., 2012) while other studies failed to find the same results (Day et al., 1991; van Gelder et al., 2010). However, most of these studies were conducted in the 1980s and 1990s and do not reflect the current potency of *Cannabis* and *Cannabis* products and the patterns of consumption. In addition, *Cannabis* contains multiple drugs besides THC and CBD, and the strains available for consumption differ vastly in their composition and potency. Indeed, a recent analysis of *Cannabis* potency in the United States (USA) and Europe reported an impressive rise in THC levels in *Cannabis* products in the past decade (Chandra et al., 2019). Therefore, it is essential to know what products people are consuming, the method of consumption, and how differently they can impact people's health. It is also difficult to interpret findings from human studies relating prenatal *Cannabis* exposure and health and behavioral outcomes due to often unmeasured confounding factors that can impact the outcomes, such as maternal age, education, income and alcohol and tobacco use.

Despite the lack of clarity on the consequences of PCE, the American College of Obstetricians and Gynecologists (ACOG) recommends that pregnant women or women who are trying to get pregnant should discontinue the use of recreational or medicinal *Cannabis* and, when reporting *Cannabis* use, women should be advised on the potential adverse health outcomes of continued use during pregnancy (American College of Obstetricians and Gynecologists, 2017). The ACOG also states that, although there is still uncertainty about the specific effects of *Cannabis* use during pregnancy, "worrisome trends do emerge" from data on PCE (ACOG, 2017). The Royal Australian and New Zealand College of Obstetricians and Gynecologists (RANZCOG), the World Health Organization (WHO), and the Society of Obstetricians and Gynecologists of Canada (SOCG) also recommend that *Cannabis* use should be discontinued in pregnancy (reviewed in Tsakiridis et al., 2021).

1.3 Cannabidiol use during pregnancy

Given what we know about women's consumption of *Cannabis* during pregnancy, we can assume that some women are consuming CBD while pregnant. A recent cross-sectional study investigated CBD use amongst Americans and Canadians and reported that CBD product use was more common among females and people aged 26-35 years old (Goodman et al., 2022). The same study also reported that the rationale for CBD was commonly stated to be the treatment of mental health conditions (e.g., anxiety and depression) and physical health (e.g., pain, headaches and sleeping problems), and CBD use was more prevalent among people who frequently used *Cannabis* (Goodman et al., 2022).

While the evidence regarding the effects of *Cannabis* and THC exposure on fetal health and behavioral outcomes is constantly growing, few studies have examined the effects of prenatal cannabidiol (CBD) exposure. Most studies investigating the specific effects of CBD have been conducted using *in vitro*, *ex vivo* or rodent models (Fish et al., 2019; Iezzi, Caceres, et al., 2022; Ochiai et al., 2021), and there is little information regarding consumption of CBD during pregnancy in humans.

Despite the lack of evidence regarding the safety of CBD use during pregnancy, women may choose to use it to treat a variety of pregnancy-related symptoms such as nausea, chronic pain, and anxiety (Sarrafpour et al., 2020). Health Canada and the FDA (Food and Drug Administration), however, strongly advises against the use of CBD during pregnancy and breastfeeding (U.S. Food & Drug Administration, 2019; Health Canada, 2022).

Most of the literature available on the effects of prenatal CBD comes from *in vitro* studies. CBD has been shown to inhibit the function of BCRP (ABCG2/Breast cancer resistance protein), an efflux transporter found in trophoblasts, both *in vitro* and *ex vivo*, albeit at high (10 μM) concentrations (Feinshtein, Erez, Ben-Zvi, Erez, et al., 2013). Feinshtein and colleagues (2013) demonstrated that exposure of trophoblast cell lines and viable placental cotyledon to CBD led to inhibition of the efflux function of the BCRP, diminishing the effectiveness of the placental barrier and potentially exposing the fetus to xenobiotics. The same group has also reported that CBD has a cell-specific effect on the expression of BCRP and P-gp (P-glycoprotein) transporters in human trophoblast-like cell lines, with the latter being an important placental protective transporter highly expressed in the first trimester (Feinshtein, Erez, Ben-Zvi, Eshkoli, et al., 2013). At the transcriptional level, long-term CBD exposure led to increase in BCRP mRNA and protein levels

and a decrease in P-gp mRNA and protein levels, potentially affecting the transplacental transport of BCRP and P-gp substrates and exposing the fetus to drugs and naturally occurring molecules that can influence pregnancy outcome (Feinshtein, Erez, Ben-Zvi, Eshkoli, et al., 2013).

CBD can also prevent the differentiation of human endometrial stromal cells into specialized decidual cells (Almada et al., 2020), a crucial step in the establishment of pregnancy that, when dysregulated, could lead to various pregnancy disorders (Okada et al., 2018). Another *in vitro* study demonstrated that CBD, when administered to placental cell models of two different trophoblast populations, disrupted apoptotic, autophagic and migration processes, which can also lead to pregnancy disorders (Alves et al., 2021). These results highlight the fact that CBD can negatively impact different aspects of placental and fetal development, which in turn can lead to infertility, miscarriages, and uteroplacental disorders.

Another considerable part of the available literature stems from studies with zebrafish. Ahmed and colleagues studied the effects of CBD on motor development by exposing zebrafish embryos to CBD during a critical stage in the embryonic development known as gastrulation (Ahmed et al., 2018). Following a 5 h exposure to the cannabinoid during the gastrulation period, embryos showed physical abnormalities; reduced survival, heart rates, and activity at the neuromuscular junction; and changes in the branching of primary and secondary motor neurons (Ahmed et al., 2018). A recent study exposed zebrafish embryos to either CBD, THC, or a combination of both during the first 10 h of embryonic development and then measured their neural and locomotor activity 4 and 5 days later, respectively(Kanyo et al., 2021). CBD decreased neural activity in a dose-dependent manner at lower concentrations than THC, and exposure to both components led to further reduction of neural activity (Kanyo et al., 2021). CBD also reduced locomotor activity, an effect likely mediated by CB1R and CB2R (Kanyo et al., 2021). Both studies suggest that exposure to CBD very early in life might alter embryonic development. Finally, CBD decreased the viability of chick embryos in a dose-dependent manner with no morphological alterations, although the CBD dose was 5 times higher than the synthetic cannabinoid HU210 (Gustafsson & Jacobsson, 2019).

In mammals, CBD can easily cross the placenta due to its high lipophilicity and low molecular weight (Ochiai et al., 2021; Sarrafpour et al., 2020). In fact, when pregnant mice were administered a single dose of CBD 10 mg/kg on gestational day 14.5 (corresponding to the second trimester in humans) the cannabinoid was transferred from the dam to the fetus 15 minutes after

drug administration, being then distributed to the fetus organs (Ochiai et al., 2021). The fetal liver showed the highest concentration of CBD followed by the brain and GI tract, with the highest half-life occurring in the brain (Ochiai et al., 2021).

Animal studies have also significantly contributed to the available literature. A single administration of 17 mg/kg of CBD to pregnant mice on day 8 of gestation (the beginning of neurulation, when the neural tube closes and the eyes and brain are formed) caused eye and facial malformations (Fish et al., 2019). However, the effects were more severe with the highest dose and less frequent than with the CB1R agonists THC and HU-210 (Fish et al., 2019). CBD also inhibited the Sonic hedgehog signaling pathway (Shh) (Fish et al., 2019), which has an essential regulatory role during development in the growth and patterning of multicellular embryos (Simpson et al., 2009). Lastly, fetal exposure to repeated CBD treatment (GD5 to GD18) led to sex-specific alterations in early-life behaviors: male mice pups prenatally exposed to the cannabinoid showed increased body weight and female pups showed a reduction in locomotor activity(Iezzi, Caceres, et al., 2022). In addition, prenatal CBD exposure caused sex-specific changes in pups communication skills, marked by alterations in the duration, frequency and types of ultrasonic vocalizations (Iezzi, Caceres, et al., 2022).

Interesting results have also been published by using a model of oral administration to study the effects of *in utero* CBD exposure. Swenson and colleagues (2022) have reported that only the male offspring of mice exposed to CBD were more sensitive to thermal pain, an activity dependent on the TRPV1 (Transient receptor potential cation channel subfamily V member 1) receptor. Female offspring exposed to CBD had impaired cognition, which the authors argue might be due to a decrease excitability of prefrontal cortical pyramidal neurons (Swenson et al., 2022). The same group, however, showed that anxiety, compulsivity, and spatial memory measurements were not altered on the CBD-exposed offspring (Swenson et al., 2022). Taken together, the evidence points to various ways that *in utero* CBD exposure can negatively affect pregnancy outcome and fetal development, emphasizing the importance of further research in the area.

1.3.1 CBD dosing

The status of CBD as a controlled and prescribed medicine *versus* an over-the-counter product is currently unclear. Although CBD has a good safety profile and is usually well tolerated in humans (Chesney et al., 2020), there is confusion around recommended CBD dosing. Different

formulations and routes of administration also impact absorption and distribution of the compound, and there is no clear direction on what CBD doses are considered therapeutic.

In clinical trials, the therapeutic doses of oral CBD used for various medical conditions range from <1 to 50 mg/kg/day, equivalent to <62-3,100 mg/day for an adult (reviewed in Millar et al., 2019). Effective doses, however, are between 300-1,500 mg/day, but the recommended doses found in non-prescription CBD products are usually bellow 150 mg/day (McGregor et al., 2020). A phase I study has reported that high doses of CBD (i.e., up to 6,000 mg in a single dose or 1,500 mg in multiple doses) are well tolerated by humans(Taylor et al., 2018). As for lower doses of CBD, the benefits and effectiveness in a clinical setting are yet to be demonstrated.

There are few human studies that provide information on the pharmacokinetics of CBD, but they are comparable to the plasma levels seen in animals. In humans, a single dose of 1,500 mg of CBD generates a mean maximum plasma concentration (C_{max}) of CBD of 292 ng/mL, and the single dose of 6,000 mg generated a mean C_{max} of 782 ng/mL (Taylor et al., 2018). Multiple dosing over a week generated higher blood levels of CBD: a two-fold increase in dose (750 to 1,500 mg) increased C_{max} 1.6-fold (330 and 541 ng/mL, respectively) (Taylor et al., 2018). However, when subjects consumed 1,500 mg CBD after eating, there was a 4.85-fold increase in the plasma levels of the cannabinoid: the C_{max} reached 335 ng/mL in a fasted state and 1,628 ng/mL in a fed state (Taylor et al., 2018). CBD was also quickly metabolized to 7-COOH-CBD, which comprised 97% of its metabolites, and 7-OH-CBD, its active metabolite (Taylor et al., 2018).

These values are consistent with plasma levels observed in rats after chronic administration of CBD. Data obtained in our lab showed that, after a single injection of 10 mg/kg CBD, dams had mean plasma levels of CBD of 239 ng/mL and 47 ng/mL for 7-OH-CBD *(*Black et al., 2023 *in preparation*). After chronic exposure, CBD C_{max} reached levels of 1,473 ng/mL for CBD and 39 ng/mL for 7-OH-CBD (Black et al., 2023 *in preparation*).

1.4 Preclinical rodent models

The use of rodent models in neurobehavioral research has made it possible to gain insight into multiple human behaviors and its underlying processes. A model, as the word defines it, is "a simple representation of a complex system" (Denayer et al., 2014) and does not fully reproduce the human condition, but rather models specific aspects of human diseases, pathways, and

processes. Although a rodent model will never fully represent the human condition, they allow the investigation of brain-behavior relations within ethically acceptable limits.

When contemplating a model, it is important to take into consideration the reliability and validity of the model. It is generally accepted that the model should have face validity, predictive validity, and construct validity, although the hierarchy of these categories differ among authors (van der Staay, 2006; Willner, 1986). Briefly, these concepts assess: the similarity between the model and the condition or process being modeled (face validity); how successfully does the model predicts the situation modeled, that is, the response seen in humans (predictive validity); and the theoretical rationale of the model, namely, how accurately the test assesses what is supposed to (construct validity). Moreover, some authors describe two other types of validity: internal validity, which refers to the relationship between cause and effect and to what extent the outcomes represent the truth in the studied population; and external validity, which refers to the extent to which the results from an experiment are generalizable to the population it is representing (Bailoo et al., 2014; Patino & Ferreira, 2018).

van der Staay defined a model to be clinically or biologically relevant to behavioral neuroscience if it permits the study of "brain–behavior relations under controlled conditions, with the final goal to gain insight into, and to enable predictions about, these relations in humans" (van der Staay, 2006). In this project, by using a rodent model of in utero exposure, we are able to investigate the outcomes of prenatal CBD exposure to both dam and pups, which in turn allows us to predict what outcomes might occur in humans, giving the model good predictive validity. Additionally, the model chosen also has good construct validity, since the tests used are common tools in the measure of neurodevelopment of rodents.

1.4.1 Neurodevelopmental testing

Neurodevelopmental tests are commonly used to identify landmarks of development and assess the impact of prenatal or postnatal insult. These developmental tests evaluate neurological reflexes, which are involuntary and reflect brain stem and spinal cord development; and postural mechanisms, which in turn are voluntary movements that depend on normal maturation of higher cortical networks (Nguyen et al., 2017). Any alterations on the normal development of the CNS can be reflected in the delay or absence of the behaviors that assessed by neurodevelopmental tests.

In humans, neurodevelopmental assessment of newborns has become a valuable tool to predict developmental disabilities (Farber et al., 1985; Zafeiriou, 2004). Newborns have complex cerebral functions that impact their movements, tone, and behavior, and there are several tests used to assess the neurological development that differ in their scoring system, time of administration and training required (reviewed in El-Dib et al., 2011). Clinicians and researchers need to choose the correct test depending on which question they are trying to answer, and different investigators will usually have a preference on which reflexes and postural reactions, or a combination of them, are used in the evaluation. Observation of the infant's general movements can be considered a valuable tool for assessment of motor development and prediction of outcomes (Zafeiriou, 2004).

In this context, it is important to have experimental models that are somewhat similar to humans and allows us to predict neurodevelopmental disabilities after prenatal or postnatal insults. Rodents are a common model used in neurodevelopmental research. The rat, at birth, is capable of some activities (e.g., suckling and vocalizing) and continues to develop throughout the postnatal period (Heyser, 2003). In rodents, markers of development can be either physical (e.g., time of eye opening, incisor eruption, and body weight) or sensorimotor. The average day for eye opening in rodents is PND13 and incisor eruption is PND7 (Heyser, 2003). They also demonstrate righting reflex (i.e., the reflex of a body to regain its normal position when displaced from it) around PND5 and negative geotaxis (i.e., a directional movement against gravity) on PND7, in average (Heyser, 2003; Ruhela et al., 2019). Average time for bar holding is PND14, crawling on PND11 and walking on PND16 (Heyser, 2003).

Although rats are highly related to humans, and we share similar organs, biochemical pathways, and physiology (Vuguin, 2007), the model used for this project has limitations that are important to note. Firstly, rat pups continue to mature outside of the uterus after they are born: the development that occurs on the first 10 PND is equivalent to the third trimester of *in utero* development in humans (Scheyer et al., 2019). Secondly, rodents are litter-bearing, i.e., the dams have multiple fetuses, which can lead to differences in nutrient supply and maternal care between pups from the same litter (Vuguin, 2007). However, when taken into consideration the cost of housing, ease of manipulation, length of gestation, and ethical considerations, rodents prove to be a very useful model to analyze the relationship between maternal CBD consumption and child outcomes.

1.5 Hypothesis

My hypothesis was that CBD exposure during pregnancy would affect fetal viability and development (e.g., number of pups per litter and pup weights) and negatively impact the neurodevelopment of the offspring during the neonatal period (PND 0 – PND 21).

1.6 Objectives

My first objective was to characterize the impact CBD had on maternal factors, litter characteristics and pup health. Secondly, I aimed to assess the developmental changes in the offspring of rat dams subjected to chronic CBD treatment via a battery of neurobehavioral assays.

2.0 NEURODEVELOPMENTAL OUTCOMES FOLLOWING PRENATAL CANNABIDIOL EXPOSURE IN MALE AND FEMALE SPRAGUE DAWLEY RAT OFFSPRING[1]

2.1 Abstract

Cannabis use has increased in recent years due to legalization in several countries. Specifically, frequency of *Cannabis* use among pregnant women has also increased. Cannabidiol, one of *Cannabis's* main components, is often seen as a natural and safe substance and has been increasingly used for treatment of medical condition such as pain, anxiety, and depression. Women report using CBD during pregnancy to alleviate pregnancy-related symptoms such as nausea, vomiting and chronic pain. However, there is very little evidence in the literature regarding the consequences of prenatal CBD exposure. In this study, we conducted various neurodevelopmental behavioral tests to assess how *in utero* exposure to CBD affects pups physical and neurodevelopment. Prenatal CBD exposure was associated with a lower body weight in offspring and a delay in neurodevelopmental outcomes in early life stages that resolved with time. More specifically, the higher dose of CBD impacted pup's reflexes in the initial days of testing (PND3-8), an effect that disappeared by PND21. This study contributes to a growing body of evidence regarding the safety of CBD use during pregnancy, and although more studies need to be conducted, pregnant women and health care professionals should be cognizant of the potential negative outcomes of prenatal CBD exposure.

2.2. Introduction

Cannabis is the most used recreational drug, with consumption rates increasing over the last decade due to its legalization in several countries in North America and Europe (Metz & Stickrath, 2015; Scheyer et al., 2019). The frequency of *Cannabis* use has increased specifically amongst pregnant women, with a reported rise in past-month use of 62% from 2002 to 2014 (Brown et al., 2017; Young-Wolff et al., 2019). In the non-scientific media, prenatal and

[1] The manuscript used as the basis for this chapter will be submitted to Neuroscience for publication and has been modified to meet formatting requirements. The manuscript was written by me – Sarah Lunardi Baccetto – with guidance from Dr. Robert Laprairie and Dr. John Howland. Experiments were conducted by me with assistance from Tallan Black, Leah McFarlane and Genre Sanfuego (University of Saskatchewan). Recordings were analyzed by me and Leah McFarlane. Data was analyzed by me with technical assistance from Dr. Robert Laprairie and Dr. John Howland.

postpartum *Cannabis* use are often portrayed as beneficial to the mother due to its antiemetic and analgesic properties and its potential to improve mental health (Jarlenski et al., 2018). Both animal and human studies show *Cannabis* use during pregnancy can lead to higher risks of physical, cognitive and emotional deficits in the offspring (reviewed in Schneider, 2009; Huizink, 2014; Higuera-Matas et al., 2015). However, mothers still report difficulty in receiving useful information about prenatal *Cannabis* use and its effects from health care providers, relying instead on the internet and personal experiences (Jarlenski et al., 2016).

Although *Cannabis* contains over one hundred cannabinoids, the two major pharmacological components of *Cannabis* are THC, the plant's main intoxicating component, and CBD (Wilkinson et al., 2016). Both THC and CBD interact directly or indirectly with the endocannabinoid system, influencing multiple physiological processes (Wu et al., 2011). The ECS is comprised of two main receptors: CB1R, largely located in the CNS, and CB2R, found mostly in the peripheral nervous system and immune cells (Cabral et al., 2008; Howlett & Abood, 2017). These receptors are activated by endocannabinoids, the two predominant ones being anandamide and 2-AG, which are catabolized by the intracellular enzymes FAAH and MAGL, respectively (di Marzo & de Petrocellis, 2012; Pertwee, 2012). Components of the ECS are also present and functional from very early developmental stages, influencing a range of physiological functions during neural development (Fride, 2008; Schonhofen et al., 2018). The ECS plays an essential role in neurogenesis, neuronal migration, synaptogenesis and synaptic pruning (Mulder et al., 2008; Maccarrone et al., 2014; Bara et al., 2021).

CBD is the major non-intoxicating compound in *Cannabis* and is reported to have analgesic, antiemetic, anxiolytic, anti-epileptic and anti-psychotic properties (Millar et al., 2019; Sarrafpour et al., 2020). There has been an increase in adult non-prescribed CBD use in recent years mostly as a treatment for medical conditions such as pain, anxiety, and depression (Corroon & Phillips, 2018). Although the pharmacology of CBD is not fully understood, there is evidence that it acts on a variety of different targets, including: CB1R, CB2R, serotonin 1a receptor (5-HT1a), G-protein coupled receptor 55 (GPR55), and TRPV1 receptors, among others (reviewed in McPartland et al., 2015). Additionally, CBD can indirectly modulate the ECS by enhancing endocannabinoid levels (McPartland et al., 2015). Although there is a lack of information about the safety of CBD use during pregnancy, pregnant women report using it to relieve nausea and vomiting, especially in the first trimester (Sarrafpour et al., 2020). CBD can also readily cross the

placenta and it is distributed throughout the fetal body, being metabolized in the fetal liver(Ochiai et al., 2021). Animal studies report negative effects to the fetus due to *in utero* CBD exposure, such as eye and midline facial malformations (Fish et al., 2019), increased anxiety behaviors in F1 female offspring, and alterations in brain DNA methylation (Wanner et al., 2021). Additionally, Iezzi et al. recently reported that pups from CBD-treated dams presented sex-specific alterations in body weight, early communication, motor skills and discrimination abilities (Iezzi, Caceres, et al., 2022). CBD also causes loss of cell viability and interferes with cell migration and apoptosis in a trophoblast model representative of placental cells (Alves et al., 2021). These processes are essential in placental development and their disruption can lead to pregnancy disorders. Importantly, a portion of the animal studies available administered single doses of CBD rather than adopting a repeated exposure paradigm (Fish et al., 2019; Ochiai et al., 2021), and limited data describe neurodevelopmental and molecular outcomes of acute or chronic CBD exposure.

Given the limited evidence available regarding the effects of prenatal CBD exposure on the ECS and neurodevelopmental outcomes, the aim of this study was to investigate the effects of CBD exposure on offspring using a preclinical rat model. In this study, we treated pregnant rats daily from GD 6-20 with CBD injected intraperitoneally (i.p.). Although the most common methods of CBD consumption in humans are sublingual, vapor and capsules/pills (Corroon & Phillips, 2018), i.p. injections are an accepted and regularly used method of assessing drug effects in animals. Indeed, Deiana et al. reported that i.p. and oral administration of CBD in rats produced similar pharmacokinetic plasma profiles, with slightly higher brain levels following oral administration (Deiana et al., 2012). We then monitored the physical development of rat pups from PND 3 to 21 using several developmental tests. We found that developmental CBD exposure impacted physical development and caused delays in neurodevelopmental outcomes early after birth that resolved as the pups matured.

2.3 Experimental procedures

2.3.1 Animals.

Twenty-four female and twelve male adult Sprague Dawley rats (Charles River) were used for breeding in this experiment. Nineteen rats had successful pregnancies, which generated 181 pups. Animals were pair housed by sex in the vivarium at the University of Saskatchewan in standard ventilated cages with plastic tubes for environmental enrichment on a 12-hour light/dark cycle.

Water and food were available ad libitum. All experiments were approved by the University of Saskatchewan Animal Research Ethics Board (AUP #20190067).

2.3.2 Breeding.

On the day of breeding, one male rat was placed in the female cage at 17:00 and left to breed overnight. The following morning (9:00), pregnancies were confirmed by presence of sperm on vaginal swab. Briefly, a vaginal swab was collected from each rat using a P200 pipette tip filled with 50 µL of phosphate-buffered saline (PBS). Samples were then visualized under a light microscope. After confirmation of pregnancy, rats were singly housed and that was considered GD0. Dams were then weighed on GD3 and left undisturbed until beginning of treatment on GD6.

2.3.3 Treatment.

Dams were treated once daily from GD6-20 (Fig. 1) with intraperitoneal (i.p.) injections of 5 mg/kg CBD (Cayman Chemical, Cat. No. 90080), 10 mg/kg of CBD, or vehicle (1:1:18 ethanol:kolliphor:PBS). The CBD used in this study contains only the (–)-CBD enantiomer, which is the naturally occurring form of CBD.

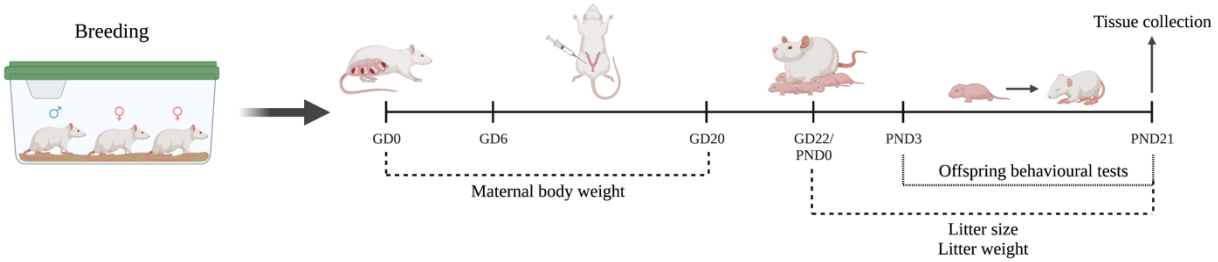

Figure 1. Schematic representation of experimental timeline. Animals were bred in house and dams were treated daily from GD6 to GD20 with i.p. injections of either vehicle (n=6), 5 mg/kg CBD (n=7) or 10 mg/kg CBD (n=6). Offspring went through behavioral testing from PND3 to PND21. Created with BioRender.com.

2.3.4 Litter measurements.

After ending of treatment on GD20, dams were left undisturbed and checked daily until litters were born. Postnatal day (PND) zero was determined if pups were found before 17:00h. On PND1, when possible, litters were culled to 5 males and 5 females.

2.3.5 Offspring physical development.

All litters were weighed weekly until PND21. Time of eye opening (opening of both eyelids) and incisor eruption (top and bottom incisors) was recorded by visual inspection performed twice daily (9:00h and 16:00h) from PND8-16 (Table 1).

Table 1. Physical developmental parameters of the offspring. Data is expressed as mean ± SEM.

Parameters	Vehicle	CBD 5 mg/kg	CBD 10 mg/kg
Eye-opening for the whole litter, PND	15.00 ± 0.26 (n=6)	15.43 ± 0.48 (n=7)	15.50 ± 0.22 (n=6)
Top incisor eruption for the whole litter, PND	11.83 ± 0.60 (n=6)	11.50 ± 0.34 (n=6)	12.00 ± 0.58 (n=4)
Bottom incisor eruption for the whole litter, PND	12.83 ± 0.31 (n=6)	13.20 ± 0.37 (n=5)	14.00 ± 0.41 (n=4)

2.3.6 Offspring behavioral testing.

For behavioral testing, one male and one female pup from each litter, when possible, were allocated to one of four groups and tested from PND3 to PND21 (Fig. 2). Each group underwent one or two tests that assess different measures of neurodevelopment. On the day of testing, the home cage was transported into the testing room and the pups that were being tested were removed from the home cage, put into the testing apparatus and replaced in the home cage immediately after. All tests were recorded with a video camera and videos were later analyzed.

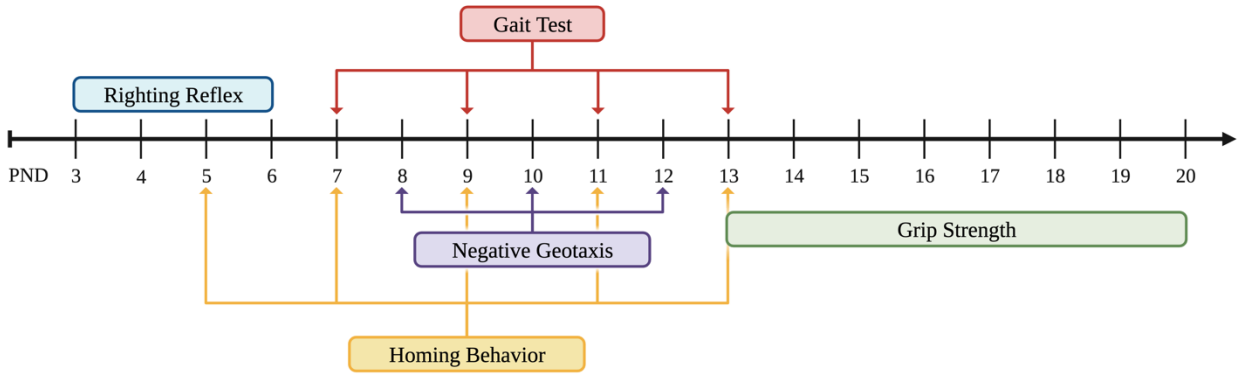

Figure 2. Schematic representation of the timeline for behavioral tests. Offspring was tested in 5 different behavioral tests: righting reflex (PND3-6); gait test (PND7, 9, 11 and 13); negative geotaxis (PND 8, 10 and 12); grip strength (PND13-20); and homing behavior (PND 5, 7, 9, 11 and 13). Created with BioRender.com.

Group A – Righting reflex and negative geotaxis: The righting reflex test is used as an indication of subcortical maturation and muscle strength (Fan et al., 2008). Briefly, the pup was placed on its back on a flat surface, held for 5s and released quickly (Fig. 3). The time required for the pup to roll over into all 4 paws was recorded with a cut-off time of 30s. Pups underwent 3 trials a day on PND3 to 6 with a cut-off time of 30s per trial. The negative geotaxis test assesses motor coordination, reflex development and vestibular function (Fan et al., 2008; Bouayed et al., 2009). Pups were placed on an inclined surface (25º) with their heads facing down and the time required for a 180º turn upward was recorded with a time limit of 120s (Fig. 4). Negative geotaxis was performed on PND 8, 10 and 12 with 3 trials per day.

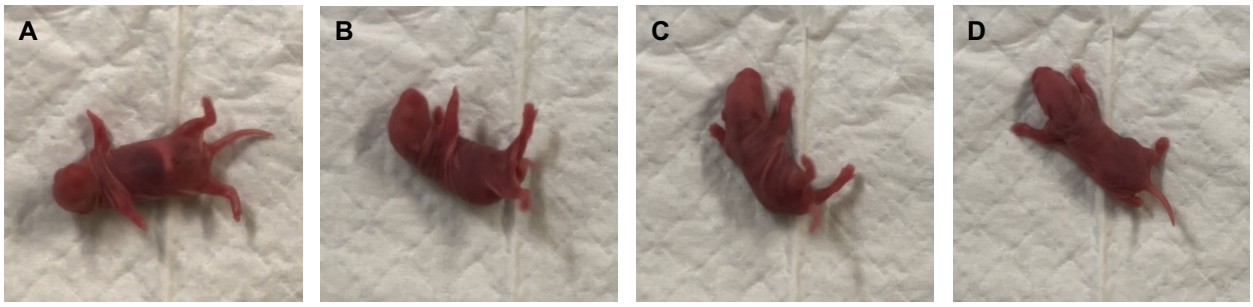

Figure 3. Righting reflex test. First, the pup is placed on its back on a flat surface, held for 5s and released quickly (**A**). The pup is then allowed roll over into all 4 paws (**D**). The time required to make the turn is recorded with a cut-off time of 30s. Pups underwent 3 trials a day on PND3 to 6.

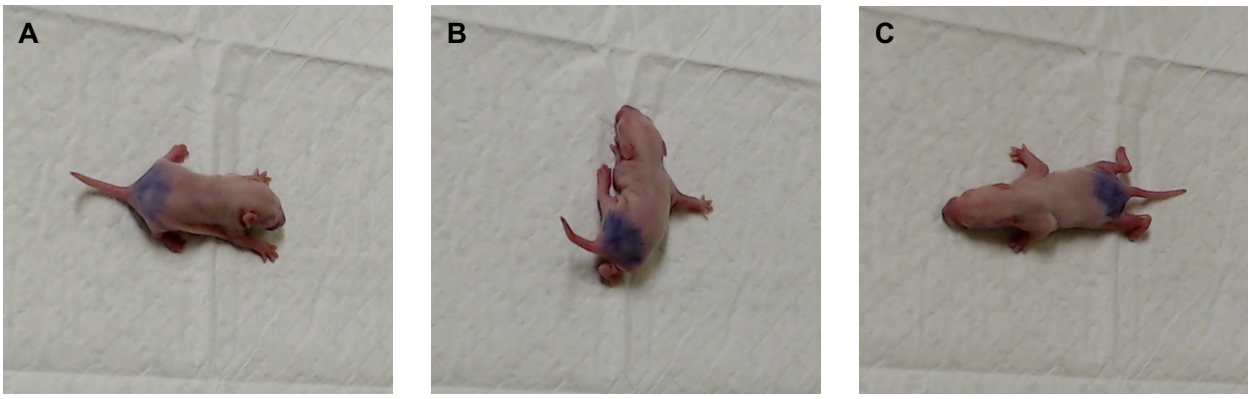

Figure 4. Negative geotaxis test. First, the pup is placed on an inclined surface (25º) with its head facing down (**A**). Then, the pup is allowed a maximum of 120s to make an 180º turn upward (**C**) and the time required for the turn is recorded. Pups underwent 3 trials a day on PND 8, 10 and 12.

Group B – Gait test: The gait test is used to measure muscle/tone equilibrium and integrity of the cerebellum (Fan et al., 2008). On PND 7, 9, 11 and 13 pups underwent the gait test. Briefly, the pup was placed in a clean cage in the center of a 15cm diameter circle and the time required for it to move all 4 paws outside of the circle was recorded, with a maximum time of 120s (Fig. 5).

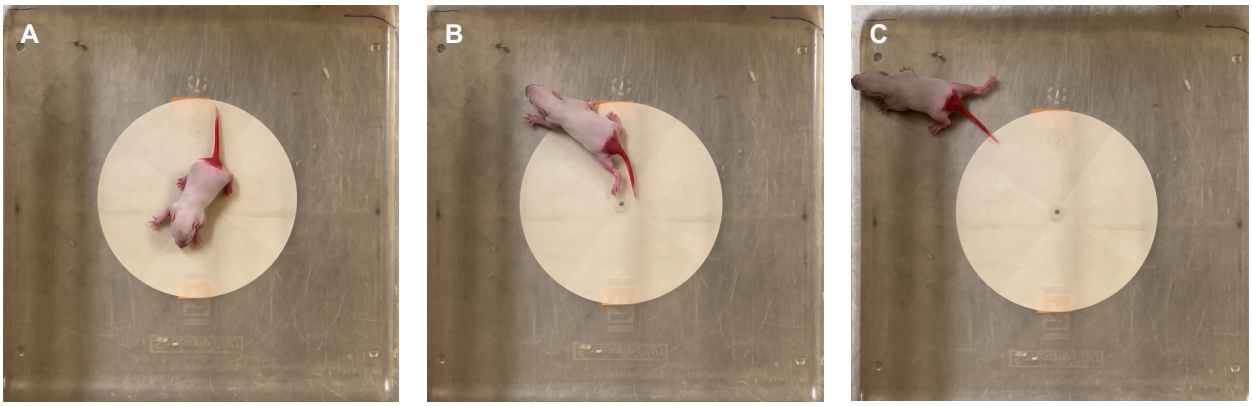

Figure 5. Gait test. The pup is placed in a clean cage in the center of a 15cm diameter circle (**A**). The test is recorded for a maximum of 120s until the pup moves all 4 paws outside of the circle (**C**). Pups were tested once a day on PND 7, 9, 11 and 13.

Group C – Grip strength: The grip strength test assesses neuromuscular and locomotor development, as well as sensory-motor coordination (Šlamberová et al., 2006; Fan et al., 2008). Pups were tested daily from PND13 to PND20. For this test, the pup's forelimbs were placed on a horizontal wire, and we recorded the total time it was able to hold onto the wire (Fig. 6).

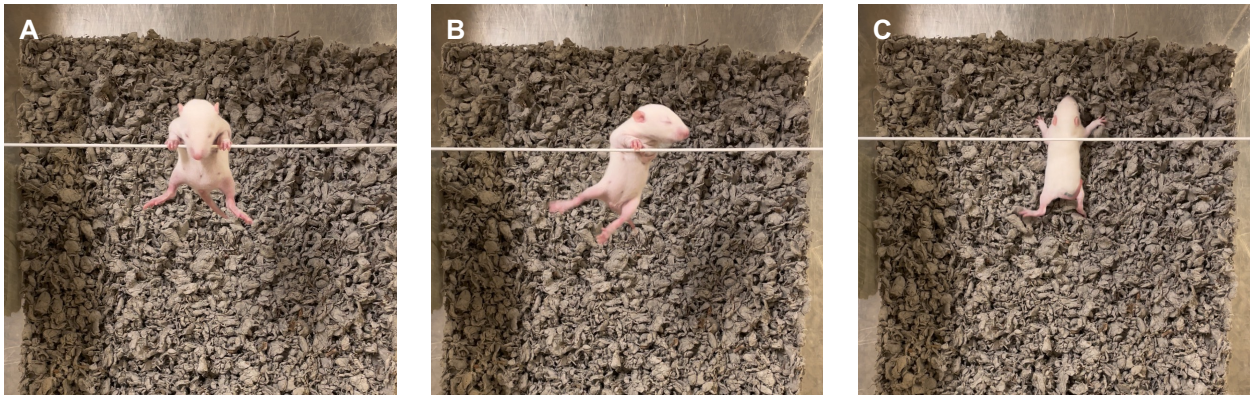

Figure 6. Grip strength test. The pup's forelimbs are placed on a horizontal wire on top of a clean cage filled with bedding (**A**). The total time the pup is able to hold onto the wire until it falls (**C**) is recorded. Pups were tested once a day on PND13-20.

Group D – Homing behavior: The homing test measures the ability of pups to locate their nest and move toward it after being displaced from it. In order to do that, the pups must rely on thermal and olfactory cues and be able to remember their nest odor and differentiate it in a different

environment (Ricceri et al., 2007; Fischer et al., 2016). Briefly, a clean cage was split into four quadrants. Fresh bedding was placed into 3 quadrants and bedding from the home cage was placed into the remaining quadrant (nest quadrant). The pups were then placed in the middle of the quadrant diagonal to the nest quadrant with their faces facing the center of the cage (Fig. 7). Three different behaviors were analyzed: (1) activity – number of quadrants crossed; (2) homing success – if they reached the nest quadrant; and (3) latency – the time required for the pup to reach the nest quadrant, with a maximum time of 3 min. Homing behavior was assessed on PND 5, 7, 9, 11 and 13.

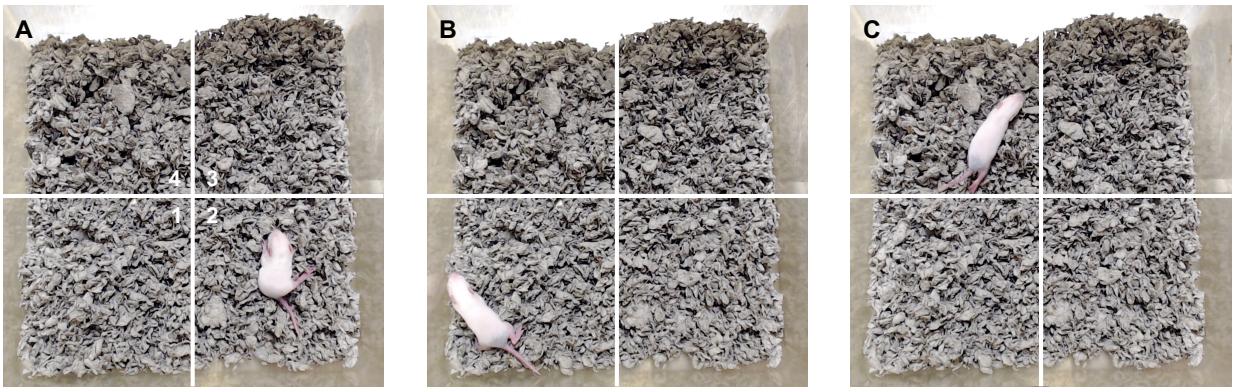

Figure 7. Homing behavior test. First, a clean cage is split into four quadrants with fresh bedding in 3 quadrants (**A1-3**) and bedding from the home cage in the nest quadrant (**A4**). The pup is then placed in the quadrant diagonal to the nest quadrant (**A2**) with their faces facing the center of the cage (**A**). The pup's activity is then recorded for 3 min and later analyzed for number of quadrats crossed and time required to reach the nest quadrant (**C**). Pups were tested once a day on PND 5, 7, 9, 11 and 13.

In order to minimize the number of confounding variables in developmental testing, some aspects should be maintained as constant as possible. These include the handling of the animals, time of days they are tested, blind testing, minimization of maternal deprivation, and equalizing of litter sizes. In this project, all viable measures were taken to control for these factors: (1) the animals were carefully handled in the same manner by the same investigators; (2) behavioral testing always occurred on the morning period; (3) the investigator running the tests was blinded to the treatment the pups were prenatally exposed to; (4) pups were only separated from their

mothers and littermates when being tested (maximum time of 3 min); and (5) when possible, litters were culled to the same number of pups.

2.3.7 Statistical analysis.

Data was analysed with GraphPad Prism 9.0. Data is expressed as mean ± standard error of the mean (SEM). The mean and SEM was calculated for maternal weights throughout gestation as there was no data for one dam of the 5 mg/kg CBD group for GD3. A two-way ANOVA followed by Bonferroni's post-hoc was then used to analyze maternal weigh throughout gestation. A one-way ANOVA was then used to analyze dam's percentage weight gain throughout gestation. A one-way ANOVA (followed by Bonferroni's post-hoc) was used to analyze litter size and litter weight on PND1, time of eye opening and time of incisors eruption. A two-way ANOVA (followed by Bonferroni's post-hoc) with factors of treatment and sex was used to analyze litter's sex ratios on PND1. The mean and SEM was also calculated for relative pup weight from PND1-21 (missing data for two 5 mg/kg CBD litters on PND21). The data was then analyzed with a two-way ANOVA followed by Bonferroni's post-hoc. For the offspring behavioral tests, a two-way ANOVA (followed by Bonferroni's post-hoc) was used to analyze negative geotaxis (3 x 3, Day x Treatment), righting reflex (4 x 3, Day x Treatment), homing latency (5 x 3, Day x Treatment) and homing activity (5 x 3, Day x Treatment). When analyzing gait test and grip strength, due to missing videos (2 and 5 values, respectively), mean and SEM were calculated. A two-way ANOVA followed by Bonferroni's post-hoc was then used to analyze gait test (4 x 3, Day x Treatment) and grip strength (10 x 3, Day x Treatment).

Estimation statistics were used to analyze the magnitude of effects on the righting reflex and negative geotaxis tests, and figures were generated with https://www.estimationstats.com/#/ (Ho et al., 2019). For both tests, Cohen's d was calculated for the initial day of testing.

2.4 Results

2.4.1 Maternal physical measurements.

Maternal weight was measured every 3 days between GD0 and GD20 and mean percentage weight gain from baseline was calculated [(final weight-baseline)/baseline*100]. The analysis showed significant main effects of Day ($F_{(7,\ 127)}$=263.2, p<0.0001) and Treatment ($F_{(2,\ 127)}$=53.67, p<0.0001) on weight, with no significant interaction ($F_{(14,\ 127)}$=0.36, p=0.98) (Fig. 8A). Vehicle-

treated dams showed increased weights throughout gestation when compared to dams treated with 5 mg/kg CBD (GD3, p=0.002; GD6, p=0.007; GD9, p=0.001; GD12, p=0.01; GD15, p=0.001; GD18, p=<0.0001; GD20, p<0.0001) and 10 mg/kg CBD (GD3, p=0.01; GD9, p=0.04; GD15, p=0.03; GD18, p=0.03; GD20, p=0.002). When comparing percentage weight gain, there was no significant difference between treatments (Fig. 8B).

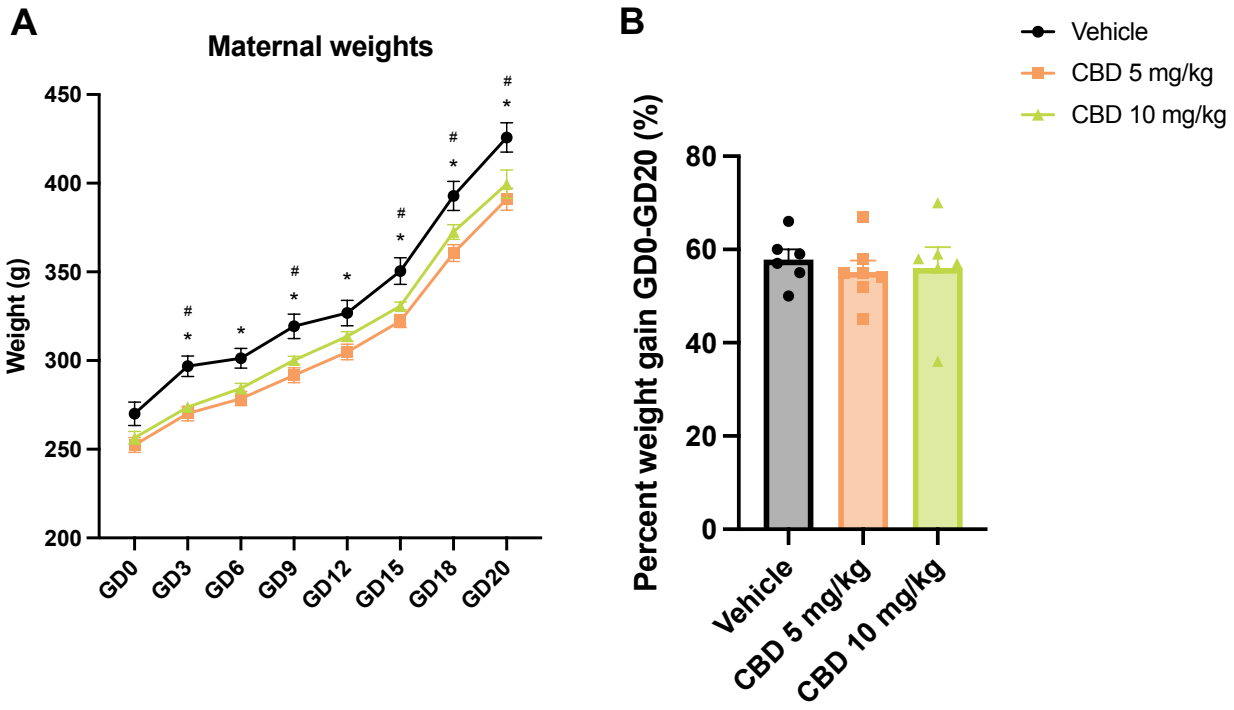

Figure 8. Effects of CBD treatment on maternal weight. (**A**) Dams were weighed every 3 days starting at GD0 until GD20. Vehicle-treated dams had consistently higher weights throughout gestation. (**B**) Percent weight gain from GD0 to GD20. All dams showed similar percentages of weight gain across treatments (Vehicle n=6, CBD 5 mg/kg n=7, CBD 10 mg/kg n=6). Data is presented as mean ± SEM. *p<0.05 (Vehicle vs. CBD 5 mg/kg); #p<0.05 (Vehicle vs. CBD 10 mg/kg).

2.4.2 Litter size, litter weight, and sex ratio.

Litter size, litter weight, and sex ratio were collected on PND1. There was no difference in litter size (Fig. 9A) or sex ratio (Fig. 9C) between treatment groups. There was a main effect of Treatment in litter weight ($F_{(2, 16)} = 4.91$, p=0.02) (Fig. 9B). According to the post-hoc analyses, treatment with 5 mg/kg CBD was associated with a decrease in litter weight (p=0.04). A similar

effect was seen with the higher dose of CBD (10 mg/kg), although the weight decrease was not statistically significant due to higher variability.

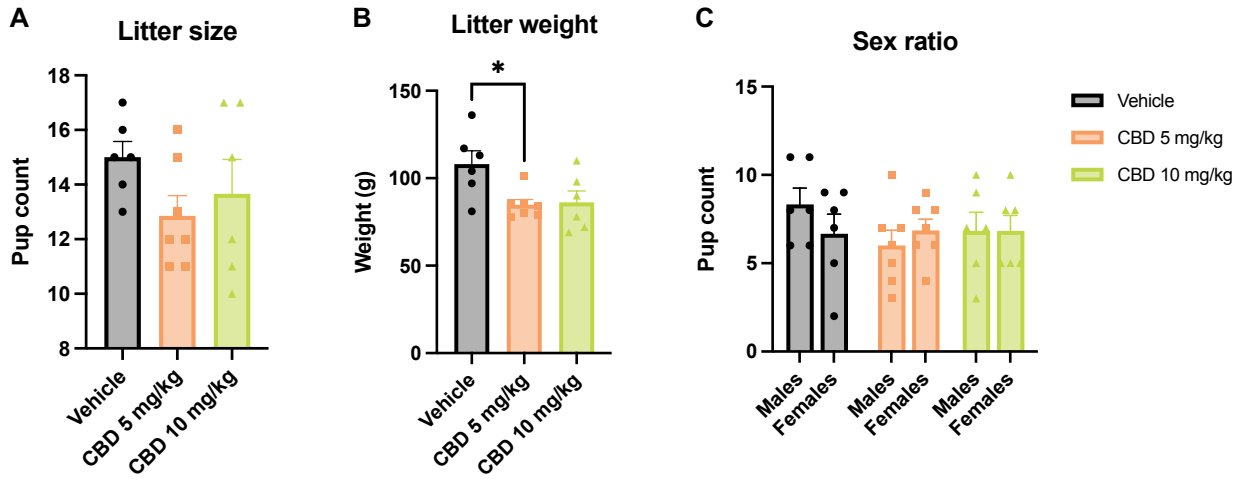

Figure 9. Effects of prenatal CBD exposure on litter. (**A**) Litter size measure on PND1. Number of pups was similar across all treatments. (**B**) Litter weight on PND1. Litters prenatally exposed to 5 mg/kg of CBD had lower weight compared to vehicle treated litters. (**C**) Number of male and female pups per litter on PND1. Sex ratio was similar across all treatments (vehicle n=6, CBD 5 mg/kg n=7, CBD 10 mg/kg n=6). Data is presented as mean ± SEM. *p<0.05.

2.4.3 Offspring physical measurements.

Litter weight was measured weekly from PND1 to PND21, and relative pup weight was calculated according to litter size (Fig. 10). There were main effects of Day ($F_{(3, 62)}$=433.70, p<0.0001) and of Treatment ($F_{(2, 62)}$=10.94, p<0.0001) on mean pup weight, with no significant interaction of Day and Treatment ($F_{(6, 6)}$=2.23, p=0.05). Post-hoc analyses showed that, when compared to control, treatment with 5 mg/kg CBD and 10 mg/kg CBD were associated with a decrease in relative pup weight on PND21 (p=0.0005 and p<0.0001, respectively). There was no difference across treatments in time of eye opening ($F_{(2, 16)}$=0.54, p=0.59), top incisor eruption ($F_{(2, 13)}$=0.24, p=0.79) and bottom incisor eruption ($F_{(2, 12)}$=2.59, p=0.12), as seen on Table 1.

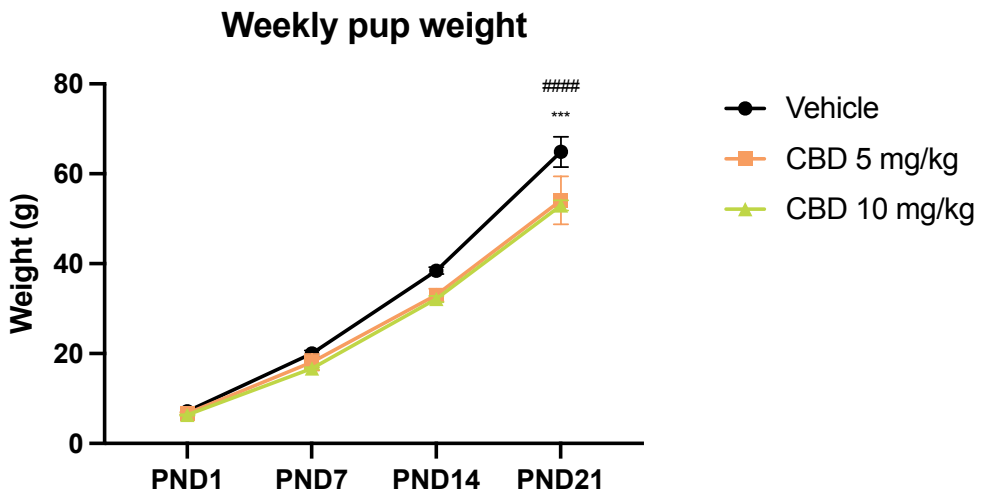

Figure 10. Effects of prenatal CBD exposure on offspring weight. Litters were weighed weekly from PND1 to PND21. Relative pup weight was calculated according to litter size. Offspring prenatally exposed to 5 mg/kg CBD and 10 mg/kg CBD showed increasingly lower weights from PND7 to PND21 when compared to vehicle (vehicle n=6, CBD 5 mg/kg n=7, CBD 10 mg/kg n=6). Data is presented as mean ± SEM. ***p=0.0005 (vehicle vs. CBD 5 mg/kg); ####p<0.0001 (vehicle vs. CBD 10 mg/kg).

2.4.4 Offspring behavioral tests.

Righting reflex. A main effect of Day ($F_{(1.548, 54.18)}$=17.56, p<0.0001) was observed in righting reflex test data (Fig. 11A). Post-hoc analyses showed no effect of treatments on time (s). *Negative geotaxis.* There was a main effect of Day ($F_{(2, 105)}$=3.39, p=0.04) on time to complete the test (Fig. 11B). Post-hoc analyses showed no differences across treatments. *Gait test.* The analysis showed a main effect of Day ($F_{(3, 138)}$=25.05, p<0.0001) on test time (Fig. 11C). Post-hoc analyses showed no differences in latency across treatments. *Grip strength.* There was an effect of Day ($F_{(9, 344)}$=12.88, p<0.0001) on grip strength (Fig. 11D). Post-hoc analyses showed no differences across treatments. *Homing latency.* Main effect of Day ($F_{(3.032, 106.1)}$=36.31, p<0.0001) was observed in latency to reach the nest quadrant in homing behavior data (Fig. 11E). Post-hoc analyses showed that treatment with 5 mg/kg CBD caused an increase in latency on PND13 when compared to vehicle (p=0.04). *Homing activity.* There was a main effect of Day on number of quadrants crossed in the homing test ($F_{(1.711, 59.90)}$=42.98, p<0.0001). Post-hoc analyses showed no differences in activity across treatment groups (Fig. 11F).

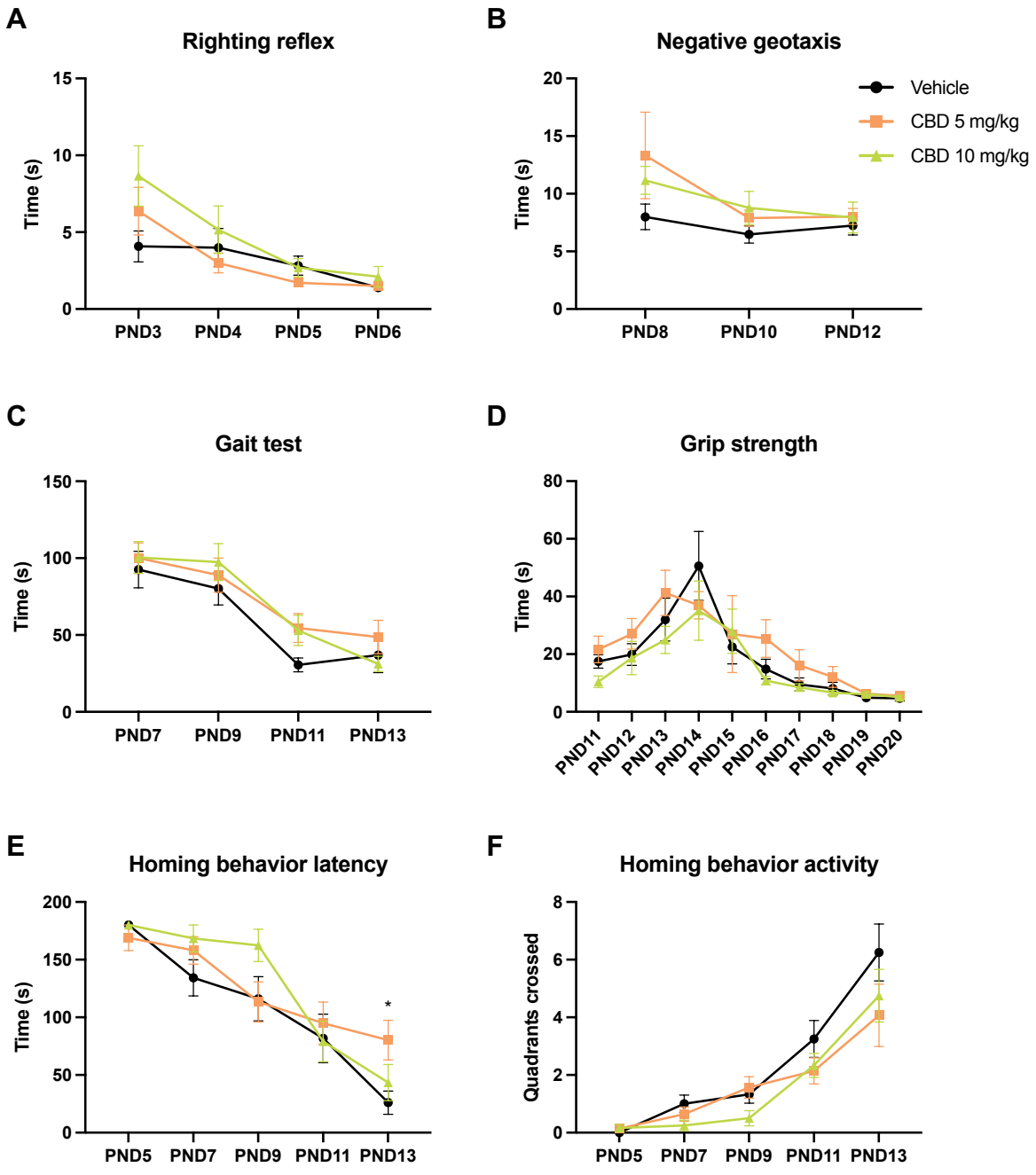

Figure 11. Effects of prenatal CBD on offspring behavioral testing. (**A**) Time (s) for pup to right in the righting reflex test. (**B**) Time (s) for pup to move out of the circle in the gait test. (**C**) Time (s) for an 180º upward turn on the negative geotaxis test. (**D**) Time (s) pups were able to hold onto the wire on the grip strength test. (**E**) Time (s) for pups to reach the nest quadrant on the

homing behavior test. (**F**) Number of quadrants crossed in the homing behavior test (vehicle n=6, CBD 5 mg/kg n=7, CBD 10 mg/kg n=6). Data is presented as mean ± SEM. *p<0.05.

2.4.5. Estimation statistics: righting reflex and negative geotaxis

Righting reflex. An analysis of the raw effect sizes showed a small effect of 5 mg/kg CBD [d=0.47, 95.0%CI (-0.423, 1.1), p=0.24] and a large effect size of 10 mg/kg CBD [d=0.85, 95.0%CI (0.0138, 1.59), p=0.05] on time to right on PND3 (Fig. 12A). *Negative geotaxis.* The analysis showed a small effect size of 5 mg/kg CBD [d=0.43, 95.0%CI (-0.44, 1.2), p=0.30] and a moderate effect size of 10 mg/kg CBD [d=0.79, 95.0%CI (-0.121, 1.74), p=0.07) on the time require for an 180º turn (Fig. 12B).

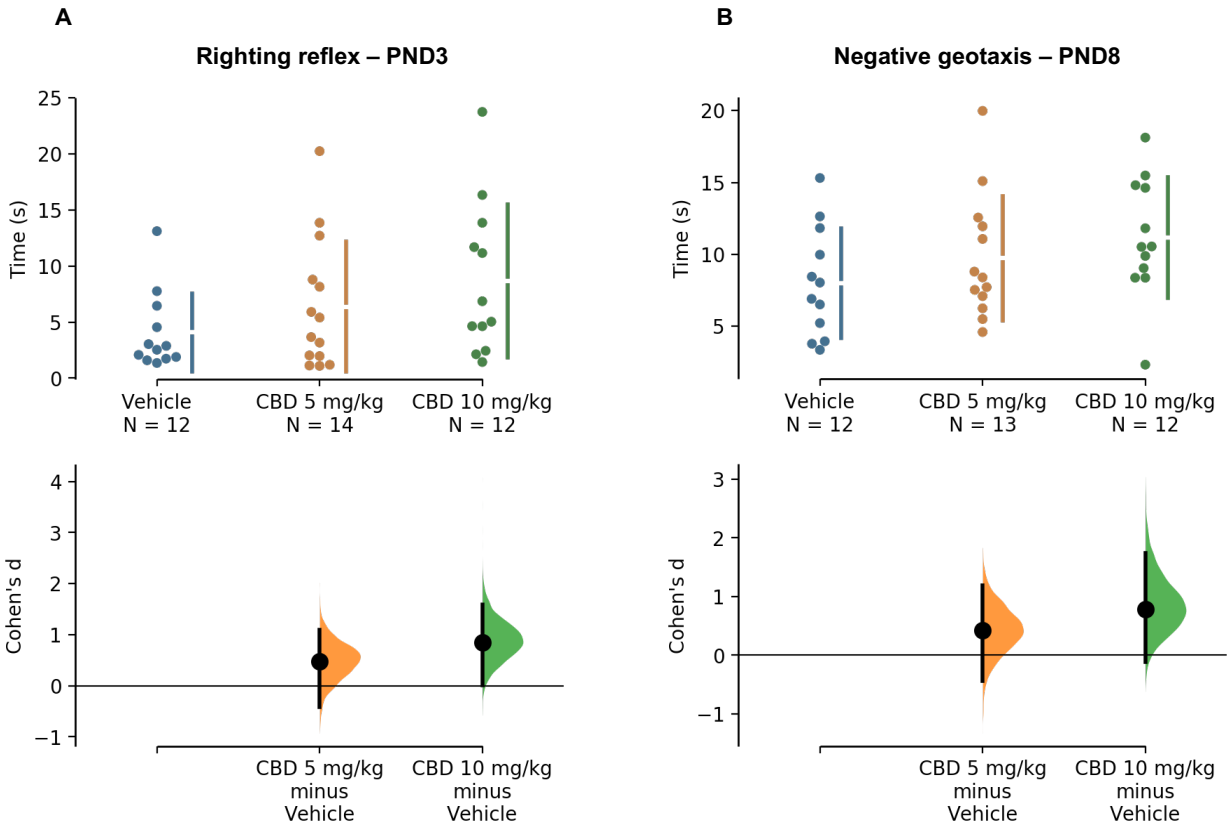

Figure 12. Effect sizes of righting reflex and negative geotaxis. Top panels for A and B show raw data points graphed in swarm plots with means ± SD (vertical lines). Bottom panels for A and B display the effect sizes (Cohen's d) with bootstrap sampling distributions (95% CI). (**A**) A small and large effect sizes are seen for 5 mg/kg CBD and 10 mg/kg CBD, respectively, on the righting

reflex test on PND3. (**B**) A small and moderate effect sizes are seen for 5 mg/kg CBD and 10 mg/kg CBD, respectively, on the negative geotaxis test on PND8.

2.5 Discussion

In this study, we investigated the neurodevelopmental consequences of *in utero* CBD exposure. Prenatal exposure to CBD was associated with a lower weight gain in pups until weaning (PND21). In both humans and animal studies, prenatal exposure to *Cannabis* or THC is associated with fetal growth restriction (Hurd et al., 2005; el Marroun et al., 2009). A cross-sectional study showed that *Cannabis* use at any point during pregnancy was associated with an increased chance of low birth weight independent of other confounding factors (T. L. Crume et al., 2018). Other studies have also reported that exposure to *Cannabis in utero* seems related to reduced fetal growth and low birth weight in a dose-dependent manner (Zuckerman et al., 1989; el Marroun et al., 2009). Prenatal exposure of rat pups to THC also consistently reduced offspring body weight during early and adolescent periods (Breit et al., 2022). Additionally, the CBD-treated offspring showed signs of delay in development, especially in the first days after birth, reflected in the righting reflex and negative geotaxis tests. An increased latency time in the homing test as well as decreased activity in the same test are also suggestive of early life locomotion deficits.

Three human studies have investigated the mental and motor development of children prenatally exposed to *Cannabis*: the Ottawa Prenatal Prospective Study (OPPS), the Maternal Health Practices and Child Development Study (MHPCD) and the Generation R study (reviewed in Huizink, 2014). Reports of the OPPS showed a negative association between prenatal *Cannabis* use and memory and verbal outcomes in children aged 4 years old (Fried & Watkinson, 1990), although there were no cognitive deficits in observed in children of the same study at ages 1 and 2 years old (Fried & Watkinson, 1988). Another clinical study has shown that the children of women who smoked *Cannabis* during the third trimester of pregnancy showed lower mental development scores at 9-months old, but this difference disappeared by 19-months of age (Richardson et al., 1995). There are also reports that *Cannabis* use during the first month of lactation led to a decrease in a motor development index at one year of age, but exposure during the first trimester of pregnancy was a confounding factor (Astley & Little, 1990). It is difficult to interpret the effects of exposure to *Cannabis* or cannabinoids during development based only on clinical studies because there are several confounding factors that can have an impact in children's

development, such as dosage, length and frequency of use, polysubstance use, maternal nutrition, genetics, socioeconomic status, sample sizes and others. Rodent models, however, allow more control over these factors and various studies have provided valuable data on the impact of prenatal *Cannabis* or cannabinoid exposure in molecular and behavioral outcomes in the offspring that are in line with findings from clinical studies (reviewed in Schneider, 2009; Calvigioni et al., 2014). Similar to the human studies mentioned above, we have reported in this study signs of detrimental effects of CBD in offspring development that occur at different time points.

Several animal studies have reported cognitive deficits in the offspring of rodents exposed to THC or other cannabinoids during pregnancy (reviewed in Higuera-Matas et al., 2015). Antonelli et al. reported that rat pups prenatally exposed to the CB1R agonist WIN55,212-2 (WIN) showed a higher latency in the homing test, indicative of a learning impairment (Antonelli et al., 2005). This test explores the pup's tendency to maintain body contact with the mother and siblings and requires sufficient olfactory and motor skills, as well as associative and discriminative abilities that allow the pup to become imprinted by the dam's odor, memorize it and recognize it among others (Bignami, 1996). A more recent study, however, did not report any differences in the latency of homing behavior in pups prenatally exposed to WIN, although WIN-exposed male pups exhibited higher frequency of crossing in the test arena, suggestive of a sex-specific negative effect of WIN on early life locomotion (Manduca et al., 2020). Shabani et. al also reported altered motor behavior in pups prenatally exposed to WIN: in the righting reflex test, they showed a decrease in the latency to turn (Shabani et al., 2011). Other studies have also demonstrated a loss in righting reflex caused by cannabinoids: Fernandes et al. reported that CBD prolonged the loss of righting reflexes caused by hexobarbital in male adult rats (Fernandes et al., 1974) and THC has been shown to cause a loss of righting reflex (Forney, 1971).

Rat pups prenatally exposed to WIN also have impaired performance in the grip strength test, specifically on PND22 (Shabani et al., 2011). Breit et al. reported that prenatal exposure to THC via e-cigarette delayed early sensorimotor development (tested in the grip strength) and motor coordination was still impaired in early adolescence (Breit et al., 2022). However, the same authors demonstrated in another study that developmental exposure (PND4-9) to the CB1R and CB2R agonist CP-55,940 (CP) advanced early motor development (Breit et al., 2019).

Synthetic cannabinoids, however, are not the same as naturally occurring cannabinoids. The CBD naturally found in the *Cannabis* plant is (–)-CBD and its analogs, while its enantiomer

(+)-CBD and other derivatives are considered synthetic structures (Morales et al., 2017). WIN is a potent CB1R agonist while CBD acts as a negative allosteric modulator of CB1R (Laprairie et al., 2015) and has multiple other pharmacological targets (reviewed in de Almeida & Devi, 2020). CBD has also been shown to target the ECS through the inhibition of FAAH (Bisogno et al., 2001). Leweke et al. reported that CBD administration was associated with FAAH inhibition and consequent increase in AEA levels (Leweke et al., 2012). Other studies, however, have shown that CBD did not inhibit human FAAH in cell homogenates, but did inhibit AEA uptake through binding to fatty acid-binding proteins (Elmes et al., 2015).

Plasma AEA and FAAH levels in pregnant women have been associated with subsequent miscarriage (Maccarrone et al., 2000; Habayeb et al., 2008). FAAH has an important role in regulating local AEA levels, which in turn directly impacts embryo development and implantation (Paria et al., 1999; Wang et al., 2006), with evidence suggesting that higher levels of uterine AEA are detrimental to the implantation process (Paria et al., 2001). In rat's placenta, FAAH expression and activity levels increase throughout pregnancy, while AEA levels fall (Cella et al., 2008). Habayeb et al. reported that levels of AEA were 3-fold higher in a group of women who had miscarried compared to a live birth group (Habayeb et al., 2008). Previous findings also show that decreased levels of FAAH in peripheral lymphocytes were associated with miscarriage in women with uncomplicated pregnancies (Maccarrone et al., 2000). Furthermore, the same authors have reported that decreased FAAH and high AEA activity were associated with failure to achieve pregnancy after in vitro fertilization and embryo transfer (Maccarrone et al., 2002). Together, these observations suggest that low circulating levels of AEA are necessary for normal pregnancy progression (Maccarrone et al., 2002). It is yet to be determined whether changes in circulating cannabinoids reflect changes in reproductive tissues, but studies demonstrating the presence of FAAH in human placenta suggest that this enzyme can modulate AEA levels in the uterus, placenta, and fetus (Park et al., 2003).

The ECS also has an important regulatory role during the initial stages of embryo development, and modulation of endocannabinoid (eCB) signaling in early stages of neural development impacts neuronal proliferation, migration, and differentiation (reviewed in Wu et al., 2011; Grant et al., 2018; Bara et al., 2021). Most studies have focused on the effects of CBR activation, reporting changes in glutamatergic, GABAergic, dopaminergic, opioidergic and serotonergic signaling (reviewed in Higuera-Matas et al., 2015). Importantly, prenatal cannabinoid

exposure can impact synaptic plasticity and those deficits can lead to altered behavioral outcomes, such as impaired cognitive functions, (e.g., memory, learning, social interaction, and aggressive behavior), depressive and anxious symptoms and addiction risk (Bara et al., 2021). Since CBD interacts both directly and indirectly with the ECS, we can reasonably assume that prenatal modulation of eCB signaling through CBD exposure can lead to deficits in neural development that will later be reflected in behavioral outcomes. Moreover, as CBD has several other pharmacological targets, its actions on the developing CNS can be even more profound.

Despite the growing literature, large gaps remain regarding outcomes of prenatal CBD exposure. For pregnant women, the information available consists mostly of online discussions and social media posts, which often perpetuate inconsistent and inaccurate information. Even when women turn to health care providers in search for guidance, there is still not enough evidence available on the consequences of developmental CBD exposure, as most of the precautions against it are based on overall *Cannabis* exposure. Future investigations are necessary to explore a more translational approach to *in utero* CBD exposure (i.e., oral administration), as well as a broader range of CBD concentrations. Based on the increasing rates of *Cannabis* and CBD consumption during pregnancy and the data provided in this study, it is crucial to expand the knowledge on the consequences of prenatal CBD exposure.

3.0 GENERAL DISCUSSION

3.1 Findings

The experiments discussed in Chapter 2.0 used a rodent model of prenatal exposure to assess the effects of CBD on health and neurodevelopment. We showed that CBD exposure during pregnancy had no impact on maternal weight gain, litter size, or sex ratios among litters, but did decrease weight gain in the offspring. *In utero* CBD exposure also did not have an effect on offspring physical health, measured by the time of eye opening and incisor eruption. In addition to measurements of physical maturation, we also assessed the neurodevelopment of the pups through different developmental tests. The results showed that all pups were able to perform the tests and their performance got better as they got older, as expected. Interestingly, we saw moderate and large effect sizes of 10 mg/kg CBD on the first day the animals were assessed in the righting reflex and negative geotaxis tests, two tests that measure their reflexes, which can be an indication to a delay in neurodevelopment. However, contrary to our hypothesis, prenatal exposure to the two different doses of CBD did not significantly impact reflex, muscular and locomotor development.

3.2 Neurodevelopmental outcomes following prenatal exposure to drugs

It is widely known that consumption of other drugs during pregnancy can lead to negative pregnancy and fetal outcomes. Alcohol, for example, is a known teratogen: prenatal alcohol exposure has been associated with intrauterine growth restriction, fetal structural abnormalities (Dejong et al., 2019) and fetal alcohol syndrome (FAS), a form of intellectual disability that dates back centuries (Calhoun & Warren, 2007). There is a very clear dose-dependent phenotype that occurs following prenatal alcohol exposure (PAE). Maternal alcohol consumption is associated with low birth weight, preterm birth and small for gestational age (SGA) and the risks increase linearly in mothers who consume 1 or more drinks per day (Patra et al., 2011). Likewise, a high and sustained level of PAE is also associated with decreased birth weight and length and lower neurodevelopmental scores at 6 and 12 months of age (Bandoli et al., 2019). Interestingly, low to moderate PAE is still associated with certain deficits if the use is continued throughout pregnancy (Bandoli et al., 2019). PAE is also associated with negative neurodevelopmental outcomes: children that were exposed to alcohol *in utero* show greater psychological and behavioral problems, including psychopathology, attention deficits and impulsivity (Lees et al., 2020).

The timing of alcohol consumption can also impact the occurrence of negative outcomes. Consumption during the second and third trimesters is associated with a higher risk of preterm delivery, a risk that increased with higher levels of alcohol consumption (Ikehara et al., 2019). However, occasional to heavy consumption of alcohol during early pregnancy (first 15 weeks) was not associated with increased risks of reduced birth weight, SGA neonates, spontaneous preterm birth or preeclampsia (McCarthy et al., 2013).

Tobacco smoking is another example of negative developmental outcomes following drug consumption in pregnancy. Tobacco use during pregnancy has been associated with increased risks for ectopic pregnancy, spontaneous abortion, preterm birth, decreased birthweight and neurobehavioral and cognitive deficits (e.g., lower IQ scores, attention deficits, aggression, and sleep disorders) (reviewed in Rogers, 2009; Banderali et al., 2015; Havard et al., 2022). Maternal smoking is also associated with other negative infant outcomes, like reduced brain size, alterations in brain functions, overweight and obesity, hypertension, type 2 diabetes, and impaired lung function (reviewed in Rogers, 2009; Banderali et al., 2015). Similar to alcohol, the effects of tobacco smoke are also dose-dependent and time specific (Banderali et al., 2015).

Tobacco smoke contains thousands of substances including nicotine, a chemical with high misuse liability (Ginzel et al., 2007). Prenatal nicotine exposure is also associated with increased risks of poor infant outcomes, such as congenital malformations, alteration in lung development, auditory-cognitive deficits, and emotional and behavioral problems (reviewed in Ginzel et al., 2007). There is also evidence that exposure to nicotine *in utero* increases the risks of nicotine dependence in the offspring (reviewed in T. Crume, 2019).

3.3 Antiemetic medications during pregnancy

Nausea and vomiting in pregnancy affect almost 80% of women in the first trimester (Lacasse et al., 2009). These symptoms can have significant impacts on women's lives, from negatively impacting their work and relationships, to increasing risks of depression, hospitalizations, weight loss, high blood pressure, and economic burden (reviewed in Figueroa Gray et al., 2018; MacDuffie et al., 2020). Despite that, there is still little evidence on the safety of anti-nausea medications during pregnancy.

MacDuffie and colleagues (2020), in their recent review, mentioned two antiemetic medications that had to be removed from the market due to their teratogenic effects: Thalidomide,

which caused severe birth defects and premature deaths; and Bendectin (doxylamine and pyridoxine), also associated with birth defects. Currently, there is only one medication approved by Health Canada and the FDA to treat NVP: Dicletin (or Diglexis, in the USA), which is also a combination of doxylamine (an antihistamine) and pyridoxine (a form of vitamin B6), and a drug that is expensive and often not covered by insurance (Health Canada, 2016; Figueroa Gray et al., 2018). However, the most commonly used medication in the USA is Ondansetron, which is not FDA-approved and, in some cases, has been associated with fetal malformations (e.g., heart defects and cleft palate) (Anderka et al., 2012; Danielsson et al., 2014) while other studies show no risk of adverse fetal outcomes (Pasternak et al., 2013; Carstairs, 2016; Figueroa Gray et al., 2018; MacDuffie et al., 2020).

Overall, there is a critical lack of evidence on the safety of anti-nausea drug use during pregnancy, which leaves women with NVP to face a dilemma when deciding whether to use medication or not. Indeed, Figueroa Gray et al. (2018) reported that women are generally careful about taking prescription medication during pregnancy, weighing the benefits *versus* the possible risks to the child's health. However, some women reported that the impact that nausea had on their lives (i.e., the inability to function socially and professionally and the uncertainty of when nausea and vomiting would occur) was too disruptive, and the treatment benefits outweighed possible risks. It is clear that NVP is a serious concern and women that suffer from it require symptom relief in some form, often preferring a more "natural" approach. Moreover, given the lack of evidence on the safety of antiemetic drugs and the small number of drugs available for this purpose, it is understandable the choice that some women are making to use CBD to treat these symptoms. However, the evidence available for the medications that are currently on the market is still superior than the literature on CBD.

3.4 CBD's pharmacological targets and potential impacts in pregnancy

As mentioned earlier, CBD has a plethora of pharmacological targets, one of them being the serotonin receptor. Russo and colleagues (2005) demonstrated in a series of *in vitro* experiments that CBD acts as an agonist at the 5-HT1a receptor and may also have actions at the rat 5-HT2a receptor. In human brain tissue, CBD also displayed affinity to 5-HT1a receptors in the hippocampus and neocortex at high concentrations (10 μm) (Martínez-Aguirre et al., 2020).

Although it is not yet clear if CBD has the same action *in vivo*, it is worthy investigating the possibility that cannabidiol might impact pregnancy via the serotoninergic system.

Different studies have shown that 5-HT signalling is directly involved in placental development and a disruption in this system can have negative impacts in placental and fetal development (reviewed in Rosenfeld, 2020). Disruptions in placental 5-HT signalling can also damage normal placental morphology and caused neurobehavioral disorders (reviewed in Rosenfeld, 2020). Additionally, 5-HT is also closely involved in a variety of neurodevelopmental processes, such as neuronal proliferation and migration (Gaspar et al., 2003; Hanswijk et al., 2020). Some author also raise the possibility that early changes in the homeostasis of 5-HT signalling can be involved in the pathophysiology of psychiatric disorders (reviewed in Gaspar et al., 2003). The 5-HT1a receptor specifically is expressed early in rat embryos (Hillion et al., 1904) and is involved in functional changes that occur in the hippocampus during development: in an earlier stage, it causes proliferation of cells; and, at a later stage, it increases the level of synaptic activity (Mehta et al., 2007). It is still unclear how each 5-HT receptor influences brain development, but changes in their activation pattern in critical stages of development can impact the formation of certain neural connections (Gaspar et al., 2003).

CBD also binds to and activates the TRPV1 receptor (Bisogno et al., 2001; de Petrocellis et al., 2011). Activation of TRPV1 channels during a critical stage of fetal development produced heart and craniofacial defects similar that are similar to those seen in first-trimester maternal fever (Hutson et al., 2017). A recent study has shown that exposure to a general anesthetic (sevoflurane) on PND7 mice increased the expression of TRPV1, reduced synaptic density in the hippocampus and led to learning and memory deficits later in life, an effect likely mediated by this receptor (Liu et al., 2021). Lastly, exposure to capsaicin (an agonist of the TRPV1 channel) during the neonatal period reduced brain weight of adult male rats and caused a reduction in several cross-sectional brain areas, as well as thinner cortices and a smaller hippocampal area, neuroanatomical changes that persisted into adulthood (P. Newson et al., 2005; P. N. Newson et al., 2014).

The 5-HT1a and TRPV1 receptors are only two examples of mechanisms by which CBD might affect placental and fetal development and lead to negative consequences later in life. Cannabidiol can be considered a promiscuous molecule and it is likely that its effects are caused by various modulations that, together, determine pregnancy and infant outcome. Therefore, we cannot yet determine the specificity of CBD's actions in a molecular level.

3.5 CBD market

Due to the recent changes around the legalization of *Cannabis* in several countries, CBD products have also become increasingly popular worldwide. Another important factor that aided in the popularization of CBD products was the passage of the Agricultural Improvement Act of 2018 (Farm Bill 2018) in the USA, which legalized products derived from hemp, i.e., the *Cannabis* plant or any parts of the plant with a THC concentration of no more than 0.3% of dry weight (Agriculture Improvement Act of 2018). These changes have made access to CBD far easier for the general public in a variety of unrestricted forms.

In 2018, CBD was the top-selling and fastest-growing herbal supplement in natural- and health-foods sales channels in the United States (Smith et al., 2019). Sales of CBD products in those channels passed US$52 million, a 332.8% increase from 2017 (Smith et al., 2019). In 2019, the CBD industry in the USA reached over US$5 billion, a 706% increase from 2018 (Brightfield Group, 2019). In the United Kingdom (UK), the CBD market in 2019 was estimated to be around £300 million and expected to reach almost £1 billion by 2025 (Gibbs et al., 2019). By 2026, the global CBD market is forecasted to reach US$17 billion (Fior Markets, 2019).

Nowadays, CBD is available either online or over the counter in many countries and in several forms, including oils and tinctures, capsules, crystals, lotions, vaporizers, beverages, bath salts, and even pet products (McGregor et al., 2020; Rubin, 2019). Although some regulatory agencies have directives regarding the marketing and sale of CBD products, there remains considerable confusion on the legal status of said products, which are widely available online and in retail stores. That confusion stems from the fact that these products currently exist in a grey area, not being considered as drugs nor as dietary supplements. While some countries prevent the sale of non-prescription CBD (e.g., Australia and New Zealand), other countries suffer from conflicting guidelines from different regulatory agencies (e.g., USA and the UK) (McGregor et al., 2020). Canada and Switzerland, on the other hand, have clear regulations regarding the access to non-prescription CBD products (McGregor et al., 2020). Interestingly, even though Canada is considerably permissive in allowing legal access to a wide range of non-medical *Cannabis* products, it has the highest quality control requirements for non-prescription CBD products (McGregor et al., 2020).

As of 2018, there is one prescription CBD product on the market (Epidiolex™) that has been approved by the FDA and the European Medicines Agency (EMA) for the treatment of specific types of seizures (McGregor et al., 2020). Additionally, Sativex™, a 1:1 THC:CBD buccal spray, is available for the treatment of spasticity in Multiple Sclerosis in many countries (McGregor et al., 2020). In Canada, there are currently two *Cannabis*-containing medications that are approved and available on the market: Sativex™ and Cesamet™ (Nabilone), which is a synthetic analogue of THC used for the treatment of nausea and vomiting (Health Canada, 2022). Health Canada also defines CBD preparations as those where: (1) CBD comprises 98% or more of the total cannabinoid content; (2) any other cannabinoids present must be naturally found in *Cannabis* and equal no more than 2%; and (3) the THC content must be lower than 1% of the total cannabinoid content (Health Canada, 2022) However, most CBD products available continue to be unregulated. Because of the lack of regulation, consumer's safety might be at risk due to inaccurate labelling of CBD products and contamination with other cannabinoids, residual solvents, and heavy metals (Bonn-Miller et al., 2017; Gardener et al., 2022; Gurley et al., 2020). Taken together, the confusion around the legality of CBD products, the lack of legal enforcement and the constantly growing consumer demand provides an ideal scenario for entrepreneurs and companies distributing these products.

3.6 CBD and the labelling inaccuracy

Despite the increase in the quantity of CBD products readily available, research shows that there is a common discrepancy in the labels of CBD-containing products and its actual contents. The FDA, over the past few years, has issued warning letters to several business regarding products that did not contain the levels of CBD they claimed to contain (U.S. Food & Drug Administration, 2022). In a study of 84 CBD products sold online, 42% contained more CBD than labeled, 26% contained less CBD, and THC was detected in 21% of products (Bonn-Miller et al., 2017). Another study of 80 CBD products sold both locally and online in the USA found that 31% of products were under-labelled and 15% were over-labelled (Johnson et al., 2022b). Finally, a study of CBD products sold in Mississippi showed that only 2 out of 20 products were within ±20% of the label claim (Gurley et al., 2020). More alarmingly, 3 products had levels of THC that exceeded 0.3%, with one product containing 45% THC, and 4 products were adulterated with synthetic cannabinoids (Gurley et al., 2020). A recent case brought attention to the gravity of the

contamination of CBD products: an eight-year-old boy, consuming CBD oil (purchased online) as a treatment for its seizure disorder, was hospitalized after 9 days of treatment due to contamination of the product with the synthetic cannabinoid AB-FUBINACA, which was consistent with the patient's symptoms (i.e., several tonic-clonic seizures, intermittent agitation, delirium, depressed mental status, tachycardia, and mydriasis) (Rianprakaisang et al., 2020).

The inconsistency between the label and the actual CBD content is not limited to the USA. In a study performed in the Netherlands, only 5 out of 16 CBD oil products contained an amount of CBD within 10% of the labelled amount (Hazekamp, 2018). Another study conducted in Italy looked at 14 commercially available CBD oils (purchased online from different European regions) and reported that 9 products had a CBD content that varied more than 10% from the label (Pavlovic et al., 2018).

Another concern is consumers being unknowingly exposed to THC when taking unregulated CBD products. The legal limit of THC allowed in CBD products varies among countries, ranging from 0% to 1% (reviewed in Dunn et al., 2021). The World Health Organization (WHO) recommend that CBD preparations that contain less than 0.2% THC are not under international drug control (ECDD, 2019). A recent study analyzed 80 CBD oil-products and showed that THC was present in 64% of the products, with concentrations ranging from 0.008 mg/mL to 2.071 mg/mL (Johnson et al., 2022a). Another study that analyzed 293 CBD products available in the German market from 2018 to 2021 showed that 10% had THC levels above the LOAEL (lowest observed adverse effect level of 2.5 mg THC/day) and 45% of samples had THC levels above the acute reference dose of 1 μg THC per kg of body weight, being classified as unsuitable for human consumption (Lachenmeier et al., 2020). It's also important to note that most people consume CBD products daily, and repeated dosing can increase the exposure to THC due to drug accumulation.

Finally, people often choose to consume CBD products without medical guidance, and it is therefore imperative that companies accurately label their products regarding CBD content so that consumers, especially vulnerable ones such as pregnant women, can be accurately informed about the doses of CBD they are consuming. This reinforces the need for a stricter regulation of CBD products, as well as the development of better manufacturing skills and testing standards.

3.7 Final considerations

Overall, the changes seen in this study are consistent with the limited evidence available. Two animal studies (Iezzi, Caceres-Rodriguez, et al., 2022; Kanyo et al., 2021) have shown a reduction in locomotor activity caused by prenatal CBD exposure. Similarly, we saw a significant increase in the latency to reach the nest quadrant in the last day of the homing behavior test, which can be indicative of locomotion deficits. There was also a delay in the pup's development of reflexes (i.e., righting reflex and negative geotaxis), an effect that resolved with time. Iezzi et al. (2022) have also shown alterations in offspring body weight following CBD exposure *in utero*, a result that was also seen in this project.

The model used has some limitations that are important to note. First, CBD exposure was limited to a specific window (GD6-20) that might not reflect the window of consumption in humans. Studies that investigated *Cannabis* consumption during pregnancy usually report that women who consume *Cannabis* while pregnant also consumed it during the preconception period (Ko et al., 2015; Volkow et al., 2019; Bayrampour & Asim, 2021; Skelton et al., 2021). Secondly, the method of administration used was i.p. injection, which is not the most translational since the main method of CBD consumption in humans is oral. Oral consumption of CBD, when compared to i.p. injections, leads to lower plasma levels of the cannabinoid and its metabolites (Deiana et al., 2012). Different methods of administration can therefore cause different CBD pharmacokinetic profiles, which can in turn lead to different effects. Lastly, this thesis was limited to investigating only physical and behavioral changes that occur during infancy (PND3-21) and did not look into alterations that might occur later in life (e.g., memory and learning impairments) that have been associated with prenatal *Cannabis* exposure (Richardson et al., 2002; reviewed in Grant et al., 2018; Corsi et al., 2020).

Besides that, the model chosen also has important strengths: we have shown in our lab that the method used for CBD administration generates plasma levels that are similar to those seen in human. In addition, the neurobehavioral tests used are good indicators of neurodevelopment in the rat that are widely used in the literature. They provide valuable information regarding the development of neural networks and are sensitive to insults that can disrupt brain development (Nguyen et al., 2017). The testing of early neurodevelopmental markers is also the only behavioral testing available for newborn rodents, since more complex tests are not yet feasible during the period investigated. Although the tests used do not directly translate to human behaviors, they

parallel the reflex testing that is conducted in human newborns and can be predictive of neurodevelopmental disorders in rodent models of diseases such as cerebral palsy and autism (Feather-Schussler & Ferguson, 2016; Nguyen et al., 2017; Ruhela et al., 2019). Moreover, several measures were taken to control for possible confounding factors, such as culling of litters, consistency in handling and time of testing, and blinding of the experimenter to the treatments.

Future research following on this work should look at a more translational method of administration, such as oral gavage. It would also be valuable to explore a wider range of CBD concentrations (lower and higher doses, such as 1 mg/kg and 30 mg/kg) to investigate the occurrence of a biphasic response to CBD. Studies show that low *versus* high doses of CBD can cause opposite effects. For example, lower doses of CBD increase wakefulness and higher doses having a sedative effect (Chagas et al., 2013). Parker and colleagues (2004) showed that low doses of CBD (5 mg/kg and 10 mg/kg) suppressed lithium-induced vomiting in shrews, while high doses (25 mg/kg and 40 mg/kg) enhanced vomiting. The biphasic effects of CBD have also been demonstrated using other measures: while a 15 mg/kg dose of CBD impaired the sexual behavior of male mice, a 30 mg/kg dose improved sexual performance (Carvalho et al., 2018). Future research investigating the molecular changes caused by prenatal CBD are also warranted, given the wide range of pharmacological targets that CBD has.

3.8 Conclusion

This thesis has demonstrated that *in utero* CBD exposure can alter offspring body weight and specific neurologic reflexes in early stages of development. Moving forward, it is critical to continue investigating the impacts of prenatal CBD exposure to maternal and infant health. With the growth of the literature on this subject, women will have access to more information and be able to make educated decisions during pregnancy.

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