Notice of Special Interest (NOSIO):

Effects of cannabis use and cannabinoids on the developing brain

Notice number: NOT-DA-20-039

Release: March 5, 2020

Expiration: September 8, 2023

Mechanism: PA-19-054, NIH Exploratory/Developmental Research Grant Program (Parent R21 Clinical trials not allowed)

Outline of notice (in bold: items that are specifically addressed by the Irit Lab proposal)

1. **Cannabis use on the rise**
	1. Reduced perceived risk
	2. Relaxed laws
2. **THC and CBD have been suggested as treatments for various health conditions**
3. Question: What are the adverse effects on the **developing brain?**
4. **Growing evidence implicated cannabis and cannabinoids in adverse psychiatric, cognitive, and behavioral consequences in children.**
5. *In utero* exposure may impact fetal brain development
	1. Neurological impairments
	2. Abnormal DA transmission
	3. Hyperactivity
	4. Poor cognitive function
6. In animal models – early development
	1. Developmental disorders
	2. Dysregulated repressive epigenetic markers that affect brain morphogenesis
	3. Neuronal and glial alteration
	4. Impaired axons pathfinding
	5. Impaired synaptic plasticity
7. **In animal models – developmental exposure and the adult**
	1. Increased impulsivity
	2. Anxiety
	3. Abnormal fear extinction
	4. Altered reward sensitivity
8. **More research is needed on the effects of cannabis at various stages during development**
	1. Replicate findings – will this proposal attempt to replicate what is already known to demonstrate credibility?
	2. **Extend findings**
	3. **Cellular and molecular mechanisms of endo and exogenous cannabinoid exposure**
	4. **\*\*\*\*\*Mechanisms by which cannabinoids affect development**

Objectives of the program:

1. **non-adult testing (fetal, neonatal, childhood, adolescent)**
2. how and when endocannabinoid system emerges
3. roles of the endocannabinoid system during development
4. **impact of endocannabinoid system on cognition and behavior**
5. timing of vulnerability to cannabis
6. effect of endocannabinoids on proliferation, migration, differentiation, axonal pathfinding, synapse formation/plasticity
7. how other substances of abuse interact with cannabis
8. **whether and how cannabis affects sex and gender specifications during development**

Submit using the **PA-19-053** mechanism – Department of Health and Human Services

NIH Exploratory/Developmental Research Grant Program (Parent R21 Clinical Trial Not Allowed)

Activity code: [R21](https://grants.nih.gov/grants/funding/ac_search_results.htm?text_curr=r21&Search.x=0&Search.y=0&Search_Type=Activity) Exploratory/Developmental Research Grant

**Due** July 16/Nov 16?

<https://grants.nih.gov/grants/how-to-apply-application-guide/due-dates-and-submission-policies/due-dates.htm>



**Page limits:**

Project Summary/Abstract 30 lines

Project Narrative 3 sentences

Specific Aims 1 page

Research strategy 6 pages

**Scoring based on:**

1. **Significance**

Does the project address an important problem or a critical barrier to progress in the field? Is the prior research that serves as the key support for the proposed project rigorous? If the aims of the project are achieved, how will scientific knowledge, technical capability, and/or clinical practice be improved? How will successful completion of the aims change the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field?



1. **Investigator(s)**

Are the PD(s)/PI(s), collaborators, and other researchers well suited to the project? If Early Stage Investigators or those in the early stages of independent careers, do they have appropriate experience and training? If established, have they demonstrated an ongoing record of accomplishments that have advanced their field(s)? If the project is collaborative or multi-PD/PI, do the investigators have complementary and integrated expertise; are their leadership approach, governance and organizational structure appropriate for the project?

1. **Innovation**

Does the application challenge and seek to shift current research or clinical practice paradigms by utilizing novel theoretical concepts, approaches or methodologies, instrumentation, or interventions? Are the concepts, approaches or methodologies, instrumentation, or interventions novel to one field of research or novel in a broad sense? Is a refinement, improvement, or new application of theoretical concepts, approaches or methodologies, instrumentation, or interventions proposed?



1. **Approach**

Are the overall strategy, methodology, and analyses well-reasoned and appropriate to accomplish the specific aims of the project? Have the investigators included plans to address weaknesses in the rigor of prior research that serves as the key support for the proposed project? Have the investigators presented strategies to ensure a robust and unbiased approach, as appropriate for the work proposed? Are potential problems, alternative strategies, and benchmarks for success presented? If the project is in the early stages of development, will the strategy establish feasibility and will particularly risky aspects be managed? Have the investigators presented adequate plans to address relevant biological variables, such as sex, for studies in vertebrate animals or human subjects?

If the project involves human subjects and/or NIH-defined clinical research, are the plans to address

1) the protection of human subjects from research risks, and

2) inclusion (or exclusion) of individuals on the basis of sex/gender, race, and ethnicity, as well as the inclusion or exclusion of individuals of all ages (including children and older adults), justified in terms of the scientific goals and research strategy proposed?



1. **Environment**

Will the scientific environment in which the work will be done contribute to the probability of success? Are the institutional support, equipment and other physical resources available to the investigators adequate for the project proposed? Will the project benefit from unique features of the scientific environment, subject populations, or collaborative arrangements?

**Must follow SF424(R&R) instructions:**

<https://grants.nih.gov/grants/how-to-apply-application-guide.html>

<https://grants.nih.gov/grants/how-to-apply-application-guide/forms-h/research-forms-h.pdf>

**Background**

**Overview.** More than 25% of people in North America report using cannabis (*Cannabis sativa*) for medicinal purposes despite limited evidence to support its [safety](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7052834/). [Recent research](https://link.springer.com/article/10.1007/s11916-020-00872-w) highlights the widespread nature of this use that includes pregnant women. Despite a warning from the [US Food & Drug Administration](https://www.fda.gov/consumers/consumer-updates/what-you-should-know-about-using-cannabis-including-cbd-when-pregnant-or-breastfeeding) strongly advising against the use by pregnant women of cannabinoids present in cannabis, use continues for an array of pregnancy-related symptoms including nausea, insomnia, anxiety, and chronic pain [[15](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9722662/#CR15)]. In light of the role of endogenously expressed cannabinoids in neurodevelopment and evidence suggesting [risks](https://jamanetwork.com/journals/jamanetworkopen/fullarticle/2788451) associated with cannabinoid exposure *in utero*, there is a critical need to understand the possible effects of CBD on fetal neurodevelopment and the mechanisms that mediate them. Our preliminary data support a novel mechanism of neurodevelopmental regulation and challenge [current attitudes](https://pubmed.ncbi.nlm.nih.gov/28843740/) toward cannabinoids that are believed to be are universally therapeutic and safe.

**Therapeutic potential of cannabis.** Cannabidiol (CBD), the primary non-psychoactive compound found in cannabis, has been [recognized](https://onlinelibrary.wiley.com/doi/full/10.1111/bcpt.13710#:~:text=Clinical%20studies%20reveal%20that%20CBD,cancer%20treatment%20(Figure%202).) for its potent [biological properties and therapeutic potential](https://onlinelibrary.wiley.com/doi/10.1111/bcpt.13710). In rodents, for example, exposure to CBD in adulthood reduces immobility and increase swimming time in the forced swim test in a model of clinical depression [[2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR2), [3](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR3) Bright and Akirav., 2023], increases time spent in the open arm of the elevated plus maze, a model of induced anxiety [[4](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR4)–[7](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR7)], and reduces responsiveness to drugs of addiction such as morphine and cocaine [[4](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR4), [8](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR8)]. In humans, CBD reduces psychotic symptoms in schizophrenia [[9](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR9), [10](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR10)] and lowers subjective measures of anxiety [[11](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR11), [12](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7789000/#CR12)]. CBD is, therefore, a strong candidate for treating diverse psychiatric conditions.

**Mechanisms mediating the effects of CBD.** The mechanisms mediating the biological effects of CBD are thought to rely on binding to [endogenous cannabinoid receptors](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5877694/). However, CBD binds to cannabinoid 1 and 2 (CB1, CB2) receptors with relatively low affinity [[2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7917759/#B2-ijms-22-01863)]. These constitutively active G protein-coupled receptors (GPCRs) are abundantly expressed in the brain and spinal cord [as early as 14 weeks gestation](https://reproductive-health-journal.biomedcentral.com/articles/10.1186/s12978-020-0880-9#:~:text=Cannabinoid%20receptors%20are%20present%20in,can%20be%20excreted%20into%20breastmilk.). Binding of CBD to cannabinoid receptors [inhibits activation](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9627301/) of several downstream signaling pathways and ion channels involved in synaptic transmission. However, additional interactions are being identified including with orphan [GPCRs and ion receptors](https://books.google.com/books?hl=en&lr=&id=djRHDgAAQBAJ&oi=fnd&pg=PP1&ots=QYL6XRy0VA&sig=WpykYxRxwlyKG-K4_zEuUw9g1Zs#v=onepage&q&f=false). [Recent evidence](https://pubmed.ncbi.nlm.nih.gov/32348868/) suggests that CBD also influences epigenetic mechanisms including methylation patterns that are responsible for its antidepressant effects in adults. Indeed, CBD administration altered [expression of microRNAs](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9953518/) in the prefrontal cortex and upregulated serotonergic receptors in a model of clinical depression. These data open the possibility of reversible epigenetic regulation as an explanation for its possible therapeutic in adults.

**The role of epigenetics in mental health.** The pathogenic role of microRNAs (miRNAs) has been implicated in the development and progression of various neuropsychiatric conditions [25, [21](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9326663/#B21-ncrna-08-00055),[55](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9326663/#B55-ncrna-08-00055),[56](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9326663/#B56-ncrna-08-00055)]. Altered levels of specific miRNAs have been observed in patients with psychiatric disorders, including major depressive disorder (Penner-Goeke and Binder, 2019, [103](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7917759/#B103-ijms-22-01863),[104](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7917759/#B104-ijms-22-01863),[105](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7917759/#B105-ijms-22-01863)]). Moreover, altered levels of miRNAs were found in postmortem brain and peripheral tissues in patients after treatment with antidepressant medication (Lopez et al., 2018), highlighting the potential of miRNAs as biomarkers for treatment response (Penner-Goeke and Binder, 2019).

Dysregulated inflammation contributes to the pathogenesis of psychiatric and neurological disorders ([Ballaz and Bourin, 2023](https://pubmed.ncbi.nlm.nih.gov/36949322/); [Beurel et al., 2020](https://pubmed.ncbi.nlm.nih.gov/32553197/)). The [off-target anti-inflammatory properties](https://pubmed.ncbi.nlm.nih.gov/10706993/#:~:text=It%20is%20concluded%20that%20(i,the%20serum%20in%20TRS%20patients.) of some psychiatric medications may play a role in their effectiveness in disorders like schizophrenia and major depression. Data are largely limited to exposure in adults but convincingly portray a role for CBD in the modulation of inflammatory signaling. CBD decreases the level of inflammatory cytokines such as interleukin (IL)-1β, IL-6, and TNF-α under systemic inflammatory responses while significantly improving performance in behavioral tasks ([Trivedi et al., 2022](https://pubmed.ncbi.nlm.nih.gov/34997430/)). Mice receiving CBD for seven days with human Aβ peptide (1–42), decreased the expression of the glial pro-inflammatory cytokine IL-1β and inducible nitric oxide synthase (iNOS) ([Esposito et al. 2007](https://pubmed.ncbi.nlm.nih.gov/17592514/)). CBD also inhibited the mRNA expression of the glial fibrillary acidic protein (GFAP), a marker of activated astrocytes, in a dose-dependent manner and diminished the expression of proinflammatory miRNAs associated with Toll-like receptor and NF-kB signaling, which is elevated by lipopolysaccharide (LPS) ([Juknat et al., 2019](https://pubmed.ncbi.nlm.nih.gov/30742662/)). Despite strong evidence to supporting a role for CBD in modulation of inflammation, very few studies have examined these effects on the developing fetus.

**CBD exposure during pregnancy.** CBD crosses the placenta and alters its very structure, significantly impacting pregnancy outcomes [[16](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9722662/#CR16)–[18](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9722662/#CR18)]. A limited number of studies have examined the long-term developmental effects on fetuses exposed to CBD during pregnancy. In animal models, CBD treatment exerts sex-specific cognitive alterations in early life, which may be predictive of the risk of developing various neuro-psychiatric and developmental disorders (Lezzi et al., 2022). A recent study showed that extended exposure of CD1 mice to CBD spanning from gestation through the first week after birth alters repetitive and hedonic behaviors in the adult progeny [[19](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9722662/#CR19)]. In another study, CBD exposure during gestation and lactation induced sex-specific changes in working spatial memory and anxiety behavior as well as genome-wide changes in brain DNA methylation in adult mouse offspring ([Wanner et al., 2021](https://clinicalepigeneticsjournal.biomedcentral.com/articles/10.1186/s13148-020-00993-4)), suggesting both modified brain’s epigenome and behavioral outcomes following developmental CBD exposure.

**MicroRNA-mediated effects of CBD.** miRNAs affected by CBD are linked to inflammatory pathways, cell cycle arrest, and Nrf2-mediated cellular stress (65). CBD downregulated specific microRNA, including miR-31, miR-127, miR-155, and miR-223, that target genes important for the induction of T regulatory cells and inhibition of Th17 cells (Elliott et al. 2015). CBD was also found to regulate specific miRNAs using lipopolysaccharide (LPS) to stimulate BV-2 microglial cells; microglia cells have been implicated in several psychiatric disorders (65). In this study [[65](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7917759/#B65-ijms-22-01863)], pretreatment with CBD (10 μM) affected the expression of miR-146a and miR-155 and enhanced the expression levels of miR-34a and miR-449a, both of which have been implicated in anxiety-related traits. miR-34a is increased in the cerebrospinal fluid (CSF) and serum of individuals with major depressive disorder [[108](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7917759/#B108-ijms-22-01863)] and targets the bipolar risk genes ankyrin-3 and voltage-dependent L-type calcium channel subunit beta-3 [[109](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7917759/#B109-ijms-22-01863)]. Developmental CBD exposure in mice has been associated with widespread changes in the brain methylome providing an epigenetic cause to its protracted effects on anxiety and memory behavior [[20](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9722662/#CR20)].

Several studies have confirmed that some miRNAs are involved in the regulation of inflammation, especially miR-21, miR-146a, and miR-155, which has been to be key in many immune and inflammatory pathways. Additionally, studies have shown that inflamma-miRs are dysregulated in neurons and have an important impact on cognitive function ([Quinn and O’Neill, 2011](https://pubmed.ncbi.nlm.nih.gov/21652514/)). Additional studies have linked miRNAs (e.g miR-126 and miR-132) to the neuroinflammatory signaling, including NF-κB signaling (Amjad et al., 2019), TLR signaling pathway (Paschon et al., 2016), B cell receptor signaling (Borbet et al., 2021), and Jak/Stat signaling ([Zhang](Dexmedetomidine%20protects%20against%20renal%20ischemia%20and%20reperfusion%20injury%20by%20inhibiting%20the%20JAK/STAT%20signaling%20activation.) et al., 2013).

**We aim to study the complex interplay between prenatal CBD exposure (PCE) and possible links to greater risks of cognitive decline, anxiety- and depression-related disorders later in life, and whether these effects are mediated via epigenetic mechanisms** **that modulate neuroinflammation**. To that end, we will examine the effects of PCE on sex-specific emotional and cognitive behaviors in later stages of life and the effects of PCE on the expression of miRNAs and neuroinflammation in the hippocampal-prefrontal (HPC-PFC) circuit. The HPC-PFC circuit plays a fundamental role in executive and emotional functions. Disruptions in HPC-PFC functional connectivity can contribute to neuropsychiatric symptoms observed in mental illnesses and neurological conditions, such as dementia, depression, and anxiety disorders (Kovner et al., 2019; Ruggiero et al., 2021). We will correlate the alterations in the expression of miRNAs and neuroinflammatory (NI) markers with the behavioral phenotype and mimic or inhibit specific miRNAs to study whether epigenetic mechanisms mediate protracted PCE-induced alterations in behavior. miRNAs are critically involved in the development and progression of various neuropsychiatric conditions [25, [21](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9326663/#B21-ncrna-08-00055),[55](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9326663/#B55-ncrna-08-00055),[56](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9326663/#B56-ncrna-08-00055)], yet many miRNAs appear to play beneficial rather than pathologic roles in settings of disease (Mendell and Olson, 2012). As such, the activation or silencing of specific miRNAs may be ideally suited to restore PCE-induced alterations in cognitive and emotional function.

**Preliminary supporting data.**  We have established a model of clinical depression in rats and obtained preliminary data to support molecular changes induced by administration of CBD. Our preliminary data demonstrate *1) CBD exerts antidepressant effects in an adult rodent model of unpredictable chronic mild stress (UCMS), 2) CBD modulates miRNAs expression that correlates with the behavioral phenotype observed with UCMS, 3) CBD induces alterations in cytokine expression that appear to be neuroprotective, and 4) neuroinflammatory cytokines are regulated by CBD.*

Antidepressant effects of CBD. In a recent study (Bright and Akirav., 2023), we studied the antidepressant effects of CBD in a rat model for depression: UCMS. We first validated the behavioral phenotype induced by UCMS. When evaluated in the forced swim test, rats exposed to UCMS exhibited significantly increased immobility. Exposure to CBD prevented this behavior response.

Expression of microRNAs with CBD. We examined the effects of UCMS and CBD on the expression of miR-16, miR-135 and miR-124, associated with resilience to stress and depression [18,26-30]. CBD restored UCMS-induced upregulation in miR-16 and miR-135 in the PFC as well as the increase in immobility time, with no effect on miR-124 (**Figure 1**). We found that the antidepressant effects of CBD in a rat model for depression that are associated with alterations with these miRNAs in the PFC, are mediated by the 5HT1a receptor ([Bright and Akirav., 2023](https://pubmed.ncbi.nlm.nih.gov/36768376/)). By affecting miRNA expression, and possibly other epigenetic mechanisms, CBD could modulate the stress response and exert anxiolytic effects.



Neuroinflammatory cytokine expression in UCMS. We also have preliminary data demonstrating alterations in neuroinflammation following exposure to shock and reminders, a rat model for PTSD, and following UCMS. Exposure to UCMS increased the expression of TNF-α in the CA1 and ventral subiculum (VS) and this increase was restored by chronic CBD administration to adult males (**Figure 2a, b**).Following exposure to shock and reminders model of PTSD, rats demonstrated increased IL-1β in the PFC (**Figure 2c**). Exposure to cannabis during the prenatal period could have long-term effects on the normal trajectory of cellular processing and neurocircuitry critical for forming behavior, representing a risk factor for the onset of neurodevelopmental and neuropsychiatric disorders [[6](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9722662/#CR6)].

We have successfully established the PCE model in our laboratory and have generated preliminary data demonstrating the impaired performance of **PCE male and female rats** in $$$ tasks? Preliminary from Shira during early August.

Figure3-SHIRA- preliminary findings with the PCE model, early-mid august

Significance

**Scoring:**

* State problem or critical barrier to progress that the proposal addresses
* Strengths and weaknesses of the rigor of the previous research
* How the findings of the proposal will advance scientific, technical, and/or clinical practice

**Significance.** The recent widespread promotion and public acceptance of CBD as a “safe” and “natural” medication, including use during pregnancy, has encouraged pregnant or nursing mothers to use CBD as a treatment for a variety of symptoms, thereby exposing the fetus to CBD. Importantly, few studies have addressed the significant and timely question of how CBD impacts the developing fetal brain.[39](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9225394/%22%20%5Cl%20%22B39),[40](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9225394/#B40)The present study will contribute new insights into the long-term consequences of PCE on emotional and cognitive function in males and females and through the delivery of miRNA inhibitors or mimics, this may suggest an epigenetic mechanism mediating the effects of PCE on behavior. Moreover, the identification of mRNA targets (e.g., NI markers) that mediate the actions of miRNAs in PCE-induced disease can provide opportunities for intervening in disease processes.

Therapeutic relevance. miRNA inhibition or delivery may provide a highly potent means to modulate a disease process. As such, the activation or silencing of specific miRNAs may be ideally suited to restore PCE-induced alterations in cognitive and emotional function. Our planned experiments will offer insight into the potential therapeutic utility of the targeted activation or silencing of specific miRNAs as an approach to restoring memory and alleviating emotional deficits, while also better defining the role that miRNAs play in the context of PCE in both males and females. Revealing cognitive impairment and emotional dysfunction associated with fetal CBD will challenge the view that CBD is a universally safe compound and will encourage further study of the developmental consequences of prenatal CBD, its underlying mechanisms and potential treatment.

Innovation

**Scoring**:

* Study aims to shift current practice paradigms
* Methodologies proposed are new
* Theoretical approaches are novel

**Innovation.** These results will yield information about long-term, selective, sex-dependent impacts on emotional and cognitive function and measure specific miRNAs in the brain that are altered in rats subjected to PCE. Our work has the potential to clarify the pathophysiological mechanisms of PCE and support the development of interventional strategies in the long-term, with further studies of the clinical manipulation of miRNA activity progresses.

A novel approach to addressing psychiatric disorders. A drug that acts on a single pathway is unlikely to diminish the complex pathological cascade that ultimately leads to a neuropsychiatric disorder. miRNAs regulate a diverse array of processes related with cognitive and emotional function and may thus offer wider-ranging benefits than the available treatments. miRNAs are interesting therapeutic targets given their ability to regulate endogenous gene expression such that one miRNA can potentially regulate entire biological pathways. As such, miRNA-based therapeutic strategies may be ideally suited to PCE-induced dysfunction. These therapeutic effects may be achieved by completely or partially ablating the functions of miRNAs of interest to downregulate the expression of targeted genes and proteins involved in disease pathogenesis.

Sex-specific clinical approaches. Identifying specific markers in males and females can guide the development of personalized, sex-specific medicine. While the miRNA therapeutic strategies employed in our animal models are too invasive for human implementation, this work will provide an invaluable and unprecedented framework for treatment by identifying specific miRNAs that can be delivered through a safer route. Understanding the mechanism of action for a given drug in greater detail has the potential to support further pharmacological development efforts and to mitigate the risk of failed clinical trials by stratifying patients to focus on subpopulations most likely to respond to such treatment.

**Research Design and Methods**

**Aim 1: To identify PCE-related patterns of miRNA and neuroinflammation dysregulation in the HPC-PFC that are associated with PCE-induced cognitive and emotional dysfunctional phenotype**

Aim 1 Rationale. The consumption of CBD during pregnancy is increasing, but the developmental consequences are still largely unknown. To address this knowledge gap and to complete our preliminary studies, we aim is to elucidate the long-term effects of PCE on cognitive and emotional phenotype in adulthood and to examine whether these alterations are associated with alterations with specific miRNAs and NI markers. To that end, in our first experiment, dams will be injected with CBD (3 or 10 mg/kg) or vehicle during days 5-18. Male and female pups will be tested on a battery of cognitive and emotional tests after which alterations in the expression of miRNAs and NI in the HPC-PFC pathway, will be assessed. We hypothesizethat PCE will have long-term effects on the behavioral phenotype, affecting cognitive and emotional behavior and will alter the expression of at least some of the miRNAs and NI markers in the HPC-PFC pathway.

Aim 1 Experimental Design. The presence of a vaginal plug will be defined as day 0 of gestation (GD0). Females will be individually housed beginning on GD0. From GD5 to GD18, dams will be injected subcutaneously (s.c.) daily with vehicle or CBD (3 or 10 mg/kg). We chose this period because CBD administered to the nursing dam may alter maternal behavior and affect pup development and later behaviors. This lower dose of CBD reaches the embryonic brain and causes behavioral changes in the offspring (Maciel et al., 2021). For each litter, the date of birth is designated as postnatal day (PND) 1. Behavioral tests are performed in male and female offspring during the PNDs. The body weight of the pups is measured every 3 days until one day after weaning (PND 23).

The behavioral battery of cognitive and emotional tests based on our previous studies (Abush and Akirav, 2012; Bauminger et al., 2022;Burstein et al., 2018) will be performed on juvenile (from PND 25 to PND 35) or adult (from PND 60 to PND 70) rats in the following order: open field (**OF**; Day 25 or 60) to assess general locomotor function (30 min., total distance, cm, divided into 5 min bins) and novelty-induced anxiogenic behavior (time in arena center, first 5 min), object location [**OL**; Day 29 or 64, after 3 days of habituation (**HABIT**) to the arena] and novel object recognition (**NOR**; Day 30 or 65) with an inter-trial interval (ITI) of 5 min, are used to measure spatial and novelty recognition and working memory; episodic-like memory (**ELM**, Day 31 or 66), in which animals spontaneously explore an environment and attempt to associate an object (What), its location (Where), and the temporal context (first or second occurrence – When) (based on Chao et al., 2014); social preference (**SP**) and social recognition (**SR**) (Day 32 or 67), include two phases: initial familiarization with an unfamiliar juvenile rat and a novel object (which constitutes a social preference test), followed by a recognition phase, with the previously familiarized rat and a novel juvenile (which constitutes a social recognition test); elevated plus maze (**EPM**, Day 33 or 68), is used to assess anxiety-related behavior; rats are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded by a video-tracking system and observer simultaneously for 5 min. Forced Swim Test (**FST**, Days 34-35 or 69-70) is a test of learned helplessness that models depressive-like behavior.

Five days after the end of the experiment (Day 40 or 75), brains will be collected for biochemical analyses in the HPC and PFC. We will target specific miRNAs based on the literature on CBD and our findings (e.g., miRNAs: miR-16, miR-31, miR-34a, miR-127, miR-135, miR-146a , miR-155, and miR-223, miR-449a)(Bright and Akirav, 2023; Elliott et al. 2015; 65) and NI markers (e.g mRNA levels of IL-6, IL-1, TNF-α, NF-kB, astrocyte and microglial markers) (Vogelzangs et al., 2013; Oliveira et al., 2016). Cognitive and emotional function will be correlated with alterations in the expression of miRNAs in the HPC-PFC pathway, suggesting a link between PCE-induced behavioral phenotype and an epigenetic mechanism.

To measure expression of the key miRNAs (noted above) involved in inflammation, we will perform a gene expression study using real-time PCR.RNA will be extracted from brains collected on days 40 or 75, and cDNA will be synthesized using qScript microRNA cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, USA). Expression will be measured using the Step One real-time PCR system (Applied Biosystems). Fold-change in expression will be reported as delta-delta Ct relative to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT; mRNA) or RNU6 (miRNA). Primer efficiency and specificity will be determined using standard curve analysis and melting curve analysis.(Portugalov et al., 2022; Zaidan et al., 2018).

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Aim 1 Anticipated results and potential problems. If we do not find long-term effects of CBD administered during gestation, dams will be injected throughout the gestation and lactation period (from GD5 to PND10; Maciel et al., 2022). In case PCE-induced alterations in miRNAs are not associated with the behavioral phenotype, we will examine PCE-induced alterations in other epigenetic mechanisms such as DNA methylation in the HPC-PFC pathway (Demaili et al., 2023).

**Aim 2: To explore whether inhibiting or activating specific candidate miRNAs can reverse PCE-related cognitive and emotional dysfunction.**

Aim 2 Rationale. In our second Aim, we will explore whether different miRNAs are critically involved in PCE-related cognitive and emotional dysfunction by using anti-miRs and miRNA mimics. Many miRNAs appear to play beneficial rather than pathologic roles in settings of disease (Mendell and Olson, 2012). As such, the activation or silencing of particular miRNAs may be ideally suited to restore PCE-induced alterations in cognitive and emotional function. Our planned experiments will offer insight into the potential therapeutic utility of the targeted activation or silencing of specific miRNAs as an approach to restoring memory and alleviating emotional deficits, while also better defining the role that miRNAs play in the context of PCE in both males and females.

Aim 2 Experimental Design. To validate the role of specific miRNAs in mediating PCE-induced alterations in behavior, we will use antagomers/agomirs to inhibit/activate specific miRNAs. Our preliminary findings demonstrated that microinjecting antagomir-16 into the right ventricle (intracerebroventricular [ICV]) significantly decreased the expression of miR-16 in the PFC, but not in the nucleus accumbens one week after microinjection (**Figure 3a, b)**.This decrease in the mPFC lasted 7 weeks after microinjection of anti-mir 16 (**Figure 3c**) but had no effect on the expression of miR-135a (**Figure 3d**). Importantly, preliminary results from a rat model for depression (early life stress, ELS) suggest that the antagomir has a potential sustained effect on behavioral performance weeks later.

Rats were exposed to ELS on PND 7-14, microinjected ICV with anti-mir 16 on PND 36. On PND 45-60 rats were treated with the fatty acid amide hydrolase (FAAH) inhibitor URB597 that increases physiological levels of anandamide and tested for depression-like behavior on PND 70. We found that URB597 restored an ELS-induced increase in immobility in the forced swim test and that anti-mir 16 blocked the restoring effect of URB597 (**Figure 4**). Findings from Aim 1 of miRNAs that are associated with PCE and the behavioral phenotype are expected to put forward specific miRNAs that are altered following PCE and that their inhibition or activation could prevent PCE-induced alterations in cognitive and emotional function.

The experimental design is similar to Aim 1, except for the antagomir/agomir injections. Briefly, from GD5 to GD18, dams will be injected subcutaneously (s.c.) daily with vehicle or CBD (3 or 10 mg/Kg). On day 50 rats will receive ICV injection of an agomir or antagomir to the right ventricle. The behavioral tests will be performed in male and female offspring during the perinatal period (PND) 60-70 as described above. After the end of the experiment (Day 75), brains will be collected for biochemical analyses in the HPC and PFC to measure the expression of miRNAs and NI mRNA.

Mimicking or inhibiting the relevant miRNAs (miR-16, miR-31, miR-34a, miR-127, miR-135, miR-146a, miR-155, and miR-223, miR-449a) will be determined based on the results from Aim 1 and from the literature described above. Hence, for example, if we find that PCE upregulated the expression levels of miR-34a and miR-449a, that are associated with depression and anxiety, in Aim 2 we will use an antagomir to inhibit their expression following PCE and examine their effects on the behavioral phenotype. We hypothesize that the antagomir will prevent at least some of the long-term effects of PCE on depression- or anxiety-like behaviors.

Cranial holes above the right ventricle will be drilled relative to bregma (anterior-posterior (AP)= +1.92 mm; medial-lateral (ML): ± 0.9 mm; dorsal-ventral (DV): -4.7 mm). After 5 min rest, 1 μl of the antagomir/agomir/PBS will be injected (Biotag, USA; 20nmol in 1 µl; 0.1 μl/min) through a 10-μl Hamilton syringe (Hamilton Co., USA) connected to a motorized nanoinjector (Stereotaxic Injector, Stoelting, IL). Animals will be allowed 7 days of recovery before behavioral experiments begin.

Anticipated results and potential problems. In case we do not find that the agomir/antagomir affects PCE-induced alterations in miRNAs or behavior, we will block inflammatory markers e.g. anti-TNF-α and IL-6 therapies and [181]. TNFα and IL-6 play crucial roles in cytokine storm pathogenesis and are likely responsible for the escalation in many Inflammation-induced diseases (Coomes et al., 2020; Liu et al., 2016; Radner et al., 2015). Hence, drugs with demonstrated anti-inflammatory effects could show improvement of mental conditions when used as add-on treatments to conventional psychiatric medications [85–91 Uzzan et al., 2021 Sominsky et al., 2012; Wang et al., 2013]. ****