**Abstract**

Alzheimer's disease (AD) is a debilitating progressive neurodegenerative disease with rising incidence rates as the average age of the global population continues to increase. AD is characterized by the accumulation of amyloid-β aggregates and tau hyperphosphorylation with concomitant oxidative stress and neuroinflammation. As the cognitive decline and dementia-like symptoms of AD take years to manifest, this disease is thought to be irreversible at the time of symptomatic diagnosis. Current treatments for AD fail to arrest or reverse disease progression, highlighting the need for novel, efficacious therapies. Dysregulated microRNA (miRNA) expression has been increasingly established as a hallmark of AD, and these non-coding transcripts have been advanced as promising diagnostic biomarkers and/or therapeutic agents.

To address the current lack of effective tools for the early detection and disease-modifying treatment of AD, the overarching aim of this study is to identify non-invasive biomarkers with the potential to aid in AD diagnosis and to guide disease-modifying treatment efforts. To that end, we will examine AD-associated patterns of miRNA dysregulation in male and female AD model rats and related regulatory mechanisms in the hippocampal-prefrontal cortex (HPC-PFC) pathway and in peripheral circulation. We will further examine promising novel therapies for AD including (i) the phytocannabinoid cannabidiol (CBD), and whether it could slow neurodegenerative processes through a bi-directional dialogue with miRNAs and the Wnt/β-catenin signaling pathway, and (ii) the targeting of specific miRNAs by silencing or activating them in the HPC-PFC. To achieve these experimental goals, we have proposed three Specific Aims:

In our **first Aim**, CBD will be administered in a streptozotocin (STZ)-induced rat model of sporadic AD, after which changes in cognitive and emotional function will be correlated with shifts in the expression of miRNAs in the HPC-PFC pathway, with an additional focus on targets related to inflammation, CBD signaling, AD pathology, and β-catenin. We will also investigate peripheral miRNAs and inflammatory cytokines as potential biomarkers of AD progression and treatment response. These findings will reveal significant AD-related genetic changes that can be blocked by CBD treatment and highlight the potential value of peripheral miRNAs as biomarkers of AD.

In our **second Aim** we will examine whether HPC input to the PFC is central to the control of behavioral phenotypes in AD rats using chemogenetic tools.

In our **third Aim** we will explore whether specific miRNAs mediate AD-related cognitive and emotional dysfunction and the therapeutic effects of CBD by using agomir and antagomir constructs to activate or inhibit specific miRNAs in the HPC-PFC pathway. The association between changes in miRNA expression, cognitive/emotional pathology, inflammatory markers, CBD targets, AD pathology-related targets, and β-catenin will then be further assessed.

We anticipate that the **successful completion of these experiments** will (i) highlight the potential therapeutic activity of activating or inhibiting specific miRNAs as an approach to overcoming emotional and memory deficits in AD, and (ii) define the importance of miRNAs as mediators of the neuroprotective benefits of CBD in both male and female AD model rats, providing a new foundation for the treatment of this and related neurodegenerative diseases. Ultimately, the establishment of a validated noninvasive biomarker of AD or associated targets will guide the future development of early diagnostic tools, preventive strategies, and effective pharmacological treatments for dementia.

Gaining a clear understanding of how a drug works before it enters clinical trials has the potential to increase the likelihood of successful treatment outcomes. CBD may protect against the pathogenesis of AD by altering miRNA expression patterns and through other complementary mechanisms. As such, the efforts of this study to both clarify the importance of miRNAs in AD and their role in the CBD treatment-related outcomes have the potential to directly benefit patient treatment efforts. The ability to reliably detect early-stage AD before the development of neurofibrillary tangles and plaques will enable the development of specific drugs that will block the progress of AD. Moreover, identifying specific AD-related biomarkers in males and females can guide the formulation of personalized, sex-specific pharmacological interventions.

**Project Narrative**

Alzheimer's disease (AD) is a progressive, devastating neurodegenerative disorder characterized by cognitive decline and neuropsychiatric symptoms that impacts the world's rapidly aging population, resulting in neurological damage that is thought to be irreversible by the time symptoms are evident. To help support earlier AD diagnosis, in this project we aim to identify specific microRNAs (miRNAs) that can predict AD development and to explore the potential viability of targeting these miRNAs to disrupt AD progression. We will further explore the ability of cannabidiol (CBD), which is an interventional drug with the potential to be immediately translated to patient treatment, to protect against AD-related neurodegeneration by modulating miRNA expression in the hippocampal-prefrontal pathway, providing an invaluable foundation for the early diagnosis and treatment of patients before the onset of AD-associated dementia symptoms.

**Specific aims**

**Background** No effective treatments for Alzheimer’s disease (AD) have yet been established, underscoring the need to identify novel effective compounds that can counteract the course of this debilitating disease. The establishment of a validated noninvasive biomarker of AD or associated targets will guide the design of early diagnostic tools, preventive strategies, and effective pharmacological interventions for AD-related dementia. MicroRNAs (miRNAs) have emerged as important regulators of AD pathogenesis and promising biomarkers in a range of pathological contexts. Other work has suggested that they phytocannabinoid cannabidiol (CBD) can provide symptomatic relief or slow AD progression through a range of neurogenic, antioxidative, and anti-inflammatory activities. As such, further work is needed to clarify the mechanisms whereby CBD can disrupt the pathogenesis of AD and for the translation of extant preclinical work into clinical settings, providing symptomatic relief to affected patients. Whether CBD influences AD through miRNA-mediated mechanisms also remains to be assessed.

**Objectives** The goal of this study is to characterize miRNA dysfunction in the hippocampal-prefrontal (HPC-PFC) pathway in AD model rats, to determine whether modulating the expression of specific miRNAs can attenuate or inhibit AD-related neurodegenerative processes, and to assess whether the phytocannabinoid CBD can attenuate associated cognitive and emotional symptoms through miRNAs silencing or activation.

**Hypotheses** We hypothesize that (i) AD is associated with impaired cognitive and emotional behaviors and the abnormal expression of miRNAs and AD-associated genes and proteins in the HPC-PFC, (ii) the early stages of AD development are associated with abnormal miRNA expression patterns in the peripheral blood, (iii) CBD can ameliorate AD-induced cognitive and emotional symptoms and AD-related changes in gene and protein expression, (iv) the activation or inhibition of specific miRNAs can protect against AD-related disease phenotypes, and (v) the neuroprotective benefits of CBD are at least partially mediated by miRNAs. To test these hypotheses, we propose three **Specific Aims**:

Aim 1: To identify AD-related patterns of miRNA, mRNA, and protein dysregulation in male and female rats that may be reversed by CBD treatment, and to explore the utility of peripheral miRNAs and inflammatory cytokines as biomarkers of AD progression and treatment responses.

Aim 2: To determine whether the HPC-PFC pathway, which is closely related to executive function and neuropsychiatric symptoms, plays a fundamental role in shaping the abnormal cognitive and emotional behaviors associated with AD.

Aim 3: To explore whether inhibiting or activating specific candidate miRNAs in the HPC-PFC can reverse AD-related cognitive and emotional dysfunction, and to determine whether CBD can protect against AD phenotypes through miRNA-mediated mechanisms.

Together, we expect that these experiments will clarify the therapeutic potential of specific miRNA activation/silencing as a means of improving memory and emotional deficits in AD, and to clarify the role that miRNAs play in mediating the beneficial effects of CBD in male and female AD model rats, thus revealing the mechanisms by which CBD slows neurodegeneration.

**Future directions** Current therapies only provide limited symptomatic relief and are ineffective in preventing AD progression. As miRNAs function as key regulatory hubs that can influence several genes and entire biological pathways, they represent attractive candidates for drug development. These miRNAs thus exhibit enormous potential for use as biomarkers for the early detection and analysis of AD disease severity, in addition to their possible therapeutic value. Several barriers to the use of miRNAs as therapeutic agents remain, including the need to develop drug formulations with increased stability and *in vivo* bioavailability. The widespread effects of miRNAs on gene regulation must also be taken into account. Accordingly, clarifying which miRNAs strongly influence AD pathogenesis in preclinical model systems may guide their clinical therapeutic development. While the silencing and activation of miRNAs in specific brain regions is invasive and only viable in animal model systems, our results will provide a robust framework for future studies of the delivery of specific miRNAs of interest via a safe, specific route.

**Other parts**

The combined budget for direct costs for the two-year project period may not exceed $275,000. No more than $200,000 may be requested in any single year.

**Earliest Start Date** April 2023, The project period may not exceed two years.

Start date: July 2023

End date: June 2025

**FACILITIES & RESOURCES**

**Contribution of the scientific environment to the probability of success**:

The University of Haifa (UoH), located on Mount Carmel, was founded in 1963 to operate under the academic auspices of The Hebrew University of Jerusalem. In 1972, the University of Haifa declared its independence and became the sixth academic institution in Israel and the fourth university. The University of Haifa is the largest comprehensive research university in Israel's northern region. Its mission is to cultivate academic excellence, create a shared Israeli experience, and promote democratic values in an environment of tolerance and multiculturalism. Such an environment contributes to outstanding research and a community of exceptional, creative, and productive alumni. Over 18,000 students study there for undergraduate, graduate, and doctoral degrees. The University of Haifa is fully committed to academic excellence, which is expressed in its many and diverse interdisciplinary and international programs and collaborations with academic institutions around the world.

The PI’s lab is part of the School of Psychological Sciences. Neuroscience research at the School of Psychological Sciences of the University of Haifa is a unique enterprise, focusing on the interface between behavior and its neural substrates in psychopathology, psychiatric and neurodegenerative disorders. Each of the research groups, ranging across diverse neuroscience disciplines and model systems, focuses on particular behaviors and seek to unveil their underlying mechanisms. These explorations are conducted on multiple levels, from the molecular and cellular mechanisms to the study of whole neuronal systems. The methods employed by the School’s faculty include molecular and cellular biology, genetic manipulations, microscopy, in vitro and in vivo electrophysiology and human functional imaging. Together, illuminating topics in learning and memory, and cognitive processes in health and disease from diverse angles, ultimately aiming at understanding the neuronal processes yielding complex behaviors. The experiments for the proposed research project will be carried out in Prof. Akirav’s Learning and Memory Lab.

**Laboratory**

The Learning and Memory Lab, directed by Prof. Irit Akirav, is affiliated to the School of Psychological Sciences and to the Integrated Brain and Behavior Center (IBBRC), University of Haifa, Israel. The research aim of this lab is to understand the neural mechanisms underlying the involvement of the endocannabinoid system in psychiatric disorders. Since the research is inherently multi-disciplinary, research approaches are combined from the fields of biology, and psychology, integrating data ranging from the molecular to whole animal level. The lab is 80m² and includes a room dedicated to biochemistry and molecular biology, several rooms for behavioral tests, and an electrophysiology room. We also use shared space in the labs’ complex for equipment.

The lab includes:

Staff

The lab includes: Staff:

Prof. Irit Akirav (Head of lab),

Lab manager

A research associate

4 Ph.D. students

3 M.A. students

3 research assistants

The lab is fully equipped with: Workstations for students, behavioral settings, 3 in vivo electrophysiology systems, Two lab-owned fully motorized stereotaxic apparatuses, Western blot equipment, and access to the departmental RT-PCR, Western blot reader…..incubators….. SHIRA

Computer: All lab members have their own personal computer with Windows 10 OS.

Akirav lab: Computer for each student + computers for equipment (total ## computers). We have licenses for all standard Office software on all computers, and licenses for Adobe Illustrator, GraphPad Prism 6.01, InStat Software (GraphPad Software, CA, USA), MATLAB, and SPSS.

**FOREIGN JUSTIFICATION**

The proposed project will be conducted by Prof. Irit Akirav, located and operating in the School of Psychological Sciences at the University of Haifa. Prof. Akirav comes with unique proprietary data, expertise, and knowledge available to her lab, for the purposes of this project.

The strength of this project is its comprehensive investigation of the research questions, using a wide range of methods, from the classical like behavioral testing, immunochemistry and pharmacology, to those at the forefront of science, like viral mediated gene transfer and chemogenetics. Such an approach is bound to yield a comprehensive picture where, if the hypotheses are correct, the outcomes from the different experimental approaches support each other, allowing greater confidence in the conclusions.

Suitability of Investigator Prof. Akirav is highly suitable for that study. For many years her research if focused on the neuronal, hormonal and molecular basis of emotions and memory. She has numerous publications on the role of the cannabinoids in regulating behaviors in models of psychiatric disorders such as stress, anxiety and post-traumatic stress disorder. Her lab is very well equipped to perform the present study, and her preliminary data presented in the proposal attest to her perfect suitability to conduct this project.

*The PI is an established researcher of the effects of cannabinoids in animal models. This proposal is considerably strengthened by the consultants, whose labs have the expertise to assist with the viral vector and chemogenetic approaches included in this proposal*

**BUDGET JUSTIFICATION (TOTAL $ for 2 years)**

a) Irit Akirav, Ph.D.: Prof. Akirav will be the Principal Investigator in this research proposal. She will have overall responsibility for the project with a special emphasis on leading the scientific team. Prof. Akirav brings unique expertise to this study through her vase expertise in bridging between behavior and neural mechanisms underlying learning and memory and emotions. Prof. Akirav will dedicate 1.2 calendar months per year to working on this project.

**Other Personnel**

b) Post doc or advanced PhD student (12 calendar months per year): A PhD student (to be recruited) will be in charge of all experiments. The PhD student will be responsible for brain microinjections and performing the molecular analysis.

c) MA student (8 calendar months per year): An MA student (to be recruited) will be responsible for behavioral experiments and pharmacology.

d) Research assistant (8 calendar months per year): A research assistant will help with the behavioral tests, i.p. injections and punching brain tissues.

**Travel**

One trip for Prof. Akirav to travel to a conference in Europe to present our findings in year two of this project. Expenses will cover the anticipated airfare and per diem cover of accommodations, meals, ground transportation, and incidentals (Cost: $2,500)

**Other Direct Costs**:

• Materials & Supplies:

Rats: We request $6,500 in year one and $6,500 in year two of this project for rat purchase and maintenance (adult and middle aged males and females, long periods of maintenance).

We request $80,000 in year one and $65,000 in year two of this project for biochemical reagents including reagents, primers, antibodies, agomirs, antagomirs, viral vectors for DREADDS, ELISA kits, western blot reagents including gel preparation antibodies, membranes, chemiluminescence reagent and general lab supplies, including: gloves, tips, tin foil, saran wrap, kimwipes, and lab glassware.

We request $2,000 per year for using departmental equipment (e.g. RT-PCR unit).

**Publication Costs**

 In accordance with NIH data sharing policies, the project’s results, collected and edited, will be published annually. We plan for one or two peer-reviewed publications for this project: 2,500 $.

**Indirect cost**

Indirect costs have been calculated at 8% of the indirect cost base that includes all costs for each Budget Period, in accordance with NIH policy for foreign applicants.

1

100 rats- M, F

Pharmacological agents (CBD, STZ, aCSF)

miRNA, mRNA, proteins, ELISA kits

2

100 rats

DREADDS

IHC

3

200 rats

Agomirs antagomirs

Pharmacological agents (CBD, STZ, aCSF)

miRNA, mRNA, proteins, ELISA kits