Spring 5-9-2024

Estrogen replacement therapy to reduce neurodegeneration and socio-cognitive deficits in a female Sprague Dawley rat model of early-onset Alzheimer's disease

Miriam Kirylo
miriamkirylo236@gmail.com

TRINITY COLLEGE

ESTROGEN REPLACEMENT THERAPY TO REDUCE NEURODEGENERATION AND SOCIO-COGNITIVE DEFICITS IN A FEMALE SPRAGUE DAWLEY RAT MODEL OF EARLY-ONSET ALZHEIMER'S DISEASE

BY

Miriam M. Kirylo

A THESIS SUBMITTED TO

THE FACULTY OF THE NEUROSCIENCE PROGRAM

IN CANDIDACY FOR THE BACCALAUREATE DEGREE

WITH HONORS IN NEUROSCIENCE

NEUROSCIENCE PROGRAM

HARTFORD, CONNECTICUT

9 May 2024

Estrogen replacement therapy to reduce neurodegeneration and socio-cognitive deficits in a female Sprague Dawley rat model of early-onset Alzheimer's disease

BY

Miriam M. Kirylo

Honors Thesis Committee

Approved:

_____

Luis Martinez, Thesis Advisor

_____

Michael Puljung, Thesis Committee

_____

Molly Helt, Acting Director, Neuroscience Program

Date: _____

Estrogen replacement therapy to reduce neurodegeneration and socio-cognitive deficits in a female Sprague Dawley rat model of early-onset Alzheimer's disease

Alzheimer's disease (AD) is a highly detrimental and increasingly prevalent neurodegenerative disease that is most commonly characterized by neurotoxic pathology and progressive cognitive decline. The population of individuals with the more typical late-onset form of AD (LOAD; diagnosed at 65 years of age or older) is exponentially increasing; estimated from 2010 census data, 4.7 million individuals in the United States were affected by AD-induced dementia, and almost 14 million Americans are estimated to be affected by AD-derived dementia by the year 2050 (Hebert et al., 2013). Globally, 50 million people in the year 2020 were estimated to have dementia, the primary cognitive hallmark of AD, and its incidence is likely to double about every two decades (Alzheimer's Disease International et al., 2020).

Early-onset Alzheimer's disease (EOAD) is the rarer subtype of the disease that is diagnosed in individuals aged less than 65 years of age and encompasses up to 6% of all AD cases (Zhu et al., 2015). Although this less-frequent form appears to be a small fraction of AD diagnoses, it nevertheless implies that, of 6 million individuals in the United States with the disease diagnosis, hundreds of thousands of Americans will be living with EOAD (Zhu et al., 2015). Symptoms of EOAD, which are likewise progressive, can begin to emerge in individuals as young as 30 years of age, but neuropathology may begin accumulating as early as 20 years-old (Gonneaud et al., 2017). Furthermore, there appears to be an equal distribution of men and women diagnosed with EOAD, but it is difficult to definitely draw a conclusion about the lack of sex differences due to the disease's low incidence (Peeters et al., 2022). Although there are no clear sex differences in EOAD prevalence, sex-specific factors like the onset of menopause

(discussed in more detail below) may still contribute substantially to EOAD's etiology in women and necessitate further investigation.

As previously mentioned, the main characteristic of AD is dementia, where memory loss is not only generalized to dysfunctional episodic and working memory but is also linked with loss of one's personality and lack of social recognition (such as for loved ones) during more severe stages of the disease (Larson et al., 1992; Storandt, 2008). The cognitive impairments affiliated with AD progression can quickly become increasingly obstructive to an individual's life. After an EOAD diagnosis, an individual who may be fully engaged with their career is likely to become unemployed, especially as the disease progresses. This may additionally create a myriad of financial burdens (Sakata & Okumura, 2017). Individuals with EOAD likely have caregiving responsibilities due to their relatively young age at the disease's onset (Chiari et al., 2021). Therefore, this indicates that the loss of independent functioning in EOAD has increased caregiving implications not only for the EOAD individual but likewise for their dependents (Chiari et al., 2021).

Although the differential diagnostic criteria for EOAD versus LOAD is the age of disease onset, there is evidence of differing symptomology and comorbidities between the two subtypes. Psychological "sub-syndromes" like depression, anxiety, apathy, and eating disorders coexist with AD's affiliated cognitive deterioration and have a higher occurrence in EOAD than LOAD (Altomari et al., 2022; Bature et al., 2017). A few studies had findings that individuals with EOAD had greater rates of cognitive decline and greater deficits in working memory, apraxias, and naming abilities compared to LOAD patients (Kaiser et al., 2012; Kalpouzos et al., 2005; Tellechea et al., 2018; Wattmo & Wallin, 2017). Furthermore, EOAD is frequently mistaken for other neuropsychiatric disorders, resulting in a significantly longer time to receive an accurate

diagnosis, likely due to the affected patients' young age at onset (Sirkis et al., 2022). This delay in diagnosis implies that the person with EOAD will not receive the necessary forms of financial and social support as well as palliative treatment until much later into the disease, potentially worsening cognitive impairment and quality of life. Despite this urgency for learning more about the disease's afflictions and catering treatments as well as diagnostic strategies to the younger individuals affected by it, there is a lack of research for EOAD in clinical settings compared to its later-onset subtype (Ayodele et al., 2021).

There is no cure for AD to date (Lin et al., 2021). This emphasizes the need for an immediate diagnosis and effective timing of medical intervention (International et al., 2011). Some early federally-approved medications focused on increasing cholinergic transmission of the basal forebrain due to its role in cognitive functioning and sensitivity to AD pathology (Z.-R. Chen et al., 2022). FDA-approved pharmacotherapies for AD include drugs that block the breakdown of acetylcholine (ie. cholinesterase inhibitors) such as tacrine and donepezil; however, it is important to consider that these drugs are palliative and facilitate minor reductions in cognitive deficits in this disease (Mayeux & Sano, 1999). Other cholinesterase inhibitors of varying selectivity (like physostigmine, metrifonate, and rivastigmine) have also been researched in clinical trials, but are intended for use in the earlier stages of AD where symptoms are not as progressed (Z.-R. Chen et al., 2022; Mayeux & Sano, 1999). Palliative treatments that intended to target the elimination of oxidative stress or supplementation of nerve growth factor synthesis, such as *ginkgo biloba* extract, the xanthine derivative propentofylline, alpha-tocopherol (or vitamin E), and the benzoquinone derivative idebenone, demonstrated inconsistent results or a lack of cognitive improvement (Mayeux & Sano, 1999). One of the most recent drug developments for AD includes the FDA-approved monoclonal antibody Lecanemab (or

Leqembi), which reduces mild cognitive deficits and diminishes plaque densities (Reardon, 2023; van Dyck Christopher H. et al., 2023). However, patient deaths in Phase III of testing have sparked significant concerns over its use.

Hormone therapy (HT) is a proposed treatment for AD, predominantly estrogen due to its neuroprotective role in the cortex (Saleh et al., 2023; Wroolie et al., 2015). This therapy has a mixed record of success in treating AD, with clinical studies reporting either improved memory and attention, no improvement in cognition, or increased risk of dementia and AD with long-term use (Mulnard et al., 2000; Savolainen-Peltonen et al., 2019; Wroolie et al., 2015). Outside of these conflicting results across numerous studies, there are risks affiliated with the use of HT, such as cardiovascular diseases (heart attacks, strokes, thromboembolisms) and cancer, when using a combination of progestin and estrogen treatments, especially during chronic use; however, women who used estrogen-only treatments did not experience a greater risk for cancers and heart attacks compared to estrogen and progestin combination users (Collaborative Group on Hormonal Factors in Breast Cancer, 1997; Institute for Quality and Efficiency in Health Care (IQWiG), 2020).

Estrogen production drastically declines during menopause, which heightens the risk for cognitive decline in women who have undergone surgical (ie. hysterectomy, ovariectomy) or age-related menopause (Hara et al., 2015; McCarthy & Raval, 2020). Premature menopause correspondingly increases the risk of dementia due to the cessation of ovarian-produced estradiol (Sochocka et al., 2023). The use of HT during a “critical window” after the halt in estrogen production but prior to the age of 60 years old has been suggested to inhibit the symptomatic onset of dementia (Sochocka et al., 2023). Delays in use of HT after the start of women’s premature menopausal period correlated with elevated tau protein levels, or a type of AD

pathology that stems from prolonged deposition of β-amyloid (Aβ) plaques, compared to women who started HT within five years after menopause (Coughlan et al., 2023; Sochocka et al., 2023). This suggests that HT concerns and drawbacks for women over the age of 65 should not be profiled onto significantly younger women who could benefit from the use of estrogen therapy (ET), particularly with respect to diminishing the risk of EOAD/LOAD and general cognitive decline. Observational clinical research found that only younger women, aged 50-63 years, benefited from ET's neuroprotective effects for the reduction of AD risk; these findings suggest that the benefits and risks behind the use of ET are likely age-dependent (Henderson et al., 2005). An inverse relationship was also suggested between women's estrogen use and development of EOAD according to population-based data, where postmenopausal women who were diagnosed with EOAD reported estrogen use or nonuse (Slooter et al., 1999). Could early and appropriately-timed activation of estrogen receptors halt or even reverse the pathology and memory deficits that are associated with EOAD? Could the risk for developing EOAD be diminished using this preventative approach?

In order to elucidate the mechanisms whereby estrogen yields neurocognitive benefits in EOAD, it is critical to first outline the relevant estrogen receptor (ER) types and signaling pathways. Estrogen synthesis occurs in both gonadal and non-gondal cells, including in neurons located in the hippocampus, hypothalamus, and cerebral cortex (Cui et al., 2013). Estradiol is the form that is most active biologically and dominates pre-menopause; the three major classifications of female estrogens are estradiol (17β-estradiol), estriol, and estrone (Cui et al., 2013). Estrogen receptor signaling is mediated by ligand binding to nuclear and/or membrane-bound estrogen receptors. Estradiol readily passes through the plasma membrane and interacts with intracellular receptors. The intracellular estrogen receptor-alpha (ERα) and

estrogen receptor-beta (ERβ) are coded by the genes ERS1 on chromosome 6 and ERS2 on chromosome 14, respectively (Enmark et al., 1997; Gosden et al., 1986). Estradiol binding to ERα or ERβ causes a conformational change in the nuclear receptors, leading to dimerization and dimer-binding to estrogen response elements (EREs) located on chromatin (Klinge, 2001; Loven et al., 2001). The complex then regulates transcription of downstream genes, ultimately inducing alterations in protein expression (Couse & Korach, 1999; Klinge, 2001; Loven et al., 2001; Marino et al., 2006; O'Malley, 2005). Indirect genomic signaling may also be managed by these nuclear receptors in some cell types, as the protein complexes that are created by the binding of estradiol to ERα or ERβ interlink with other intracellular factors that then influence gene expression (O'Lone et al., 2004).

Estradiol's role in neuroprotection has been highlighted in a variety of *in vitro* and *in vivo* studies. In cultured primary hippocampal neurons from embryonic rats, 17β-estradiol protected neurons from excessive calcium influx-induced neurotoxicity caused by glutaminergic activation (Nilsen & Brinton, 2003). 17β-estradiol was found to utilize mechanisms like protective storage of mitochondrial calcium and upregulation of the regulator protein, Bcl-2, for this protection against excessive-calcium neurotoxicity (Nilsen & Brinton, 2003). Neuroprotective properties of estradiol were likewise observed in ovariectomized C57BL/6 mice that had infusions of the ERα agonist 1,3,5-tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) and the ERβ agonist 2,3-bis (4-hydroxyphenyl) propionitrile (DPN) into the dorsal hippocampus, where cognitive features such as novel object recognition were rescued in groups receiving estradiol, PPT, or DPN (Boulware et al., 2013). Another study found that ovariectomized Swiss female mice exhibited replenished object recognition memory when treated with PPT, but not DPN, infusions into the CA1 subregion of the dorsal hippocampus (Pereira et al., 2014). However, C57/BL6 mice treated

with DPN did exhibit memory-protection properties, demonstrating that the Swiss mouse strain was differentially sensitive to these nuclear receptor agonists (Pereira et al., 2014). These collective results support nuclear ERs' roles in object memory preservation.

As discussed above, both nuclear ER subtypes are implicated in cognitive processes relevant for EOAD, but there is growing evidence that ERα may be particularly critical. ERα-knockout (ERαKO) mice that were administered a gene encoding for ERα via lentiviral delivery exhibited a rescue of spatial memory (Foster et al., 2008). Electrophysiological measurements likewise demonstrated that the ERαKO mice had lower synaptic responses in the hippocampus upon treatment with estradiol benzoate compared to wildtype mice, reinforcing the notion that ERα is critical for memory maintenance and hippocampal synaptic transmission (Foster et al., 2008). Another study utilizing ERαKO mice implemented an intracerebroventricular infusion of Aβ42 peptides and investigated the knockout's effect on Aβ42 plaque densities as well as neuroinflammation and cognition (Hwang et al., 2015). ERαKO mice infused with the peptides exhibited significantly elevated plaque densities compared to peptide-infused C57BL/6 wildtype mice both in the cortex and the hippocampus; similarly, neuroinflammation, detected via the expression of inflammatory proteins Cox-2, iNOS, and GFAP, was likewise elevated in the peptide-infused knockout mice in comparison to the peptide-infused wildtype mice (Hwang et al., 2015). These two factors can directly influence the degree to which cognitive properties are impacted. Memory deficits were predictably observed in the ERαKO mice infused with Aβ42 peptides, as depicted by higher escape distances and escape latencies during the Water Maze Test in comparison to the wildtype mice that were infused with the peptides during all four testing days (Hwang et al., 2015). Furthermore, ERα is more transcriptionally active than ERβ in the hippocampus (Bean et al., 2014). This reinforces the

notion of ERα's role for memory preservation and regulation of proteinous factors that facilitate neuroprotection and preservation of cognition.

In addition to mediating direct genomic effects of estradiol, ERα and ERβ may also signal at the cell membrane via a transactivational mechanism involving metabotropic glutamate receptors (mGluRs) (Gross & Mermelstein, 2020). Specifically, these ERs couple directly with mGluR1, a G_q-coupled receptor whose activation ultimately stimulates phospho-ERK/MAPK signaling (Gross & Mermelstein, 2020). A study found that PPT and DPN infused directly into the hippocampus of female mice improves spatial learning/memory and elevates hippocampal phospho-ERK levels, effects that are blocked by pretreatment with an mGluR1 antagonist (Boulware et al., 2013).

A similarly rapid form of estradiol signaling occurs via binding to the G-protein coupled receptor 1 (GPER1) (Barton et al., 2018). GPER1 has a lower affinity for estradiol than its nuclear receptor peers (Barton et al., 2018; Fuentes & Silveyra, 2019; Prossnitz & Barton, 2014). The actions of the membrane G-protein coupled estrogen receptor's transcriptional mechanism is induced upon binding by estradiol, causing a conformational change in the receptor that leads to activation of specific G-proteins and downstream intracellular signaling cascades via second messenger systems (Fuentes & Silveyra, 2019). Specifically, estradiol-binding stimulates adenylyl cyclase, which then induces the conversion of intracellular adenosine triphosphate into the second messenger cyclic adenosine monophosphate (cAMP) (Fuentes & Silveyra, 2019; Lösel & Wehling, 2003). Phosphorylation of cAMP response element binding protein (CREB) by cAMP's activation of protein kinase A (PKA) has been shown to increase dendritic spine formation and prevalence of synaptic proteins in various subregions of the hippocampus (Leuner et al., 2003; Murphy & Segal, 1996). A pathway induced directly by GPER1 is activation of the

phosphoinositide 3-kinase/protein kinase-B (PI3K/Akt) pathway, facilitating neuroprotection by upregulating the formation of further downstream synaptic proteins (Du et al., 2004; Ivanova et al., 2002; Mannella & Brinton, 2006; Spencer et al., 2008). The GPER1-facilitated calcium/calmodulin protein kinase II (CamKII) pathway has also been noted to have a critical role for memory by upregulating spinophilin in hippocampal neurons (Lee et al., 2004).

An important downstream factor that may be induced by both nuclear and/or membrane ER signaling is neprilysin (NEP). NEP is a type II integral membrane, zinc-dependent metallopeptidase that has shown to possess Aβ-degrading activity (Marr et al., 2004; Meems et al., 2018; Nalivaeva & Turner, 2013). In addition to studies observing its clearance abilities *in vitro*, the enzyme also degrades peptides *in vivo* (Iwata et al., 2004; Rofo et al., 2022; Shirotani et al., 2001). In comparison to other peptidases, NEP is most efficient in clearing Aβ from both 40 and 42 fragment classifications (Shirotani et al., 2001). Catabolic features of NEP for Aβ-clearance broadly stem from its density of disulfide bonds for structural integrity, a critical Glu584 residue located in a zinc-binding region within its C-terminus, and its cleavage of peptides from hydrophobic amino acids' N-terminus (Hersh & Rodgers, 2008; Klein et al., 2006; Nalivaeva & Turner, 2013). Interestingly, the Aβ42 fragment has been theorized to be the more neurotoxic form relative to the more commonly-observed Aβ40 (Hersh & Rodgers, 2008; Klein et al., 2006; Nalivaeva & Turner, 2013; Wang et al., 2021). Their main structural difference resides in Aβ42's two additional amino acid residues located at the C-terminus, which are the hydrophobic alanine and isoleucine residues (Hersh & Rodgers, 2008; Klein et al., 2006; Nalivaeva & Turner, 2013; Wang et al., 2021). An inverse relationship has been suggested between NEP and Aβ levels, especially in the hippocampus, which is a major subcortical region for learning and memory (Yasojima et al., 2001; Anand & Dhikav, 2012). According to the

Amyloid Hypothesis, Aβ plaques are the driving factor that induce AD and its affiliated downstream cognitive deficits (Hardy & Selkoe, 2002). This may imply that neurodegeneration caused by Aβ peptides reduces the number of NEP-expressing neurons, further elevating plaque deposition within AD. Reciprocally, NEP expression may be generally downregulated in EOAD and/or AD, permitting plaque deposition that then leads to significant neuronal loss. It is important to note that these two proposed mechanisms are not mutually exclusive; in fact, they may work synergistically to dramatically increase Aβ accumulation in individuals with AD.

Estrogen has been shown to upregulate NEP expression. A study utilizing human neuroblastoma SH-S5Y5 cells demonstrated that overexpression of ERα and ERβ significantly increased NEP levels in culture (Liang et al., 2010). Similarly, NEP levels were found to be elevated in ovariectomized APP23 mice treated with 17β-estradiol, which non-selectively targets ERs (Li et al., 2013). Furthermore, ERαKO mice infused with Aβ had reduced NEP expression and fewer NEP-expressing neurons compared to WT mice infused with Aβ (Hwang et al., 2015). Thus, it is implied that ERα holds a critical role in regulating NEP for the degradation of Aβ peptides and opposing EOAD and AD pathology, meanwhile likewise being important for cognitive maintenance.

There has been limited investigation into using estradiol replacement therapy to reduce risk and deficits of EOAD and virtually no studies that have employed an EOAD animal model to study the disease and how estradiol may affect its progression. As previously mentioned, HT has been linked to numerous detrimental health outcomes when prescribed to women >65 years of age; however, no such implications have been made for younger women aged <65 years of age. I hypothesize that estradiol binding to ERα upregulates NEP expression and Aβ degradation, ultimately reducing the density of plaque deposits. Because estradiol signaling ceases

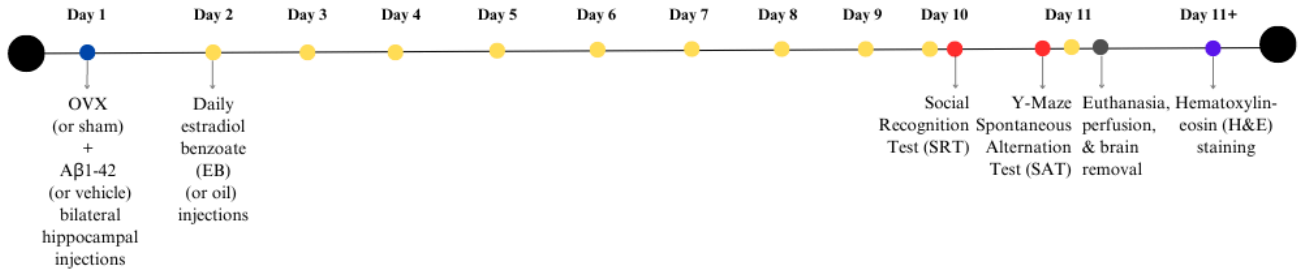
post-menopause, the resulting downregulation of NEP may permit the initiation and perpetuation of Aβ aggregation in EOAD, initiating neurodegeneration and cognitive deficits. I investigated the implementation of estradiol replacement therapy in a female Sprague Dawley (SD) rat model of EOAD with the intention of observing reduced neurodegeneration and social recognition deficits. I predicted that animals with EOAD pathology that were treated with estradiol would perform in cognitive assessments similarly to a healthy reference group and better than animals with EOAD pathology that were not treated with estradiol. EOAD with treatment animals were likewise predicted to have reduced neurodegeneration and elevated NEP expression compared to EOAD animals without treatment.

Materials and Methods

Outline of Experimental Design

The goal of the study was to identify the neurobiochemical mechanisms whereby β-estradiol-3-benzoate (EB) hormone treatment diminishes neurodegeneration of hippocampal neurons and rescues facets of cognition in a young-adult SD rat model of EOAD. An overview of the experimental timeline is provided in Figure 1. EB-treated EOAD (htEOAD) rats underwent a single surgical event, where they were ovariectomized to eliminate endogenous sources of estrogen and had aggregated $A\beta_{1\text{-}42}$ peptides injected bilaterally into the hippocampal CA1 subregion to induce EOAD-associated pathology and cognitive deficits. Behavioral and neural assessments were then performed in htEOAD rats as well as in no treatment (ntEOAD) and healthy reference (HREF) rats.

(A)



(B)

Figure 1. (A) Outline of experimental procedures, hormone injections, behavioral assessments, and assays for hormone-treated early-onset Alzheimer's disease (htEOAD), no treatment early-onset Alzheimer's disease (ntEOAD), and healthy reference (HREF) animals. (B) Key for chronological outline of experiment. Repetition of each colored symbol indicates each day which event took place.

Table 1. Surgical procedures, hormone injections, molecular/pathological assessments, and memory assessments per treatment group.

Treatment Group	Surgical Procedures	Hormone Injections	Behavioral Assessments	Histology
Hormone-treated early-onset Alzheimer's Disease (htEOAD)	Ovariectomy (OVX) Aggregated $A\beta_{1\text{-}42}$ peptides CA1 hippocampal injection	Estradiol benzoate	Social Recognition Test Y-Maze Spontaneous Alternation Test	Perfusion Hematoxylin & Eosin staining for Neurodegeneration
No hormone treatment early-onset Alzheimer's Disease (ntEOAD)	OVX Aggregated $A\beta_{1\text{-}42}$ peptides CA1 hippocampal injection	Oil	Social Recognition Test Y-Maze Spontaneous Alternation Test	Perfusion Hematoxylin & Eosin staining for Neurodegeneration
Healthy Reference (HREF)	Sham OVX Vehicle CA1 hippocampal injection	Oil	Social Recognition Test Y-Maze Spontaneous Alternation Test	Perfusion Hematoxylin & Eosin staining for Neurodegeneration

Animal Model Description

Experimental 10-week-old female Sprague Dawley (SD) rats bred from Charles River Laboratory (Kingston, NY, USA) were utilized for the early-onset Alzheimer's Disease (EOAD) model. Stimulus animals were 5-7 weeks of age for use during behavioral assessments. All

animals were maintained on a 12:12 light-dark cycle with the dark period starting at 12:00 pm EDT/11:00 AM EST. Animals were housed in polycarbonate cages with wire-top lids and provided with *ad libitum* access to food (LabDiet 5001; F. Fischer & Son, Somerville, NJ, USA) and water. All experimental and stimulus animals were pair-housed throughout the experiment, with the exception that experimental animals were briefly single-housed from surgery through the end of the post-surgical monitoring period. Procedures and testing were administered in strict accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council (US) Committee for the Update of the Guide for theCare and Use of Laboratory Animals, 2011) and approved by the Trinity College Animal Care and Use Committee.

Preparation of the Aggregated $A\beta_{1\text{-}42}$ peptides

Lyophilized $A\beta_{1\text{-}42}$ peptides (120301; Abcam, Cambridge, UK) were initially dissolved in 99% 1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP) (ICN15124505; Fisher Scientific, Pittsburgh, PA, USA) to a concentration of 2 μg/μl for half of the $A\beta_{1\text{-}42}$ animals from each treatment group. This concentration was then altered to 1 μg/μl after being assessed as too neurotoxic due to severe lesions observed in the sites of injection. After a 60 min incubation at room temperature with intermittent vortexing, vial contents were transferred to 2 mL low-binding microfuge tubes. Peptide samples were dried using a centrifugal concentrator (78100-00; Labconco, Kansas City, MO, USA) without heat for 15 mins. The film was resuspended at 5 mM in dimethyl sulfoxide (DMSO) (J66650.AE; Scientific, Waltham, MA, USA). If a vial was allocated for an upcoming surgical event, the solution was resuspended for a second time with sterile-filtered phosphate buffered saline (PBS) to a final concentration of 2 μg/μl or 1 μg/μl. PBS was filtered prior to resuspension using a hydrophilic, 33 mm diameter filter with a 0.22 μm pore size (SFPES033022S; Membrane Solutions, Auburn, WA, USA) that was fastened onto a syringe. To

allow aggregation, the peptide solutions were stored at 37°C in an incubator for 72 hours (Zussy et al., 2011). After the 72-hour incubation period, aggregates were placed on ice and then stored at -20°C until ready for intracranial injection.

Transmission electron microscopy (TEM) (Philips CM12 TEM/(S)TEM; Cambridge, MA, USA) was used to confirm peptide aggregation. 4 µl samples of aggregated peptides or vehicle (9% DMSO and 91% PBS) were deposited on a copper grid and stained with three 2% uranyl acetate and deionized water washes. Images were taken at 25,000X magnification and a 120kV HV for aggregates and at 110,000X magnification and 120kV HV for vehicle control samples due to significantly differing sizes of observed molecules.

Surgical Procedures

Overview of Surgical Procedures

In a single surgical event, htEOAD and ntEOAD animals underwent an ovariectomy (OXV) procedure and a bilateral injection of aggregated $A\beta_{1\text{-}42}$ peptides (8 µg/hemisphere or 4 µg/hemisphere) into the CA1 subregion of the hippocampus; HREF animals received a sham OVX in addition to a bilateral injection of vehicle. Animals were anesthetized with isoflurane gas (14043-704-06; Patterson Veterinary, Greeley, CO, USA) maintained at 1.5-4% in oxygen via the SomnoSuite Small Animal Anesthesia System (SS6346B; Kent Scientific, Torrington, CT, USA) throughout the surgery and had anesthetic depth assessed via a toe pinch. A subcutaneous injection of 2.5 mg/kg meloxicam (137590E; Pivotal, Liberty, MO, USA) diluted in 0.9% saline was administered as an analgesic. The flank and head were shaved and ophthalmic ointment (701039; LubriFresh™, Major Pharmaceuticals, Livonia, MI, USA) was applied to the eyes to prevent drying out during the surgery. The shaved regions had three sets of veterinary betadine

(67618-154-16; Aviro Health, Stamford, CT, USA) and 70% ethanol washes, with the final wash being betadine that was left to dry, in preparation of the surgical fields. At the conclusion of the surgical procedures, the skin incisions were closed with wound clips and topical triple antibiotic ointment was applied to all incision sites. The animals were single-housed in a clean cage and observed by surgical personnel until they achieved ambulatory posture. Post-operative care assessments occurred in the mornings and afternoons following the surgery for three days, where weights (morning assessment only), observations of food and water consumption, as well as whether animals were bright, alert, and responsive, were noted. A second injection of 2.5 mg/kg meloxicam was given on the morning following the surgery. Animals were pair-housed again after the third postoperative day, and wound clips were removed on the seventh postoperative day.

OVX Procedure

After surgical preparation, animals had an incision (<1 cm) in the skin, fascia, and muscle made post-lateral to the intersection of the vertebral column and the 13th rib. A sac containing the fat pad and ovary was isolated and externalized from the peritoneal cavity through the intramuscular incision. A curved hemostat, heated in a glass bead sterilizer (Germinator 500; Braintree Scientific, Braintree, MA, USA) was used to cauterize and remove the ovaries from the fat pad and uterine horn. The cauterized fat pad and uterine horn were then returned to the peritoneal cavity through the same incision, which was then closed with simple, uninterrupted 3x2x1 surgical knot sutures with violet braided, polyglycolic acid absorbable 4-0 sutures (60045; Oasis, Mettawa, IL, USA).

Hippocampal $A\beta_{1\text{-}42}$ Injections

Immediately after the OVX procedure, animals were placed into a stereotaxic apparatus (51603U; Stoelting, Wood Dale, IL, USA) with their incisors secured against an incisor bar, and the snout was secured into the apparatus nose cone, which delivered anesthesia for the duration of the procedure. Ear bars were secured anterior to the ears and superior to the end of the jaw to fasten the skull in the apparatus. A 3 cm incision was made along the midline of the scalp, beginning between the eyes, and the wooden ends of two cotton swabs (51688; Dynarex, Corporation, Orangeburg, NY, USA) were used to scrape the exposed skull of pericranial tissue. A 24 gauge, 5 µl Hamilton microinjection syringe (Model 7105; 0.566 mm diameter) (88000; Hamilton Company Inc., Reno, NV, USA) was secured to the syringe controller of the microinjection unit (Model 5001; David Kopf Instruments, Tujunga, CA, USA) that was attached to the stereotaxic apparatus. The stereotaxic coordinates were identified for the bregma intersection and lambda intersection, and the incisor bar was adjusted to dorsoventral (DV) height deviation of within ±0.05 mm for the two skull suture intersections. The CA1 region of the hippocampus was bilaterally targeted according to the following (modified from previous literature) coordinates: anteroposterior (AP) = -3.6 mm and mediolateral (ML) = ± 2.5 mm, DV = -3.2 mm (Song et al., 2018). The ML and AP coordinates were adjusted relative to bregma, and the DV coordinates were relative to the dura. The bilateral holes were drilled with a pedal-controlled, manual drill (G170773; Foredom, Bethel, CT, USA) and 1.35 mm-diameter drill bit (514555; Stoelting, Wood Dale, IL, USA). The Hamilton microinjection syringe loaded with $A\beta_{1\text{-}42}$ peptide aggregates or vehicle was slowly inserted into the brain. Bilateral injections were made at a flow rate of 0.5 µL/min over 8 minutes (8 µg of aggregated peptides per hemisphere and later 4 µg of aggregated peptides per hemisphere). The microinjection needle

was left *in situ* for an additional 5 minutes post-injection to permit any remaining solution to disperse away from the needle tip. After removal of the syringe, each skull hole was sealed with bone wax (MedPlus Services, Nashville, TN, USA). Throughout the duration of the surgery, a piece of Surgifoam (1972; Ethicon, Somerville, NJ, USA) was applied to halt profuse bleeding at the incision sites on a need-basis. Prior to closing, two small pieces of the Surgifoam were applied at the anterior and posterior ends of the incision. The incisions were closed, antibiotic ointment was applied, and the animals were allowed to recover as previously described.

Hormone Treatments

Following the surgery, animals were subcutaneously injected daily from Day 2 of the experimental procedure until the final testing day (Day 11). Animals receiving estradiol replacement therapy were injected with 0.1 mL of 132.8 μM of EB (Sigma-Aldrich, St. Louis, MO, USA). All other females were injected with 0.1 mL cottonseed oil vehicle (177110025; Acros Organics, Janssen Pharmaceuticalaan, Geel, BE). EB or vehicle were stored identically in glass vials that were covered with aluminum foil to protect from ultraviolet ray-induced degradation.

Behavioral Assessments

Social Recognition

The social recognition test (SRT) (Mathiasen & DiCamillo, 2010) occurred in a series of three phases: the Habituation Phase, Trial I, and Trial II. Trial I and Trial II, each lasting a total of 5 minutes, were recorded on an overhead camera. Animals were assessed for social recognition in a clean polycarbonate cage with bedding on Day 10. The experimental animals were placed into separate cages for the duration of the assessment; testing animals remained housed in the test

environment, while stimulus animals were returned to their home cage until use during trials. Each testing session was recorded on an overhead camera in dim lighting (adjusted to exactly 3.7 lux in the center of testing home cage after time for warming-up) (LX1330B; Dr. Meter) during the animals' dark cycle. Prior to the onset of the recordings, experimental and stimulus animals (unfamiliar juvenile female SD rats) were habituated separately for two hours in their polycarbonate cages. The first hour of the Habituation Phase occurred during the light phase, and the second hour of the phase occurred in dim lighting. For Trial I of the assessment, a cage containing the experimental animal was placed underneath the overhead camera with the wire-top lid removed, and one stimulus animal was placed in the cage with the testing animal. Afterwards, the stimulus animal was removed, and a 30-minute inter-trial-interval (ITI) period took place. Memory consolidation occurs within this 30-minute period, so an animal with intact social recognition would be able to recognize, and investigate for a shorter duration, a familiar stimulus animal that was introduced immediately before and removed during the ITI. After the 30-minute interval, the same stimulus animal was reintroduced to the same testing animal by being placed in its polycarbonate cage for 5 minutes for Trial II of the assessment. After the conclusion of the trial, animals were returned to their home cages. Videos were manually scored by trained laboratory personnel using the computer software BORIS (Friard & Gamba, 2024) and/or JWatcher (Blumstein et al., 2020) for the duration of time each test subject spent investigating the stimulus animal and grooming.

Spatial Memory

Animals were assessed for spatial memory using an eight minute Spontaneous Alternation Test (SAT) in a gray polycarbonate Y-Maze apparatus, which consisted of three arms (45.5 cm l x 10.5 cm w x 30.0 cm h, each angled at 120°) (Maze Engineers, Skokie, IL, USA).

After warming up, lights were adjusted to a lux of 16.0 with a deviation of 0.5 lux at the end of each of the three arms. The entire assessment took place during the light phase and was likewise recorded on an overhead camera. It was scored using the software BORIS and/or JWatcher for transitions from an arm into the center of the apparatus, and vice versa. Initial placements were alternated within the three arms between animals. A spontaneous alternation (SAT) was defined as a testing animal's consecutive entry into three different arms, where an entry into a specific arm did not overlap within the most previous entry.

The apparatus was initially wiped down with 70% ethanol prior to the testing. The testing animal was placed into a designated arm and allowed to move freely between the center and arms of the Y-Maze. After eight minutes, the animal was removed and returned to its home cage. The apparatus was initially cleaned with SaniCloth wipes and then wiped for a second time with 70% ethanol between animals. Videos were scored using BORIS for duration in the center and three arms of the apparatus and for transitions between them. Following, a spontaneous alternation percentage, derived from a ratio of successful alternations to all possible alternations, was calculated for the assessment of the animals' spatial memory.

Brain Sectioning and Histology

Perfusion and Brain Removal

Animals were deeply anesthetized by inhalation of isoflurane gas in a glass euthanasia chamber. After making a long abdominal incision and cutting the diaphragm, each animal underwent a transcardial perfusion via the left ventricle with 4% paraformaldehyde in PBS (19210; Electron Microscopy Sciences, Hatfield, PA, USA) and decapitated to remove the brain,

which was stored in 4% paraformaldehyde in a glass vial overnight. Fixative was replaced by 30% sucrose in PBS solution, and the tissue was stored at around 4°C for at least 48 hours.

Cryostat Sectioning

Brains were cut using a razor blade at the posterior end between the intersection of the occipital lobe and the cerebellum to achieve a flat and level surface to place the brain for mounting with OCT compound (23-730-571; Fisher Scientific, Waltham, MA, USA) solution onto a metal sectioning plate. After cutting at the intersection, a cylindrical mold was placed over the brain and filled with the OCT compound. The OCT mold was permitted to freeze on the cryostat's (CryoStar Nx50 Cryostat; Thermo Fisher Scientific, Waltham, MA, USA) cryo-bar. Tissue was sectioned at -18°C, collecting 35 μm-thick sections of tissue. Each tissue section was stored in cryoprotectant solution within a well of a 24-well plate at -20°C until mounting onto slides.

Neurodegeneration Assay

In assessment of neuronal degeneration, tissue sections were mounted on Superfrost Plus microscope slides (1255015; Thermo Fisher Scientific, Waltham, MA, USA) and left to dry in the hood for two days prior to the nuclear and cytoplasmic hematoxylin-eosin (H&E) staining procedure. Slides were first dipped in bovine gelatin solution prior to mounting (G9391; Sigma-Aldrich, St. Louis, MO, USA), and mounted sections on slides were stained using an H&E staining kit (H-3502; Vector Laboratories, Inc., Newark, CA, USA). Staining protocol was adjusted and customized from the kit's provided procedure steps. First, hematoxylin was applied to the tissue on the slides with a transfer pipette, and the sections were incubated in the solution for 5 minutes. Slides were then rinsed twice in distilled water in Coplin jars for 15 seconds each.

Bluing reagent was applied to the sections with a transfer pipette for a 25-second incubation, and the slides underwent another two rinses with distilled water for 15 seconds each. Slides were rinsed in 100% ethanol for 10 seconds prior to the eosin reagent incubation. Tissue was incubated with Eosin Y solution for two minutes, and the slides were rinsed again in Coplin jars containing 100% ethanol for 10 seconds. Slides were then dehydrated in three series of 100% ethanol in Coplin jars for 1.5 minutes each. Subsequently, the slides were cleared for five minutes each in two series of CitriSolv solution. Cleared slides were coated with DPX mounting solution (13512; Electron Microscopy Sciences, Hatfield, PA, USA) prior to coverslipping and left to dry in the hood overnight for at least two days prior to imaging and characterization.

Imaging and Data Analysis

Imaging for Neurodegeneration

Stained and coverslipped slides were first cleaned using glass cleaner prior to the onset of imaging. A Nikon DS-Ri2 digit camera connected to a Nikon Eclipse E600 microscope was used to image tissue containing the CA1 and CA3 subregions of the hippocampus at 20X magnification. The Nikon NIS Elements software for windows was used to obtain photomicrographs of the hippocampal subregions of interest. Images were imported into ImageJ (Version 2.0.0) for cell counting within a consistent 323 mm^2 area of the left and right CA1 and CA3 subregions. Densely eosinophilic neuronal somas with pyknotic nuclei were assessed as degenerating neurons and counted using ImageJ's cell-counter. Degenerating neuron densities were determined by dividing the cell count by the area of the 323 mm^2 counting domain.

Data Analysis

Statistical analyses were performed using the JASP computer software for Macintosh, version 0.17.1 (JASP Team, 2023). Independent samples t-tests and one-sample t-tests were used with data from pilot experiments to examine effects of inter-trial-interval (ITI) lengths on investigation durations during both SRT trials and for comparison of IRs to a 50% threshold, respectively. One-way ANOVAs were used to examine the effects of estradiol replacement therapy between treatment groups on social recognition and spatial memory. Throughout all analyses, a *p* value of less than 0.05 was considered to be statistically significant.

Results

Determination of Stereotaxic Coordinates for Targeting the Hippocampal CA1 Subregion

To establish consistent and reliable stereotaxic coordinates for intracranial injections into the CA1 subregion of the hippocampus (Figure 2A), pilot stereotaxic surgeries were conducted on female Sprague Dawley rats aged around 10 weeks of age. After a number of adjustments, we confirmed ML: ± 2.5 mm and AP: -3.6 mm from bregma and DV: - 2.7 mm from the dura to precisely target the hippocampal CA1 (Figure 2B,C).

A B C

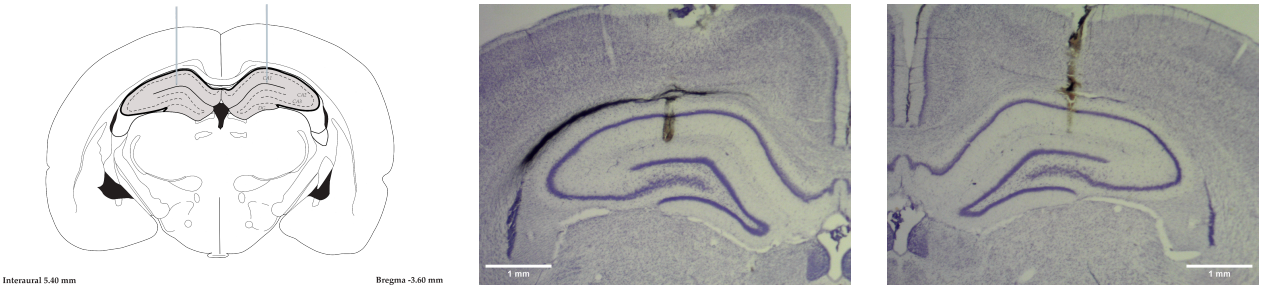


Figure 2. (A) An example of a bilaterally-targeted hippocampal CA1 subregion (Paxinos & Watson, 2009). Cresyl violet stained neural section depicting injections targeting the CA1 region of the hippocampus of the (B) left and (C) right hemispheres.

Establishment of an Assessment for Social Recognition

A paired-samples *t*-test was run to analyze durations of stimulus animal investigation per trial in the SRT, in order to determine whether different ITIs impacted retention of social recognition in our healthy pilot animals. One study reported that a 30-minute ITI retained odor recognition memory, but 60, 120, and 180-minute ITIs failed to do so (Manella et al., 2013). In the present study, pilot animals spent significantly less time investigating stimulus animals during Trial II than during Trial I within the SRT with a 30-minute ITI, illustrating intact social recognition, $t(5) = 5.461$, $p = 0.003$ (Figure 3A). Expectedly, animals did not depict an intact social recognition in the SRT with a 180-minute ITI, as there was no significant difference in the amount of time healthy pilot animals spent investigating the stimulus animal between Trial I and Trial II, $t(5) = 0.921$, $p = 0.399$ (Figure 3B). Additionally, a one-sample t-test was conducted for the 30-minute and 180-minute ITI SRTs to discern how investigation ratios (IRs) derived from each ITI would relate to a 50% threshold, denoting equal stimulus investigation in both trials, and hence no social recognition. The 30-minute ITI had an IR that was significantly lower than the 50% threshold value, $t(5) = -5.361$, $p = 0.003$ (Figure 3C). The 180-minute ITI had an IR that was not statistically different from the 50% threshold value, $t(5) = -0.330, 0.755$ (Figure 3C).

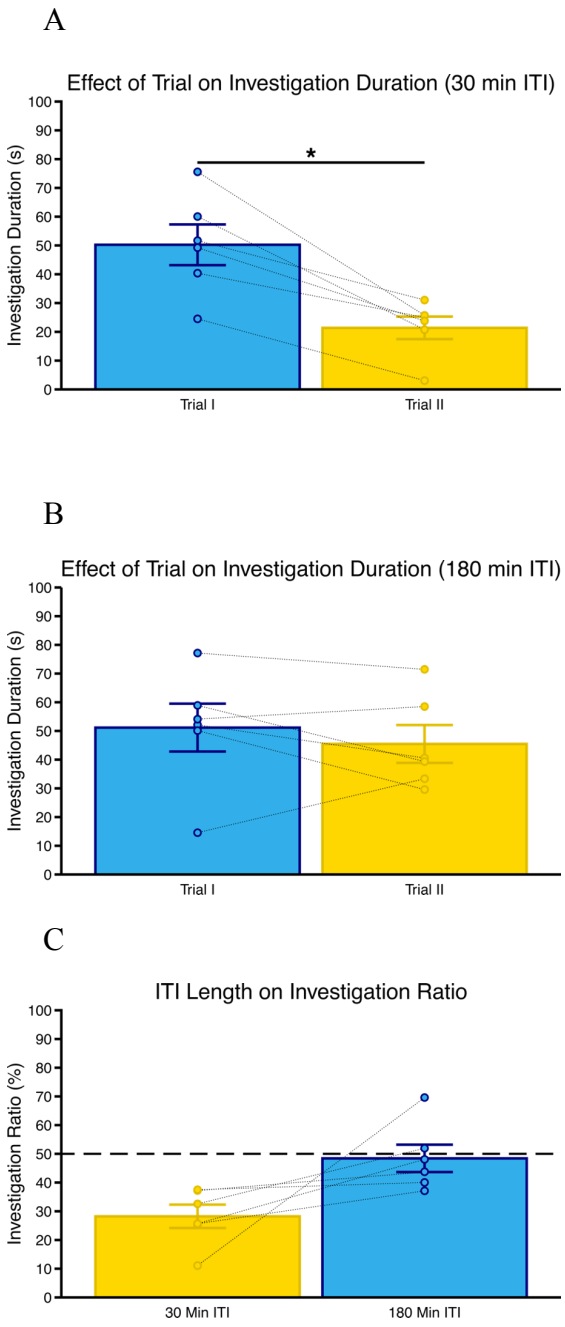


Figure 3. (A) Mean (+/–) SEM of investigation durations (s) of Trial I and Trial II for a 30-minute inter-trial-interval (ITI) in healthy, intact pilot animals. Animals depicted a significantly lower investigation duration in Trial II than in Trial I. (B) Mean (+/–) SEM of investigation durations (s) of Trial I and Trial II for an 180-minute ITI. There was no significant difference in investigation duration between Trial I and Trial II (C) Mean (+/–) SEM

investigation ratio for 30-minute ITI and 180-minute ITI. The investigation ratio for the 30-minute ITI was significantly lower than the 50% threshold, but there was no statistically significant difference between the 180-minute ITI's investigation ratio and the 50% threshold. *$p < 0.05$

Establishment of an Assessment for Spatial Memory

The SAT was utilized as an assessment of working spatial memory. Subsequent studies demonstrated that healthy control rats exhibited a spontaneous alternation percentage of about 75% (Faradila et al., 2020). Healthy female pilot animals depicted a spontaneous alternation percentage of ~71%, which was comparable to the control animals' SAT percentage of the reference study (Faradila et al., 2020) (Figure 4).

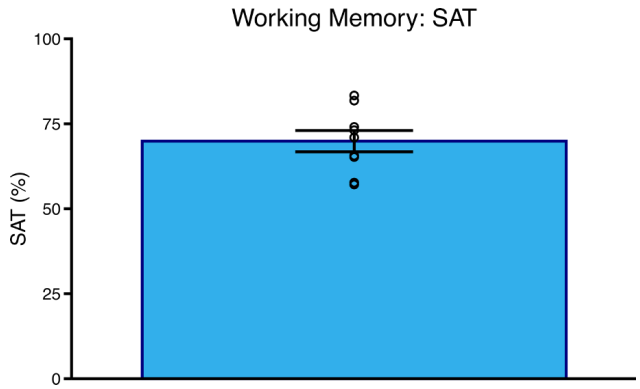
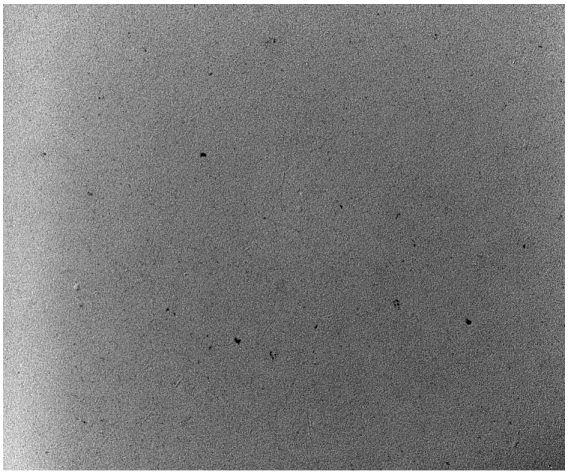


Figure 4. Mean (+/–) SEM spontaneous alternation percentage for pilot animals.

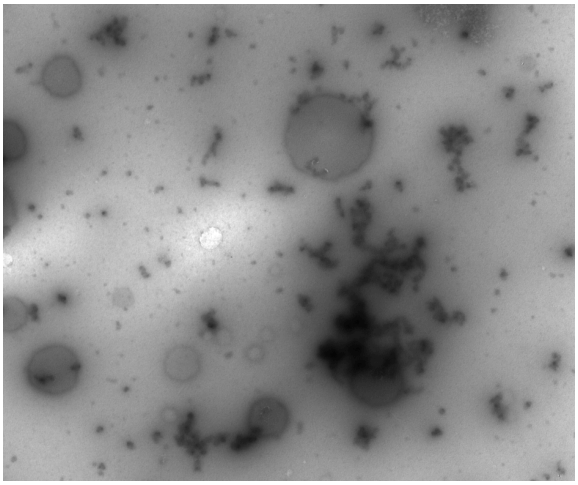
Confirmation of $A\beta_{1\text{-}42}$ Peptide Aggregation

TEM images were analyzed in order to confirm that our protocol was successfully inducing aggregation of the monomeric $A\beta_{1\text{-}42}$. In contrast to vehicle-only (PBS and DMSO) samples (Figure 5A,B), samples containing 72-hour incubated $A\beta_{1\text{-}42}$ showed clear evidence of successful aggregation (Figure 5C-E). Furthermore, upon confirmation of aggregation, peptides were measured and characterized by length, where peptides of the length of 60-220 nm were characterized to be in the protofibrillation stage, and peptides with a length of greater than 220 nm were in the fibrillation stage (Dubnovitsky et al., 2013). The vast majority of aggregates were characterized to be in the protofibrillation stage, with aggregation progressing further into fibrils (Walsh et al., 1999) (Figure 5D). Protofibrils are suggested to be the most neurotoxic form of these peptides (Z.-L. Chen et al., 2023; Lannfelt et al., 2014). This characterization of aggregation stages also provided insight into the state of neurotoxicity that the aggregates were in when injected into EOAD-pathology animals, indicating that aggregates were being incubated, and injected, at a point where the peptides should be most neurotoxic.

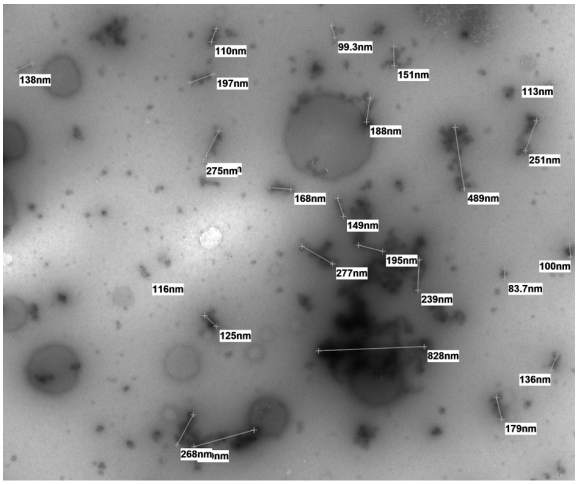
A



B



C



D

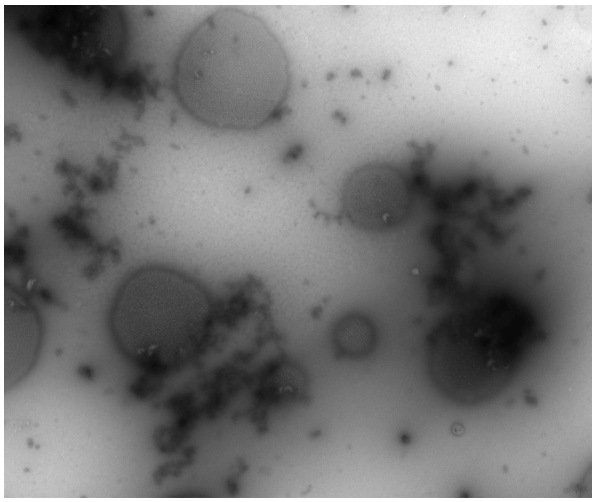


Figure 5. Confirmation and characterization of human β-amyloid42 ($A\beta_{1\text{-}42}$) peptide (2 μg/μl) aggregation with TEM. (A) Vehicle (PBS and DMSO) control imaged at 110,000X magnification. (B) $A\beta_{1\text{-}42}$ peptides post-72 hour incubation period imaged at 25,000X. (C) $A\beta_{1\text{-}42}$ peptides imaged at 25,000X with length (nm) measurements post-72 hour incubation. (D) $A\beta_{1\text{-}42}$ peptides imaged at 25,000X demonstrating fibrillogenesis and early plaque formation.

Effects of Estradiol Replacement Therapy on Social Recognition

After successfully establishing key experimental parameters, additional female cohorts underwent the complete experimental protocol (Figure 1). Examination of the effect of treatment group (HREF/htEOAD/ntEOAD) on social recognition data from Day 10 was conducted with a one-way ANOVA. Animals with hippocampal lesions (n=3) were excluded from the dataset. Data analyzed depicted a statistically significant difference between treatment groups' IRs, $F(2, 19) = 5.009$, $p = 0.018$ (Figure 6). Tukey's Post-Hoc comparisons at the front end demonstrated that ntEOAD animals had a significantly higher IR than HREF animals, but there were no differences in IRs between the HREF and htEOAD animals nor between the ntEOAD and htEOAD animals.

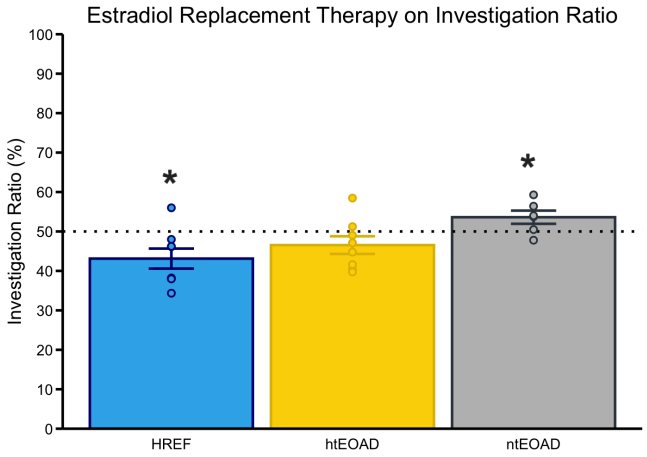


Figure 6. Mean (+/–) SEM investigation ratio (IR) (%) of healthy reference (HREF), hormone-treated early-onset Alzheimer's disease (htEOAD), and no treatment early-onset Alzheimer's disease (ntEOAD) animals. There was a statistically significant difference observed between the IRs of HREF and ntEOAD animals, but there was no statistically significant

difference between HREF and htEOAD animals nor between ntEOAD and htEOAD animals. *$p < 0.05$

Effects of Estradiol Replacement Therapy on Spatial Memory

Females undergoing the full experimental protocol also underwent SAT testing on Day 11. As previously mentioned, animals with hippocampal lesions ($n = 3$) were excluded from analyzed data. A one-way ANOVA examining the effect of treatment (HREF/htEOAD/ntEOAD) found no significant difference in the spontaneous alternation percentages between HREF, htEOAD, and ntEOAD animals, $F(2, 19) = 1.194$, $p = 0.325$ (Figure 7). Despite the establishment of what was previously deemed to be a reliable assessment for spatial memory (Figure 4), HREF animals did not perform as expected for reference animals with intact spatial memory, as their percentages were considerably below the 71% that was outlined as a reference within healthy animals.

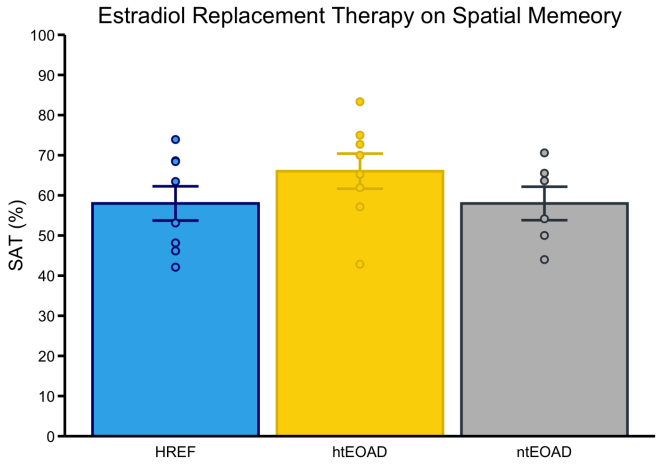


Figure 7. Mean (+/–) SEM Spontaneous Alternation percentages (%) of healthy reference (HREF), hormone-treated early-onset Alzheimer's disease (htEOAD), and no treatment

Alzheimer's disease (ntEOAD animals). There was no statistically significant difference observed between groups for spontaneous alternation percentages. *$p < 0.05$

Effects of Estradiol Replacement Therapy on Neurodegenerating Hippocampal Cells

In assessment of the effects of estradiol replacement therapy on the quantity of neurodegenerating cells in the hippocampal CA1 and CA3 subregions, cell-counting of neurodegenerating cells in the regions occurred in imaged H&E-stained tissue. Images of CA3 included small sections of the dentate gyrus. Non-statistical, preliminary quantitative data demonstrated a higher average degenerating cell density in the bilateral CA1/CA3 of ntEOAD ($n = 2$) compared to HREF ($n = 2$) animals (Table 2). The degenerating cell density in the CA1/CA3 of htEOAD ($n = 2$) animals tended to fall in between the values accounted for in HREF and ntEOAD animals (Table 2). These preliminary data suggest that our EOAD rat model was able to induce neurodegeneration, and that estradiol treatment may reduce the number of degenerating cells in these hippocampal subregions.

	HREF	**htEOAD**	**ntEOAD**
Left CA1	0.045	0.135	0.251
Right CA1	0.088	0.132	0.221
Left CA3	0.031	0.124	0.385
Right CA3	0.077	0.255	0.125

Table 2. Non-statistical, preliminary quantification of neurodegenerating cell densities (cells/mm^2) in the left and right hippocampal CA1 and CA3 subregions. Early-onset Alzheimer's

disease (EOAD) animals that did not receive treatment (n =2) had a greater degenerating cell density than healthy reference animals in all four regions. Hormone-treated early-onset Alzheimer's disease (htEOAD) animals that received estradiol treatment had a degenerating cell density in the left and right CA1 and left CA3 that was in between the density of healthy reference (HREF) and no treatment early-onset Alzheimer's disease (ntEOAD) animals, but not in the right CA3.

Discussion

The overarching goal of the study was to investigate the extent to which estradiol's neurocognitive benefits in EOAD are mediated via NEP. Systemic administration of EB for the activation of both membrane and nuclear ERs showed to be effective in partially reducing deficits in social recognition in htEOAD animals, as our SRT findings demonstrated no difference in their IRs between HREF nor ntEOAD animals. However, the lack of a significant difference in the SAT percentages between HREF and ntEOAD animals reflects a failure to validate the cognitive deficits typically observed in a rodent model of AD (Faradila et al., 2020). Preliminary, quantitative results of neurodegeneration in the CA1 and CA3 demonstrated a greater neurodegenerating cell density in the CA1 and CA3 subregions within ntEOAD than in HREF animals. Furthermore, htEOAD animals had a density of neurodegenerating cells in the CA1 that was in between the ntEOAD animals' and HREF animals'. These outcomes suggest the therapeutic potential of estradiol replacement for women under the age of 65 years at risk for EOAD immediately following menopause.

The lack of a significant difference between all treatment groups for their spatial memory, as depicted within the outcomes of the SAT, does not support our underlying predictions for the test. SAT results demonstrated that all treatment groups performed equivalently within the assessment. The main underlying issue within the test is that HREF animals did not perform as expected of a healthy reference group within the SAT. Despite pilot data delineating that healthy animals would be expected to achieve an SAT percentage of around 70%, which is comparable to the outcomes of reference animals in previous studies (Faradila et al., 2020), our experimental reference animals had an SAT percentage considerably below that threshold. Because reference animals did not perform as expected within the test, and because there was no significant difference in SAT percentages between HREF and ntEOAD animals, it was unclear as to whether our EOAD model successfully induced cognitive and/or spatial memory deficits. Therefore, no implications may be drawn for estradiol replacement therapy's ability to rescue spatial memory. There may be an inherent complication within the test itself, limiting our ability to differentiate cognitive capabilities between reference and EOAD animals. One difference between the experimental reference animals and the preceding pilot animals is that experimental animals received a bilateral vehicle injection into the hippocampal CA1, whereas pilot animals did not undergo any form of surgical event. Although the vehicle is not expected to induce any form of cognitive decline and/or deficit, the injection needle may have induced a small lesion while penetrating past cortical tissue and into the hippocampus. However, results from a previous study demonstrated that SD rats injected bilaterally with saline vehicle into the hippocampus did not portray spatial learning or memory deficits (Ahmed et al., 2010). Thus, it is unlikely that the bilateral vehicle injections into the hippocampi of our reference animals would be causing their unsuccessful demonstration of intact spatial memory. Furthermore, a different issue within the

test may be the protocol and/or apparatus, itself. Recent, preliminary data from our lab that utilized a restricted Y-Maze test exhibited expected spatial memory in healthy pilot animals, so future work could implement this test in our EOAD model. Previous studies have used a Morris water maze test or reward alternating T-Maze and successfully assessed working and/or spatial memory between WT and transgenic models of AD in rats (Berkowitz et al., 2018; Deacon & Rawlins, 2006; Saré et al., 2020). Therefore, future work could likewise utilize the Morris water maze test or the reward alternating T-Maze test as a measure of working memory and spatial memory in our EOAD model.

The induction of amyloidosis was fundamentally based on our bilateral injections of Aβ42 peptides into the hippocampi of female SD rats, aged 10 weeks, to generate our EOAD model. Previous literature that prepared and injected Aβ42 aggregates into the hippocampus utilized a 1 μg/μl concentration with a 1 μl volume (1 μg of peptide) bilaterally, whereas other researchers used 5 μg/hemisphere (Cetin & Dincer, 2007; Karthick et al., 2019). A study reported using 3 μl injections of 0.5, 1.0, and 2.5μg/μl Aβ42 fibrils (1.5 μg, 3.0 μg, or 7.5 μg of peptide/hemisphere) bilaterally into the hippocampus (Aquino et al., 2023). The rationale for initially utilizing a 2 μg/μl concentration (8 μg/hemisphere) of Aβ42 aggregates in the present study was to induce maximal potential neurodegeneration for substantial cognitive deficits within our EOAD model, consistent with the Amyloid Hypothesis, allowing us to assess the furthest extent to which estradiol replacement therapy could rescue social recognition and working spatial memory in EOAD-pathology animals that received the treatment (Makin, 2018). However, out of five animals that received the bilateral injections of the 2 μg/μl peptide concentration and daily oil injections, three of the animals (excluded from dataset) demonstrated severe hippocampal lesions bilaterally. This then prompted the decision to reduce the peptide

concentration to 1 μg/μl. Interestingly, one animal that received 2 μg/μl and estradiol treatment did not portray the lesions, suggesting a potential neuroprotective role of estradiol. However, because it was the only animal that received EB with the higher peptide concentration, and hence, the only animal that depicted these qualitative results, a decisive conclusion cannot be drawn from this observation. Furthermore, subsequent animals that received the 1 μg/μl bilateral concentration of the peptides and no treatment did not present lesions resembling the preceding animals', as only mild cortical and hippocampal damage was observed.

The Aβ protocol was successful in inducing aggregation of Aβ42 from its monomeric form, as observed from pre-injection samples. TEM confirmation of aggregation likewise provided insight into the stages of aggregation that the peptides were assembling into with the preparation and incubation protocol; stages of aggregation were characterized according to peptides' lengths (Dubnovitsky et al., 2013; Walsh et al., 1999). Images depicted the majority of developing aggregates to be in the protofibrillation stage, as most peptides in the imaged 2 μg/μl sample had a length of <250 nm, whereas remaining peptides advanced into developing fibrils, having a length of >250 nm. Because protofibrils are denoted to be in their most neurotoxic stage, this implied that the preparation and three-day incubation established the right conditions for aggregating most of the peptides to potentially induce maximal neurodegeneration in the CA1 (Z.-L. Chen et al., 2023; Lannfelt et al., 2014). The evidence of fibril formation additionally confirmed that the peptides' aggregation progressed beyond protofibrils, which likewise mimicked the natural advancement of pathology within EOAD. Future work should verify further *in vivo* incubation and accumulation of peptides in bilaterally-injected animals with antibody targeting of plaques in sectioned neural tissue. Additionally, TEM should confirm and characterize aggregation within our 1 μg/μl Aβ42 samples.

In further exploration of estradiol replacement therapy's capacity in serving as either a preventative therapy or curative therapy for women at risk for or with EOAD, varying dosages, treatment lengths, and treatment onset timing could be implemented as additional parameters within our female EOAD rat model. Previous studies have used subcutaneous injections of 0.2 μg 17β-estradiol in 0.1 ml cottonseed oil as an exogenous replacement of estradiol in ovariectomized female rats (Martinez et al., 2014; Peterson et al., 2016). This replacement does not mimic the natural fluctuations of estradiol within the menstrual cycle, where estradiol levels are lowest during the follicular phase and peak during ovulation (Sacher et al., 2013). Previous randomized, controlled trials have utilized single, daily oral doses of estradiol as a means of estradiol replacement therapy in postmenopausal and/or post-hysterectomy women (Mulnard et al., 2000; Taylor et al., 1996). Alternative, non-daily treatment protocols include a transdermal patch or vaginal rings, creams, or inserts, which should be replaced once or twice weekly but still deliver relatively stable and high levels of estradiol during the delivery time period (Mehta et al., 2021). To parallel the oral administration route, our EOAD model of estradiol replacement therapy featured single, daily doses of EB, being in injection form. Nonetheless, future experimentation with estradiol replacement therapy could examine the dosing/timing of EB within the EOAD rat model. It is important to consider that ovariectomized female rats that were administered higher (eg. 10 μg in 0.1 ml sesame oil) injection doses of EB had worse non-spatial working memory than rats that received lower doses (Holmes et al., 2002; Wide et al., 2004). A dose-response study may be necessary to fully elucidate the specific EB dose for treatment parameters that are most effective in rescuing social cognition in EOAD. This approach could be effectively complemented by also evaluating estradiol serum levels throughout the experimental protocol.

Additionally, given that systemic EB was administered immediately following an animal's ovariectomy in htEOAD animals in our study, it would be interesting to consider differential onset and duration of treatment. Implementing a delay in the onset of estradiol replacement therapy after the start of estradiol deficiency may provide different outcomes than in our current model; this would more accurately model the therapy when it is applied in a clinical population. A further consideration is that in contrast to our 10-day treatment protocol, one study used a 40-day capsular exposure of 25% 17β-estradiol and reported enhanced working memory in female rats (Rodgers et al., 2010). Thus, another approach in evaluating estradiol replacement therapy in potentially completely reversing EOAD-affiliated deficits could be implementing differential treatment lengths.

Preliminary quantitative results suggest the potential moderate reduction in the neurodegenerating cell density in EOAD animals treated with estradiol replacement therapy. Furthermore, there were more quantified degenerating cells in the CA1 and CA3 hippocampal subregions in EOAD animals not receiving treatment than in healthy, reference animals. Limitations of the data include the small sample size that was analyzed, as this quantification occurred in two animals per treatment group. Thus, the remaining animals' stained tissue must be quantified prior to drawing a definitive conclusion, and a second (blind) scorer of neurodegenerating cells should likewise be implemented. Moreover, differential staining intensities of H&E may be a confounding factor in the staining quality of tissue, as manual administration stains using a pipette caused some error in incubation times between tissue sections. Future work will implement Coplin jars for staining incubations within the protocol, instead. Assuming we can validate these preliminary observations by taking the steps discussed above, estradiol may be suggested to moderately reduce the quantity of degenerating cells in the

CA1. Because nuclear estrogen receptors upregulate NEP expression, and NEP efficiently degrades Aβ42 plaques, this potential neuroprotection of estradiol replacement therapy may be supported by ERα's indirect role in reducing neurotoxicity and neurodegeneration (Humpel, 2021; Liang et al., 2010; Marr et al., 2004). Alternatively, GPER1 may likewise have a prominent role in enhancing cell viability and resistance against neurodegeneration via the PI3K/Akt pathway's enhancement of hexokinase-2 for elevated glycolytic activity (Agostini et al., 2016; Kumar et al., 2011). Thus, ER-specific pharmacological agonists may be utilized in future work to pinpoint which particular ER is responsible for this potentially diminished neurodegeneration.

Due to time constraints, quantification of NEP-expressing cells in the hippocampal CA1 and CA3 subregions did not occur in the present experiment. Future work will conduct immunohistochemical analysis of NEP-expressing cells in our regions of interest. If the current experimental predictions support the study's hypothesis, we would expect to see more NEP-expressing cells in htEOAD animals, which would be a quantity that is comparable to HREF animals, than in ntEOAD animals. Future studies could also examine how pharmacological inhibition of NEP or a NEP-knockout might impact social recognition deficits and neurodegeneration within our EOAD model.

In conclusion, estradiol replacement therapy in our EOAD model animals showed to be effective in moderately reducing deficits in social recognition in comparison to EOAD-pathology animals that did not receive treatment. Differences in IRs between HREF and ntEOAD animals could likewise be attributed to the induction of social anxiety or social apathy within our EOAD model. Additionally, the assertion that the therapy was effective in rescuing spatial recognition could not be made, as HREF animals did not perform as expected within the test. Future work

within the study aims to identify and validate a more reliable assessment of spatial cognition, as well as expand the sample size of all treatment groups. These future studies should also quantify expression of NEP in the hippocampus via immunohistochemistry and look to more directly assess NEP's causal role via administration of a NEP inhibitor. Finally, future projected work could implement adeno-associated virus-induced overexpression of ERα and ERβ to ascertain the primary mechanism by which NEP expression is regulated.

References

Agostini, M., Romeo, F., Inoue, S., Niklison-Chirou, M. V., Elia, A. J., Dinsdale, D., Morone, N., Knight, R. A., Mak, T. W., & Melino, G. (2016). Metabolic reprogramming during neuronal differentiation. *Cell Death & Differentiation*, *23*(9), 1502–1514. https://doi.org/10.1038/cdd.2016.36

Ahmed, T., Enam, S. A., & Gilani, A. H. (2010). Curcuminoids enhance memory in an amyloid-infused rat model of Alzheimer's disease. *Neuroscience*, *169*(3), 1296–1306. https://doi.org/10.1016/j.neuroscience.2010.05.078

Altomari, N., Bruno, F., Laganà, V., Smirne, N., Colao, R., Curcio, S., Di Lorenzo, R., Frangipane, F., Maletta, R., Puccio, G., & Bruni, A. C. (2022). A Comparison of Behavioral and Psychological Symptoms of Dementia (BPSD) and BPSD Sub-Syndromes in Early-Onset and Late-Onset Alzheimer's Disease. *Journal of Alzheimer's Disease*, *85*(2), 691–699. https://doi.org/10.3233/JAD-215061

Alzheimer's Disease International, Guerchet, M., Prince, M., & Prina, M. (2020). *Numbers of people with dementia worldwide: An update to the estimates in the World Alzheimer Report 2015*. https://www.alzint.org/resource/numbers-of-people-with-dementia-worldwide/

Anand, K. S., & Dhikav, V. (2012). Hippocampus in health and disease: An overview. *Annals of Indian Academy of Neurology*, *15*(4), 239–246. https://doi.org/10.4103/0972-2327.104323

Aquino, R., de Concini, V., Dhenain, M., Lam, S., Gosset, D., Baquedano, L., Forero, M. G., Menuet, A., Baril, P., & Pichon, C. (2023). Intrahippocampal Inoculation of Aβ1–42 Peptide in Rat as a Model of Alzheimer's Disease Identified MicroRNA-146a-5p as

Blood Marker with Anti-Inflammatory Function in Astrocyte Cells. *Cells*, *12*(5), 694. https://doi.org/10.3390/cells12050694

Ayodele, T., Rogaeva, E., Kurup, J. T., Beecham, G., & Reitz, C. (2021). Early-Onset Alzheimer's Disease: What Is Missing in Research? *Current Neurology and Neuroscience Reports*, *21*(2), 4. https://doi.org/10.1007/s11910-020-01090-y

Barton, M., Filardo, E. J., Lolait, S. J., Thomas, P., Maggiolini, M., & Prossnitz, E. R. (2018). Twenty years of the G protein-coupled estrogen receptor GPER: Historical and personal perspectives. *The Journal of Steroid Biochemistry and Molecular Biology*, *176*, 4–15. https://doi.org/10.1016/j.jsbmb.2017.03.021

Bature, F., Guinn, B., Pang, D., & Pappas, Y. (2017). Signs and symptoms preceding the diagnosis of Alzheimer's disease: A systematic scoping review of literature from 1937 to 2016. *BMJ Open*, *7*(8), e015746. https://doi.org/10.1136/bmjopen-2016-015746

Bean, L. A., Ianov, L., & Foster, T. C. (2014). Estrogen Receptors, the Hippocampus, and Memory. *The Neuroscientist*, *20*(5), 534–545. https://doi.org/10.1177/1073858413519865

Berkowitz, L. E., Harvey, R. E., Drake, E., Thompson, S. M., & Clark, B. J. (2018). Progressive impairment of directional and spatially precise trajectories by TgF344-Alzheimer's disease rats in the Morris Water Task. *Scientific Reports*, *8*(1), 16153. https://doi.org/10.1038/s41598-018-34368-w

Blumstein, D., Daniel, J., & Evans, C. (2020). *Welcome to JWatcher*. https://www.jwatcher.ucla.edu/

Boulware, M. I., Heisler, J. D., & Frick, K. M. (2013). The memory-enhancing effects of hippocampal estrogen receptor activation involve metabotropic glutamate receptor signaling. *The Journal of Neuroscience: The Official Journal of the Society for*

Neuroscience, *33*(38), 15184–15194. https://doi.org/10.1523/JNEUROSCI.1716-13.2013

Cetin, F., & Dincer, S. (2007). The Effect of Intrahippocampal Beta Amyloid (1-42) Peptide Injection on Oxidant and Antioxidant Status in Rat Brain. *Annals of the New York Academy of Sciences*, *1100*(1), 510–517. https://doi.org/10.1196/annals.1395.056

Chen, Z.-L., Singh, P. K., Calvano, M., Norris, E. H., & Strickland, S. (2023). A possible mechanism for the enhanced toxicity of beta-amyloid protofibrils in Alzheimer's disease. *Proceedings of the National Academy of Sciences*, *120*(36), e2309389120. https://doi.org/10.1073/pnas.2309389120

Chen, Z.-R., Huang, J.-B., Yang, S.-L., & Hong, F.-F. (2022). Role of Cholinergic Signaling in Alzheimer's Disease. *Molecules*, *27*(6), 1816. https://doi.org/10.3390/molecules27061816

Chiari, A., Pistoresi, B., Galli, C., Tondelli, M., Vinceti, G., Molinari, M. A., Addabbo, T., & Zamboni, G. (2021). Determinants of Caregiver Burden in Early-Onset Dementia. *Dementia and Geriatric Cognitive Disorders EXTRA*, *11*(2), 189–197. https://doi.org/10.1159/000516585

Collaborative Group on Hormonal Factors in Breast Cancer. (1997). Breast cancer and hormone replacement therapy: Collaborative reanalysis of data from 51 epidemiological studies of 52 705 women with breast cancer and 108 411 women without breast cancer. *The Lancet*, *350*(9084), 1047–1059. https://doi.org/10.1016/S0140-6736(97)08233-0

Coughlan, G. T., Betthauser, T. J., Boyle, R., Koscik, R. L., Klinger, H. M., Chibnik, L. B., Jonaitis, E. M., Yau, W.-Y. W., Wenzel, A., Christian, B. T., Gleason, C. E., Saelzler, U. G., Properzi, M. J., Schultz, A. P., Hanseeuw, B. J., Manson, J. E., Rentz, D. M., Johnson, K. A., Sperling, R., … Buckley, R. F. (2023). Association of Age at Menopause and

Hormone Therapy Use With Tau and β-Amyloid Positron Emission Tomography. *JAMA Neurology*, *80*(5), 462–473. https://doi.org/10.1001/jamaneurol.2023.0455

Couse, J. F., & Korach, K. S. (1999). Estrogen Receptor Null Mice: What Have We Learned and Where Will They Lead Us? *Endocrine Reviews*, *20*(3), 358–417. https://doi.org/10.1210/edrv.20.3.0370

Cui, J., Shen, Y., & Li, R. (2013). Estrogen synthesis and signaling pathways during ageing: From periphery to brain. *Trends in Molecular Medicine*, *19*(3), 197–209. https://doi.org/10.1016/j.molmed.2012.12.007

Deacon, R. M. J., & Rawlins, J. N. P. (2006). T-maze alternation in the rodent. *Nature Protocols*, *1*(1), 7–12. https://doi.org/10.1038/nprot.2006.2

Du, B., Ohmichi, M., Takahashi, K., Kawagoe, J., Ohshima, C., Igarashi, H., Mori-Abe, A., Saitoh, M., Ohta, T., Ohishi, A., Doshida, M., Tezuka, N., Takahashi, T., & Kurachi, H. (2004). Both estrogen and raloxifene protect against beta-amyloid-induced neurotoxicity in estrogen receptor alpha-transfected PC12 cells by activation of telomerase activity via Akt cascade. *The Journal of Endocrinology*, *183*(3), 605–615. https://doi.org/10.1677/joe.1.05775

Dubnovitsky, A., Sandberg, A., Rahman, M. M., Benilova, I., Lendel, C., & Härd, T. (2013). Amyloid-β Protofibrils: Size, Morphology and Synaptotoxicity of an Engineered Mimic. *PLoS ONE*, *8*(7), e66101. https://doi.org/10.1371/journal.pone.0066101

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjöld, M., & Gustafsson, J.-Å. (1997). Human Estrogen Receptor β-Gene Structure, Chromosomal Localization, and Expression Pattern1. *The Journal of Clinical Endocrinology & Metabolism*, *82*(12), 4258–4265.

https://doi.org/10.1210/jcem.82.12.4470

Faradila, F., Syafrita, Y., & Lipoeto, N. I. (2020). *Relationship between Amyloid-beta 42 Levels and Y-maze Alternation Values in Sprague Dawley Alzheimer's Induction Received Medium-Chain Triglycerides Therapy | Open Access Macedonian Journal of Medical Sciences*. https://oamjms.eu/index.php/mjms/article/view/3243

Foster, T. C., Rani, A., Kumar, A., Cui, L., & Semple-Rowland, S. L. (2008). Viral Vector–mediated Delivery of Estrogen Receptor-α to the Hippocampus Improves Spatial Learning in Estrogen Receptor-α Knockout Mice. *Molecular Therapy*, *16*(9), 1587–1593. https://doi.org/10.1038/mt.2008.140

Friard, O., & Gamba, M. (2024). *About BORIS - BORIS*. https://www.boris.unito.it/about/

Fuentes, N., & Silveyra, P. (2019). Estrogen receptor signaling mechanisms. *Advances in Protein Chemistry and Structural Biology*, *116*, 135–170. https://doi.org/10.1016/bs.apcsb.2019.01.001

Gonneaud, J., Arenaza-Urquijo, E. M., Mézenge, F., Landeau, B., Gaubert, M., Bejanin, A., de Flores, R., Wirth, M., Tomadesso, C., Poisnel, G., Abbas, A., Desgranges, B., & Chételat, G. (2017). Increased florbetapir binding in the temporal neocortex from age 20 to 60 years. *Neurology*, *89*(24), 2438–2446. https://doi.org/10.1212/WNL.0000000000004733

Gosden, J. R., Middleton, P. G., & Rout, D. (1986). Localization of the human oestrogen receptor gene to chromosome 6q24—Q27 by in situ hybridization. *Cytogenetics and Cell Genetics*, *43*(3–4), 218–220. https://doi.org/10.1159/000132325

Gross, K. S., & Mermelstein, P. G. (2020). Chapter Nine—Estrogen receptor signaling through metabotropic glutamate receptors. In G. Litwack (Ed.), *Vitamins and Hormones* (Vol. 114, pp. 211–232). Academic Press. https://doi.org/10.1016/bs.vh.2020.06.003

Hara, Y., Waters, E. M., McEwen, B. S., & Morrison, J. H. (2015). Estrogen Effects on Cognitive and Synaptic Health Over the Lifecourse. *Physiological Reviews*, *95*(3), 785–807. https://doi.org/10.1152/physrev.00036.2014

Hardy, J., & Selkoe, D. J. (2002). The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. *Science*, *297*(5580), 353–356. https://doi.org/10.1126/science.1072994

Hebert, L. E., Weuve, J., Scherr, P. A., & Evans, D. A. (2013). Alzheimer disease in the United States (2010–2050) estimated using the 2010 census. *Neurology*, *80*(19), 1778–1783. https://doi.org/10.1212/WNL.0b013e31828726f5

Henderson, V., Benke, K., Green, R., Cupples, L., Farrer, L., & t for. (2005). Postmenopausal hormone therapy and Alzheimer's disease risk: Interaction with age. *Journal of Neurology, Neurosurgery, and Psychiatry*, *76*(1), 103–105. https://doi.org/10.1136/jnnp.2003.024927

Hersh, L. B., & Rodgers, D. W. (2008). Neprilysin and amyloid beta peptide degradation. *Current Alzheimer Research*, *5*(2), 225–231. https://doi.org/10.2174/156720508783954703

Holmes, M. M., Wide, J. K., & Galea, L. A. M. (2002). Low levels of estradiol facilitate, whereas high levels of estradiol impair, working memory performance on the radial arm maze. *Behavioral Neuroscience*, *116*(5), 928–934. https://doi.org/10.1037/0735-7044.116.5.928

Humpel, C. (2021). Intranasal Delivery of Collagen-Loaded Neprilysin Clears Beta-Amyloid Plaques in a Transgenic Alzheimer Mouse Model. *Frontiers in Aging Neuroscience*, *13*. https://doi.org/10.3389/fnagi.2021.649646

Hwang, C. J., Yun, H.-M., Park, K.-R., Song, J. K., Seo, H. O., Hyun, B. K., Choi, D. Y., Yoo, H.-S., Oh, K.-W., Hwang, D. Y., Han, S.-B., & Hong, J. T. (2015). Memory Impairment in Estrogen Receptor α Knockout Mice Through Accumulation of Amyloid-β Peptides. *Molecular Neurobiology*, *52*(1), 176–186. https://doi.org/10.1007/s12035-014-8853-z

Institute for Quality and Efficiency in Health Care (IQWiG). (2020). Menopause: What are the benefits and risks of long-term hormone therapy? In *InformedHealth.org [Internet]*. Institute for Quality and Efficiency in Health Care (IQWiG). https://www.ncbi.nlm.nih.gov/books/NBK564986/

International, A. D., Ferri, C., Prince, M., & Bryce, R. (2011). *World Alzheimer Report 2011: The benefits of early diagnosis and intervention*. https://www.alzint.org/resource/world-alzheimer-report-2011/

Ivanova, T., Mendez, P., Garcia-Segura, L. M., & Beyer, C. (2002). Rapid stimulation of the PI3-kinase/Akt signalling pathway in developing midbrain neurones by oestrogen. *Journal of Neuroendocrinology*, *14*(1), 73–79. https://doi.org/10.1046/j.0007-1331.2001.00742.x

Iwata, N., Mizukami, H., Shirotani, K., Takaki, Y., Muramatsu, S., Lu, B., Gerard, N. P., Gerard, C., Ozawa, K., & Saido, T. C. (2004). Presynaptic Localization of Neprilysin Contributes to Efficient Clearance of Amyloid-β Peptide in Mouse Brain. *The Journal of Neuroscience*, *24*(4), 991–998. https://doi.org/10.1523/JNEUROSCI.4792-03.2004

Kaiser, N. C., Melrose, R. J., Liu, C., Sultzer, D. L., Jimenez, E., Su, M., Monserratt, L., & Mendez, M. F. (2012). Neuropsychological and Neuroimaging Markers in Early Versus Late-Onset Alzheimer's Disease. *American Journal of Alzheimer's Disease & Other Dementias®*, *27*(7), 520–529. https://doi.org/10.1177/1533317512459798

Kalpouzos, G., Eustache, F., de la Sayette, V., Viader, F., Chételat, G., & Desgranges, B. (2005). Working memory and FDG–PET dissociate early and late onset Alzheimer disease patients. *Journal of Neurology*, *252*(5), 548–558. https://doi.org/10.1007/s00415-005-0685-3

Karthick, C., Nithiyanandan, S., Essa, M. M., Guillemin, G. J., Jayachandran, S. K., & Anusuyadevi, M. (2019). Time-dependent effect of oligomeric amyloid-β (1-42)-induced hippocampal neurodegeneration in rat model of Alzheimer's disease. *Neurological Research*, *41*(2), 139–150. https://doi.org/10.1080/01616412.2018.1544745

Klein, A., Kowall, N., & Ferrante, R. (2006). *Neurotoxicity and Oxidative Damage of Beta Amyloid 1–42 versus Beta Amyloid 1–40 in the Mouse Cerebral Cortex—KLEIN - 1999—Annals of the New York Academy of Sciences—Wiley Online Library*. https://nyaspubs.onlinelibrary.wiley.com/doi/abs/10.1111/j.1749-6632.1999.tb07845.x?sid=nlm%3Apubmed

Klinge, C. M. (2001). Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Research*, *29*(14), 2905–2919.

Kumar, R., Zakharov, M. N., Khan, S. H., Miki, R., Jang, H., Toraldo, G., Singh, R., Bhasin, S., & Jasuja, R. (2011). The Dynamic Structure of the Estrogen Receptor. *Journal of Amino Acids*, *2011*, e812540. https://doi.org/10.4061/2011/812540

Lannfelt, L., Möller, C., Basun, H., Osswald, G., Sehlin, D., Satlin, A., Logovinsky, V., & Gellerfors, P. (2014). Perspectives on future Alzheimer therapies: Amyloid-β protofibrils - a new target for immunotherapy with BAN2401 in Alzheimer's disease. *Alzheimer's Research & Therapy*, *6*(2), 16. https://doi.org/10.1186/alzrt246

Larson, E. B., Kukull, W. A., & Katzman, R. L. (1992). Cognitive impairment: Dementia and

Alzheimer's disease. *Annual Review of Public Health*, *13*, 431–449. https://doi.org/10.1146/annurev.pu.13.050192.002243

Lee, S. J., Romeo, R. D., Svenningsson, P., Campomanes, C. R., Allen, P. B., Greengard, P., & McEwen, B. S. (2004). Estradiol affects spinophilin protein differently in gonadectomized males and females. *Neuroscience*, *127*(4), 983–988. https://doi.org/10.1016/j.neuroscience.2004.05.049

Leuner, B., Falduto, J., & Shors, T. J. (2003). Associative memory formation increases the observation of dendritic spines in the hippocampus. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *23*(2), 659–665. https://doi.org/10.1523/JNEUROSCI.23-02-00659.2003

Li, R., He, P., Cui, J., Staufenbiel, M., Harada, N., & Shen, Y. (2013). Brain endogenous estrogen levels determine responses to estrogen replacement therapy via regulation of BACE1 and NEP in female Alzheimer's transgenic mice. *Molecular Neurobiology*, *47*(3), 857–867. https://doi.org/10.1007/s12035-012-8377-3

Liang, K., Yang, L., Yin, C., Xiao, Z., Zhang, J., Liu, Y., & Huang, J. (2010). Estrogen Stimulates Degradation of β-Amyloid Peptide by Up-regulating Neprilysin*. *Journal of Biological Chemistry*, *285*(2), 935–942. https://doi.org/10.1074/jbc.M109.051664

Lin, P., Sun, J., Cheng, Q., Yang, Y., Cordato, D., & Gao, J. (2021). The Development of Pharmacological Therapies for Alzheimer's Disease. *Neurology and Therapy*, *10*(2), 609–626. https://doi.org/10.1007/s40120-021-00282-z

Lösel, R., & Wehling, M. (2003). Nongenomic actions of steroid hormones. *Nature Reviews. Molecular Cell Biology*, *4*(1), 46–56. https://doi.org/10.1038/nrm1009

Loven, M. A., Wood, J. R., & Nardulli, A. M. (2001). Interaction of estrogen receptors alpha and

beta with estrogen response elements. *Molecular and Cellular Endocrinology*, *181*(1–2), 151–163. https://doi.org/10.1016/s0303-7207(01)00491-9

Makin, S. (2018). The amyloid hypothesis on trial. *Nature*, *559*(7715), S4–S7. https://doi.org/10.1038/d41586-018-05719-4

Manella, L., Alperin, S., & Linster, C. (2013). Stressors impair odor recognition memory via an olfactory bulb-dependent noradrenergic mechanism. *Frontiers in Integrative Neuroscience*, *7*, 97. https://doi.org/10.3389/fnint.2013.00097

Mannella, P., & Brinton, R. D. (2006). Estrogen Receptor Protein Interaction with Phosphatidylinositol 3-Kinase Leads to Activation of Phosphorylated Akt and Extracellular Signal-Regulated Kinase 1/2 in the Same Population of Cortical Neurons: A Unified Mechanism of Estrogen Action. *The Journal of Neuroscience*, *26*(37), 9439–9447. https://doi.org/10.1523/JNEUROSCI.1443-06.2006

Marino, M., Galluzzo, P., & Ascenzi, P. (2006). Estrogen Signaling Multiple Pathways to Impact Gene Transcription. *Current Genomics*, *7*(8), 497–508.

Marr, R. A., Guan, H., Rockenstein, E., Kindy, M., Gage, F. H., Verma, I., Masliah, E., & Hersh, L. B. (2004). Neprilysin regulates amyloid Beta peptide levels. *Journal of Molecular Neuroscience: MN*, *22*(1–2), 5–11. https://doi.org/10.1385/JMN:22:1-2:5

Martinez, L. A., Peterson, B. M., Meisel, R. L., & Mermelstein, P. G. (2014). Estradiol facilitation of cocaine-induced locomotor sensitization in female rats requires activation of mGluR5. *Behavioural Brain Research*, *271*, 39–42. https://doi.org/10.1016/j.bbr.2014.05.052

Mathiasen, J. R., & DiCamillo, A. (2010). Social recognition assay in the rat. *Current Protocols in Neuroscience*, *Chapter 8*, Unit 8.5I. https://doi.org/10.1002/0471142301.ns0805is53

Mayeux, R., & Sano, M. (1999). Treatment of Alzheimer's Disease. *New England Journal of Medicine*, *341*(22), 1670–1679. https://doi.org/10.1056/NEJM199911253412207

McCarthy, M., & Raval, A. P. (2020). The peri-menopause in a woman's life: A systemic inflammatory phase that enables later neurodegenerative disease. *Journal of Neuroinflammation*, *17*(1), 317. https://doi.org/10.1186/s12974-020-01998-9

Meems, L., Chen, Y., & Burnett, J. C. (2018). Chapter 9 - The Cardiac Natriuretic Peptide System: Linking the Heart and Kidney in Cardiorenal Homeostasis and Therapeutics. In A. K. Singh & G. H. Williams (Eds.), *Textbook of Nephro-Endocrinology (Second Edition)* (pp. 163–171). Academic Press. https://doi.org/10.1016/B978-0-12-803247-3.00009-X

Mehta, J., Kling, J. M., & Manson, J. E. (2021). Risks, Benefits, and Treatment Modalities of Menopausal Hormone Therapy: Current Concepts. *Frontiers in Endocrinology*, *12*, 564781. https://doi.org/10.3389/fendo.2021.564781

Mulnard, R. A., Cotman, C. W., Kawas, C., van Dyck, C. H., Sano, M., Doody, R., Koss, E., Pfeiffer, E., Jin, S., Gamst, A., Grundman, M., Thomas, R., & Thal, L. J. (2000). Estrogen replacement therapy for treatment of mild to moderate Alzheimer disease: A randomized controlled trial. Alzheimer's Disease Cooperative Study. *JAMA*, *283*(8), 1007–1015. https://doi.org/10.1001/jama.283.8.1007

Murphy, D. D., & Segal, M. (1996). Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *16*(13), 4059–4068. https://doi.org/10.1523/JNEUROSCI.16-13-04059.1996

Nalivaeva, N. N., & Turner, A. J. (2013). Chapter 127—Neprilysin. In N. D. Rawlings & G.

Salvesen (Eds.), *Handbook of Proteolytic Enzymes (Third Edition)* (pp. 612–619). Academic Press. https://doi.org/10.1016/B978-0-12-382219-2.00127-7

National Research Council (US) Committee for the Update of the Guide for theCare and Use of Laboratory Animals. (2011). *Guide for the Care and Use of Laboratory Animals* (8th ed.). National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK54050/

Nilsen, J., & Brinton, R. D. (2003). Mechanism of estrogen-mediated neuroprotection: Regulation of mitochondrial calcium and Bcl-2 expression. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(5), 2842–2847. https://doi.org/10.1073/pnas.0438041100

O'Lone, R., Frith, M. C., Karlsson, E. K., & Hansen, U. (2004). Genomic Targets of Nuclear Estrogen Receptors. *Molecular Endocrinology*, *18*(8), 1859–1875. https://doi.org/10.1210/me.2003-0044

O'Malley, B. W. (2005). A Life-Long Search for the Molecular Pathways of Steroid Hormone Action. *Molecular Endocrinology*, *19*(6), 1402–1411. https://doi.org/10.1210/me.2004-0480

Paxinos, G., & Watson, C. (2009). *The Rat Brain in Stereotaxic Coordinates*. Elsevier/Academic. https://books.google.com/books?id=sQNfmwEACAAJ

Peeters, G., Katelekha, K., Lawlor, B., & Demnitz, N. (2022). Sex differences in the incidence and prevalence of young-onset Alzheimer's disease: A meta-analysis. *International Journal of Geriatric Psychiatry*, *37*(1). https://doi.org/10.1002/gps.5612

Pereira, L. M., Bastos, C. P., de Souza, J. M., Ribeiro, F. M., & Pereira, G. S. (2014). Estradiol enhances object recognition memory in Swiss female mice by activating hippocampal estrogen receptor α. *Neurobiology of Learning and Memory*, *114*, 1–9.

https://doi.org/10.1016/j.nlm.2014.04.001

Peterson, B. M., Martinez, L. A., Meisel, R. L., & Mermelstein, P. G. (2016). Estradiol impacts the endocannabinoid system in female rats to influence behavioral and structural responses to cocaine. *Neuropharmacology*, *110*(Pt A), 118–124. https://doi.org/10.1016/j.neuropharm.2016.06.002

Prossnitz, E. R., & Barton, M. (2014). Estrogen biology: New insights into GPER function and clinical opportunities. *Molecular and Cellular Endocrinology*, *389*(1), 71–83. https://doi.org/10.1016/j.mce.2014.02.002

Reardon, S. (2023). FDA approves Alzheimer's drug lecanemab amid safety concerns. *Nature*, *613*(7943), 227–228. https://doi.org/10.1038/d41586-023-00030-3

Relationship between Amyloid-beta 42 Levels and Y-maze Alternation Values in Sprague Dawley Alzheimer's Induction Received Medium-Chain Triglycerides Therapy | Open Access Macedonian Journal of Medical Sciences. (2020). https://oamjms.eu/index.php/mjms/article/view/3243

Rodgers, S. P., Bohacek, J., & Daniel, J. M. (2010). Transient Estradiol Exposure during Middle Age in Ovariectomized Rats Exerts Lasting Effects on Cognitive Function and the Hippocampus. *Endocrinology*, *151*(3), 1194–1203. https://doi.org/10.1210/en.2009-1245

Rofo, F., Metzendorf, N. G., Saubi, C., Suominen, L., Godec, A., Sehlin, D., Syvänen, S., & Hultqvist, G. (2022). Blood–brain barrier penetrating neprilysin degrades monomeric amyloid-beta in a mouse model of Alzheimer's disease. *Alzheimer's Research & Therapy*, *14*(1), 180. https://doi.org/10.1186/s13195-022-01132-2

Sacher, J., Okon-Singer, H., & Villringer, A. (2013). Evidence from neuroimaging for the role of the menstrual cycle in the interplay of emotion and cognition. *Frontiers in Human*

Neuroscience, *7*, 374. https://doi.org/10.3389/fnhum.2013.00374

Sakata, N., & Okumura, Y. (2017). Job Loss After Diagnosis of Early-Onset Dementia: A Matched Cohort Study. *Journal of Alzheimer's Disease*, *60*(4), 1231–1235. https://doi.org/10.3233/JAD-170478

Saleh, R. N. M., Hornberger, M., Ritchie, C. W., & Minihane, A. M. (2023). Hormone replacement therapy is associated with improved cognition and larger brain volumes in at-risk APOE4 women: Results from the European Prevention of Alzheimer's Disease (EPAD) cohort. *Alzheimer's Research & Therapy*, *15*(1), 10. https://doi.org/10.1186/s13195-022-01121-5

Saré, R. M., Cooke, S. K., Krych, L., Zerfas, P. M., Cohen, R. M., & Smith, C. B. (2020). Behavioral Phenotype in the TgF344-AD Rat Model of Alzheimer's Disease. *Frontiers in Neuroscience*, *14*. https://doi.org/10.3389/fnins.2020.00601

Savolainen-Peltonen, H., Rahkola-Soisalo, P., Hoti, F., Vattulainen, P., Gissler, M., Ylikorkala, O., & Mikkola, T. S. (2019). Use of postmenopausal hormone therapy and risk of Alzheimer's disease in Finland: Nationwide case-control study. *The BMJ*, *364*, l665. https://doi.org/10.1136/bmj.l665

Shirotani, K., Tsubuki, S., Iwata, N., Takaki, Y., Harigaya, W., Maruyama, K., Kiryu-Seo, S., Kiyama, H., Iwata, H., Tomita, T., Iwatsubo, T., & Saido, T. C. (2001). Neprilysin Degrades Both Amyloid β Peptides 1–40 and 1–42 Most Rapidly and Efficiently among Thiorphan- and Phosphoramidon-sensitive Endopeptidases*. *Journal of Biological Chemistry*, *276*(24), 21895–21901. https://doi.org/10.1074/jbc.M008511200

Sirkis, D. W., Bonham, L. W., Johnson, T. P., La Joie, R., & Yokoyama, J. S. (2022). Dissecting the clinical heterogeneity of early-onset Alzheimer's disease. *Molecular Psychiatry*,

27(6), Article 6. https://doi.org/10.1038/s41380-022-01531-9

Slooter, A. J. C., Bronzova, J., Witteman, J. C. M., Broeckhoven, C. V., Hofman, A., & Duijn, C. M. van. (1999). Estrogen use and early onset Alzheimer's disease: A population-based study. *Journal of Neurology, Neurosurgery & Psychiatry*, *67*(6), 779–781. https://doi.org/10.1136/jnnp.67.6.779

Sochocka, M., Karska, J., Pszczołowska, M., Ochnik, M., Fułek, M., Fułek, K., Kurpas, D., Chojdak-Łukasiewicz, J., Rosner-Tenerowicz, A., & Leszek, J. (2023). Cognitive Decline in Early and Premature Menopause. *International Journal of Molecular Sciences*, *24*(7), 6566. https://doi.org/10.3390/ijms24076566

Song, X., Liu, B., Cui, L., Zhou, B., Liu, L., Liu, W., Yao, G., Xia, M., Hayashi, T., Hattori, S., Ushiki-Kaku, Y., Tashiro, S., & Ikejima, T. (2018). Estrogen Receptors Are Involved in the Neuroprotective Effect of Silibinin in Aβ1–42-Treated Rats. *Neurochemical Research*, *43*(4), 796–805. https://doi.org/10.1007/s11064-018-2481-3

Spencer, J. L., Waters, E. M., Romeo, R. D., Wood, G. E., Milner, T. A., & McEwen, B. S. (2008). Uncovering the Mechanisms of Estrogen Effects on Hippocampal Function. *Frontiers in Neuroendocrinology*, *29*(2), 219–237. https://doi.org/10.1016/j.yfrne.2007.08.006

Storandt, M. (2008). Cognitive Deficits in the Early Stages of Alzheimer's Disease. *Current Directions in Psychological Science*, *17*(3), 198–202. https://doi.org/10.1111/j.1467-8721.2008.00574.x

Taylor, A. E., Adams, J. M., Mulder, J. E., Martin, K. A., Sluss, P. M., & Crowley, W. F., Jr. (1996). A randomized, controlled trial of estradiol replacement therapy in women with hypergonadotropic amenorrhea. *The Journal of Clinical Endocrinology & Metabolism*,

81(10), 3615–3621. https://doi.org/10.1210/jcem.81.10.8855811

Tellechea, P., Pujol, N., Esteve-Belloch, P., Echeveste, B., García-Eulate, M. R., Arbizu, J., & Riverol, M. (2018). Early- and late-onset Alzheimer disease: Are they the same entity? *Neurología (English Edition)*, *33*(4), 244–253. https://doi.org/10.1016/j.nrleng.2015.08.009

van Dyck Christopher H., Swanson Chad J., Aisen Paul, Bateman Randall J., Chen Christopher, Gee Michelle, Kanekiyo Michio, Li David, Reyderman Larisa, Cohen Sharon, Froelich Lutz, Katayama Sadao, Sabbagh Marwan, Vellas Bruno, Watson David, Dhadda Shobha, Irizarry Michael, Kramer Lynn D., & Iwatsubo Takeshi. (2023). Lecanemab in Early Alzheimer's Disease. *New England Journal of Medicine*, *388*(1), 9–21. https://doi.org/10.1056/NEJMoa2212948

Walsh, D. M., Hartley, D. M., Kusumoto, Y., Fezoui, Y., Condron, M. M., Lomakin, A., Benedek, G. B., Selkoe, D. J., & Teplow, D. B. (1999). Amyloid β-Protein Fibrillogenesis: STRUCTURE AND BIOLOGICAL ACTIVITY OF PROTOFIBRILLAR INTERMEDIATES *. *Journal of Biological Chemistry*, *274*(36), 25945–25952. https://doi.org/10.1074/jbc.274.36.25945

Wang, L., Eom, K., & Kwon, T. (2021). Different Aggregation Pathways and Structures for Aβ40 and Aβ42 Peptides. *Biomolecules*, *11*(2), 198. https://doi.org/10.3390/biom11020198

Wattmo, C., & Wallin, Å. K. (2017). Early- versus late-onset Alzheimer's disease in clinical practice: Cognitive and global outcomes over 3 years. *Alzheimer's Research & Therapy*, *9*(1), 70. https://doi.org/10.1186/s13195-017-0294-2

Wide, J. K., Hanratty, K., Ting, J., & Galea, L. A. M. (2004). High level estradiol impairs and

low level estradiol facilitates non-spatial working memory. *Behavioural Brain Research*, *155*(1), 45–53. https://doi.org/10.1016/j.bbr.2004.04.001

Wroolie, T. E., Kenna, H. A., Williams, K. E., & Rasgon, N. L. (2015). Cognitive Effects of Hormone Therapy Continuation or Discontinuation in a Sample of Women at Risk for Alzheimer Disease. *The American Journal of Geriatric Psychiatry*, *23*(11), 1117–1126. https://doi.org/10.1016/j.jagp.2015.05.009

Yasojima, K., Akiyama, H., McGeer, E. G., & McGeer, P. L. (2001). Reduced neprilysin in high plaque areas of Alzheimer brain: A possible relationship to deficient degradation of β-amyloid peptide. *Neuroscience Letters*, *297*(2), 97–100. https://doi.org/10.1016/S0304-3940(00)01675-X

Zhu, X.-C., Tan, L., Wang, H.-F., Jiang, T., Cao, L., Wang, C., Wang, J., Tan, C.-C., Meng, X.-F., & Yu, J.-T. (2015). Rate of early onset Alzheimer's disease: A systematic review and meta-analysis. *Annals of Translational Medicine*, *3*(3), 38. https://doi.org/10.3978/j.issn.2305-5839.2015.01.19

Zussy, C., Brureau, A., Delair, B., Marchal, S., Keller, E., Ixart, G., Naert, G., Meunier, J., Chevallier, N., Maurice, T., & Givalois, L. (2011). Time-Course and Regional Analyses of the Physiopathological Changes Induced after Cerebral Injection of an Amyloid β Fragment in Rats. *The American Journal of Pathology*, *179*(1), 315–334. https://doi.org/10.1016/j.ajpath.2011.03.021