Elucidating the Effects of Dietary Choline Deficiency on the Hippocampal and Plasma Proteomes of Non-Transgenic and 3xTg-AD Mice

by

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ABSTRACT

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases worldwide, with no effective treatments or preventions. Evidence suggests that environmental factors, including dietary nutrients, contribute to the etiology of AD. Choline is an essential nutrient found in many common foods. Choline is produced endogenously, but not at levels sufficient for healthy metabolic function and thus requires dietary supplementation. Literature shows that ~90% of Americans do not meet the adequate intake threshold for dietary choline consumption and therefore are dietary choline-deficient. While dietary choline supplementation throughout life has been shown to have significant health benefits, such as reducing AD pathology and improving cognition in a mouse model of AD, the impacts of dietary choline deficiency are unknown. Experiments were designed to understand the effects of dietary choline deficiency in healthy, non-transgenic mice (NonTg) and in the 3xTg-AD mouse model of AD. From 3 to 12 months of age, mice received either adequate choline (ChN) in the diet or were put on a choline-deficient (Ch-) diet. A Ch- diet leads to significant weight gain throughout life in both the NonTg and 3xTg-AD mice, with AD mice showing a greater increase. Additionally, impaired glucose metabolism, which is a risk factor for AD, was induced in both NonTg Ch- and 3xTg-AD Ch- mice. Interestingly, Ch- induced cardiomegaly in 3xTg-AD mice and elevated markers of cardiac dysfunction in NonTg mice to similar levels in 3xTg-AD mice. Finally, Ch- exacerbated amyloid-β plaque pathology and tau hyperphosphorylation in the hippocampus and cortex of 3xTg-AD mice. Proteomic analyses revealed Ch- induced changes in hippocampal proteins associated with postsynaptic receptor regulation, microtubule stabilization, and neuronal

development, as well as well-known AD-associated proteins (MAPT, BACE1, MECP2, CREBBP). Proteomic analyses also revealed Ch- induced changes of plasma proteins associated with secondary pathologies of AD including inflammation, immune response insulin metabolism, and mitochondrial dysfunction (SAA1, SAA2, IDE, HSPD1, VDAC-1, VDACE-2). Taken together, these data suggest that dietary choline deficiency induces system-wide cellular and molecular dysfunction associated with AD across several pathogenic axes, through proteomic changes not only in the hippocampus but also in the plasma.

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CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) is one of the most prevalent aging-related neurodegenerative disorders across the United States, with more than 6 million Americans currently living with AD and a projected 16 million Americans by 2050.[1] AD also has significant economic implications; in 2021 alone, AD and other dementias cost the United States an estimated $355 billion.[1] The number one risk factor for AD is aging, and the United States population is continually increasing in age.[2] From 2009 to 2019, the segment of Americans from 45-64 has increased by 4%, and this rate is expected to continue throughout the coming decades.[3] Moreover, the landscape of FDA-approved effective treatments or preventions from AD is critically limited, and there is still significant uncertainty throughout the scientific community surrounding the etiology of AD. This highlights the immediate need to identify the causes of AD and develop interventions to reduce AD cases.

AD is characterized by two hallmark brain pathologies, extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles (NFT), resulting in a progressive decline of cognitive ability.[4] Both pathologies are the result of the aggregation of misfolded proteins. Aβ plaques are formed by the amyloidogenic processing of amyloid precursor protein (APP), where APP is cleaved by beta-secretase (BACE1) forming a long-secreted form of APP (sAPPβ) and C99. C99 is subsequently cleaved by gamma-secretase creating Aβ fragments, which then oligomerize and fibrilize into Aβ plaques.[4] NFT pathology is the result of the disassociation of tau protein (MAPT) from neuronal microtubules, where its nonpathogenic function is to the microtubule.[5] Tau undergoes

several post-translational modifications, such as acetylation and phosphorylation, which cause it to dissociate from microtubules, leading to disintegrated tubulin and compromised axonal transport.[5] Following its detachment, tau undergoes further acetylation, hyperphosphorylation, and subsequent aggregation into neurofibrillary tangles.[5] Myriad other pathologies also exist in AD, including microglial hyperactivation and neuroinflammation, cardiac impairment, insulin dysregulation, and energy dyshomeostasis.[2] While a wealth of literature exists around these pathologies, it is still unclear how exactly they contribute to AD pathogenesis or when they chronologically coincide with Aβ and NFT pathology. These pathologies support the notion that AD is not simply a neurological disease. It is a complex, systems-wide disease that affects several different metabolic and cellular processes throughout the human body.

In addition to the complexity of AD pathologies, less than 5% of AD cases occur because of genetic abnormalities associated with Aβ (known as familial AD), leaving over 95% of cases characterized as sporadic AD.[6] While there is no clear cause of sporadic AD, a wealth of evidence suggests that environmental factors, including exercise and diet, may play a role in sporadic AD pathogenesis.[7]

Choline, an essential nutrient found in a variety of foods, is an essential part of the metabolic pathway responsible for the creation of choline phospholipids, betaine, and acetylcholine, a key neurotransmitter involved in neurogenesis, synapse formation, learning, and memory.[8] Moreover, betaine serves as a methyl group donor in the conversion of homocysteine (Hcy) to methionine.[8] Methionine plays a critical role in

histone and DNA methylation, which have both been implicated in AD pathogenesis. [9] Additionally, Hcy has been shown to play a role in the formation of Aβ plaques and elevated Hcy levels in human patients have been tied to increased risk for developing AD.[10] There is significant evidence showing that maternal choline supplementation (at 4.5x the adequate intake levels) has important cognitive benefits for offspring, and these findings have been corroborated in several mouse studies.[11-13] More specifically, maternal choline supplementation in mouse models of both AD and Down syndrome has shown to improve behavioral deficits and neuropathology.[14-16] Additionally, a more recent study has shown that lifelong choline supplementation in a mouse model of AD significantly improves Aβ plaque density, learning and memory deficits, and brain inflammation.[17] Alongside previous literature demonstrating the link between dietary choline intake and Aβ and NFT pathology, additional work has highlighted the relationship between choline and dysfunction of systems-wide cellular and molecular processes that are also implicated in AD. For example, high dietary choline is associated with attenuated microglial activation and low insulin resistance.[17,18] Further, choline is known to have system-wide effects, as it is involved in cardiovascular development, liver toxicity, and hypertension.[19]

While choline is produced endogenously in both mice and humans, endogenous production of choline is not sufficient for normal metabolic functioning and thus, dietary supplementation is necessary.[20] The United States established an adequate intake threshold for choline consumption in 1998 particularly to prevent fatty liver disease, stating that adult men and women should consume 550 mg/day and 425mg/day,

respectively.[8] Additionally, pregnant women should consume 550 mg/day given the evidence showing a need for choline supplementation during fetal development.[8] However, studies have shown that a staggering ~10% of Americans meet this daily threshold, with ~90% of Americans consuming a diet deficient in choline.[21] Interestingly, previous work has shown that a functional single nucleotide polymorphism (rs7964) in the gene encoding phosphatidylethanolamine N-methyltransferase (PEMT), a protein responsible for the endogenous production of choline, is associated with AD in a Chinese population.[22] This suggests that abnormalities in endogenous choline production are associated with AD, implying that reduced levels of choline may elevate the risk of AD.

Despite the myriad publications demonstrating the positive effects of dietary choline supplementation, there is a limited understanding of the health and cognitive impacts of dietary choline deficiency throughout life. To this end, we sought to understand the impacts of a choline-deficient diet in healthy wildtype (NonTg) mice and in a mouse model of AD (3xTg-AD) containing Aβ and NFT pathology, to elucidate the effects of dietary choline deficiency in healthy aging and AD. We conducted neuropathological and systems-pathology analyses to understand the pathological effects of adulthood dietary choline deficiency throughout the body, and further conducted comparative proteomic analyses of the hippocampus, a key brain region involved in learning and memory that is affected in AD, and in the blood plasma of these mice to identify potential mechanisms by which dietary choline deficiency may be modulating AD pathology and system-wide cellular and molecular dysfunction.

CHAPTER 2

MATERIALS AND METHODS

Mice Strain Generation and Housing

3xTg-AD mice, which harbor homozygous pathological mutations in APP (APP Swedish), PSEN1 (PSEN1 M146V), and MAPT (MAPT P301L) genes, are maintained in colonies by breeding homozygous 3xTg-AD mice with one another. These mice were generated on a C57BL6/129Svj hybrid background as previously described.[23] C57BL6/129Svj mice were used as the healthy wildtype control (NonTg) to maintain the same genetic background as 3xTg-AD mice. It is important to note that 3xTg-AD mice show significant neuropathological variability across sex, whereas female 3xTg-AD mice consistently display neuropathology and cognitive deficits that are predictable based on age and males do not. Thus, male mice were excluded from this study, consistent with previous experiments using 3xTg-AD mice.[23] All mice were kept on a 12-hour light/dark cycle at 23 degrees Celsius with *ad libitum* access to food and water at four to five mice per cage. All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of Arizona State University.

Choline Deficiency Diet and Sample Size

Due to choline's well-known role in the developing brain[24,25], NonTg and 3xTg-AD mice were kept on a choline normal (ChN) diet until three months of age (equivalent to 18 years in humans) to avoid confound, followed by some mice remaining on a ChN diet

and other mice consuming a choline-deficient (Ch-) diet until the tissue was harvested at 12 months of age (*Fig. 1A*). From 0-3 months of age, all mice were placed on a standard laboratory chow (Envigo Teklab Diets, Madison WI) with normal choline (ChN; 2.0g/kg; #TD.180228) based on the human adequate intake. At 3-months of age, mice were either kept on the ChN diet or placed on a laboratory chow choline deficit diet (Ch-; 0.0g/kg; #TD.110617). All other diet components are equivalent (i.e.., protein, carbohydrates, fat, and Kcal/g; *Fig. 1B*). This resulted in the generation of four experimental groups (NonTg ChN, n = 20; 3xTg-AD ChN, n = 15; NonTg Ch-, n = 16; 3xTg-AD Ch-, n = 16; *Fig. 1C*). Four animals were randomly selected per group to be used for hippocampal and plasma proteomic analyses.

Weight and Glucose Tolerance Test

The weight of each animal was measured in grams biweekly, beginning at 3 months of age until animals were euthanized at 12 months of age. Weight gain for each animal was calculated as the percent change between an animal's baseline weight at 3 months of age and their weight at the time of death.

To administer the glucose tolerance test, animals fasted overnight for 16 hours, and their tails were nicked using a razor blade to obtain blood for measurement of baseline fasting blood glucose levels. Animals then received an intraperitoneal 2 mg/kg glucose injection and blood glucose was sampled from the tail using a TRUEtrack glucose meter and

TRUEtrack test strips (Trividia Health) at 15, 30, 45, 60, 90, 120, and 150 minutes following the injection.

Tissue Harvesting and Processing

Mice were euthanized at 12 months of age and the brain, including hippocampal and cortical tissue, was dissected out, separately flash-frozen, and homogenized in a T-PER tissue protein extraction reagent supplemented with protease (Roche Applied Science) and phosphatase inhibitors (Millipore). Homogenized tissue was then centrifuged at 4 degrees Celsius for 30 minutes, and the supernatant was decanted to be used as the soluble fraction for enzyme-linked immunosorbent assay (ELISA) and liquid chromatography tandem mass spectrometry (LC-MS/MS). The pellet was also homogenized in 70% formic acid solution and centrifuged at 4 degrees Celsius for 30 minutes to be used as the insoluble fraction for ELISA.

For LC-MS/MS, solubilized brain proteins were quantified using EZQ Protein Quantitation Kit (Thermo Fisher) and 2.25ug alkylated (Pierce) using 40mM final concentration freshly prepared iodoacetamide for 30 minutes in the dark at room temperature. Samples were processed using the Protifi S-trap Micro Columns and instructions were given via the S-trap Ultra High Recovery Protocol (Protifi). Briefly, samples were acidified by the addition of 12% phosphoric acid to a final concentration of ~1.2% phosphoric acid. Proteins were digested by addition of 2.0 µg of porcine trypsin (MS grade, Pierce) and incubated at 30ºC for 2 hours. S-trap buffer (90% methanol, 100 mM TEAB final) was also added in volumes

7X our total sample volume. Acidified sample and the S-trap buffer were filtered through columns. Columns were washed 3X with S-trap buffer. An additional 0.5 µg of trypsin and 25 µL of 50 mM TEAB were added to the top of each column and incubated for 1 hour at 47ºC. Samples were eluted off the S-trap columns using three elution buffers: 50 mM TEAB, 0.2% formic acid in water, and 50% acetonitrile/50% water + 0.2% formic acid. Samples were dried down via speed vac and resuspended in 20-30 µL of 0.1% formic acid.

Enzyme-linked Immunosorbent Assay

We used commercially available ELISA kits (Invitrogen-ThermoFisher Scientific) to detect hippocampal levels of soluble and insoluble fractions of $A\beta_{40}$ and $A\beta_{42}$, Aβ oligomers, and soluble and insoluble fractions of pTau Ser181 and pTau Ser396 as previously described.[17] pTau Ser181 and pTau Ser396 have been identified as early events in tau pathogenesis and specific biomarkers for AD.

Cardiac Pathology

Hearts were harvested at tissue collection and flash frozen to extract RNA. Total RNA was extracted from left ventricular extract using the RNeasy Mini Kit (Qiagen) as previously described.[28,29] All qPCR probes were obtained from Integrated DNA Technologies.

Statistical Analyses

Two-way factorial Analysis of variance (ANOVA; independent variables genotype and diet), were used to analyze the physiological and pathological experiments, including weight, RNA markers from cardiac tissue, and area under the curve for the GTT output, followed by Bonferroni's corrected post hoc tests when appropriate. A repeated measure ANOVA was used to analyze the GTT data. Student's unpaired t-tests were utilized for comparison of 3xTg-AD mice for ELISAs. Examination of ROUT test (to identify statistical outliers) and Levene's test (to assess homogeneity of variance) revealed no significant effects necessitating the use of statistical tests other than the ones used. For all analyses, the variance was approximately the same among groups. Significance was set at $p < 0.05$.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

All LC-MS analyses were performed at the Biosciences Mass Spectrometry Core Facility at Arizona State University. All data-dependent mass spectra were collected in positive mode using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) coupled with an UltiMate 3000 UHPLC (Thermo Scientific). One µL of peptides was fractionated using an Easy-Spray LC column (50 cm × 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size, Thermo Scientific) equipped with an upstream 300um x 5mm trap column. Electrospray potential was set to 1.6 kV and the ion transfer tube temperature to 300ºC. The mass spectra were collected using the "Universal" method optimized for peptide

analysis provided by Thermo Scientific. Full MS scans (375–1500 m/z range) were acquired in profile mode with the Orbitrap set to a resolution of 120,000 (at 200 m/z), cycle time set to 3 seconds and mass range set to “Normal”. The RF lens was set to 30% and the AGC set to “Standard”. Maximum ion accumulation time was set to “Auto”. Monoisotopic peak determination (MIPS) was set to “peptide” and included charge states 2-7. Dynamic exclusion was set to 60s with a mass tolerance of 10ppm and the intensity threshold set to 5.0e3. MS/MS spectra were acquired in a centroid mode using a quadrupole isolation window set to 1.6 (m/z). Collision-induced fragmentation (CID) energy was set to 35% with an activation time of 10 milliseconds. Peptides were eluted during a 240-minute gradient at a flow rate of 0.250 uL/min containing 2-80% acetonitrile/water as follows: 0-3 minutes at 2%, 3-75 minutes 2-15%, 75-180 minutes at 15-30%, 180-220 minutes at 30-35%, 220-225 minutes at 35-80% 225-230 at 80% and 230-240 at 80-5%.

Label-free Quantification (LFQ)

We analyzed raw files against the Uniprot (www.uniprot.org) *Mus musculus* database (Mmus_UP000000589.fasta) using Proteome Discover 2.4 (Thermo Scientific). Raw files were searched using SequestHT that included Trypsin as the enzyme, maximum missed cleavage site 3, min/max peptide length 6/144, precursor ion (MS1) mass tolerance set to 20 ppm, fragment mass tolerance set to 0.5 Da, and a minimum of 1 peptide identified. Carbamidomethyl (C) was specified as fixed modification, and dynamic modifications set to Acetyl and Met-loss at the N-terminus, and oxidation of Met. A concatenated target/decoy strategy and a false-discovery rate (FDR) set to 1.0% were calculated using

Percolator (19). The data was imported into Proteome Discoverer 2.4, and accurate mass and retention time of detected ions (features) using Minora Feature Detector algorithm. The identified Minora features were then used to determine the area-under-the-curve (AUC) of the selected ion chromatograms of the aligned features across all runs and relative abundances calculated.

Gene Ontology

Gene ontology analysis was performed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) Version 11.5. An in-depth description of how protein networks are classified in STRINGv11.5 has been previously described.[30]

CHAPTER 3

RESULTS

Dietary Choline Deficiency Increases Weight and Induces Glucose Dyshomeostasis in NonTg and 3xTg-AD Mice

Choline has been shown to play an essential role in energy homeostasis and glucose tolerance, another cellular and molecular system which is dysfunctional in AD.[18] Thus, we examined the weight (*Fig. 2A-C*) and performed a glucose tolerance test (GTT; *Fig. 2D, E*) of all four groups of mice, to understand if Ch- throughout life promotes weight gain and impairs glucose metabolism. For % weight change from baseline, we found significant main effects of genotype ($F_{(1,65)} = 51.96$, $p < 0.0001$) and diet ($F_{(1,65)} = 52.86$, $p < 0.0001$), where 3xTg-AD mice had a higher % weight change than NonTg mice and Ch- mice had a higher % change than their ChN counterparts (*Fig. 2B, C*). We also found a significant genotype by diet interaction ($F_{(1, 65)} = 10.91$, $p = 0.0016$). Post hoc analysis revealed that the NonTg Ch- had a significantly higher % weight change from baseline than their ChN counterparts ($p = 0.026$) and that the 3xTg-AD Ch- mice show a higher % weight change from baseline than 3xTg-AD ChN mice ($p < 0.0001$). Notably, NonTg Ch- and 3xTg-AD ChN mice show no significant difference ($p > 0.99$), indicating the weight increase in NonTg Ch- mice that mimics that of the AD mouse.

Next, for the GTT, we found a significant main effect for genotype ($F_{(1, 65)} = 26.02$, $p < 0.0001$) and diet ($F_{(1,65)} = 47.01$, $p < 0.0001$), where the 3xTg-AD mice show higher glucose levels than NonTg mice, and Ch- mice had higher glucose levels than the ChN

mice (*Fig. 2D*). We also found a significant genotype by diet by time interaction ($F_{(7,455)}$ = 2.674, p = 0.0101). Post hoc analysis revealed that 3xTg-AD Ch- mice had significantly higher glucose levels from the 15 through 150 min time points compared to the ChN counterparts ($p < 0.05$). Strikingly, NonTg Ch- mice had higher glucose levels than their ChN counterparts at the 15 through 60 min timepoints ($p < 0.05$). Lastly, we analyzed the area under the curve (AUC) for the GTT as it provides a better assessment of glucose tolerance.[31] We found a significant main effect for genotype ($F_{(1, 65)} = 26.04$, $p < 0.0001$) and diet ($F_{(1,65)} = 47.25$, $p < 0.0001$), where the glucose AUC for 3xTg-AD mice was higher than NonTg mice, and higher for Ch- mice than the ChN mice (*Fig. 2E*). We also found a significant genotype by diet interaction ($F_{(1,65)} = 4.393$, $p = 0.040$). Post hoc analysis revealed that AUC was higher in NonTg Ch- than their ChN counterparts ($p = 0.0042$), and in 3xTg-AD Ch- compared to 3xTg-AD ChN mice ($p < 0.0001$). Notably, AUC was similar between the NonTg Ch- and 3xTg-AD ChN mice ($p > 0.9999$).

Collectively, these results show that a Ch- diet increases weight and results in impaired glucose metabolism in both the control and the AD mouse model, with the 3xTg-AD mice showing greater deficits. This is notable as weight gain and impaired glucose metabolisms are risk factors for AD[31], highlighting the importance of dietary choline to deter metabolic deficits.

AD Pathology and Dietary Choline Deficiency Induce Cardiac Dysfunction

Given the role of choline in cardiac dysfunction and previous reports of cardiac dysfunction in AD[32-34], we examined cardiac pathology in all four groups. We first measured heart weight normalized by tibia length as a measure of cardiomegaly, a symptom of cardiac dysfunction[35]; NonTg ChN (n = 9), NonTg Ch- (n = 7), 3xTg-AD ChN (n = 4) , and 3xTg-AD Ch- (n = 6). We found significant main effects for genotype ($F_{(1,22)} = 29.63$, $p < 0.0001$) and diet ($F_{(1,22)} = 13.78$, $p = 0.001$), where 3xTg-AD mice had a higher heart weight than NonTg mice, and the Ch- groups had higher heart weights than the ChN mice (*Fig. 2F*). We also found a significant genotype by diet interaction ($F_{(1,22)} = 13.78$, $p = 0.001$). Post hoc analysis revealed that the 3xTg-AD Ch- mice had a higher heart weight than the other groups ($p < 0.01$). Next, total RNA was extracted from snap frozen left ventricular extracts and subjected to qRT-PCR for transcript analysis; NonTg ChN (n = 6), NonTg Ch- (n = 6), 3xTg-AD ChN (n = 4), and 3xTg-AD Ch- (n = 6). We examined the expression levels of *Col1a1*, *Myh7*, and *Nppa*, which are all genes whose expression is associated with cardiac pathology.[36-38] For *Colla1*, we found a significant genotype by diet interaction ($F_{(1,18)} = 27.85$, $p < 0.0001$; *Fig. 2G*). Post hoc analysis revealed that the NonTg ChN mice had a lower expression of Colla1 than the other groups ($p < 0.05$). Similarly for *Myh7*, we found a significant genotype by diet interaction ($F_{(1,18)} = 9.743$, $p = 0.006$), and post hoc analysis revealed that the NonTg ChN mice had a lower RNA expression than the other groups ($p < 0.05$). Lastly, for *Nppa*, we found a significant genotype by diet interaction ($F_{(1,18)} = 19.17$, $p = 0.0004$). Post hoc analysis revealed that the NonTg ChN mice had a lower

expression than the other groups ($p < 0.05$). Taken together, these data demonstrate that cardiac dysfunction can be caused by both AD pathogenesis and dietary choline deficiency.

Dietary Choline Deficiency Exacerbates Aβ Pathology and Tau Hyperphosphorylation in 3xTg-AD Mice

To understand the effects of dietary choline deficiency on AD pathogenesis, we used ELISAs to quantify soluble and insoluble Aβ and pTau fractions in 3xTg-AD Ch- (n = 8) and ChN (n = 7) mice. Mice without familial AD mutations (NonTg) do not display Aβ or tau pathology, and therefore were excluded from these analyses.[14] The two major isoforms of Aβ are $A\beta_{40}$ and $A\beta_{42}$, found in both insoluble and soluble fractions.[4] These isoforms of Aβ aggregate into Aβ oligomers, which are toxic to neurons and serve as the precursor for insoluble Aβ plaques.[39] For soluble $A\beta_{40}$ fractions, we found no significant differences in hippocampal ($t_{(13)} = 1.712$, $p = 0.111$) or cortical ($t_{(13)} = 1.685$, $p = 0.116$) levels between 3xTg-AD ChN and Ch- mice (*Fig. 3A*). For soluble $A\beta_{42,}$ we found a significant difference where 3xTg-AD Ch- mice had higher levels in the hippocampus ($t_{(13)} = 2.833$, $p = 0.014$) and cortex ($t_{(13)} = 11.59$, $p < 0.0001$) compared to their ChN counterparts (*Fig. 3B*). Similarly, we found a significant difference in toxic soluble Aβ oligomers, where 3xTg-AD Ch- mice had higher levels in the hippocampus ($t_{(13)} = 3.335$, $p = 0.054$) and cortex ($t_{(13)} = 2.648$, $p = 0.021$) compared to their ChN counterparts *(Fig. 3C*). For insoluble $A\beta_{40}$ fractions in the hippocampus, we found a non-significant trend between 3xTg-AD Ch- and ChN mice ($t_{(13)} = 1.919$, $p = 0.077$; *Fig. 3D*). In the cortex ($t_{(13)} = 2.398$, $p = 0.032$), we found that 3xTg-AD Ch- mice exhibited higher levels of insoluble $A\beta_{40}$ compared to their

ChN counterparts (*Fig. 3D*). Lastly, for insoluble $A\beta_{42}$ fractions, we found a significant difference where 3xTg-AD Ch- mice had higher levels in the hippocampus ($t_{(13)} = 2.357$, $p = 0.035$) and cortex ($t_{(13)} = 13.90$, $p < 0.0001$) compared to the ChN counterparts (*Fig. 3E*). These data demonstrate that simply being deficient in dietary choline throughout life increases the levels of toxic Aβ pathology in 3xTg-AD mice.

We also sought to understand the effects of dietary choline deficiency on tau pathogenesis in 3xTg-AD mice. We used ELISAs to detect tau phosphorylation at serine 181 (pTau ser181) and serine 396 (pTau ser396). These two tau phosphorylation sites are known to be early events in tau pathogenesis and specific markers of AD pathology.[26, 27] We detected the levels of these two markers in the hippocampus and cortex for soluble fractions and only in the hippocampus for insoluble fractions due to the scarcity of sample available. For soluble pTau ser181, we found a significant difference where 3xTg-AD Ch- mice had higher levels in the hippocampus ($t_{(13)} = 9.045$, $p < 0.0001$) and cortex ($t_{(13)} = 7.691$, $p < 0.0001$) compared to the ChN counterparts (*Fig. 3F*). For soluble pTau ser396, we found a significant difference where 3xTg-AD Ch- mice had higher levels in the hippocampus ($t_{(13)} = 13.92$, $p < 0.0001$) and cortex ($t_{(13)} = 4.245$, $p < 0.001$) compared to the ChN counterparts (*Fig. 3G*). For insoluble pTau ser181 in the hippocampus, we found a significant difference where the 3xTg-AD Ch- mice had higher levels in the hippocampus than the ChN counterparts ($t_{(13)} = 2.228$, $p = 0.0442$; *Fig. 3H*). No significant differences between 3xTg-AD ChN and Ch- mice were detected in hippocampal insoluble pTau ($t_{(13)} = 0.622$, $p = 0.545$; *Fig. 3I*). These data suggest that dietary choline deficiency also worsens tau pathology in 3xTg-AD mice. Taken together, these data illustrate that dietary choline

deficiency significantly exacerbates the two hallmark pathologies of AD, amyloidosis, and tau phosphorylation, in a mouse model with a predisposition to AD.

Dietary Choline Deficiency Alters Protein Networks Associated With Neurodevelopment and Metabolic Processing in NonTg Hippocampi

To further understand how choline deficiency affects the hippocampus and exacerbates AD pathology, we performed liquid chromatography tandem mass spectrometry (LC-MS/MS) followed by label-free quantification (LFQ) of the hippocampal proteome. We conducted two analyses, comparing the NonTg ChN hippocampal proteome (n = 4) to the NonTg Ch- hippocampal proteome (n = 4), and comparing the 3xTg-AD ChN hippocampal proteome (n = 4) to the 3xTg-AD Ch- hippocampal proteome (n = 4). LC-MS/MS followed by LFQ identified a total of 4,436 proteins, 4,459 proteins, 4,525 proteins, and 4,379 proteins in the NonTg ChN, NonTg Ch-, 3xTg-AD ChN, 3xTg-AD Ch- hippocampi respectively. In the NonTg ChN and NonTg Ch- comparison, 195 differentially abundant proteins (adj. p-value < 0.05; $-1 > |\text{Log2 FC}| > 1$) were identified, and in the 3xTg-AD ChN and 3xTg-AD Ch- comparison, 468 differentially abundant proteins (adj. p-value < 0.05; $-1 > |\text{Log}_2 \text{FC}| > 1$) were identified (*Fig. 4A & 4B, respectively)*. Of these two sets of differentially abundant proteins, 55 were commonly identified as differentially abundant due to dietary choline deficiency in both the NonTg and 3xTg-AD hippocampi (*Fig. 4C)*.

We performed gene ontology on the two sets of differentially abundant proteins to understand what altered biological processes were represented in these data sets. In the

NonTg ChN and NonTg Ch- comparison, gene ontology revealed changes in pathways associated with neuronal development, cell adhesion, neuron projection development and neuron differentiation (*Fig. 4D)*. These findings are consistent with choline's well-known role in neurodevelopment.[24] Additionally, gene ontology revealed changes in metabolic pathways including oxoacid metabolic process and carboxylic acid metabolic process, which also corroborate choline's well-documented role in metabolic processing.[40]

Dietary Choline Deficiency Alters Protein Networks Associated With Microtubule Activity and Postsynaptic Membrane Regulation in 3xTg-AD Hippocampi

In the dataset of differentially abundant proteins between 3xTg-AD ChN and 3xTg-AD Ch- hippocampi, gene ontology revealed changes in protein networks closely associated with AD pathology (*Fig. 4E)*. Altered biological processes included regulation of microtubule motor activity, intermediate filament organization, and positive regulation of ATP-dependent microtubule motor activity (plus-end-directed). These pathways are particularly interesting as they are related to microtubule function, which is closely tied to tau pathology in AD.[5] Tau's disassociation and subsequent hyperphosphorylation are known to disrupt microtubule activity [5], and these data suggest that dietary choline deficiency also alters microtubule function in 3xTg-AD hippocampi.

Additionally, gene ontology of the list of differentially abundant hippocampal proteins in the 3xTg-AD ChN mice compared to the 3xTg-AD Ch- mice revealed a series of altered biological processes associated with postsynaptic membrane regulation, including long-

term synaptic potentiation, protein localization to postsynaptic membrane, and regulation of postsynaptic membrane neurotransmitter levels. These findings corroborate previous work showing that lifelong dietary choline supplementation modulates the abundance of critical neuroreceptors in the 3xTg-AD hippocampus.[17]

Dietary Choline Deficiency Alters Protein Networks Associated With Immune Response and Inflammation in NonTg Plasma

Given the systems-wide dysfunction observed in dietary choline-deficient mice, we also performed liquid chromatography tandem mass spectrometry (LC-MS/MS) followed by label-free quantification (LFQ) of the plasma proteome, to understand the mechanisms by which dietary choline deficiency may be causing systems-wide dysfunction. We again performed two analyses, comparing the NonTg ChN plasma proteome (n = 4) to the NonTg Ch- plasma proteome (n = 4), and comparing the 3xTg-AD ChN plasma proteome (n = 4) to the 3xTg-AD Ch- plasma proteome (n = 4). LC-MS/MS followed by LFQ identified a total of 687 proteins, 698 proteins, 702 proteins, and 697 proteins in the NonTg ChN, NonTg Ch-, 3xTg-AD ChN, 3xTg-AD Ch- plasma respectively. In the NonTg ChN and NonTg Ch- comparison, 74 differentially abundant proteins (adj. p-value < 0.05; -1 > |Log2 FC| > 1) were identified, and in the 3xTg-AD ChN and 3xTg-AD Ch- comparison, 67 differentially abundant proteins (adj. p-value < 0.05; -1 > |Log2 FC| > 1) were identified (*Fig. 5A & 5B, respectively)*. Of these two sets of differentially abundant proteins, 25 were commonly identified as differentially abundant due to dietary choline deficiency in both the NonTg and 3xTg-AD plasma (*Fig. 5C)*.

We then performed gene ontology analysis of the two datasets to understand what biological processes were represented in these datasets. Gene ontology of the list of differentially abundant plasma proteins in the NonTg ChN and NonTg Ch- comparison revealed that choline deficiency altered biological processes related to inflammatory response, including acute-phase response, acute inflammatory response, response to inorganic substance, complement activation (alternative pathway), and defense response (*Fig. 5D*). This is particularly interesting, as inflammation and immune dysfunction are relevant in a variety of diseases that affect all organs throughout the body, including AD.[41-43]

Dietary Choline Deficiency Alters Protein Networks Associated With Insulin Binding and Porin Activity in 3xTg-AD Plasma

Gene ontology of the list of differentially abundant plasma proteins in the 3xTg-AD ChN and 3xTg-AD Ch- mice revealed altered biological processes associated with insulin binding (*Fig. 5E)*. This finding corroborates the dysfunction we have identified in insulin resistance and glucose homeostasis in the 3xTg-AD Ch- mice, suggesting that dietary choline deficiency modulates insulin binding protein networks in the plasma, and thereby causes glucose metabolism impairments.

Interestingly, gene ontology also revealed an altered protein network associated with porin activity, specifically including the VDAC-1 and VDAC-2 proteins (*Fig. 5E)*. These

proteins and their role in mitochondrial dysfunction have been previously associated with AD pathology, cell death in the AD brain, and cognitive decline in AD patients.[44-46]

CHAPTER 4

DISCUSSION

Our results demonstrate that dietary choline deficiency causes system-wide dysfunction, including weight gain, impaired glucose metabolism, cardiac stress, cardiomegaly, and exacerbated AD-like pathology. These findings are particularly important given previous work indicating that ~90% of Americans fail to meet the daily adequate intake threshold for dietary choline.[21] Thus, dietary choline deficiency poses significant health risks for a large majority of the American population.

Our results also demonstrate that dietary choline deficiency exacerbates AD pathology in a mouse model of AD. In the 3xTg-AD mouse model, dietary choline deficiency increased Aβ pathology and tau hyperphosphorylation, demonstrating that dietary choline deficiency induces cellular and molecular dysfunction associated with AD. These findings are particularly interesting within the context of recently published literature demonstrating the positive effects of lifelong choline supplementation on AD pathology.[17] Further, proteomics analyses revealed dietary choline deficiency-induced changes in key proteins related to AD-pathogenesis. The abundance of MAPT, as well as two other proteins within the MAPT family (MAP2, MAP4), are altered in dietary choline-deficient 3xTg-AD hippocampi. These findings corroborate worsened tau pathology seen in 3xTg-AD Ch- mice compared to 3xTg-AD ChN mice. Levels of BACE1, a key protein in the amyloidogenic processing of APP[4], as well as levels of MECP2, an epigenetic regulator linked to AD[47], are also altered in dietary choline-deficient 3xTg-AD hippocampi. The modulation of these proteins in future mechanistic studies may show that dietary choline

deficiency increases the risk of AD. While there are several means by which dietary choline deficiency can modulate these proteins, we speculate that these changes occur due to epigenetic and transcriptional dysregulation. Choline, through its metabolism by betaine homocysteine methyltransferase (BHMT), is a methyl-donor that regulates the methylation of DNA and histones.[48]

Proteomics analysis also revealed dietary choline deficiency-induced modulation of hippocampal protein networks associated with postsynaptic membrane receptor regulation, suggesting that dietary choline deficiency may also be linked to the synaptic dysfunction observed in AD. Interestingly, CREBBP, a crucial protein in long-term synaptic potentiation that is linked to AD[49,50], as well as NPTN, another critical protein for long-term potentiation at hippocampal excitatory synapses that is also linked to AD[51], are both altered by dietary choline deficiency in 3xTg-AD hippocampi. Notably, these proteins are also key in learning and memory which is one of the earliest affected behaviors in AD.[52] Moreover, proteins that modulate AMPA receptors (CACNG8, LRRTM1, FRRS1L) and NMDA receptors (MAIAP2, GRIN2A) are also altered by dietary choline deficiency[53-57], suggesting that dietary choline deficiency may contribute to postsynaptic membrane dysfunction in AD. Previous work has shown that choline, a precursor to acetylcholine, modulates the expression of alpha7 nicotinic acetylcholine receptor (α7nAchR) and Sigma-1R receptors (σ1R).[17] Our results corroborate these previous findings that lifelong choline supplementation regulates the expression of postsynaptic receptors and suggests that choline alters postsynaptic receptor abundance through additional pathways outside of acetylcholine. Altogether, these results provide

significant evidence that choline deficiency exacerbates AD pathology and synaptic dysfunction in those with a predisposition to AD, and thus increases the risk of AD.

There is significant evidence that AD pathology extends beyond Aβ plaques, neurofibrillary tangles, and synaptic dysfunction, including increased neuroinflammation, mitochondrial dysfunction, energy dyshomeostasis, and insulin resistance.[2] These pathologies are not unique to the brain and are seen across the entire body. Our results indicate that both AD pathology and choline deficiency cause cardiac dysfunction and promote weight gain and that choline deficiency induces glucose dyshomeostasis in healthy aging and AD. Given the systems-wide dysfunction observed, we performed comparative proteomics on plasma from dietary choline normal and dietary choline-deficient NonTg and 3xTg-AD mice. Interestingly, dietary choline deficiency modulates key inflammation and immune response pathways. More specifically, within the acute inflammatory response pathway, dietary choline deficiency alters SAA1 and SAA2 in both NonTg and 3xTg-AD mice. These two serum amyloid proteins are produced systemically as an inflammatory response to environmental insult.[58] SAA1 has been shown to prime microglia for ATP-dependent interleukin-1B release, which is associated with AD onset.[58] These findings are particularly insightful, as they corroborate previous literature demonstrating that lifelong choline supplementation in an AD mouse model decreases disease-associated microglial activation.[17] Previous literature also indicates that central nervous system glial cell populations are responsive to SAA1 secretion in the blood and that SAA1 overexpression increases amyloid aggregation and glial activation in an AD mouse model.[58,59] This suggests that choline deficiency modulates SAA1/2

secretion in the plasma, causing glial hyperactivation, increased neuroinflammation, and even increasing Aβ aggregation. Future work should aim to elucidate the effects of modulating plasma SAA1 and SAA2 to slow neuronal death and cognitive decline seen in AD.

With respect to increased insulin resistance caused by dietary choline deficiency in healthy aging and AD, we find that a plasma protein network associated with insulin binding is altered by dietary choline deficiency. More specifically, levels of IDE and HSPD1 (also known as HSP60) were altered due to dietary choline deficiency. IDE, an insulin-degrading enzyme, degrades insulin and is directly related to insulin resistance.[60] Moreover, previous literature has shown that IDE can also degrade Aβ and that mice that lack the gene encoding IDE display a 50% decrease in Aβ degradation.[61,62] Previous literature has also tied IDE and its impact on insulin resistance to cognitive impairment.[63] Additionally, there is growing evidence that HSPD1 modulates diabetes-induced inflammation and protects against Aβ oligomer-induced synaptic toxicity.[64,65] This is particularly important given the large amounts of literature that demonstrate the negative effects of type 2 diabetes mellitus on brain insulin resistance, oxidative stress, and cognitive decline.[66] Taken together, dietary choline deficiency may modulate key insulin binding proteins contributing to the diabetes-like pathology observed in AD cases. Future work should seek to understand how modulating these insulin-binding plasma proteins may improve or exacerbate AD pathogenesis and glucose dyshomeostasis seen in AD.

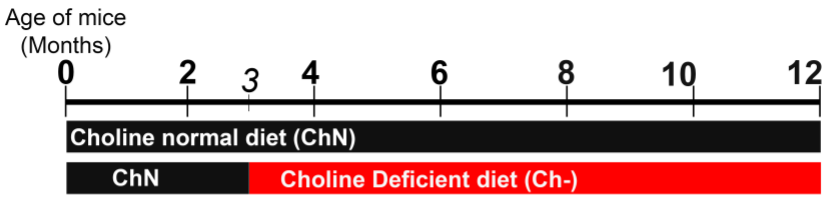
Finally, mitochondrial dysfunction has recently emerged as another driver of AD pathology.[67] Our results indicate that dietary choline deficiency modulates two plasma proteins involved in porin binding, VDAC-1 and VDAC-2, that have also been linked to mitochondrial dysfunction and AD pathology.[68,69] VDAC-1 has previously been implicated in mitochondrial dysfunction seen in APP transgenic mice[68] and VDAC-2 has been implicated in mitochondrial dysfunction seen in 3xTg-AD mice[69], suggesting that VDAC-1 and VDAC-2 modulation by dietary choline deficiency may induce mitochondrial dysfunction in AD mouse models. Future work should seek to confirm this hypothesis by investigating the impacts of dietary choline deficiency on mitochondrial dysfunction in AD models. VDAC-1 has been shown to mediate Aβ toxicity in the brain, and its overexpression triggers cell death.[70] Additionally, VDAC-1 interacts with several AD-relevant proteins, including phosphorylated tau, Aβ, and gamma-secretase.[68] Interestingly, VDAC-1 serum expression has been strongly correlated with Mini-Mental State Exam (MMSE) scores of AD patients, a clinical measure of cognition.[71] Future work should also seek to modify VDAC-1 and VDAC-2 in the plasma of AD mice, to understand if its peripheral alteration may improve mitochondrial dysfunction, AD pathology, and cognitive decline.

In conclusion, dietary choline deficiency incudes weight gain, impairs glucose metabolism, and promotes cardiac stress and cardiomegaly in NonTg mice and exacerbates these pathologies in 3xTg-AD mice. Dietary choline deficiency also worsens Aβ pathology and tau hyperphosphorylation in 3xTg-AD mice. Mechanistically, dietary choline deficiency induces changes in key hippocampal protein networks involved in microtubule activity,

amyloidogenic processing of APP, and postsynaptic membrane receptor regulation, as well as key plasma protein networks associated with inflammation and immune response, insulin binding, and mitochondrial function. Our data suggest that dietary choline deficiency induces system-wide dysfunction in NonTg and 3xTg-AD mice and exacerbates Aβ plaque and neurofibrillary tangle pathology as well as neuroinflammation, glucose dyshomeostasis, and mitochondrial dysfunction in a mouse model of AD. Taken together, our results indicate that dietary choline deficiency poses significant health risks and increases the risk for AD across several pathogenic axes.

FIGURES

A.



B.

Formula g/Kg			
Control Normal Diet (ChN)		Choline Deficient Diet (Ch-)	
Casein	200	Casein	200
DL-Methionine	3	DL-Methionine	3
Sucrose	494.89	Sucrose	496.99
Corn Starch	150	Corn Starch	150
Corn Oil	50	Corn Oil	50
Cellulose	50	Cellulose	50
Mineral Mix, AIN-76 (170915)	35	Mineral Mix, AIN-76 (170915)	35
Vitamin Mix, AIN-76A (40077)	15	Vitamin Mix, AIN-76A (40077)	15
Choline Bitartrate	2	Choline Bitartrate	0
Ethoxyquin, antioxidant	0.01	Ethoxyquin, antioxidant	0.01

C.

Experimental groups		
Genotype	Diet	N
NonTg	**ChN**	**20**
3xTg-AD	**ChN**	**15**
NonTg	**Ch-**	**16**
3xTg-AD	**Ch-**	**16**

Figure 1. Experimental Design. (A) Beginning at 3 months of age, NonTg and 3xTg mice were placed on either a choline normal diet (ChN) or a choline-deficient diet (Ch-) until 12 months of age. (B) All diet components are equivalent except for choline bitartrate, which was either 2g/Kg for ChN mice or 0g/kg for Ch- mice. (C) This resulted in the generation of four experimental groups: NonTg ChN (n = 20), 3xTg-AD ChN (n = 15), NonTg Ch- (n = 16), and 3xTg-AD Ch- (n = 16).

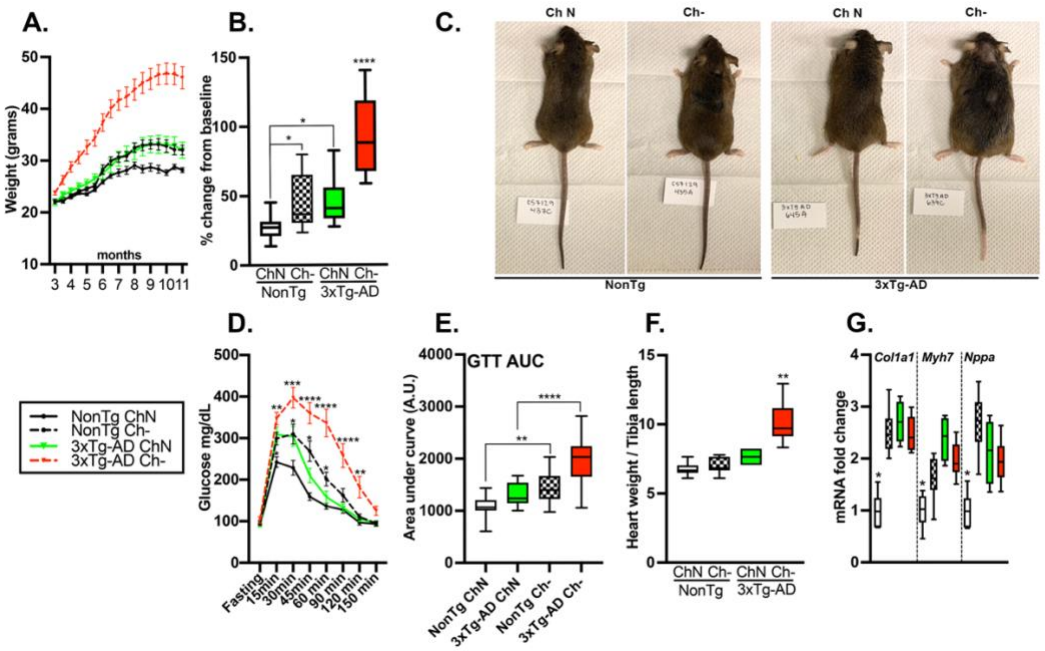


Figure 2. Dietary Choline Deficiency Promotes Weight Gain and Induces Glucose Dyshomeostasis and Cardiac Dysfunction. We examined the effects of Ch- on weight gain and glucose dyshomeostasis (NonTg ChN, n = 20; NonTg Ch-, n = 16; 3xTg-AD ChN, n = 15; 3xTg-AD Ch-, n = 16), as well as cardiomegaly and cardiac dysfunction (NonTg ChN, n = 9; NonTg Ch-, n = 7; 3xTg-AD ChN, n = 4; 3xTg-AD Ch-, n = 6). (A-C) Weight change analysis revealed that 3xTg-AD mice had a higher percent weight change than NonTg mice ($F_{(1,65)} = 51.96$, $p < 0.0001$), and Ch- mice had a higher percent weight change than their ChN counterparts ($F_{(1,65)} = 52.86$, $p < 0.0001$). (D-E) In the glucose tolerance test, we that 3xTg-AD mice show higher glucose levels than NonTg mice ($F_{(1, 65)} = 26.02$, $p < 0.0001$), and Ch- mice had higher glucose levels than the ChN mice ($F_{(1,65)} = 47.01$, $p < 0.0001$). We also found a significant genotype by diet by time interaction ($F_{(7,455)} = 2.674$, $p = 0.0101$). We analyzed the area under the curve (AUC) for the GTT, as it provides a better assessment of glucose tolerance, and found a significant main effect for genotype ($F_{(1, 65)} = 26.04$, $p < 0.0001$) and diet ($F_{(1,65)} = 47.25$, $p < 0.0001$). We also found a significant genotype by diet

interaction ($F_{(1,65)}$ = 4.393, p = 0.040). (F) Measuring heart weight normalized by tibia length as a measure of cardiomegaly, we found significant main effects for genotype ($F_{(1,22)}$ = 29.63, p < 0.0001) and diet ($F_{(1,22)}$ = 13.78, p = 0.001), and a significant genotype by diet interaction ($F_{(1,22)}$ = 13.78, p = 0.001). (G) We examined the expression levels of Col1a1, Myh7, and Nppa, genes that are altered by cardiac dysfunction). We found a significant genotype by diet interaction for Col1a1 mRNA expression ($F_{(1,18)}$ = 27.85, p < 0.0001), Myh7 mRNA expression ($F_{(1,18)}$ = 9.743, p = 0.006), and Nppa mRNA expression ($F_{(1,18)}$ = 19.17, p = 0.0004). *p < 0.05; **p < 0.01; **** p < 0.0001

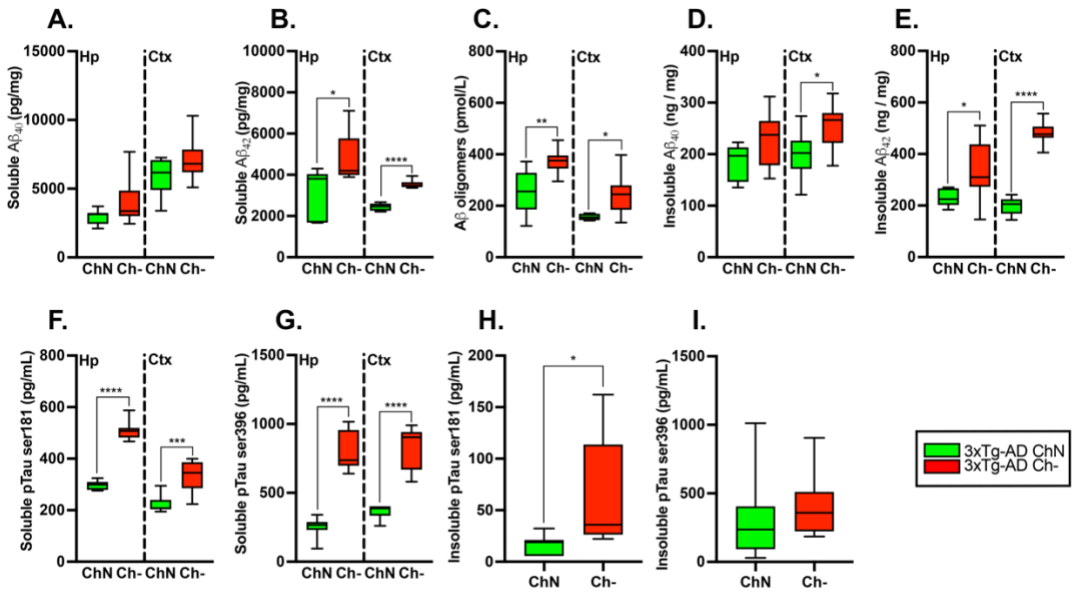
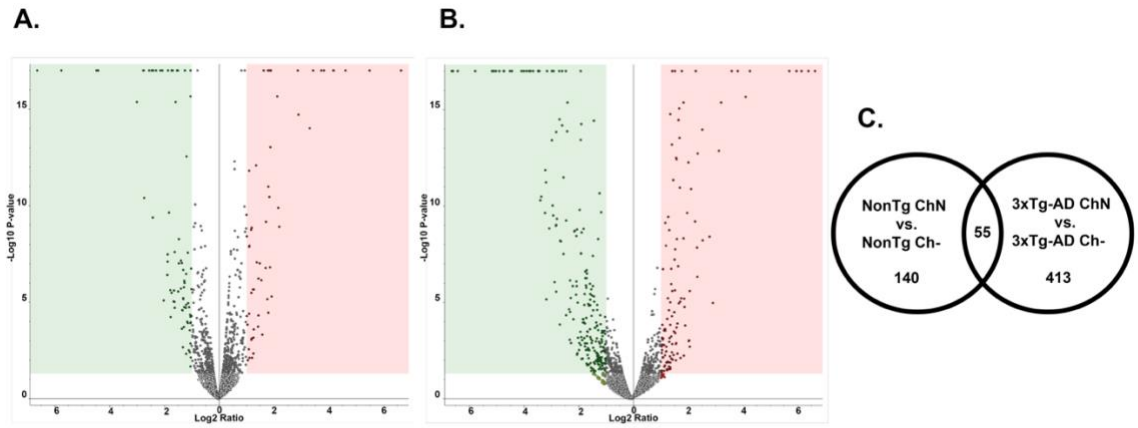


Figure 3. Dietary Choline Deficiency Exacerbates Aβ Pathology and Tau Hyperphosphorylation in 3xTg-AD Mice. We used ELISAs to quantify soluble and insoluble Aβ and pTau fractions in 3xTg-AD Ch- (n = 8) and ChN (n = 7) mice. (A) We found no significant differences in hippocampal ($t_{(13)}$ = 1.712, p = 0.111) or cortical ($t_{(13)}$ = 1.685, p = 0.116) levels of soluble $A\beta_{40}$ between 3xTg-AD ChN and Ch- mice. (B) 3xTg-AD Ch- mice had significantly higher levels of soluble $A\beta_{42}$ in the hippocampus ($t_{(13)}$ = 2.833, p = 0.014) and cortex ($t_{(13)}$ =11.59, p < 0.0001) compared to their ChN counterparts. (C) 3xTg-AD Ch- mice had significantly higher levels of Aβ oligomers in the hippocampus ($t_{(13)}$ = 3.335, p = 0.054) and cortex ($t_{(13)}$ = 2.648, p = 0.021) compared to their ChN counterparts. (D) We found no significant difference between 3xTg-AD Ch- and ChN mice in hippocampal insoluble $A\beta_{40}$ levels ($t_{(13)}$ = 1.919, p = 0.077). In the cortex, 3xTg-AD Ch- mice exhibited significantly higher levels of insoluble $A\beta_{40}$ compared to their ChN counterparts ($t_{(13)}$ = 2.398, p = 0.032). (E) 3xTg-AD Ch- mice had significantly higher levels of insoluble $A\beta_{42}$ in the hippocampus ($t_{(13)}$ = 2.357, p = 0.035) and cortex ($t_{(13)}$ = 13.90, p < 0.0001) compared to

their ChN counterparts. (F) 3xTg-AD Ch- mice had significantly higher levels of soluble pTau ser181 in the hippocampus ($t_{(13)} = 9.045$, $p < 0.0001$) and cortex ($t_{(13)} = 7.691$, $p < 0.0001$) compared to the ChN counterparts. (G) 3xTg-AD Ch- mice had significantly higher levels of soluble pTau ser396 in the hippocampus ($t_{(13)} = 13.92$, $p < 0.0001$) and cortex ($t_{(13)} = 4.245$, $p < 0.001$) compared to their ChN counterparts. (H) 3xTg-AD Ch- mice had significantly higher levels of soluble pTau ser396 in the hippocampus compared to their ChN counterparts ($t_{(13)} = 2.228$, $p = 0.0442$). (I) No significant differences between 3xTg-AD ChN and Ch- mice were detected in hippocampal insoluble pTau ser396 ($t_{(13)} = 0.622$, $p = 0.545$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$



D.

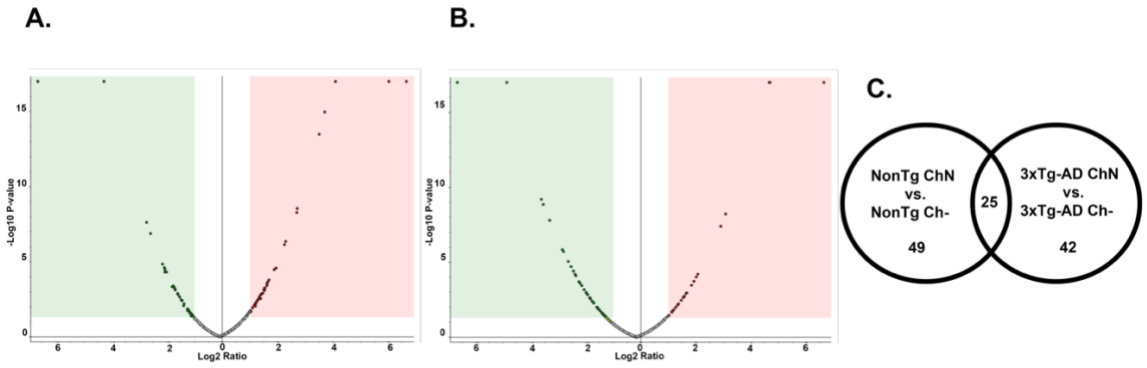
Biological Process (Gene Ontology)	Count in Network	Strength	False Discovery Rate
Negative regulation of proteolysis	12 of 361	0.59	0.0333
Neuron development	23 of 855	0.49	0.0015
Cell adhesion	20 of 744	0.49	5.00E-03
Carboxylic acid metabolic process	19 of 754	0.47	0.0148
Neuron differentiation	26 of 1056	0.46	0.0015
Oxoacid metabolic process	20 of 800	0.46	0.0115
Neuron projection development	17 of 697	0.45	0.0448
Cell development	38 of 1730	0.41	0.00018
Intracellular transport	25 of 1166	0.4	0.0127
Establishment of localization in cell	32 of 1518	0.39	0.002

E.

Biological Process (Gene Ontology)	Count in Network	Strength	False Discovery Rate
positive regulation of ATP-dependent microtubule motor activity, plus-end-directed	3 of 6	1.38	0.0443
Regulation of microtubule motor activity	4 of 14	1.14	0.0322
intermediate filament organization	7 of 25	1.13	5.40E-04
Peptide cross-linking	5 of 24	1	0.0234
intermediate filament cytoskeleton organization	9 of 46	0.97	0.00032
signal transduction involved in DNA damage checkpoint	5 of 27	0.95	0.0335
Neurotransmitter receptor transport	6 of 39	0.87	0.024
long-term synaptic potentiation	8 of 57	0.83	0.0061
protein localization to postsynaptic membrane	6 of 43	0.83	0.0347
Regulation of postsynaptic membrane neurotransmitter receptor levels	8 of 62	0.79	0.0091

Figure 4. Dietary Choline Deficiency Alters Potein Networks Associated With Neurodevelopment and Metabolic Processing in NonTg Hippocampi and Microtubule Activity and Postsynaptic Membrane Regulation in 3xTg-AD Hippocampi. (A-B) LC-MS/MS followed by LFQ identified 195 differentially abundant proteins (adj. p-value < 0.05; -1 > |Log2 FC| > 1) between NonTg ChN (n = 4) and NonTg Ch- (n = 4) hippocampi, and 468 differentially abundant proteins (adj. p-value < 0.05; -1 > |Log2 FC| > 1) between 3xTg-AD ChN (n = 4) and 3xTg-AD Ch- (n = 4) hippocampi. (C) 55 proteins were commonly identified as differentially abundant due to dietary choline deficiency in both NonTg and 3xTg-AD hippocampi. (D) In the NonTg ChN and NonTg Ch- comparison, gene ontology revealed changes in hippocampal pathways associated with neuronal development, cell adhesion, neuron projection development, neuron differentiation, oxoacid metabolic process, and carboxylic acid metabolic process. (E) In the 3xTg-AD ChN and 3xTg-AD Ch- comparison, gene ontology revealed changes in hippocampal protein networks associated with regulation of microtubule motor activity, intermediate filament organization, positive

regulation of ATP-dependent microtubule motor activity (plus-end-directed), long-term synaptic potentiation, protein localization to postsynaptic membrane, and regulation of postsynaptic membrane neurotransmitter levels.



D.

Biological Process (Gene Ontology)	Count in Network	Strength	False Discovery Rate
Porin activity	2 of 5	2.2	0.0424
Insulin binding	2 of 6	2.12	0.0471
Unfolded protein binding	5 of 82	1.38	0.0015
Identical protein binding	23 of 1962	0.66	0.00000041
Small molecule binding	23 of 2406	0.58	0.0000108
Nucleotide binding	17 of 2021	0.52	0.0032
Anion binding	21 of 2678	0.49	0.00092
Protein binding	38 of 6764	0.34	0.000013
Heterocyclic compound binding	25 of 4771	0.31	0.0378
Organic cyclic compound binding	25 of 4858	0.31	0.0424

E.

Biological Process (Gene Ontology)	Count in Network	Strength	False Discovery Rate
Complement activation, alternative pathway	3 of 10	2.05	0.0163
Fructose metabolic process	3 of 11	2.01	0.0163
Cytolysis	5 of 24	1.89	0.00017
Acute-phase response	4 of 40	1.57	0.0163
Acute inflammatory response	5 of 76	1.39	0.0147
Response to inorganic substance	10 of 556	0.83	0.0147
Defense response	13 of 1133	0.63	0.0163

Figure 5. Dietary Choline Deficiency Alters Protein Networks Associated With Immune Response and Inflammation in NonTg Plasma and Insulin Binding and Porin Activity in 3xTg-AD Plasma. (A-B) LC-MS/MS followed by LFQ identified 74 differentially abundant proteins (adj. p-value < 0.05; -1 > |Log2 FC| > 1) between NonTg ChN (n = 4) and NonTg Ch- (n = 4) plasma, and 67 differentially abundant proteins (adj. p-value < 0.05; -1 > |Log2 FC| > 1) between 3xTg-AD ChN (n = 4) and 3xTg-AD Ch- (n = 4) plasma. (C) 25 proteins were commonly identified as differentially abundant due to dietary choline deficiency in both NonTg and 3xTg-AD plasma. (D) In the NonTg ChN and NonTg Ch- comparison, gene ontology revealed changes in plasma pathways associated with inflammatory response, including acute-phase response, acute inflammatory response, response to inorganic substance, complement activation (alternative pathway), and defense response. (E) In the 3xTg-AD ChN and 3xTg-AD Ch- comparison, gene ontology revealed changes in plasma protein networks associated with insulin binding and porin activity.

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