**Research strategy- 6 pages Innovation. Approach (research design, add preliminary results here)**

Progressive impairment of memory and cognition is a key clinical feature of **Alzheimer’s disease (AD**), which is characterized by extracellular **amyloid β-protein (Aβ)** deposits in the brain (plaques), intraneuronal tau pathology, neuronal cell death, and inflammation that ultimately manifest in the form of neuropsychiatric symptoms including depression and anxiety (Galts et al., 2019; Mendez, 2021). Around 95% of hospitalized patients with AD have the sporadic form of the disease known as the late-onset AD (Diniz et al., 2017). No effective cure for AD has been established to date, underscoring the need to identify novel effective compounds that can counteract the AD course.

Dementia is more than twice as common in women as in men, even at middle age (Chêne et al., 2015; Koran et al., 2017). Estrogen regulates key processes implicated in AD pathogenesis, in particular the accumulation of β-amyloid protein (Scheyer et al 2018). Understanding the biology of sex differences in cognitive function will not only provide insight into AD prevention, but also is integral to the development of personalized, sex-specific medicine (Li and Singh, 2014).

A fundamental problem in AD is accurate diagnosis as it relies on the clinical criteria and presence of symptoms such as memory loss and cognitive difficulties (Sabbagh et al., 2017). In the search for better biomarkers, epigenetic modifications have emerged as important players in the pathogenesis of AD, with potential implications for treatment (Perkovic et al., 2021). **MicroRNAs (miRNAs(** are short noncoding RNAs that modulate gene expression and are closely linked to AD pathogenesis (Kou et al., 2020). Importantly, miRNA expression is differently expressed in AD patients compared to control subjects (Perkovic et al., 2021), suggesting a possible role as a biomarker and/or a novel therapeutic target for AD. miRNAs can be detected in the peripheral circulation or at brain tissues. Several miRNAs (e.g. miR-9, miR-29abc, miR-34a, miR-107, miR-125b, miR-132, miR-146a, miR-155) have a significant biomarker potential as they are highly associated with AD and detected peripherally and in the CNS (Perkovic et al., 2021). However, the mechanisms underlying how miRNAs may slow neurodegenerative processes are largely unknown.

The hippocampus-prefrontal cortex (**HPC-PFC**) pathway 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 AD, depression and anxiety disorders (Kovner et al., 2019; Ruggiero et al., 2021). Exposing hippocampal and cortical neurons to Aβ peptide activates glycogen synthase kinase 3β (GSK-3β) and thereby drives Wnt/β-catenin signaling pathway degradation, contributing to neurofibrillary tangle formation and impaired neuronal survival. One of the target genes of β-catenin is Dicer that encodes the Dicer protein, which is involved in the cleaving and formation of all miRNAs. Hence, miRNAs are modified as a function of AD whereupon they can regulate genes involved in the Wnt signaling pathway. Dysregulation of miRNA expression in AD and depression has been reported in patients’ CSF and in the hippocampus in animal models (Bisrat et al 2016; Teo and Soga, 2018 Chopra et al 2020; Muller et al 2014’ Walgrave et al 2021).

**Cannabidiol (CBD)** is a safe, non-psychoactive phytocannabinoid that reportedly exhibits immunomodulatory activity in neurodegenerative disease, may ameliorate the symptoms of AD and retard cognitive decline (Li et al., 2020). CBD is able to promote PI3K/Akt signaling, which in turn inhibits GSK-3β. The GSK-3β inhibition induces an increase of the Wnt/β-catenin pathway, thus exerting a neuroprotective action against the oxidative stress and neurotoxicity induces of Aβ in AD (Cassano et al., 2020). In line with this evidence, in an *in vitro* model of AD, CBD treatment suppressed the hyperphosphorylation of tau protein-mediated to β-catenin and GSK-3β, in Aβ-stimulated PC12 neuronal cells (Esposito et al., 2006). Moreover, CBD decreased Aβ levels in SH-SY5Y cells transfected with the amyloid precursor protein (SH-SY5YAPP+) (Scuderi et al., 2014), and, in a mouse model, CBD administration ameliorated cognitive impairment (Cheng et al., 2014). In a recent study, CBD enhanced interleukin (IL)-33 and triggering receptor expressed on myeloid cells 2 (TREM2), which were associated with improved cognitive function and ameliorated AD symptoms (Khodadadi et al., 2021).

As there is an urgent need to develop new medications with a disease-modifying effect to reduce the progress of AD, the aims of this study are to improve diagnosis and early treatment of AD. In our planned studies, we propose to examine in male and females rats, AD-induced alterations in miRNAs associated with AD and related regulatory mechanisms in the HPC-PFC pathway and to provide novel therapies against AD in the short- (CBD) and long-term (miRNAs).

In our **first experiment**, CBD is administered in a streptozotocin (**STZ**)-induced rat model of sporadic AD, after which cognitive and emotional function are correlated with alterations in the expression of miRNAs in the HPC-PFC pathway, as well as targets related to inflammation, CBD signaling, AD pathology and β-catenin. We also investigate peripheral miRNAs and inflammatory cytokines as potential biomarkers of AD 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 microRNAs as biomarkers of AD. Male and female STZ rats may demonstrate similar cognitive and emotional patterns of dysfunction, but may show differential sex- and brain region-dependent alterations in the expression of miRNAs in response to STZ and to CBD treatment (Manolli and Tollkuhn, 2018).

In our **second experiment** we ask whether HPC input to PFC is central in controlling the behavioral phenotype in AD rats; to that end we will use a chemogenetic dual-virus approach (Boender et al 2014) to selectively inhibit the glutamatergic projections from HPC to the PFC and examine whether reducing glutamate release can induce cognitive decline and emotional dysfunction as observed in our STZ rats. This would suggest that the HPC-PFC pathway plays a fundamental role in abnormal cognitive and emotional behaviors associated with AD.

In our **third experiment**, we will explore whether different miRNAs are critically involved in AD-related cognitive and emotional dysfunction and the therapeutic effects of CBD by using agomirs and antagomirs to activate or inhibit specific miRNAs in the HPC-PFC pathway; after which the association between changes in miRNA expression, cognitive/emotional pathology, inflammatory markers, CBD targets, AD pathology-related targets, and β-catenin will be assessed.

Together, these experiments (i) may suggest that targeting specific miRNAs (activating or silencing) may have therapeutic potential to restore memory and emotional deficits, and (ii) may define miRNA-regulated therapeutic role of CBD in the treatment of AD in males and females and thus propose how CBD may slow neurodegenerative processes. 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 could increase the likelihood for drug success. CBD may have a therapeutic effect via mediation of microRNAs, but probably also through other different pathways. Elucidating specific microRNAs that predict the disease before the appearance of plaques and tangles will allow the development of specific drugs that will block the development of AD. Perhaps a combination of both drugs (CBD and miRNA-based) may prove to be even more effective.

 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?*

AD is the world's leading cause of dementia and the population of patients with AD continues to grow. However, none of the available treatments for AD prevent or reverse the progression of the disease but rather they treat the disease symptoms with limited efficacy and have been associated with adverse effects (Salomone et al., 2012). Hence, finding non-invasive and readily available biomarkers for early diagnosis of AD as well as finding drug treatments that are disease-modifying, and not only attenuating symptoms, are of crucial importance.

CBD may represent a clinically-safe and efficacious disease-modifying therapy to attenuate neurocognitive decline and emotional dysfunction; findings from this study may suggest that CBD may slow neurodegenerative processes through a bi-directional dialogue with miRNAs and Wnt/β-catenin signaling. A drug that works on a single pathway will probably not diminish the complex cascade that leads to AD (Watt and Karl, 2017). CBD has a wide range of targets which indicates on its potential as a multimodal drug for AD treatment. The translation of our findings into clinical setting could be quite rapid as CBD is readily available, appears to only have limited side effects (Bergamaschi et al., 2011) and is safe for human use (Leweke et al., 2012). Our study will provide "proof of principle" that CBD is a valid candidate for novel AD therapies.

miRNAs regulate a variety of processes related to AD and may provide wider-ranging benefits than the available treatments. miRNAs are interesting therapeutic targets because of their ability to regulate the endogenous gene expression with the possibility of only one miRNA regulating entire biological pathways. Therefore, miRNA-based therapy can be highly relevant in AD, in which the cause is complex and related to a number of genes and biological processes. The therapeutic effects might be achieved by completely or partially eliminating the function of the miRNA of interest in order to downregulate the expression of targeted genes and proteins that are involved in disease pathogenesis. The findings from this study may provide new insights into biomarker candidates for the early diagnosis of AD so that we can use non-invasive and readily available peripheral miRNAs biomarkers for early diagnosis, and perhaps start treatment before the outbreak of the disease. While the delivery procedure of agomir or antagomir miRNAs’ silencing and activation into the brain is invasive and only applied in animal studies, our study can identify specific miRNAs to be delivered in a safe and specific route.

We use different drugs without a clear idea of how they work, which targets they hit, what processes they alter and which of these actions are required for therapeutic efficacy. Nevertheless, understanding a drug's mechanism in animal models could guide drug development and help to prevent failures in the transfer to clinical trials. Learning how a drug works can help stratify clinical trials to focus them on those patients most likely to respond. Moreover, identifying specific markers in males and females can guide the development of personalized, sex-specific medicine.

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?*

Early diagnosis of AD will enhance our understanding of the pathological mechanisms underlying the disease, and support the development of innovative preventive strategies. As there is no effective cure for AD, and the treatments available can reduce only the symptoms in the initial phase of the disease, it is of paramount importance to identify novel effective compounds for counteracting the AD course and treat the disease. Our research will refine existing approach by significantly impacting early diagnosis of AD, elucidation of its pathophysiological mechanisms, and using intervention strategies in the short- and long-term. CBD treatment can be immediately transferred to humans to relief symptoms while research on the activation and inhibition of miRNAs progresses.

**Approach**

**Experiment 1:** Reviewing the role of miRNAs in AD puts forward several miRNAs implicated in regulating multiple proteins/processes involved in dementia (including: miR-9, miR-29abc, miR-34a, for review see: Perkovic et al., 2021). However, the molecular and cellular mechanisms underlying how miRNAs may slow neurodegenerative processes are largely unknown. Here, male and female adult and middle-aged rats are subjected to a rat model of AD, using intracerebroventricular (icv) injection of STZ, a widely used method for modelling neuroinflammation and neurodegenerative processes. We have preliminary data demonstrating impaired performance of STZ adult male and female rats in the objet recognition and object location tasks (figure 1). STZ rats are treated with vehicle or CBD and assessed for cognitive and emotional function. Preliminary data from our lab in female naïve middle-aged (15 months) rats demonstrate that CBD may be beneficial for the elderly in the relief of emotional function and improvement of memory performance (figure 2).

*Add figure 2 from Nadya: CBD may be beneficial for the elderly*

*Add figure 1 from Shira: Impaired performance in STZ rats in the object recognition and object location tasks*

Cognitive and emotional function will be correlated with alterations in the expression of **miRNAs in the HPC-PFC pathway** and several interacting systems and targets including **inflammation markers** [inducible nitric oxide synthase (iNOS), glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (Iba-1), and arginase-1 (ARG-1)], **CBD main targets** [CB1 and CB2 receptors, the enzyme that hydrolyzes the endocannabinoid anandamide: fatty acid amide hydrolase (FAAH), and 5HT1a serotonergic receptor), **markers associated with AD pathology** [Aβ protein, phosphorylated Tau (p-Tau Ser396) protein], and **β-catenin**. We will also investigate **peripheral miRNAs as potential biomarkers** of AD and treatment response and **pro-inflammatory cytokines** (IL-1b, IL-6 and TNF-α) in whole blood and correlate these alterations with the ones observed in the brain. Findings from this initial experiment will identify significant dysregulation of genes that are associated with AD and that may be restored with CBD treatment. Peripheral microRNAs have the potential to be used as biomarkers of AD and to predict response to treatment. Preliminary data from our lab suggest that CBD restores stress-induced increase in miR-16 and miR-135 levels in the medial PFC in a rat model for depression [unpredicted chronic mild stress (UCMS)] (figure 3).

*figure 3 from Uri demonstrating that CBD normalized stress- induced increase in miR-16 and miR-135 levels in the medial PFC in a rat model for depression*.

CBD normalized UCMS- induced increase in miR-16 and miR-135a levels in the mPFC. Rats exposed to UCMS + Veh demonstrated increased miR-16 and miR-135a expression compared to No UCMS-Veh (p<.001) and to UCMS-CBD (p<.01) rats (n=8-10 in each group).

Research design of experiment 1:



To explore the neuro-protective effects of of CBD on AD onset and progression in STZ rats, CBD is injected for two weeks after STZ or repeatedly (after STZ, at 3 and 6 months). Adult (2 months) and middle-aged (15 months) male and female rats are randomly divided into 4 groups: aCSF+Vehicle, aCSF+CBD, STZ+Vehicle and STZ+CBD. Rats receive ICV injection of STZ or artificial cerebrospinal fluid (aCSF; day 0) to the left ventricle. One day after the stereotaxic procedure (day 1), all rats receive daily i.p. injections of CBD (10 mg/kg) or Vehicle for 14 consecutive days. Tail blood is collected on day 1 and day 14. Behavioral tests are performed from Day 15 to Day 29 at the following order: open field (OF; day 15), object location [OL; day 19, after 3 days of habituation (HABIT) to the arena], novel object recognition (NOR; day 20), episodic-like memory (ELM, day 21), social preference and social recognition (day 22), elevated plus maze (EPM, day 23), Morris water maze (MWM, days 24-28). At the end of the experiment (day 30), brains and blood are collected for biochemical analysis. In another set of rats, adult (2 months) males and females receive ICV injection of STZ or aCSF to the left ventricle (day 0). Then, rats are treated with CBD (10 mg/kg) or Vehicle for 14 days, followed by behavioral and cognitive evaluations as described. The treatment and behavioral assessment are performed three times during the experiment, at the following time points: one day after the stereotaxic procedure (day 1), 3 months afterwards and 6 months thereafter. At the end of the experiment, rats’ tissues will be collected for biochemical assessment.

**Experiment 2:** A monosynaptic unidirectional projection of neurons extending from the ventral subiculum of the HPC to the medial PFC [mPFC, infralimbic (IL), prelimbic (PL)] is critically involved in executive functions and emotional regulation (Godsil et al., 2013). Hence, disruptions in HPC-PFC functional connectivity using Designer Receptors Exclusively Activated by Designer Drugs (DREADD)-based chemogenetic tools can contribute to cognitive decline and neuropsychiatric symptoms such as depression and anxiety. Chemogenetic technology is frequently used to investigate the neural mechanisms of behavior (Smith et al., 2016), and is particularly suitable for studying the role played by HPC-PFC excitatory transmission since: (i) it allows for cell-type specificity in distinct anatomical brain regions, through the use of viral constructs carrying a cell-type specific promoter (e.g., CamKII); (ii) it enables relatively long-term reduction in activity [up to 6 hrs (Zhu et al., 2014)], which is particularly suitable for conducting a series of behavioral studies; and (iii) rather than complete excitation or inhibition of all cells in a desired population, DREADD-induced changes lead to more physiologically meaningful changes in the patterns of activity, since their effects are mediated by endogenous intracellular signaling mechanisms (Armbruster et al., 2007). Our preliminary studies, collaborating with Prof. Inna Gaisler-Salomon at our department, indicate that a DREADD-carrying virus can be directed to a specific cell population and expressed following viral delivery (figure 4).

10x magnification of fluorescence following injection of ssAAV-8/2-mCaMKIIa-hM4D(Gi)mCherry-WPRE-hGHp(A) (0.3 microL) into the hippocampus. Brains were perfused 21 days post injection. 35micron slices were cut and stained with 0.5% DAPI.



Research design of experiment 2:



A Cre-dependent AAV2 vector (AAV2-hSyn-DIO-hm3D-mcherry, 5.5 × 1012 vg/ml; 0.5 μl/side at 0.1 μl/min) will be injected bilaterally into the ventral subiculum. The retrograde-traveling CAV2-Cre virus (1–1.2 × 1012 vg/ml; 0.75 μl/side at 0.15 μl/min) or a control mCherry virus (1–1.2 × 1012 vg/ml; 0.75 μl/side at 0.15 μl/min) will be microinjected into the IL or PL (in different groups) (Boender et al., 2014; Kerstetter et al., 2016; Marchant et al., 2016). Rats will be allowed to recover for 4 weeks before STZ to allow for robust gene expression in target regions. The DREADD agonist clozapine N-oxide (CNO; dissolved in drinking water at a concentration of 0.25 mg/ml; Carvalho Poyraz et al 2016) is chronically administered to rats within drinking water for 2 weeks after which behavioral testing will be performed without CNO )from Day 15 to Day 28, as described in Experiment 1(. Also, sham/CNO controls will be included since the CNO metabolite clozapine may affect physiology and behavior by binding to receptors other than DREADDs (Gomez et al 2017; MacLaren et al 2016). The experiment will thus consist of the following groups in aCSF and STZ injected rats: hM3D (HPC) –Cre (IL); hM3D (HPC) –Veh (IL); hM3D (HPC) –Cre (PL) and hM3D (HPC) –Veh (IL). Rats are decapitated at the end of behavioral testing. We will use immunohistochemistry analysis to verify virus expression. We hypothesize that glutamatergic inhibition in these pathways may induce AD-like alterations in cognitive and emotional function, suggesting that the HPC-PFC pathway is crucial for these effects.

**Experiment 3:** miRNAs are crucial regulators of gene expression and promising candidates for biomarker development. MiRNAs activation or silencing may be promising candidates for cognitive disorders and dementia (Bahlakeh et al., 2021). To determine whether specific miRNAs can have therapeutic-like effects in STZ rats and to determine whether the therapeutic-like effects of CBD are mediated by specific miRNAs, we will use a viral approach to inhibit/activate specific miRNAs in the HPC-PFC pathway. We have preliminary findings that microinjecting antagomir-16 (anti-mir, 20 nm) into the right ventricle significantly decreases the expression of mir-16 in the PFC (figure 5). Based on the findings from experiment 1, a specific miR will be activated or inhibited to ameliorate STZ-induced alterations in cognitive and emotional function. In another set of rats, a specific miR will be activated or inhibited to exacerbate STZ-induced alterations in memory and emotional function or to block therapeutic-like effects of CBD in STZ males and females.

Rats were microinjected with antagomir-16 (anti-mir, 20 nm) into the mPFC and decapitated after one week. Left:  histological verification of the site of microinjection. Right: A significant decrease was observed in the expression of mir-16 in the PFC (\*, p<0.05).



Research design of experiment 3:

Adult (2 months) and middle-aged (15 months) male and female rats receive ICV injection of STZ or aCSF to the left ventricle and an agomir or antagomir to the right ventricle (day 0) and the experiment continues as in Exp. 1. Rats are decapitated at day 30 and the association between changes in miRNA expression, cognitive/emotional pathology, inflammatory markers, CBD targets, AD pathology-related targets, and β-catenin will be assessed. In case we find that silencing/activating miRNAs does not ameliorate STZ-induced dysfunctional cognitive and emotional function, we will increase Wnt/β-catenin pathway by blocking the phosphorylation of GSK-3β or overexpressing β-catenin in the HPC-PFC pathway.

 **Methods:**

**Subjects:** Adult (60 days old) and middle-aged (15 months)male and female Sprague-Dawley rats are grouped housed at 22 ± 2°C under 12-hour light/dark cycles (lights turned on at 07:00). Rats are allowed water and laboratory rodent chow ad lib. Appropriate measures are taken to minimize the number of animals used and their suffering.

**STZ model for sporadic AD:** Rats are anaesthetized with a mixture of ketamine/xylazine solution and placed in a stereotaxic frame (Stoelting). STZ (3 mg, 10μl) or aCSF are ICV injected to the left ventricle (AP: −0.8 mm, ML: +1.5 mm, DV: −3.6 mm from dura). **Agomir and Antagomir** are ICV injected to the right ventricle (20 nmol). For **chemogenetic inhibition** viruses are delivered to the ventral subiculum. The injection volume and flow rate are controlled by a micromanipulator (2 µL/min; Viral Vector Facility, ETH Zurich), after which the syringe needle remains in place for additional 10 min to prevent reflux.

**Behavioral battery** (Abush and Akirav, 2012; Bauminger et al., 2022;Burstein et al., 2018):The **OF** test assess general locomotor function (total distance, cm, divided into 5 min bins) and novelty-induced anxiogenic behavior (time in arena center, first 5 min). The **NOR** test, with an inter-trial interval (ITI) of 5 min, is used to measure novelty recognition and working memory. The **OL** version of this test assess spatial memory. We assess total exploration time (s) and the mean discrimination-index (DI), calculated as TN/TN+TF (TN = novel object exploration time, TF = familiar object exploration time) in the test phase. In the **ELM**, animals spontaneously explore an environment and attempt to associate an object (What), its location (Where) and the temporal context (first or second occurrence – When). The task consists of two sample trials and a test session of object exploration of 5 min each. 1h intervenes between each trial. Compared to samples, objects could be stationary (A1 and B1) or in different locations (A2 and B2). Animals tend to explore A1 > B1 (“temporal pattern”), B2 > B1 (“spatial pattern”) and A1 > A2 (“integrative pattern”). A1: old stationary; A2: old displaced; B1: recent stationary; B2: recent displaced (Chao et al., 2014). The **SP and SR** task includes two phases: first 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). The **EPM** 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. The **MWM** test is also a test of spatial learning that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform.

**Drugs:** CBD (10 mg/kg), freshly prepared and administered in 1 mL/kg of vehicle. The dose is based on previous work (Campos et al 2013) and our preliminary results.

**Real-time (RT) PCR and western blots (WB):** Brain tissues are collected to detect miRNAs expression. A 50–100 mg tissue sample is mixed with 1ml Trizol (Invitrogen) to extract total RNA from frozen samples. One microliter of RNA is used to measure the expression of miRNAs using RT-PCR. The expression of U6 is used as internal control. The expression of miRNAs is calculated according to the threshold cycle (CT). The CT of the target gene for each sample is corrected by subtracting the CT of the internal control (ΔCT). The controls are chosen as reference samples with mean ΔCT for the control samples being subtracted from the ΔCT for all experimental samples (ΔΔCT). Finally, the relative expression levels are calculated as 2−[(CT of a specific miRNA) − (CT of U6)].

Rats are euthanized and brain tissues of the IL, PL, ventral subiculm and dorsal hippocampus are harvested and whole blood is collected for biochemical analysis. RNA extraction, cDNA preparation and qRT-PCR are performed as previously described (Bauminger et al., 2022) to detect the expression of miRNAs and mRNA; protein levels are determined using WB as previously described (Alteba et al., 2021). miRNAs: miR-9, miR-29abc, miR-34a, miR-107, miR-125b, miR-132, miR-146a, miR-155Mrna; **CBD main targets**: cannabinoid receptors 1 and 2 (*CNR1* and *CNR2*, respectively), FAAH, the enzyme that hydrolyzes the endocannabinoid anandamide, and the serotonergic receptor 5HT1A (*htr1a*). **Proteins markers associated with AD pathology**: Aβ protein, phosphorylated Tau (p-Tau Ser396) protein; **β-catenin** (1:5,000; abcam, UK; ab32572 [E247]). We will also investigate **peripheral miRNAs as potential biomarkers** of AD and treatment response and **pro-inflammatory cytokines** (IL-1b, IL-6 and TNF-α) in whole blood. For WB, samples are resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Blots are incubated with the specific primary antibodies (abcam) overnight at 4°C, followed by washing and 1h incubation with an HRP-linked secondary antibody. Protein samples are standardized with β-actin (cell signaling; 1:1,000).

**Estrus cycle and estrogen (females):** The estrous cycle is examined daily in the morning by collecting vaginal cytology samples as previously described (Zer-Aviv and Akirav 2016). Levels of pro-inflammatory cytokines IL-1b, IL-6 and TNF-α in whole blood are evaluated using sandwich ELISA according to the manufacturer’s instructions (Abcam and R&D systems).