INVESTIGATIONS INTO THE RELATIONSHIP BETWEEN MENOPAUSE AND ALZHEIMER'S DISEASE PATHOLOGY IN A MOUSE MODEL WITH OVARIAN FOLLICLE DEPLETION

By

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Abstract:

Alzheimer's Disease (AD) currently affects about 6.9 million Americans, with over two-thirds of those individuals being women. Interestingly, changes in the brain indicative of AD coincide with the onset of menopause. Post-menopausal women have significantly higher levels of AD pathologies, including amyloid beta and hyperphosphorylated tau, than pre-menopausal women. To investigate the relationship between AD pathologies and menopause, we induced menopause-like state in an AD mouse model, APP/PS1, by treatment with 4-vinylcyclohexene diepoxide (VCD) to accelerate ovarian follicle depletion. To monitor the transition to menopause, vaginal lavages and cytology was performed to determine if mice reached anestrous. Both female APP/PS1 mice and controls reached anestrous and tissues were evaluated for AD related changes. Female APP/PS1 mice treated with VCD had significantly reduced levels of AB-42 in the hippocampus. Overall, this study demonstrates the utility of using VCD in AD study to investigate the association with menopause. These studies could further research into novel biomarkers and therapeutics for women with AD.

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Table of Contents

List of Tables

List of Figures

1. Introduction and Background

1.1 Overview of Alzheimer's Clinical Presentation and Pathology

An estimated 6.9 million Americans, age 65 or older, are currently living with Alzheimer's Disease (AD) (Alzheimer's Association, 2024). By 2060, this number is predicted to almost triple to 14 million in the U.S. (Matthews et al., 2019). Of those affected, about two-thirds are women, making sex a risk factor for the disease (Alzheimer's Association, 2024). The lifetime risk of a man over the age of 65 to develop AD is 11.6%, while women have a risk of 21.2% (Aggarwal & Mielke, 2023). Clinically, AD is described by progressive cognitive decline, including memory loss, difficulty completing familiar tasks, confusion, and changes in mood and personality (Alzheimer's Association, 2023a).

Pathologically, AD is characterized by the presence of senile plaques composed of beta-amyloid (Aβ), neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein (p-tau), and chronic neuroinflammation. The amyloid precursor protein (APP), involved in cell health and growth, is degraded by enzymes alpha-secretase and gamma-secretase in a nonamyloidogenic pathway (O'Brien & Wong, 2011). In AD, APP is cleaved at a different site via beta-secretase and gamma-secretase, producing insoluble peptides of Aβ (Chow et al., 2010). Amyloidogenic processing produces two Aβ isoforms that are toxic to neurons, Aβ-40 and Aβ-42 (Chen et al., 2017). Insoluble Aβ peptides bind together chemically to form oligomers, fibrils, and ultimately senile plaques (Chen et al., 2017). Aβ plaque accumulation disrupts cell signaling and communication (O'Brien & Wong, 2011) and is observed in patients over a decade prior to the onset of clinical symptoms, suggesting Aβ as an early target to prevent AD (Jack et al., 2013).

The presence of p-tau pathology has a greater relationship with cognitive decline (Ossenkoppele et al., 2016). Neuron stabilization and trafficking of resources is maintained by microtubules, consisting of tau protein. Phosphorylation of tau allows for detachment from microtubules, enabling the trafficking of

cargo throughout the cell. In turn, dephosphorylation of tau is critical for reattachment and re-stabilization. In AD, tau protein becomes hyperphosphorylated (p-tau) and cannot reconnect to the microtubules, resulting in the collapse of structure and stabilizing proteins. These debris accumulate into NFTs and ultimately lead to neurodegeneration. Phosphorylation of specific epitopes have been investigated as strong candidate biomarkers for AD, with particularly promising data currently in p-tau 181 and p-tau 217 (Thijssen et al., 2021). Additionally, total tau (t-tau) levels are accepted as a biomarker for neurodegeneration (Jack et al., 2018).

Chronic neuroinflammation is another hallmark of AD, as observed in several other neurodegenerative diseases, including Parkinson's Disease, Huntingtin's Disease, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) (Amor et al., 2010; Kinney et al., 2018). Inflammation is vital to the central nervous system (CNS) for protection against insult, sustaining a healthy environment, and allowing adequate brain function (Yong et al., 2019). Microglia are the resident immune cells of the CNS, responsible for the regulation of brain development, supporting neuronal networks, and protection against disruption and attack (Colonna & Butovsky, 2017). In a resting state, microglia are ramified, using processes to communicate with other cells and monitor the environment (Augusto-Oliveira et al., 2019). When microglia are activated, such as when they identify AD pathologies or cellular debris, they undergo phenotypic change into an amoeboid state, allowing them to migrate and phagocytose (Daniel Lee & Landreth, 2010). When activated, microglia release cytokines, signaling for additional activated glia cells and assistance in protecting the brain, a process known as reactive microgliosis (Daniel Lee & Landreth, 2010). In AD, activated microglia are observed surrounding Aβ plaques, phagocytosing, and degrading Aβ, a benefit early in the disease (Fan et al., 2017; Salazar et al., 2021). As the disease progresses, microglia fail to return to a resting state, sustaining chronic activation, leading to excess synaptic pruning, imbalance of cytokines, and depletion of resources. The inability to continue degradation of Aβ, NFTs, and cellular

debris drive the inflammatory response even more, while also exacerbating pathogenesis, a critical cycle targeted in AD research.

1.2 Sex Influences Alzheimer's Disease Pathology

It is important to note, as defined by the World Health Organization (WHO), gender refers to characteristics of men and women that are socially constructed, such as norms, behaviors, and roles, varying through societies and over time (World Health Organization, 2023). As the terms "man" and "woman" are typically in relation to gender, the paper uses these terms to describe an individual who was born biologically male and an individual born as biologically female, respectively, to describe differences between biological sexes.

Twice as many women are diagnosed with AD than men, and the difference in prevalence is the first indication that sex influences the disease. Research supports this by demonstrating disparities in pathogenesis and related mechanisms between men and women. During associative memory encoding, men with greater amyloid burden have greater hippocampal-prefrontal connectivity, while the connectivity does not vary by amyloid burden in women (Wu et al., 2019). Mixed results of Aβ burden have been reported, with studies showing both increased levels of Aβ in women, and also no differences between sexes; this requires further investigation (Palta et al., 2021; Wisch et al., 2021).

There has been more of a consensus on the influence of sex in tau pathology, with several studies demonstrating females having higher tau burden in both cognitively normal and individuals with dementia. In cognitively normal females, research shows significantly higher tau burden in several brain regions, including the rostral middle frontal and superior and middle temporal regions, when compared to men (Wisch et al., 2021). In women with mild cognitive impairment (MCI), tau pathology is more widespread compared to men with MCI than in men with MCI (Shokouhi et al., 2020). Furthermore, women on the trajectory for developing AD, who are cognitively normal but have higher Aβ burden, also

had elevated levels of early tau deposition, when compared to corresponding men (Buckley et al., 2019). One study reported no significant differences in tau-PET signal between premenopausal women and men, however, postmenopausal women had significantly higher levels of tau in several brain regions (Buckley et al., 2022). This topic requires additional research into sex differences in tau pathology, with emphasis on menopause. Additionally, collecting data on menopausal status data could assist with comparisons across studies.

Chomosomes determine biological sex and influences the innate and adaptive immune response in a sex-specific manner. With women having XX and men having XY chromosomes, every cell has a sex (Osborne et al., 2018). Interestingly, the X chromosome contains most of the immune-related genes in the human genome and can impact the risk for developing autoimmune disorders (Migliore et al., 2021; Yanguas-Casás, 2020). In aged female mice, microglia have higher expression of genes related to activation, specifically *Sppl, Gpnmb, Lgals3, Apoe, Ccl3, Clec7a, and Ccl4* (Lynch, 2022). Using pathway analysis to further distinguish changes, female microglia have upregulation of genes involved in components of the complement cascade and the microglial sensome (Mangold et al., 2017). Assessment of human brain tissues agreed with these findings by demonstrating greater immune activation genes expressed in women; this study also revealed sex disparities in gene expression related to aging (Berchtold et al., 2008). Microglia are essential in the critical period of sexual differentiation through immune signaling, establishing dimorphic neural circuits (Lenz et al., 2013). The abundance and morphology of microglia are reported to be different between males and females. Rodent models demonstrate males to have a greater number of microglia in the parietal cortex, CA1, CA3, dentate gyrus, and amygdala of rodent models, and a larger percent of them in an amoeboid state (Schwarz et al., 2012). Female microglia express an elevated number of cytokines, including IL-10 and IL-1β (Osborne et al., 2018). Interestingly, female microglia transplanted into a male brain, following microglia depletion, maintained a “female” profile, suggesting that cells have a sex even in the presence/absence of different hormones (Kodama & Gan,

2019). A limitation to these data is that most of the research has been done in rodent models, however, new methods, including single-cell analysis will further our understanding of sex differences in the human immune response.

1.3 Diagnosis and Risk Factors of Alzheimer's Disease

Alzheimer's Disease is diagnosed by medical history, cognitive and functional tests, physical and neurological exams, and neuroimaging (Alzheimer's Association, 2023b). As described earlier, AD is displayed as progressive cognitive decline, along with changes in mood and behaviors, confusion, and difficulty completing common tasks. Interestingly, women with AD have been reported to have higher rates and greater severity of depressive symptoms, psychotic symptoms, including delusions and hallucinations, and aberrant motor behavior; in contrast, men display more severe apathy and aggression, and are more likely to have wondering tendencies (Colombo et al., 2018; Eikelboom et al., 2022; Lee et al., 2017). Until recently, there has only been treatment for symptom management through cholinesterase inhibitors (galantamine, rivastigmine, and donepezil), for mild to moderate AD, and N-methyl-D-aspertate (NMDA) antagonist (memantine), for moderate to severe AD (NIA, 2023). Within the last few years, two disease-modifying therapies (DMT) have been approved by the Food and Drug Administration (FDA) for treatment of AD. These include anti-amyloid monoclonal antibodies, aducanumab (Aduhelm) and lecanemab (Leqembi), with another under FDA standard review, donanemab. Outcomes from the Phase 2 trial evaluating donanemab resulted in a drug-placebo difference that led to an approximate 5-month delay in cognitive decline (Dickson 2023). Modeling of the lecanamab outcomes beyond the clinical trial treatment period estimated a 2.5-year delay to mild AD (Tahami Monfared 2023). These data demonstrate that the earlier a patient can be treated with a DMT, the greater the impact it can have on "time-saved", by slowing the disease progression (Dickson et al., 2023). However, reports describe new challenges in diagnosing women with aMCI or AD, which would delay the treatment of women and contribute to the sex difference

in prevalence. Cognitive tests used to diagnosis aMCI/AD are not sex-specific. These include measures of verbal memory, in which women tend to perform better than men, even with moderate hippocampal atrophy (Sundermann et al., 2016). One study investigated the use of sex-specific norms and cut off scores in determining aMCI, revealing that when sex-specific criteria were not used, there was about 10% false negatives (missed aMCI) among women, and 10% false positives in men (Sundermann et al., 2019). Additionally, when evaluating patients using ADAS-Cog and CDR-SB, women with MCI progress at faster rates than men (Lin et al., 2015; Mielke et al., 2014). The inability to adequately detect and diagnose women with aMCI/AD, delays opportunity for treatment by DMT, highlighting the need for sex-specific cognitive testing and/or scores, as well as predictive and diagnostic biomarkers.

As stated above, the lifetime risk of a man over the age of 65 to develop AD is 11.6%, while women have a risk of 21.2% (Aggarwal & Mielke, 2023). Age is the greatest risk factor for Alzheimer's Disease, though, being a women increases the risk as well. Differences between men and women in several aspects can account for the disproportion of AD risk, including sex differences in overall risk factors (i.e. APOE status, immune response, lifestyle, physical and mental health), as well as sex-specific risk factors (i.e. pregnancy, menopause). For example, APOE status is the strongest genetic risk factor for LOAD (Liu et al., 2013). In general, APOE4 homozygotes have an increased risk for developing AD up to 15 fold, with APOE2 reducing the risk by about half (Raulin et al., 2022). However, when evaluated by sex, one APOE4 allele increases the risk in females 4 times higher than in men (Altmann et al., 2014; Farrer et al., 1997). A recent study reported that APOE2 may only have protective effects in men (Wood et al., 2023). Transitioning to menopause also influences the risk for developing AD, demonstrating a sex-specific risk factor, and one I will elaborate on in future sections. Overall, table 1 presents data that support diverse risk for AD between sexes.

Table 1. Risk factors that may contribute to sex-differences in AD

Genetic	APOE Status	• One APOE4 allele increases risk 4 fold higher than in men (Altmann et al., 2014; Farrer et al., 1997) • APOE2 may have protective effects in only men (Wood et al., 2023)
	Chromosomes	• The X chromosome contains the most immune-related genes in the human genome, with females having two (XX) ("Physiological Sex Differences in Microglia and Their Relevance in Neurological Disorders," 2020) • The number of X chromosomes can influence the risk for developing autoimmune disorders (Migliore et al., 2021)
Immune Response	Cytokines	• Greater levels of IL-1β related to poorer verbal learning than men (Caldwell et al., 2021)
	Microglia	• Preclinical models demonstrate alterations in the abundance and morphology of microglia between male and female (VanRyzin et al., 2018) • Sex differences in transcriptomic and proteomic profiles in microglia have been reported in preclinical models (Guneykaya et al., 2018)
	Response to treatment	• Women generally have a stronger immune response than men, which may lead to greater efficacy of therapies than in men (Klein & Morgan, 2020; S. Wang et al., 2019) • Women tend to experience more adverse reaction to immunotherapies than men (Klein & Morgan, 2020)
Brain Connectivity		• Overall, women show lower magnitude on measures of resting state functional network topology and connectivity (Z. Yang et al., 2022)
		• 54% of connections showed sex differences through resting-state fMRI (Ritchie et al., 2018)
Mental Health	Stress	• Women show association between psychological stress in middle age and the development of dementia (Johansson et al., 2010) • Women are at greater risk for developing anxiety, trauma, and stress-related disorders than men, and may sex hormones may play a role (S. H. Li & Graham, 2017)
	Depression	• Women are about twice as likely to have depression than men (Kessler, 2003) • Late life and severe depression in women increases the risk for developing dementia (Hickey et al., 2023)
Physical Health	Metabolic Health	• Women with AD, and that are APOE4+, have greater impairment of mitochondrial energy production (Arnold et al., 2020) • Prevalence of diabetes is higher in men but there are more women with diabetes after menopause (Mauvais-Jarvis, 2018)
	Head Trauma	• Head injuries and TBI are more prevalent in men (Biegon, 2021)

		• Women are underrepresented in TBI research (Gupte et al., 2019)
	Cardiovascular Health	• Women with coronary heart disease were 1.6 times more likely to develop AD than men (Dong et al., 2022) • Cardiovascular mortality is higher in men and selective survival of men with better cardiovascular health may contribute to higher risk of dementia in women (Chêne et al., 2015)
Lifestyle	Education	• Women tend to have lower education levels, however as education disparities in education are decrease, this may attenuate AD risk in women (Bloomberg et al., 2021)
	Diet	• Research suggests that men may be more sensitive to an unhealthy Western diet than women (D'Amico et al., 2020)
	Physical Activity	• Women tend to engage in less physical activity than men (Kaplan et al., 2001)
	Sleep	• Women have 40% greater risk of insomnia throughout life (Mong & Cusmano, 2016) • Women have increased levels of melatonin and cortisol (Gunn et al., 2016)
Sex-specific risk factors	Pregnancy	• Preeclampsia is associated with increased risk for dementia (Basit et al., 2023) • There have been studies investigating number of children/pregnancies and the risk of AD, however, mixed results have been reported (Beeri et al., 2009; Jang et al., 2018)
	Menopause	• AD risk is associated with the age of menarche, age of menopause, and length of time between the two (Sochocka et al., 2023) • Vasomotor symptoms ("hot flashes") during sleep are associated with greater white matter hyperintensity volume and lower levels of plasma Aβ, implying higher levels of Aβ plaque ((Thurston et al., 2023)Presented at the Menopause Society Annual Meeting, 2023)),

1.4 Disparities in women-focused research and inclusion in clinical and preclinical studies

The 1977 FDA guidelines, "General Considerations for the Clinical Evaluation of Drugs," advised that women of childbearing age be excluded from Phase 1 and early Phase 2 drug trials, limiting research to only postmenopausal women. In 1993, the FDA reversed the guidelines, and together with NIH,

recommended women be included in clinical trials, and that the trials comprise of both genders in the same study; it was also recommended that early drug trials include both sexes to evaluate safety, efficacy, and dosing. The updates to guidelines on clinical trials was influential, and as of 2015, there were higher rates of women in clinical trials than men, though there are no requirements to stratify data based on sex (Klein & Flanagan, 2016).

Of great concern, between 2002 and 2012, there was a 99.6% failure rate in AD drugs being evaluate in clinical trials, with a mere 0.4% success rate (J. L. Cummings et al., 2014). While the recruitment of women are higher than men in AD clinical trials, with 58% of the 110469 participants being female, it is still disproportioned to the prevalence of the disease (64%) and varies widely based on the study (2.2%-90.7%) (Pinho-Gomes et al., 2022). Moreover, of the 118 clinical trials that reported results between 2010 and 2021, only 8 included sex stratified outcomes; none of the trials presented screen fails or adverse events data by sex (Pinho-Gomes et al., 2022). As discussed above, studies show that women tend to have greater immune response, which could lead to increased adverse events to treatment, as well as the possibility of sex-specific effects (i.e. based on APOE4 status). For example, of drugs that were removed from market between 1997-2000, 8 out of 10 of them had greater adverse effects in women (Klein & Flanagan, 2016). Omission of sex-disaggregated data may account for some of the failure rate identified in AD drug testing and historical views of excluding women due to hormone fluctuations, reproduction, and cost associated with including female participants remains an issue. The simple inclusion of sex-specific data analyses could provide insights into disease processes or response to treatment in a sex-specific manner.

Before even getting to clinical trials, there is an even greater gap in the inclusion of sexes in preclinical models used in neuroscience research, though recent data on this topic is more limited. According to a publication evaluating neuroscience and biomedical studies, published in 2009, the ratio of articles reporting on only males samples compared to only females was most skewed in the neuroscience

field, 5.5:1; sex was omitted in over 20% of the neuroscience articles (Beery & Zucker, 2011) (Figure 1A). Historically, a greater amount of research unspecified the sex used in neuroscience and biomedical research until about 1969, when a shift occurs, with a decrease in unspecified studies, and a substantial increase in studies investigating males, suggesting the previously unspecified reports focused primarily on males as well (Beery & Zucker, 2011) (Figure 1B). Research using cell culture lines are also bias towards males, or often not specifying the sex of the cell line. In 2018, about 50% of publications specified the sex of the cells used, a positive increase from just 25% in 2013 (J. Y. Kim et al., 2021). Furthermore, of the 53

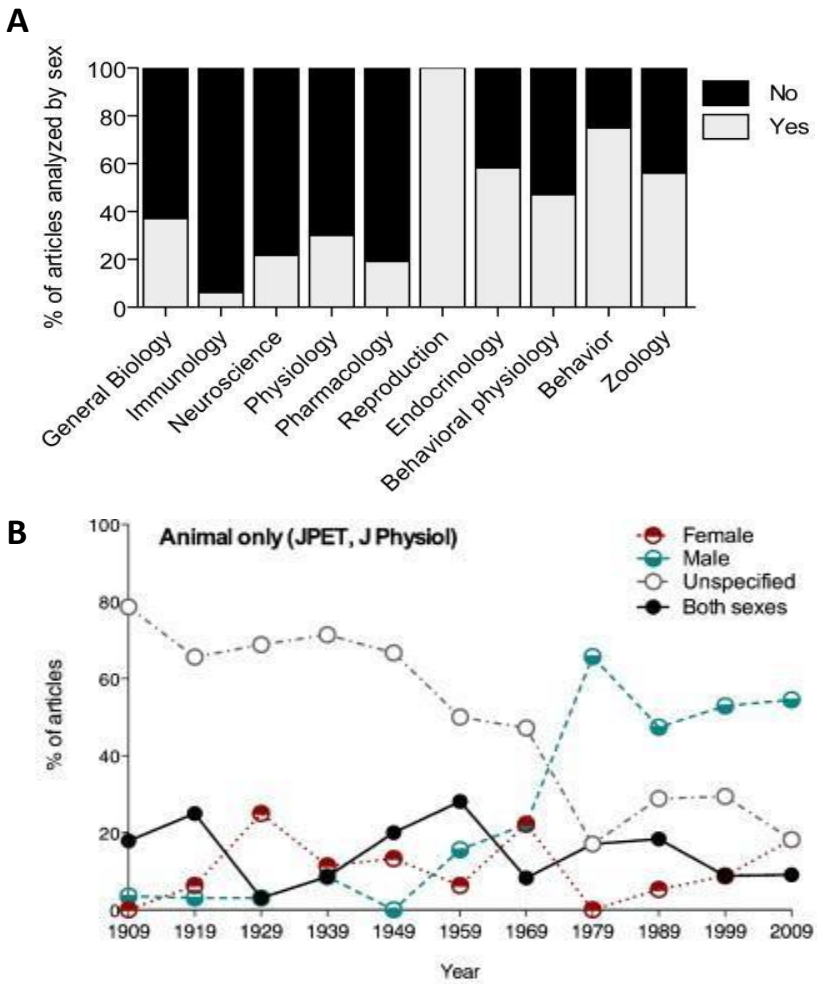

Figure 1. Quantification of studies that reported sex (Beery & Zucker, 2011). A.) The percent of articles that analyzed sex in each of the fields B.) Changes in the number of articles reporting sex over time in animal studies

that reported the sex, 18 used both male and female cells, 23 used male only, and 12 used female only (J. Y. Kim et al., 2021). Current research on this topic would be of interest. These findings imply that AD research and related mechanisms were/are primarily being investigated in male preclinical models, possibly contributing to the failure rate in AD drugs. My proposed study aims to contribute to the understanding of cellular and molecular mechanisms in a sex-specific manner and improve the gap in female-focused research by providing a novel approach in investigations of menopause and AD.

1.5 Female reproductive hormones and replacement therapy

Regular menstrual cycle in humans consist of two phases: the follicular phase (including menses) and the luteal phase (Figure 2A). During the follicular phase, estrogen levels increase, and progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) stay relatively low, lasting about 13 days. Around day 14, estrogen levels plummet, LH and FSH spike, and progesterone steadily increases; ovulation occurs. Following ovulation, the luteal phase lasts about 14 days with estradiol and progesterone rising and falling, and LH and FSH quickly returning to low levels. This cycling continues in a healthy woman until the perimenopausal period, when irregular cycling and fluctuating hormones occur. This transition is marked by overall decreases in estradiol and substantial increases in FSH; progesterone levels tend to drop at a faster pace than estradiol. In a menopausal state, FSH and LH are higher, while estradiol and progesterone are low (Figure 2B).

There are three types of estrogen, estrone (E1), estradiol (E2), and estriol (E3). E2 is the most abundant during the reproductive years, however, following menopause, E2 levels decrease and E1 is the primary estrogen. With the large drop in E2 and progesterone, hormone replacement therapy (HRT) has been a compelling therapeutic for AD, however, controversial. Inconsistencies with study results on whether HRT has beneficial or deleterious effects (as recently reviewed by Mills et al. 2023), led to the "critical window hypothesis," proposing that HRT must be started within a window of time as related to

menopause for therapeutic benefit, demonstrating a need for further research (Maki, 2006; Mills et al., 2023). Additional factors that must be considered include reproductive history, drug formulation, dose, regimen, and route of administration (Mills et al., 2023).

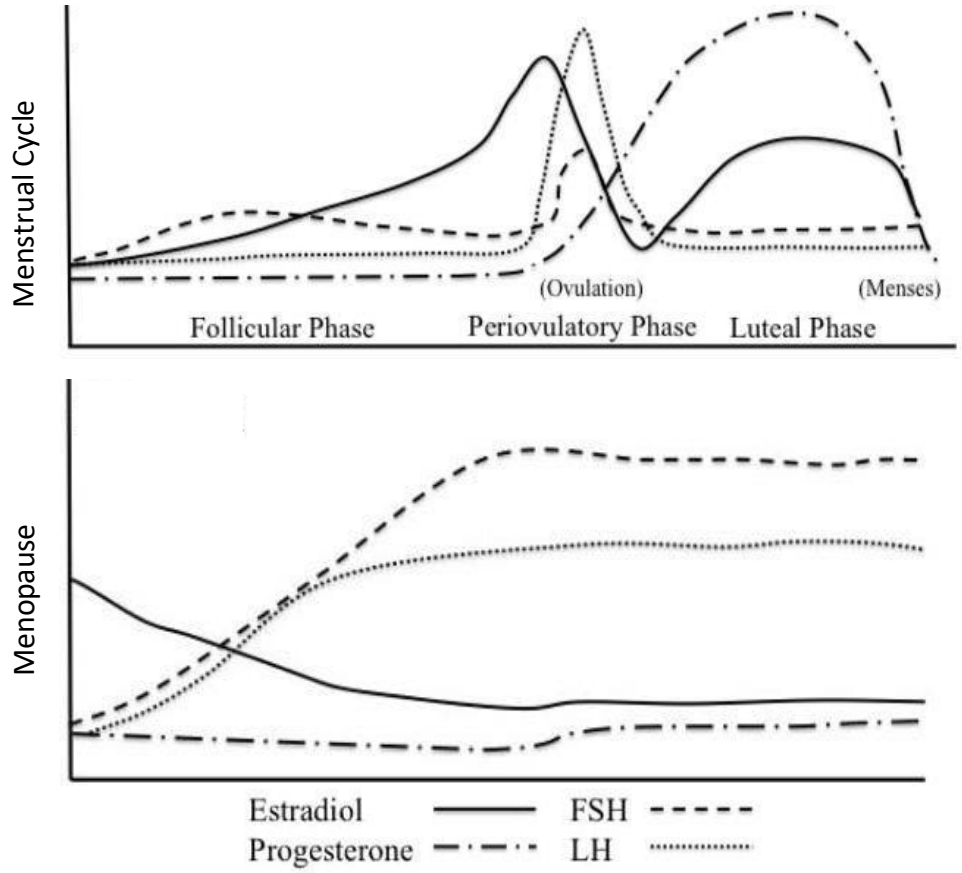

Figure 2. Alterations in hormones levels through the menstrual cycle and during menopause (Koebele & Bimonte-Nelson, 2016)

As HRT with the introduction of estrogen and progesterone has been controversial, blocking of FSH may be a novel target in AD research. Gonadotrophins, including FSH, have been shown to be

increased in AD patients and demonstrates a relationship with Aβ pathology (Bowen et al., 2000; Short et al., 2001). In 3xTg AD mice with menopause (ovariectomized), blocking FSH led to decreases in Aβ pathology and improvement of cognitive deficits (Xiong et al., 2022). The variability in HRT, and potential of FSH as a therapeutic target, supports the need to better understand the mechanistic alterations that happen during the menopause transition and the impact it has on brain health.

1.6 Perimenopause and Menopause

Brain changes associated with AD have been reported to start over a decade before the onset of clinical symptoms, suggesting that AD is not only a consequence of old age (Jack et al., 2013); these midlife brain changes correspond with menopausal age. The median age of menopause in developing countries is 48-52 years old, with the average age in the US being 51; a metanalysis determined the mean menopausal age being 48.8 years across 35 counties (Davis et al., 2015). While men's testosterone levels also decrease through aging, it is at a gradual slope and usually asymptomatic (Hägg & Jylhävä, 2021). In contrast, women have a pronounced shift in hormones and these changes influence AD risk and disease progression. A great contribution of research by Drs. Lisa Mosconi, Roberta Brinton, and their teams regarding this topic, advances our understanding of the relationship between menopause and brain processes by demonstrating alterations in Aβ and tau burden, glucose metabolism, brain structure, connectivity, and influence of APOE status during the menopausal transition (Mosconi et al., 2021). However, it is first necessary to describe the stages of menopause to appreciate the findings.

Menopause is a cession of menstruation, due to the loss of ovarian follicle function, resulting in the inability to reproduce. This process happens in every biological woman either through the natural process of aging or iatrogenic (i.e. ovariectomy, chemotherapy/radiation). Menopause is defined as being without a menstrual period for 12 months. The phase before menopause, referring to the time between the onset of symptoms to true menopause, is the perimenopausal period, lasting about 8-10 years. Many

symptoms associated with perimenopause are neurological symptoms, including hot flashes, insomnia, mood changes, brain fog, headaches, anxiety, and depression. Typically onset of symptoms and change in menstrual patterns are indications of perimenopause/menopause, not needing a formal diagnosis; however, healthcare providers can test hormone levels, including E2, FSH, and LH for indication and predication of menopause (NIH-NICHD, 2021).

With the timeframe of menopause coinciding with the timing of AD-related brain changes, investigations into the alterations of biomarkers during the menopause transition are of great importance. Studies show that in postmenopausal women, Aβ deposition is greater than premenopausal women, with Aβ levels being more pronounced in menopausal women that were APOE4 positive (Mosconi et al., 2021). As indicated earlier, tau-PET demonstrated significantly higher levels of tau burden in postmenopausal women in several brain regions as compared to premenopausal (Buckley et al., 2022). Alterations in bioenergetics were also reported across menopause transition. Postmenopausal women have reduced cerebral glucose metabolism compared to perimenopausal women; lower cerebral glucose metabolism was also described in the perimenopausal women compared to pre-, demonstrating a gradient through the transition (Mosconi et al., 2017). Alterations in brain processes through menopause makes research into the relationship between AD and menopause a compelling target for prevention and treatment of AD.

1.7 Mouse Models of Menopause

Animal models can assist with advancing basic understanding of menopause as it relates to aging and brain health. While rodents experience reproductive senescence, it is not consistent with human reproductive aging. To begin, mice have an estrous cycle, when the lining of the uterus is reabsorbed rather than shed through menstruation, as it is in human menstrual cycles. Additionally, the estrous cycle lasts about 4-5 days, consisting of 4 phases: proestrus, estrus, metestrus, and diestrus. The menstrual cycle spans about 28 days, consisting of two phases, the follicular phase and the luteal phase. In rodents,

estrogen levels peak during proestrus, followed by ovulation, whereas in humans, there is a more gradual increase in estrogen over the follicular phase; estrogen levels peaking before ovulation in both cycles (Hong & Choi, 2018). Fluctuation of reproductive hormones are similar through the estrous cycle as observed in the menstrual cycle, providing an avenue for cross-species comparisons (Figure 3). Rodents do not transition into menopause but rather experience irregular cycling around 9-12 months known as estropause (Koebele & Bimonte-Nelson, 2016) (Figure 4A). Estropause is variable in several ways between mice, including timing and duration of estropause, fluctuation in irregular cycling, and in some cases, mice can return to cycling following estropause. Due to the inconsistencies between mice and human reproductive aging, as well as the variability between mice, natural aging of mice is not the best representation for the study of human menopause (Figure 4A and 4D comparison).

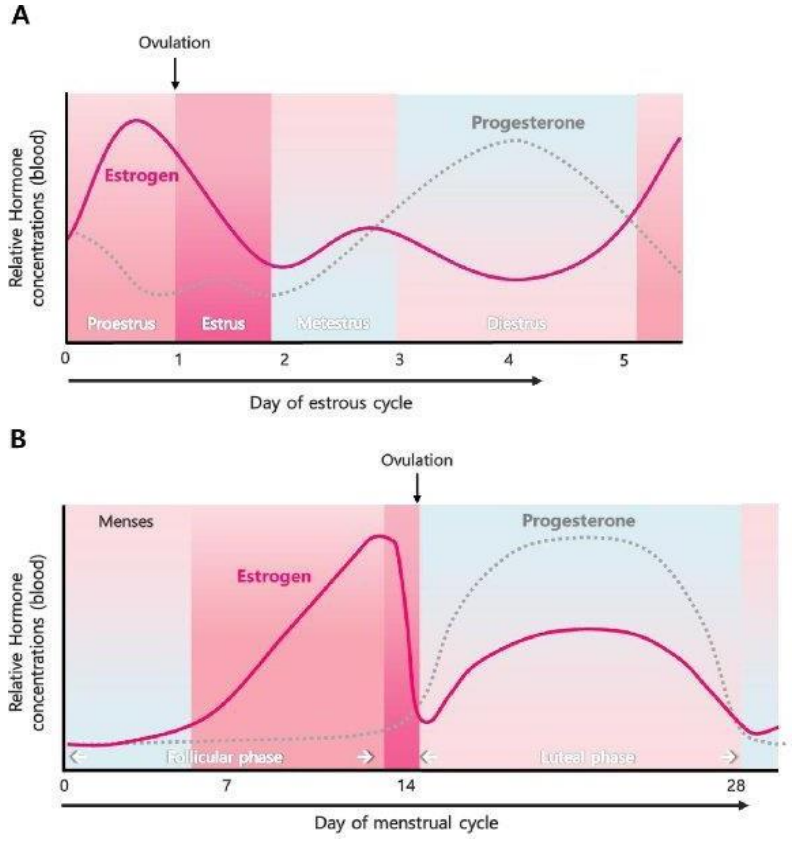

Figure 3. Similarities between the estrous and menstrual cycles allow for cross-species comparisons (Hong & Choi, 2018). A.) Estrous cycle of a rodent B.) Menstrual cycle in humans

Traditionally, the use of ovariectomized (OVX) rodents, a model in which the ovaries are surgically excised, have been employed in menopause-related investigations (Souza et al., 2019). Ovariectomies in mice lead to cession of estrous cyclicity, rapid drop in estrogen levels, and increases in FSH, resulting in similar levels observed in human menopause (Marongiu, 2019a) (Figure 4B). This model has been fundamental to many discoveries surrounding aging, reproduction, and AD research. However, substantial, rapid changes of hormones in the OVX model does not mimic the gradual transition of human menopause, lacking a perimenopausal period. The perimenopausal period could be important in the relationship between menopause and AD and it is necessary for studies to use an animal model that encompasses the gradual hormone transition of human menopause to better understand the effects.

The chemical 4-vinylcyclohexene diepoxide (VCD) is a compound used commercially as a reactive diluent for diepoxides and epoxy resins (Kappeler & Hoyer, 2012). Studies by the National Toxicology Program screening for carcinogens found VCD causes direct damage to ovarian follicles (Kappeler & Hoyer, 2012). Specifically, VCD depletes primary and primordial follicles through acceleration of naturally occurring atresia, with ovaries still intact (Brooks et al., 2016) (Figure 5). Consecutive intraperitoneal (i.p) injections results in lengthening of the estrous cycle and hormone fluctuations with low levels of estrogen and high levels of FSH, mimicking a perimenopausal state (Figure 4C). Once the ovarian follicles are fully depleted, stabilization of hormone levels occur, leaving the mice unable to ovulate, representing menopause. Studies show that various dosing regimen can lead to longer or shorter perimenopausal periods, allowing researchers to answer questions related to timing of brain alterations or treatment opportunities (Brooks et al., 2016). A common VCD regimen is 15 consecutive days of i.p. injections at 160mg/kg/mL, with an incubation time of about 135 days until cessation of cycling (anestrous, as discussed below) (Brooks et al., 2016; Fernandes et al., 2019; Perez et al., 2013). While no animal model can truly represent humans, the VCD model is beneficial in providing a way to incorporate the

perimenopausal period and gradual transition into menopause that the OVX model cannot offer, allowing a translational approach from animal discoveries into humans.

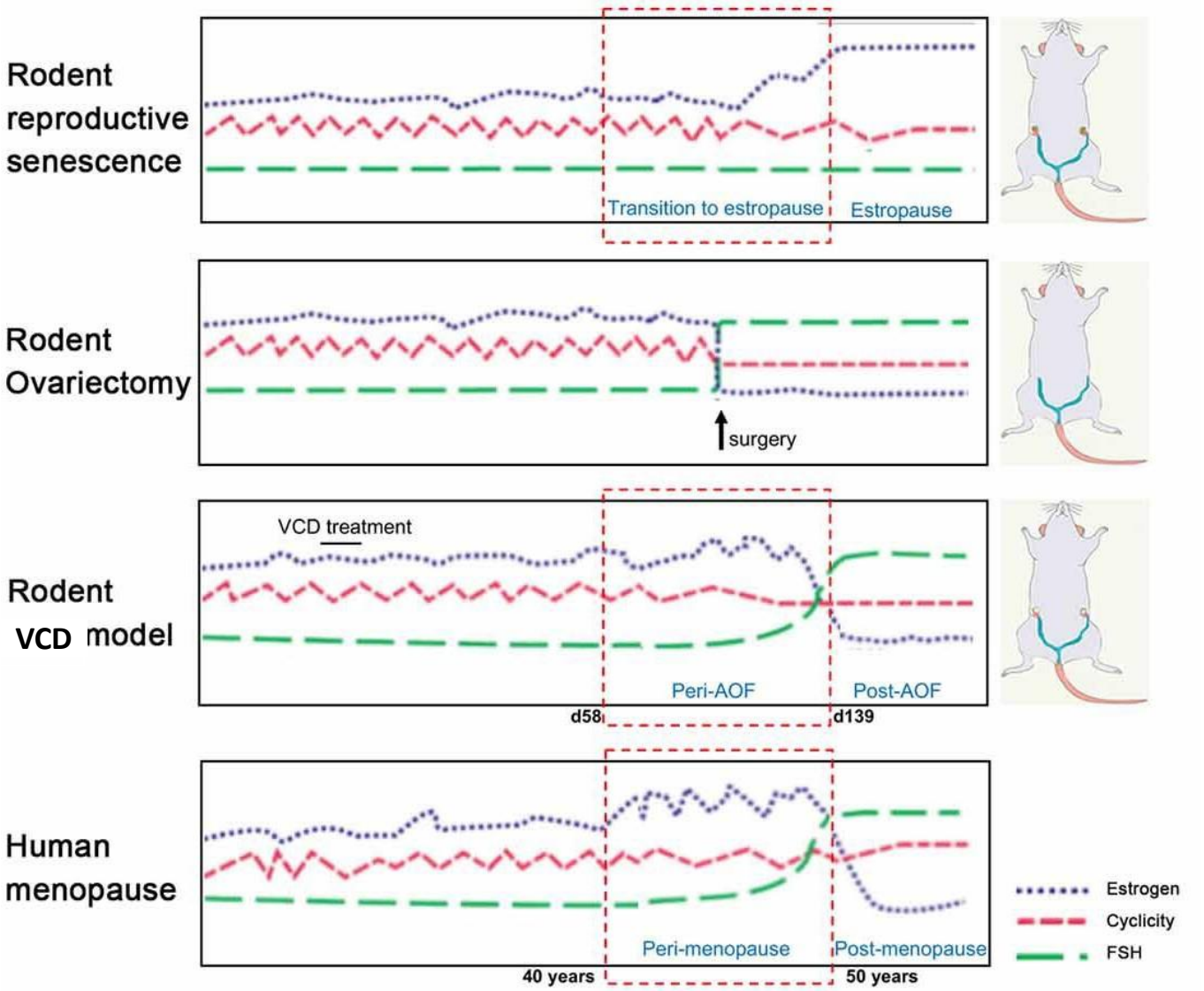

Figure 4. Comparison of female reproductive aging models compared to human menopause (Marongiu, 2019a) A.) Natural aging of a rodent leads to a transition into estropause with varying levels of hormones B.) Ovariectomized rodents have a sudden drop in estrogen and spike in FSH immediately following surgery C.) The VCD model has a gradual hormone transition into a menopause-like state with a perimenopausal period D.) Human menopause has a gradual hormone transition with a perimenopausal period (AOF – accelerated ovarian failure)

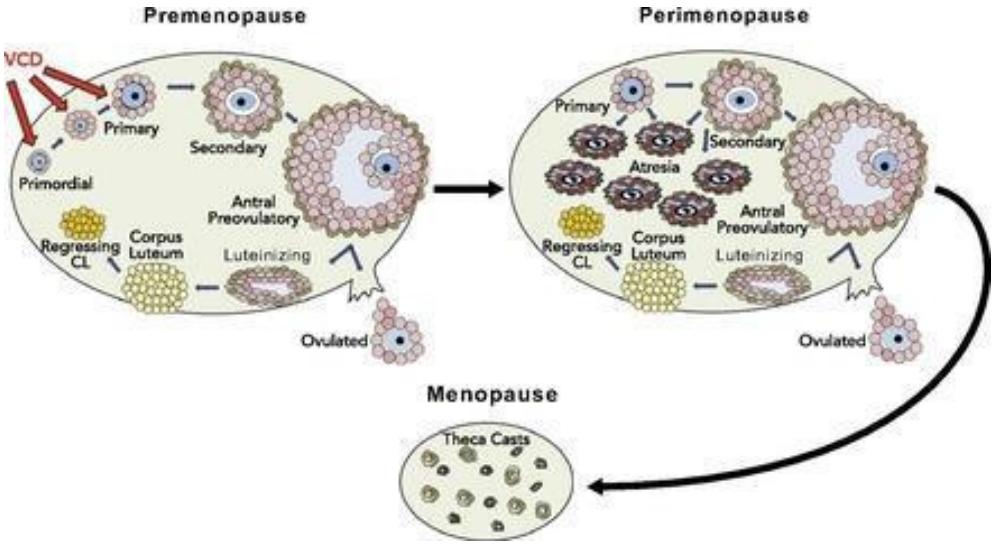

Figure 5. VCD drug targets primordial and primary ovarian follicles (Brooks et al., 2016)

1.8 Vaginal Cytology

Hormone levels are commonly evaluated for transition into menopause, marked by overall decreases in estradiol and substantial increases in FSH. In humans, a simple blood test can be performed to quantify the levels. This method can also be employed in mouse models by either performing regular blood draws or collecting blood via cardiac puncture during sacrifice. With such large fluctuations and variability in hormone levels, the limited amount of blood that can be collected from mice, and the time and cost associated with several tests, a great method to determine the reproductive state of mice is through vaginal cytology.

As previously introduced, mice have four stages of the estrous cycle: proestrus, estrus, metestrus, and diestrus (Figure 6). The proestrus phase is the preparatory stage for the animal coming into heat and is determined by the presence of nucleated epithelial cells (Ajayi & Akhigbe, 2020) (Figure 6D). The estrus

stage is when receptivity of the female is the highest and is characterized as the presence of cornified epithelial cells (H. Kim et al., 2016) (Figure 6E). The metestrus stage occurs in the absence of conception, having large amounts of both cornified epithelial cells and leukocytes, as well as the presence of nucleated epithelial cells (Ajayi & Akhigbe, 2020) (Figure 6F). Diestrus is the phase between breeding opportunity before cycling back into proestrus; the stage has majority leukocytes (Figure 6G). While each phase has anticipated cell types, the samples may be collected during the transition from one phase to another; additionally, cells types can overlap into other phases. When determining the stage of the estrous cycle that the mouse is in, it is important to consider the ratio of cell types. Figure 6H demonstrates the overlap of cell types in each of the phases and can be used as a guide when determining the stages.

Following VCD treatment and through incubation, vaginal lavages can be conducted to collect cells and monitor the reproductive ability through estrous cycle staging of the mice. During perimenopausal-like state, the estrous cycle will become irregular, with change in duration of time mice are spending in each of the stages, skipping stages, and lengthening of the estrous cycle. When the reproductive organs become inactive through complete ovarian follicle depletion, mice enter a persistent diestrus state, as known as anestrus, representing human menopause.

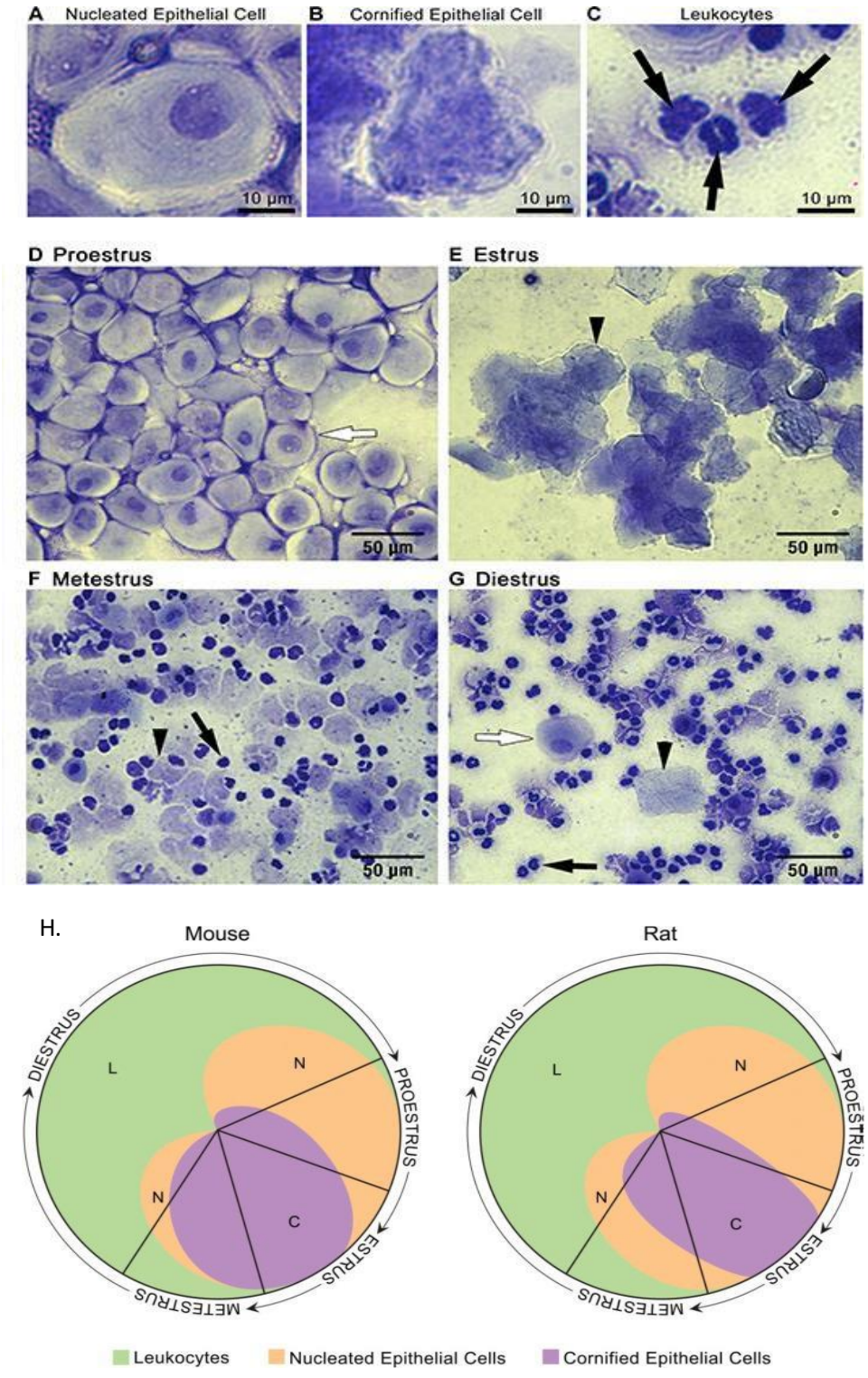

Figure 6. Vaginal cytology of cell types through the estrous cycle (Byers et al., 2012; McLean et al., 2012) A-C.) Cell types found in vaginal smears from rodents, nucleated epithelial cells, cornified epithelial cells, and leukocytes, respectively D.) Proestrus stage with a majority of nucleated epithelial cells E.) Estrus stage with a majority of cornified epithelial cells F.) Metestrus stage with a combination of all three cell types G.) Diestrus stage with a majority of leukocytes H.) Demonstration of the ratio of cell types found in each of the estrous cycle stages in rodents

1.9 G Protein-coupled Estrogen Receptor 1 (GPER1)

There are three types of estrogen receptors: ERα and ERβ, which are nuclear transcription factors, and G protein-coupled receptor 1 (GPER1; previously orphan receptor G protein-coupled receptor 30, or GPR30). GPER1 is a seven transmembrane G protein-coupled receptor (GPCR) found on the cell membrane and endoplasmic reticulum of several cell types, including neurons, microglia, and astrocytes. The presence of GPER1 has been identified in several brain regions, with the highest levels reported in the hypothalamic-pituitary axis, hippocampus, cortex, and thalamus (Roque et al., 2019). GPER1 is physiologically activated by E2, whereas other steroids, including progesterone, testosterone, and cortisol, do not activate the receptor. Activation has been associated with several functions, including cAMP production, activation of protein kinases, activation of ion channels, and modulation of gene expression (Villa et al., 2016). Synthetic ligand agonist, 1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro-^{3}H-cyclopenta[c]quinolin-8-yl]-ethanone (G-1) crosses the blood brain barrier and is used to investigate GPER1 mechanisms (Bologa et al., 2006). In a mouse with lipopolysaccharide (LPS) induced inflammation, G-1 inhibited cytokine production of TNF-α and IL-6 (Blasko et al., 2009). In a traumatic brain injury (TBI) mouse model, G-1 inhibited injury-related increases in pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α, and increased levels of anti-inflammatory IL-4 in male and OVX female mice; additionally, G-1 promoted an anti-inflammatory microglial profile (Pan et al., 2020). Together, these studies demonstrate the anti-inflammatory effects of GPER1. In cell cultured BV2 cells, GPER1 activation by G-1 results in decreased phagocytosis through AnxA1 pathways (Loiola et al., 2019). Inflammatory processes and AD pathology has a role in myelin damage and, interestingly, one study demonstrated enhanced remyelination of oligodendrocytes by G-1 (Hirahara et al., 2013; M. Zhao et al., 2021).

GPER1 is involved in memory and cognition through neurogenesis, synaptic plasticity, and synaptic transmission (Luo et al., 2023). G-1 administered to OVX rats following global cerebral ischemia, led to

increases in neurogenesis in the hippocampus, with greater expression of synaptic proteins, decreases in neuronal impairment and improvements in cognitive function (L. Wang et al., 2021). One group published findings showing activation of GPER1 demonstrated neuroprotection through modulation of PI3K-AKT-mTOR pathways, reducing glutamate-induced excessive autophagy (Yue et al., 2019). Furthermore, the same authors reported different effects of GPER1 activation in astrocytes through G-1 treatment, restoring autophagy in glutamate-treated astrocytes and demonstrating cell specific effects (X. Wang et al., 2020).

There have been investigations into the role of GPER1 in neurodegenerative diseases and some of the findings are included in Table 2; however, studies in AD have been limited and the table demonstrates this gap (Roque et al., 2019). In the 5xFAD mouse model, novel object recognition (NOR) tests were performed and deficits were reported in both males and females; administration of G-1 ameliorated the impairment only in the female mice (Kubota et al., 2016). In primary neuronal cell cultures treated with Aβ and G-1, activation of GPER1 led to increases in cell viability and decreases in Aβ induced DNA fragmentation, nitric oxide, and total oxidant status, demonstrating a protective effect against Aβ toxicity (Kurt et al., 2019). Overall, these findings demonstrate a difference in GPER1 between sexes and highlights the relationship of GPER1 in neurodegenerative diseases, including AD. With about 34% of FDA approved drugs targeting GPCRs, this supports the utility GPER1 could have in AD therapeutics (Hauser 2018).

Table 2. Effects induced by GPER1 activation in neurodegenerative diseases (Roque et al., 2019)

	Major conclusions	Models	Reference
AD	Selective GPER activation ameliorated object recognition memory in female but not male mice;	5XFAD mice (intact female and male); Exposure to G1 and G15;	(Kubota et al., 2016)
PD	Increased concentration of dopamine and its metabolites, and DAT and VMAT2 specific binding in the striatum; Increased DAT specific binding in the substantia nigra;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 10 days - before and after dopaminergic lesion;	(Bourque et al., 2013)
	Increased dopamine and DOPAC concentration and specific binding of DAT and VMAT in the striatum; Increased anti-apoptotic Bcl-2 protein and activation of the pro-survival kinase Akt in the striatum;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of raloxifene twice daily for 10 days - before and after dopaminergic lesion;	(Bourque et al., 2014)
	Increased dopamine concentration and DAT and VMAT-2 specific binding in the striatum; Increased DAT specific binding in the substantia nigra; Increased GDNF, BDNF and Bcl-2 protein levels in the striatum;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 10 days - before and after dopaminergic lesion;	(Bourque et al., 2015)
	Prevention of the dopaminergic neuron loss in a GDNF-dependent process;	Wistar rat midbrain neuron-glia cultures; MPP^+ model; Exposure to G1;	(Bessa et al., 2015)
	Increased dopaminergic fibers density in the striatum; Prevention of the dopaminergic neurons loss in the substantia nigra; Decreased microglial cells number and IL-1β, TNF-α and IL-6 protein and mRNA levels in the midbrain;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 12 days - before and after dopaminergic lesion;	(Guan et al., 2017)
	Prevention of the dopaminergic neurons loss in the substantia nigra; Protection of the motor functions; Decreased IL-1β, CD68 and iNOS mRNA levels in the substantia nigra;	C57BL/6 mice (male); Unilateral injections in the substantia nigra with LPS on 5th day of G1 treatment; Subcutaneous injection of G1 twice daily for 12 days - before and	(Mendes-Oliveira et al., 2017)

		after dopaminergic lesion;	
	G1 reduced the MPP^+-induced cell death through the increase of GDNF, effects that were abrogated by G15;	Neuroblastoma cell line SH-SY5Y; MPP^+ model; Exposure to G1;	(Cheng et al., 2017)
MS	Selective GPER activation mediates protection against MS, which is significantly impaired in GPER gene-deficient mice;	C57Bl/6J mice (female); GPER KO mice; EAE; Exposure to G1;	(C. Wang et al., 2009)
	Selective GPER activation reduces the severity of MS through the decrease of pro-inflammatory cytokines, including IFNγ and IL-17; G1 inhibits the production of cytokines such as TNFα and IL-6 in a dose-dependent manner;	Primary culture of macrophages, microglia and a murine macrophage cell line (RAW264.7); EAE; Exposure to G1;	(Blasko et al., 2009)
	E_2 reduced disease severity in wild-type and ERα KO mice, but had no impact on GPER KO group; These different effects were associated to the production of anti-inflammatory IL-10; GPER have an important but still undefined role in regulating immune reactivity in MS severity;	C57Bl/6J mice (intact female); Ethinylestradiol treatment; WT, ERαKO and GPERKO mice;	(Yates et al., 2010)
	GPER is expressed throughout oligodendrocyte differentiation and promyelinating stages; Selective GPER activation enhanced oligodendrocyte maturation and remyelination after demyelination;	Primary oligodendrocyte cultures from Wistar rat spinal cord; Demyelination model; Exposure to G1 and G15;	(Hirahara et al., 2013)

Abbreviations: Bcl-2 – B-cell lymphoma 2; BDNF – brain-derived neurotrophic factor; DAT – dopamine transporter; DOPAC - 3,4-Dihydroxyphenylacetic acid; EAE - autoimmune encephalomyelitis; G-15 - (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline; GDNF - glial cell-derived neurotrophic factor; iNOS - inducible nitric oxide synthase; KO – knockout; MPP+ - 1-methyl-4-phenylpyridinium; MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; VMAT - vesicular monoamine transporter

1.10 Relationships between Diabetes, Sex, and AD

About 49-52% of Americans are estimated to have either diabetes mellitus or prediabetes (Menke et al., 2015). Diabetes mellitus type 2 (DM2) increases the lifetime risk for developing AD by 1.5 to 4-fold; approximately 80% of patients with AD also have insulin resistance or abnormal fasting glucose levels (J. Cummings et al., 2022). The prevalence of DM2 by gender varies through reproductive stages. More men have diabetes earlier in life, whereas following menopause, women are more likely to have DM2, resulting in a greater number of women having DM2 globally (Ciarambino et al., 2022). Women who have transitioned to menopause early, have a greater risk for developing DM2, consistent with the risk for developing AD (Ciarambino et al., 2022). HRT has been shown to delay the onset of DM2 by improving beta-cell insulin secretion, glucose effectiveness, and insulin sensitivity, demonstrating a relationship between reproductive hormones and metabolic pathways (Mauvais-Jarvis et al., 2017).

Insulin degrading enzyme (IDE) has the primary responsibility of breaking down insulin. It is also involved in degradation of glucagon, atrial natriuretic peptide, and Aβ, and is a regulator of proteasomal degradation, supporting IDE as a link between DM2 and AD pathogenesis (Pivovarova et al., 2016). IDE is secreted by neurons and microglia and degrades Aβ *in vitro* and *in vivo;* this was demonstrated through use of IDE-/- mouse models, in which IDE deficiency resulted in over 50% decrease in Aβ degradation (Farris et al., 2003; Vekrellis et al., 2000). Furthermore, naturally occurring IDE missense mutations in a rodent model of DM2 led to 15-30% decreases in insulin and Aβ degradation, demonstrating IDE gene mutations to be a risk gene for LOAD (Farris et al., 2004). In humans, patients with MCI had significantly lower levels of membrane-bound IDE in the hippocampus compared to controls (Z. Zhao et al., 2007). Furthermore, IDE concentrations and activity continued to decrease through the conversion from MCI to mild-severe AD (Z. Zhao et al., 2007). The lower levels of IDE activity was also negatively correlated with Aβ in MCI and AD patients (Z. Zhao et al., 2007).

IDE expression is modulated by estrogens, indicating a relationship between menopause, DM2 and AD. *In vitro*, E2 increases mRNA and protein levels of IDE through ER-β and activation of PI3K pathways (L. Zhao et al., 2011). In OVX rodents there is a significant decrease in IDE in the hippocampus, which is prevented by E2 treatment (L. Zhao et al., 2011). Additionally, in an OVX 3xTg mouse model of AD, E2 increased hippocampal IDE and attenuated Aβ accumulation and plaque formation (L. Zhao et al., 2011). In diabetic mouse models, IDE is negatively correlated with GSK3-β, a serine-threonine kinase, activated through insulin receptor pathways (PI3K/Akt/GSK3-β) (Lauretti et al., 2020). GSK3-β is involved in AD pathogenesis by modulation of APP cleavage through BACE1, with GSK3-β inhibition leading to attenuation of Aβ production (DaRocha-Souto et al., 2012; Lauretti et al., 2020). Furthermore, GSK3-β is directly involved in phosphorylation of tau proteins and a target in the prevention of tau pathology (Lauretti et al., 2020). IDE may influence the activation of insulin receptors/GSK3-β pathways and attenuate or exacerbate AD pathology through insulin levels. In mice, the administration of sodium orthovanadate, a tyrosine phosphatase inhibitor which activates PI3K/Akt/GSK3-β pathways, resulted in increased levels of IDE, decreasing p-Tau and improving cognition (Akhtar et al., 2020; Tian et al., 2023). A recent paper highlights the possible therapeutic effects of IDE against AD pathologies through the PI3K/Akt/GSK3-β pathway supporting this idea (Tian et al., 2023).

It is important to acknowledge the relationship between GPER1 and metabolic functions. Female GPER1 (-/-) mice are reported to have hyperglycemia and impaired glucose tolerance in plasma, reduced skeletal growth, and increased blood pressure (Mårtensson et al., 2009). Estrogens have been shown to regulate insulin release and investigations determined a link between GPER1 and insulin secretion. Through E2 treatment of OVX GPER1 (+/+) and OVX GPER1(-/-) mice, knockdown of GPER1 decreased insulin, demonstrating that GPER1 mediates E2-stimulated insulin release, which could be contributing to impaired glucose metabolism in GPER1(-/-) mice (Mårtensson et al., 2009). Consistent with these findings, treatment with G-1 leads to insulin release in pancreatic islets (Balhuizen et al., 2010; Nilsson et al., 2011).

This research is focused on pancreatic tissues and peripheral effects; however, it would be of interest to investigate the role of GPER1 in metabolic brain health.

1.11 Beta-Amyloid Mouse Model of Alzheimer's Disease, APP/PS1

Early-onset AD (EOAD; as known as familial AD) accounts for approximately 5% of all cases (Mendez, 2019). Patients develop EOAD before the age of 65 and are positive for familial gene mutations in APP, presenilin 1 (PS1) and presenilin 2 (PS2). The other 95% of cases are late-onset AD (LOAD; as known as sporadic AD), associated with aging, is typically diagnosed after the age of 65 and does not have a direct cause, but several risk factors, including APOE status. With no known direct cause of LOAD, the use of EOAD animal models is vital to research into the pathologies associated with both types: Aβ, p-tau, neuroinflammation, and related-mechanisms. The APP/PS1 double transgenic mouse model is derived by the insertion of APP and PS1 mutations, allowing Aβ pathology to develop in a mouse, which would otherwise not arise. Studies using this model have reported Aβ deposits in the hippocampus at about 3-4 months old, hyperphosphorylated tau-positive neuritic structures and activated microglia surrounding plaques, synaptic loss at about 4 months and cognitive deficits in several learning and memory tasks (ALZFORUM, 2013; Bittner et al., 2012; Jackson Laboratory, 2023; Radde et al., 2006; Serneels et al., 2009). Sex differences have been reported in the APP/PS1 mice, with female mice having higher levels of Aβ accumulation and worse cognitive processing (X. Li et al., 2016). Furthermore, glucose and insulin tolerance were reported to develop earlier in APP/PS1 male mice, with male mice having significantly lower plasma insulin levels and higher total cholesterol (X. Li et al., 2016). Use of the APP/PS1 mouse model is important to investigate the influence of menopause on Aβ pathology.

2. Dissertation Project Proposal

2.1 Premise and rational

Majority of AD patients are women and there is a critical need for research into this disparity. AD-related brain changes have been reported over a decade prior to the onset of clinical decline. This timeline coincides with women going through perimenopause and menopause. Reproductive hormones play a role in brain functions and processes, supporting a link between AD and menopause. Using the VCD mouse model would allow us to evaluate changes in the brain through gradual transition with the inclusion of the perimenopausal period. Comparison of FSH levels would demonstrate translation from the VCD model to human menopause. By using VCD in the well-established APP/PS1 mice, assessment of Aβ levels would assist in determining the relationship between Aβ and menopause. Furthermore, p-tau 396/404 would be measured to see if a menopausal-like state in the mice would alter tau pathways. In our previous research, we found significant decreases in the GPCR, GABA-B receptor, in the APP/PS1 with the presence of Aβ (Osse et al., 2023). GPER1 is a GPCR estrogen receptor that may contribute to the difference in AD prevalence between sexes. I proposed to investigate GPER1 levels in male and female APP/PS1 mice to determine if Aβ alters GPER1, as we previously observed in another GPCR, GABA-B (Osse et al., 2023). Additionally, I would quantify levels of GPER1 in the VCD verses non-VCD treated mice to further understand the connection between menopause and GPER1. DM2 is higher in postmenopausal women than in premenopausal women and men, suggesting a relationship between metabolic processes and reproductive aging. IDE is involved in several processes that link AD and DM2, including glucose/insulin balance, degradation of Aβ, upstream pathways of p-tau, and a relationship with female reproductive hormones. Quantification of IDE in the APP/PS1 mice, as well as with or without VCD treatment, would be intriguing to better understand the connections with AD, DM2 and menopause. These upstream and downstream targets are demonstrated in figure 7.

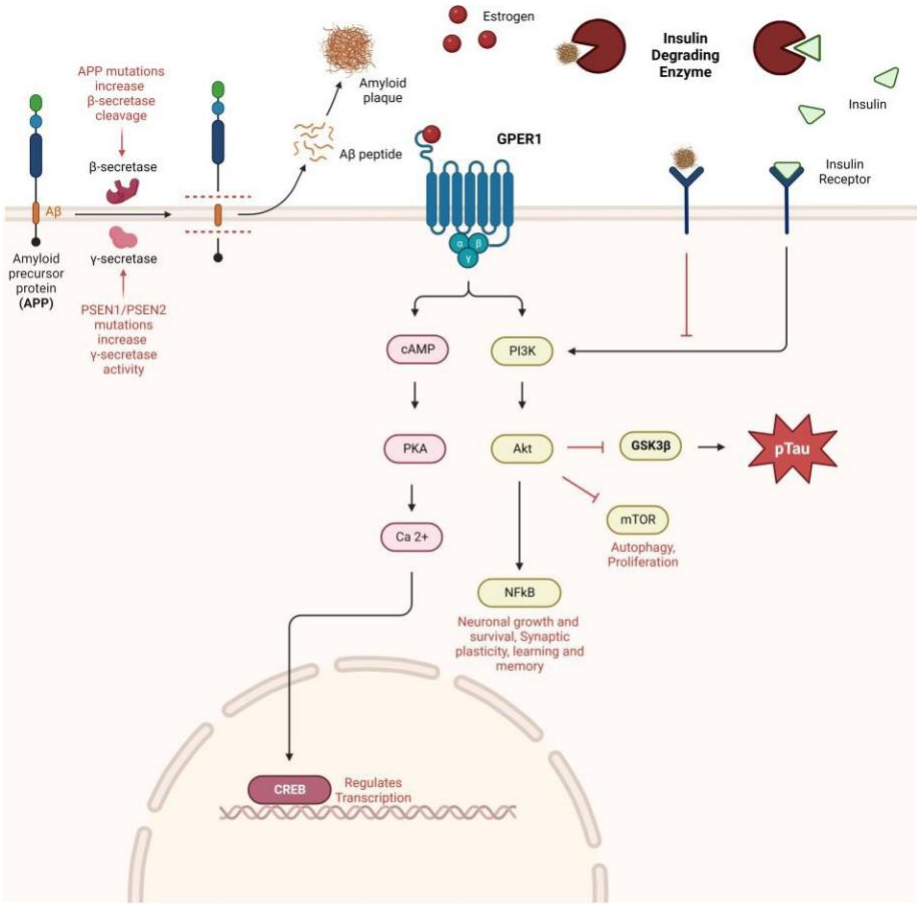

Figure 7. Pathway demonstrating that relationship between the targets investigated in this study (Made with BioRender)

2.2 Primary Investigation and Outcome Measures

For this project, I planned to utilize female and male APP/PS1 and C57BL/6J (WT) mice at 7 months of age and randomly assign them to a treatment group: C57BL/6J with sesame oil (WT + Oil), C57BL/6J with VCD (WT + VCD), APP/PS1 with sesame oil (APP/PS1 + Oil), or APP/PS1 with VCD (APP/PS1 + VCD), for both sexes (indicated by (F) for female and (M) for males). VCD would be administered at 160mg/kg/mL for 15 consecutive days by i.p. injection to induce ovarian follicle toxicity in the female mice leading to a menopausal-like state. A subset of male mice would also receive VCD to eliminate the possibility of a drug

effect on Aβ and Aβ-related pathways. Female mice would be monitored by vaginal lavages and cytology until anestrous is observed in the VCD treated mice. Mice would be defined as being in a menopausal-like state (anestrous) when they reach diestrus for two full cycles. Due to the age of the mice, some irregular cycling may occur in the control female mice, though they will be excluded if they reach persistent diestrus. All groups would be sacrificed for cellular and molecular evaluations of brain tissues. FSH would be measured in the plasma using enzyme-linked immunosorbent assay (ELISA) to demonstrate translation of the VCD menopausal mouse model to humans. Quantification of Aβ-40 and Aβ-42 in hippocampi using Luminex multiplex assay would be the primary measure to evaluate whether the VCD mice have alterations in Aβ burden. Furthermore, we would use western blot to quantify GPER1, IDE, and p-tau 396/404. These investigations would be done to compare VCD and non-VCD mice in APP/PS1 and WT to determine the influence of menopause on AD pathology in female mice; male mice would also be evaluated to distinguish whether findings are due to a drug effect rather than ovarian follicle toxicity.

2.3 Anticipated Outcome

By using VCD to induce a menopausal-like state, I anticipated mice would successfully undergo ovarian follicle toxicity, demonstrated by an anestrous state. This would be determined through vaginal cytology, in which I would observe a persistent diestrus phase for two full cycles in (F)APP/PS1 + VCD and (F)WT + VCD mice, and regular estrous cycling in the (F)WT + Oil and (F)APP/PS1 + Oil mice. FSH levels were expected to be greater in the mice treated with VCD (Lohff et al., 2006). Aβ-40 and Aβ-42 quantification was hypothesized to be altered in (F)APP/PS1 + VCD compared to (F)APP/PS1 + Oil. (M)APP/PS1 + Oil mice are predicted to have less Aβ burden than (F)APP/PS1 + Oil mice; (M)APP/PS1 + VCD mice are also expected to have no change in Aβ compared to (M)APP/PS1 + Oil, since they would not undergo ovarian follicle depletion, ruling out a drug effect (X. Li et al., 2016).

I anticipated no change in GPER1 in the hippocampus of (M/F)WT + VCD mice compared to (M/F)WT + Oil, since data from a recent study suggested that there would be no effect (Gannon et al., 2023). However, previous studies from our lab investigating GPCR GABA-B levels in APP/PS1 mice demonstrated alterations with the presence of Aβ (Osse et al., 2023). With these findings, I hypothesized that we would see alterations in GPER1 in (M/F)APP/PS1 + Oil mice compared to (M/F)WT + Oil. Additionally, I predicted an interaction between the presence of Aβ and menopausal-like state of VCD may result in alterations of GPER1.

IDE was reported to have a strong relationship with Aβ pathology, through the degradation of Aβ monomers, and is regulated through estrogens. With APP/PS1 mice previously demonstrating increases in IDE, potentially through the presence of Aβ, I predicted that we would observe a significant increase in IDE in the (M/F)APP/PS1 + Oil mice compared to the (M/F)WT + Oil mice (Zhang & Wang, 2018). It was also expected that a difference in IDE would be observed in the (F)APP + Oil and (M)APP/PS1 + Oil, due to greater Aβ burden in female mice. Based on the predicted findings of significant alterations of Aβ in female APP/PS1 + VCD, I expected consistent changes in IDE, with no differences in the corresponding male mice.

A relationship between GPER1, IDE, and p-tau may exist through similar pathways of PI3K/Akt mediated GSK3β. It was predicted that alterations in GPER1 in APP/PS1 + Oil mice would lead to significant changes in p-tau 396/404, compared to WT + Oil. With decreases in circulating hormones, (F)APP/PS1 + VCD mice were expected to have a greater change in p-tau 396/404 compared to (F)APP/PS1 + Oil. (M)APP/PS1 + VCD mice were expected to have no difference in p-tau 396/404 compared to (M)APP/PS1 + Oil, since the drug was expected to have little impact on the male mice. Overall, I anticipated females having greater AD related changes compared to males, with menopause exacerbating AD core pathologies and related pathways.

3. Methods

3.1 Care of Animals

Founder mice, B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax (APP/PS1) and C57BL/6J (wildtype controls), were purchased through The Jackson Laboratory (Bar Harbor, ME). The APP/PS1 colony was maintained as a heterozygous colony per breeding considerations (male hemizygous APP/PS1 mice are breed with female C57BL/6J). Wildtype controls were maintained as homozygous. All animals were group-housed with a 12-12-hour light–dark cycle in a temperature (22 ± 1°C) and humidity-controlled vivarium, with standard rodent chow and water available *ad libitum*. Animals were handled once per week to reduce stress and anxiety (Hurst & West, 2010). All procedures were performed during the light phase and in accordance with the University of Nevada, Las Vegas Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH) guidelines for ethical treatment of research subjects.

3.2 Genotyping

Tail samples from the mice were acquired and used for isolation of genomic DNA. Genotype was determined through polymerase chain reaction (PCR) and gel electrophoresis. All mice were genotyped for both the APP and PS1 transgenes. The APP gene is 397bp and was determined using (forward) 5'- AGG ACT GAC CAC TCG ACC AG -3' and (reverse) 5'- CGG GGG TCT AGT TCT GCA T -3' primers. The PS1 gene, 608bp in size, was determined through (forward) 5'- AAT AGA GAA CGG CAG GAG CA -3' and (reverse) 5'- GCC ATG AGG GCA CTA ATC AT -3'primers. Wildtype control genes were included using primers (forward) 5' – CTA GGC CAC AGA ATT GAA AGA TCT - 3'and (reverse) 5' – GTA GGT GGA AAT TCT AGC ATC ATC C – 3', resulting in a gene size of 324bp. Only mice that tested positive for both the APP and PS1 genes were used in the completion of the study.

3.3 4-Vinylcyclohexene Diepoxide (VCD) Administration

4-Vinylcyclohexene Diepoxide (VCD) was purchased from Millipore Sigma (Cat: 110159) and dissolved in sesame oil at a stock concentration of 40mg/mL. Mice (9.5 months old) were injected i.p. at a dose of 160mg/kg/mL for 15 consecutive days (24 hours apart) (Figure 8). Animals were monitored throughout treatment and no signs of illness or discomfort was observed.

3.4 Vaginal Lavages

To determine the stage of the estrous cycle, and evaluate them for a menopausal-like state, vaginal lavages were performed. A micropipette and a narrow pipette tip containing 20µL of saline was used to flush the vaginal cavity about 3-4 times. The samples were placed on a microscope slide coated with 0.1% Poly-L-lysine solution diluted 1:10. Samples were allowed to dry overnight. Vaginal lavages were performed early in the light phase for 14 days, to avoid transition periods between phases (Cora, Kooistra, and Travlos 2015). Saline was used for cell collection to maintain an isotonic solution.

3.5 Giemsa Staining

For vaginal cytology, Giemsa stain (Genesee Scientific, Cat: 72-411) was utilized to visualize the cells. The slides containing dried samples from vaginal lavages were placed into methanol for three minutes to ensure adherence of the sample to the slide. Slides were placed into 5% Giemsa stain for 20 minutes. Slides were rinsed with water and then placed into 1x phosphate-buffered saline (PBS) (pH 7.0) for one minute. Rinses with 1x PBS and water were repeated and the slides were allowed to dry prior to imaging.

3.6 Microscopy and Vaginal Cytology

Following staining, cells were imaged with the Echo Revolve Microscope (Echo, A Bico Company, San Diego, CA, USA) using 10x and 20x brightfield. Estrous cycle stages were interpreted as estrus, metestrus, diestrus, or proestrus based on the ratio of cornified epithelial cells, leukocytes, and nucleated epithelial cells, consistent with previous protocols (McLean et al, 2012). We collected two full weeks of samples to accurately interpret if mice are in anestrous. To ensure mice they were in anestrous, vaginal cytology was performed two consecutive weeks, once per month, to determine if mice transitioned to the menopause state (defined as two full cycles in anestrous). Previous literature reports that the incubation time for VCD at this drug regimen leads to menopause-like changes in approximately 135 days, (Kao, S. W., Sipes, I. G., & Hoyer, P. B., 1999). All mice were in an anestrous state at 186 days of incubation, so our mice were well into reported "menopausal state" at time of sacrifice (Figure 8).

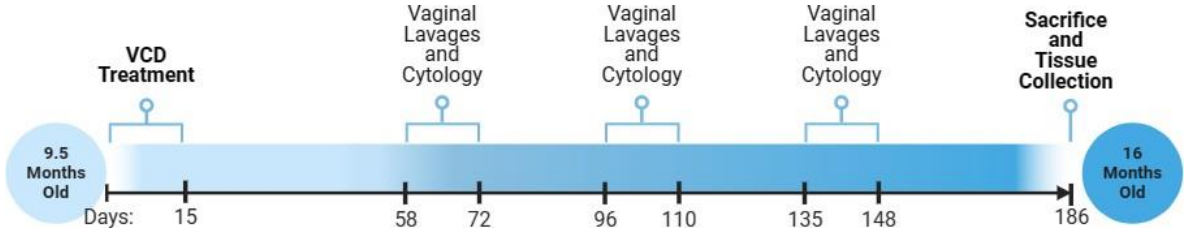

Figure 8. Experimental Timeline (Made with BioRender)

3.7 Blood and Tissue Collection

For collection of blood and brain tissue, mice were individually euthanized by an i.p. injection of Somnasol (Henry Schein Animal Health, Dublin, Ohio). Blood samples were collected through cardiac

puncture. Mice were transcardially perfused with 20mL sterile 1x PBS and decapitated. Brains were removed and dissected for hippocampal, cortical, and cerebella tissues from both right and left hemispheres. Tissues were flash frozen with liquid nitrogen and stored in -80°C until used for study. Blood was centrifuged to collect plasma and stored in -80°C until use.

3.8 Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma samples were used to quantify the amount of FSH in the mice by ELISA (Estradoil ELISA, Calbiotech, CA, USA). Briefly, 25uL of plasma and standard were added to the appropriate wells. The manufacture protocol was followed with no modifications. The ELISA plate was read on a microplate reader at an absorbance of 450nm. Samples failed quality control for ELISA analysis and results were not analyzed or interpretated.

3.9 Protein Isolation

Whole protein lysates were extracted from frozen hippocampal tissue using Bio-Plex Cell Lysis Kit (Cat. No. 171304011, Bio-rad, CA, USA) following manufacturer's protocol, consistent with a recent 2022 study using Luminex assays (Pillon et al., 2022). Complete cell lysis buffer was added to the frozen tissues, homogenized (Kinematica Polytron 1300D, Luzern, CHE), and incubated overnight at -80°C. The following day, the homogenates were allowed to thaw on ice and immediately sonicated (Branson SFX 150, Branson Ultrasonics, Brookfield, Connecticut). The samples were centrifuged for 10 minutes at 4,500g. Supernatants were transferred to a new, low-binding centrifuge tube. Total protein concentrations were measured using Pierce Bicinchoninic Assay Kit (Cat. No. 23255, Thermo Fisher Scientific, MA, USA) per the manufacturer's protocol. Prior to storage, protein lysates were diluted to the highest, consistent concentration of 200,000ug/µL with complete cell lysis buffer, to facilitate equal volume protein loading. Lysates and dilutions were stored at -80°C until use.

3.10 Luminex

Milliplex Mouse Amyloid Beta Magnetic Bead Panel (Millipore Sigma, Cat. No. MABMAG-83K) was used to quantify Aβ-40 and Aβ-42 levels in the hippocampal tissue from the mouse models. Standards were prepared in Assay Buffer provided by the kit. Samples were prepared and assay was performed per the manufacture's protocol without modifications. Concentrations will be determined by simultaneously evaluated Aβ-40 and Aβ-42 using Luminex multiplex immunoassay (Bio-Plex 200 system, Bio-Rad, Hercules, California). Raw data was exported and statistically analyzed using SPSS statistical software version 25 (IBM, Armonk, New York) and ANOVA analysis was performed (see results for details) to determine statistical values, reaching statistically significance of $p < 0.05$.

3.11 Western Blot

Samples were prepared by mixing 10µg of protein and equal volume of Laemelli protein loading dye (Cat. No. 1610747, Bio-rad, CA, USA), incubated at 95-100°C for five minutes, and centrifuged for one minute. Protein samples were loaded in 8% discontinuous polyacrylamide gel electrophoresis (PAGE) gel and resolved in a stepwise voltage method for three hours. Resolved proteins will be transferred to a polyvinylidene fluoride (PVDF) membrane (Cat. No. IPVH00010, Millipore-Sigma, MO, USA) for 45 minutes at 90 volts. Membranes were blocked with PBS-based blocking buffer (Cat. No. 927-40000, LI-COR, NE, USA) with gentle shaking at 4°C overnight. Membranes were incubated with a primary antibody (1:5000, p-tau 396/404, Abcam, MA, USA; 1:5000, total tau, Millipore Sigma, MA, USA; GPR30/GPER1, 1:1000, Abcam, MA, USA; IDE, 1:1000, Abcam, MA, USA; β-actin 1:20k, ThermoFisher Scientific, MA, USA) mixture, including 0.1% Tween-20 for 4°C for three hours. Membranes were rinsed/washed five times with 1x PBS with Tween-20 (PBST) at room temperature with five minutes of gentle shaking each time. Samples were incubation with secondary antibody mixture (IRDye 680RD Rabbit, 1:20k, LI-COR, NE, USA; IRDye 800CW Mouse, 1:40k, LI-COR, NE, USA) with 0.1% Tween-20 for one hour at room temperature with gentle

shaking. Rinses were repeated with 1x PBST; additional two rinses with 1x PBS were performed. Membranes were scanned using the ChemiDoc MP Imaging System (BioRad, Hercules, CA, USA) and files were imported into Image Lab software (BioRad, Hercules, CA, USA). Normalization of the target bands was performed using β-actin signal (Herrera et al., 2019; Perez-Gonzales et al., 2014; Toth et al., 2013; Wang et al., 2014). Normalization values were used to calculate the proportion to control ((F)WT + Oil). Data was statistically analyzed using SPSS statistical software version 25 (IBM, Armonk, New York) and three-way ANOVA was performed to determine statistical values, reaching statistically significance of $p < 0.05$.

4. Results

4.1 VCD induced anestrous in APP/PS1 and WT mice, representing menopause

To induce a menopausal state, female APP/PS1 and WT mice were injected at a dose of 160mg/kg/mL for 15 consecutive days. Vaginal lavages were performed two consecutive weeks every month. Vaginal cytology was used to determine the estrous cycle stage for every day collected. The full estrous cycle was assessed and determined as normal cycling, abnormal, or anestrous. With mouse estrous cycles lasting 4-6 days, I defined two full estrous cycles as two weeks. We evaluated (F)WT + Oil and found that all the mice continued their estrous cycle as normal throughout the study (Figure 9). To assess whether the (F)APP/PS1 + Oil mice had altered estrous cycle at baseline attributed to the genetic modification or the presence of Aβ, the estrous cycle was evaluated and we determined that all the mice had normal cycling, consistent to (F)WT + Oil (Figure 10). Following 186 days of incubation, (F)WT + VCD and (F)APP/PS1 + VCD mice all reached two full estrous cycles of diestrus (anestrous) (Figure 11 and Figure 12 respectively); we did not observe any differences between the two groups. Overall, we found normal cycling in the WT and APP/PS1 injected with oil as drug vehicle; VCD led to anestrous over a two-

week period, in both WT and APP/PS1 mice. Anestrous indicates ovarian follicle depletion and represents menopause in the (F)WT + VCD and (F)APP/PS1 + VCD mice.

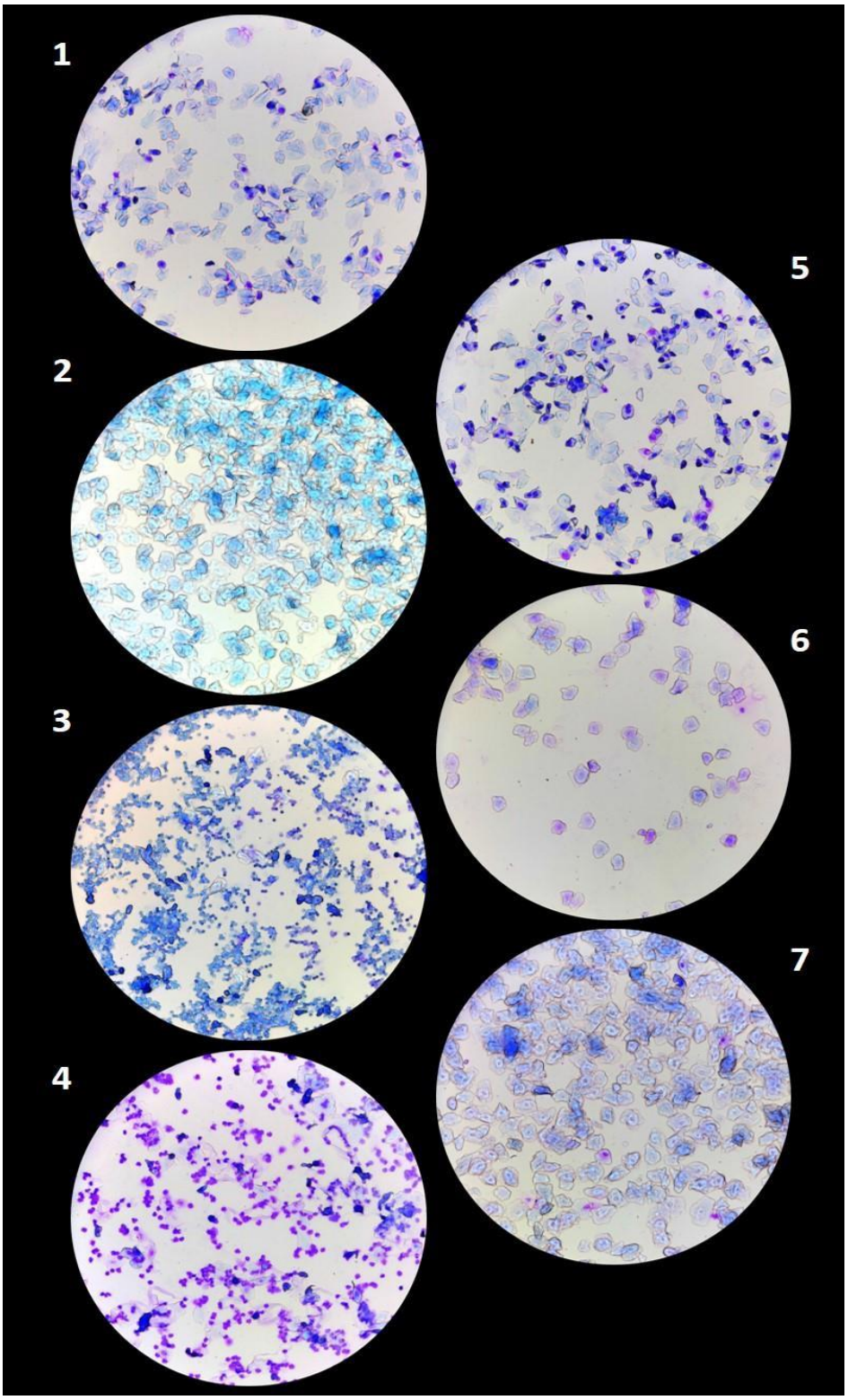

Figure 9. Representative figure of estrous cycle in WT control mice. Cells were collected via vaginal lavage for one week prior to sacrifice and stained with Giemsa stain. Cytology was performed to determine the stage of the estrous cycle. 1) estrus, 2) estrus, 3) diestrus, 4) diestrus, 5) proestrus, 6) estrus, 7) estrus. Mouse WT 1557 at 20x magnification

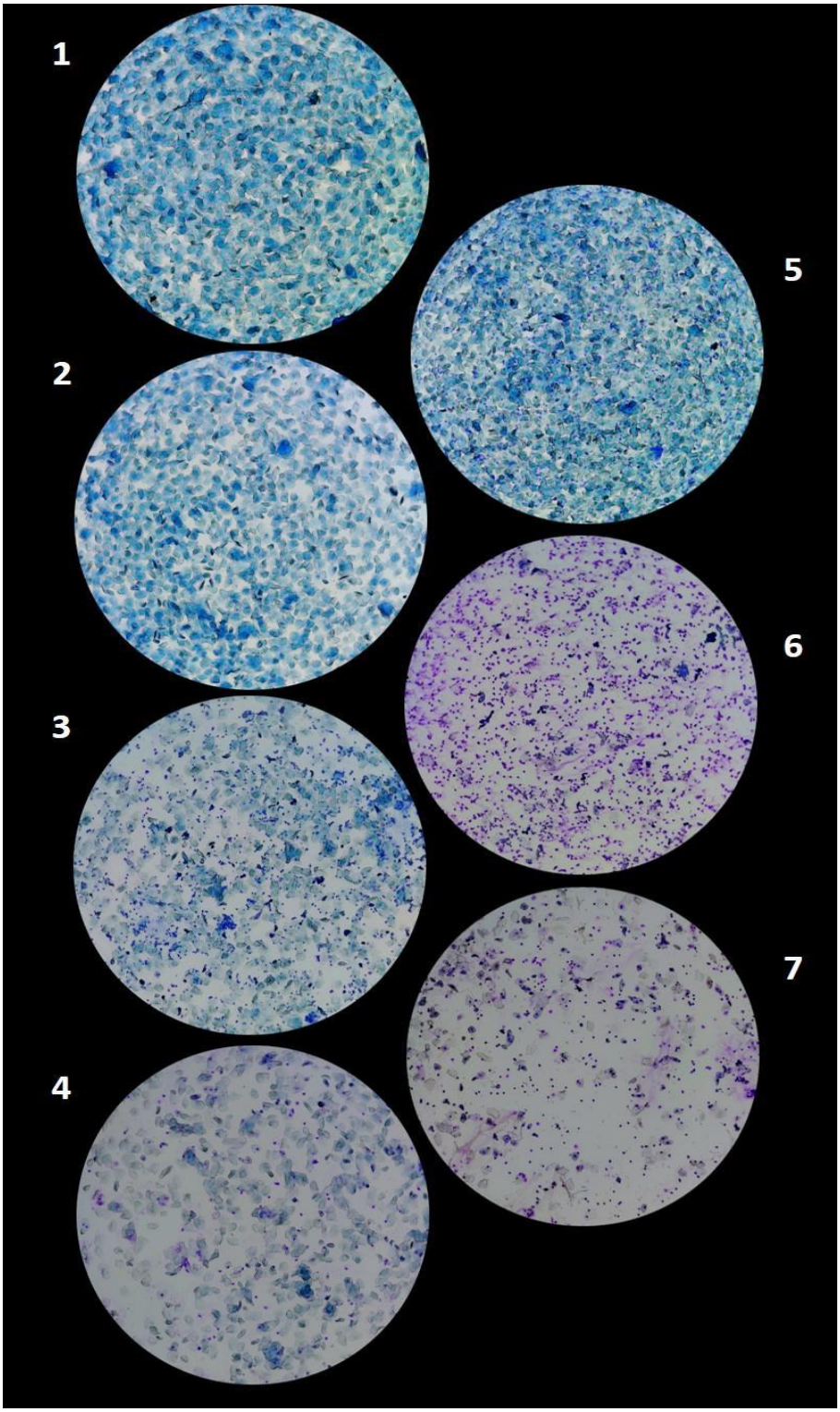

Figure 10. Representative figure of estrous cycle in APP/PS1 mice. Cells were collected via vaginal lavage for one week prior to sacrifice and stained with Giemsa stain. Cytology was performed to determine the stage of the estrous cycle. 1) estrus, 2) estrus, 3) metestrus, 4) proestrus/early estrus, 5) estrus, 6) diestrus, 7) diestrus/early proestrus. Mouse APP/PS1 594 at 20x magnification

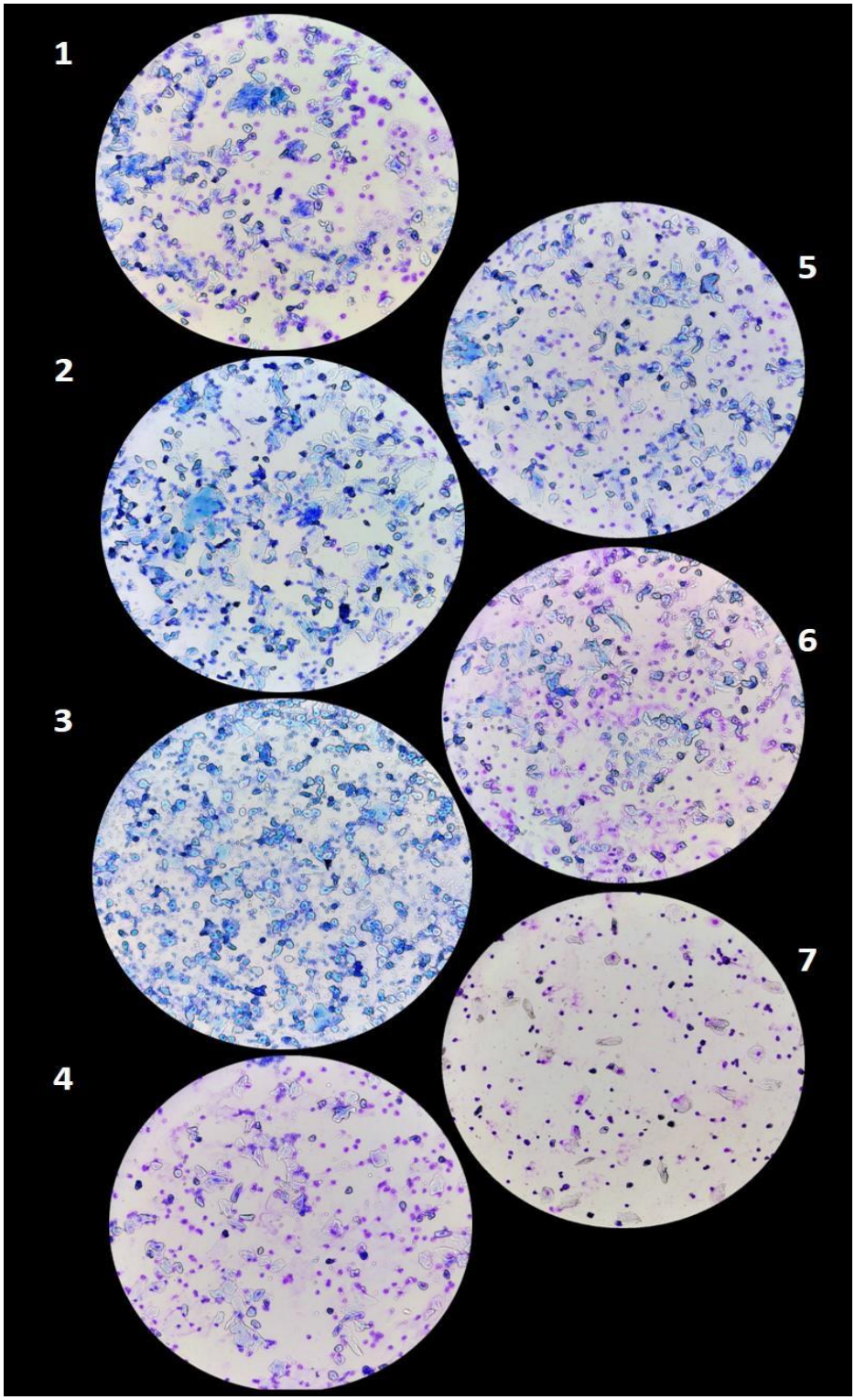

Figure 11. Representative figure of estrous cycle in WT control mice with VCD. Cells were collected via vaginal lavage for one week prior to sacrifice and stained with Giemsa stain. Cytology was performed to determine the stage of the estrous cycle. 1) diestrus, 2) diestrus, 3) diestrus, 4) diestrus, 5) diestrus, 6) diestrus, 7) diestrus. Mouse WT 1555 at 20x magnification

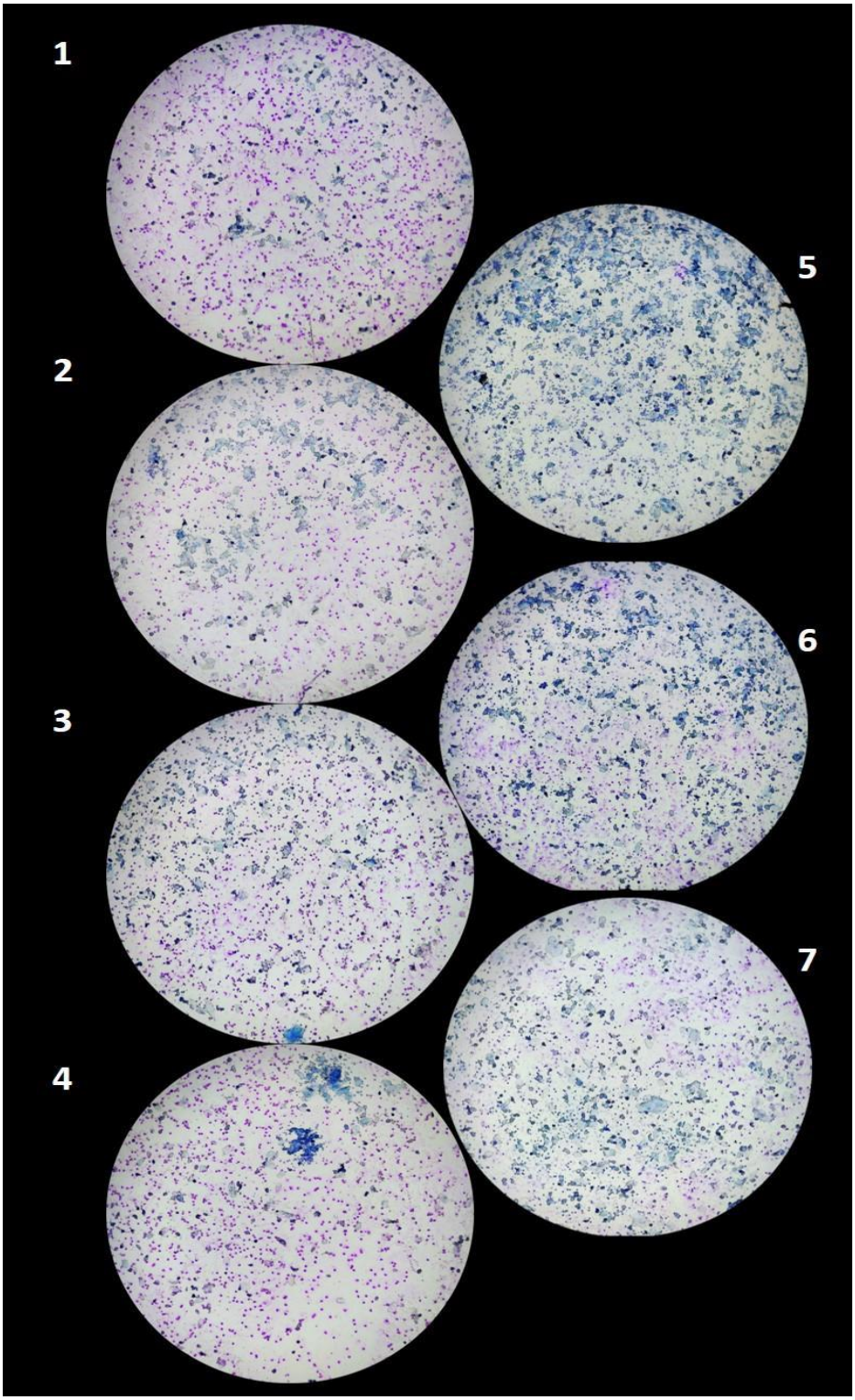

Figure 12. Representative figure of estrous cycle in APP/PS1 mice with VCD. Cells were collected via vaginal lavage for one week prior to sacrifice and stained with Giemsa stain. Cytology was performed to determine the stage of the estrous cycle. 1) diestrus, 2) diestrus, 3) diestrus, 4) diestrus, 5) diestrus, 6) diestrus, 7) diestrus. Mouse APP/PS1 612 at 20x magnification

4.2 VCD alters Aβ levels in the hippocampus of female APP/PS1 mice

The first aim of this study was to investigate if menopause alters Aβ levels by evaluating the (F)APP/PS1 + VCD and (F)APP/PS1 + Oil mice. We used Luminex multiplex assay to quantify Aβ-40 and Aβ-42 levels in the hippocampus of the mice. To answer this question, we ran a one-way ANOVA to compare (F)APP/PS1 + Oil and (F)APP/PS1 + VCD groups; we decided it would be best to do the same in the male mice to be able to determine if any findings were attributed to a drug effect, rather that ovarian follicle depletion. VCD treatment did not have a significant effect in Aβ-40 levels in female or male mice ($F_{5,28}$ = 20.308, $p < 0.05$, Tukey post-hoc ns) (Figure 13A). However, Aβ-42 levels were significantly decreased in the (F)APP/PS1 + VCD mice compared to the (F)APP/PS1 + Oil ($F_{5,28}$ = 20.308, $p < 0.05$, Tukey post-hoc $p < 0.05$) (Figure 13B). There were no significant differences in the (M)APP/PS1 + VCD mice compared to (M)APP/PS1 + Oil, confirming no off-target drug effects ($F_{5,28}$ = 20.308, $p < 0.05$, Tukey post-hoc ns) (Figure 13A and Figure 13B). We found (F)APP/PS1 + Oil to have significantly higher levels of both Aβ-40 ($F_{5,28}$ = 20.308, $p < 0.05$, Tukey post-hoc $p < 0.05$) and Aβ-42 ($F_{5,28}$ = 36.808, $p < 0.05$, Tukey post-hoc $p < 0.05$) compared to (M)APP/PS1 + Oil (Figure 13A and Figure 13B). Greater levels of Aβ in female APP/PS1 mice compared to male APP/PS1 mice has been previously reported, demonstrating our findings to be consistent with the literature (J. Wang et al., 2003). Due to the cost and space of samples available in the assay kit, (M)WT + Oil and (M)WT + VCD were not included, though we included the (F)WT + Oil groups to demonstrate the very low levels of Aβ in WT mice (Figure 14A and 14B). (F)WT + VCD also had low levels of Aβ, showing no drug effect on Aβ levels in WT mice, as we would expect with the absence of a genetic mutation (Figure 14A and 14B). Overall, female APP/PS1 treated with VCD led to significant decreases in Aβ-42, demonstrating a relationship between ovarian follicle depletion and Aβ pathology. In the mouse model of menopause, the observed changes in Aβ can provide insight into how menopause influences AD.

To be thorough in our analyses, we also performed a two-way ANOVA to investigate interaction effects. And found no significant interaction in Aβ-40 ($F_{1,58}$ = 0.704, ns) or main effects (sex: $F_{1,58}$ = 0.079, ns; treatment: $F_{1,58}$ = 0.051, ns). In Aβ-42 there were also no significant interactions ($F_{1,58}$ = 1.702, ns) or main effects (sex: $F_{1,58}$ = 0.341, ns; treatment: $F_{1,58}$ = 0.447, ns). However, plots of the estimated marginal means revealed an interaction effect for both Aβ-40 and Aβ-42 (Figure 14C). Using the Shapiro-Wilk test, the data distribution did not depart significantly from normality ((F)Aβ-40: W = 0.914, ns; (M)Aβ-40: W = 0.854, ns; (F)Aβ-42: W = 0.922, ns; (M)Aβ-42: W = 0.94, ns), concluding normal distribution. Further evaluation of skewness and kurtosis supported this, with values falling between +/-2 ((F)Aβ-40: Skew [1.175], Kurt [1.593]; (M)Aβ-40: Skew [1.097], Kurt [0.31]; (F)Aβ-42: Skew [0.606], Kurt [-0.729]; (M)Aβ-42: Skew [0.275], Kurt [-0.941]), which is considered acceptable (George & Mallery, 2006). Graphing of the means reveal differences between the (F)APP/PS1 + Oil and (M)APP/PS1 + Oil at baseline, which could be contributing to the inconsistent effects observed in the two-way ANOVA test.

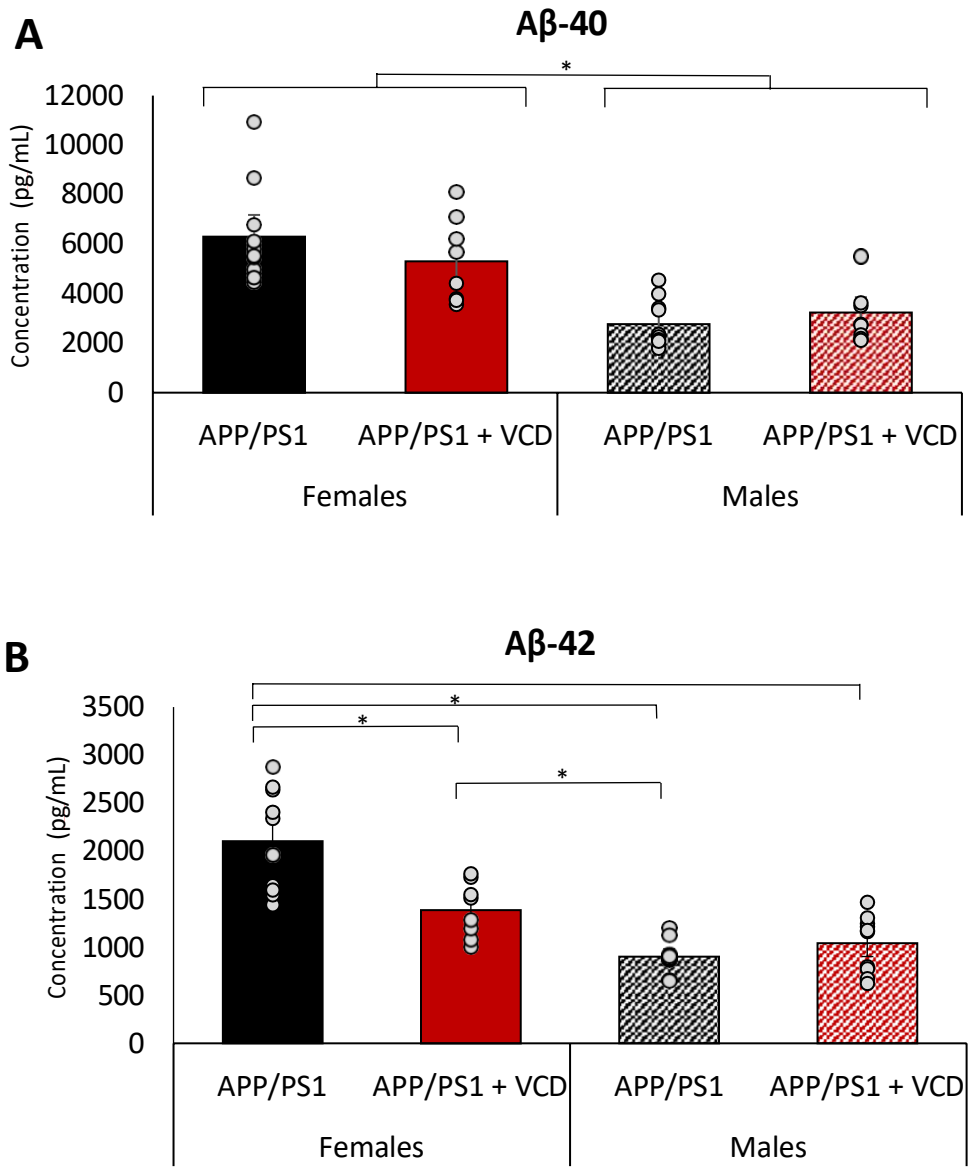

Figure 13. One-way ANOVA analysis of Aβ levels in APP/PS1 mice with VCD treatment. A) concentration of Aβ-40 levels B) concentration of Aβ-42 levels; * $p < 0.05$

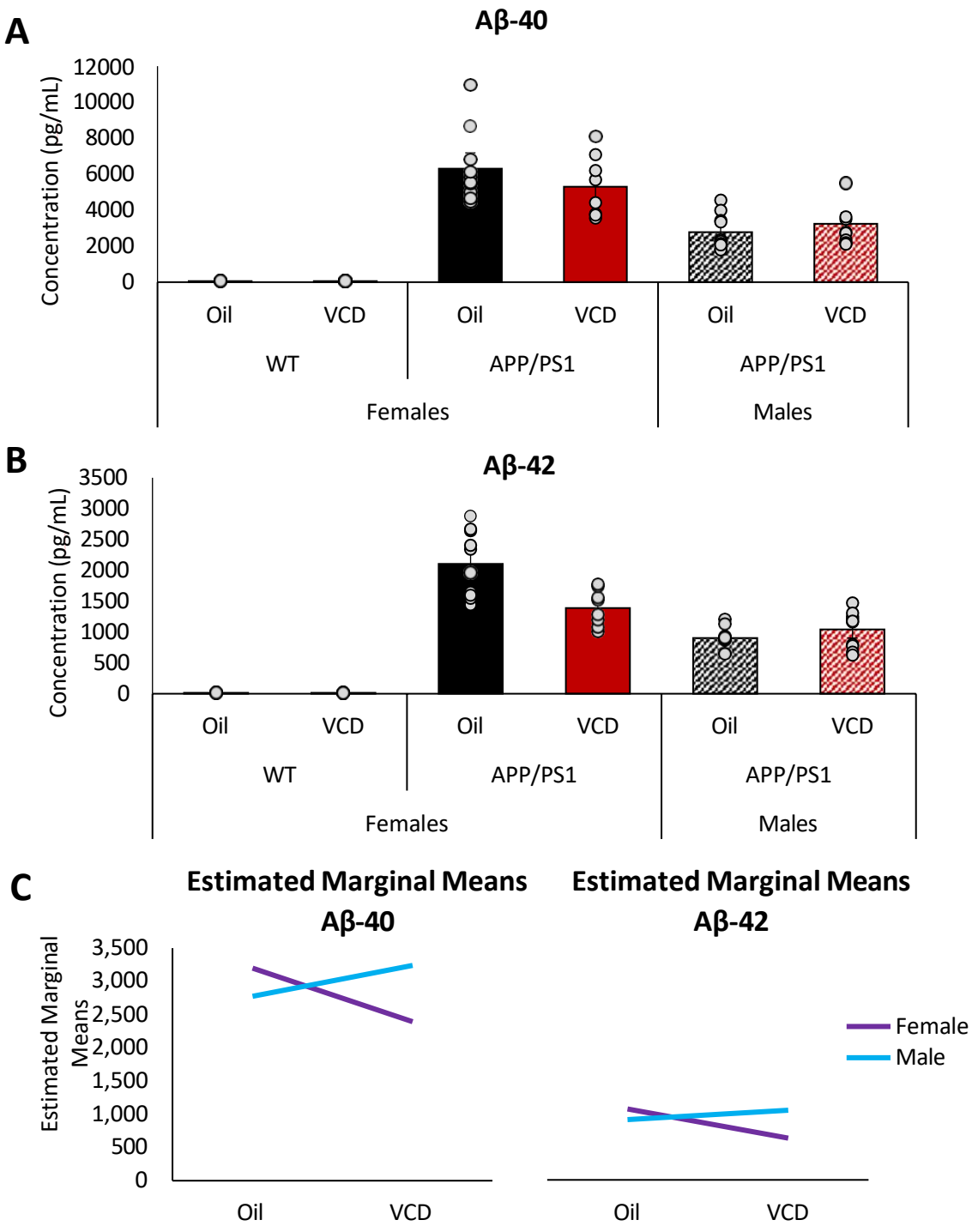

Figure 14. Two-way ANOVA analysis of Aβ levels in APP/PS1 mice with VCD treatment. A) concentration of Aβ-40 levels B) concentration of Aβ-42 levels C) plots of the estimated marginal means demonstrating an interaction effect between Sex*Treatment in Aβ-40 and Aβ-42

4.3 VCD treatment had no effect on p-tau and t-tau levels in WT or APP/PS1 mice

To investigate whether VCD treatment altered tau pathology, we performed western blot experiments to measure the total protein levels of p-tau 396/404 and t-tau in the hippocampus of the mice. Three-way ANOVA for p-tau 396/404 revealed no significant interaction (Sex*Genotype*Treatment: $F_{1,86} = 2.653$, ns; Genotype*Treatment: $F_{1,86} = 0.633$, ns; Sex*Treatment: $F_{1,86} = 0.002$, ns; Sex*Genotype: $F_{1,86} = 2.021$, ns) or main effects (Sex: $F_{1,86} = 1.383$, ns; Genotype: $F_{1,86} = 0.028$, ns; Treatment: $F_{1,86} = 0.026$, ns) (Figure 15A). Three-way ANOVA for t-tau also showed no significant interaction (Sex*Genotype*Treatment: $F_{1,98} = 0.241$, ns; Genotype*Treatment: $F_{1,98} = 3.252$, ns; Sex*Treatment: $F_{1,98} = 0.005$, ns; Sex*Genotype: $F_{1,98} = 2.895$, ns) or main effects (Sex: $F_{1,98} = 2.244$, ns; Genotype: $F_{1,98} = 0.25$, ns; Treatment: $F_{1,98} = 3.342$, ns) (Figure 15B). These findings demonstrate that there was no significant difference between the sexes, male and female, or the genotype, WT and APP/PS1. It also shows that VCD did not influence phosphorylation of tau at the 396/404-epitope site, or the overall levels of total tau. Further investigations into VCD treatment and tau pathology would be beneficial to measure additional epitope sites and how VCD may influence tau pathology in an AD tau mouse model.

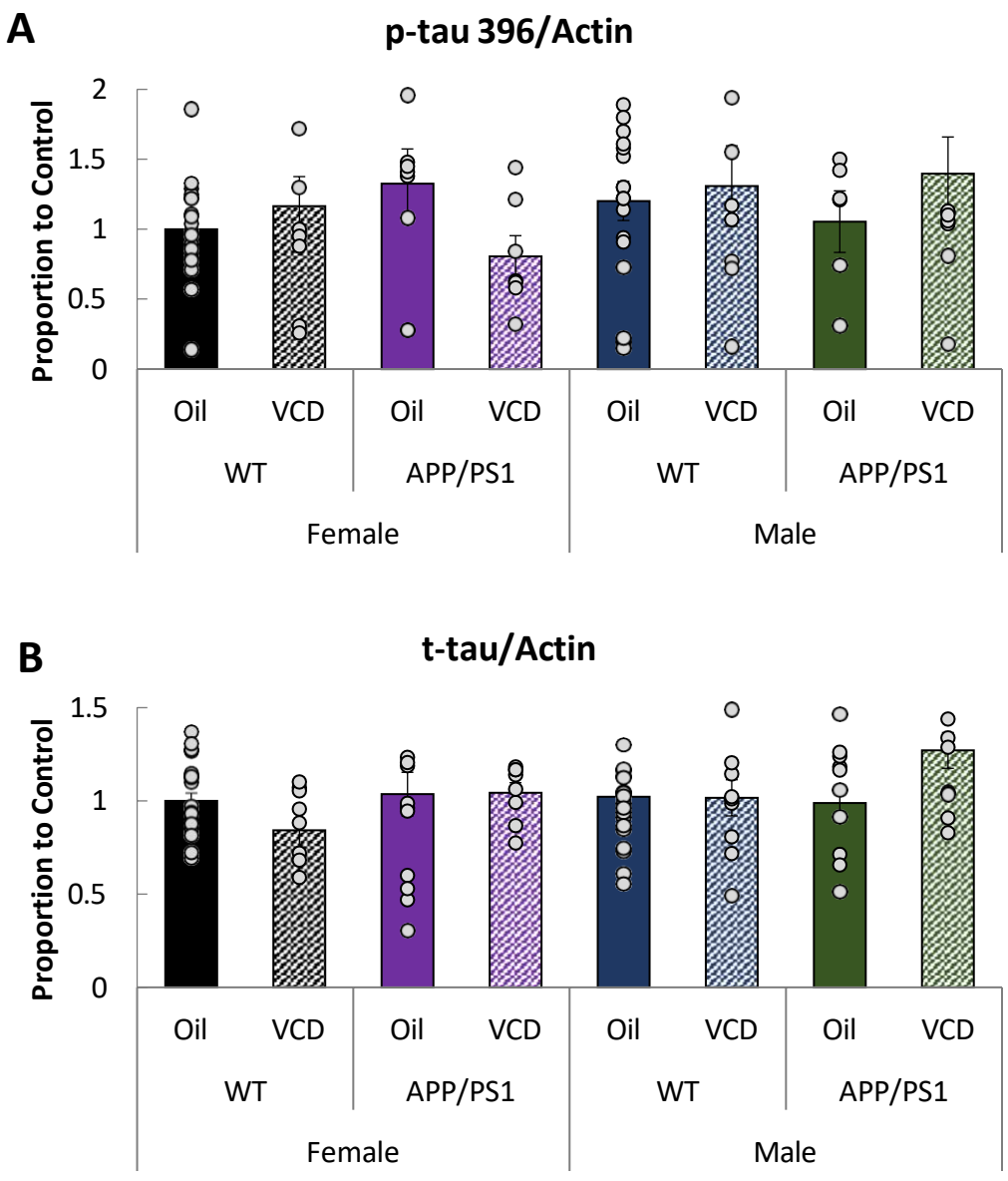

Figure 15. Tau Associated Changes A) Three-way ANOVA analysis of p-tau 396 normalized to beta-actin levels between treatment groups. No significant interactions or main effect were observed B) Three-way ANOVA analysis of p-tau 396 normalized to beta-actin levels between treatment groups. No significant interactions or main effect were observed; * $p < 0.05$

4.4 VCD treatment had no effect on GPER1 levels in WT or APP/PS1 mice

We aimed to identify a mechanism which could be altered in the VCD model and explain the changes we observed in the Aβ-42 levels. As described above, there are previous reports of alterations in GPCRs, including GABA-B from our lab, that could help result in significant changes in Aβ-42 in the (F)APP/PS1 + VCD mice (Salazar et al., 2021; Thathiah & De Strooper, 2011). Furthermore, literature describes the relationship between GPER1 and hormones, Aβ pathology, inflammation, and metabolic risk factors associated with AD (Blasko et al., 2009; Kurt et al., 2019; Mårtensson et al., 2009; Pan et al., 2020; Villa et al., 2016). Western blot evaluations and three-way ANOVA analysis showed no significant interaction (Sex*Genotype*Treatment: $F_{1,97} = 0.131$, ns; Genotype*Treatment: $F_{1,97} = 0.157$, ns; Sex*Treatment: $F_{1,97} = 0.044$, ns; Sex*Genotype: $F_{1,97} = 0.028$, ns) or main effects (Sex: $F_{1,97} = 0.164$, ns; Genotype: $F_{1,97} = 0.201$, ns; Treatment: $F_{1,97} = 0.521$, ns) in GPER1 (Figure 16A). This furthers our knowledge in how ovarian follicle depletion does not lead to changes in GPER1 and is not directly involved in the mechanism associated with the decreased levels of Aβ-42 in the (F)APP/PS1 + VCD mice.

4.5 VCD treatment had no effect on IDE in WT or APP/PS1 mice

To further our investigation into the relationship between altered Aβ levels and VCD, we measured IDE levels using western blot. IDE is shown to be involved in degradation of Aβ pathology and could account for some of the changes in Aβ-42 in the (F)APP/PS1 + VCD mice (Z. Zhao et al., 2007). Three-way ANOVA of the data showed no significant interaction (Sex*Genotype*Treatment: $F_{1,95} = 0.112$, ns; Genotype*Treatment: $F_{1,95} = 0.896$, ns; Sex*Treatment: $F_{1,95} = 0.527$, ns; Sex*Genotype: $F_{1,95} = 1.327$, ns) or main effects (Sex: $F_{1,95} = 0.17$, ns; Genotype: $F_{1,95} = 0.089$, ns; Treatment: $F_{1,95} = 0.025$, ns) in IDE (Figure 16B). Our data demonstrates that IDE is also not directly involved in the mechanism associated with the decreased levels of Aβ-42 in the (F)APP/PS1 + VCD mice. Additional research is needed to further understand the relationship between ovarian follicle depletion and Aβ pathology.

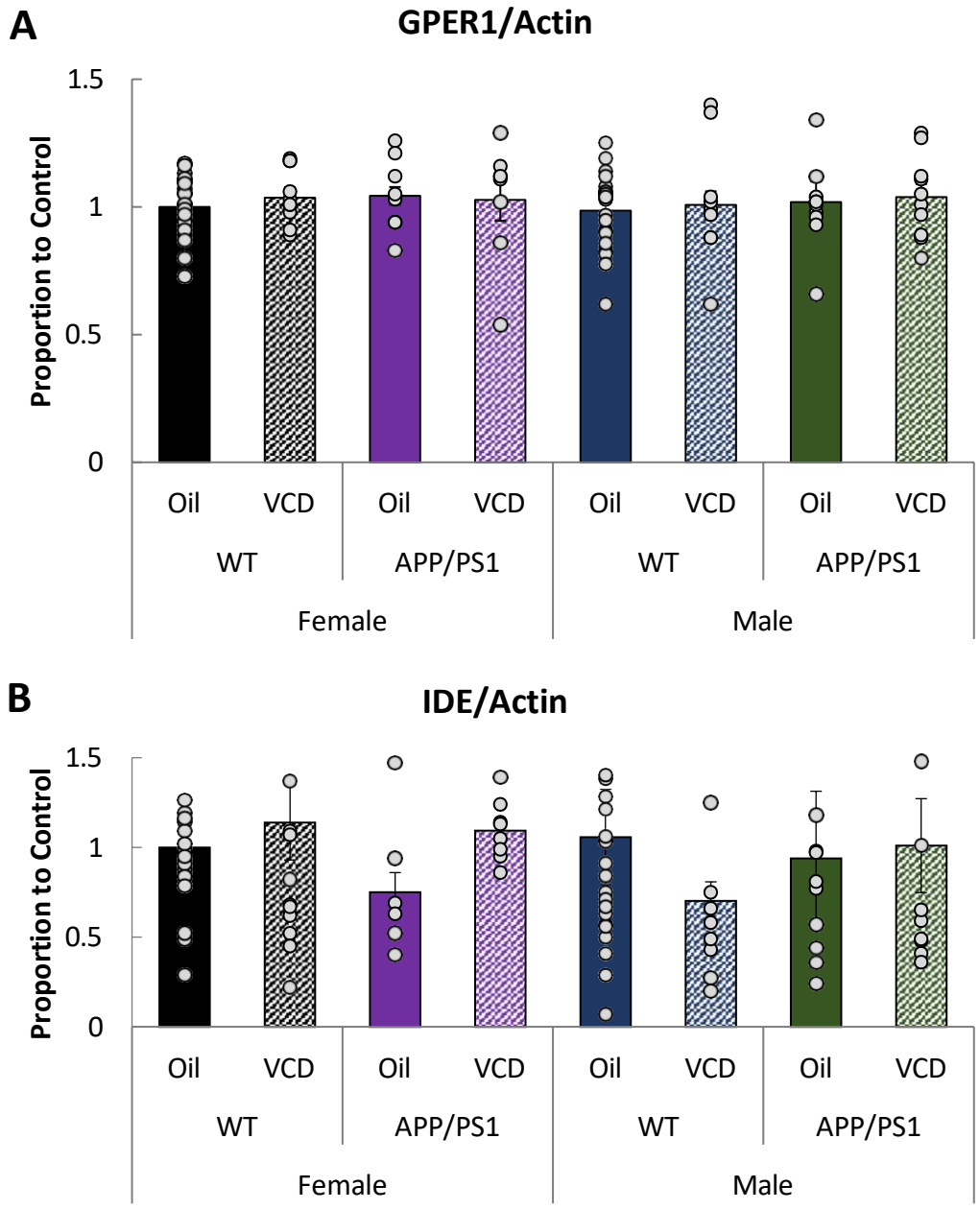

Figure 16. Proteins GPER1 and IDE Analysis to investigate mechnisms involved in the AB changes. A) Three-way ANOVA analysis of GPER1 normalized to beta-actin levels between treatment groups. No significant interactions or main effect were observed B) Three-way ANOVA analysis of IDE normalized to actin levels between treatment groups. No significant interactions or main effect were observed B) Three-way ANOVA analysis of p-tau 396 normalized to beta-actin levels between treatment groups. No significant interactions or main effect were observed; * $p < 0.05$

5. Discussion

Two-thirds of AD patients are women, making sex a risk factor for AD. Neuroscience research has historically focused on men, slowing progression of knowledge, and understanding of the female brain. There is a critical need for research into women's brain health and aging; with the high prevalence in women, AD is predicted to affect an estimated 9.3 million women in the U.S. by 2060 (Matthews et al., 2019). While societal and cultural factors play a role, there are several biological risk factors for AD in women, including genetic composition, immune response, metabolic health, mental health, and sex-specific factors. One of the greatest risk factors of AD in women is the transition to menopause, demonstrated by increases in AD pathogenesis in peri- and postmenopausal women (Buckley et al., 2022; Mosconi et al., 2021). As described earlier, women transition to menopause at about 50 years old, around the same time as AD-related brain changes are reported (Davis et al., 2015; Jack et al., 2013). Investigations into the relationship between menopause and AD in women could lead to a greater understanding of risk, novel biomarkers, protective measures, therapeutic treatments, and overall healthier aging for women. My study aims to reduce the prevalence of AD in women by using a novel mouse model of menopause, advancing basic science underlaying these processes and assisting with translation between animal models and human menopause.

Traditionally, preclinical studies of menopause use OVX rodents, surgically removing the ovaries, resulting in immediate and rapid changes in sex hormones. While the endpoint of the OVX model demonstrates similarities in human menopause, this model more closely represents bilateral oophorectomy-induced menopause, rather than natural menopause. In a 2022 paper estimating the number of bilateral oophorectomies performed in a geographically defined area in Minnasota, U.S., only about 0.1% of women have bilateral oophorectomies before the age of 50, suggesting that less than 0.1% of women have surgically-induced menopause (Erickson et al., 2022). While data is limited, this

estimate demonstrates a critical need for a mouse model of naturally occurring menopause, rather than a model of surgically-induced menopause. In recent years, a novel model of menopause has emerged, using VCD as a pharmacological treatment leading to advanced ovarian follicle depletion. Previous studies using VCD demonstrated it to be successful in depleting ovarian follicles, leading to gradual reductions in E2 and progesterone, and increases in FSH, constant with the transition to menopause in humans (Lohff et al., 2005). Unique to the VCD model, the pharmacological treatment results in a gradual transition into a menopause-like state, with ovarian tissues still intact, as in natural human menopause. This method also allows the ability to control the timing of transition by increasing or decreasing the dosage and regimen. Overall, VCD treatment in rodents is a better model of human menopause for research.

Ovarian follicle depletion results in anestrous (persistent diestrus), a way to indicate a menopause-like state in mice. To verify the mice in our project had transitioned into anestrous, we demonstrated vaginal cytology to be an effective method for monitoring the estrous cycle. Vaginal lavages were performed as a cost-effective, quick, and painless way to collect vaginal cell samples. Optimization of this protocol included sample collection medium and volume, staining concentration, and slide coating for cell adhesion (Cora et al., 2015). Quality sample collection, staining, and imaging ensures correct evaluation of cell types for accurate estrous cycle determination. To support the vaginal cytology data, I proposed to perform a terminal blood collection on the mice to measure FSH in plasma using ELISA. Unfortunately, the blood collection resulted in hemolyzed samples, failing quality control for the assay. Optimization of the blood collection is currently underway, including testing of a smaller gage needle and adjustment to the centrifugation parameters. Our lab will evaluate the FSH levels in the VCD treated mice in our upcoming project to measure the transition to a menopause-like state, and support our cytological evaluations. While we were hoping to have these data for this project, increases in FSH have already been previously reported in the VCD model by Dr. Patricia Hoyer's group as a reliable

indication of menopause-like state in mice using VCD treatment, providing a translational measure consistent with human menopause (Lohff et al., 2005).

In humans, the transition to menopause has demonstrated alterations to AD pathologies (Buckley et al., 2022; Mosconi et al., 2021). While it is well-documented that there is a relationship (described above), the mechanisms and timing surrounding AD changes in the brain through menopause is not well understood. Majority of studies involved in menopausal and AD research utilize the OVX model. A paper by Dr. Roberta Marongiu (2019) presents the VCD model of accelerated ovarian failure as a useful tool in studies of menopausal transition in relation to AD (Marongiu, 2019b). Though, to my knowledge, only one study has been done using this model in AD research. Golub et al investigated Aβ in APPswe VCD-induced mice, with interest in estrogen replacement therapy, and a focus on cognition through behavior testing (Golub et al., 2008). Mice in this study were treated with VCD at the same dose and duration as my present study, though the mice were allowed to incubate for 60-70 days, while my study investigated changes following 180 days of incubation. The researchers used immunohistochemistry to evaluate the percent of area positive for an n-terminal Aβ antibody, as a measure of overall Aβ levels, and found no significant changes (Golub et al., 2008). As these two papers represent the literature of VCD in AD research, my study fills a gap by using the well-established APP/PS1 mouse model with VCD treatment to further investigate Aβ changes using advanced methods of Luminex multiplexing for higher specificity and sensitivity to quantify Aβ.

Before I could begin menopausal related studies on the APP/PS1 mice, I first wanted to establish normal estrous cycling in the transgenic mouse model to determine whether there were any alterations due to genetic insertions or Aβ presence. Vaginal cytology in the (F)APP/PS1 + Oil mice showed estrous cycling consistent with the (F)WT + Oil mice, demonstrating the transgenes to not influence estrous cycling. Inducing accelerated ovarian failure in the (F)APP/PS1 + VCD mice successfully lead to anestrous, consistent with the (F)WT + VCD, representing menopause in the AD model. This demonstrated the

ability to use VCD in the APP/PS1 mouse model and allowed me to continue the studies on gradual menopausal transition and its influence on Aβ pathology, which is influential to AD research.

Using Luminex multiplex assay, Aβ-40 and Aβ-42 levels were measured simultaneously in the mice. We demonstrated no significant changes in the (M)APP/PS1 + VCD and (M)APP/PS1 + Oil, showing that the drug did not have off target effects directly related to Aβ levels. Compared to (M)APP/PS1 + Oil, (F)APP/PS1 + Oil had significantly higher levels of Aβ-40 and Aβ-42, consistent with the literature (J. Wang et al., 2003). In (F)APP/PS1 + VCD, there was a significant decrease in Aβ-42 compared to (F)APP/PS1 + Oil; we report no significant differences in Aβ-40. As Aβ-42 is the more toxic amyloidogenic peptide, this is an interesting finding (Vadukul et al., 2017). Human postmenopausal women show elevated levels of Aβ, and I hypothesized increases in Aβ in the mice following VCD treatment as well. This unexpected finding may be related to timing, as studies on HRT suggest that treatment at different times throughout the menopausal transition, and even the years following, can affect results, indicating changes to the underlying pathways. With this mind, a full study of APP/PS1 mice with VCD treatment should be performed at different timepoints to capture the pathogenic and mechanistic changes throughout the process. More closely modeling human menopause by intact ovarian tissue and gradual transition to ovarian failure, the findings from these VCD studies could be applied to human research through a translational approach, leading to novel biomarkers and targets for therapeutic treatments.

HRT has been a compelling but controversial topic. One of the most well-known HRT studies is the Women's Health Initiative Estrogen plus Progestin (WHI-EPT) clinical trial, enrolling 16,608 postmenopausal women ages 50 to 79. Following the report of trial results in 2002, the trial was discontinued by the data and safety monitoring board due to adverse effects in cardiovascular disease and breast cancer, with additional evidence of increased risk of coronary heart disease, stroke, and pulmonary embolism, outweighing data showing benefits in bone health and colon cancer (Rossouw et al., 2002). Data from an ancillary study to the WHI-EPT, the Women's Health Initiative Study of Cognitive

Aging (WHIMS), evaluated cognitive function following the HRT (Shumaker et al., 2004). This study reported that HRT did not decrease MCI or dementia, and authors recommended against using HRT for prevention of dementia or treatment of cognitive decline (Shumaker et al., 2004). This led to significant drops in the prescriptions and usage of hormone therapies, including a 46% reduction in the U.S. within just 5 months after the announcement of the early termination of WHI clinical trial (Buist et al., 2004). However, evaluations of the trials led to controversy surrounding the study (Cagnacci & Venier, 2019). The WHI-EPT clinical trial treated patients daily with a combined oral tablet of 0.625mg of equine estrogen and 2.5mg of medroxyprogesterone acetate (Manson et al., 2003). The use of equine estrogens were typical for HRT treatments during that time period, however, the WHI findings led to development of safer new estrogens derived from plants and synthesized in the lab (Files et al., 2011). Another question that emerged was the timing of treatment in relation to menopause. Eligibility criteria for this study was postmenopausal women ages 50-79; with menopause occurring at about 50 years old, at average, this sample greatly varied in time spent in menopause prior to treatment. New studies have investigated HRT in menopausal women within 5 years of menopausal transition and found benefits to cognition, leading to the "critical window hypothesis" (Maki, 2013; Mills et al., 2023; Shao et al., 2012). Other factors that need to be considered for HRT is the formulation of the drug, dose, regimen, and route of administration.

Important to the drug development and testing process, pre-clinical investigations of novel therapeutics are necessary before clinical trials. Use of OVX rodents limits the ability for human menopause to be accurately modelled, with no perimenopausal period and the excision of the ovarian tissue. The VCD model allows investigations into preventive treatment, early interventions during pre- and perimenopausal periods, and treatment following a more natural menopausal transition. There are similarities in the hormones altered between the VCD model and human menopause, allowing basic science advancements in biomarker discovery and novel treatments targeting specific mechanisms

involved in menopause in relation to AD. Controlling dose and duration of VCD treatment can extend or shorten the perimenopausal period, allowing for more specific studies of mechanistic changes throughout the transition.

Discrepancies on findings across menopausal studies related to AD and brain processes are common. One reason for this variability could be the absence of timing details collected and reported not only in clinical trials, but also in the menopausal rodent models. A recent 2020 time course study demonstrated that even in OVX mice, brain changes occur over 8 weeks following ovariectomies, using novel object recognition, step-through passive avoidance, and Morris water maze cognitive tests (Tao et al., 2020). Furthermore, alterations in hippocampal proteins, brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) demonstrated changes in synaptic plasticity, while alterations in unc-51 like autophagy activating kinase 1 (ULK1) and microtubule-associated protein 1 light chain 3 (LC3)-II/LC3-I indicated changes in autophagy in the OVX mice 8-weeks post-surgery (Tao et al., 2020). Together, this demonstrates the plasticity of the brain and how pathways evolve and adapt, even to peripheral changes. However, this also highlights the need for better reporting of study timelines related to hormone changes and timing of treatments. Several studies fail to report the type of surgery being performed (unilateral vs bilateral), how soon after surgery mice undergo drug treatment, cognitive testing, and sacrifice, limiting reproducibility, interpretation, and comparisons between studies. With both the OVX and VCD models, reporting of details regarding the study timeline is vital.

GPCRs are cell surface receptors involved in a range of processes in the CNS, with over 90% of GPCRs being expressed in the brain (Vassilatis et al., 2003). About 34% of FDA approved drugs target GPCRs, and 60% of candidate drugs in clinical trials are aimed at these receptors, demonstrating the promise they have in DMT (Hauser et al., 2017; D. Yang et al., 2021). GPCRs are involved in many stages of AD progression, showing relationships with Aβ production, aggregation, and clearance (Thathiah & De Strooper, 2011). Previous data from our lab demonstrated significant decreases in the GPCR GABA-B in

male APP/PS1 mice with the presence of Aβ pathology (Salazar et al., 2021). Furthermore, our recent publication demonstrated that knockdown of the GABA-B receptor in APP/PS1 mice exacerbated pathology (Osse et al., 2023). With these findings, I investigated changes in GPER1, an estrogen GPCR in the APP/PS1 mice, as well as its relationship to menopause through VCD. Alterations in this receptor could account for changes in Aβ-42 in the (F)APP/PS1 + VCD mice observed. This study showed no significant alterations in GPER1 between sexes, genotype, or treatment, suggesting that GPER1 may not be involved in the mechanisms associated with Aβ pathology and greater risk of AD in women. Changes in sex hormones involved with menopause also does not influence GPER1 levels. Altogether, our findings highlight the special role of GABA-B in Aβ pathology, not observed in all GPCRs. Interestingly, research has described a relationship between GABA-B and hormone levels. Chronic E2 treatment in rats results in significant decreases in the abundance of GABA-B receptors in the brain (François-Bellan et al., 1989). Furthermore, using a GABA-B agonist, baclofen, and progesterone treatment in female OVX rats showed altered GABA-B receptor binding in relation to progesterone levels (al-Dahan & Thalmann, 1996). Comparison of progesterone levels to the estrous cycle suggested greater GABA-B receptor binding during proestrus and lowest binding during estrus (al-Dahan & Thalmann, 1996). Future directions should include evaluations of the GABA-B receptor in female APP/PS1 mice treated with VCD to better understand this relationship between this receptor and menopause.

With connections between metabolic health and menopause, as well as metabolic risk factors for AD, I evaluated IDE levels in this project and found no significant changes between sex, genotype, or treatment. This was another unexpected finding, as there are previous reports of IDE alterations in OVX mice (L. Zhao et al., 2011). As discussed earlier, this may be due to timing differences between the OVX and VCD models. A time course study comparing IDE and Aβ levels through VCD induced menopausal transition would be interesting. I evaluated p-tau 396/404 and t-tau levels as downstream effects of GPER1 and IDE, and found no significant differences in either measure, consistent with no changes in the

upstream proteins. Further research into downstream effects, including markers of inflammation, cell death, and bioenergetics would be worthy to help explain the effects of Aβ-42 alterations in the (F)APP/PS1 + VCD mice.

Employing the VCD model into other AD and risk factor models is essential. Currently, we induced menopause in a tauopathy AD mouse model using VCD and are evaluating changes in tau. In addition, our lab developed a protocol to model DM2 by inducing hyperglycemia in mice using staggering injections of streptozotocin (STZ), a pancreatic beta-cell toxin (Murtishaw et al., 2018). We combined the STZ and VCD models to investigate the link between DM2 and menopause. Preliminary data demonstrated remarkable changes in blood-glucose levels, critical to ongoing research. As I have highlighted, timing of brain and hormone changes is important to AD research, and utilization of EOAD mouse models are not ideal for studies, as these models tend to have differences in the progression of AD pathology based on their genetic mutations, and may have alterations in pathways not constant to LOAD. Pathogenesis in these mice may also be more aggressive than in human AD. It would be worthy to employ the VCD model into LOAD mouse models to better understand the mechanisms associated with menopause and AD, such as the Model Organism Development and Evaluation for Late-onset Alzheimer's Disease (MODEL-AD) Consortium's genetic risk factor models TREM2*R47H and APOE4 knock-ins (*APOE4 Knock-In (Lamb) | ALZFORUM*, n.d.; *Trem2 R47H KI (Lamb/Landreth) | ALZFORUM*, n.d.).

The novel VCD model will advance research by using a translational approach. More closely modeling human menopause by maintaining ovaries intact and a gradual transition to ovarian failure, findings from VCD studies could be more carefully applied to human research. Investigations into mechanistic changes through menopausal transition could lead to new developments in AD biomarkers and therapeutic treatments. Knowledge of how sex hormones influence AD, could advance research, mitigating the gender gap in AD prevalence. Currently, there are only three drugs in the AD pipeline for

treatment of AD targeting growth factor and hormone pathways, 1) CORT108297, a glucocorticoid receptor modulator used for the reduction of corticosterone (murine cortisol) pertaining to stress (NCT04601038), 2) Gonadotrophin releasing hormone (GnRH) being investigated in patients with Down syndrome (NCT04390646), and 3) Leuprolide (Leuprorelin; Lupron), a synthetic analog of GnRH (NCT03649724) (J. L. Cummings et al., 2023). Leuprolide is a GnRH agonist with indirect effects on reducing LH and FSH. Pre-clinical models demonstrated improvement in cognition in aged OVX WT animals and female Tg2576 mice, while reducing Aβ (Bryan et al., 2010; Casadesus et al., 2006). Pre-clinical testing of novel drugs in the VCD model may lead to greater translational findings for AD drug development.

In HRT clinical studies, inclusion criteria are typically broad to assist with recruitment, allowing postmenopausal women over a specific age to be recruited into the study. As basic science data shows and previous clinical trials support, timing of treatment is important, and the number of years postmenopausal may influence drug effects on the brain, which should be accounted for. Furthermore, inclusion into the study may not be the concern, but collection of menopausal details (i.e. years that an individual has been postmenopausal) and/or stratification of data should be performed, which could undercover effects otherwise masked, and allow better understanding of HRT benefits on cognition and AD. While this seems obvious for studies pertaining to HRT, there is a need to apply these practices across all clinical trials and drug development. As personalized medicine and combination therapies continue to expand, treatment in a sex-specific manner may be especially beneficial for women in relation to menopausal status. Creating awareness and understanding that there are fundamental differences between the male and female brain that should be considered is vital. As described previously, female participation is higher in AD clinical trials than men (58%), though it is still disproportionate to the prevalence of the disease in women (64%), ranging from 2.2%-90.7% women (Pinho-Gomes et al., 2022). Data from studies should be stratified, especially in studies with many

participants, to tease out sex effects; neglecting to do so may be contributing to the high failure rate in AD drugs (J. L. Cummings et al., 2014). Reproductive history is generally not collected, but would be an easy measure to include to further research for AD therapeutics. Overall, there is a need to bring awareness and focus to women's brain health and aging, and the possibility of sex-specific biomarkers and treatment for AD.

Overall, the aim of my study was to investigate cellular and molecular alterations in AD pathogenesis in relation to menopause. I employed a novel mouse model of advanced ovarian follicle depletion using VCD to provide a more translational menopausal model for AD research. Quantification of Aβ in (F)APP/PS1 + VCD mice resulted in significant decreases in Aβ-42 compared to (F)APP/PS1 + Oil mice. Evaluations of related proteins GPER1, IDE, p-tau 396/404 and t-tau showed no significant differences, demonstrating a need for further research into the mechanisms associated with Aβ pathology and menopausal transition. My study successfully exhibits how the VCD rodent model in AD research can be used to inform and advance biomarker discovery, drug development, and clinical trials, mitigating the prevalence of AD in women.

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Osse, A.M.L., Nguyen, A., Moeller, S., Cummings, J.L., Kinney, J.W., John, S.E., blood-based biomarkers for Alzheimer's disease among ethnoracial minority samples – a scoping review. In-progress

Ortiz, A.A., Murtishaw A.S., Salazar, A.M., **Osse, A.M.L.,** Kinney, J.W., Hyperglycemia and high-fat diet differentially impact Alzheimer's disease-related changes in a fractalkine receptor knockout mouse model. Submitted for publication

Calvin-Dunn, K., Mcneela, A., **Osse, A.M.L.,** Bhasin, G., Ridenour, M., Kinney, J.W., Hyman, J., Electrophysiological Insights from animal models in human studies of Alzheimer's disease: An exhaustive review. Submitted for publication

Yang, Z., Sreenivasan, K.R., Strom, E.N., **Osse, A.M.L**., Pasia, L.G., Cosme, C.G., Mugosa, M.N., Chevalier, E.L., Ritter, A., Miller, J.B., Cordes, D., Cummings, J., Kinney, J.W. Clinical and biological relevance of glial fibrillary acidic protein in Alzheimer's disease. Alzheimer's Research & Therapy. 2023

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Osse, **A.M.L,** Pandey, R.S., Wirt, R.A., Ortiz, A.A., Salazar, A., Kimmich, M., Strom, E.N., Oblak, A., Lamb, B., Hyman, J.M., Carter, G.W., Kinney, J.W., (2023) Reduction in GABAB on glia induce Alzheimer's disease related changes. Brain, Behavior, and Immunity

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Salazar, A.M., **Leisgang, A.M.,** Ortiz, A.A., Murtishaw, A.S., Kinney, J.W., (2020). Alterations of GABA B receptors in the APP/PS1 mouse model of Alzheimer's Disease. Neurobiology of Aging.

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CONFERENCE PRESENTIONS:

Ortiz, A.A., **Osse, A.M.L.,** Wang, J., Pasia, L., Balsamo, B., Suk, K., Sharma, S., Shanchez, R., Cortez, L., Hernandez, K., Kinney, J.W. (TBD) Exacerbation of Beta-Amyloid and Diabetes-Associated Pathology due to Chronic Hyperglycemia in an Alzheimer's disease mouse model. Poster accepted for presentation at UNLV OUR Fall 2023 Undergraduate Research Symposium (Las Vegas, NV-Symposium rescheduled)

Hernandez, K., Ortiz, A.A., **Osse, A.M.L.,** Kinney, J.W. (TBD) Measuring Tau Protein Phosphorylation in a Humanized Mouse Model of Alzheimer's disease under Chronic hyperglycemic Conditions. Poster accepted for presentation at UNLV OUR Fall 2023 Undergraduate Research Symposium (Las Vegas, NV-Symposium rescheduled)

McCurn, K., **Osse, A.M.L.,** Ortiz, A.A., Balsamo, B., Kinney, J.W. (TBD) Evaluating sex differences in a Tau Model of Alzheimer's disease. Poster accepted for presentation at UNLV OUR Fall 2023 Undergraduate Research Symposium (Las Vegas, NV-Symposium rescheduled)

Mamales, C., **Osse, A.M.L.,** Ortiz, A.A., Suk, K., Osario, J., Omidiji, L., McCurn, K., Kinney, J.W. (TDB) Evaluation of a Mouse Model with Loss of GABA (B) Receptors Crossed with a Tau P301S Model. Poster accepted for presentation at UNLV OUR Fall 2023 Undergraduate Research Symposium (Las Vegas, NV-Symposium rescheduled)

McCurn, K., **Osse, A.M.L.,** Ortiz, A.A., Balsamo, B., Kinney, J.W. (2023) Evaluating sex differences in a Tau Model of Alzheimer's disease. Poster presented at the Society for Neuroscience (SfN) Annual Conference (Washington D.C.)

Osse, A.M.L., Ortiz, A.A., Mamales, C., Suk, K., Osario, J., Omidiji, L., McCurn, K., Kinney, J.W. (2023) Evaluation of a Mouse Model with Loss of GABA (B) Receptors Crossed with a Tau P301S Model. Poster presented at the Society for Neuroscience (SfN) Annual Conference (Washington D.C.)

Ortiz, A.A., **Osse, A.M.L.,** Wang, J., Pasia, L., Balsamo, B., Suk, K., Sharma, S., Shanchez, R., Cortez, L., Hernandez, K., Kinney, J.W. (2023) Exacerbation of Beta-Amyloid and Diabetes-Associated Pathology due to Chronic Hyperglycemia in an Alzheimer's disease mouse model. Poster presented at the Society for Neuroscience (SfN) Annual Conference (Washington D.C.)

Hernandez, K., Ortiz, A.A., Romero, L., **Osse, A.M.L.,** Kinney, J.W. (2023) Assessing the role of neuroinflammation in a mouse model of hyperglycemia with relevance to Alzheimer's disease. Poster presented at the Society for Neuroscience (SfN) Annual Conference (Washington D.C.)

Ortiz, A.A., **Osse, A.M.L.,** Balsamo, B., Suk, K.S., Cruz, K.S., Cortez, L.E., Godinez-Cardona, M., Kinney, J.W. (2023) Investigating Alzheimer's Disease-Related Pathology in a Chronic Hyperglycemic Mouse Model. Poster presented virtually at Alzheimer's Association International Conference (AAIC) (Amsterdam, Netherlands)

McCurn, K., **Osse, A.M.L.,** Oritz, A.A., Kinney, J.W., (2023) Investigating Alterations of the GABA-B Receptor in a Tau Model of AD. Poster presented at Center for Academic Enrichment and Outreach: Minority-Serving Institution Week 2023 Research Symposium (Las Vegas, NV)

Hernandez, K., Ortiz, A.A., Romero, L., Platt, A., **Osse, A.M.L.,** Godinez-Cardona, M., Kinney, J.W., (2023) Examining Neuroinflammation Severity in the Hippocampus of a Chronic Hyperglycemic Mouse Model Related to Alzheimer's Disease. Poster presented at Center for Academic Enrichment and Outreach: Minority-Serving Institution Week 2023 Research Symposium (Las Vegas, NV)

McCurn, K., **Osse, A.M.L.,** Oritz, A.A., Kinney, J.W., (2023) Investigating Alterations of the GABA-B Receptor in a Tau Model of AD. Poster presented at Center for Academic Enrichment and Outreach: Minority-Serving Institution Week McNair National Conference (College Park, MD)

Hernandez, K., Ortiz, A.A., Romero, L., Platt, A., **Osse, A.M.L.,** Godinez-Cardona, M., Kinney, J.W., (2023) Examining Neuroinflammation Severity in the Hippocampus of a Chronic Hyperglycemic Mouse Model Related to Alzheimer's Disease. Poster presented at Center for Academic Enrichment and Outreach: Minority-Serving Institution Week McNair National Conference (College Park, MD)

Wang, E.H., Kurzyniec S., Ortiz, A.A., Strom, E.N., Jones-Lepp, T., **Osse, A.M.L**., Kinney, J.W., Okamura, Y., (2023). Media Component Analysis during Human Primary T Cell Culture using a Triple Quadrupole Mass Spectrometer. Poster presented at 71st ASMA Conference on Mass Spectrometry and Allise Topics (Houston, TX)

Romero Yusti, I., **Osse, A.M.L**., Ortiz, A.A., Kinney, J.W., (2023) Evaluation of GABA-B receptor levels on glia cells in the novel GAB/CX3ert mouse model. Poster presented at the National Collegiate Research Conference (NCRC) (Harvard University, Boston, MA)

Osse, A.M.L., Ortiz, A.A., Strom, E.N., Kinney J.W. (2023). Evaluation of sex differences in a novel mouse model with loss of GABAB receptors. Poster presented at the UNLV's 25th Annual Graduate and Professional Student Research Forum (Las Vegas, NV) – **2nd Place Poster Session Winner**

Osse, A.M.L., Ortiz, A.A., Strom, E.N., Kinney J.W. (2022). Evaluation of sex differences in a novel mouse model with loss of GABAB receptors. Poster presented at the Society for Neuroscience (SfN) Annual Conference, (San Diego, CA)

Strom, E.N., **Osse, A.M.L.**, Ortiz, A.A., Jones-Lepp, T., Kurzyniec, S. Kinney J.W. (2022). Evaluating proteomic and metabolomic differences from glial cell cultures in neurodegenerative disease mouse models. Poster presented at the Society for Neuroscience (SfN) Annual Conference (San Diego, CA)

Ortiz, A.A., **Osse, A.M.L.**, Hernandez, K., Pineda, C., Romero, L., Wang, J., Platt, A., Godinez Cardona, M., Strom, E.N., Salazar, A., Kinney J.W. (2022). Age-dependent changes in a hyperglycemic mouse model relevant to Alzheimer's disease. Poster presented at the Society for Neuroscience (SfN) Annual Conference (San Diego, CA)

Hernendez, K.S., Ortiz, A.A., Romero, L.E., **Osse, A.M.L**., Platt, A.R., Godinez-Cardona, M.N., Kinney, J. W., (2022) Examining Neuroinflammation Severity in the Hippocampus of a Chronic Hyperglycemic Mouse Model Related to Alzheimer's Disease. Lightning Talk given at the UNLV Fall Undergraduate Research Symposium (Las Vegas, NV)

Romero Yusti, I., **Osse, A.M.L**., Ortiz, A.A., Kinney, J.W., (2022) Evaluation of GABA-B receptor levels on glia cells in the novel GAB/CX3ert mouse model. Poster presented at the UNLV Fall Undergraduate Research Symposium (Las Vegas, NV)

Romero Yusti, I., **Osse, A.M.L**., Ortiz, A.A., Kinney, J.W., (2022) Evaluation of estrous cycle and menopause induction in the novel GAB/CX3ert mouse model. Poster presented at the UNLV Fall Undergraduate Research Symposium (Las Vegas, NV)

Romero, L.E., Ortiz, A.A., **Osse, A.M.L.** Hernandez, K.S., Godinez-Cardon, M.N., Kinney, J.W., (2022) Investigating microglial activity in the prefrontal cortex of a mouse model of sustained hyperglycemia in relation to Alzheimer's Disease. Poster presented at the UNLV Fall Undergraduate Research Symposium (Las Vegas, NV)

Hernendez, K.S., Ortiz, A.A., Romero, L.E., **Osse, A.M.L**., Platt, A.R., Godinez-Cardona, M.N., Kinney, J. W., (2022) Examining Neuroinflammation Severity in the Hippocampus of a Chronis Hyperglycemic Mouse Model Related to Alzheimer's Disease. Poster presented at the Nevada INBRE Symposium (Las Vegas, NV)

Ortiz, A.A., Wang, J., Pineda, C., **Osse, A.M.L**., Platt, A., Kinney, J.W., (2022) Diabetes – a major risk factor for Alzheimer's Disease. Poster presented at the UNLV Graduate and Professional Student Research Forum (Las Vegas, NV)

Platt, A.R., Wirt, R.A., **Leisgang, A.M.,** Flores, E., Crew, L., Kinney, J.W., Hyman, J.M. (2021, November). GABAergic Signaling in Microglia on Hippocampal-cortical Network Activity and Remote Recall. Poster presented virtually at the UNLV OUR's Fall Undergraduate Research Symposium

Ferguson, S.J., **Osse, A.M.L**, Ortiz, A.A., Mamales, C., Poston, W., Kinney, J.W. (2021, November). Behavioral Evaluation of a Novel Mouse Model with the Loss of GABAB Receptors. Poster presented virtually at the UNLV OUR's Fall Undergraduate Research Symposium

Ferguson, S.J., **Osse, A.M.L**, Ortiz, A.A., Mamales, C., Poston, W., Kinney, J.W. (2021, September). Behavioral Evaluation of a Novel Mouse Model with the Loss of GABAB Receptors. Poster presented at the American Indian Science and Engineering Society (Phoenix, AZ)

Ortiz, A. A**.**, **Osse, A.M.L.,** Platt, A.M., & Kinney, J.W. (2021, July) Examining the effects of a blood glucose rescue on Alzheimer's disease-related pathology, in young vs. aged hyperglycemic mice. Poster presented at the Alzheimer's Association International Conference (Virtual)

Leisgang, A.M., Salazar, A.M., Chuapoco, R.L., Chowdhry, A., Ortiz, A.A., Edwards, P. J., Kinney J.W. (2021, February). Evaluation of a novel mouse model with loss of GABAB receptors through an immune challenge. Poster presented virtually at the UNLV's 23rd Annual Graduate & Professional Student Research Forum (Las Vegas, NV)

Platt, A., Ortiz, A., **Leisgang, A**., Kinney, J. (2020, November). Effects of a blood glucose rescue on Alzheimer's-related pathology in young verses aged hyperglycemic mouse models. Poster presented virtually at UNLV OUR's 2020 Fall Undergraduate Research Symposium (Las Vegas, NV)

Leisgang, A.M., Salazar, A.M., Chuapoco, R.L., Chowdhry, A., Ortiz, A.A., Edwards, P. J., Kinney J.W. (2020, February). Evaluation of a novel mouse model with loss of GABAB receptors through an immune challenge. Poster presented at the UNLV's 22nd Annual Graduate & Professional Student Research Forum (Las Vegas, NV)

Chowdhry, A., **Leisgang, A.,** Chuapoco, R., Salazar, A., Kinney, J. (2019, November). An Examination of Alzheimer's Disease Pathology: Neuroinflammatory Marker Levels in GABA-B Knockout Mice. Poster presented at the UNLV OUR's 2019 Fall Undergraduate Research Symposium (Las Vegas, NV)

Leisgang, A.M., Salazar, A.M., Chuapoco, R.L., Chowdhry, A., Ortiz, A.A., Edwards, P. J., Kinney J.W. (2019, October). Evaluation of a novel mouse model with loss of GABAB receptors through an immune challenge. Poster presented at the Society for Neuroscience (SfN) Annual Conference, Chicago, IL.

Leisgang, A.M., Salazar, A.M., Chuapoco, R.L., Chowdhry, A., Ortiz, A.A., Edwards, P. J., Kinney J.W. (2019, October). Evaluation of a novel mouse model with loss of GABAB receptors through an immune challenge. Poster presented at the 2019 NIH IDeA Western Regional Conference, Las Vegas, NV.

Chuapoco, R., **Leisgang, A.,** Chowdhy, A., Salazar, A., Kinney, J. (2019, October). An Examination of Alzheimer's Disease Pathology: Neuroinflammation in GABA Knockout Mouse Models. Poster presented at the 2019 NIH IDeA Western Regional Conference (Las Vegas, NV)

Perez, M., Ortiz, A.A., **Leisgang, A.M**., Tran, C.M., Chuapoco, R.L., Salazar, A.M., Kinney, J.W. (2019, October). Examination of Alzheimer's Disease-related Pathology as a Result of Hyperglycemia in Young Versus Aged Mice. Poster presented at the 2019 NIH IDeA Western Regional Conference (Las Vegas, NV)

Chuapoco, R., **Leisgang, A.,** Chowdhy, A., Salazar, A., Kinney, J. (2019, August). An Examination of Alzheimer's Disease Pathology: Neuroinflammation in GABA Knockout Mouse Models. Poster presented at the UNLV OUR's 2019 Summer Undergraduate Research Symposium (Las Vegas, NV)

Guese, C., Ortiz, A.A., **Leisgang, A.M**., Salazar, A.M., Perez, M., Platt, A., Kinney, J. (2019, August). Examining Alterations of GABAB Receptors in Hyperglycemia and Alzheimer's Disease Related Pathology. Poster presented at the UNLV OUR's 2019 Summer Undergraduate Research Symposium (Las Vegas, NV)

Perez, M., Ortiz, A.A., **Leisgang, A.M**., Tran, C.M., Chuapoco, R.L., Salazar, A.M., Kinney, J.W. (2019, August). Examination of Alzheimer's Disease-related Pathology as a Result of Hyperglycemia in Young Versus Aged Mice. Poster presented at the UNLV OUR's 2019 Summer Undergraduate Research Symposium (Las Vegas, NV)

Leisgang, A.M., Salazar, A.M., Ortiz, A.A., Boren, A.J., Kinney J.W. (2019, February). GABA-specific changes in a mouse model of Alzheimer's Disease. Poster presented at the UNLV's 21st Annual Graduate & Professional Student Research Forum (Las Vegas, NV)

Leisgang, A.M., Salazar, A.M., Ortiz, A.A., Boren, A.J., Kinney J.W. (2018, November). GABA-specific changes in a mouse model of Alzheimer's Disease. Poster presented at the Nevada Regional Neuroscience Meeting (Las Vegas, NV)

Leisgang, A.M., Salazar, A.M., Ortiz, A.A., Boren, A.J., Kinney J.W. (2018, November). GABA-specific changes in a mouse model of Alzheimer's Disease. Poster presented at the Society for Neuroscience (SfN) (San Diego, CA)

Ortiz, A.A, **Leisgang, A.M**., Tran, C.M., Chuapoco, R.L., Perez, M., Salazar, A.M., Kinney, J.W. (2018, November). Examination of Alzheimer's disease – related pathology as a result of hyperglycemia in young versus aged mice. Poster presented at the Society for Neuroscience (SfN) (San Diego, CA)

Leisgang, A.M., Salazar, A.M., Ortiz, A.A., Boren, A.J., Kinney J.W. (2018, June). GABA-specific changes in a mouse model of Alzheimer's Disease. Poster presented at the NIH, NIGMS Seventh Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE) (Washington D.C.)

Thu, S., Christensen, Z., Holman, E., Chang, K., Phillips, M., **Leisgang, A.,** Hodkiewicz, E., Palmer, R., Sandhu, D. (2015, May). Map based cloning of five male-sterility genes in Soybean. Poster presented at the UWSP College of Letters & Science Undergraduate Research Symposium (Stevens Point, WI)

Thu, S., Christensen, Z., Holman, E., Chang, K., Phillips, M., **Leisgang, A**., Hodkiewicz, E., Palmer, R., Sandhu, D. (2015, April). Map based cloning of five male-sterility genes in Soybean. Poster presented at the UW System Symposium for Undergraduate Research & Creative Activity (Milwaukee, WI)

TRAININGS & CERTIFICATION:

IDeA National Resource for Proteomics Faculty and Student Workshop,

- Accepted through application process for all-expense paid trip
- National Institutes of Health IDeA Program, Arkansas, February 2020

Flow Cytometry Training, Expert Cytometry's ExCyte Mastery Class, August 2019

Responsible Conduct of Research (RCR) Certification, UNLV, April 2019

RESEARCH EXPERIENCE:

Graduate Student, Department of Brain Health, UNLV June 2017-Present

- Mentor: Dr. Jefferson Kinney
- Studies focus on biomarker discovery for Alzheimer's disease, as well as risk factors including biological sex and diabetes. My interest is specifically in women and understanding the role of menopause in the Alzheimer's disease, with the goal of reducing the risk and develop better diagnostic tests and therapeutic treatments for women with Alzheimer's disease
- Cellular and molecular techniques including polymerase chain reaction (PCR), quantitative real-time PCR (qRT-PCR), western blot, flow cytometry, Luminex multiplex assay, Quanterix multiplex assay, mass spectrometry, tissue sectioning, immunohistochemistry, fluorescence and confocal microscopy, cell culture, analysis of data/statistics, biobanking, animal behavior testing, animal drug treatments, and animal colony maintenance.
- Additional skills obtained includes academic writing (peer reviewed research and review publications, book chapter, grant applications), involvement in Institutional Animal Care and Use Committee (IACUC) protocol update and review, research presentations, mentoring research assistants, and collaboration with faculty members and students

Assistance with Clinical Study, Madison Medical Affiliates, Milwaukee
March 2016-June 2017

- Mentor: Dr. Manish Gharia,
- Assisted research team with the clinical study *Optimization of a Device Using the Cole Relaxation Frequency to Find Cancer in Excised Human Dermal Tissue: Feasibility Study,* to improve Mohs surgery and reduce the wait time patients endure during treatment of squamous and basal cell carcinomas
- Responsibility in documenting study patient appointments, providing consent forms, communicating with the clinical research coordinator, providing research team with correct and accurate documentation, medical record input, understanding and following study guidelines

Research Assistant, Biology Department, UWSP May 2014-May 2015

- Mentor: Dr. Devinder Sandhu
- Researching male-sterile soybean genes to fine map and isolate the gene for application in hybrid seed production
- Techniques including polymerase chain reaction (PCR), gel electrophoresis, gel imaging, DNA/RNA isolation and purification, designing primers, and conference presentations

Field Assistant, Biology Department, UWSP August 2014

- Mentor: Dr. Devinder Sandhu in collaboration with the University of Iowa
- Assisted in the harvest of genetically modified soybean plants for transposon research gaining experience in attention to detail, precision and accuracy, patience, and independence in the research field

HONORS AND AWARDS:

Summer Doctoral Fellowship, UNLV Summer 2023

- Awarded $7500.00 for summer support

25th Annual Graduate and Professional Student Research Forum April 2023

- 2nd Place Poster Session Winner ($250)

Nathan J. Lindsay Brain Health Endowed Fellowship February 2023

- Awarded $4500.00 for support

Graduate and Professional Student Association (GPSA) Travel Award, UNLV

- Awarded $1178.01 to attend the Society for Neuroscience (SfN) Conference in San Diego, CA, November 2022 October 2022

The Mary S. and D. Keith Kleven P.T., M.S. Endowed Scholarship September 2022

- Awarded $1500.00 for support

Summer Session Scholarship, UNLV April 2022

- Awarded $2000.00 for summer support

Featured on UNLV Student Spotlight September 2021

Summer Doctoral Fellowship, UNLV Summer 2021

- Awarded $7000.00 for summer support

Graduate and Professional Student Association (GPSA) Research Award, UNLV

- Awarded $1174.60 for research project October 2020

Accepted to the IDeA National Resource for Proteomics Faculty and Student Workshop

- All-expense paid trip to Little Rock, AR to attend the workshop February 2020

Southwest Travel Award, UNLV October 2019

- Awarded $400.00 roundtrip to attend the Society for Neuroscience (SfN) Conference in Chicago, IL, October 2019

Graduate and Professional Student Association (GPSA) Travel Award, UNLV July 2019

- Awarded $700.00 to attend the Society for Neuroscience (SfN) Conference in Chicago, IL, October 2019

Graduate and Professional Student Association (GPSA) Travel Award, UNLV April 2018

- Awarded $680.00 to attend the Society for Neuroscience (SfN) Conference in San Diego, CA, November 2018

ACEDEMIC SERVICE:

UNLV Women's Brain Health Initiative March 2020-present
Graduate and Professional Student Association (GPSA) Sponsorship Committee Member
- Includes evaluation of funding applications and organization of annual research forum Dec 2020-August 2021

Leadership and Professional Development Academy Advisory Board Member Sep 2020-August 2021
Graduate and Professional Student Association (GPSA) Rep Sep 2020-Aug 2021
- School of Integrated Health Science Representative
- Department of Brain Health Representative

Experimental Student Committee May 2018-May 2020
- Vice President (2019-2020)
- Neuroscience Emphasis Representative (UNLV) (2018-2019)

Neuroscience Journal Club Member (UNLV) September 2017-2018
- Presentation of research in February 2018

Scholar Society of UWSP September 2013 – May 2015
- Chair Position: Secretary (September 2014-May 2015)

UWSP Society of Leadership and Success Inducted 2013

PROFESSIONAL MEMBERSHIPS:

Association for Women in Science (AWIS) – Membership Director 2023-present
International Society to Advance Alzheimer's Research and Treatment (ISTAART)
- Alzheimer's Association 2020-present

Society for Neuroscience (SfN) 2018-present
Association for Psychological Science (APS) 2018-2019

COMMUNITY OUTREACH:

Desert Oasis High School Career Talk coordinator and volunteer 2019-present
Brain Health and You Lunchtime Forum Panelist
- Women's Brain Health September 2022
- Get Moving, Stay Balanced November 2021

Department of Brain Health Social Media Manager 2020-2022
Walk to End Alzheimer's Disease participant 2019, 2020, 2021, 2022
Nevada Brain Bee coordinator of volunteers January 2019
Brain Awareness Program (Elementary school visits) volunteer November 2017

PROFESSIONAL REFERENCES:

References provided upon request