Published in final edited form as:

Mol Psychiatry. 2020 October ; 25(10): 2620–2629. doi:10.1038/s41380-018-0322-z.

Maternal choline supplementation ameliorates Alzheimer's disease pathology by reducing brain homocysteine levels across multiple generations

Ramon Velazquez¹, Eric Ferreira¹, Wendy Winslow¹, Nikhil Dave¹, Ignazio S. Piras², Marcus Naymik², Matthew J Huentelman², An Tran¹, Antonella Caccamo¹, Salvatore Oddo^{1,3}

¹Arizona State University-Banner Neurodegenerative Disease Research Center at the Biodesign Institute, Arizona State University, Tempe, Arizona.

²Translational Genomics Research Institute, Phoenix, Arizona.

³School of Life Sciences, Arizona State University, Tempe, Arizona.

Abstract

The lack of effective treatments for Alzheimer's disease (AD) is alarming considering the number of people currently affected by this disorder and the projected increase over the next few decades. Elevated homocysteine levels double the risk of developing AD. Choline, a primary dietary source of methyl groups, converts homocysteine to methionine and reduces age-dependent cognitive decline. Here, we tested the transgenerational benefits of maternal choline supplementation (ChS; 5.0 g/kg choline chloride) in two generations (Gen) of APP/PS1 mice. We first exposed 2.5-month-old mice to the ChS diet and allowed them to breed with each other to generate Gen-1 mice. Gen-1 mice were exposed to the ChS diet only during gestation and lactation; once weaned at postnatal day 21, Gen-1 mice were then kept on the control diet for the remainder of their life. We also bred a subset of Gen-1 mice to each other and obtained Gen-2 mice; these mice were never exposed to ChS. We found that ChS reduced A β load and microglia activation, and improved cognitive deficits in old Gen-1 and Gen-2 APP/PS1 mice. Mechanistically, these changes were linked to a reduction in brain homocysteine levels in both generations. Further, RNA-Seq data from APP/PS1 hippocampal tissue revealed that ChS significantly changed the expression of 27 genes. These genes were enriched for inflammation, histone modifications, and neuronal death functional classes. Our results are the first to demonstrate a transgenerational benefit of ChS and suggest that modifying the maternal diet with additional choline reduces AD pathology across multiple generations.

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

*To whom correspondence should be addressed: Salvatore Oddo, Ph.D., Neurodegenerative Disease Research Center, Biodesign Institute, School of Life Sciences, Arizona State University, 1001 S McAllister Ave, Tempe, AZ 85287, 480-727-3490, oddo@asu.edu.

Conflict of Interest

The authors have no conflict of interest.

INTRODUCTION

The prevalence of Alzheimer's disease (AD) is anticipated to increase significantly over the next few decades¹. This is alarming given that no effective treatment options are available to prevent, treat or manage AD. Thus, there is an unmet and urgent need to develop new therapeutic approaches to mitigate this disorder.

Environmental factors such as diet are associated with AD risk^{2, 3} and dietary interventions have been tested in patients with some success. For instance, patients following the Mediterranean-Dietary Approaches to Stop Hypertension (DASH) diet for 4.5 years had significantly lower rate of AD by 54%⁴. A more recent clinical study using 70 subjects found that consumption of a Mediterranean diet reduced A β -amyloid load⁵. These results highlight how nutritional interventions may be key to reducing the development of AD pathogenesis; however, more work is necessary to understand the mechanisms by which dietary interventions lower AD pathology.

Homocysteine (Hcy) is a neurotoxic amino acid that accumulates in various neurological disorders, including AD⁶⁻⁸. Notably, elevated plasma Hcy doubles the risk of developing AD and dementia^{9, 10}. Recent studies suggest that Hcy induces cognitive and memory decline by increasing neuronal calcium influx that promotes amyloid and tau protein accumulation, apoptosis, and neuronal death^{6, 11, 12}. Consistent with these observations, increasing Hcy exacerbates A β pathology in animal models of AD^{13, 14}. Thus, reducing Hcy may be a valid approach to mitigate AD pathology.

Choline, a nutrient part of the vitamin B complex, converts Hcy to methionine^{15, 16}. Increasing choline intake during gestation and lactation improves cognition in mouse models of Down syndrome¹⁷⁻¹⁹. However, it has yet to be determined if maternal choline supplementation (ChS) improves cognitive deficits and reduces brain Hcy levels in mouse models of AD. Additionally, it has yet to be determined whether the benefits of maternal ChS are transgenerational. To this end, there is growing literature suggesting that specific dietary compounds induce benefits that may persist across multiple generations^{20, 21}.

Materials and Methods

Animals and behavioral testing.

We have backcrossed the APP/PS1 mice²² for 12 generations into a pure 129/SvJ background. All protocols were approved by the Institutional Animal Care and Use Committee of Arizona State University. Both male and female APP/PS1 mice were used for breeding and testing. The Morris water maze task was conducted as previously described^{23, 24}.

Diet Regimen and sample size

We exposed 2.5-month-old NonTg and APP/PS1 breeders to either a control (CTL) diet containing 1.1 g/kg of choline or to a choline supplemented (ChS) diet containing 5.0 g/kg of choline (Fig. 1a). The concentration of choline was obtained from previous publications¹⁷⁻¹⁹. The offspring (Gen-1) were exposed to either the CTL or ChS diet during

gestation and lactation (until postnatal day 21), and then kept on the CTL diet for the remainder of their lives: (i) APP/PS1 CTL = 18, (ii) NonTg CTL = 26, (iii) APP/PS1 ChS = 24, (iv) NonTg ChS = 17. The sample size was chosen based on previous experience performing similar experiments in these mice. We bred a subset of Gen-1 mice with each other and obtained Gen-2, which were never exposed to ChS (Fig. 1b): (i) APP/PS1 CTL-CTL = 17, (ii) NonTg CTL-CTL = 20, (iii) APP/PS1 ChS-CTL = 15, and (iv) NonTg ChS-CTL = 14. The ChS-CTL mice were derived from mothers who were exposed to ChS during gestation and lactation. The CTL-CTL mice were derived from mothers who were never exposed to ChS. The behavior experiments were performed in all mice. Mice were randomly selected for the gene expression, biochemical and histological analyses and n's are shown in the corresponding results section.

Western blots, ELISA, and Immunohistochemistry.

Western blots were performed under reducing conditions as we previously detailed²⁵. To assess soluble and insoluble levels of A β , we used the Life Technologies ELISA kit as previously described²⁶. The immunohistochemistry was performed as we previously described²⁶.

Antibodies.

The following antibodies were purchased from Abcam: BHMT (#ab96415), CD68 (#ab955), Rbm25 (#ab72237), Synaptophysin (#ab32127). Cell signaling: β -actin (#4967), Pin1 (#3722), PSD95 (#3409). Millipore: anti-A β ₄₂ (#5078P), 6E10 (#MAB1560), APP C-terminal (#171610). Santa Cruz Biotechnology: H2-Ab1 (#sc-71201). Wako: Iba1 (NCNP24).

Homocysteine and methionine detection in the brain.

Hemispheres were homogenized and processed using Liquid Chromatography-Mass Spectrometry as previously described^{27, 28}.

RNA sequencing and differential expression.

RNA was isolated from the hippocampus using the RNeasy Kit (Qiagen). Sequencing libraries were prepared with 250 ng of total RNA using TruSeq Stranded Total RNA with Ribo-Zero Gold Kit (Illumina Inc). PCR-enriched fragments were validated on a 2200 TapeStation (Agilent Technologies) and quantitated via qPCR. The final library was sequenced by 50bp paired-end sequencing on a HiSeq 2500 (Illumina). The reads were aligned to the mouse reference genome (mm9 build 37) as described in²⁹. Outliers and batch effects detection were conducted through Principal Component Analysis (PCA). Gene expression differential analyses were performed using the R-package DESeq2 v1.14.1³⁰. Raw RNA-Seq data will be publicly available on Gene Expression Omnibus repository.

Enrichment and protein-protein interaction network analyses.

Pathway enrichments were assessed using KEGG³¹, Panther³², and REACTOME³³. Gene Ontology enrichment was conducted using the same statistical method as for the pathways³⁴.

All the results were corrected with the Bonferroni's method, considering pathways significant at adj $p < 0.01$.

We conducted a Network Topology-based analysis (NTA) using the Network Expansion method³⁵ referencing to BioGRID 3.4 database (<https://thebiogrid.org/>) and setting the number of top-ranking neighbors to 10. The generated network was investigated for enriched Gene Ontology classes, correcting the p-values with the FDR method³⁶. The NTA was conducted using WEBGESTALT³⁷.

Statistical analyses.

Repeated measures ANOVAs were used to analyze the behavioral experiments followed by Bonferroni's corrected post hoc tests when appropriate. Two-way factorial ANOVAs were used to analyze probe trial dependent measures, body weight, western blot, Hcy, and methionine levels. Student's unpaired t-tests were employed for comparison of APP/PS1 mice within each generation. Examination of descriptive statistics revealed no violation of any test assumptions necessitating use of statistical test other than the ones used. For all analyses, the variance was approximately the same among groups.

RESULTS

Maternal ChS ameliorates spatial memory deficits and amyloid pathology in APP/PS1 mice.

We assessed body weight in 12-month-old Gen-1 mice and found that APP/PS1 mice were heavier ($38.53 \pm 1.762\text{g}$) than NonTg mice ($31.95 \pm 2.002\text{g}$; $F_{(1, 81)} = 12.224$, $p < 0.01$). These changes were independent of the diet (Fig. 1c). We then tested 12-month-old Gen-1 mice on the Morris water maze (MWM). For the five-day learning phase, we found a significant effect of day for distance traveled ($F_{(1, 4)} = 32.967$, $p < 0.0001$; Fig. 1d) and escape latency ($F_{(1, 4)} = 29.714$, $p < 0.0001$; Fig. 1e) to the platform, indicating that all groups learned at the same pace. On day 6, we performed a probe trial and found a significant genotype by diet interaction for platform crossings ($F_{(1, 81)} = 4.308$, $p < 0.05$; Fig. 1f) and latency to first cross the platform location ($F_{(1, 81)} = 4.840$, $p < 0.05$; Fig. 1g). Post-hoc analyses revealed that APP/PS1 ChS mice performed significantly better than APP/PS1 CTL mice and as well as the NonTg groups in both measurements ($p < 0.05$). Lastly, we found no significant differences in swim speed on day 6 of testing among the four groups (Fig. 1h). These results demonstrate that maternal ChS improves spatial reference memory deficits in adult APP/PS1 mice.

We immunostained sections from Gen-1 APP/PS1 CTL and APP/PS1 ChS ($n = 6/\text{group}$) mice with an $A\beta_{42}$ -specific antibody. We found a $44.01 \pm 21.69\%$ decrease of $A\beta$ load within the hippocampus of ChS mice compared with the CTL counterparts ($t_{(10)} = 19.739$, $p < 0.01$; Fig. 1i–k). We measured $A\beta$ levels by sandwich ELISA and APP processing by western blot. Soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ levels were significantly lower in ChS mice compared to the CTL mice (soluble $A\beta_{40}$: $t_{(10)} = 16.408$, $p < 0.01$; soluble $A\beta_{42}$: $t_{(10)} = 14.438$, $p < 0.01$; insoluble $A\beta_{40}$: $t_{(10)} = 9.863$, $p < 0.05$; insoluble $A\beta_{42}$: $t_{(10)} = 6.727$, $p < 0.05$; Fig. 1l–m). While APP and C83 levels were similar between the two groups, C99

levels were lower in APP/PS1 ChS mice ($t_{(10)} = 11.014$, $p < 0.01$; Fig. 1n–q). These results are consistent with a recent report showing that maternal ChS reduces A β levels.³⁸

To determine the effects of ChS on synaptic function, we measured the levels of the synaptic markers synaptophysin and PSD95. Synaptophysin levels were similar among the four groups in Gen-1 and Gen-2 (Supplementary Fig. 1a–b). In contrast, in Gen-1 there was a significant genotype by diet interaction for PSD95 ($F_{(1, 20)} = 4.400$, $p < 0.05$; Supplementary Fig. 1a, c). Post hoc analyses indicated that the APP/PS1 ChS mice had significantly higher levels of PSD95 than APP/PS1 CTL mice ($p < 0.05$). In Gen-2, we found a significant main effect of diet for PSD95 ($F_{(1, 20)} = 4.590$, $p < 0.05$; Supplementary Fig. 1d–f), which indicated that ChS-CTL mice had significantly higher levels of PSD95 than the CTL-CTL counterparts.

Transgenerational effects of ChS on AD-like pathology

To determine the transgenerational effect of ChS, we used 14.5-month-old Gen-2 mice. We found a significant difference in body weight between APP/PS1 (37.559 ± 2.073 g) and NonTg mice (30.394 ± 1.532 g; $F_{(1, 62)} = 12.737$, $p < 0.01$; Fig. 2a). This difference was independent of diet. We then tested Gen-2 mice in the MWM. During learning, we found a significant effect of day for distance traveled ($F_{(1, 4)} = 12.021$, $p < 0.0001$; Fig. 2b) and for escape latency to the platform location ($F_{(1, 4)} = 25.598$, $p < 0.0001$, Fig. 2c). Notably, we found a significant effect of diet for escape latency ($F_{(1, 62)} = 4.213$, $p < 0.05$; Fig. 2c), indicating that the ChS-CTL mice learned faster. On the day 6 probe trial, there were no statistically significant differences among the groups for all measurements (Fig. 2d–f).

At the end of the behavioral testing, we immunostained sections from Gen-2 APP/PS1 CTL-CTL and APP/PS1 ChS-CTL ($n = 6$ /group) mice with an A β_{42} -specific antibody (Fig. 2g–h). There was a $29.37 \pm 21.03\%$ decrease in A β_{42} plaque load in APP/PS1 ChS-CTL mice compared with APP/PS1 CTL-CTL mice ($t_{(10)} = 7.627$, $p < 0.05$; Fig. 2i). Next, we measured A β levels by sandwich ELISA and found that soluble A β_{40} levels ($t_{(10)} = 7.937$, $p < 0.05$), insoluble A β_{40} ($t_{(10)} = 5.648$, $p < 0.05$), and insoluble A β_{42} levels ($t_{(10)} = 7.937$, $p < 0.05$) were significantly lower in APP/PS1 ChS-CTL mice compared to APP/PS1 CTL-CTL mice (Fig. 2j–k). No significant differences were detected for soluble A β_{42} levels (Fig. 2j), full-length APP, C99, and C83 (Fig. 2l–o). Although further experiments are needed to address this difference, it is tempting to speculate that the effects of ChS are stronger in the Gen-1 mice because these mice were directly exposed to ChS during gestation and lactation, compared to Gen-2 mice. Indeed, the latter group was never directly exposed to the ChS diet.

ChS alters the gene expression profile in key signaling pathways.

We performed RNA sequencing (RNA-Seq) using the hippocampus of 12-month-old Gen-1 APP/PS1 CTL ($n = 8$) and APP/PS1 ChS mice ($n = 9$). We found 27 differentially expressed genes between the two groups (Supplementary Table 1). To validate the RNA-Seq data, we measured three randomly-selected genes by qRT-PCR: *Pin1*, *H2-Ab1* and *Rbm25*. Consistent with the RNA-Seq data, we found a significant upregulation of *Pin1* ($p = 0.048$) and downregulation of *H2-Ab1* ($p = 0.034$) and *Rbm25* ($p = 0.041$) in APP/PS1 ChS

compared to APP/PS1 CTL mice (Fig. 3a). We also measured the steady-state levels of these proteins in both Gen-1 and Gen-2 APP/PS1 mice ($n = 6/\text{group}$) and found a significant upregulation of Pin1 protein levels in Gen-1 APP/PS1 ChS ($t_{(10)} = 5.388$, $p < 0.05$, Fig. 3b–c) mice compared to CTL counterparts. We also found a significant downregulation of H2-Ab1 ($t_{(10)} = 6.632$, $p < 0.05$) and Rbm25 ($t_{(10)} = 5.352$, $p < 0.05$) in Gen-1 APP/PS1 ChS mice compared to CTL (Fig. 3b–c). We found consistent results in Gen 2 [Pin1: $t_{(10)} = 5.292$, $p < 0.05$; H2-Ab1: $t_{(10)} = 5.153$, $p < 0.05$; Rbm25: $t_{(10)} = 6.054$, $p < 0.05$; Fig. 3b, d]. These results highlight consistent changes in protein levels in both Gen-1 APP/PS1 ChS and Gen-2 APP/PS1 ChS-CTL mice.

To determine the functional profile of our gene set, we performed a Gene Ontology analysis. We obtained a total of 57 enriched processes (adj $p < 0.01$) with a range of 4 to 8 genes included in each process. For each Gene Ontology category, we observed that immune-related processes were most significantly enriched, namely “peptide antigen binding” (adj $p = 5.59\text{E-}06$), “negative regulation of immune system process” (adj $p = 1.96\text{E-}04$), and “major histocompatibility complex proteins” (adj $p = 1.00\text{E-}08$) (Supplementary Table 2). We next performed pathway analyses and obtained a total of 22 enriched pathways, which also showed a prevalence of immune system related processes (Supplementary Table 3). We then analyzed the upregulated ($n = 8$) and downregulated ($n = 19$) gene sets independently. When we only analyzed the upregulated genes, we did not identify any significant processes. In contrast, when we only analyzed the downregulated genes, we identified the same significant processes that we identified when we included all 27 genes (Supplementary Tables 4–5). This suggests that the downregulated genes drive the Gene Ontology results.

Using a protein-protein interaction analysis, we obtained an expanded sub-network of 14 genes, including 4 of the input genes from the initial 27 differentially expressed genes (Pin1, Rpl19, Rbm25, and H2-Ab1). The network was enriched for two Gene Ontology processes related to “neuron death” (FDR adj $p < 0.05$; Fig. 3e, Supplementary Table 6). We repeated the analysis using only the downregulated genes ($n = 19$) and obtained an extended subnetwork of 11 genes, including two input genes (*Rbm25* and *RunX1*; Fig. 3f). This network was significantly enriched for Gene Ontology processes related to histone and chromatin modification, histone deacetylation and gene expression regulation (FDR adj $p < 0.01$; Supplementary Table 7). We repeated the network analysis including the upregulated genes ($n = 8$) and found an extended sub-network weakly enriched in Gene Ontology processes related to neuronal death (FDR adj $p < 0.05$; Fig. 3g, Supplementary Table 8). These results illustrate that maternal ChS alters gene expression throughout life, predominantly those genes involved in the brain immune response, histone modifying processes and regulation of neuronal death.

Transgenerational reduction of activated microglia in APP/PS1 mice.

We examined microglia activation by double-staining tissue with an antibody against Iba1, a marker of total microglia, and CD68, a lysosomal marker ($n = 3/\text{group}$). The levels of CD68 are increased in activated microglia³⁹. Thus, CD68 and Iba1 colocalization signals can be used to determine the ratio of activated versus total microglia. In Gen-1 mice, we found a significant reduction in microglia activation as indicated by a decreased intensity of yellow

pixels of CD68/Iba1 colocalization in the ChS mice ($t_{(4)} = 3.562$, $p < 0.05$, Fig. 4a–b). In Gen-2 mice, microglia activation was reduced in APP/PS1 ChS-CTL mice compared to CTL-CTL counterparts ($t_{(4)} = 3.787$, $p < 0.05$, Fig. 4c–d).

Transgenerational reduction of brain Hcy levels in APP/PS1 mice.

High Hcy levels double the risk of developing AD and dementia^{7, 9}, and increase A β aggregation^{6, 7, 14, 40}. Hcy is converted to methionine through betaine-homocysteine-methyltransferase (BHMT)⁹. We used liquid chromatography-mass spectrometry to measure the brain Hcy and methionine levels ($n = 6/\text{group}$). In Gen-1 mice, we found a significant effect of diet where the ChS groups had significantly lower Hcy levels than the CTL groups ($F_{(1, 20)} = 10.059$, $p < 0.01$, Fig. 5a). We also found a significant genotype by diet interaction for Hcy ($F_{(1, 20)} = 6.984$, $p < 0.05$, Fig. 5a). Post-hoc analysis revealed that the APP/PS1 ChS mice had a $56.27 \pm 21.34\%$ reduction in brain Hcy levels compared to the APP/PS1 CTL mice ($p < 0.05$). Notably, there were no significant differences between the APP/PS1 ChS and NonTg CTL mice. Consistent with these results, we found that ChS groups had significantly higher levels of methionine than the CTL groups ($F_{(1, 20)} = 21.403$, $p < 0.001$, Fig. 5b). Next, we measured the expression levels of BHMT ($n=4$ for CTL and CTL-CTL/genotype; $n = 5$ for ChS and ChS-CTL/genotype) and found that both APP/PS1 ($t_{(7)} = 5.851$, $p < 0.05$, Fig. 5c, d) and NonTg ChS mice ($t_{(7)} = 8.709$, $p < 0.05$, Fig. 5c, e) had significantly higher expression of this enzyme than the CTL counterparts. In Gen-2 mice, we found a significant effect for diet where Hcy levels were lower in the ChS-CTL groups than the CTL-CTL groups ($F_{(1, 20)} = 17.852$, $p < 0.001$; Fig. 5f). Additionally, we found a significant genotype by diet interaction ($F_{(1, 20)} = 5.059$, $p < 0.05$; Fig. 5f). Post-hoc analysis revealed that APP/PS1 ChS-CTL mice, whose parents were exposed to ChS, show a $35.21 \pm 8.25\%$ reduction in brain Hcy levels compared to the APP/PS1 CTL-CTL mice ($p < 0.05$). We also found a significant effect of diet for methionine levels where ChS-CTL groups had significantly higher levels than the CTL-CTL groups ($F_{(1, 19)} = 8.876$, $p < 0.01$; Fig. 5g), and a significant genotype by diet interaction ($F_{(1, 19)} = 13.976$, $p < 0.01$; Fig. 5g). Post-hoc analysis revealed that APP/PS1 ChS-CTL mice show a significant increase in brain methionine levels compared to the APP/PS1 CTL-CTL mice ($p < 0.05$). Consistent with the Hcy data, BHMT levels were increased in APP/PS1 ($t_{(7)} = 8.709$, $p < 0.05$, Fig. 5h–i) and NonTg ChS-CTL mice ($t_{(7)} = 10.398$, $p < 0.05$, Fig. 5h, j).

DISCUSSION

Our results show that maternal ChS produces life-long improvements in cognition and AD-like pathology. We demonstrate a reduction in AD-like pathology in Gen-2 APP/PS1 mice, which were never exposed to ChS, highlighting the transgenerational effect of choline. The decline in AD pathology was associated with a decrease in brain Hcy levels. This is remarkable as high Hcy levels double the AD risk and facilitate A β aggregation^{6,9}. We further show that maternal ChS alters the expression of genes within networks involved in brain immune response, histone modifications and regulation of neuronal death months following the initial supplementation. Consistent with the changes in the expression of genes linked to the immune response, we find reduced microglia activation in both APP/PS1

generations. The changes in the expression of genes involved in histone modification could explain the long-lasting effects observed in Gen-1 and the transgenerational effects of ChS.

Our results show that ChS reduced A β pathology in both generations of APP/PS1 mice who were directly or indirectly exposed to ChS. This is consistent with one report indicating that early gestation ChS reduces amyloidosis in APP/PS1 mice [38]. We also find that ChS improves spatial memory in Gen-1 APP/PS1 mice and spatial learning in Gen-2 ChS-CTL NonTg and APP/PS1 mice. While this could appear as a discrepancy, it must be noted that the Gen-1 mice were behaviorally tested at 12 months of age while Gen-2 mice were tested at 14.5 months of age. 12-month-old APP/PS1 CTL mice are not impaired in spatial learning; thus, at this age, there is no deficit to improve. However, 14.5-month-old APP/PS1 CTL-CTL were significantly impaired compared to age-matched NonTg CTL-CTL mice. Under these conditions, ChS rescued learning deficits.

Our results show a transgenerational reduction of brain Hcy levels with maternal ChS. Substantial evidence indicates that high Hcy levels are toxic and increase A β aggregation^{41–43}. Specifically, homocysteine binds to A β and facilitates its aggregation and accumulation. Thus, it is tempting to speculate that the reduced A β levels in the APP/PS1 mice exposed to ChS are linked to lower homocysteine levels, which in turn reduce A β aggregation and accumulation. It is also plausible that reduced microglia activation may contribute to lower A β levels in APP/PS1 mice.

Choline converts homocysteine to methionine through the donation of methyl groups by choline's metabolite, betaine^{15,16}. During development, choline is a primary dietary source of methyl groups to produce S-adenosylmethionine, which is a substrate for epigenetic mechanisms^{44, 45}. Enriching the pool of methyl donors in early development leads to changes in epigenetic patterns that can be inherited through cell replication and persist throughout multiple generations⁴⁴. For example, Waterland and colleagues found that a high methyl donor diet was sufficient to prevent the transgenerational amplification of obesity in an obesity-prone mouse; benefits that were seen in two subsequent generations²¹. Notably, our results show downregulation of genes involved in histone modifications and chromatin remodeling suggesting that maternal ChS may have induced alterations in gene expression that led to reductions in brain Hcy and AD-pathology.

In conclusion, the present study demonstrates that supplementing the maternal diet with additional choline improves spatial memory and reduces A β pathology in APP/PS1 mice. The reduction in AD pathology is associated with decreased brain Hcy levels. Remarkably, we provide compelling evidence that maternal ChS also produces transgenerational benefits. If these findings generalize to humans, ChS may help mitigate the estimated increased prevalence rate of this insidious disease. Moreover, because the animal literature indicates beneficial effects of ChS for normal cognitive aging^{46, 47}, and recent evidence shows benefits in humans⁴⁸, this type of nutritional advice could be given to the general population, thereby providing substantial benefits of cognitive function throughout life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants to S.O. from the National Institute of Aging (2R01AG037637-07) and R.V. from the National Science Foundation (1606833).

References

1. Association As. 2018 Alzheimer's Association disease facts and figures. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association* 2018; 14: 367–429.
2. Dosunmu R, Wu J, Basha MR, Zawia NH. Environmental and dietary risk factors in Alzheimer's disease. *Expert Rev Neurother* 2007; 7(7): 887–900. [PubMed: 17610395]
3. Hu N, Yu JT, Tan L, Wang YL, Sun L, Tan L. Nutrition and the risk of Alzheimer's disease. *Biomed Res Int* 2013; 2013: 524820. [PubMed: 23865055]
4. Morris MC, Tangney CC, Wang Y, Sacks FM, Bennett DA, Aggarwal NT. MIND diet associated with reduced incidence of Alzheimer's disease. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association* 2015; 11(9): 1007–1014.
5. Berti V, Walters M, Sterling J, Quinn CG, Logue M, Andrews R et al. Mediterranean diet and 3-year Alzheimer brain biomarker changes in middle-aged adults. *Neurology* 2018; 90(20): e1789–e1798. [PubMed: 29653991]
6. Li J-G, Chu J, Barrero C, Merali S, Praticó D. Homocysteine Exacerbates β -Amyloid Pathology, Tau Pathology, and Cognitive Deficit in a Mouse Model of Alzheimer Disease with Plaques and Tangles. *Annals of neurology* 2014; 75(6): 851–863. [PubMed: 24644038]
7. Morris MS. Homocysteine and Alzheimer's disease. *The Lancet Neurology* 2003; 2(7): 425–428. [PubMed: 12849121]
8. Isobe C, Murata T, Sato C, Terayama Y. Increase of total homocysteine concentration in cerebrospinal fluid in patients with Alzheimer's disease and Parkinson's disease. *Life Sciences* 2005; 77(15): 1836–1843. [PubMed: 15935398]
9. Shen L, Ji H-F. Associations between Homocysteine, Folic Acid, Vitamin B12 and Alzheimer's Disease: Insights from Meta-Analyses. *Journal of Alzheimer's disease: JAD* 2015; 46(3): 777–790. [PubMed: 25854931]
10. Whalley LJ, Duthie SJ, Collins AR, Starr JM, Deary IJ, Lemmon H et al. Homocysteine, antioxidant micronutrients and late onset dementia. *European Journal of Nutrition* 2014; 53(1): 277–285. [PubMed: 23625136]
11. Obeid R, Herrmann W. Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia. *FEBS Letters* 2006; 580(13): 2994–3005. [PubMed: 16697371]
12. Ho PI, Collins SC, Dhitavat S, Ortiz D, Ashline D, Rogers E et al. Homocysteine potentiates β -amyloid neurotoxicity: role of oxidative stress. *Journal of Neurochemistry* 2001; 78(2): 249–253. [PubMed: 11461960]
13. Zhuo J-M, Portugal GS, Kruger WD, Wang H, Gould TJ, Pratico D. Diet-induced hyperhomocysteinemia increases amyloid-beta formation and deposition in a mouse model of Alzheimer's disease. *Current Alzheimer Research* 2010; 7(2): 140–149. [PubMed: 19939226]
14. Pacheco-Quinto J, Rodriguez de Turco EB, DeRosa S, Howard A, Cruz-Sanchez F, Sambamurti K et al. Hyperhomocysteinemic Alzheimer's mouse model of amyloidosis shows increased brain amyloid beta peptide levels. *Neurobiology of Disease* 2006; 22(3): 651–656. [PubMed: 16516482]
15. Olthof M J Brink E, B Katan M, Verhoeve P. Choline supplemented as phosphatidylcholine decreases fasting and postmethionine-loading plasma homocysteine concentrations in healthy men, vol. 822005, 111pp.
16. Steenge GR, Verhoeve P, Katan MB. Betaine supplementation lowers plasma homocysteine in healthy men and women. *The Journal of Nutrition* 2003; 133(5): 1291–1295. [PubMed: 12730412]

17. Ash JA, Velazquez R, Kelley CM, Powers BE, Ginsberg SD, Mufson EJ et al. Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice. *Neurobiology of Disease* 2014; 70: 32–42. [PubMed: 24932939]
18. Powers BE, Kelley CM, Velazquez R, Ash JA, Strawderman MS, Alldred MJ et al. Maternal choline supplementation in a mouse model of Down syndrome: Effects on attention and nucleus basalis/substantia innominata neuron morphology in adult offspring. *Neuroscience* 2017; 340: 501–514. [PubMed: 27840230]
19. Velazquez R, Ash JA, Powers BE, Kelley CM, Strawderman M, Luscher ZI et al. Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome. *Neurobiology of Disease* 2013; 58: 92–101. [PubMed: 23643842]
20. Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Molecular and Cellular Biology* 2003; 23(15): 5293–5300. [PubMed: 12861015]
21. Waterland RA, Travisano M, Tahiliani KG, Rached MT, Mirza S. Methyl donor supplementation prevents transgenerational amplification of obesity. *International Journal of Obesity* (2005) 2008; 32(9): 1373–1379. [PubMed: 18626486]
22. Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA et al. Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet* 2004; 13(2): 159–170. [PubMed: 14645205]
23. Caccamo A, Ferreira E, Branca C, Oddo S. p62 improves AD-like pathology by increasing autophagy. *Molecular Psychiatry* 2017; 22(6): 865–873. [PubMed: 27573878]
24. Velazquez R, Ferreira E, Tran A, Turner EC, Belfiore R, Branca C et al. Acute tau knockdown in the hippocampus of adult mice causes learning and memory deficits. *Aging Cell* 2018; e12775. [PubMed: 29749079]
25. Velazquez R, Tran A, Ishimwe E, Denner L, Dave N, Oddo S et al. Central insulin dysregulation and energy dyshomeostasis in two mouse models of Alzheimer's disease. *Neurobiology of Aging* 2017; 58: 1–13. [PubMed: 28688899]
26. Velazquez R, Shaw DM, Caccamo A, Oddo S. Pim1 inhibition as a novel therapeutic strategy for Alzheimer's disease. *Molecular Neurodegeneration* 2016; 11(1).
27. Ueland PM, Helland S, Broch OJ, Schanche JS. Homocysteine in tissues of the mouse and rat. *The Journal of Biological Chemistry* 1984; 259(4): 2360–2364. [PubMed: 6698970]
28. Holm PI, Ueland PM, Kvalheim G, Lien EA. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. *Clin Chem* 2003; 49(2): 286–294. [PubMed: 12560353]
29. Dobin SM, Malone VS, Lopez L, Donner LR. Unusual histologic variant of a low-grade fibromyxoid sarcoma in a 3-year-old boy with complex chromosomal translocations involving 7q34, 10q11.2, and 16p11.2 and rearrangement of the FUS gene. *Pediatr Dev Pathol* 2013; 16(2): 86–90. [PubMed: 23075075]
30. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; 15(12): 550. [PubMed: 25516281]
31. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 2000; 28(1): 27–30. [PubMed: 10592173]
32. Mi H, Poudel S, Muruganujan A, Casagrande JT, Thomas PD. PANTHER version 10: expanded protein families and functions, and analysis tools. *Nucleic Acids Res* 2016; 44(D1): D336–342. [PubMed: 26578592]
33. Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R et al. The Reactome pathway Knowledgebase. *Nucleic Acids Res* 2016; 44(D1): D481–487. [PubMed: 26656494]
34. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000; 25(1): 25–29. [PubMed: 10802651]

35. Chatr-Aryamontri A, Oughtred R, Boucher L, Rust J, Chang C, Kolas NK et al. The BioGRID interaction database: 2017 update. *Nucleic Acids Res* 2017; 45(D1): D369–D379. [PubMed: 27980099]
36. Benjamini YaH Yosef. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of Royal Statistical Society* 1995; 57(1): 289–300.
37. Wang J, Duncan D, Shi Z, Zhang B. WEB-based GENE SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res* 2013; 41(Web Server issue): W77–83. [PubMed: 23703215]
38. Mellott TJ, Huleatt OM, Shade BN, Pender SM, Liu YB, Slack BE et al. Perinatal Choline Supplementation Reduces Amyloidosis and Increases Choline Acetyltransferase Expression in the Hippocampus of the APPswePS1dE9 Alzheimer's Disease Model Mice. *PloS One* 2017; 12(1): e0170450. [PubMed: 28103298]
39. Zotova E, Holmes C, Johnston D, Neal JW, Nicoll JA, Boche D. Microglial alterations in human Alzheimer's disease following Abeta42 immunization. *Neuropathol Appl Neurobiol* 2011; 37(5): 513–524. [PubMed: 21166690]
40. Oikonomidi A, Lewczuk P, Kornhuber J, Smulders Y, Linnebank M, Semmler A et al. Homocysteine metabolism is associated with cerebrospinal fluid levels of soluble amyloid precursor protein and amyloid beta. *Journal of Neurochemistry* 2016; 139(2): 324–332. [PubMed: 27507672]
41. Agnati LF, Genedani S, Leo G, Forni A, Woods AS, Filafferro M et al. Abeta peptides as one of the crucial volume transmission signals in the trophic units and their interactions with homocysteine. Physiological implications and relevance for Alzheimer's disease. *J Neural Transm (Vienna)* 2007; 114(1): 21–31. [PubMed: 16969627]
42. Li JG, Chu J, Barrero C, Merali S, Pratico D. Homocysteine exacerbates beta-amyloid pathology, tau pathology, and cognitive deficit in a mouse model of Alzheimer disease with plaques and tangles. *Ann Neurol* 2014; 75(6): 851–863. [PubMed: 24644038]
43. Ho PI, Collins SC, Dhitavat S, Ortiz D, Ashline D, Rogers E et al. Homocysteine potentiates beta-amyloid neurotoxicity: role of oxidative stress. *J Neurochem* 2001; 78(2): 249–253. [PubMed: 11461960]
44. Jiang X, West AA, Caudill MA. Maternal choline supplementation: a nutritional approach for improving offspring health? *Trends in endocrinology and metabolism: TEM* 2014; 25(5): 263–273. [PubMed: 24680198]
45. Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. *The Journal of Nutrition* 2002; 132(8 Suppl): 2333S–2335S. [PubMed: 12163687]
46. Meck WH, Williams CL. Metabolic imprinting of choline by its availability during gestation: implications for memory and attentional processing across the lifespan. *Neuroscience and Biobehavioral Reviews* 2003; 27(4): 385–399. [PubMed: 12946691]
47. Meck WH, Williams CL, Cermak JM, Blusztajn JK. Developmental periods of choline sensitivity provide an ontogenetic mechanism for regulating memory capacity and age-related dementia. *Frontiers in Integrative Neuroscience* 2007; 1: 7. [PubMed: 18958235]
48. Caudill MA, Strupp BJ, Muscalu L, Nevins JEH, Canfield RL. Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: a randomized, double-blind, controlled feeding study. *The FASEB Journal* 2018; 32(4): 2172–2180. [PubMed: 29217669]

Figure 1. Maternal ChS ameliorates spatial memory deficits and reduces Aβ pathology. (a-b) Experimental design. Gen-1 offspring were exposed to either the CTL or ChS diet during gestation and lactation and kept on the CTL diet for the remainder of their lives. Mice were behaviorally tested on the MWM at 12 months of age. Gen-2 mice were never exposed to ChS and were on the CTL diet for their entire lives. These mice were tested on the MWM task at 14.5 months of age. (c) Body weight of 12-month-old Gen-1 mice. (d-e) Distance traveled and escape latency to the platform during the learning phases of the MWM. (f-h) Probe trials for the MWM. (i-j) Sections from APP/PS1 mice stained with an Aβ₄₂-specific antibody. (k) Quantitative analysis of the Aβ₄₂ load. (l-m) ELISA measurements for soluble and insoluble Aβ. (n-q) Western blot and quantitative analyses showing APP, C83 and C99. Data are presented as box plots. The center line represents the median value, the limits represent the 25th and 75th percentile, and the whiskers represent the minimum and maximum value of the distribution. * $p < 0.05$, ** $p < 0.01$.

Figure 2. Reduced AD-like pathology in Gen-2 mice.

(a) Body weight of 14.5-month-old Gen-2 mice. (b–c) Distance traveled and escape latency to the platform during the learning phases of the MWM. (d–f) Probe trials for the MWM. (g–h) Sections from APP/PS1 mice stained with an Aβ₄₂-specific antibody. (i) Quantitative analysis of the Aβ₄₂ load. (j–k) Soluble and insoluble Aβ levels, measured by ELISA. (l–o) Western blot and quantitative analyses showing APP, C83 and C99. Data are presented as box plots. The center line represents the median value, the limits represent the 25th and 75th percentile, and the whiskers represent the minimum and maximum value of the distribution.

* $p < 0.05$, ** $p < 0.01$.

Figure 3. Validation of altered hippocampal gene expression and protein-protein network analysis in APP/PS1 mice.

(a–d) qRT-PCR data and western blot analyses for *Pin1*, *H2-Ab1*, and *Rbm25*. (e–g) Protein-Protein Network analyses that included (e) the 27 genes that were differentially expressed, (f) the 19 genes that were significantly downregulated and (g) the genes that were significantly upregulated. The large circles are the input genes (differentially expressed genes in our input list), while the others are top-ranking neighbors. Data are presented as box plots. The center line represents the median value, the limits represent the 25th and 75th percentile, and the whiskers represent the minimum and maximum value of the distribution.

* $p < 0.05$.

Figure 4. Transgenerational reduction of activated microglia in APP/PS1 mice. (a, c) Photomicrographs of hippocampal tissue from Gen-1 and Gen-2 APP/PS1 mice fluorescently stained for Iba1 and CD68. (b, d) Quantitative analysis of CD68/Iba1 colocalization. Data are presented as scatterplots with mean $\pm$ SE. * $p < 0.05$.

Figure 5. Transgenerational reduction of brain homocysteine by altering methionine cycle. Transgenerational reduction of brain homocysteine by altering methionine cycle. (a–b) Hcy and methionine levels in Gen-1 mice. (c–e) Western blot analysis of betaine-homocysteine-methyltransferase (BHMT) in Gen-1 mice. (f–g) Hcy and methionine levels in Gen-2 mice. (h–i) Western blot analysis for BHMT in Gen2 mice. For box plots the center line represents the median value, the limits represent the 25th and 75th percentile, and the whiskers represent the minimum and maximum value of the distribution. For scatterplots, data are presented as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$