2022

The neuroprotective actions of perinatal choline supplementation on amyloidosis in APP.NLGF knock-in Alzheimer's disease model mice

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

THE NEUROPROTECTIVE ACTIONS OF PERINATAL CHOLINE SUPPLEMENTATION ON AMYLOIDOSIS IN APP.NLGF KNOCK-IN ALZHEIMER'S DISEASE MODEL MICE

by

JAY CHOU

B.S., University of Texas, 2017

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2022

Approved by

First Reader _____

Jan K. Blusztajn, Ph.D.
Professor of Pathology & Laboratory Medicine

Second Reader _____

Tiffany J. Mellott, Ph.D.
Associate Professor of Pathology and Laboratory Medicine

ACKNOWLEDGMENTS

My special thanks to Dr. Jan K. Blusztajn, Ph.D., and Dr. Tiffany J. Mellott, Ph.D. and their Pathology and Laboratory Medicine Lab at Boston University School of Medicine. I could not have undertaken and completed this endeavor without their knowledge, guidance, and generosity. Their contributions to the research and writing of this thesis were invaluable and for them, I am indebted.

THE NEUROPROTECTIVE ACTIONS OF PERINATAL CHOLINE SUPPLEMENTATION ON AMYLOIDOSIS IN APP.NLGF KNOCK-IN ALZHEIMER'S DISEASE MODEL MICE

JAY CHOU

ABSTRACT

Alzheimer's Disease is a growing public health problem, with the number of Americans suffering from the disease projected to more than double from 5.8 million today to 13.8 million in 2050. While there is still no cure for Alzheimer's Disease, a preventative strategy may mitigate its cost to society in the future. Previous studies have shown an ameliorative effect of perinatal choline supplementation on amyloidosis in the hippocampus of APP.PS1 mice. In this study, we test the effects of perinatal choline supplementation on the APP.NLGF strain of mice – which uses a gene knock-in strategy to avoid the non-physiologic overexpression of amyloid precursor protein and better recapitulate the disease in humans. When compared to APP.NLGF mice raised on a control diet, the perinatal choline supplemented APP.NLGF mice exhibited: i) an amelioration of learning and memory deficits in 9- and 12-months old mice as measured by contextual fear conditioning, ii) reduced amyloidosis in the cortex of 9- and 12-months old mice, and iii) an age- and brain region-dependent response to perinatal choline supplementation. These results suggest that increasing the dietary intake of choline during pregnancy may protect the offspring from AD-associated cognitive decline and amyloidosis.

Table of Contents

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACH	ACETYLCHOLINE
Aβ	AMYLOID-BETA
AD	ALZHEIMER'S DISEASE
AI	ADEQUATE INTAKE
APP	AMYLOID PRECURSOR PROTEIN
β-CTF	BETA-CARBOXYL-TERMINAL FRAGMENTS
CDC	CENTERS FOR DISEASE CONTROL AND PREVENTION
CFC	CONDITIONAL FEAR CONDITIONING
DAB	DIAMINOBENZIDINE
DNA	DEOXYRIBONUCLEIC ACID
IHC	IMMUNOHISTOCHEMISTRY
NHANES	NATIONAL HEALTH AND NUTRITION EXAMINATION SURVEY
NMDA	N-METHYL D-ASPARTATE
PBS	PHOSPHATE-BUFFERED SALINE
US	UNITED STATES
VLDL	VERY LOW-DENSITY LIPOPROTEIN

CHAPTER 1: INTRODUCTION

Alzheimer's Disease

Alzheimer's Disease (AD) is a chronic neurological disorder that is the leading cause of dementia – the decline of a person's memory and critical thinking faculties accompanied by changes in personality and loss of independence in daily life. In 2020, there were 5.8 million Americans living with AD; By 2050, that number is projected to reach 13.8 million.[1] It is estimated that a new person is diagnosed with AD every 68 seconds in the United States alone and the CDC lists AD as the 6th leading cause of death in the United States.[2, 3]

The burden of this disease to our society is staggering. In 2019, 16 million family members or other uncompensated caregivers spent 18.6 billion hours caring for AD patients. This is estimated to be worth almost $244 billion in lost wages.[1] The cost is not limited to time and money but even extends into an increased chance for emotional distress and negative physiological outcomes for caregivers.[1] Since age is the most important risk factor for AD, our increasing life expectancy and 65+ population means the burden of AD will only grow heavier in the future. The scarcity of specialists trained in dementia-care means the brunt will be carried by a primary care sector that is already stretched thin.[1]

There are 4 recognized stages of Alzheimer's Disease.[4] Stage I is the preclinical stage, characterized by early signs of pathology and minor memory loss, but no clinical symptoms of AD and no loss of function in daily life activities. Stage II is early AD, when the classic dementia symptoms of temporospatial disorientation,

concentration and memory impairment, personality change, and depression appear. Stage III is moderate AD, characterized by the spread of pathology through the cerebral cortex along with worsening dementia symptoms to the point where patients may have difficulty communicating and recognizing family members. Stage IV is late-stage AD, characterized by heavy accumulation of pathology throughout the brain resulting in major decreases in cognitive function. Patients suffer from severe dementia, fail to recognize family members, and have difficulty performing basic life functions. They become bedridden and ultimately succumb to complications due to AD. Preclinical Stage I often lasts for years – neuropathologic brain changes can even appear decades before the onset of clinical symptoms. Because of this delay, there is much interest in implementing prevention strategies during the early stages of AD.[2]

The two most prominent pathological features of AD are extracellular amyloid plaques and intracellular tau neurofibrillary tangles. The accumulation of plaques and tangles in the brain tissue disrupts synaptic connections, impedes signal propagation, and kills neurons. Clinical symptoms of AD directly arise from the death of neurons associated with memory and cognition.[5] The death of neurons produces marked atrophy and reduced weight of the brain, especially in the cortices and hippocampus.[6] Cortical sulci will appear to grow as the gyri shrink; Ventricles will also enlarge as the surrounding brain tissue recedes. While brain atrophy is highly suggestive of AD, it is also a characteristic shared by disorders such as Huntington's Disease and Multiples Sclerosis. Therefore, amyloid plaques and tau

neurofibrillary tangles remain necessary for a proper diagnosis.[6] Neurofibrillary tangles are found just beneath a neuron's cell membrane and are composed of thick bundles of hyperphosphorylated tau-protein.[6] Tau protein is typically bound to microtubules and plays a role in structural stability and transport within a neuron. If hyperphosphorylated, it unbinds from microtubules and clumps together into tangles that disrupt cellular transport and disassemble microtubules, eventually resulting in neuron death. Tau pathology was once thought to be a downstream effect of amyloid-beta (Aβ) accumulation but is now considered by some to be a parallel pathologic pathway in AD.[7] Despite this, Aβ remains a focus of research efforts thanks to the "amyloid hypothesis" – which holds the amyloid-beta molecule responsible for AD pathogenesis.[8,9]

The extracellular deposition of amyloid plaques begins with amyloid precursor protein (APP) – a ubiquitous transmembrane molecule found in cells throughout the body, but especially abundant in the central nervous system. The function of APP is still unclear, but it is thought to be involved in cell adhesion and cell growth.[10,11] There are two branches of APP processing, mediated by α-secretase and β-secretase. β-secretase is more abundant in neurons and only the β-secretase pathway generates Aβ. Figure 1 illustrates the pathogenic processing of APP into Aβ. First, APP is cleaved by β-secretase into the intermediate product β carboxyl terminal fragment (βCTF). A second enzyme, γ-secretase, cleaves βCTF into Aβ40 and Aβ42. These Aβ isoforms are highly insoluble and are the main components of

amyloid plaques. Aβ42 has two more amino acid residues than Aβ40 and is the more prone to aggregation. Because of its critical role in AD pathogenesis, γ-secretase is a target for therapeutic interventions.[12] Unfortunately, γ-secretase appears to also cleave a broad range of other transmembrane proteins, and as such, γ-secretase inhibitors produce serious side-effects and have been deemed unsafe for use.[7]

There is currently no cure for AD. Current medical treatments aim to ameliorate the symptoms and fall into two classes: cholinesterase inhibitors and N-methyl D-aspartate (NMDA) antagonists.[4] In AD, deterioration of cholinergic neurons results in a decrease in the levels of the neurotransmitter acetylcholine (ACh). Cholinesterase inhibitors prevent the breakdown of ACh, thus increasing availability of the neurotransmitter at synapses and delaying cognitive decline.[7] In contrast, NMDA antagonists work by ameliorating pathologically high glutamate levels that are theorized to lead to neuronal dysfunction.[13] They have limited clinical benefit alone, so they're usually administered in combination with cholinesterase inhibitors.[4,13] Pharmacological drugs have thus far only been able to delay the progression of AD, and unsuccessful clinical trials for therapeutic agents in recent years indicates little promise in this direction.[4] Future research is aimed towards an individually-tailored approach towards patients with AD that incorporates elimination of risk factors, treating comorbidities, and personalized lifestyle interventions to suit every individual.[4] Until the day precision medicine or a cure for AD becomes available, we must look for alternative strategies to prevent the

disease.[14,15] In this context, nutrition may be one of the most simple lifestyle interventions to implement.

Choline

Nutrition is as, if not more, important for our brain as it is the rest of our body, as adequate nutrition is necessary for cognitive development, brain maintenance, and function. It is especially important during gestation and early development – the peak periods of brain formation that lay the foundations for long-term healthy brain function through childhood and into adulthood. Nutrient deficiencies during brain development can negatively impact neuron proliferation, axon and dendrite growth, the formation and pruning of synapses, axon myelination, and neuron apoptosis.[16] Post-early-childhood, the brain continues to develop at a slower rate until about 30 years of age, after which it enters a stage of natural atrophy for the rest of its life.[15] The long time window over which this occurs gives plenty of opportunity to employ nutrition as a protective strategy against age-related disorders such as AD and dementia. Epidemiologic studies suggest certain dietary patterns as well as a handful of specific nutrients as good candidates for healthy brain aging outcomes.[15,17]

For example, a review of 56 articles across scientific literature in 2019 linked high adherence to the Mediterranean Diet to higher cognitive performance and lower rates of neurodegenerative diseases.[18] The Mediterranean Diet consists of fruits, vegetables, nuts, legumes, whole grains, fish, olive oil, and moderate meat,

low-fat dairy, and alcohol consumption.[14,17] It emphasizes healthy fats and a high intake of fish and vegetables. The traditional Japanese diet is also high in fish and vegetables. Though less well-studied, it is interesting that Japan's transition from a traditional Japanese diet to a Western diet high in meat, dairy, and sugar coincides with a jump in AD prevalence in the country from 1% in 1985 to 7% in 2008.[14,19] In both cases, the Mediterranean Diet and the Japanese diet suggest that certain dietary habits may be protective against cognitive decline and AD. Indeed, most dietary recommendations for cognitive health will recommend foods high in antioxidants and omega-3 fatty acids (particularly fish, vegetables, and olive oil) to combat systemic oxidative stress and inflammation that is thought to be the biological origin of AD.[20] Few currently include a recommendation of increasing choline intake despite the evidence supporting choline's cognitive benefits.

Choline is an essential nutrient acquired through our diet with a small, but inadequate, amount of endogenous production. It is a multirole nutrient that is important throughout life for cellular growth and maintenance, neurotransmission, membrane synthesis, lipid transport, and single-carbon metabolic processes.[29] The foods with the highest concentrations of choline are liver, egg yolk, red meat, and poultry, with vegetables generally containing less choline than do animal products. The Adequate Intake (AI) was only established in 1998 by the Food and Nutrition Board of the National Academy of Medicine at 550 mg/day for men and 425 mg/day for women based on the minimum levels required to prevent liver damage.[21,22] However, reports between 2009-2014 from NHANES found only about 8.03% of

adults in the US meet their AI, and choline deficiency is quite prevalent across genders and age groups.[24] The recent dietary shift towards more green vegetables and whole grains and away from meat and dairy products following healthy diet recommendations such as the Mediterranean Diet may contribute to deficiency. It may also be, in part, a side effect of the avoidance of high-cholesterol foods, which tend to also be high in choline.[25] Healthy human adults fed a choline-deficient diet developed liver and muscle damage, which were resolved when choline was restored to their diet.[21,26] Long-term choline deficiency can cause non-alcoholic fatty liver disease, as the liver cannot transport very low-density lipoproteins (VLDL) out of itself without the choline product, phosphatidylcholine.[25,26] It is also a risk factor for cardiovascular disease, as low choline results in elevated homocysteine levels.[23]

For pregnant and lactating women, the AI is increased to 450 mg/day and 550 mg/day, respectively, to meet the needs of the developing fetus and infant.[29] This may still be inadequate, as studies have shown that increasing maternal choline intake to 930 mg/day still did not bring biomarkers for choline metabolism in pregnant women up to par with levels observed in non-pregnant women.[22] Additionally, there is no increase in choline excretion into the urine – indicating that 930 mg/day does not exceed metabolic requirements.[22] Choline is pumped across the placenta and against the concentration gradient so that there is a 10-times greater concentration in the amniotic fluid than in the mother's blood.[23] Fetal serum has a 6-7-times greater concentration of choline than adult serum.[23] These observations suggest how critical choline is to the developing fetus, yet reports

between 2005-2014 from the National Health and Nutrition Examination Survey (NHANES) found that only 8.51% of pregnant women in the United States achieve an adequate intake of choline.[24]

This statistic is cause for concern considering the high demand for choline during neurodevelopment. Figure 2 illustrates the physiological roles of choline. Seventy to eighty percent of choline is used for the biosynthesis of cellular membranes, including in neurons.[22,23] Choline is converted into phosphatidylcholine and sphingomyelin, which make up over 50% of the phospholipid bilayer.[22,23] Nineteen to twenty-nine percent of choline is oxidized into betaine, a methyl group donor that can be used for the epigenetic modification of DNA and subsequent gene expression.[22,25] About 1% of choline is used for the synthesis of the neurotransmitter acetylcholine (ACh) at nerve terminals by the enzyme choline acetyl acetyltransferase which uses an acetyl group donated by acetyl coenzyme A.[25,42] ACh is involved in many important brain functions including: regulation of neuron proliferation, differentiation, migration, maturation, plasticity, and survival, as well as synapse formation.[22] It is also the means of chemical signal propagation at synapses and neuromuscular junctions and has a role in the regulation of organs such as the heart, lungs, blood vessels, the digestive tract, and bladder.[36,37] The availability of choline determines the rate of ACh synthesis and increased ACh levels augments its release into synapses.[23] Thus, choline availability influences signal transmission. In addition, cell culture studies have shown that phosphatidylcholine in the neuronal membranes acts as a choline reservoir during times of choline

deficiency for use in acetylcholine synthesis.[30,31] Consequently, choline deficiency may deplete the major structural component in neuronal membranes and lead to the reduced viability or death of neurons.[30,31]

There is evidence in both animal and human studies to support that choline supplementation provides lasting cognitive benefits as well as protection against AD pathology. A 2006 review of 34 animal studies using rodents found a link between choline availability during development and better cognitive performance.[27] Studies of perinatal choline supplementation in AD model mice found alterations in APP processing that led to reduced number and area of amyloid plaques.[43] Another study on AD model mice found that lifelong choline supplementation decreased plaque number and improved their spatial memory through decreasing APP processing and attenuating chronic activation of microglia.[44] Other rodent studies have shown maternal choline supplementation during periods of high hippocampal development in the fetus to be associated with significant changes, including increased hippocampal progenitor cell proliferation, decreased hippocampal cell death, and a 30% improvement in visuospatial and auditory memory throughout life versus animals with no maternal choline supplementation.[23, 53-56] The effects of maternal choline supplementation in human offspring requires more study, but current evidence supports the same cognitive benefits as seen in rats and mice. In a human study of maternal choline supplementation on infant cognition, mothers that were given 930 mg/day instead of 480 mg/day of choline during their last trimester had infants who performed significantly better in information processing speed,

visuospatial memory, and mean reaction time.[28] For reference, 300-350 mg/day is the average choline intake of pregnant women in the United States.[28] More abundant choline for the fetus likely improves cognitive performance by augmenting structural and organizational development in the brain which protects cholinergic neurons from atrophy and promotes neurogenesis in the hippocampus.[22] The effects of choline availability may be mediated by methylation of DNA which alters the expression of genes involved in cognitive processes.[23]

Mouse Models of AD

Animal models are indispensable for studying the mechanisms of brain amyloidosis as well as for testing therapeutic interventions in lieu of human subjects. A good model must be an appropriate analog of humans, have low genetic variation for consistent and reproducible results, cheap and easy to raise, and will recapitulate AD in humans as closely as possible.[38] A wild-type mouse is a poor animal model of amyloidosis because the murine Aβ molecule does not aggregate into plaques as mice age, which is likely due to three different amino acid substitutions between the sequences for human and murine Aβ.[39] Many transgenic mouse models of AD produce human Aβ and also includes several genetic mutations that induce AD-like pathology. Mice are relatively easy to keep and, because they have a short lifespan and yet develop AD-like phenotypes, it is possible to follow neuropathological processes within a period of months. However, they do not fully replicate the disease since mice don't model tau pathology or neurodegenerative

brain atrophy observed in human AD.[39] Another discrepancy is that the genetic mutations used to induce pathology originate from human familial forms of early-onset AD, which only account for a small minority (~1%) of cases in humans, whereas most cases (>95%) of AD are late-onset and arise sporadically.[40,41] Because sporadic late-onset AD does not arise from genetic mutation, no transgenic mouse model can be engineered.[40]

One of the most ubiquitous mouse genotypes used to model AD is the APP.PS1 strain because of its stable and well-characterized genetics, high reproductive rate, and the early onset of disease.[33] As such, changes in memory and pathology in the APP.PS1 strain have been extensively studied.[33] This genotype combines two genetic mutations designed to produce pathologically elevated Aβ42 levels. The Swedish KM670/671 NL mutation causes APP to be a more favorable substrate for β-secretase, the enzyme which cleaves APP into the intermediate β-CTF, and results in elevated levels of Aβ40 and Aβ42 while not affecting the Aβ42:Aβ40 ratio.[57] The PS1dE9 mutation increases the amount of β-CTF that comes in contact with γ-secretase that preferentially cleaves β-CTF into Aβ42, resulting in an aggressive increase to the Aβ42:Aβ40 ratio without changing Aβ40 levels which in turn accelerates amyloid deposition.[32,33] These APP.PS1 mice produce high amounts of Aβ which accumulate into plaques by 4-6 months of age.[32,43] Working memory begins to deteriorate at 5-6 months, and spatial memory at 15 months.[33] While widely used in research labs as a proxy for human AD, there is a concern that

the non-physiological overexpression of APP results in over-production of other amyloid fragments besides Aβ40/42.[33] This makes it difficult to establish a direct causative link between Aβ40/42 and the neuropathology and behavioral changes of AD. Overexpression of APP may also inadvertently disrupt its physiological function in the APP.PS1 strain.[33]

To combat those problems, the APP.NLGF strain was recently developed with the aim of replicating AD amyloid pathology while avoiding the non-physiological overexpression of APP using a gene "knock-in" strategy.[33,34] Gene knock-in involves changing the mouse DNA sequence encoding APP so that there is a direct substitution of 3 amino acids in the murine Aβ that effectively "humanizes" it.[33,34] Because the mouse gene locus and apparatus controlling the expression of APP is preserved, the knock-in strategy avoids the non-physiological over-expression of APP. In addition, the APP.NLGF strain harbors three mutations associated with AD within its APP locus: i) the Swedish NL mutation that elevates Aβ production, ii) the Arctic G mutation that promotes Aβ aggregation, and iii) the Beyreuther/Iberian F mutation that increases Aβ42:Aβ40 ratio to encourage pathology.[33,34] This genotype exhibits aggressive amyloid deposition in the brain starting at 2 months of age, reaching near saturation by 7 months of age.[34] An important feature of APP.NLGF mice is that they exhibit abnormal anxiety, depression, and impaired social interaction affective disorders. Ninety percent of AD cases in humans are coupled with non-cognitive affective disorders including anxiety and depression, so an

animal model that permits study of both memory loss and changes in behavior is ideal.[33] When compared to the APP.PS1 strain, the APP.NLGF strain had fewer total plaques, smaller plaque size, and less percentage of brain area covered by plaques.[33] Despite the seemingly weaker presentation of pathology in the APP.NLGF mice as compared to some of the other AD models, they have markedly worse symptoms of affective disorder than APP.PS1 which suggests that amyloid plaques differentially contribute to AD symptoms between the two strains.[33] There have been only a handful of studies that compare the APP.NLGF mouse to previously-developed strains, and none to my knowledge that have studied the effect of maternal choline supplementation on amyloid plaque pathology. Since the APP.NLGF strain more closely resembles human AD pathology, any therapeutic results from maternal choline supplementation using this animal model may be more pertinent.

Hypothesis

In the new strain of APP.NLGF transgenic mice modeling human Alzheimer's Disease amyloidosis, perinatal choline supplementation will result in less amyloidosis in the brain and more mild cognitive impairment when compared to mice that did not receive perinatal choline supplementation. Our hypothesis is based on previous perinatal choline supplementation studies done on APP.PS1 mice which yielded these results.

Objectives and Specific Aims

This study will test the effect of perinatal choline supplementation on cerebral cortical and hippocampal plaque size, plaque number, percent of area covered by plaques, as well as behavior change in APP.NLGF mouse models of Alzheimer's Disease at four ages. Its specific aims are:

- To quantify percentage of area covered by plaques, mean plaque size, and mean plaque count for comparison between perinatal choline supplemented and control diet groups at 3, 6, 9, and 12 months of age.
- To quantify Contextual Fear Conditioning data for assessing the benefits of perinatal choline supplementation on cognitive performance and affective disorder.

CHAPTER 2: MATERIALS AND METHODS

Mouse Model

This study was performed using the APP knock-in mouse model of AD, APP.NLGF (strain: C57BL/6-App<tm3(NL-G-F)Tcs>/TcsRbrc (RBRC06344), developed by Takashi Saito et. al at the RIKEN Brain Science Institute in Japan.[34] This genotype has a "humanized" APP amino acid sequence and includes: i) the Swedish (NL) mutation which elevates Aβ production, ii) the Arctic (G) mutation that promotes Aβ aggregation, and iii) the Beyreuther/Iberian mutation (F) that increases Aβ42: Aβ40 ratio to encourage pathology.[33,34] The endogenous mouse APP locus which controls APP expression is preserved so that the APP.NLGF mouse model overproduces Aβ42 without overexpressing APP.[34] At the start of the experiment, breeding pairs of homozygous APP.NLGF mice were fed either a control AIN76A (containing 1.1g/kg of choline chloride) diet or choline-supplemented AIN76A (containing 5g/kg of choline chloride) diet. The AIN76A diet is a defined rodent chow (Dyets #110098) made up of 20.73% protein, 66% carbohydrate, and 5% fat.[43] After 7 days, the mice were bred and the pregnant dams were maintained on their respective diets all the way through the birth of their offspring and through 22 days of weaning their pups. After day 22, all the offspring are fed on the control diet. This supplementation strategy is meant to provide choline during the most critical period of neurodevelopment when the hippocampus and other brain structures are forming. The study design is laid out in Figure 3. At 3, 6, 9, and 12 months of age, groups of the offspring are put through behavioral tests and subsequently euthanized using

CO_2. After the mice are decapitated, the brains were divided at the midline: one hemisphere used for immunohistochemical analysis (IHC) (right) and one hemisphere dissected into frontal cortex, septum, and hippocampus and flash frozen for protein, RNA, and DNA analysis (left). The hemispheres for IHC were fixed in PLP fixative (4% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate; pH 7.4) at 4°C for 24 hours, and then cryoprotected serially in a 10% then 20% glycerol/2% dimethyl sulfoxide in 0.1M PBS, pH 7.3 solution for later immunohistochemistry. The number of APP.NLGF animals in each group are as follows: 3M male control (n=6), 3M female control (n=6), 3M male supplemented (n=8), 3M female supplemented (n=3). 6M male control (n=7), 6M female control (n=6), 6M male supplemented (n=8), 6M female supplemented (n=5). 9M male control (n=8), 9M female control (n=8), 9M male supplemented (n=10), 9M female supplemented (n=10). 12M male control (n=8), 12M female control (n=4), 12M male supplemented (n=9), 12M female supplemented (n=9). The hippocampi of two mice (a 6M control diet male and a 3M supplemented female) were too damaged during the staining and mounting process and excluded from quantitation.

Immunohistochemistry

Brains were frozen and cut coronally into 40 micrometer sections using a sliding microtome. Sections were stored in a PBS and Na-Azide solution until use. For Aβ42 immunohistochemistry, brain sections were selected for the anterior hippocampus at roughly Bregma -2.12 mm. First, they were washed 10 minutes in

PBS on a Bellydancer shaker machine to remove Na-Azide. They were then transferred into a 70% Formic Acid solution for 1 minute with gentle agitation for antigen retrieval, before transfer back into fresh PBS for 3X5 minute washes. The sections were then incubated in a 10% goat serum diluted in PBS solution for 1 hour at room temperature on the Bellydancer for blocking. Finally, sections were transferred into wells with rabbit anti-Aβ42 primary antibody (Invitrogen #700254) diluted 1:1000 in a solution of 0.3% Triton-X 100, 2% goat serum, and 0.008% Na-Azide in PBS and allowed to incubate at room temperature on the Bellydancer overnight. The following day, sections were transferred into fresh PBS for 3X10 minute washes before being incubated in a goat-anti-rabbit-conjugated with horseradish peroxidase secondary antibody (Millipore) diluted 1:750 in a 2% goat serum in PBS solution for 3 hours at room temperature on the Bellydancer. Staining was developed in a diaminobenzidine (DAB) solution prepared by dissolving 50 mg of DAB into 45.5 mL of 50 mM tris buffer, 4.5 mL of sodium imidazole, and two drops (approximately 6.25 μL) of 95% hydrogen peroxide solution with gentle agitation before transfer into fresh PBS for wash and storage until mounting. The tissue sections were mounted onto subbed slides using double-distilled water as the mounting medium. Once the tissue has dried, the mounted slides were immersed into fresh ethanol 2X1 minute, followed by immersion into fresh xylene 2X5 minutes. Permount (Fisher SP15-100) was used to coverslip. Once dried, tissue sections were examined under the 2x magnification objective of an

Olympus B061 microscope which allowed us to fit the entire hippocampus and cortex into a single photographic image.

Image Analysis

Image analysis was performed in the ImageJ software. The regions of interest were outlined using the freehand tool to include the entire hippocampus and a consistent region of the somatosensory cortex as pictured in Figure 6. Staining intensity threshold was set to maintain a consistent level of plaque detection in every image, and plaques under 10 pixels in size were not counted. Plaque count, mean plaque size, and percent area covered by plaques was measured by ImageJ. Three sections of the somatosensory cortex and hippocampus per animal were analyzed, and the data averaged to obtain a single data point for our measured outcomes of percent area covered by plaques, mean plaque size, and mean plaque count. The averaged datapoint of the three sections were graphed in JMP Pro 15 software.

Data Analysis

All statistical analysis was analyzed in JMP Pro 15 using one- or two-way ANOVA where appropriate.

CHAPTER 3: FIGURES AND RESULTS

Figures

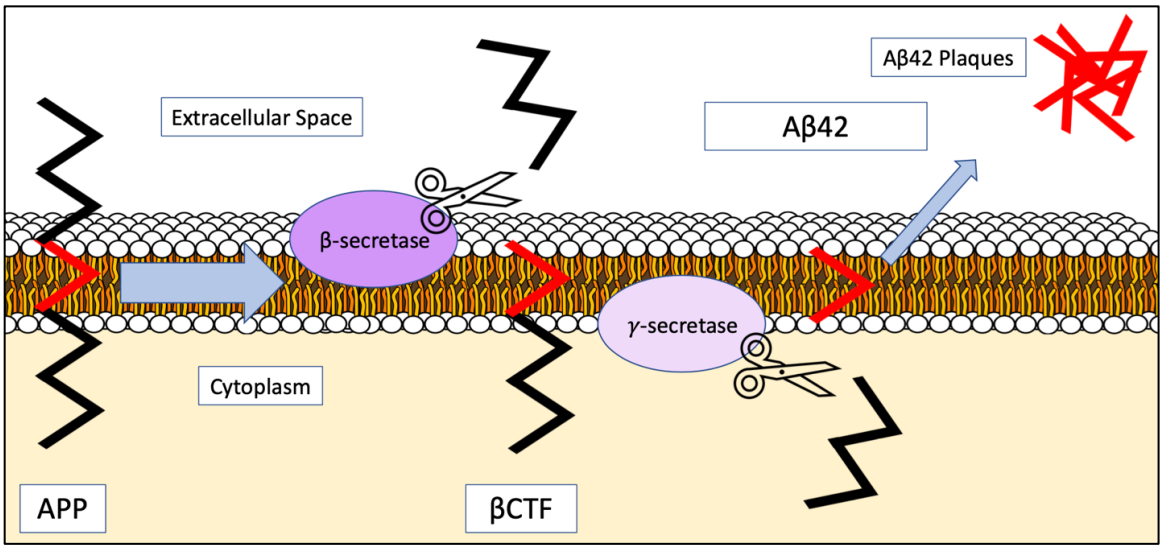

Figure 1. Cleavage of Amyloid Precursor Protein (APP) into Aβ Fragments. APP is a transmembrane protein found in cells throughout the body but especially abundant in the central nervous system. It is first cleaved by β-secretase into the intermediate molecule β-CTF. β-CTF is then cleaved by γ-secretase into between the 42nd and 43rd peptides into Aβ42. Aβ42 is highly insoluble and aggregates into Aβ plaques.

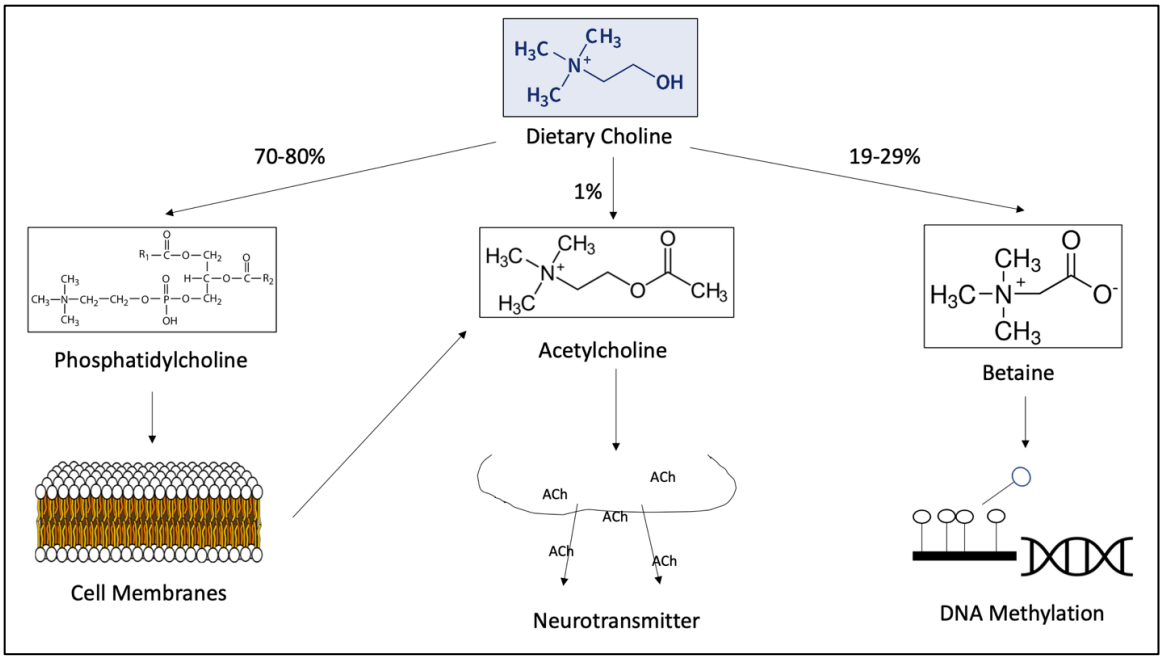

Figure 2. Roles of Choline. 70-80% of choline intake is converted to phosphatidylcholine and its derivative, sphingomyelin, for use in the structure of cell membranes. ~1% of choline intake is converted into the neurotransmitter acetylcholine. The remaining 19-29% is converted into the methyl-donor betaine for use in the epigenetic modification of DNA expression.

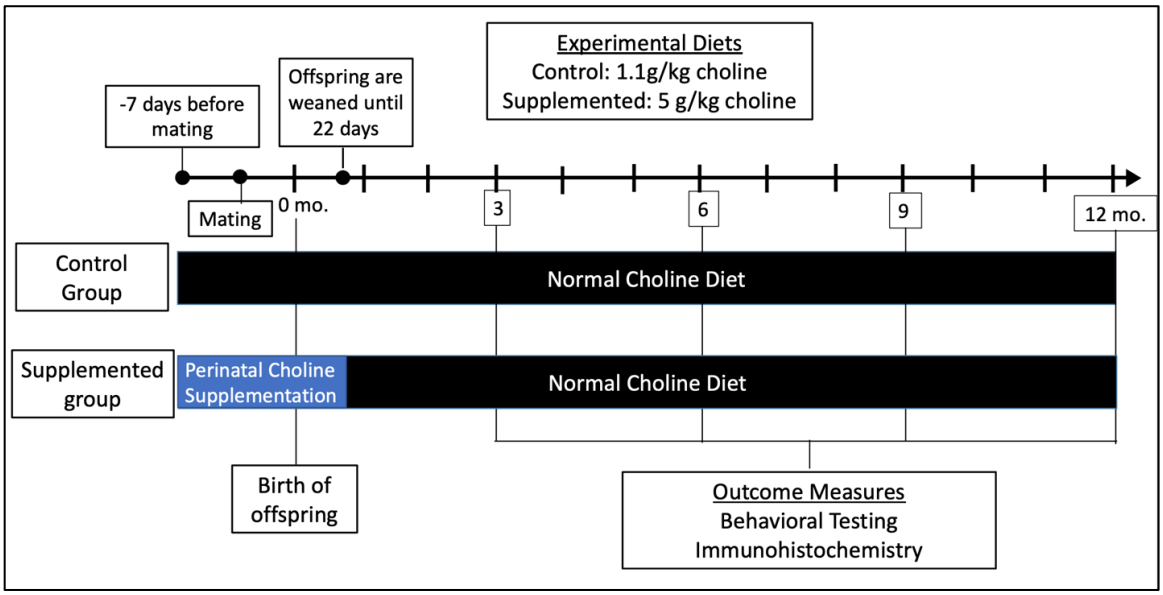

Figure 3. Timeline and Study Design.

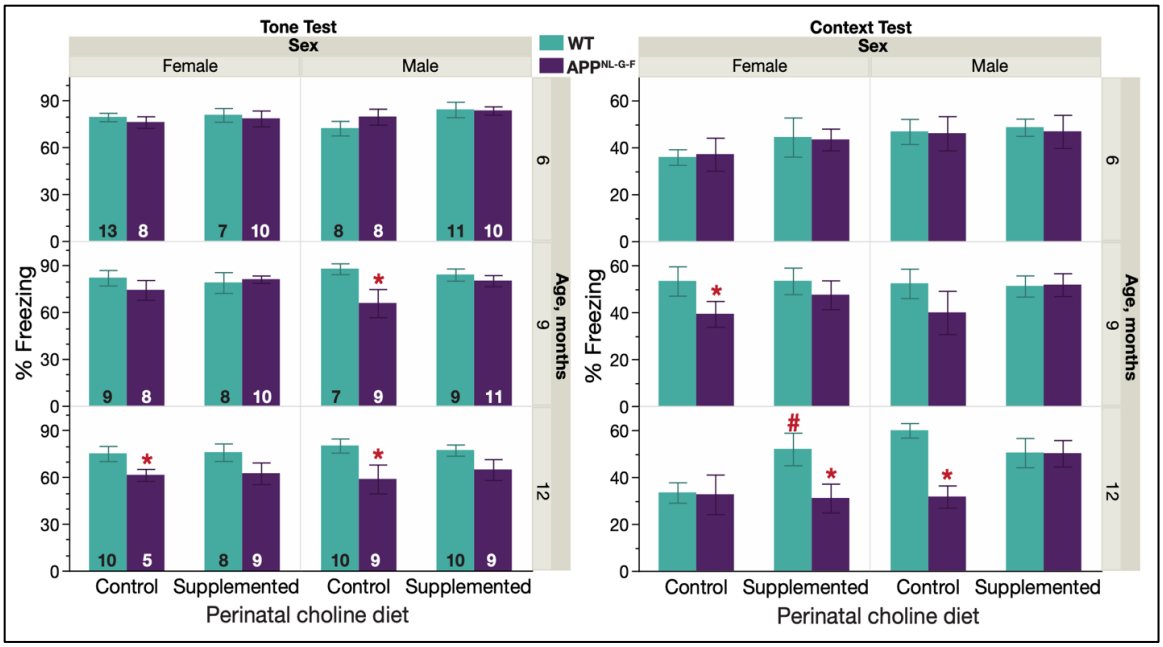

Figure 4. Measures of Memory Deficits from Contextual Fear Conditioning (CFC) Results. CFC exposes APP.NLGF and C57 subjects on day 1 to an environmental context followed by a neutral tone and an electric shock. Through 5 conditioning trials, subjects will learn to associate that environmental context and tone with the electric shock and that fear will elicit the mouse fear response of freezing in place. On day 2, subjects go through the tone test, in which they are placed into a box without environmental context and the tone is played, but there is no shock. On day 3, subjects go through the context test, in which subjects are placed into a box with the environmental context, but there is no tone or shock. The % of time subjects were frozen in each test was recorded to assess their memory of the contextual fear conditioning. The number of animals in each group is indicated in the left panel. * indicates a significant effect of diet ($p<0.05$) on % freezing. # indicates a significant effect of genotype ($p<0.05$) on % freezing.

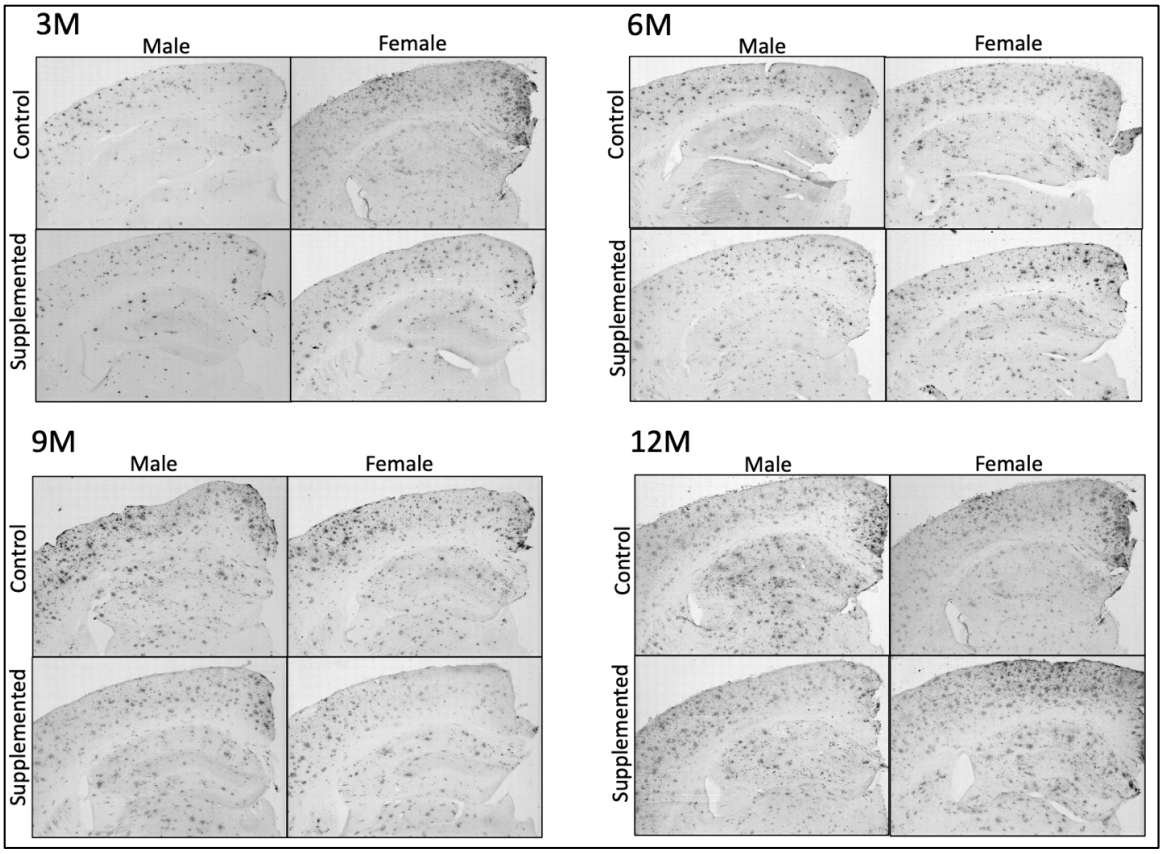

Figure 3. Representative Images of Brain Amyloidosis at 2x Magnification from Each Experimental Group.

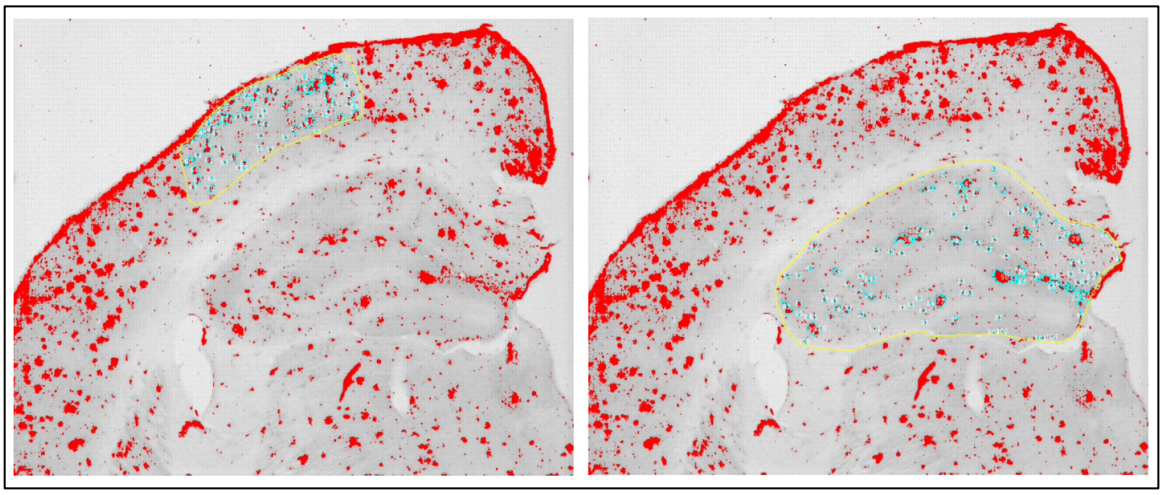

Figure 4. Image Analysis in ImageJ for the Quantitation of Amyloidosis. Images of the cerebral cortex and anterior hippocampus were used for image analysis in ImageJ. The cortical area was selected so that the lateral boundary lines up with the lateral boundary of the hippocampus, while the medial boundary was set at the divide between the somatosensory area

and the primary motor area. The entirety of the hippocampus was used for image analysis. In both brain regions, we elected to exclude edge effects and any plaques less than 10 pixels in size from our analysis. Red indicates which plaque pass the threshold, blue indicates which plaques within the designated area fit the size parameters and are counted.

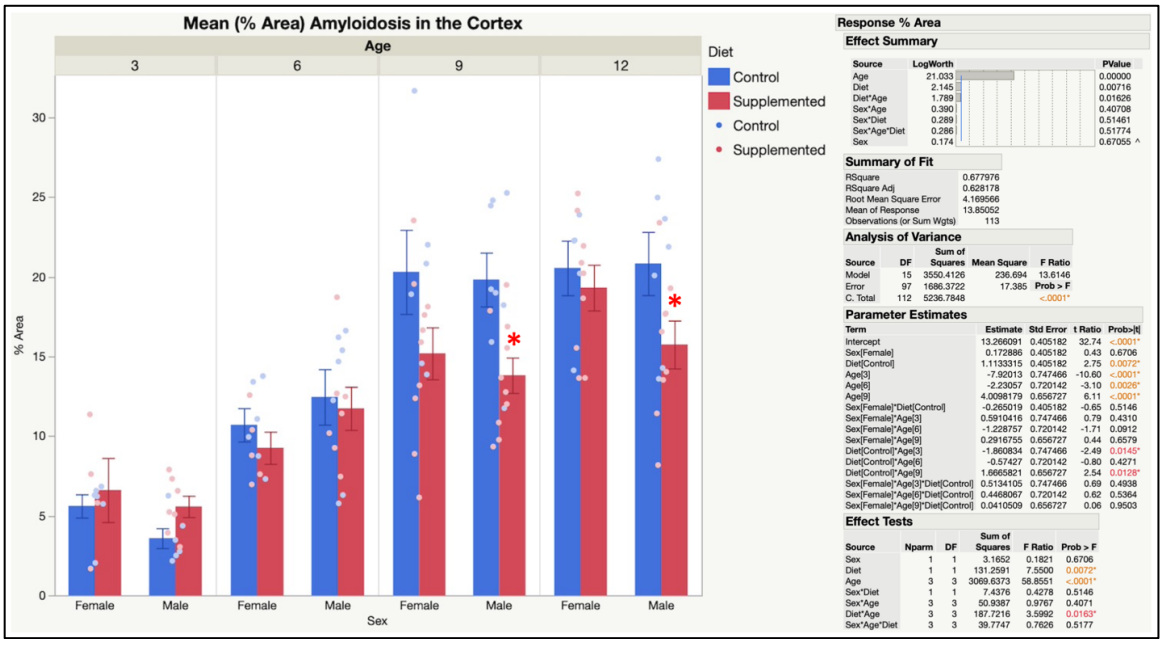

Figure 5. Amyloidosis of the APP.NLGF Mouse Cerebral Cortex Measured by % Area Covered by Plaques. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in a consistent region of cortex. Three tissue sections per animal were used to calculate a single data point for mean % area covered by plaques, and then graphed against age, sex, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant effect of age ($p<0.0001$) and diet ($p=0.0072$) on the % area covered by plaques in the cortex, along with an age by diet interaction ($p=0.0163$). * indicates $p<0.05$ related to the effect of diet on % area covered by plaques.

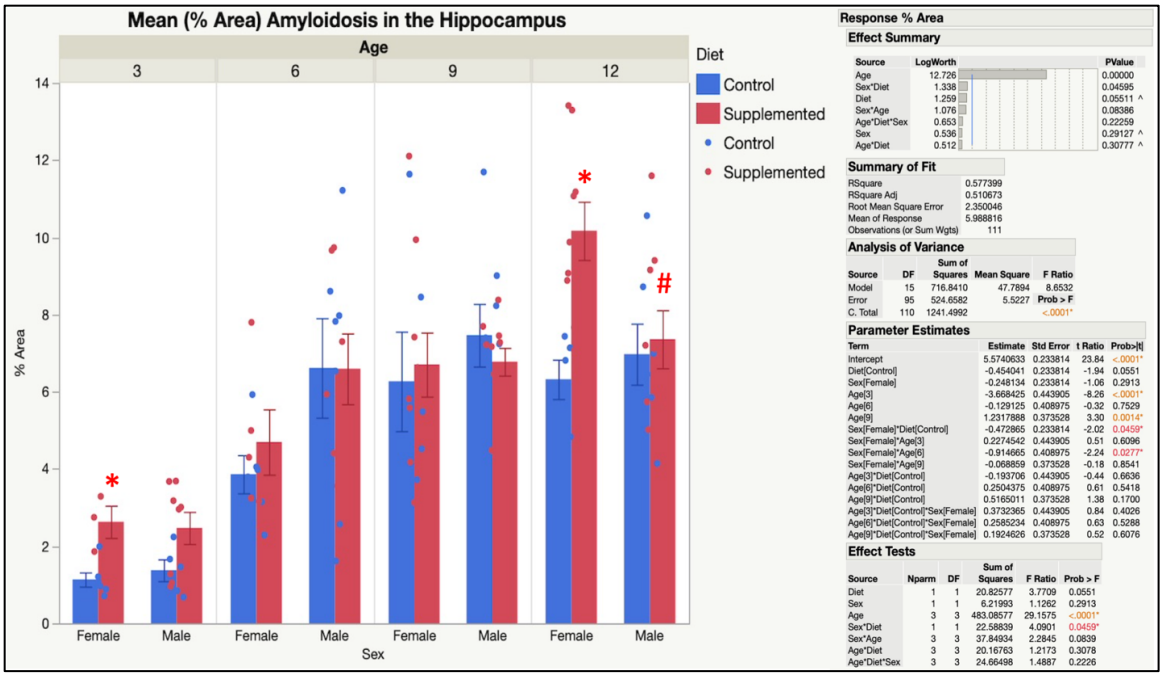

Figure 6. Amyloidosis of the APP.NLGF Mouse Hippocampus Measured by % Area Covered by Plaques. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in the hippocampus. Three tissue sections per animal were used to calculate a single data point for mean % area covered by plaques, and then graphed against age, sex, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant effect of age ($p<0.0001$) on % area covered by plaques in the hippocampus, along with a sex by diet interaction ($p=0.0459$). * indicates $p<0.05$ related to the effect of diet on % area covered by plaques. # indicates $p<0.05$ related to the effect of sex on % area covered by plaques.

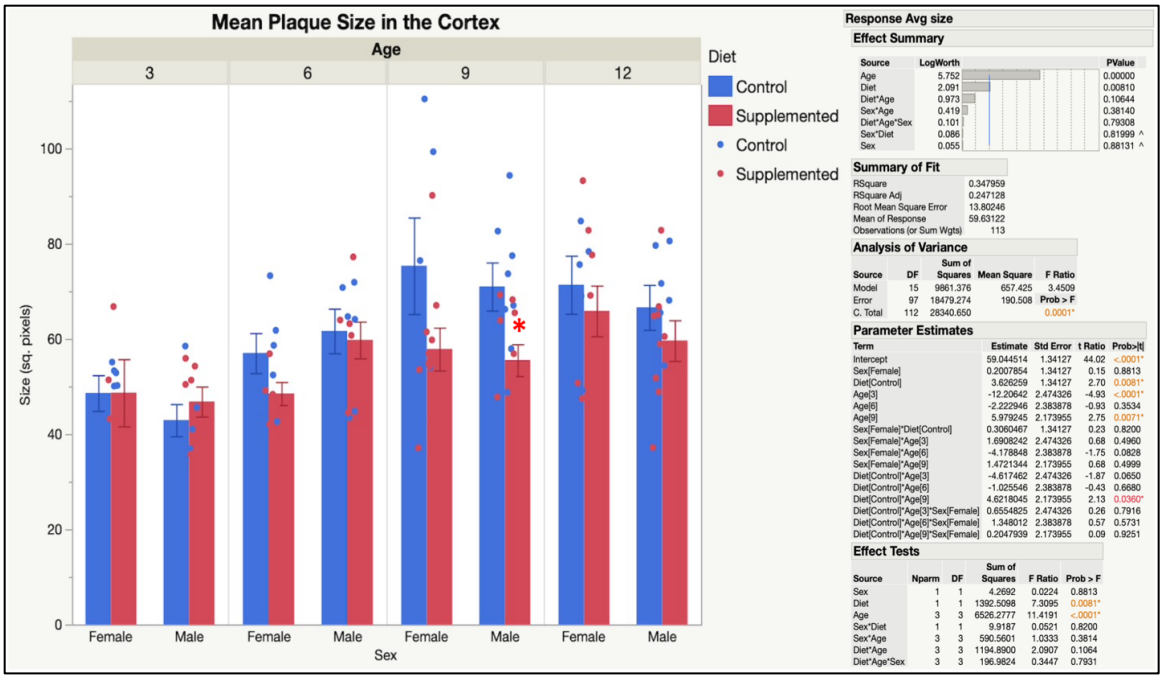

Figure 7. Mean Plaque Size (pixels) in the APP.NLGF Mouse Cerebral Cortex. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in a consistent region of cortex. Three tissue sections per animal were used to calculate a single data point for mean plaque size in pixels2, and then graphed against age, sex, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant effect of age ($p<0.0001$) and diet ($p=0.0081$) on mean plaque size in the cortex. * indicates $p<0.05$ related to the effect of diet on mean plaque size.

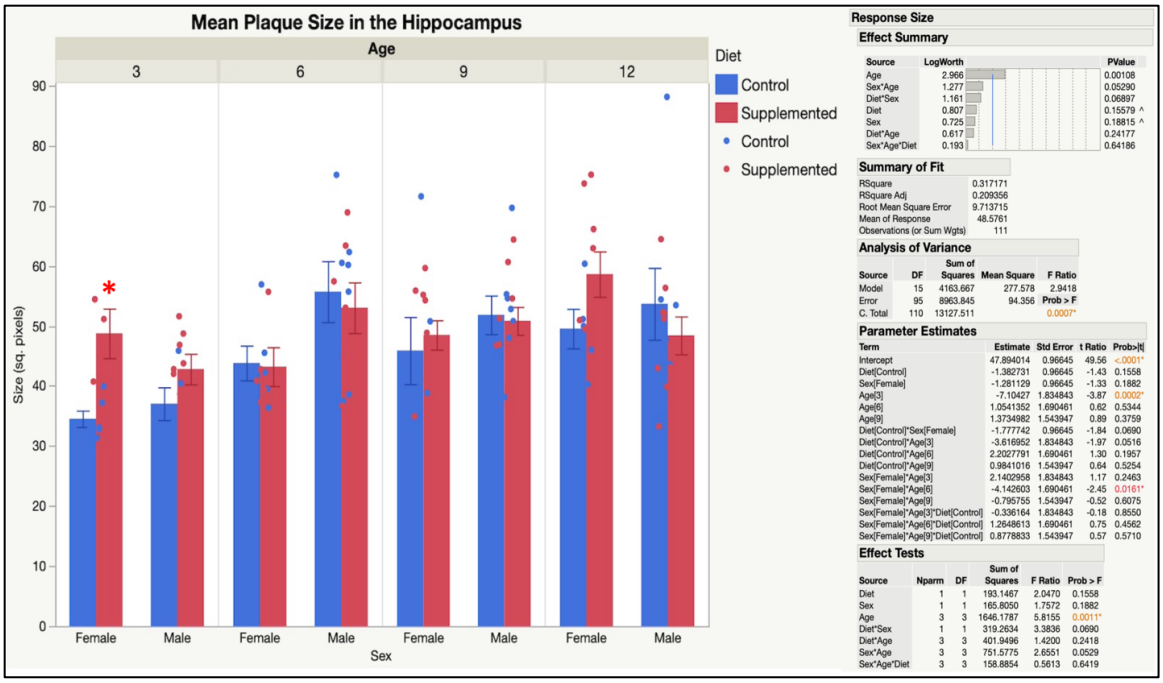

Figure 8. Mean Plaque Size (pixels) in the APP.NLGF Mouse Hippocampus. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in the hippocampus. Three tissue sections per animal were used to calculate a single data point for mean plaque size in pixels2, and then graphed against age, sex, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant effect of age ($p=0.0011$) on mean plaque size in the hippocampus. * indicates $p<0.05$ related to the effect of diet on mean plaque size.

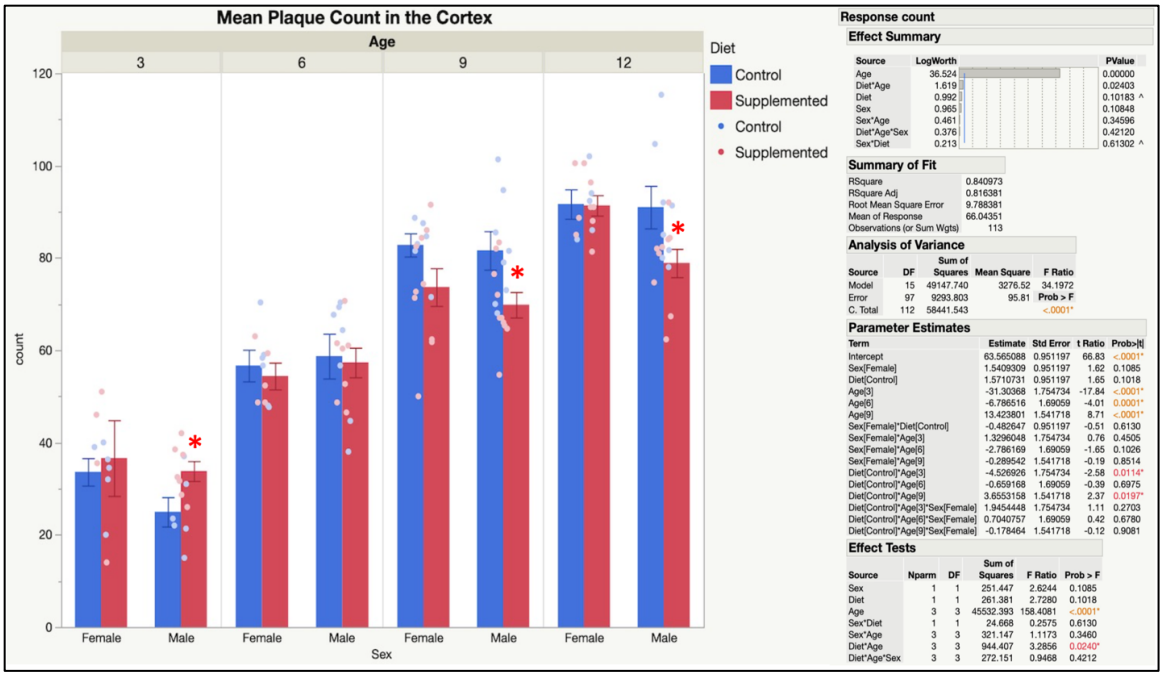

Figure 9. Mean Plaque Count in the APP.NLGF Mouse Cerebral Cortex. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in a consistent region of cortex. Three tissue sections per animal were used to calculate a single data point for mean plaque count, and then graphed against age, sex, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant effect of age ($p<0.0001$) on mean plaque count in the cortex, along with an age by diet interaction ($p=0.024$). * indicates $p<0.05$ related to the effect of diet on mean plaque count.

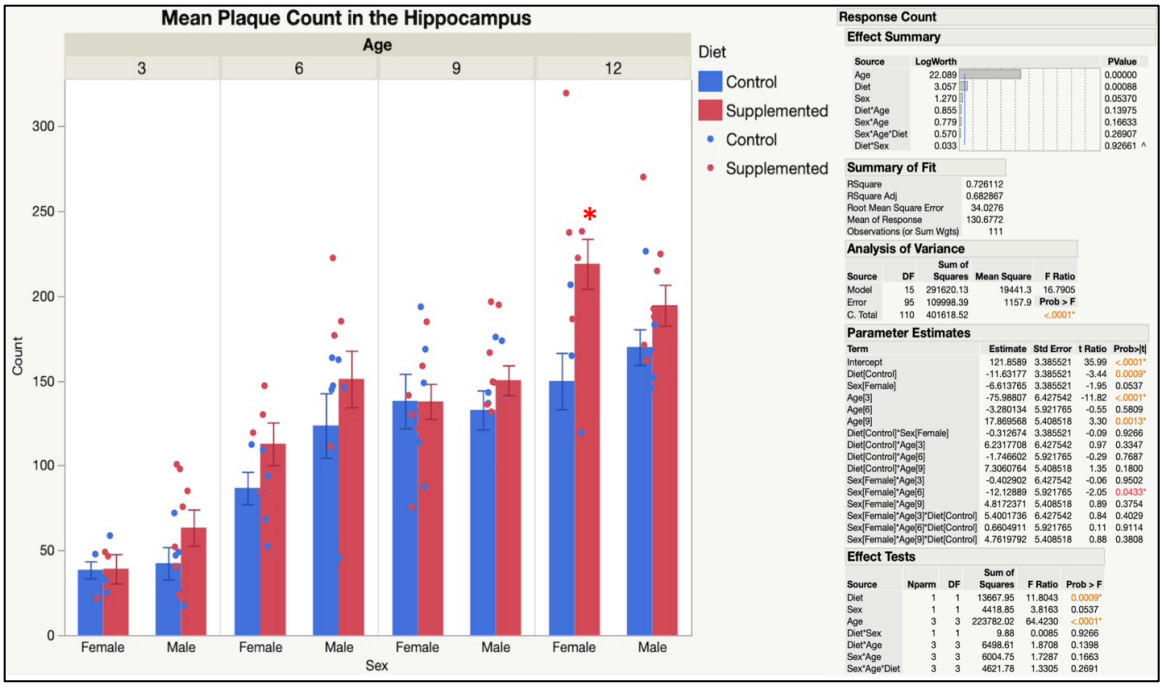

Figure 10. Mean Plaque Count in the APP.NLGF Mouse Hippocampus. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in the hippocampus. Three tissue sections per animal were used to calculate a single data point for mean plaque count, and then graphed against age, sex, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant effect of age ($p<0.0001$) and diet ($p=0.0009$) on mean plaque count in the hippocampus. * indicates $p<0.05$ related to the effect of diet on mean plaque count.

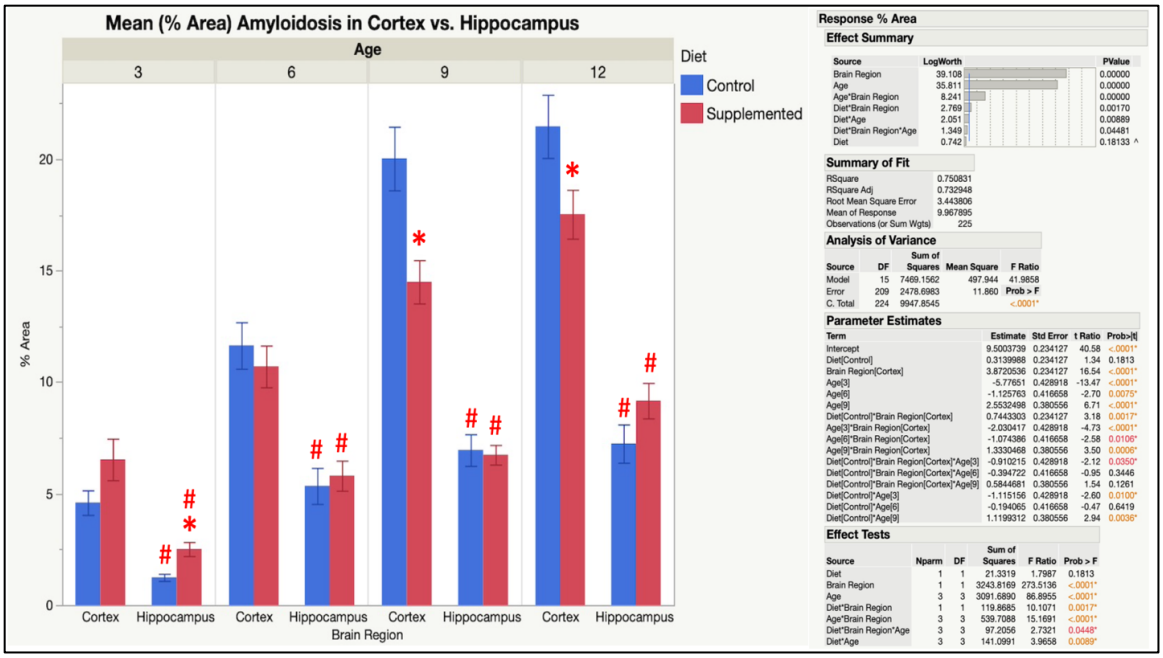

Figure 11. Comparison of Amyloidosis in the Cerebral Cortex versus Hippocampus Measured by % Area Covered by Plaques. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in a consistent region of the cortex and the hippocampus. Three tissue sections per animal were used to calculate a single data point for mean % area covered by plaques, and then graphed against age, brain region, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant overall effect of age ($p<0.0001$) and brain region ($p=0.0001$) on the % area covered by plaques, along with an age by diet interaction ($p=0.0163$), an age by brain region interaction ($p<0.0001$), a diet by brain region interaction ($p=0.0017$), and a diet by brain region by age interaction ($p=0.0448$). * indicates $p<0.05$ related to the effect of diet on % area covered by plaques. # indicates $p<0.05$ related to the effect of brain region on % area covered by plaques.

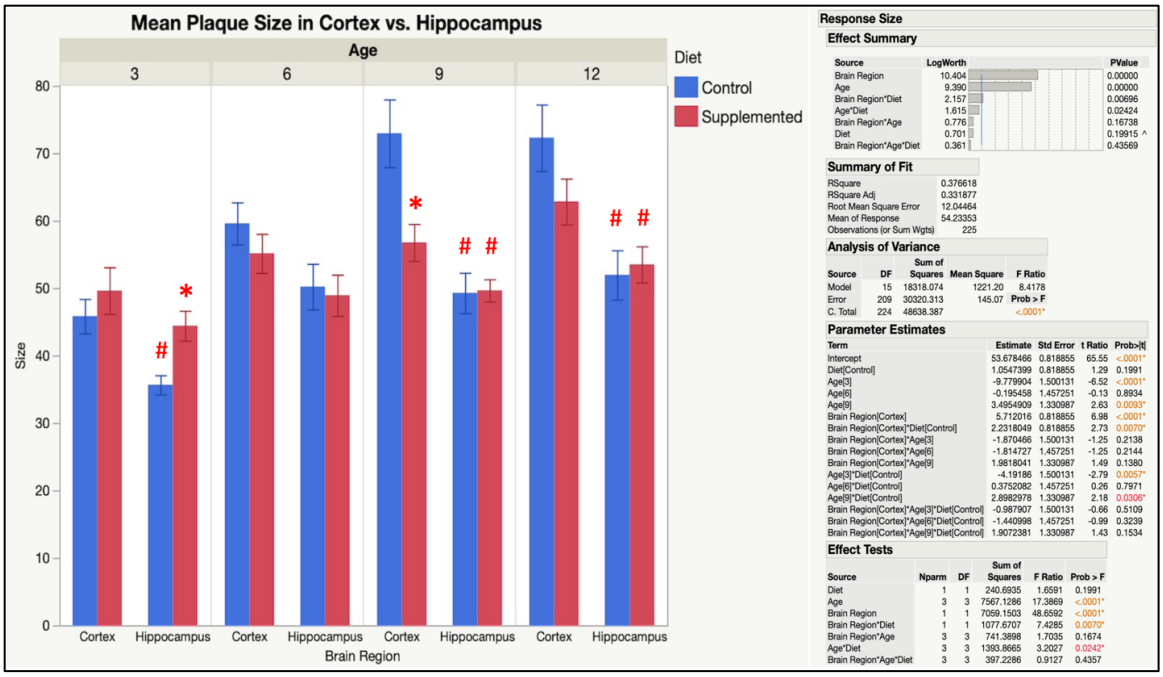

Figure 12. Comparison of Mean Plaque Size in the Cerebral Cortex versus Hippocampus. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in a consistent region of the cortex and the hippocampus. Three tissue sections per animal were used to calculate a single data point for mean % area covered by plaques, and then graphed against age, brain region, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant effect of age ($p<0.0001$) and brain region ($p<0.0001$) on the mean plaque size, along with an age by diet interaction ($p=0.0242$), and a brain region by diet interaction ($p=0.0070$). * indicates $p<0.05$ related to the effect of diet on mean plaque size. # indicates $p<0.05$ related to the effect of brain region on mean plaque size.

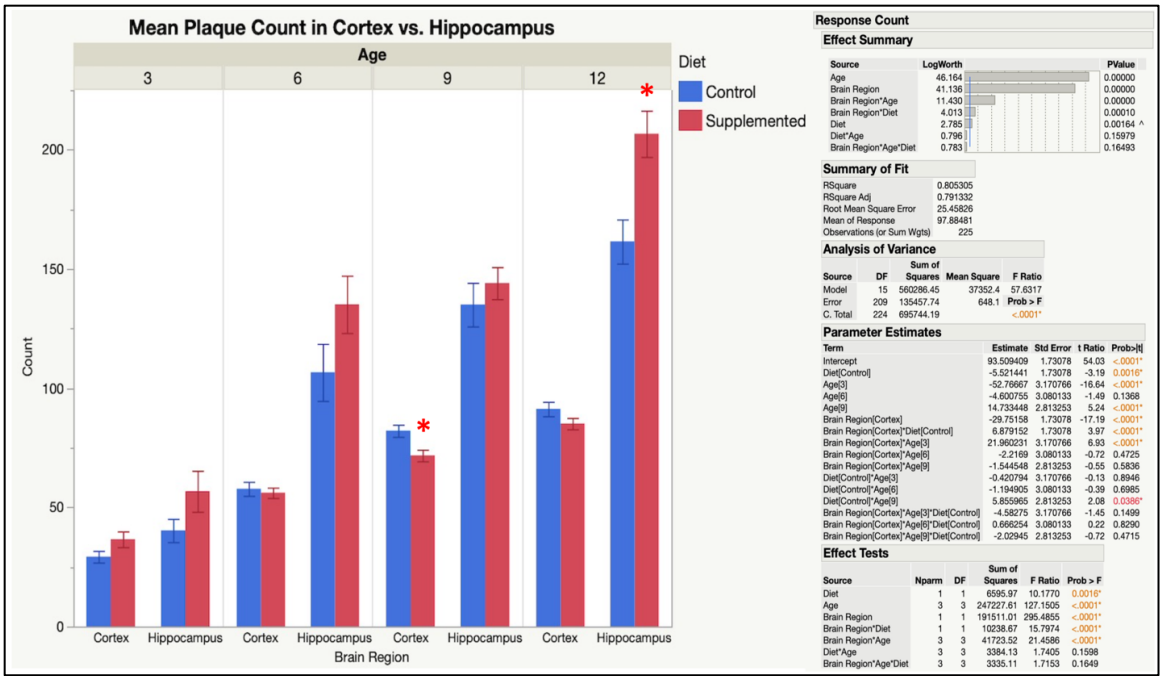

Figure 13. Comparison of Mean Plaque Count in the Cerebral Cortex versus Hippocampus. Brain tissue sections were stained using a rabbit anti-Aβ42 primary antibody and a goat-anti-rabbit conjugated with HRP secondary antibody exposed to a DAB, sodium imidazole, and hydrogen peroxide solution. Stained sections were imaged under a microscope, then analyzed using ImageJ software to quantitate amyloid plaques in a consistent region of the cortex and the hippocampus. Three tissue sections per animal were used to calculate a single data point for mean % area covered by plaques, and then graphed against age, brain region, and diet using JMP Pro 15 software to produce this figure. An ANOVA analysis was run and found a significant effect of age ($p<0.0001$), diet ($p=0.0016$), and brain region ($p<0.0001$) on the mean plaque count, along with an age by diet interaction ($p=0.0242$), and a brain region by diet interaction ($p=0.0070$). * indicates $p<0.05$ related to the effect of diet on mean plaque count.

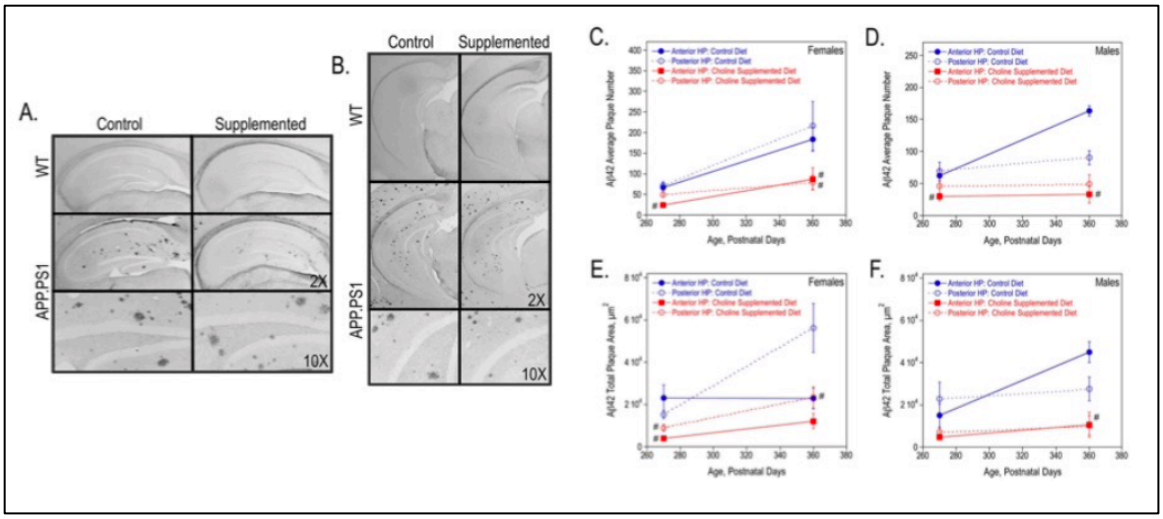

Figure 14. Aβ42 Plaques in the Hippocampus of 9M and 12M WT and APP.PS1 Mice. Used with permission from Mellott, et. Al. (2017).

Results

Previous work done in the lab used Conditional Fear Conditioning (CFC) to assess differences in memory between APP.NLGF and C57 "wild-type" mice on control and perinatal choline-supplemented diets at 3, 6, 9, and 12 months of age. CFC involved teaching subjects to associate a contextual setting and sound with a painful stimulus so that either one would elicit the murine fear response – freezing in place. On Day 1, the mice were placed into a conditioning chamber with white walls and a wire floor. They were subjected to 5 trials in which a 20 second tone was played and followed by two seconds of electric shock. Day 2 is the tone test, in which the mice were placed into a chamber with striped walls and a solid floor. The 20-second tone was played 5 times, but there was no tone or electric shock. Day 3 is the context test, in which the mice were placed into a chamber with white walls and a wire floor once again, but there was no electric shock. The percentage of time that the mouse

was frozen was recorded for days 2 and 3, and the results are presented in Figure 4. All 3M (data not shown) and 6M mice responded to fear conditioning and there were no differences between experimental groups. In the 9M age group, male APP.NLGF mice under the control diet were impaired in their fear response to the tone test. Choline supplemented male APP.NLGF mice did not suffer the same impairment. Similar results were observed for male APP.NLGF mice in the context test but these were not statistically significant. Female APP.NLGF mice had no impairment in the tone test. In the context test, female mice under the control diet had impaired fear response. Choline supplementation protected female mice from impairment. In the 12M age group, both male and female APP.NLGF mice under the control diet had impaired fear response to the tone test. Under choline supplementation, fear response in the APP.NLGF mice was only marginally improved, but enough so that there was no significant difference from the WT mice. In the context test, perinatal choline-supplemented female WT mice exhibited a significantly stronger fear response compared to female WT mice under the control diet. However, choline supplementation failed to produce the same response in female APP.NLGF mice. In 12M males, APP.NLGF mice under the control diet suffered a dramatically impaired fear response to the context test when compared to diet-matched WT mice. Choline supplementation protected male APP.NLGF mice from that impairment.

Because the memory deficits and affective disorders associated with AD are thought to in part result directly from the accumulation of amyloid plaques in the

cerebral cortex and hippocampus of the brain, we have elected to perform an immunohistochemical study of amyloidosis in the brains of APP.NLGF mice to assess that correlation and what benefits, if any, choline supplementation may bring. In order to investigate the effects of perinatal choline supplementation on brain amyloidosis, we quantitated amyloid plaques in the cortex and the hippocampus of APP.NLGF AD model mice of 3, 6, 9, and 12 months of age. Figure 3 is a timeline and overview of the study design, which is based on a previous study on perinatal supplementation of APP.PS1 mice done in this lab.[43] The brains collected from each age group were first cut into coronal sections 40 microns (μm) thick, then stained using the immunohistochemical process described in our methods above and imaged under a microscope to produce qualitative data. Figure 5 includes representative images of brain amyloidosis at 2x magnification from each experimental group differentiated by age, sex, and diet. Images of three brain sections from the anterior hippocampus and somatosensory cortex of each animal were analyzed using ImageJ to produce data for plaque count, plaque size, and percent area covered by plaques. Figure 6 shows how the image analysis was performed, where yellow outlines denote the area of analysis and blue outlines denote the plaques that were counted. The entire hippocampus was used for quantitation. The cortical area was defined by the lateral boundary of the hippocampus and the divide between the somatosensory area and the primary motor area. We elected to exclude edge effects and plaques that were smaller than 10 pixels in size. The quantitative data for each result were then averaged to create

a single data point for each animal and analyzed by the statistical software JMP Pro 15 to generate Figures 7-14.

Figure 7 is a graph of the percent area covered by plaques in the cerebral cortex of male and female APP.NLGF mice under control and supplemented conditions at 3, 6, 9, and 12 months of age. ANOVA analysis found a significant overall effect of age ($p<0.0001$) and diet ($p=0.0072$) on percent area covered by plaques in the cortex. There was no significant effect of sex on amyloidosis. As the mice age, we observe increasing plaque coverage in both the control and supplemented diet groups and plateaus at 9M and 12M. Perinatally choline-supplemented mice had less percent area covered by plaques as compared to controls in all age groups except the 3M. From our data, it appears that mice under supplemented conditions start life with greater amyloidosis in their cerebral cortex. While not statistically significant, perinatal choline supplementation appears to be detrimental in the 3M group. This effect is reversed by 6M, after which choline supplementation decreases amyloidosis when compared to mice under control conditions. Indeed, there was significant age by diet interaction ($p=0.0163$) in the ANOVA, which indicates that depending on the age of the animal, the effect of choline supplementation is different. The "*" symbols in Figure 7 indicate where in the 9M ($p=0.0270$) and 12M ($p=0.0414$) males that diet made a significant difference in percent area covered by plaques in the cortex.

Figure 8 shows the percent area covered by plaques in the hippocampus of male and female APP.NLGF mice under control and supplemented conditions at 3, 6,

9, and 12 months of age. There was a significant overall effect of age ($p<0.0001$) on the percentage of the total area covered by plaques in the hippocampus. There was no significant effect of sex or diet on amyloidosis. The observed increasing percent area covered by plaques in the hippocampus as the mice age was not as high as that in the cortex (Figure 7). The "*" symbol indicates where diet made a significant difference in percent area covered by plaques in the hippocampus of 3- and 12-month females ($p=0.0059$ and $p=0.0133$, respectively). Just like we observed in the cortex, mice under supplemented conditions appear to start life with greater amyloidosis in their hippocampus – although only the females were statistically significant. Mice under control conditions catch up by 6M, after which there is no significant difference in percent area covered by plaques between the control and supplemented groups. Mice under both dietary conditions plateau at about 6-7% coverage of the hippocampus by 9M. However, 12M perinatally choline-supplemented female mice had, on average, 10% plaque coverage of their hippocampus. The "#" symbol in Figure 8 indicates where sex made a significant difference in percent area covered by plaques in the hippocampus within the 12M age group ($p=0.0177$). This sexually dimorphic response to choline supplementation in the oldest mice likely accounts for the significant sex by diet interaction ($p=0.0459$) detected in the ANOVA analysis. Unlike in the cortex, ANOVA analysis did not find any significant overall effect of diet, nor an age by diet interaction.

Figure 9 shows mean plaque size in pixels in the cortex of male and female APP.NLGF mice under control and supplemented conditions at 3, 6, 9, and 12 months of age. ANOVA analysis found a significant overall effect of age ($p<0.0001$) and diet ($p=0.0081$) on mean plaque size in the cortex. There was no significant effect of sex on mean plaque size. Plaques grew larger with age, as expected. In control mice, plaques started at 44-48 pixels in size at 3M and steadily grow by about 46-59% until 9M when they plateau at about 70 pixels in size. In perinatally choline-supplemented diet mice, plaques start at 46-48 pixels in size at 3M and only grow by about 25-40% until they are 60-64 pixels in size by 12M. The "*" indicates where diet made a significant difference in mean plaque size in the males of the 9M age group ($p=0.0177$). These data suggest that in the cortex, choline supplementation does slow the growth rate and decrease mean plaque size over the animal's lifetime, which is consistent with our findings in Figure 7 that perinatal choline supplementation produce an overall decrease in % area covered by plaques in the cortex.

Figure 10 is a graph of mean plaque size in pixels in the hippocampus of male and female APP.NLGF mice under control and supplemented conditions at 3, 6, 9, and 12 months of age. ANOVA analysis found a significant overall effect of age ($p=0.0011$) on mean plaque size in the hippocampus. There was no significant overall effect of either diet or sex on mean plaque size. Just as in the cortex, plaques grew larger with age in the hippocampus. However, average plaque size in the hippocampus appears to be 10-20% smaller than in the cortex, reaching 50-58

pixels in size by 12M. As in Figures 7 and 8, we find that choline supplemented mice start out with worse amyloid pathology when compared to control mice in the youngest age group – though, as indicated by "*", only the females ($p=0.0038$) were statistically significant. The difference in plaque size is quickly attenuated, and by 6M both supplemented and control mice have roughly the same mean plaque size. Choline supplementation appears to have no effect on mean plaque size within the hippocampus.

Figure 11 is a graph of mean plaque count in the cortex of male and female APP.NLGF mice under control and supplemented conditions at 3, 6, 9, and 12 months of age showing a significant overall effect of age ($p<0.0001$). There was no significant effect of either sex or diet. Mean plaque count in the cortex increases steadily with age, starting at 25-36 plaques at 3M and reaching 78-90 plaques at 12M. Although there is no overall effect of diet on mean plaque count in the hippocampus, stratifying the data by age allows the observation of a significant diet by age interaction ($p=0.024$). At 3M, perinatal choline supplementation resulted in 2-10 more plaques than in control diet animals. At 6M and 9M, choline supplementation results in 1-2 and 8-10 fewer plaques, respectively. And at 12M, there is no difference in mean plaque count in the females while there are 10 fewer plaques in the males under supplemented conditions. The "*" symbols in Figure 11 indicate where diet made a significant difference in mean plaque count within each age group. Only the 3M ($p=0.0381$), 9M ($p=0.027$), and 12M ($p=0.0414$) males saw a statistically significant difference in mean plaque count relative to diet.

Figure 12 is a graph of mean plaque count in the hippocampus of male and female APP.NLGF mice under control and supplemented conditions at 3, 6, 9, and 12 months of age. There was significant overall effect of age ($p<0.0001$) and diet ($p=0.0009$). There was no significant effect of sex. Mean plaque count in the hippocampus increases with age, starting at 40-60 plaques at 3M and reaching 150-220 plaques by 12M. Choline supplementation results in either no difference in plaque number, or a greater plaque number when compared to control diet mice in the same age group. At 12M, supplemented male mice have 9% more plaques in their hippocampus, and supplemented female mice have 47% more plaques in their hippocampus when compared to age-matched control diet mice. The difference between sexes in the 12M group was not statistically significant. Our data suggests that, at least in the hippocampus, perinatal choline supplementation may be detrimental for the brain in terms of mean plaque count.

Figures 7-12 revealed two trends within the data that informed the generation of Figures 13-15. First, no ANOVA analyses of percent area covered by plaques, mean plaque size, and mean plaque count found a significant overall effect of sex. Second, there appears to be a differential response to diet depending on whether we're looking in the mouse cerebral cortex or the hippocampus. With these trends in mind, we performed another analysis so that the sex variable was replaced by the brain region variable.

Figure 13 is a graph of the percentage of the total area covered by plaques in the cortex and hippocampus of APP.NLGF mice under control and supplemented

conditions at 3, 6, 9, and 12 months of age. ANOVA analysis found a significant overall effect of age ($p<0.0001$) and brain region ($p=0.0001$) on the percent area covered by plaques. No significant overall effect of diet was found. Stratifying by age group, the "*" symbols indicates where diet made a significant difference in percent area covered by plaques in 3M hippocampus ($p=0.0017$), 9M cortex ($p=0.0022$), and 12M cortex ($p=0.0335$). Amyloidosis increases with age in both the cortex and the hippocampus. However, there is greater percent area covered by plaques in the cortex than in the hippocampus across both diets and all age groups, and most dramatically in the 9M and 12M mice. The "#" symbols indicate where brain region made a significant difference in percent area covered by plaques at 3M control and supplemented ($p<0.0001$ and $p=0.0007$, respectively), 6M control and supplemented ($p<0.0001$ and $p=0.0003$, respectively), 9M control and supplemented ($p<0.0001$ and $p<0.0001$, respectively) and 12M control and supplemented ($p<0.0001$ and $p<0.0001$, respectively). ANOVA analysis also found significant age by diet interaction ($p=0.0163$), age by brain region interaction ($p<0.0001$), diet by brain region interaction ($p=0.0017$), and diet by brain region by age interaction ($p=0.0448$). The level of interaction between variables suggests that the effect of perinatal choline supplementation on amyloidosis in the brain is highly complex and may change based on the age of the mouse and what region of its brain is under examination. However, Figure 13 also suggests that perinatal choline supplementation produces no significant overall difference in amyloidosis when compared to control diet conditions. It appears that the significant reduction in

amyloid burden that we observed in the cortex (Figure 7) is accompanied by the lack of significant effect or even a slight worsening in the percentage of total area covered by plaques observed in the hippocampus (Figure 8).

Figure 14 is a graph of mean plaque size in the cortex and hippocampus of APP.NLGF mice under control and supplemented conditions at 3, 6, 9, and 12 months of age. ANOVA analysis found a significant overall effect of age ($p<0.0001$) and brain region ($p<0.0001$) on mean plaque size. No significant effect of diet was found. Amyloid plaques grew in size as the mice age, and across all age groups and regardless of diet, plaques in the cortex were larger than plaques in the hippocampus. Stratifying by age, the "*" symbols indicate where perinatal choline supplementation significantly increased mean plaque size by 26% in 3M hippocampus ($p=0.0033$) and reduced mean plaque size by 24% in 9M cortex ($p=0.0046$) when compared to control diet conditions. Stratifying by brain region, the "#" symbols indicate where plaque size was significantly different between the cortex and the hippocampus in control diet 3M mice ($p=0.0028$), control diet and supplemented 9M mice ($p=0.0004$ and $p=0.0325$, respectively), and control diet and supplemented 12M mice ($p=0.0004$ and $p=0.0077$, respectively). ANOVA analysis also found significant age by diet interaction ($p=0.0242$) and brain region by diet interaction ($p=0.0070$). The effect of diet is different depending on the age of the animal. At 3M, we observe that supplemented conditions resulted in plaques that were larger than plaques in control conditions. By 6M, there is a reversal and plaque size in the control group either equals or overtakes plaque size in the supplemented

groups. The effect of diet is also different depending on brain region. In the cortex, choline supplementation increases mean plaque size in the 3M age group and reduces mean plaque size in the 6M, 9M, and 12M age groups. In the hippocampus, choline supplementation also increases mean plaque size in the 3M age group but does not significantly change the mean plaque size in the 6M, 9M, and 12M age groups when compared to the control diet. From these data, we find that in the cortex, choline supplementation may result in larger plaques in the beginning but will also decrease plaque size in 9M and 12M mice when compared to the control diet. This therapeutic effect is not observed in the hippocampus.

Figure 15 is a graph of mean plaque count in the cortex and hippocampus of APP.NLGF mice under control and supplemented conditions at 3, 6, 9, and 12 months of age. ANOVA analysis found a significant overall effect of age ($p<0.0001$), diet ($p=0.0016$), and brain region ($p<0.0001$) on mean plaque count. Although we observe more plaques in the hippocampus than in the cortex across all four age groups, it must be pointed out that because the areas of analysis between the cortex and the hippocampus are not equal (Figure 6), the mean plaque counts cannot be directly compared. The data presented in Figure 15 are valuable for illustrating how the effect of age and diet varies with respect to brain region, as reflected by the significant age by brain region interaction ($p<0.0001$) and diet by brain region interaction ($p<0.0001$). Mean plaque count increases with age between the youngest and oldest age groups, by 129-183% in the cortex, and by 281-300% in the hippocampus. For diet, choline supplementation results in increased mean plaque

count in both the cortex and the hippocampus in the 3M age group. After that, choline supplementation appears to have either no significant effect or a small therapeutic effect on mean plaque count in the cortex, while having a marked detrimental effect and increasing mean plaque count in the hippocampus when compared to control diet animals.

To summarize the results from our immunohistochemical experiment, the effects of choline supplementation on amyloidosis (% area), plaque size, and plaque count largely depends on age and brain region. Generally speaking, choline supplementation appears to: i) worsen amyloid pathology at first, then becoming protective against severe pathology later in life, and ii) be therapeutic in the cortex and inconclusive to detrimental in the hippocampus with regard to amyloid pathology.

CHAPTER 4: DISCUSSION

Perinatal Choline Supplementation Produces Opposite Effects in the Hippocampi of APP.PS1 and APP.NLGF strains of AD Model Mice

The APP.NLGF strain of AD model mouse uses a knock-in strategy to avoid the non-physiological overexpression of APP that may occlude the direct link between AD pathology and its behavioral symptoms. The neuroprotective effects of perinatal choline supplementation have been studied before in the APP.PS1 strain of AD model mouse, but never in APP.NLGF.[43,44] Our study found reduced amyloidosis in the cerebral cortex, but not the hippocampus, of 9- and 12-months old APP.NLGF mice on a perinatal choline supplemented diet when compared against APP.NLGF mice on a control diet. These results indicate that in the APP.NLGF mouse strain, there is a complex effect of perinatal choline supplementation on AD-associated amyloidosis that is dependent on both age and brain region. Importantly, repeating this experiment in APP.PS1 and APP.NLGF animals yields distinctly different results. Panels C and D in Figure 16 present the average plaque count in the 9M and 12M hippocampi of perinatal choline supplemented and control diet APP.PS1 mice. We see that perinatal choline supplementation has decreased the plaque number in both males and females when compared to age matched mice on the control diet. The opposite effect is seen in the hippocampi of APP.NLGF mice, presented in Figure 12, where perinatal choline supplementation has had no effect in the 9M hippocampi and increased mean plaque count in the 12M hippocampi. The differences in their response to choline

supplementation as well as in their phenotypic presentation of amyloid pathology suggests that the APP.PS1 and APP.NLGF strains are very different models of AD. The underlying cause of this is unclear but is likely linked to the knock-in strategy preserving the murine promoter of the APP sequence. The implications of this are that researchers will need to carefully consider which strain of mouse will best fit the needs of their experiments.

Perinatal Choline Supplementation Reduced Percent Area Covered by Plaques in the Cortex of APP.NLGF Mice

The percentage of the total area covered by plaques is our primary measure of amyloidosis because it standardizes both mean plaque size and mean plaque count against the total pixels in the brain region used for analysis. From Figure 13, we can see that the effect of perinatal choline supplementation is dependent on age group and brain region. Starting with age, perinatal choline supplementation in APP.NLGF mice appears to worsen amyloidosis in the youngest 3M age group while ameliorating amyloidosis in the 9M and 12M age groups when compared to age-matched APP.NLGF mice on the control diet. An increase in percent area covered by plaques after perinatal choline supplementation is unexpected based on previous studies done on APP.PS1 transgenic mice that found a disruptive effect on amyloidogenic APP processing, possibly through γ-secretase, that attenuates plaque accumulation.[43,44] Because our study was performed using APP.NLGF mice, this leads to the notion that either the gene knock-in strategy which preserves the murine APP promoter, and/or the combination of (NL), (G), and (F)

genetic mutations may underly the initial worsening in Aβ pathology.[43,44] It is also possible that there is some other unknown mechanism behind this observation. Nevertheless, by 6 months of age, this effect of perinatal choline supplementation has reversed and now either decreases or produces no change in percent area covered by plaques. Which one of the two results we observe is also dependent on brain region. In the cortex, perinatal choline supplementation reduces amyloidosis in 9M and 12M mice, while in the hippocampus, perinatal choline supplementation does not significantly alter amyloidosis when compared to control diet mice. This finding is reaffirmed when we stratify the data in Figure 13 by brain region to produce Figures 7 and 8 and find a statistically significant effect of diet on percent area covered by plaques in the cortex only. Further research is necessary to illuminate why the neuroprotective action of perinatal choline supplementation may be region-specific.

Perinatal Choline Supplementation Reduced Mean Plaque Size in the Cortex of 9M and 12M APP.NLGF Mice

Although not as useful as percent area covered by plaques as a measure of amyloidosis, it is still worth analyzing the effect of choline supplementation on mean plaque size because of its therapeutic implications. Previous studies done on both mice and humans have found that amyloid plaques do not grow in size with age nor clinical stage of AD, but tend to stay the same.[47] Anti-Aβ immunization therapy in human AD patients results in fewer and smaller amyloid plaques in their hippocampus, suggesting clearance of existing plaques as the underlying mechanism.[58] In contrast, γ-secretase

inhibitors do not reduce plaque size but work by preventing the pathogenic cleavage of βCTF into Aβ40 and Aβ42, thus preventing an increase in plaque count.[59] From our data in Figure 14, we observe that perinatal choline supplementation resulted in a reduction in mean plaque size in the cortex of 9M and 12M mice, though only 9M reached statistical significance. Additionally, perinatal choline supplementation failed to prevent an increase in mean plaque count in either the cortex or hippocampus. Together, these results point towards an amyloid-clearing mechanism like that from anti-Aβ immunization therapy, yet a previous study from our lab reported that perinatal choline supplementation in APP.PS1 mice resulted in reduced Aβ peptides and increased βCTF – reminiscent of mice treated with γ-secretase inhibitors.[60] This suggests that perhaps choline supplementation may have more than one therapeutic mechanism. In any case, the benefits of this intervention are restricted to the cortex of APP.NLGF mice, as our results in Figure 14 shows no difference in mean plaque size between the hippocampi of control diet and choline supplemented group.

Perinatal Choline Supplementation Increases Mean Plaque Count in the Hippocampus of 12M APP.NLGF Mice

Except for in the cortex of the 9M age group (Figure 15), perinatal choline supplementation appears to have no therapeutic effect on mean plaque count in the brains of APP.NLGF mice. No significant difference in mean plaque count was found in any other brain region across all four age groups except in the hippocampus of the 12M mice, where choline supplementation significantly increased the mean plaque count when

compared to the control diet. This is an unexpected result that prompts further examination. Stratifying our results in Figure 15 by brain region shows no change or a mildly significant decrease in mean plaque count in the cortex of 9M and 12M males (Figure 11). Conversely, it shows the hippocampus as the brain region where perinatal choline supplementation appears to be birthing new plaques (Figure 12). This effect is especially pronounced in the hippocampus of 12M females, and together with a mild increase in mean plaque size (Figure 10), results in the 4% more area covered by plaques in the hippocampus of 12M choline supplemented females over control diet females (Figure 8). In this manner, we have identified the hippocampus of 12M female APP.NLGF mice as anomalous in its response to choline supplementation. Further study or a larger population size may prove this result an outlier.

Reduced Amyloidosis in 9M and 12M Mice Correlates with Amelioration of Memory Deficits in 9M and 12M Mice

The contextual fear learning (CFC) test is a measure of fear learning and memory. The learned association of a painful experience with an audible tone or an environmental context depends on the coordinated activity of the hippocampus, amygdala, and somatosensory cortex.[46,48] While our study did not quantitate amyloid plaques in the amygdala, we did quantitate plaques in the hippocampus and the somatosensory cortex (Figure 6). Analysis of those results found that perinatal choline supplementation ameliorated amyloidosis in the cortex of 9M and 12M APP.NLGF mice but had little effect in the hippocampus except increasing amyloidosis in 12M females. Earlier work

done in our lab also found that APP.NLGF mice on the control diet had an impaired reaction during the CFC test at 9M and 12M, which is indicates abnormal fear learning and memory deficits that are suggestive of AD-associated dysfunction in the hippocampus, amygdala, and/or somatosensory cortex. The impaired reaction was ameliorated in APP.NLGF mice under perinatal choline supplementation. The results from our CFC and immunohistochemistry experiments support the effectiveness of perinatal choline supplementation in ameliorating learning impairment, memory deficits, and amyloidosis in the cortex. However, as correlation is not causation, we must be cautious about making any causative links between the decrease in amyloidosis and the rescue of cognitive function. With the rescue of learning and memory in the CFC results, we might expect to see a decrease in amyloidosis of the hippocampus, yet our immunohistochemistry experiments do not show this. One of the oldest ongoing inconsistencies in AD research is the common finding that there is little association between regional plaque burden and which brain regions are dysfunctional.[49] Emblematic of this are neuroimaging studies of human cases of AD that find amyloid plaque deposition does not begin in the hippocampus until late in course of the disease, even though hippocampal function is the first to fail.[49] Additionally, other regions like the medial prefrontal cortex that might suffer severe plaque buildup may be functionally spared.[49] Indeed, some studies find that amyloid plaque deposition even in patients without any significant cognitive impairment.[51] Despite this, amyloidosis remains a pathological hallmark of AD and an initiating factor of pathology, making it a prime target for therapeutic interventions.

Limitations

While the APP.NLGF mouse was conceived to avoid the non-physiologic overexpression of APP in current transgenic mice used to study AD, like any animal model, it has its share of drawbacks. Among these is the inclusion of the human familial Arctic (G) mutation for the purposes of promoting Aβ aggregation. A side effect of this mutation is the appearance of amyloid plaques in the subcortical brain structures of the APP.NLGF mouse – consistent with the presentation of pathology in human carriers of the Arctic mutation.[35,39,50] This is not representative of most cases of AD, in which amyloidosis is typically limited to the corticolimbic structures of the brain – although a recent study has found that Aβ may originate deep in the brain at the mammillary bodies before migrating out to the hippocampus and cortex.[51] Adding on to this misrepresentation is the fact that all mouse models of AD are built off human familial genetic mutations that cause early-onset AD that make up less than 1% of human cases. More than 95% of human cases are sporadic, late-onset AD that arise spontaneously. With these points in mind, APP.NLGF may not be the best animal model for every study, but it provides an option for scientists who need to avoid the overexpression of APP. A final weakness of our study is the relatively small population size. More mice in each study group would give us more statistical power and may temper some outliers in our data – most notably in the 12M female hippocampus.

CHAPTER 5: CONCLUSION AND FUTURE DIRECTION

These data show that perinatal choline supplementation is a viable preventative strategy against Alzheimer's Disease based on the amelioration of amyloidosis in the cortex and amelioration of learning and memory deficits in the contextual fear conditioning test. The effect of perinatal choline supplementation on amyloidosis is both age-dependent and brain-region dependent. Compared to their control diet counterparts, choline supplemented mice start out with increased amyloidosis during their first three months after birth but are protected from more severe amyloidosis later in life. Our study also reveals that perinatal choline supplementation resulted in a smaller mean plaque size and had little effect on mean plaque count. This pattern is reminiscent of anti-Aβ immunization therapy, which was a promising AD therapy that was previously scrapped due to hazardous side effects. Recently, in a reversal on position the controversial human monoclonal antibody aducanumab was approved for use in treating AD by the U.S. Food and Drug Administration despite disagreements over clinical efficacy and a risk of brain swelling and bleeding posed to some patient.[61,62] In a cohort study of 165 patients with prodromal or mild AD, Aducanumab selectively bound to Aβ and resulted in the clearance of amyloid plaques, though the clinical benefits were questionable.[63,64] The results of our study, in which perinatal choline supplementation reduced percent of area covered by plaques and mean plaque size but not mean plaque count, though less robust, resembles the results of aducanumab, suggesting that this dietary intervention may be a safer alternative.

Finally, our results shows that perinatal choline supplementation has a differential effect based on brain region, being ameliorative in the cortex and generally ineffective in the hippocampus.

Based on evidence from this study and others, increasing our intake of choline to meet or exceed adequate intake may be a simple and effective measure to mitigate the increasing prevalence of AD that we expect with the increasing life expectancy of our population. The inclusion of choline in daily multivitamins or as a supplement for pregnant women is a particularly advisable course of action. Future avenues of study include studying the role of choline in the expression of insulin-like growth factor 2 (IGF2), which has been shown to enhance memory, stimulate the release of acetylcholine, as well as reduce amyloidosis. The therapeutic effects of choline may be mediated via IGF2 as choline availability alters the methylation pattern of genes encoding IGF2. Homocysteine is another molecule of interest, as it binds to Aβ peptides and facilitates their aggregation into plaques. Choline, in the form of the methyl donor betaine, converts homocysteine into methionine and may thus decrease the accumulation of amyloid plaques. As dietary trends in our society move further away from choline rich sources of food, it is more important now than ever to raise awareness of this essential nutrient and its implications for cognitive health.

BIBLIOGRAPHY

1. John Wiley & Sons, Ltd. (2020, March 10). 2020 Alzheimer's Disease Facts and figures. *Alzheimer's & Dementia*, 16: 391-460. https://doi.org/10.1002/alz.12068

2. Oboudiyat C, Glazer H, Seifan A, Greer C, Isaacson RS. Alzheimer's disease. *Seminars in Neurology*. 2013 Sep;33(4):313-29. doi: 10.1055/s-0033-1359319. Epub 2013 Nov 14. PMID: 24234352.

3. Kochanek KD, Xu JQ, Arias E. Mortality in the United States, 2019. *NCHS Data Brief, no 395*. Hyattsville, MD: National Center for Health Statistics. 2020.

4. Breijyeh, Z., & Karaman, R. (2020). Comprehensive review on Alzheimer's disease: Causes and treatment. *Molecules*, *25*(24), 5789. https://doi.org/10.3390/molecules25245789

5. Bloom G. S. (2014). Amyloid-β and tau: the trigger and bullet in Alzheimer disease pathogenesis. *JAMA Neurology*, *71*(4), 505–508. https://doi.org/10.1001/jamaneurol.2013.5847

6. DeTure, M. A., & Dickson, D. W. (2019). The neuropathological diagnosis of Alzheimer's disease. *Molecular Neurodegeneration*, *14*(1), 32. https://doi.org/10.1186/s13024-019-0333-5

7. Yiannopoulou, K. G., & Papageorgiou, S. G. (2020). Current and Future Treatments in Alzheimer Disease: An Update. *Journal of Central Nervous System Disease*. https://doi.org/10.1177/1179573520907397

8. Zheng, H., Koo, E.H. (2011). Biology and pathophysiology of the amyloid precursor protein. *Molecular Neurodegeneration* 6, 27 (2011). https://doi.org/10.1186/1750-1326-6-27

9. Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis of Alzheimer's Disease at 25 years. *EMBO Molecular Medicine (2016) 8:595-608*. https://doi.org/10.15252/emmm.201606210

10. Wilkins, H. M., & Swerdlow, R. H. (2017). Amyloid precursor protein processing and bioenergetics. *Brain Research Bulletin*, *133*, 71–79. https://doi.org/10.1016/j.brainresbull.2016.08.009

11. O'Brien, R. J., & Wong, P. C. (2011). Amyloid precursor protein processing and Alzheimer's disease. *Annual Review of Neuroscience*, *34*, 185–204. https://doi.org/10.1146/annurev-neuro-061010-113613

12. Satoru Funamoto, Shinji Tagami, Masayasu Okochi, Maho Morishima-Kawashima (2020). Successive cleavage of β-amyloid precursor protein by γ-secretase. *Seminars in Cell & Developmental Biology*. Volume 105, Pages 64-74. ISSN 1084-9521. https://doi.org/10.1016/j.semcdb.2020.04.002

13. Matsunaga, S., Kishi, T., & Iwata, N. (2015). Memantine monotherapy for Alzheimer's disease: a systematic review and meta-analysis. *PloS one*, *10*(4), e0123289. https://doi.org/10.1371/journal.pone.0123289

14. Muñoz Fernández, S. S., & Lima Ribeiro, S. M. (2018). Nutrition and Alzheimer Disease. *Clinics in Geriatric Medicine*, *34*(4), 677–697. https://doi.org/10.1016/j.cger.2018.06.012

15. Flanagan, E., Lamport, D., Brennan, L., Burnet, P., Calabrese, V., Cunnane, S. C., de Wilde, M. C., Dye, L., Farrimond, J. A., Emerson Lombardo, N., Hartmann, T., Hartung, T., Kalliomäki, M., Kuhnle, G. G., La Fata, G., Sala-Vila, A., Samieri, C., Smith, A. D., Spencer, J., Thuret, S., … Vauzour, D. (2020). Nutrition and the ageing brain: Moving towards clinical applications. *Ageing Research Reviews*, *62*, 101079. https://doi.org/10.1016/j.arr.2020.101079

16. Prado, E. L., & Dewey, K. G. (2014). Nutrition and brain development in early life. *Nutrition Reviews*, *72*(4), 267–284. https://doi.org/10.1111/nure.12102

17. Moore, K., Hughes, C. F., Ward, M., Hoey, L., & McNulty, H. (2018). Diet, nutrition and the ageing brain: current evidence and new directions. *The Proceedings of the Nutrition Society*, *77*(2), 152–163. https://doi.org/10.1017/S0029665117004177

18. van den Brink, A. C., Brouwer-Brolsma, E. M., Berendsen, A., & van de Rest, O. (2019). The Mediterranean, Dietary Approaches to Stop Hypertension (DASH), and Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) Diets Are Associated with Less Cognitive Decline and a Lower Risk of Alzheimer's Disease-A Review. *Advances in Nutrition (Bethesda, Md.)*, *10*(6), 1040–1065. https://doi.org/10.1093/advances/nmz054

19. Grant W. B. (2016). Using Multicountry Ecological and Observational Studies to Determine Dietary Risk Factors for Alzheimer's Disease. *Journal of the American College of Nutrition*, *35*(5), 476–489. https://doi.org/10.1080/07315724.2016.1161566

20. National Academies of Sciences, Engineering, and Medicine, Health and Medicine Division, Board on Health Sciences Policy, Committee on Preventing Dementia and Cognitive Impairment, Downey, A., Stroud, C., Landis, S., &

Leshner, A. I. (Eds.). (2017). *Preventing Cognitive Decline and Dementia: A Way Forward*. National Academies Press (US). https://www.ncbi.nlm.nih.gov/books/NBK436397/ doi: 10.17226/24782

21. Wallace, T. C., Blusztajn, J. K., Caudill, M. A., Klatt, K. C., Natker, E., Zeisel, S. H., & Zelman, K. M. (2018). Choline: The Underconsumed and Underappreciated Essential Nutrient. *Nutrition Today*, *53*(6), 240–253. https://doi.org/10.1097/NT.0000000000000302

22. Strupp, B. J., Powers, B. E., Velazquez, R., Ash, J. A., Kelley, C. M., Alldred, M. J., Strawderman, M., Caudill, M. A., Mufson, E. J., & Ginsberg, S. D. (2016). Maternal Choline Supplementation: A Potential Prenatal Treatment for Down Syndrome and Alzheimer's Disease. *Current Alzheimer Research*, *13*(1), 97–106. https://doi.org/10.2174/1567205012666150921100311

23. Zeisel S. H. (2006). Choline: critical role during fetal development and dietary requirements in adults. *Annual Review of Nutrition*, *26*, 229–250. https://doi.org/10.1146/annurev.nutr.26.061505.111156

24. Wallace, T. C., & Fulgoni, V. L. (2017). Usual Choline Intakes Are Associated with Egg and Protein Food Consumption in the United States. *Nutrients*, *9*(8), 839. https://doi.org/10.3390/nu9080839

25. Zeisel S. H. (2021, October 29). *Precision Nutrition: Lessons from the Study of Choline*. Seminar presented at the Nancy L.R. Bucher Seminar Series at Boston University Medical School, Boston, MA.

26. Zeisel, S. H., Klatt, K. C., & Caudill, M. A. (2018). Choline. *Advances in Nutrition (Bethesda, Md.)*, *9*(1), 58–60. https://doi.org/10.1093/advances/nmx004

27. McCann, J. C., Hudes, M., & Ames, B. N. (2006). An overview of evidence for a causal relationship between dietary availability of choline during development and cognitive function in offspring. *Neuroscience and Biobehavioral Reviews*, *30*(5), 696–712. https://doi.org/10.1016/j.neubiorev.2005.12.003

28. Caudill, M. A., Strupp, B. J., Muscalu, L., Nevins, J., & Canfield, R. L. (2018). Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: a randomized, double-blind, controlled feeding study. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *32*(4), 2172–2180. https://doi.org/10.1096/fj.201700692RR

29. Wiedeman, A. M., Barr, S. I., Green, T. J., Xu, Z., Innis, S. M., & Kitts, D. D. (2018). Dietary Choline Intake: Current State of Knowledge Across the Life Cycle. *Nutrients*, *10*(10), 1513. https://doi.org/10.3390/nu10101513

30. Blusztajn, J. K., Holbrook, P. G., Lakher, M., Liscovitch, M., Maire, J. C., Mauron, C., ... & Wurtman, R. J. (1986). " Autocannibalism" of membrane choline-phospholipids: physiology and pathology. *Psychopharmacology Bulletin*, *22*(3), 781-786.

31. Blusztajn, J. K., Liscovitch, M., & Richardson, U. I. (1987). Synthesis of acetylcholine from choline derived from phosphatidylcholine in a human neuronal cell line. *Proceedings of the National Academy of Sciences*, *84*(15), 5474-5477. https://doi.org/10.1073/pnas.84.15.5474

32. Jankowsky, J. L., Fadale, D. J., Anderson, J., Xu, G. M., Gonzales, V., Jenkins, N. A., ... & Borchelt, D. R. (2004). Mutant presenilins specifically elevate the levels of the 42 residue β-amyloid peptide in vivo: evidence for augmentation of a 42-specific γ secretase. *Human Molecular Genetics*, *13*(2), 159-170. https://doi.org/10.1093/hmg/ddh019

33. Locci, A., Orellana, H., Rodriguez, G., Gottliebson, M., McClarty, B., Dominguez, S., Keszycki, R., & Dong, H. (2021). Comparison of memory, affective behavior, and neuropathology in APP^{NLGF} knock-in mice to 5xFAD and APP/PS1 mice. *Behavioural Brain Research*, *404*, 113192. https://doi.org/10.1016/j.bbr.2021.113192

34. Saito, T., Matsuba, Y., Mihira, N., Takano, J., Nilsson, P., Itohara, S., Iwata, N., & Saido, T. C. (2014). Single App knock-in mouse models of Alzheimer's disease. *Nature Neuroscience*, *17*(5), 661–663. https://doi.org/10.1038/nn.3697

35. Sakakibara, Y., Sekiya, M., Saito, T., Saido, T. C., & Iijima, K. M. (2018). Cognitive and emotional alterations in App knock-in mouse models of Aβ amyloidosis. *BMC Neuroscience*, *19*(1), 46. https://doi.org/10.1186/s12868-018-0446-8

36. Suryanarayanan, A., Wexler, P. (2014). Acetylcholine. *Encyclopedia of Toxicology (Third Edition)*, Academic Press. Pages 49-50. ISBN 9780123864550. https://doi.org/10.1016/B978-0-12-386454-3.00218-9

37. Whitehouse, P.J., Aminoff, M. J., Daroff R. B. (2014). Acetylcholine. *Encyclopedia of the Neurological Sciences (Second Edition)*, Academic Press. Pages 19-21. ISBN 9780123851581. https://doi.org/10.1016/B978-0-12-385157-4.00039-7

38. Davidson, M. K., Lindsey, J. R., & Davis, J. K. (1987). Requirements and selection of an animal model. *Israel Journal of Medical Sciences*, *23*(6), 551–555.

39. Kitazawa, M., Medeiros, R., & Laferla, F. M. (2012). Transgenic mouse models of Alzheimer disease: developing a better model as a tool for therapeutic interventions. *Current Pharmaceutical Design*, *18*(8), 1131–1147. https://doi.org/10.2174/138161212799315786

40. Rao, C. V., Farooqui, M., Zhang, Y., Asch, A. S., & Yamada, H. Y. (2018). Spontaneous development of Alzheimer's disease-associated brain pathology in a Shugoshin-1 mouse cohesinopathy model. *Aging Cell*, *17*(4), e12797. https://doi.org/10.1111/acel.12797

41. Masters, C. L., Bateman, R., Blennow, K., Rowe, C. C., Sperling, R. A., & Cummings, J. L. (2015). Alzheimer's disease. *Nature Reviews. Disease Primers*, *1*, 15056. https://doi.org/10.1038/nrdp.2015.56

42. Purves D, Augustine GJ, Fitzpatrick D, et al., editors. Neuroscience. 2nd edition. Sunderland (MA): Sinauer Associates; 2001. Acetylcholine. Available from: https://www.ncbi.nlm.nih.gov/books/NBK11143/

43. Mellott, T. J., Huleatt, O. M., Shade, B. N., Pender, S. M., Liu, Y. B., Slack, B. E., & Blusztajn, J. K. (2017). Perinatal Choline Supplementation Reduces Amyloidosis and Increases Choline Acetyltransferase Expression in the Hippocampus of the APPswePS1dE9 Alzheimer's Disease Model Mice. *PloS One*, *12*(1), e0170450. https://doi.org/10.1371/journal.pone.0170450

44. Velazquez, R, Ferreira, E, Knowles, S, et al. Lifelong choline supplementation ameliorates Alzheimer's disease pathology and associated cognitive deficits by attenuating microglia activation. *Aging Cell*. 2019; 18:e13037. https://doi.org/10.1111/acel.13037

45. Velazquez, R., Ferreira, E., Winslow, W., Dave, N., Piras, I. S., Naymik, M., Huentelman, M. J., Tran, A., Caccamo, A., & Oddo, S. (2020). Maternal choline supplementation ameliorates Alzheimer's disease pathology by reducing brain homocysteine levels across multiple generations. *Molecular Psychiatry*, *25*(10), 2620–2629. https://doi.org/10.1038/s41380-018-0322-z

46. Yokoyama, M., & Matsuo, N. (2016). Loss of Ensemble Segregation in Dentate Gyrus, but not in Somatosensory Cortex, during Contextual Fear Memory Generalization. *Frontiers in Behavioral Neuroscience*, *10*, 218. https://doi.org/10.3389/fnbeh.2016.00218

47. Serrano-Pozo, A., Mielke, M. L., Muzitansky, A., Gómez-Isla, T., Growdon, J. H., Bacskai, B. J., Betensky, R. A., Frosch, M. P., & Hyman, B. T. (2012). Stable size distribution of amyloid plaques over the course of Alzheimer disease. *Journal of Neuropathology and Experimental Neurology*, *71*(8), 694–701. https://doi.org/10.1097/NEN.0b013e31825e77de

48. Mehla, J., Lacoursiere, S. G., Lapointe, V., McNaughton, B. L., Sutherland, R. J., McDonald, R. J., & Mohajerani, M. H. (2019). Age-dependent behavioral and biochemical characterization of single APP knock-in mouse ($APP^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$) model of Alzheimer's disease. *Neurobiology of Aging*, *75*, 25–37. https://doi.org/10.1016/j.neurobiolaging.2018.10.026

49. Altmann, A., Ng, B., Landau, S. M., Jagust, W. J., Greicius, M. D., & Alzheimer's Disease Neuroimaging Initiative (2015). Regional brain hypometabolism is unrelated to regional amyloid plaque burden. *Brain: a Journal of Neurology*, *138*(Pt 12), 3734–3746. https://doi.org/10.1093/brain/awv278

50. Kalimo, H., Lalowski, M., Bogdanovic, N., Philipson, O., Bird, T. D., Nochlin, D., Schellenberg, G. D., Brundin, R., Olofsson, T., Soliymani, R., Baumann, M., Wirths, O., Bayer, T. A., Nilsson, L. N., Basun, H., Lannfelt, L., & Ingelsson, M. (2013). The Arctic AβPP mutation leads to Alzheimer's disease pathology with highly variable topographic deposition of differentially truncated Aβ. *Acta Neuropathologica Communications*, *1*, 60. https://doi.org/10.1186/2051-5960-1-60

51. Aizenstein, H. J., Nebes, R. D., Saxton, J. A., Price, J. C., Mathis, C. A., Tsopelas, N. D., ... & Klunk, W. E. (2008). Frequent amyloid deposition without significant cognitive impairment among the elderly. *Archives of Neurology*, *65*(11), 1509-1517. doi:10.1001/archneur.65.11.1509

52. Gail Canter, R., Huang, WC., Choi, H. *et al.* 3D mapping reveals network-specific amyloid progression and subcortical susceptibility in mice. *Communications Biology* 2**,** 360 (2019). https://doi.org/10.1038/s42003-019-0599-8

53. Meck, W. H., Smith, R. A., & Williams, C. L. (1988). Pre- and postnatal choline supplementation produces long-term facilitation of spatial memory. *Developmental Psychobiology*, *21*(4), 339–353. https://doi.org/10.1002/dev.420210405

54. Meck, W. H., & Williams, C. L. (2003). Metabolic imprinting of choline by its availability during gestation: implications for memory and attentional processing across the lifespan. *Neuroscience and Biobehavioral Reviews*, *27*(4), 385–399. https://doi.org/10.1016/s0149-7634(03)00069-1

55. Albright, C. D., Friedrich, C. B., Brown, E. C., Mar, M. H., & Zeisel, S. H. (1999). Maternal dietary choline availability alters mitosis, apoptosis and the localization of TOAD-64 protein in the developing fetal rat septum. *Brain Research. Developmental Brain Research*, *115*(2), 123–129. https://doi.org/10.1016/s0165-3806(99)00057-7

56. Albright, C. D., Tsai, A. Y., Friedrich, C. B., Mar, M. H., & Zeisel, S. H. (1999). Choline availability alters embryonic development of the hippocampus and septum in the rat. *Developmental Brain Research*, *113*(1-2), 13-20. https://doi.org/10.1016/S0165-3806(98)00183-7

57. Citron, M., Vigo-Pelfrey, C., Teplow, D. B., Miller, C., Schenk, D., Johnston, J., Winblad, B., Venizelos, N., Lannfelt, L., & Selkoe, D. J. (1994). Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(25), 11993–11997. https://doi.org/10.1073/pnas.91.25.11993

58. Serrano-Pozo, A., William, C. M., Ferrer, I., Uro-Coste, E., Delisle, M. B., Maurage, C. A., Hock, C., Nitsch, R. M., Masliah, E., Growdon, J. H., Frosch, M. P., & Hyman, B. T. (2010). Beneficial effect of human anti-amyloid-beta active immunization on neurite morphology and tau pathology. *Brain: a Journal of Neurology*, *133*(Pt 5), 1312–1327. https://doi.org/10.1093/brain/awq056

59. Kreft, A. F., Martone, R., & Porte, A. (2009). Recent advances in the identification of γ-secretase inhibitors to clinically test the Aβ oligomer hypothesis of Alzheimer's disease. *Journal of Medicinal Chemistry*, *52*(20), 6169-6188. 10.1021/jm900188z

60. Mitani, Y., Yarimizu, J., Saita, K., Uchino, H., Akashiba, H., Shitaka, Y., Ni, K., & Matsuoka, N. (2012). Differential effects between γ-secretase inhibitors and modulators on cognitive function in amyloid precursor protein-transgenic and nontransgenic mice. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *32*(6), 2037–2050. https://doi.org/10.1523/JNEUROSCI.4264-11.2012

61. Tolar, M., Abushakra, S., Hey, J. A., Porsteinsson, A., & Sabbagh, M. (2020). Aducanumab, gantenerumab, BAN2401, and ALZ-801-the first wave of amyloid-targeting drugs for Alzheimer's disease with potential for near term approval. *Alzheimer's Research & Therapy*, *12*(1), 95. https://doi.org/10.1186/s13195-020-00663-w

62. Knopman, D. S., & Perlmutter, J. S. (2021). Prescribing aducanumab in the face of meager efficacy and real risks. *Neurology*, 97(11), 545-547. https://doi.org/10.1212/WNL.0000000000012452

63. Schneider, L. (2020). A resurrection of aducanumab for Alzheimer's disease. *The Lancet Neurology*, *19*(2), 111-112. https://doi.org/10.1016/S1474-4422(19)30480-6

64. Sevigny, J., Chiao, P., Bussière, T. *et al.* The antibody aducanumab reduces Aβ plaques in Alzheimer's disease. *Nature* 537, 50–56 (2016). https://doi.org/10.1038/nature19323

CURRICULUM VITAE