**RESEARCH PLAN:**

**#** **2023047: Mechanisms underlying the long-term effects of prenatal exposure to cannabidiol (CBD) on behavior in the adult progeny.**

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1. **Description of the subject and the scientific and technological background**

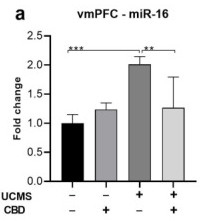
**Overview.** More than 25% of people in North America report using cannabis (*Cannabis sativa*) for medicinal purposes despite limited evidence to support its safety (Huestis et al., 2019). Recent research highlights the widespread nature of this use that includes pregnant women (Sarrafpour et al., 2020). Despite a warning from the US Food & Drug Administration and others advising against the consumption of cannabidiol (CBD) by pregnant women, use continues for an array of pregnancy-related symptoms including nausea, insomnia, anxiety, and chronic pain (Iezzi et al., 2022; US Food & Drug Administration, 2023). Up to 5% of pregnant women, especially those with lower educational attainment and socioeconomic backgrounds, use CBD to treat these symptoms. In light of the role of endogenously expressed cannabinoids in neurodevelopment combined with evidence suggesting significant risks associated with cannabinoid exposure *in utero*, there is a critical need to understand the effects of CBD on fetal neurodevelopment and the mechanisms that mediate them (Jarlenski et al., 2017; Marchand et al., 2022).

**CBD in the modulation of mental health.** CBD, the primary non-psychoactive compound found in cannabis, has been recognized for its potent biological properties and therapeutic potential, particularly as a modulator of psychiatric disorders (Peng et al., 2022). In rodents, for example, exposure to CBD in adulthood improves performance in the forced swim test (FST), a model of clinical depression, reduces anxiety-like behaviors in the elevated plus maze test, and reduces responsiveness to drugs of addiction, including morphine and cocaine (Luján et al., 2018; Resstel et al., 2009). In humans, CBD reduces psychotic symptoms in schizophrenia and lowers subjective measures of anxiety (Leweke et al., 2012; McGuire et al., 2018; Zuardi et al., 1993). CBD has, therefore, been a strong candidate for treating diverse psychiatric conditions, and public uptake of this idea has been robust.

**Mechanisms mediating the effects of CBD.** CBD exerts its molecular and behavioral effects through various molecular targets. For example, it exhibits a low affinity for the cannabinoid 1 and 2 (CB1, CB2) receptors (Pertwee, 2008), G protein-coupled receptors (GPCRs) that are abundantly expressed in the brain and spinal cord as early as 14 weeks gestation (Roncero et al., 2020), while also inhibiting fatty acid amide hydrolase (FAAH), thereby preventing the catabolism of the endogenous cannabinoid anandamide (De Petrocellis et al., 2011; Ligresti et al., 2016). CBD also acts as an allosteric modulator of the serotonin type 1A (5-HT1A) receptor, promoting the agonist-related stimulation of GTPgammaS binding (Russo et al., 2005) in addition to activating and desensitizing the transient receptor potential cation channel subfamily V 1-2 (TRPV1-2) proteins (Bisogno et al., 2001). CBD activates the extracellular signal-regulated kinase (ERK) pathway through the 5-HT1a receptor (Fogaça et al., 2016), resulting in β-catenin accumulation in the cytosol and therefore in the cell nucleus. β-catenin is a multi-functional protein that plays an important role in the mature central nervous system; its dysfunction has been implicated in several neuropsychiatric disorders, including depression (Teo et al., 2018). We have recently found that β-catenin downregulation via viral-mediated gene transfer in the nucleus accumbens (NAc), blocked the therapeutic-like effects of the FAAH inhibitor URB597 on anxiety- and depression-like behaviors in adult rats exposed to a model of post-traumatic stress disorder (PTSD) (Mizrachi Zer-Aviv et al., 2022). We found a similar effect in the PFC; β-catenin downregulation in the PFC blocked the effects of URB597 on anxiety-like behavior (**Figure 1**); together, these findings suggest a novel mechanism for the therapeutic-like effects of the FAAH inhibitor in a PTSD rat model.

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**Figure 1: β-catenin downregulation (DR β) in the PFC of rats exposed to the shock and reminders model of PTSD blocks the preventive effects of the FAAH inhibitor URB597 on anxiety-like phenotypes.** (a) Rats in the Shock-GFP-Veh group exhibited an increased acoustic startle response compared to the No Shock-GFP-Veh and Shock-GFP-URB groups. (b) Compared to rats in the Shock-GFP-Veh and Shock-DRβ-URB groups, those in the Shock-GFP-URB group demonstrated decreased freezing during extinction day 5. (n=8-9/group) \*p<0.05, indicate statistically significant effects after post-hoc comparisons; □p<0.05, □□p<0.01, indicate significant differences between the shocked and non-shocked groups. Taken from Sbarski, Portugalov, Parise, Nestler, Mizrachi Zer-Aviv & Akirav, in preparation.



There is evidence that CBD also influences epigenetic mechanisms including methylation patterns that are responsible for its antidepressant effects in adults. Indeed, we found that CBD administration in rodents alters the expression of microRNAs (miRNAs) in the PFC via serotonergic receptors in a model of clinical depression (Bright & Akirav, 2023). **Figure 2** shows that CBD modulates miRNA expression in adult rats exposed to the unpredictable chronic mild stress (UCMS) model for depression, supporting reversible epigenetic regulation as a mechanism mediating CBD’s possible therapeutic effects in adults.

**CBD exposure during pregnancy.** CBD can efficiently cross the maternofetal placental barrier and

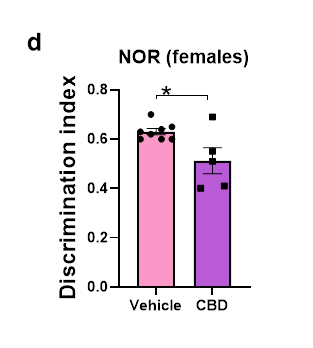
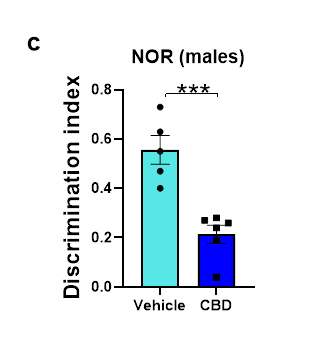
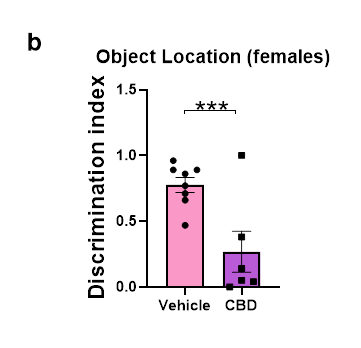
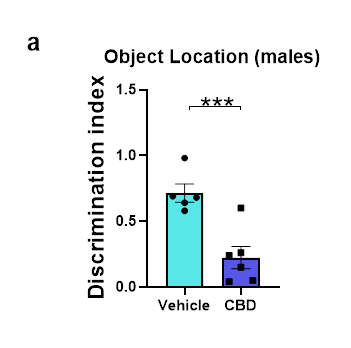
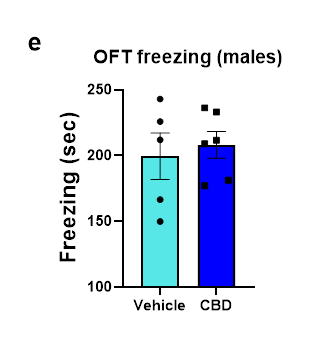
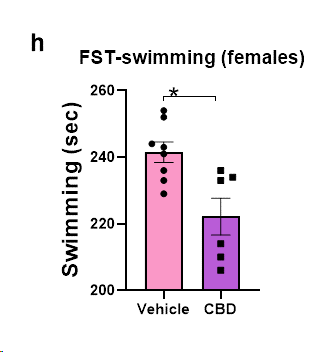
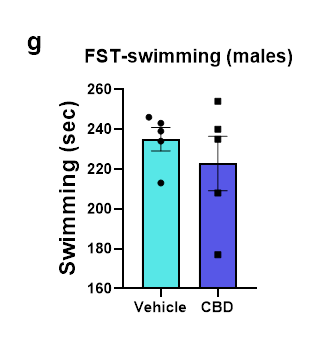
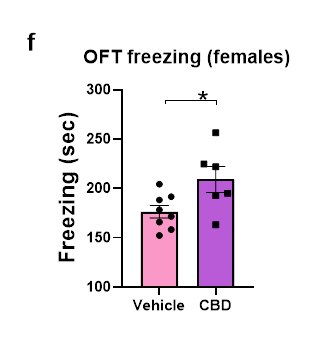
accumulate in fetal tissues including the brain (Ochiai et al., 2021). There is basis to challenge current attitudes toward cannabinoids that are believed to be universally therapeutic and safe. A limited number of studies have examined the long-term developmental effects on fetuses exposed to CBD during pregnancy. In animal models, CBD treatment exerts sex-specific cognitive alterations in early life, which predict the risk of developing various neuropsychiatric and developmental disorders (Iezzi et al., 2022). A recent study showed that exposure of CD1 mice to CBD from gestational day 5 to postnatal day 10 alters repetitive and hedonic behaviors in the adult progeny (Maciel et al., 2022). CBD exposure during gestation and lactation also induces sex-specific changes in working spatial memory and anxiety-like behaviors as well as genome-wide changes in brain DNA methylation in adult offspring (Wanner et al., 2021). However, conflicting data exist in the literature to suggest that developmental CBD is associated with mixed behavioral outcomes. Fetal CBD (50 mg/kg) exposure to pregnant mice from embryonic day 5 through birth did not impact offspring anxiety-like behavior or compulsivity (open field test, light-dark box, elevated zero maze), and did not alter offspring spatial memory, despite sensitizing males to thermal pain, decreasing females problem-solving behaviors, and reducing excitability of PFC pyramidal neurons (Swenson et al., 2023). Similarly, CBD (20 mg/kg) from two weeks prior to mating through gestation and lactation induced a significant sexual dimorphism when measuring spontaneous alternation in the Y-maze (Wanner et al., 2021). These results emphasize that fetal CBD exposure disrupts neurodevelopment and postnatal behavior in a sex-specific manner. Identifying potential sex differences in the risks associated with prenatal CBD is a public health imperative.



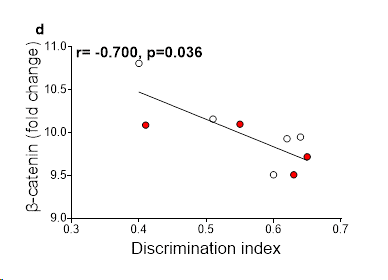
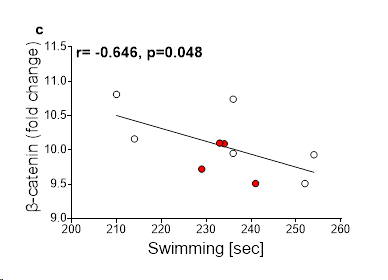
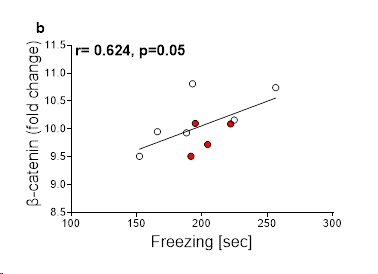
**Figure 2: CBD reverses the effects of unpredictable chronic mild stress (UCMS) model for depression on miRNA expression in the PFC.** UCMS rats treated with vehicle demonstrated upregulation in PFC of (a) miR-16, and (b) miR-135 compared **to** all groups. (n=9-10/group) \*\*, p < 0.01, \*\*\*, p < 0.001; Bright & Akirav, 2023

Prenatal CBD exposure-induced alterations in emotion and cognition. We have successfully established the prenatal CBD exposure (PCE) model in our laboratory and have generated preliminary data demonstrating sexually dimorphic effects of PCE on behavioral performance. Dams were injected with vehicle or CBD (10 mg/kg) from gestational day 5 to 18 and their pups were tested for emotional and cognitive performance starting postnatal day 23. PCE-exposed pups demonstrated impaired performance in the object location and novel object recognition tasks; moreover, PCE females, but not males, demonstrated increased freezing in a novel environment and decreased swimming in the FST, suggesting an anxiogenic- and depressive-like phenotype (**Figure 3**).

**The role of WNT/β-catenin pathway and microRNAs in mental health.** Wnt/β-catenin signaling was found to have a main role in various psychiatric conditions (Teo et al., 2018; Vallée, 2022; N. Xu et al., 2023). It seems to be implicated in synaptic plasticity, particularly involving emotional learning and memory processes, and appears to be involved in disorders associated with strong memory formation such as fear learning in PTSD (Maguschak & Ressler, 2012) acting on fear retrieval through the medial PFC (mPFC) (Narvaes et al., 2022). Decreased β-catenin protein levels were found in postmortem human brain samples of suicide victims suffering from major depressive disorder in the mPFC and hippocampus (Karege et al., 2012; Pilar-Cuellar et al., 2014; Ren et al., 2013). Accordingly, studies have shown that exposure to chronic stress reduces β-catenin in the PFC and hippocampus (Chen et al., 2012; Cuesta et al., 2020; Habib et al., 2020; Leem et al., 2018; Mohamed et al., 2020) and β-catenin overexpression was found to normalize depression- and anxiety-like behaviors (Dahlhoff et al., 2010; Dias et al., 2014; Kaidanovich-Beilin et al., 2004; Ochiai et al., 2021; Vidal et al., 2019; Zotova et al., 2013). Moreover, lowered β-catenin protein levels, but not mRNA levels were found in the NAc of depressed patients, suggesting that depression may be associated with reduced activity of β-catenin, and perhaps not a defect at the transcriptional level (Karege et al., 2012; Vidal et al., 2019). We have demonstrated that β-catenin in D2-type medium spiny neurons (MSNs) activates a network in the NAc that mediates behavioral resilience, whereas deficits in this pathway contribute to depression-related pathology, suggesting that PFC inputs to NAc appear to be particularly important in controlling this β-catenin regulation (Dias et al., 2014).

   ***Figure 3:*** *PCE impaired object location in males (a) and females (b), novel object recognition (NOR) in males (c) and females (d), no effect on freezing in males (e), increased freezing in females (f), no effect in the FST in males (g), but decreased swimming in females (h); (n=5-6) (\*, p<0.05; \*\*\*, p<0.001).*

CBD increase Wnt/β-catenin activation, possibly via its control over the PI3K/Akt/GSK-3β axis; CBD downregulates the expression of GSK-3β through the promotion of the PI3K/Akt signaling; GSK-3β downregulates the canonical WNT/β-catenin pathway by inhibiting β-catenin cytosolic stabilization and its translocation in the nucleus (Libro, Bramanti, et al., 2016; Libro, Diomede, et al., 2016); hence, CBD enhances Wnt/β-catenin pathway activity by downregulating the expression of GSK-3β through the promotion of the PI3K/Akt signaling (Ozaita et al., 2007). We have preliminary data demonstrating that prenatal CBD exposure upregulated mRNA expression of β-catenin in the PFC of the female offspring (**Figure 4**) and that this upregulation was significantly correlated with impaired recognition memory, and with a depressive and an anxiogenic phenotype as measured in the FST and open field test. As far as we know, this is the first evidence that female offspring of dams exposed to CBD during gestation show alterations in the β-catenin gene in the PFC.



It has been shown that β-catenin is a critical regulator of a network that includes downstream microRNAs (Dias et al., 2014). A pathogenic role for miRNAs has been suggested in the development and progression of several neuropsychiatric conditions (Azevedo et al., 2016).Altered levels of specific miRNAs have been observed in patients with disorders, including major depressive (Azevedo et al., 2016; Maffioletti et al., 2016; Penner-Goeke & Binder, 2019; Smalheiser et al., 2014) disorder. Moreover, altered levels of miRNAs were found in the postmortem brain and peripheral tissues of patients after treatment with antidepressant medication, highlighting the potential of miRNAs as biomarkers for treatment response (Lopez et al., 2018; Penner-Goeke & Binder, 2019).

***Figure 4:******Prenatal CBD exposure promotes mRNA β-catenin upregulation associated with the behavioral phenotype in females.*** *Compared to the control group, females exhibited increased mRNA levels of β-catenin )CTNNB1((a) that was positively correlated with freezing behavior (b), and negatively correlated with swimming behavior in the FST (c) and the decline in performance in the novel object recognition task (d). (n=4-6/group). Red circles represent the CBD group. \*, p< 0.05*

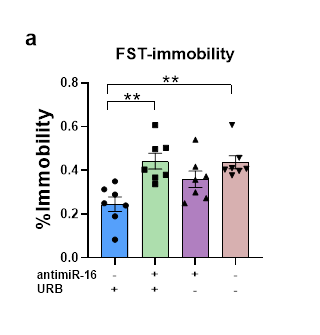
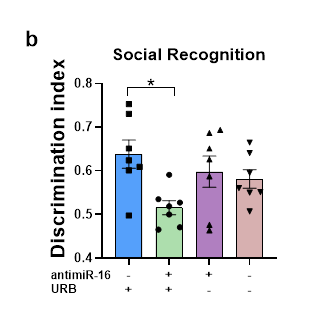
**miRNAs involved in the β-catenin signaling pathway and stress/stress adaptation**. The mechanisms between the alterations in β-catenin and the onset of anxiety and depressive-related symptoms have not been clearly elucidated in most studies; however, it has been suggested that a key role may lie with Dicer1, one of the target genes for β-catenin (Dias et al., 2014; Teo et al., 2018). Dicer1 codes for the Dicer protein, which is involved in the cleaving and formation of all miRNA end-products. MiRNAs such as miR-124, miR-135, and miR-15a have been shown in separate studies to play a role in stress adaptations (Higuchi et al., 2016; Issler & Chen, 2015; Volk et al., 2016). In addition to that, a general decrease in miRNA expression throughout the brain has been found to be consistent with lowered activation of the frontal cortex in depressed human subjects (Smalheiser et al., 2012), while up-regulation of the miR-16 miRNA in the raphe nuclei and hippocampus was found to induce depressive behavior (Bai et al., 2012). The levels of miR-214-3p, which targets β-catenin transcripts, were significantly increased in the mPFC of mice exposed to chronic social defeat stress (CSDS) and 214-3p antagomir-214-3p, a neutralizing inhibitor of miR-214-3p, increased the levels of β-catenin and reversed the depressive-like behavior in CSDS mice (Deng et al., 2019). MiR-155 has been suggested to be involved in regulating depression-like behaviors through the inhibition of the Wnt/β-catenin signaling pathway; chronic unpredictable mild stress mice increased expression levels of miR-155 and GSK-3β in the hippocampus and decreased expression of β-catenin (Dai et al., 2020).

**Our working hypothesis is that prenatal CBD exposure increases the risk of developing cognitive decline, anxiety- and depression-related disorders later in life, via mechanisms involving β-catenin and specific miRNAs in the PFC**. miRNAs are critically involved in the development and progression of various neuropsychiatric conditions. Yet many miRNAs appear to play beneficial rather than pathologic roles in settings of disease (Geaghan & Cairns, 2015; Issler & Chen, 2015; Martins & Schratt, 2021; Mendell & Olson, 2012; B. Xu et al., 2012). As such, the *activation or silencing* of specific miRNAs may be ideally suited to restore PCE-induced alterations in cognitive and emotional function. Moreover, 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, mRNA sequencing will be used to identify and quantify genes that are expressed following PCE and are correlated with the PCE-induced emotional and cognitive phenotype.

The silencing of specific miRNAs has long-term effects on behavior. Our preliminary findings show that microinjecting antagomir-16 into the right ventricle decreases the expression of miR-16 in the PFC and affects depression-like behavior. Intracerebroventricular (ICV) injection of antagomir-16 into the right ventricle significantly decreased the expression of miR-16 in the mPFC, but not in the NAc one week after microinjection (**Figure 5a, b**).This decrease in the mPFC lasted 7 weeks after microinjection of anti-mir 16 (**Figure 5c**) but had no effect on the expression of miR-135 (**Figure 5d**). Importantly, using the early life stress (ELS) rat model of depression, we demonstrate that silencing miR-16 has a potential sustained effect on behavioral performance weeks later. Rats were exposed to ELS on postnatal day (PND) 7-14 and received ICV anti-mir-16 on PND 36. On PND 45-60, rats were treated with the 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 and decrease in social recognition and that anti-mir-16 reverses the effects of URB597 (**Figure 6**). One of the main effects of CBD is to increase anandamide by FAAH inhibition (Bisogno et al., 2001).

***Figure 5****: Antimir-16 microinjection into the right ventricle specifically and durably silences miR-16 in the mPFC. (a-d) Antimir-16 administration into the right ventricle decreased the expression of mir-16 in the PFC (a) but not the Nac (b), and the effect in the mPFC lasted at least 7 weeks (c) without any corresponding change in miR-135a expression (d) (n=5/group) \*p<0.05.*



***Figure 6****.* ***miR-16 silencing modifies behavioral phenotypes.*** *Anti-mir-16 blocks the effects of URB597 on ELS-induced increases in immobility (a) and decrease in social recognition (b) (n=7/group) \*p<0.05, \*\*, p<0.01*

**2.** **Objectives and significance of the research**

**The main objective** is to enhance our understanding of the pathological mechanisms underlying the effects of PCE while also supporting the development of innovative preventive strategies. Specifically, we: 1) 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, 2) better define the role that β-catenin and miRNAs play in the context of prenatal CBD exposure in males and females, and 3) help inform clinical recommendations for pregnant women seeking symptom relief. Revealing cognitive impairments and emotional dysfunction associated with fetal CBD exposure 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 for prenatal CBD exposure-exposed individuals. We propose three specific aims:

***Aim 1 will identify*** ***prenatal CBD exposure-related patterns of miRNA and Wnt/*β-catenin *dysregulation in the PFC that are associated with PCE-induced cognitive and emotional dysfunctional phenotypes*.** The consumption of CBD during pregnancy is increasing, but the developmental consequences are still largely unknown. To address this knowledge gap, we will characterizelong-term effects of prenatal CBD exposure on the behavioral phenotype, affecting cognitive and emotional behavior, and the expression of miRNAs and β-catenin in the PFC circuit.

***Aim 2 will determine whether β-catenin in the PFC can reverse prenatal CBD exposure-related cognitive and emotional dysfunction.*** To that end, we will use viral-mediated β-catenin downregulation in the PFC to block PCE-induced cognitive and emotional dysfunction. This would suggest that β-catenin is a key mediator of PCE.

***Aim 3 will explore whether inhibiting or activating specific candidate miRNAs can protect against PCE phenotype and their effects on related transcripts.*** miRNAs are interesting therapeutic targets given their ability to regulate endogenous gene expression such that one miRNA can potentially regulate entire biological pathways. 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. We hypothesize thatthe *activation or silencing* of specific miRNAs will restore prenatal CBD exposure-induced alterations in cognitive and emotional function in a sex-dependent manner. Elucidating the mechanisms by which CBD affects development would support miRNA-based interventional strategies in the long-term, as further studies of the clinical manipulation of miRNA activity progress.

Due to their established role in disease development, it is critically important to understand the mechanisms by which various miRNAs mediate the post-transcriptional regulation of gene expression. Therefore, the identification of mRNA targets that mediate the actions of miRNAs in PCE-induced disease will be assessed using mRNA sequencing. Identifying specific related transcripts would make them interesting targets for potential therapeutic intervention. Together, the findings will determine whether and how prenatal CBD exposure affects the developing brain.

**Expected significance**: The recent widespread promotion and public acceptance of CBD as a “safe” and “natural” compound, 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 or early postnatal babies to CBD. Importantly, few studies have addressed the significant and timely question of how CBD impacts the developing fetal brain (Schonhofen et al., 2018). The present study will contribute new mechanistic insights into the long-term consequences of PCE on emotional and cognitive function in males and females. Through the delivery of miRNA inhibitors or mimics, we aim to identify and validate an epigenetic mechanism mediating the effects of PCE on behavior. Moreover, due to their established role in disease development, it is critically important to understand the mechanisms by which various miRNAs mediate the post-transcriptional regulation of gene expression. Therefore, the identification of mRNA targets that mediate the actions of miRNAs in PCE-induced disease can provide opportunities for clinically intervening in disease processes. We expect that these experiments will elucidate the therapeutic potential of specific miRNA activation/silencing as a means of improving memory and emotional deficits following PCE, and to clarify the role that miRNAs play in mediating the effects of CBD in female rats. Hence, our work has the potential to significantly contribute to the understanding of the pathophysiological mechanisms of PCE and support the future development clinical manipulation of miRNA activity.

Therapeutic relevance. Our proposed 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. We should note that manipulating the Wnt/β-catenin pathway is not a therapeutic option at the moment due to its involvement in cancer-related processes.

Revealing cognitive impairments and emotional dysfunction associated with fetal CBD exposure 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 for PCE exposed individuals.

**Innovation.** These results will yield information about **long-term, selective, sex-dependent** negative impacts on emotional and cognitive function induced by prenatal CBD exposure and the mediating mechanisms involved. Our work has the potential to clarify the pathophysiological mechanisms of prenatal CBD exposure and support the development of miRNA-based interventional strategies in the long-term, as 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 to cognitive and emotional function and may therefore 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. Given the evidence regarding sex differences in psychiatric disorders (Swaab & Bao, 2020), identifying specific targets 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 mechanisms of action for a given compound 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.

1. **Comprehensive description of the methodology and plan of operation**

Akirav’s lab will complete the behavioral, pharmacological, and viral experiments, and activate/inhibit specific miRNAs using agomirs/antagomirs (Aims 1-3). Brains will be sent from Haifa to Nestler’s lab, that will be responsible for the mRNA sequencing experiments, and will also provide the β-catenin viral vectors (Aims 2-3).

**Aim 1: To identify prenatal CBD exposure-related patterns of miRNA and Wnt/β-catenin dysregulation in the PFC circuit that are associated with prenatal CBD exposure-induced cognitive and emotional dysfunctional phenotypes.**

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 to elucidate the long-term effects of PCE on cognitive and emotional phenotype in adulthood and to examine whether these changes are associated with alterations with specific miRNAs and Wnt/β-catenin signaling. To this end, in our first experiment, dams will be injected with CBD or vehicle during days 5-18 of gestation. Male and female pups will be tested in a battery of cognitive and emotional tests, after which alterations in the expression of miRNAs and β-catenin signaling in the PFC circuit will be assessed. We hypothesizethat PCE will have long-term effects on the behavioral phenotype, affect cognitive and emotional behavior, and will alter the expression of miRNAs and β-catenin in the PFC.

Aim 1 Experimental Design (**Figure 7**). The presence of a vaginal plug will be defined as gestational day (GD) 0. Females will be individually housed beginning on GD0. From GD5 to GD18, dams will be injected subcutaneously (s.c.) daily with vehicle or CBD (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 low dose of CBD reaches the embryonic brain and causes behavioral changes in the offspring (Maciel et al., 2022). The sample size was chosen based on a priori power analysis (n=13 per group; effect size=0.25, power=0.8, α error prob= 0.05) (Faul et al., 2007). For each litter, the date of birth will be designated as postnatal day (PND) 1. The body weight of the pups will be measured every 3 days until one day after weaning (PND 22) and then once a week. The behavioral battery of cognitive and emotional tests based on our previous studies will be performed on adult males and females (from PND 60 to PND 70) rats in the following order: open field (OF; Day 60) to assess general locomotor function and novelty-induced anxiogenic behavior, social interaction with a novel juvenile (SI; Day 61); object location (OL; Day 62), and novel object recognition (NOR; Day 63); episodic-like memory (ELM, Day 64), 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) (Chao et al., 2014); social preference (SP) and social recognition (SR) (Day 65); elevated plus maze (EPM, Day 66) to assess anxiety-related behavior; and Forced Swim Test (FST, Days 67-68) to model depressive-like behavior (Abush & Akirav, 2012; Bauminger et al., 2022; Burstein, 2015). After the end of the experiment (Day 75), brains will be collected for biochemical analyses in the mPFC. Due to feasibility reasons, we will also dissect other relevant brain areas (ventral and dorsal hippocampus, NAc, basolateral amygdala) for future analysis.

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Figure 7: Experimental Design

Using real-time PCR, we will measure the expression of specific miRNAs involved in the β-catenin signaling pathway and stress/stress adaptation described in the literature (e.g., miR-124, miR-135, miR-15a, miR-16, miR-214-3p, and miR-155) (Bai et al., 2012; Dai et al., 2020; Deng et al., 2019; Higuchi et al., 2016; Issler & Chen, 2015; Smalheiser et al., 2012; Volk et al., 2016). We will also measure mRNA and protein levels of the Wnt/β-catenin signaling pathway (e.g., β-catenin, Dicer 1, GSK-3β).

Using Pearson bivariate correlation tests, performance in cognitive and emotional tests will be correlated with alterations in the expression of miRNAs and mRNAs in the PFC to quantify a link between PCE-induced behavioral phenotype and an epigenetic mechanism and β-catenin.

**RT-PCR**: RNA will be extracted, 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 and melting curve analyses (Portugalov et al., 2022; Zaidan et al., 2018a). **Estrous Cycle:** The estrous cycle is examined in the morning (between 07:30–09:00), the day before the first behavioral tests. Vaginal cytology samples will be collected by brief introduction and immediate extraction of a small amount of phosphate buffer with a micropipette in the rat's vagina. The phase of the cycle (metaestrous, diestrous, pro-estrous, or estrous) will be determined based upon the presence of leukocytes, nucleated epithelial, or cornified epithelial cells (Zer-Aviv and Akirav, 2016).

**Risk analysis and alternative paths for Aim 1**. The experiments proposed in Aim 1 will complete the study for which we already show preliminary data, including evidence for the long-term effects of PCE on behavior (**Figure 3**). If 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 (Zaidan et al., 2018b). Previously, we found that following exposure to ELS, changes in CB1 and FAAH gene expression were accompanied by altered DNA methylation patterns in the promoter regions of these genes (Demaili et al., 2023).

**Aim 2: To determine whether β-catenin in the PFC can reverse prenatal CBD exposure-related cognitive and emotional dysfunction.**

Aim 2 Rationale. The WNT/β-catenin pathway is dysregulated in numerous disorders such as depression, schizophrenia, bipolar disorder, autism, and Alzheimer’s disease (De Ferrari et al., 2003; Zhang et al., 1998). CBD, among other effects, activates the ERK pathway through the 5-HT1a receptor (Fogaça et al., 2016), resulting in β-catenin accumulation in the cytosol and therefore in the cell nucleus. We have preliminary findings demonstrating that PCE upregulated mRNA expression of β-catenin in the PFC of the female offspring (Figure 4) and that this upregulation was significantly correlated with impaired recognition memory, and with a depressive and an anxiogenic phenotype as measured in the FST and open field test. Hence, in our second Aim, we will explore whether β-catenin downregulation in the PFC will block the PCE induced effects on cognitive and emotional dysfunction of the offsprings.

Aim 2 Experimental Design (**Figure 8**): From GD5 to GD18, dams will be injected daily with vehicle or CBD as described in Aim 1. Adult male and female rats receive a total of 1μL of the HSV viral vector or green fluorescent protein (GFP) into the PFC (Day 55) (Stoelting, Wood Dale, IL, USA) at a rate of 0.1 μL/min (coordinates relative to Bregma: anterior-posterior (AP): +2.9 mm; medial-lateral (ML): ± 0.6 mm; dorsal-ventral (DV): -5 mm). The vector is used to downregulate (DR) the expression of β-catenin compared to a GFP control; the vector is expressed in vivo within 2–3 h, with maximal expression from 3–5 days post-injection that lasts 8 days in vivo (see Mizrachi Zer-Aviv et al., 2022 for more details). Next, the behavioral battery of cognitive and emotional tests based on our previous studies will be performed as follows: OFT (Day 60), NOR (Day 61), and FST (Days 62-63). After the end of the experiment (Day 68), brains will be collected for biochemical analyses in the PFC. Due to feasibility reasons, we will dissect other relevant brain areas for future analysis.

A diagram of a delivery of dr / gf

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Figure 8: Experimental Design

**Viral Mediated Gen Transfer**: Rats are anesthetized with a mixture of ketamine/xylazine solution and placed in a stereotaxic frame (Stoelting). Holes are drilled into the skull, and viruses are delivered bilaterally using a 10-μl syringe and metal needle (Hamilton, Reno, NV). The injection volume and flow rate are controlled by a micromanipulator at a volume of 1 μl for HSV viral vector, a high titer range of 3 to 5 × 10ʌ8 transduction unit, TU/ml) and a rate of 0.1 μl/ min. Injection needles are left in place for 10 min following all injections to ensure adequate viral delivery. The viral vectors are provided by Prof. Nestler’s lab.

**Risk analysis and alternative paths for Aim 2:** Based on our preliminary findings, we expect PFC β-catenin downregulation to reverse PCE-induced alterations in behavior. This would suggest that the effects of PCE are regulated by β-catenin. In this case we will run another cohort of animals and test the other behavioral tasks (e.g., object location, social preference and social recognition, etc.). If we do not find that β-catenin mediates the effects of PCE, we will examine the involvement of Dicer1, as it is a protein that produces miRNAs within cells and it is a β-catenin target gene (Teo et al., 2018).

**Aim 3: To explore whether inhibiting or activating specific candidate miRNAs can reverse PCE-related cognitive and emotional dysfunction and their effects on related transcripts.**

Aim 3 Rationale. We will explore whether different miRNAs are required for PCE-related cognitive and emotional dysfunction by using anti-miRs (antagomirs) and miRNA mimics (agomirs). Many miRNAs appear to have beneficial rather than pathologic effects in the setting of disease (Mendell & Olson, 2012). As such, the activation or silencing of particular miRNAs may be ideally suited to reverse PCE-induced alterations in cognitive and emotional function. Our proposed experiments will offer insight into the potential therapeutic utility of the targeted activation or silencing of specific miRNAs as an approach to restoring cognitive function and alleviating functional deficits while also better defining the role that miRNAs play in the context of PCE in both males and females. Due to their established role in disease development, it is critically important to understand the mechanisms by which various miRNAs mediate the post-transcriptional regulation of gene expression. Hence the identification of mRNA targets that mediate the actions of miRNAs in PCE-induced disease will be assessed using mRNA sequencing. This is significant because it will provide a unique list of transcripts that may be important in mediating the effects of PCE, and may prove informative for understanding neurobiological mechanisms underlying behavioral pathology caused by it. Identifying specific related transcripts would make them interesting targets for future study and excellent candidates for potential therapeutic intervention.

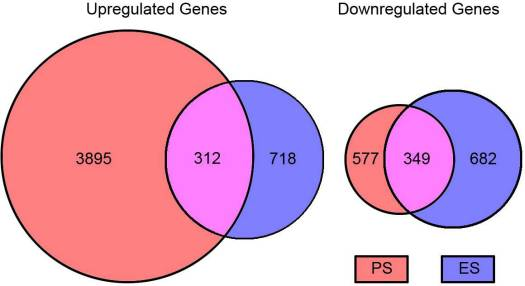
Aim 3 Experimental Design (**Figure 9**). To validate the role of specific miRNAs in mediating PCE-induced alterations in behavior, we will use antagomirs/agomirs to inhibit/activate specific miRNAs. We found that antagomir-16 microinjected into the ventricle decreases the expression of miR-16 in the PFC (**Figure 5**) and that this antagomir has a long-term effect on depression-like behavior (**Figure 6**). The experimental design is similar to Aim 1, except the antagomir/agomir injections will validate the roles of specific miRs. Briefly, from GD5 to GD18, dams will be injected s.c. daily with vehicle or CBD (10 mg/kg). On PND 50 rats will receive ICV injection of an agomir or antagomir to the right ventricle. 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; 20 nmol in 1 µL; 0.1 μL/min) through a 10-μL Hamilton syringe (Hamilton Company, Reno, NV) connected to a motorized nanoinjector (Stereotaxic Injector, Stoelting, Wood Dale, IL). Animals will be allowed 7 days of recovery before behavioral experiments begin. The behavioral tests will be performed in male and female offspring during PND 60-70, as described above. After the end of the experiment (Day 75), brains will be collected and sent to Nestler’s lab for mRNA sequencing. Mimicking or inhibiting the relevant miRNAs will be determined based on the results from Aim 1 and from the literature described above (e.g., miRNAs: miR-31, miR-34a, miR-127, miR-135, miR-146a, miR-155, and miR-223, miR-449a). For example, if we find that PCE upregulates the expression levels of miR-34a and miR-449a and is associated with behavioral phenotypes, in Aim 3 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 the long-term effects of PCE on memory and depression- or anxiety-like behaviors. The brains will be sent to Nestler’s lab for mRNA sequencing to examine gene expression changes between prenatal exposure to CBD and vehicle. This will provide a unique list of transcripts that may be important in mediating the effects of PCE on behavior and may prove informative for understanding neurobiological mechanisms underlying behavioral pathology caused by PCE. We have previously compared genome-wide mRNA expression patterns in the ventral tegmental area of mice exposed to an emotional or a physical stressor using RNA-seq. We found a significant overlap in gene expression changes between emotional and physical stress conditions, which suggests several potential gene targets for mediating the behavioral abnormalities observed (Warren et al., 2013) (**Figure 10**).

Figure 9: Experimental Design

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**Figure 10**: Venn diagrams showing overlap in significantly upregulated or downregulated mRNAs within the ventral tegmental area of mice exposed to emotional (ES) or physical (PS) stress conditions 24-hr after the last exposure to stress (p< 0.05) Warren et al., 2013).



**Agomir/Antagomir microinjection**: Rats are anesthetized, and holes are drilled above the right ventricle (AP: +1.92 mm; ML: ˗0.9 mm; DV: -4.7 mm). The antagomir/agomir/PBS (Biotag, USA) is injected (20nmol in 1 µl; 0.1 μl/min) through a 10-μl Hamilton syringe (Hamilton Co., USA) connected to a motorized nanoinjector (Stoelting). **RNA sequencing (Nestler)**: RNA will be isolated from the PFC of male and female rats exposed to CBD or vehicle prenatally, 24-hr after the last behvaioral test and analyzed by RNA-seq using Illumina’s HiSeq 2000. Quantitative PCR will be used to validate results from the RNA-seq experiment, using the same cDNA (for details see Warren et al., 2013).

**Aim 3: Risk analysis and alternative paths:** Our preliminary data (**Figure 6**) demonstrate the sustained effect of antagonizing miRs on behavioral performance. Yet, in the event that agomir/antagomir infection does not modulate PCE-induced alterations in miRNAs or behavior, we will use an alternative approach of silencing multiple miRNAs at once, or we will combine different approaches (e.g., combining the silencing or mimicking miRNAs with a pharmacological treatment). For example, we can pharmacologically block inflammatory markers, e.g., TNF-α, IL-6 (Sheridan, 2007). TNFα and IL-6 play crucial roles in cytokine storm pathogenesis and are likely responsible for the escalation in many inflammation-induced diseases (Coomes & Haghbayan, 2020; Liu et al., 2016; Radner & Aletaha, 2015). Hence, drugs with demonstrated anti-inflammatory effects could show improvement of mental conditions when used as add-on treatments to conventional psychiatric medications (Sominsky et al., 2012; Uzzan & Azab, 2021). We have preliminary data demonstarting that PCE decreased the levels of hippocampal CA1 TNFα in males and increased in females, suggesting sex-dependent effects of PCE on neuroinflammatory markers (**Figure 11**).

Figure 11: PCE decreased the levels of CA1 TNFα in males (a) but increased in females (b) (n=5-6) (\*, p<0.05; \*\*, p<0 01).



**Expected cooperation between Israeli and American teams**: Research in Nestler’s lab aims to better understand the molecular mechanisms of addiction and depression. We use animal models of these disorders to identify the ways in which long-term exposure to drugs of abuse or stress changes the brain to lead to addiction- or depression-like syndromes. Research in Akirav’s lab aims to better understand the role of the endocannabinoids system and cannabinoids in psychiatric disorders, memory and plasticity. The two labs have an ongoing collaboration on cannabinoids-β catenin interaction in a rat model for PTSD (Mizrachi Zer-Aviv et al., 2022) and we would be happy to extend our collaboration to the proposed study. The collaboration between the two labs will involve complementary contributions of the expertise and research of each of the involved laboratories. Akirav’s lab will be responsible for the behavioral paradigms, pharmacology and the viral vectors and miRNA silencing/mimicking experiments. Brains will be sent to Nestler’s lab to complete mRNA sequencing, that currently cannot be carried out in Haifa. Nestler’s lab will also provide the viral vectors for Aim 2.

**An account of available U.S. and Israeli resources, including all personnel and equipment relevant to the research**

**Akirav’s Lab**: We have the equipment, space, and expertise to perform the proposed experiments. Experiments will be carried out by PhD students, under the close supervision of the PI and of the lab manager. The behavioral rooms are equipped with NOLDUS tracking systems (EthoVision XT8 video tracking system). The surgery room is fully equipped for conducting small animal surgeries. Viral vector injection unit operating within a BSL2 area is fully equipped with syringe pumps and stereotaxic frames devoted for stereotaxic surgery, cannula implantation, and stereotaxic injections. Three fully equipped electrophysiology set-ups are also available. The Lab is equipped with GeneExplorer Thermal Cycler 96 (Bioer Technology, Hangzhou, Zhejiang, China), Western blotting equipment, ECL immunoblotting detection system (Bio-Rad Laboratories, Hercules, CA), and XRS charge-coupled device camera (Bio-Rad Laboratories, Hercules, CA). RT-PCR machine (Applied Biosystems, Carlsbad, CA), Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), computational microscope (Olympus, Tokyo, Japan), analytical scale, biological safety cabinets, chemical fume hoods, virus room, cryostat, benchtop centrifuge, microplate reader ELISA Plate Reader (Agilent BioTek Epoch, Santa Clara, CA, USA), conventional freezer, ultra-low temperature freezer, and an autoclave are available as shared resources in our department.

**Nestler’s Lab:**

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