Investigation of cellular and molecular targets in the brain of mice given intranasal GHK peptide to treat age-related cognitive decline

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Abstract

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There are currently 1.2 billion people worldwide over the age of 60 and this number is projected to double by 2050. With the world population over 60 reaching 22% within the next 50 years, there is an increasing need to understand the mechanisms and processes of aging. An area of major interest is brain aging given that approximately 2 out of 3 Americans experience some level of cognitive impairment by age 70. Neuropathology associated with this cognitive impairment can lead to more severe neuropathology associated with Alzheimer's disease and other dementias providing a need to develop interventions to treat and potentially prevent or reverse age related cognitive decline. One particular therapeutic of interest is a naturally occurring peptide called glycyl-L-histidyl-L-lysine (GHK) as recent studies suggest that GHK in its Cu bound form has the potential to age related cognitive decline. In order to further investigate the potential of GHK-Cu as a therapeutic for age related cognitive decline, mouse hippocampal tissue from 20-month-old C57BL/6 mice treated with 15mg/kg of intranasal GHK-Cu daily for 8 weeks were used for immunohistochemistry and RNA sequencing to discover cellular and molecular targets of this peptide. Results suggested that mice treated with intranasal GHK-Cu had lower expression of potentially harmful astrocytes. RNA sequencing results revealed that mice treated with GHK-Cu had upregulation or downregulation of various gene pathways in a way that supports healthy brain aging. Sex differences were also observed with certain pathways being affected differently in males and females. These findings provide rationale for further investigation of GHK-Cu as a therapeutic for age related cognitive decline in preclinical and clinical studies.

Investigation of cellular and molecular targets in the brain of mice given intranasal GHK peptide to treat age-related cognitive decline

Introduction

With the proportion of people over 60 reaching 22% by 2050, it is essential to understand the molecular, cellular and system processes that occur as we age (World Health Organization). One aspect of aging of major interest is brain aging and cognitive decline. Brain aging and age-related cognitive decline is associated with varying degrees of dysfunction ranging from a minimal decline in memory as a result of aging to more severe dysfunction that is not due to the normal processes of aging. This more severe dysfunction can be categorized into mild cognitive impairment (MCI) and dementia (UCSF Memory and Aging). MCI is used in cases where an individual presents increased dysfunction compared to typical age related decline but is still able to function in their daily lives without any major impairments or need for assistance. Dementia is used when individuals can no longer function independently. Age related cognitive decline (ARCD) describes the early stages of cognitive decline that have not yet impacted the individual to the point of impairing day to day life. There is a crucial need to understand ARCD and develop therapeutic interventions given that neuropathology related to ARCD can increase the risk for more severe neuropathology associated with MCI and dementias such as Alzheimer's Disease (Sierra, 2017). ARCD is very common and while it is not at the level of cognitive incapacitation, it still results in memory deficits that may have adverse effects on well-being in older people.

Unfortunately, few interventions to treat and reverse ARCD have been developed. Many lifestyle changes, medications and therapies have been studied to treat ARCD and dementia, however, each treatment comes with challenges. One lifestyle change that has been given a significant amount of attention is physical exercise, specifically aerobic exercise. Some studies have shown positive effects of aerobic exercise on treating MCI, but as a whole, studies of aerobic exercise have demonstrated conflicting outcomes. High intensity resistance training has also been shown to promote cognition as well as protect AD vulnerable hippocampal subfields for at least 12 months intervention (Broadhouse, 2020). While various forms of exercise have been shown to slow cognitive decline and potentially delay dementia onset, not all individuals are able to exercise often or consistently enough to see these positive effects. Many drugs also have the potential to be used in order to slow or prevent the progression of cognitive decline. Some of these drugs include cholinesterase inhibitors, lecanemab and donanemab. Cholinesterase inhibitors are commonly prescribed for MCI, however, studies have not shown benefit on cognitive outcomes or progression from MCI to AD (Petersen, 2018). Lecanemab is an amyloid sequestering agent which received accelerated Food and Drug Administration approval for the treatment of mild dementia due to AD MCI. Even though lecanemab has statistically significant effects on cognition these effects may not be clinically significant. Another drawback is the cost which would total $126 billion dollars per year if the entire target population was treated (Burke,

2023). Donanemab is a monoclonal antibody used for the treatment of MCI. While donanemab showed clinical significance, it requires IV infusions every 4 weeks (Sims, 2023). An intense treatment such as this may not be accessible to many individuals for a variety of reason. Although there are many interventions for cognitive decline, there is a lack of treatments that are clinically effective affordable and widely accessible.

Aside from the previously mentioned limitations, another limitation of these drugs is that they target processes involved with neurons. While this is important, it also important to acknowledge and target other cell types in the brain. Astrocytes are the most abundant cell type in the brain and play a role in maintaining homeostasis in the central nervous system (CNS) including preserving the integrity of the blood-brain barrier (BBB), the reuptake of neurotransmitters, synaptogenesis, the production of trophic factors supporting neurons and oligodendrocytes, and the control of the immune system (Vu, 2023). Although astrocytes do not conduct electrical signals like neurons and other cell types in the CNS, they are important cells in maintaining function of neurons by providing metabolic and structural support (Wei, 2023). Astrocytes also promote neuronal remodeling through the secretion of growth factors and modulation of the extracellular environment, producing the majority of extracellular matrix (ECM) components in the brain (Dzyubenko, 2025). The mechanism by which astrocytes function in the ECM is similar to that of fibroblasts in systemic organs. As we age there is a loss of morphological structure in star-shaped astrocytes that can lead to a decline in their functionality (Zhang, 2025). In addition, there is an accumulation of reactive astrocytes and aged astrocytes which trigger neuroinflammation and have detrimental impacts on the tissue microenvironment, ultimately leading to ARCD.

Given that astrocytes play a vital role in maintaining brain health and cognitive function specifically in the ECM, it is of interest to investigate drugs that target astrocytes and the ECM. Targeting specific components of the ECM can be used as a way to treat a variety of different diseases. Drugs in various classes such as nonsteroidal anti-inflammatory drugs (NSAIDs), corticoids, hypolipidemic drugs, antibiotics, TNF inhibitors and anti-cancer drugs have been shown to target the ECM in various ways to target multiple disease conditions such as inflammation, lung disease, cardiac disease, cancer, and pulmonary fibrosis (Ahmad, 2020). While drugs targeting components of the ECM have shown to be effective in many of the diseases listed previously, there aren’t any targeting the brain and cognitive decline. Developing a safe novel therapeutic targeting the ECM and astrocytes is of great interest.

One particular therapeutic of interest is glycyl-L-histidyl-L-lysine (GHK). GHK is a copper binding tripeptide which is naturally found in human plasma but declines as we age (Pickart, 2018). It has also been shown to target fibroblasts in the skin. In irradiated human dermal fibroblasts, GHK-Cu increased the expression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Dou, 2022). Inflammation is involved in wound and skin healing with excessive inflammation delaying healing and leading to scar formation. GHK-Cu has been shown to reduce TNF-alpha induced secretion of proinflammatory cytokine IL-6 in normal human dermal fibroblasts thus reducing harmful inflammation. GHK has also been shown to restore replicative vitality

to fibroblasts from patients after anticancer radiation therapy that damages cellular DNA (Pickart, 2015). Studies have also shown that GHK may be able to improve tissue regeneration by restoring activity of genes involved in the TGF-beta pathway.

TGF- β expression increases in the aging brain thus contributing to a decline in neurogenesis by exacerbating pathomechanisms such as reactive astrogliosis that are responsible for the development of dementia (Kandasamy, 2020). There is evidence that TGF- β plays a role in synaptogenesis. This is of interest as astrocytes facilitate both inhibitory and excitatory synapse formation. TGF- β regulates astrocyte reactivity through several different mechanisms. One mechanism is through directly activating the GFAP promoter which increases GFAP expression in astrocytes. TGF- β also activates many of the known molecular triggers and modulators of reactive astrogliosis (Luo, 2022). TGF- β expression is increased in astrocytes in the aging brain and has been linked to breakdown of the BBB which is an early biomarker of cognitive decline. Taken together, these findings provide rationale for the use of GHK-Cu as a clinical treatment for ARCD.

In order to conduct preclinical studies and investigate interventions such as GHK-Cu for age related cognitive decline, a mouse model can be used. One particular model of value is using the mouse strain C57BL/6 as ARCD occurs in old C57BL/6 mice allowing for a model to test drugs and therapeutics for cognitive decline, impairment and dementia (Daneshjoo, 2022). Characterization of ARCD in C57BL/6 mice includes age dependent cognitive decline measured by reduction in assessments such as learning ability, working memory. This model also provides translational value as there have been well established comparisons between mouse and human ages (Fig.3). Given that aging is the greatest risk factor for the development of age related diseases, targeting processes of aging is necessary to prevent the onset of severe disease such as dementia.

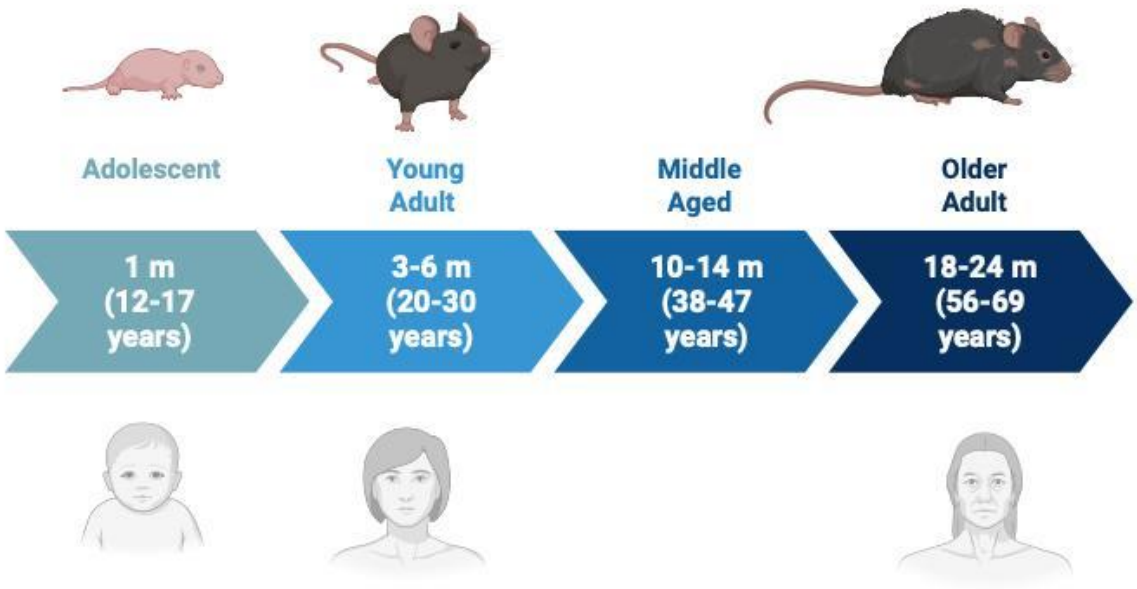

Figure 1. Comparison between mouse age and human age. Mouse age is quantified in months and human age in years.

One unpublished study has already demonstrated the positive effects of GHK-Cu on ARCD (Tucker, 2023). In this study, male and female 20-month-old C57BL/6 mice were obtained from the National Institute on Aging Aged Rodent tissue colony, contracted by Charles River, Inc. Mice were group housed with up to five mice per cage in a specific pathogen-free (SPF) facility at the University of Washington under a 12-hour light and 12-hour dark cycle with a room temp of 25 ± 4°C and reverse osmosis water in an automatic watering system. Mice were group housed with up to five mice per cage. Each cage was given three nestlets (Ancare Corp, Bellmore, NY) in order to provide activity and stimulation. Mice were monitored daily for health issues. Cages and nestlets were replaced bi weekly. Upon arrival mice were acclimated to handling procedures for two weeks prior to the start of the study in order to reduce stress response and acclimate the mice to the intranasal drug delivery. One week after acclimation, treatment began and was continued for 8 weeks. All procedures were approved by the University of Washington IACUC (protocol 2174-31).

GHK-Cu was administered intranasally as a GHK-Cu complex (Active Peptide, Cambridge, MA) at a dose of 15mg/kg. Mice were given 15 mg/kg in 20 µL saline daily between 7 AM and 10 AM over the 8-week period.

At 0, 4, and 8 weeks of treatment a spatial navigation learning task called Box Maze was performed. Mice were placed in the center of a clear box lined with reflective material, using a bright overhead light as a stimulus. The box contained seven false exits and one true exit. Each side of the box contained a large image as well as a smaller image directly above each exit hole. For each of the four trials, mice were placed starting in a different direction making sure each mouse was placed in the same sequence. The mice were allowed 120 seconds to find the escape exit which lead to a dark rest cage lined with paper towels where they rested for 30 seconds before being transferred to their home cage for 90 seconds. If mice did not find the escape exit within the allotted 120 seconds, they were shown and encouraged to enter the escape exit. A successful escape was defined as the mouse having its head and all four paws in the escape tube. If the mouse failed to find the escape hole within 120 seconds, 120 seconds was recorded. Each false exit hole and the true escape hole was thoroughly cleaned with 70% ethanol between trials. The tube leading to the rest cage was washed with soap and water between cages. Y maze was administered as a behavioral spatial test of working memory. In this task, mice were placed in the cross section of a Y-shaped maze with three arms spaced 120° apart and allowed to explore the maze freely for 5 minutes during which the animal's position is recorded, noting which arm the mouse entered. Each arm was assigned a letter (A, B or C) for recording purposes. Each mouse was placed in the same arm to begin the trial. Total number of entries into arms of the maze, number of same-arm entries, number of previous-arm entries and number of new-arm entries were recorded. Data was recorded as percent spontaneous alternation which is calculated as the number of times the mouse completed a triad or loop through all three arms divided by total number of arm entries minus two. Spontaneous alternation is the capacity of a mouse to explore a new arm of the maze rather than returning to an arm they have more recently visited. This can be used as an indicator of working memory over a short period of time. Y-maze was administered at 0, 4 and 8 weeks of treatment.

In the y-maze, male and female mice treated with GHK-Cu for 8 weeks showed a higher spontaneous alternation percentage than control cohorts. This indicates increased cognitive ability. After 4 weeks of treatment only male mice showed percent alternation suggesting that females require a longer treatment period. In the box maze male and female mice treated with GHK-Cu showed decreased escape times by the fourth trial compared to mice treated with saline. Given these positive effects of GHK-Cu on behavioral measures of cognition, it is of interest to further investigate the role GHK-Cu plays in cognition in terms of cellular and molecular targets.

The expectation is that astrocytes will be targeted, but the molecular pathways being affected by GHK-Cu are not known. One approach to discover molecular targets is through RNA sequencing. In 2017, Pickart et al used the Broad Institute's Connectivity Map (cMap) to acquire gene expression data of three gene expression profiles of cells

treated with 1 micromolar of GHK. The PC3 (human prostate cancer) cell line was used for two of the profiles, and the MCF7 (human breast cancer) was used for the third. GenePattern was used to analyze the data obtained from cMap. The purpose of this study was to investigate the effect of GHK on expression relevant to nervous system function and cognitive decline. Results from this study showed that GHK induces a 50% or greater change of expression in 31.2% of human genes, affecting genes linked to multiple biochemical pathways including the nervous system. They found that 18 genes with significant antioxidant activity were affected by GHK. They also found that GHK is primarily stimulatory for gene expression of DNA repair genes suggesting an increase in DNA repair activity. Astrocytes were also affected by GHK with 15 astrocyte associated genes being upregulated and 6 being downregulated. This study provided promising results and rationale for the use of GHK-Cu as a treatment for cognitive decline.

My project is designed to further explore the role of astrocytes in alleviating ARCD in mice treated with intranasal GHK peptide as well as identify specific pathways associated with the reduction of ARCD in mice treated with intranasal GHK peptide. It is proposed that GHK-Cu will alleviate age-related cognitive decline by targeting astrocytes and pathways of aging.

Materials and Methods

Sample Processing. After the 8 week treatment period, mice were euthanized via CO2 inhalation followed by decapitation as a secondary method of euthanasia. Brain and systemic organs were collected and placed into cassettes for formalin fixation. Brains were sectioned sagittally with the left side being frozen at -80°C and the right side being fixed. Brain tissue was also flash frozen with the brain being divided into four sections: hippocampus, cerebellum, frontal cortex, and remainder of the brain. Body tissues and organs were also sectioned for formalin fixation and flash freezing. After 72 hours, the formalin fixed tissues were transferred to 70% alcohol and brain tissue was placed in 1X PBS. Formalin fixed organs were embedded into paraffin blocks and slides with 5 um brain slices were made for immunohistochemistry by the Ladiges Histology Core.

Immunohistochemistry. Staining was performed using an AbCam IHC kit (HRP/DAB Rabbit Kit: ab64261, HRP). Slides were first rehydrated with xylene in two separate baths for 10 minutes each, then 100% ethanol for twice for 10 minutes. Slides were then immersed in 95% ethanol, 70% ethanol and deionized water twice for 5 minutes each. Following rehydration, slides were incubated in a 1X Citrate Buffer Antigen Retrieval solution at 98°C in a hot water bath for 20 minutes. After the antigen retrieval, slides were incubated with a hydrogen peroxide block for 10 minutes followed by two 5-minute washes in TBST. The slides were then incubated in a protein block for 10 minutes followed by two 5-minute TBST washes. The slides were incubated overnight with primary antibodies in a Tris-buffered saline, 0.1% Tween® 20 Detergent solution (TBST) in a humidified chamber at 4°C. The next day slides were washed with three 5-minute TBST washes. Then the slides were incubated with Biotinylated Goat Anti-Mouse followed by three 5-minute TBST washes and Streptavidin Peroxidase followed by three 5-minute TBST washes. Finally, DAB chromogen was applied to the slides for

various amounts of time depending on the antibody used. This was followed by three washes with TBST and one with deionized water. Slides were then dehydrated with 70% ethanol, 100% ethanol and xylene for one minute each before being mounted with mounting media and a coverslip. The antibodies used were GFAP (1:1500, Invitrogen: PA1-10019), TGF- β (1:50, Abcam ab215715), synaptophysin (1:250, Invitrogen MA5-16402), PSD95 (1:250, Abcam ab18258), MCP1 (1:800, Novus NBP1-07035) and S100β (Abcam ab52642). All antibodies had either been previously optimized by the Ladiges Lab or were optimized for this study. Once dry, slides were cleaned and imaged at 40X under a Nikon Eclipse E400 microscope with a Nikon D7100 camera through a microscope camera adaptor. Images were then uploaded onto a Google drive where slides are separated into folders organized by stain, sex and treatment. Next images were uploaded to Qupath for quantitative analysis of DAB staining. Once images are in the QuPath app H-DAB (diaminobenzidine) was selected. The region of interest (ROI) was then selected. A threshold was then set to measure positive percent DAB of the total ROI area.

RNA isolation, sequencing, and analysis. Flash frozen hippocampus tissue was used. Twelve samples were selected for both males and females with six treated and six control samples for each sex. The tissue was first weighed then placed in tubes with ceramic beads and trizol which was then homogenized. RNA was then separated from non RNA material through centrifugation. The supernatant containing the RNA was then incubated with chloroform and centrifuged. The aqueous phase was then precipitated with isopropyl alcohol and centrifuged. This left a pellet of RNA which was washed with ethanol. The washed pellet was then incubated in Rnase free water at room temperature and then at 55°C. The RNA concentration was then determined via nanodrop. The RNA was then further purified via RNA cleanup buffers using the RNA Mini kit (Qiagen). RNA concentration was then determined via the nanodrop. If the concentration was below 20 ng/ul, the sample was discarded. Samples were stored at -80°C. Samples were sent to Novogene for sequencing. Once received by Novogene, samples underwent quality control before being approved for sequencing. Samples were processed by Novogene using the Illumina Novoseq X-Plus platform resulting in bulk-mRNA paired-end fastq files. For analysis, the browser-based cluster computing platform SciDAP (SciDAP.com) was used. The SciDAP analysis pipeline is available online at https://github.com/datirium. Experimental samples were aligned using Trim-Galore and Star with the mm10 mouse genome as the reference genome. Differential expression between sample cohorts was generated using DESeq2, where the false discovery rate was set to 0.1 and adjusted using the Wald test. GSEA analysis was generated using the Hallmark gene sets (GSEA) and significance for the false discovery rate (FDR) was set at 0.1. All RNA quantification was performed within the SciDAP pipeline. All RNA statistics were performed within the SciDAP pipeline. All DESeq2 and GSEA data visuals were generated within the SciDAP pipeline.

Statistics. Differences between cohorts were calculated using one-way ANOVA. T-test was used for comparisons between treated and control cohorts for each sex. GraphPad Prism was used to create all graphs and statistical analysis. Significance was defined as $p < 0.05$, and $FDR < 0.1$.

Results

This study focused on the effects of GHK-Cu on pathways of aging, cells and mechanisms associated with brain aging specifically in the hippocampus. This brain region was chosen as it presents neural stem cells in the sub granular zone of the dentate gyrus, which declines with age (Gaspar-Silva, 2023). This decline has implications for learning and memory in rodents which makes them more susceptible to ARCD. Several markers of aging were decreased in mice treated with GHK-Cu. Markers for reactive astrocytes were also decreased in in mice treated with GHK-Cu.

Mice treated with GHK-Cu showed a decrease in astrocytes

Aging leads to a functional decline in astrocytes. There is also an increase in reactive astrocytes which lead to inflammation leading to ARCD. Two markers of astrocyte reactivity were selected: glial fibrillary acidic protein (GFAP) and S100beta. GFAP is primary used to identify astrocytes and is considered a marker for reactive astrogliosis. S100beta is a calcium binding protein primary found in astrocytes. It plays a stimulatory role in astrocyte proliferation and has been shown to be higher in patients with AD. GFAP showed significant differences between treated and control groups in both males and females. Density was decreased in treated cohorts compared to control cohorts (Figure 2). Treated and control males had decreased density of GFAP compared to females in both the treated and control groups. This indicates that GHK-Cu is targeting astrocyte reactivity. There were no significant differences observed in the staining density of S100beta between treated and control cohorts. Females showed a non-significant trend with lower density expressed in the treated cohort compared to the male cohort. Males did not show any differences between treated and control cohorts. This suggests that GHK-Cu does not play a role in the expression of S100beta in the hippocampus.

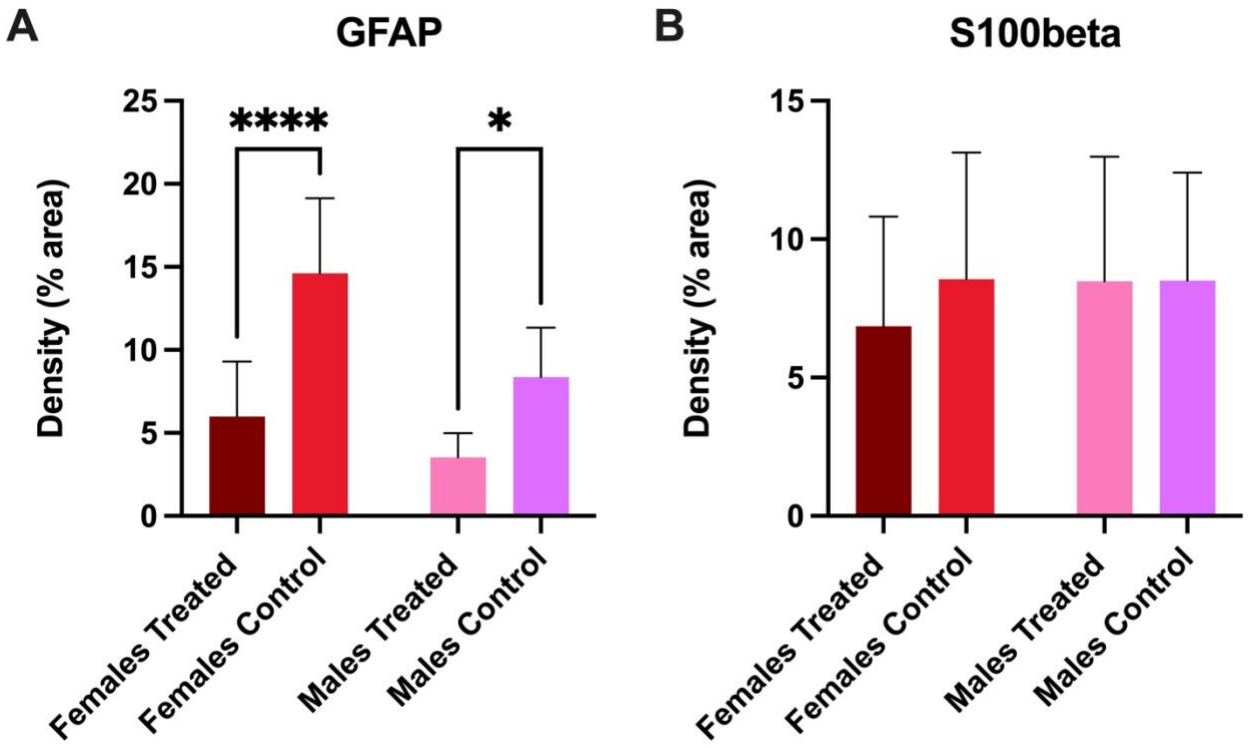

Figure 2. Density of IHC staining for astrocytes in the hippocampus quantified by Qupath (A) Male and female mice treated with GHK-Cu showed a decrease in GFAP. (B) There were no significant differences observed between treated and untreated cohorts in S100beta expression.

Presynaptic integrity was increased in female mice treated with GHK-Cu

Astrocytes are closely associated with synapses, playing a role in regulating synapse formation, function and plasticity. Two markers were chosen to look at synaptic integrity. Synaptophysin was used as a pre-synaptic marker and PSD95 was used as a post synaptic marker. Female mice treated with GHK-Cu had increased density of synaptophysin compared to control mice (Figure 3). There were no significant differences observed in males between treated and control mice. Neither males nor females showed significant differences in PSD95 staining densities between cohorts. These results suggest that GHK-Cu does not have an effect on pre and post synaptic integrity in females and on post synaptic integrity in females. Female mice showed a much more robust difference in GFAP in treated mice compared to control mice which could be correlated to the increase in synaptophysin. Even though male mice also showed a significant difference in GFAP, the positive effects of GHK-Cu may not have been enough to make a difference on synaptic integrity.

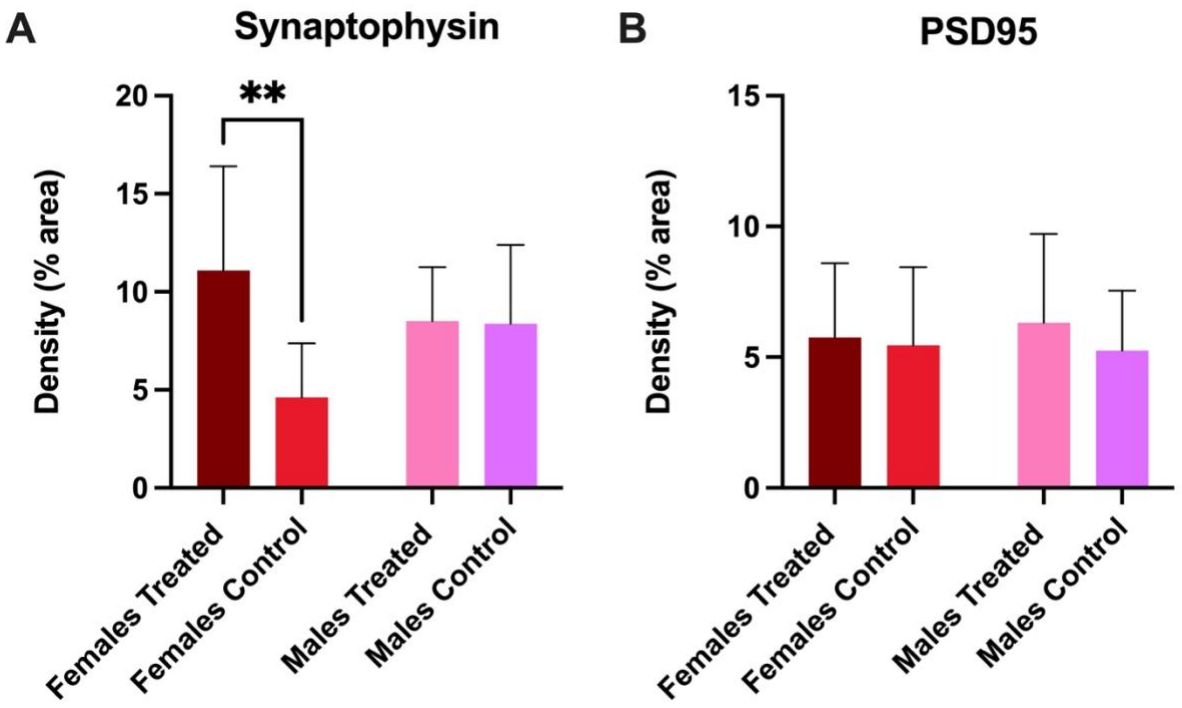

Figure 3. Density of IHC staining for markers of pre and post synaptic integrity in the hippocampus. (A) Female mice showed a significant increase of synaptophysin in treated mice compared to control mice. No differences were observed between male cohorts. (B) No significant differences were observed in PSD95 between treated and control in both males and females.

The TGFbeta signaling pathway was not affected by GHK-Cu treatment

Aging leads to increased expression of TGF- β. This increased expression leads to an increase in reactive astrocyte's which in turn leads to cognitive decline. No significant differences were observed between treated and control groups in both males and females, however, there were non-significant trends observed (Figure 4). Both treated males and females showed a decrease in density compared to controls. This suggests that GHK-Cu is not having an effect on the TGF- β pathway.

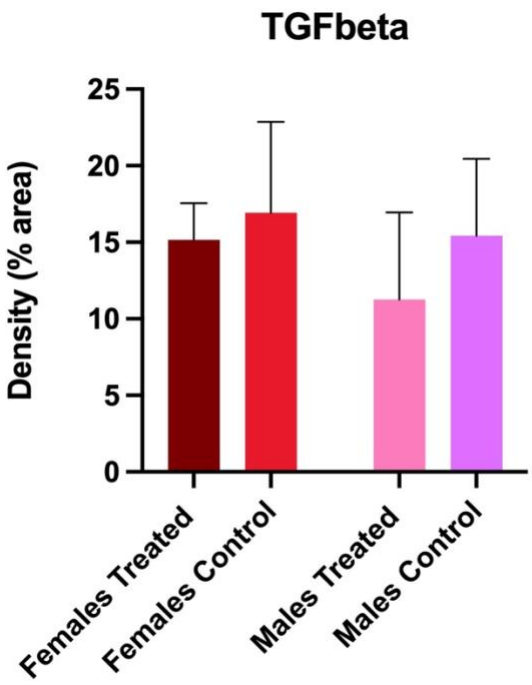

Figure 4. Density of IHC staining for TGFbeta in the hippocampus. Male and female mice treated with GHK-Cu showed a nonsignificant decrease in TGFbeta compared to control cohorts.

With these observations, it was decided to actively look for genes that were affected by GHK using RNA sequencing. RNA sequencing data revealed many pathway and gene differences between treated and control mice. Upregulation and downregulation of hallmark pathways was found using Gene Set Enrichment Analysis (GSEA) to determine whether an a priori set of genes shows statistically significant differences between the treated and control groups. The hallmark gene sets were chosen as they summarize and represent specific well-defined biological states or processes and display coherent expression. The GSEA analysis provided an enrichment score (ES) as the primary readout. The ES reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes. A positive ES indicates gene set enrichment at the top of the ranked list and a negative ES indicates gene set enrichment at the bottom of the ranked list. A greater magnitude ES indicates the genes within the set have a higher correlation with the phenotype. In order the analyze the GSEA results, the normalized enrichment score (NES) is most commonly used as it accounts for differences in gene set size and correlations between gene sets. This allows for comparison across gene sets. Table 1 ~~below~~ lists hallmark pathways that were significantly (p-value <0.05, FDR < 0.1) up or down regulated in treated mice compared to control mice. Male mice showed up regulation of 5 pathways and down regulation of 9 pathways. Females showed up regulation in 12 pathways and down regulation in 3 pathways.

Male			Female		
Pathway	**Regulation**	**NES**	**Pathway**	**Regulation**	**NES**
UV Response DN	Up	4.932	IL2 STAT5 Signal	Up	3.563
Mitotic Spindle	Up	3.413	Interferon Alpha	Up	3.546
Androgen Response	Up	3.234	Interferon Gamma	Up	3.499
Hedgehog Signaling	Up	3.192	Epithelial Mesenchymal Transition	Up	3.425
G2M Checkpoint	Up	3.17	TGFbeta	Up	3.112
UV Response UP	Down	-2.766	Bile Acid Metabolism	Up	3.102
Cholesterol Homeostasis	Down	-2.8	Estrogen Response	Up	3.022
Fatty Acid Metabolism	Down	-2.806	Xenobiotic Metabolism	Up	3.012
MYC Targets V1	Down	-2.809	Coagulation	Up	2.864
Xenobiotic Metabolism	Down	-3.047	Androgen Response	Up	2.789
Adipogenesis	Down	-3.087	ILK JAK STAT3 Signaling	Up	2.784
ROS	Down	-3.409	P53	Up	2.732
DNA Repair	Down	-4.076	P13k AKT MTOR	Down	-3.15
Oxidative Phosphorylation	Down	-5.438	Oxidative Phosphorylation	Down	-4.203
			MYC Targets V1	Down	-4.312

Table 1. Summary of regulation and NES scores of hallmark pathways in mice treated with GHK-Cu. Male mice showed up regulation of 5 pathways and down regulation of 9 pathways. Females showed up regulation in 12 pathways and down regulation in 3 pathways. All pathways listed had p-value <0.05 and FDR <0.1.

Genes associated with the TGFbeta pathway were upregulated in female mice treated with GHK-CU

Female mice treated with GHK-Cu had a positive NES score indicating upregulation of genes associates with the TGFbeta signaling pathway. Males treated with GHK-Cu also showed upregulation of genes in this pathway, however the upregulation was not statistically significant. These results were unexpected as it was predicted that GHK-Cu

would down regulate genes in this pathway. This was also the expected result given that astrocyte reactivity was decreased in mice treated with GHK-Cu. While GHK-Cu may be decreasing TGFbeta, there could be other factors at play that caused the upregulation of the TGFbeta signaling pathway.

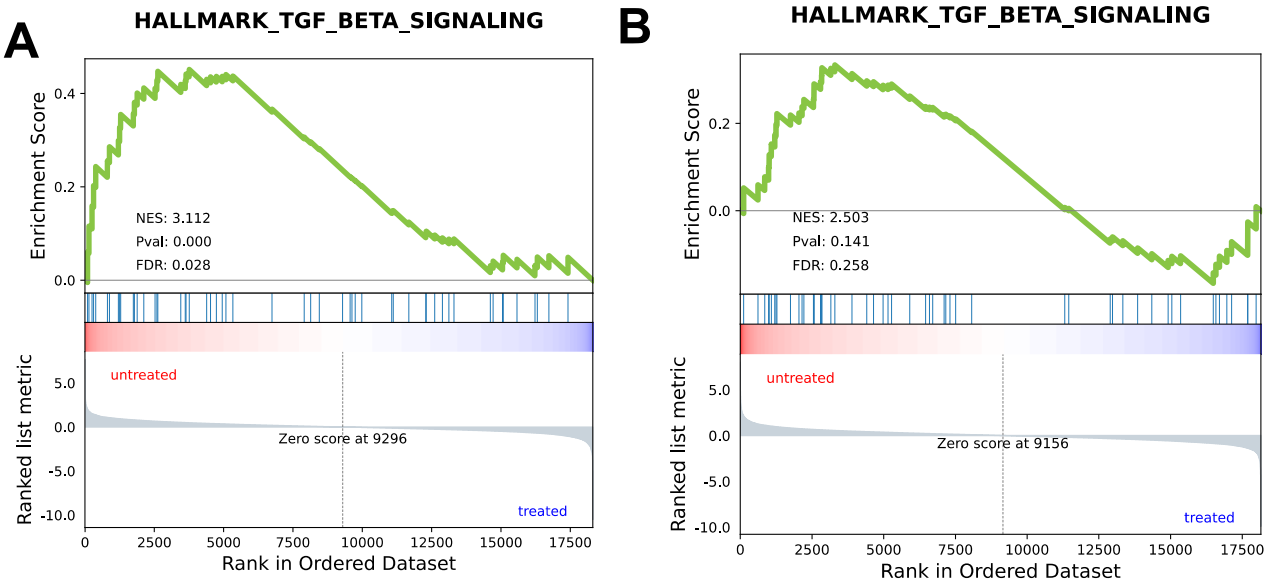

Figure 5. GSEA enrichment plots for the TGFbeta signaling pathway(A) Genes associated with the TGFbeta pathway were upregulated in female treated mice (p-val = 0.000) (B) Genes associated with the TGFbeta pathway were upregulated in male treated mice.

In male mice treated with GHK-Cu cell Cycle associated genes were upregulated and DNA repair associated genes were downregulated

As humans age there in an increase in DNA damage and lower ability to repair DNA which can harm the ability of a cell to successfully proceed through the cell cycle. The G2M checkpoint is a cell cycle checkpoint that ensures DNA integrity before a cell enters mitosis. P53 is involved in functions such as tumor suppression, DNA repair, cell cycle regulation (G1, G2, S phase), apoptosis and stress response. The mitotic spindle is necessary during cell division to ensure that chromosomes segregate accurately. Hedgehog signaling plays a role in regulating cell cycle progression and has been shown to be downregulated with age contributing to age related inflammation. Upregulation of hedgehog signaling has been shown to have potential in increasing DNA damage tolerance. Females treated with GHK-Cu showed downregulation of genes associated with DNA repair (Figure 6). Males treated with GHK-Cu showed significant downregulation of genes associates with DNA repair. Females treated with GHK-cu showed downregulation of genes associated with the G2M checkpoint. Males treated with GHK-cu showed significant upregulation of genes associated with the G2M checkpoint. Females treated with GHK-cu showed upregulation of genes associated with hedgehog signaling. Males treated with GHK-cu showed significant upregulation of genes associated with hedgehog signaling. Females treated with GHK-cu showed downregulation of genes associated with the mitotic spindle. Males treated with GHK-cu showed significant upregulation of genes associated with the mitotic spindle. Females

treated with GHK-cu showed significant upregulation of genes associated with the p53 pathway. Males treated with GHK-cu showed downregulation of genes associated with the p53 pathway. These results show that GHK-Cu is positively affecting certain pathways associated with the cell cycle and DNA damage while negatively affecting other pathways. The downregulation of genes associated with DNA in males suggests that GHK-Cu is negatively affecting the ability to repair damaged DNA in the hippocampus. The upregulation of genes associated in various aspects of the cell cycle in males suggests that GHK-Cu is aiding in the progression of the cell cycle. Although there is downregulation of DNA repair, it is not necessarily having a negative impact on cells ability to progress through the cell cycle. Even though there were no significant effects of GHK-Cu on the DNA repair hallmark genes in females, p53 was upregulated suggesting that GHK-Cu is aiding in DNA repair as well as other processes such as genome stability. There are also sex differences observed with some pathways being differently regulated between males and females.

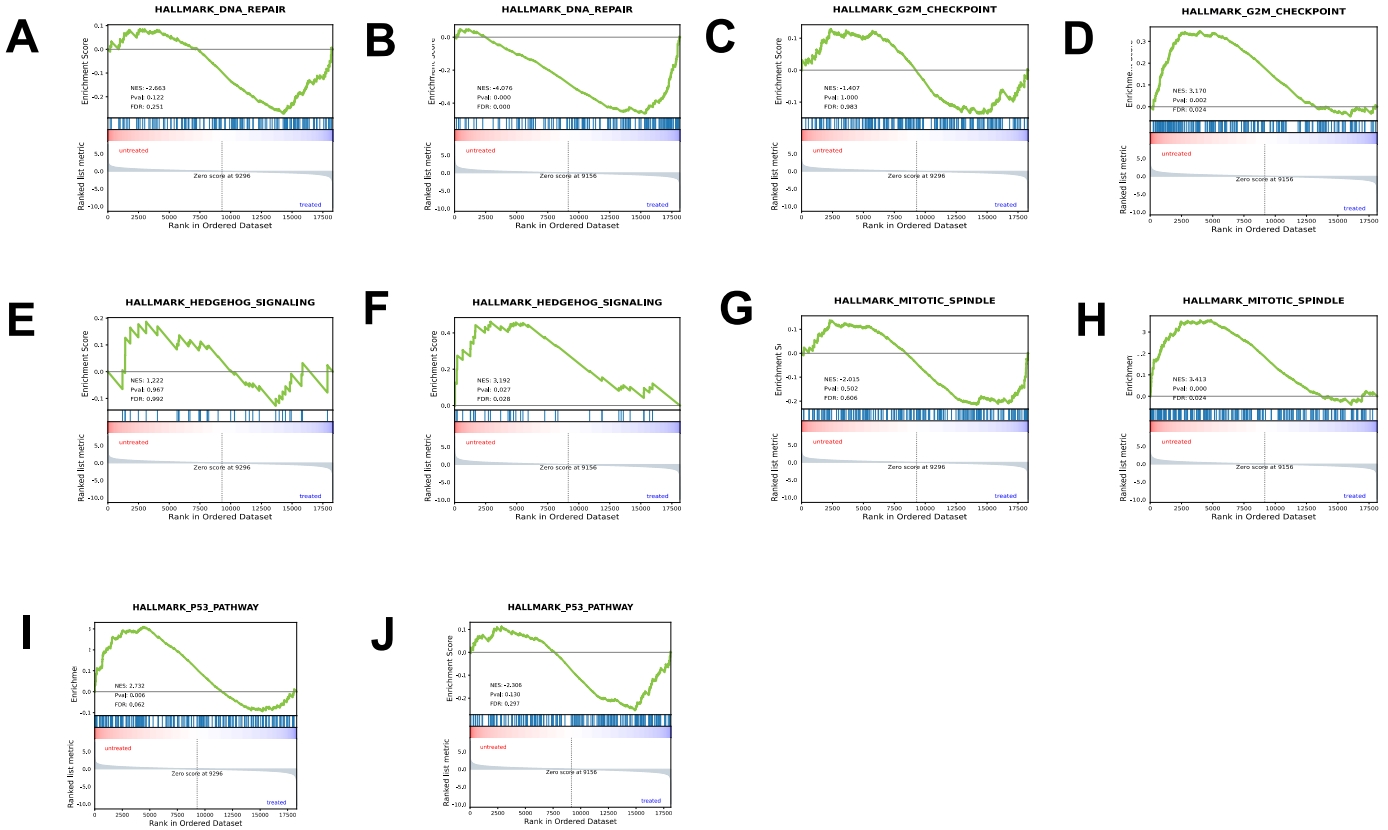

Figure 6. GSEA enrichment plots for pathways associated with the cell cycle and DNA damage. (A) Females treated with GHK-Cu showed downregulation of genes associates with DNA repair. (B) Males treated with GHK-Cu showed downregulation of genes associates with DNA repair. (C) Females treated with GHK-cu showed downregulation of genes associated with the G2M checkpoint (D) Males treated with GHK-cu showed upregulation of genes associated with the G2M checkpoint (E) Females treated with GHK-cu showed upregulation of genes associated with hedgehog signaling (F) Males treated with GHK-cu showed upregulation of genes associated with hedgehog signaling (G) Females treated with GHK-cu showed downregulation of genes associated with the mitotic spindle (H) Males treated with GHK-cu showed upregulation of genes associated with the mitotic spindle (I) Females treated with GHK-cu

showed upregulation of genes associated with the p53 pathway (J) Males treated with GHK-cu showed downregulation of genes associated with the p53 pathway

Mitochondrial Function was reduced in treated mice and ROS was decreased

Oxidative phosphorylation is a cellular process that generates ATP. As we age there a decrease in oxidative phosphorylation which leads to impaired energy production of cells. This decrease in energy production is associated with reduced mitochondrial function and an increase in ROS. Male and female mice treated with GHK-Cu showed downregulation of genes associated with oxidative phosphorylation and ROS (Figure 7). Males showed significance in both pathways while females only showed significance in the oxidative phosphorylation pathway. It was expected that GHK-Cu would increase oxidative phosphorylation and reduce ROS. These results suggest that GHK-Cu is reducing ROS but this reduction may not be linked to oxidative phosphorylation.

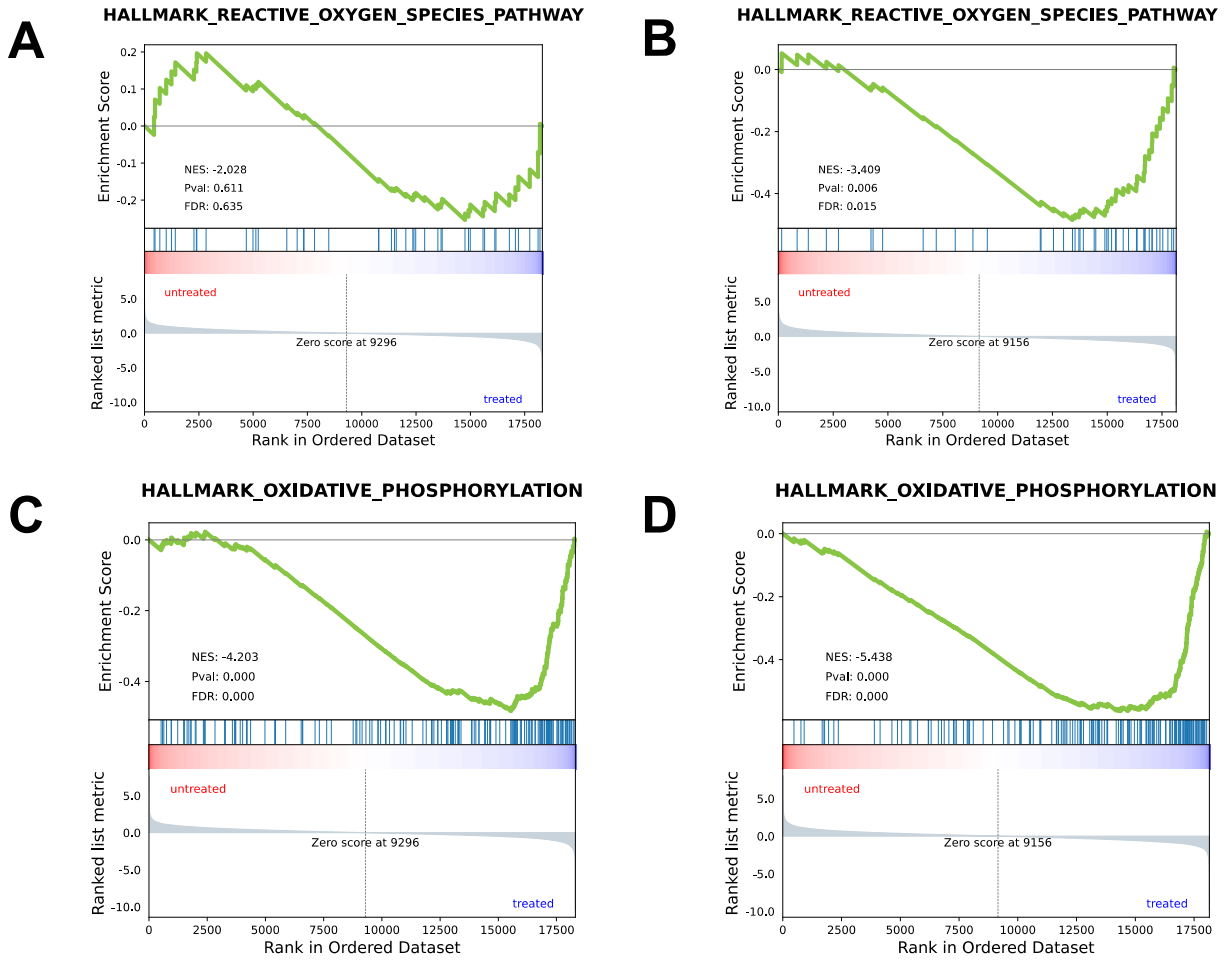

Figure 7. GSEA enrichment for pathways of mitochondrial function (A) Females treated with GHK-Cu showed downregulation of genes associated with the ROS pathway (B) Males treated with GHK-Cu showed downregulation of genes associated with the ROS pathway (C)Females treated with GHK-Cu showed downregulation of genes associated with oxidative phosphorylation (D) Females treated with GHK-Cu showed downregulation of genes associated with oxidative phosphorylation.

Cytokines associated genes were upregulated in female mice treated with GHK-Cu

Interferons are a group of cytokines which are signaling proteins produced by the body in response to viral infections. Interferon alpha is primarily produced by leukocytes and binds to cell surface receptors leading to the activation of genes that promote biological responses such as antiviral, antiproliferative and natural killer (NK) cell activation. Aging impairs the production of interferon gamma which may impact the early non-specific NK cell response to viral infection. Interferon gamma is produced by various immune cells such as T cells and plays a role in regulating immune response against pathogens, tumors and modulating inflammation. In general aging is associated with upregulated interferon gamma response. Interleukins (IL) are another type of cytokine that play a role in inflammation. The IL2 STAT 5 signaling pathway is dysregulated during aging with potential of leading to a decline in memory T cell generation. The IL6 JAK STAT3 signaling pathway is also involved in inflammation and plays a role in neuroinflammation and brain aging. Females treated with GHK-Cu showed significant up regulation of genes associated with the interferon alpha response (Figure 8). Males treated with GHK-Cu showed downregulation of genes associated with the interferon alpha response. Females treated with GHK-Cu showed significant upregulation of genes associated with the interferon gamma response. Males treated with GHK-Cu showed downregulation of genes associated with the interferon gamma response. Females treated with GHK-Cu showed significant upregulation of genes associated with the IL2 STAT5 signaling. Males treated with GHK-Cu showed downregulation of genes associated with the IL2 STAT5 signaling. Females treated with GHK-Cu showed significant upregulation of genes associated with the IL6 JAK STAT3 signaling. Males treated with GHK-Cu showed downregulation of genes associated with the IL6 JAK STAT3 signaling. These results suggest that GHK-Cu has a negative effect in females by increasing inflammation through interferon activation.

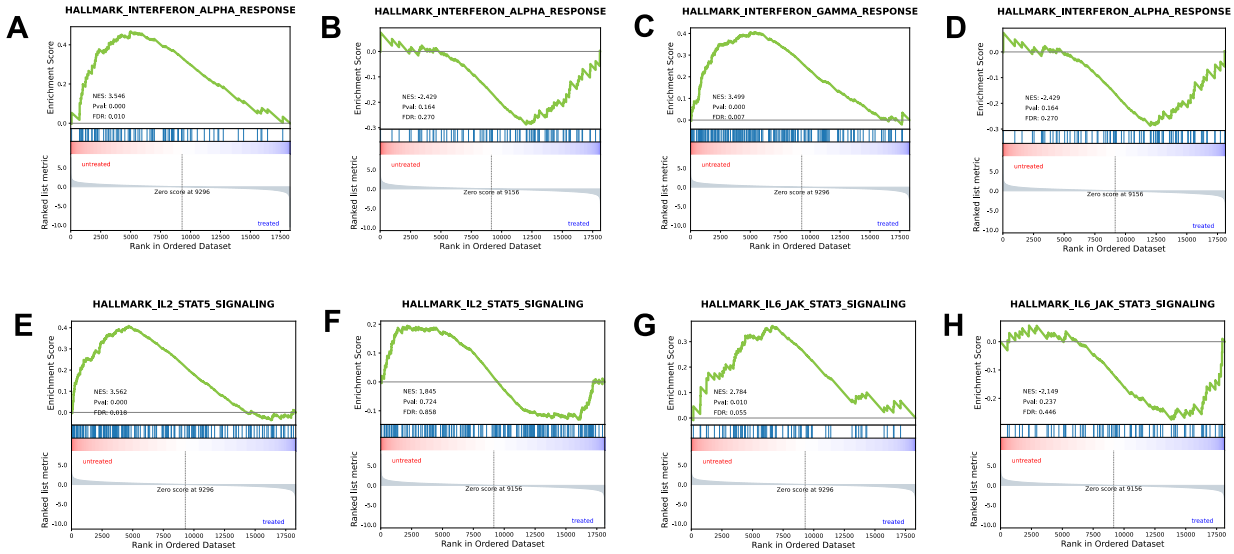

Figure 8. GSEA enrichment plots for cytokine pathways. (A) Females treated with GHK-Cu showed significant up regulation of genes associated with the interferon alpha response. (B) Males treated with GHK-Cu showed downregulation of genes associated with the interferon alpha response. (C) Females treated with GHK-Cu showed significant upregulation of genes associated with the interferon gamma response. (D) Males treated with GHK-Cu showed downregulation of genes associated with the interferon gamma response. (E) Females treated with GHK-Cu showed significant upregulation of genes associated with the IL2 STAT5 signaling. (F) Males treated with GHK-Cu showed downregulation of genes associated with the IL2 STAT5 signaling. (G) Females treated with GHK-Cu showed significant upregulation of genes associated with the IL6 JAK STAT3 signaling. (H) Females treated with GHK-Cu showed downregulation of genes associated with the IL6 JAK STAT3 signaling.

MTOR showed downregulation in mice treated with GHK-Cu

mTor complex 1 (mTORC1) is a protein complex that regulates protein synthesis modulated lipid synthesis, suppresses autophagy, senses nutrients and energy, and balances anabolic and catabolic processes. As we age there is an increase in mTORC1 activity which leads to increased protein synthesis and altered mitochondrial function. The P13 AKT MTOR pathway is a cellular signaling pathway that regulates cell growth, proliferation, survival and metabolism. Overactivation of this pathway in neurons can lead to neurogenerative diseases such as Alzheimer's Disease. Female mice treated with GHK-Cu showed significant downregulation of genes in both the mTORC1 pathway and P13 AKT MTOR pathway (Figure 9). Males treated with GHK-CU also showed downregulation, however, it was not statistically significant. These results suggest that treatment with GHK-Cu in females is regulating these pathways in a way that supports healthy brain aging.

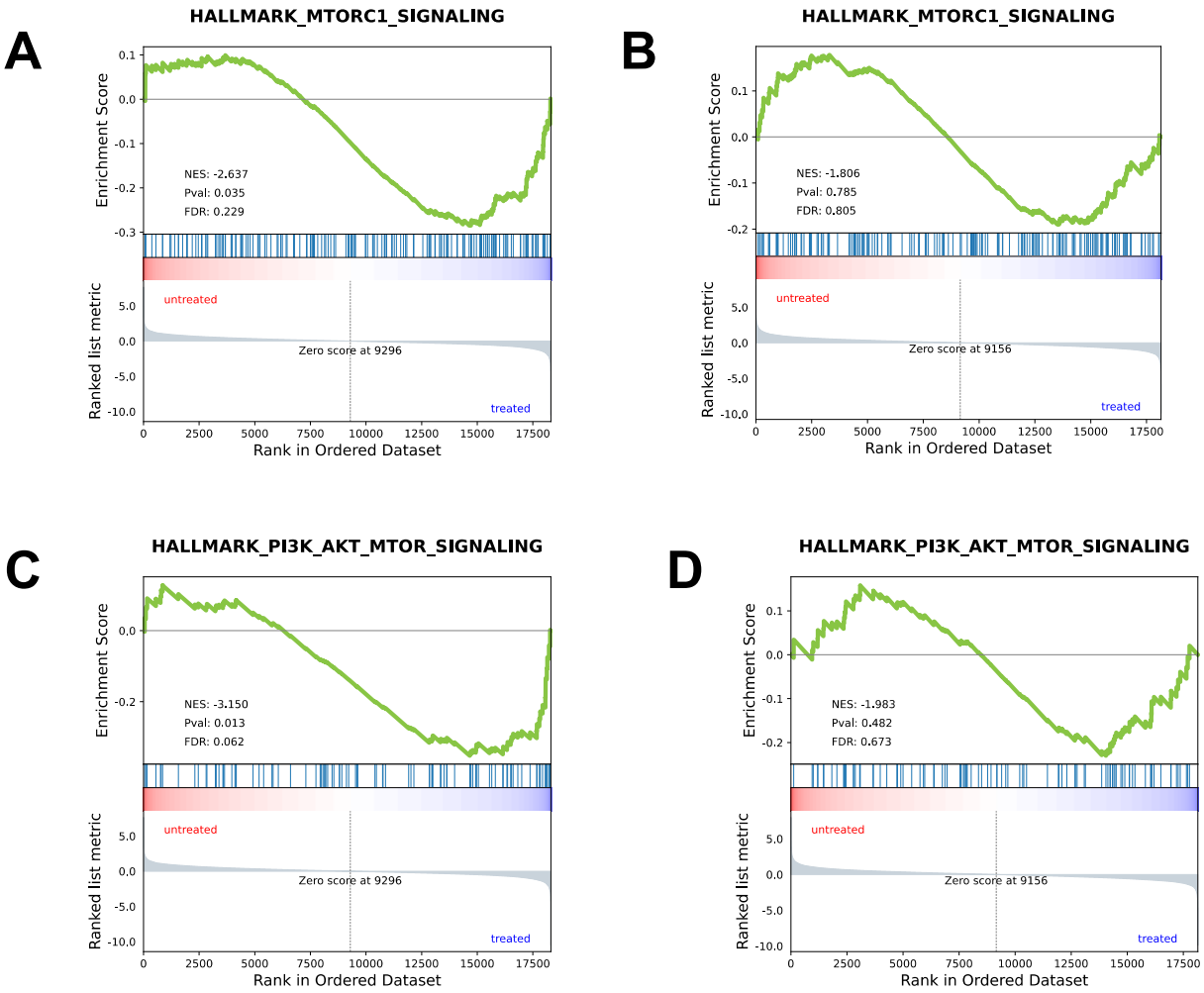

Figure 9. GSEA enrichment plots for pathways associated with MTOR signaling. (A) Female mice treated with GHK-Cu showed significant downregulation of genes associated with the MTORC1 pathway. (B) Male mice treated with GHK-Cu showed downregulation of genes associated with the MTORC1 pathway. (C) Female mice treated with GHK-Cu showed significant downregulation of genes associated with the P13K AKT MTOR pathway. (D) Male mice treated with GHK-Cu showed downregulation of genes associated with the . (C) Female mice treated with GHK-Cu showed significant downregulation of genes associated with the pathway.

Genes Associated with hormone response were upregulated in mice treated with GHK-Cu

Androgens are a group of hormones mainly consisting of male sex hormones that promote the development of male physical characteristics. While males usually have higher levels of androgens, they play other roles such as bone health and red blood cell production making them important for females as well as males. Both male and female mice treated with GHK-Cu showed significant up regulation of genes associated with androgen response. Estrogen is a group of hormones that is necessary for female sexual and reproductive development. Like androgens, it also plays a role in bone health for males and females. Female mice treated with GHK-Cu showed significant upregulation of genes associated with late estrogen response (Figure 10). Male mice showed non-significant down regulation of genes associated with the late estrogen

response. Given that aging leads to a decline in hormones such as testosterone resulting in decreased muscle mass and strength, these results suggest that GHK-Cu is having a positive effect. The upregulation of genes associated with the estrogen response shows a positive effect of GHK-Cu.

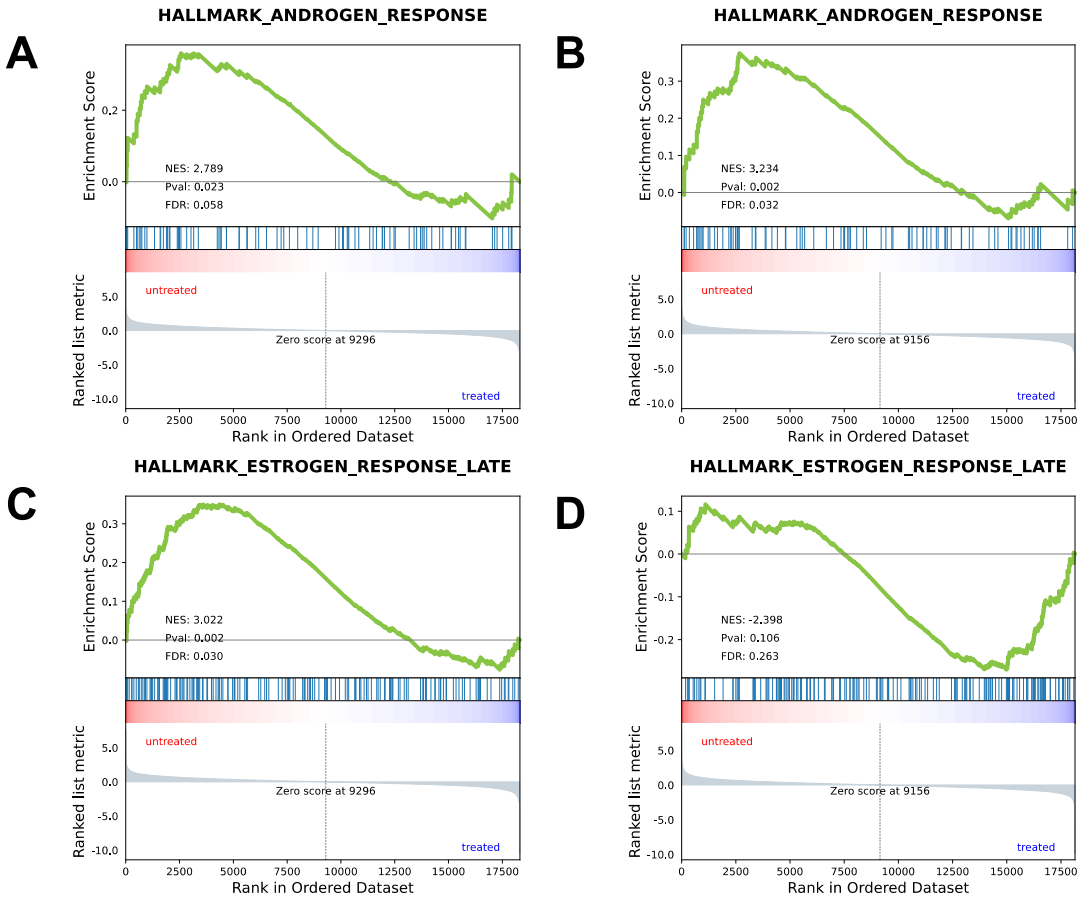

Figure 10. GSEA enrichment plots for androgen response and estrogen response. (A) Females treated with GHK-Cu showed significant upregulation of genes associated with the androgen response. (B) Males treated with GHK-Cu showed significant upregulation of genes associated with the androgen response. (C) Females treated with GHK-Cu showed significant upregulation of genes associated with the late estrogen response. (D) Males treated with GHK-Cu showed downregulation of genes associated with the late estrogen response.

Males treated with GHK-Cu showed downregulation of genes associated with adipogenesis and the fatty acid metabolism

Adipogenesis is the process by which adipocytes are formed from precursor cells leading to the development of adipose tissue. As we age, adipogenesis is impaired. There is also disruption of beige adipogenesis which converts white fat into brown fat. These impairments lead to increased visceral fat, reduced thermogenesis and adipose tissue dysfunction. The fatty acid metabolism is a series of pathways that involve

breaking down or creating fatty acids. Aging leads changes in this metabolism such as increased adiposity, increased lipotoxicity and decreased capacity to use fatty acids. Female mice treated with GHK-Cu showed a non-significant upregulation of genes in both adipogenesis and the fatty acid metabolism (Figure 11). Treated male mice showed significant downregulation of genes in both of these pathways. These results suggest that male mice have a reduction in adipogenesis and therefore a reduction in the formation of adipose tissue. This reduction is beneficial in the context of obesity, insulin sensitivity and inflammation but harmful in other ways as it could cause energy deficiency. The downregulation of the fatty acid metabolism in male mice suggests that GHK-Cu is affecting genes that either break down or create fatty acids.

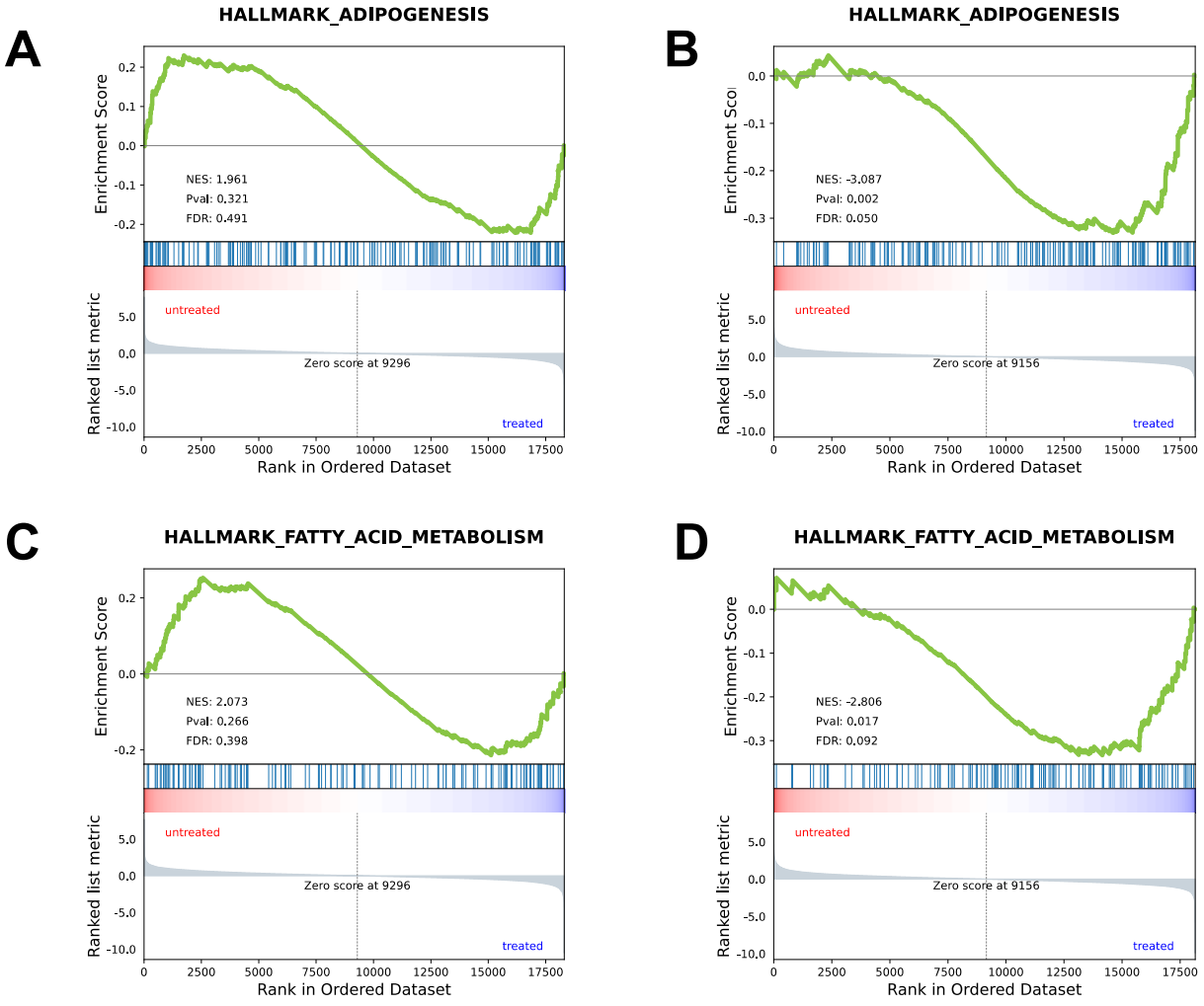

Figure 11. GSEA enrichment plots for adipogenesis and fatty acid metabolism. (A) Female mice treated with GHK-Cu showed upregulation of genes associated with adipogenesis. (B) Male mice treated with GHK-Cu showed significant downregulation of genes associated with adipogenesis. (C) Female mice treated with GHK-Cu showed upregulation of genes associated with the fatty acid metabolism. (D) Male mice treated with GHK-Cu showed significant downregulation of genes associated with the fatty acid metabolism.

Epithelial Mesenchymal Transition is upregulated in treated female mice

The epithelial mesenchymal transition is a process where epithelial cells transform in epithelial cells. This process involves the downregulation of epithelial markers and the upregulation of mesenchymal markers. EMT is important during embryogenesis but can be reactivated for wound healing and tissue regeneration, however, pathological activation of EMT can adversely cause organ fibrosis as well as being implicated in cancer. Treated female mice showed significant upregulation of EMT genes (Figure 12). Treated males also showed upregulation of EMT genes. GHK-Cu has been shown to reduce the expression of EMT leading to decreased fibroblast accumulation which could benefit conditions like pulmonary fibrosis. Given that increased levels of EMT are associated with fibrosis, these results suggest that GHK-Cu is having a negative effect on EMT in the hippocampus.

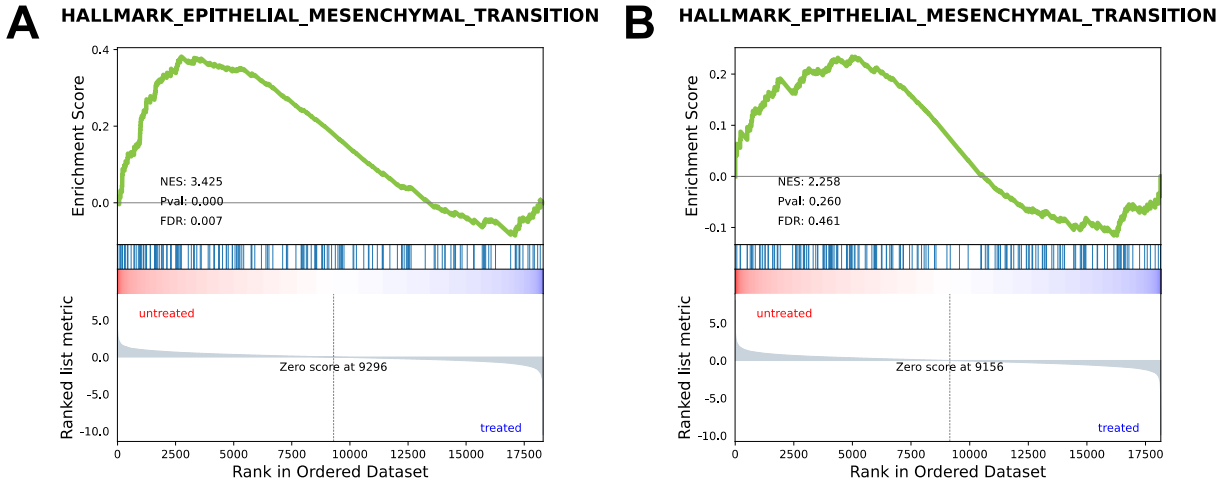

Figure 12. GSEA enrichment plots for epithelial mesenchymal transition (EMT). (A) Females treated with GHK-Cu showed significant upregulation of EMT associated genes. (B) Males treated with GHK-Cu showed upregulation of EMT associated genes.

Coagulation genes were upregulated in females and downregulated in males

Coagulation is an essential process of hemostasis in which blood changes from a liquid to a semi solid state as a way to stop bleeding. Aging tends to increase the risk of blood clots as there are increased levels of coagulants and decreased levels of anticoagulants. Female treated mice show a significant up regulation of coagulation genes while treated males show significant downregulation (Figure 13). The hallmark coagulation gene set includes genes that are involved in coagulation as well genes involved in anticoagulation so the regulation of genes within this set needs to be investigated as individual genes rather than as a whole in order to assess whether upregulation or downregulation of this gene set is positive or negative.

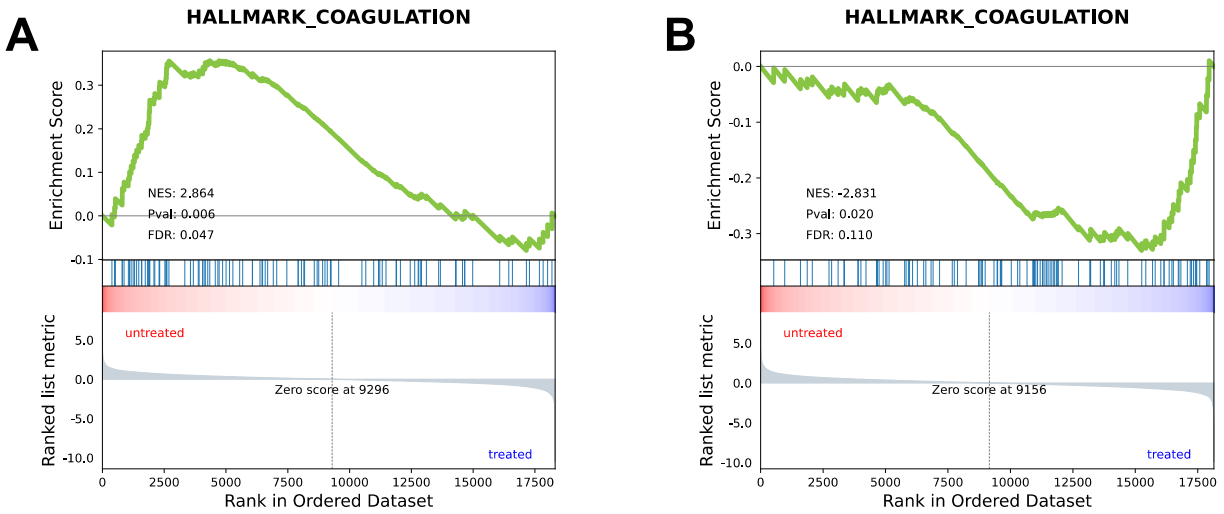

Figure 13. GSEA enrichment plots of coagulation. (A) Female mice treated with GHK showed significant upregulation of genes associated with coagulation. (B) Male mice treated with GHK showed significant upregulation of genes associated with coagulation.

Discussion

As the world population becomes increasingly older, there is an urgent need to study the molecular and cellular process that are affected as humans age. The brain, like all other organs, undergoes age related changes and having an understanding of these changes will allow for treatment and hopefully prevention of diseases associated with brain aging. One of the main symptoms seen with brain aging is a decline in cognition. This decline can lead to more serious conditions such as dementia so developing therapeutics for ARCD is vital. This study focused on GHK-Cu as a potential therapeutic for treating ARCD using hippocampal tissue from aged mice treated with GHK-Cu daily for 8 weeks.

Astrocytes, Synaptic Integrity and TGFbeta. While often overlooked, astrocytes play many important roles in the brain. They perform many tasks including clearing excess neurotransmitters, stabilizing and regulating the BBB and promoting synapse formation (Wei, 2023). GFAP is a commonly used marker of reactive astrocytes and has shown upregulation at the mRNA and protein level in aging mice, primates and humans (Labarta-Bajo, 2025). GFAP levels are also upregulated in neurodegenerative diseases. Mice treated with GHK-Cu showed a decrease in GFAP compared to control mice. Given that increased levels of GFAP are indicative of neurogenerative diseases, these findings show promise for the use of GHK-Cu as a treatment to slow the progression of ARCD with implications of preventing more server cognitive decline such as dementia. S100beta is a protein that is mainly produced by astrocytes in the brain. There were no differences observed in S100beta between treated and control mice. This doesn't mean that GHK-Cu doesn't have an effect as S100beta is typically measured in biological fluids such as blood serum or cerebrospinal fluid (CSF) and might not have been shown in protein expression in the hippocampus.

Considering that GFAP was reduced in treated mice, it was expected that there would have been an increase in markers of synaptic integrity, however, there were no changes observed in synaptophysin or PSD95. One possible explanation for this is that GHK-Cu is not affecting the synapses in the hippocampus. Expression changes of genes encoding synapse modifying proteins have been shown to be regional with thrombospondin changes being detected in the hypothalamus and cerebellum and *SPARC* changes being detected in the cortex (Labarta-Bajo, 2025). Although GHK-Cu does not show an effect in synapses in the hippocampus, it could be affecting synapses in other brain regions.

It was also expected that levels of TGFbeta would be decreased in mice treated with GHK-Cu based on the connection between astrocyte reactivity and TGFbeta. With reduced levels of GFAP it was expected that there would also be decreased levels of TGFbeta, however, no difference was observed between treated and control mice with IHC staining. RNA sequencing data showed the opposite of the expected results with genes associated with the TGFbeta signaling pathway being upregulated in female mice treated with GHK-Cu. Since GFAP was decreased in treated mice this suggests that TGFbeta was not contributing to reactive astrogliosis. Some studies have shown that a deficit in TGFbeta is an early event in the pathophysiology of cognitive impairment (Grass, 2021). TGF-β1 also plays a role in synaptogenesis and can enhance synaptic plasticity by promoting the expression of brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB). An impairment of TGF-β1 signaling has been reported in AD pathogenesis (Caraci, 2018). Given that the upregulation of TGFbeta does not appear to be contributing to reactive astrogliosis, the upregulation of genes associated with the TGFbeta signaling pathway in female mice treated with GHk-Cu may be positive and contribute to the reduction of cognitive decline seen in the behavioral phenotype data in the study by Tucker, 2023.

Mitochondrial function and oxidative stress and connections with adipogenesis and fatty acid metabolism. Mitochondrial dysfunction and oxidative stress increase with increasing age leading to decreased ATP production and increased ROS resulting from impaired electron transport chain function. While the brain is constantly producing ROS, there are relatively few antioxidants which creates an environment that is favorable for oxidative damage. Many dietary antioxidants cannot enter the brain due to the BBB, so it largely relies on endogenous antioxidants such as Cu- and Zn-dependent superoxide dismutase (SOD). GHK-Cu has been shown to SOD mimetic activity (Pickart, 2017). In male mice treated with GHK-Cu genes associated with oxidative phosphorylation were downregulated. These results were unexpected as GHK-Cu was proposed to increase ATP production through oxidative phosphorylation. It would be expected that with a downregulation of oxidative phosphorylation there would be upregulation of ROS pathway genes, however the opposite was observed with downregulation of ROS genes in treated mice. The downregulation of ROS genes in treated male and female mice demonstrated the antioxidant activity of GHK-Cu. The findings suggest that the decrease in oxidative phosphorylation is being affected by a mechanism or pathway other than ROS. One possible explanation for these findings is that oxidative phosphorylation is decrease due to a deficiency in one or more of the molecules needed

in this process. In male mice there was downregulation in genes associated with adipogenesis and the fatty acid metabolism. This downregulation could lead to a decrease in adipocytes and fatty acids. Adipocytes are needed to release fatty acids and fatty acids are need in order to produce NADH and FADH2. Given that treated male mice showed downregulation of adipogenesis and fatty acid metabolism as well as downregulation of oxidative phosphorylation, these processes could be linked. This also correlates with the reduction in ROS genes as ROS typically promotes adipogenesis.

Inflammation. Chronic inflammation generally refers to a low-grade, chronic, systemic condition in the absence of overt infection and is a significant risk factor for morbidity and mortality in the elderly. This inflammation can come from various sources such as the accumulation of oxidative stress, mitochondrial dysfunction, and cellular senescence (Jin, 2022). There is also an increase in ROS which triggers DNA damage leading to inflammation. Animal studies using spatial leaning tasks in mice have shown that systemic inflammation can cause cognitive impairment (Cunningham, 2009). Human studies have shown that there are increased levels of inflammatory markers in people with MCI or AD (Darweesh, 2018). Females treated with GHK-Cu showed significant up regulation of genes associated with the interferon alpha response. Males treated with GHK-Cu showed downregulation of genes associated with the interferon alpha response. Females treated with GHK-Cu showed significant upregulation of genes associated with the interferon gamma response. Males treated with GHK-Cu showed downregulation of genes associated with the interferon gamma response. A study conducted by Shavlakdze et al found progressive upregulation of IFN responses in the hippocampus of seven different age points of rats (Cao, 2022). Females treated with GHK-Cu showed significant upregulation of genes associated with the IL2 STAT5 signaling. Males treated with GHK-Cu showed downregulation of genes associated with the IL2 STAT5 signaling. The IL2 STAT 5 signaling pathway is dysregulated during aging with potential of leading to a decline in T cell generation. A study by Lemaitre proposed that delivering IL to the brain of aged mice would increase the endogenous brain resident population of regulatory T cells. They found that treatment of aged mice with IL2 partially reversed the aging signature in glial cells, restoring key pathways to the state observed in young mice. Treatment also prevented the age induced decline in spatial learning with aged mice performing almost as well as young mice. Females treated with GHK-Cu showed significant upregulation of genes associated with the IL6 JAK STAT3 signaling. Males treated with GHK-Cu showed downregulation of genes associated with the IL6 JAK STAT3 signaling. The JAK STAT signaling pathway is one of the critical factors that promotes neuroinflammation in neurogenerative diseases (Rusek, 2023). While GHK-Cu did not have an effect on male mice, and had a negative effect in female mice by upregulating interferon alpha and gamma responses as well as the IL6 JAK STAT3 pathways, GHK-Cu did have a positive effect on the IL2 STAT5 signaling pathway. The upregulation of the IL2 STAT5 pathway demonstrates a positive effect by potentially increasing the population of T cells in the hippocampus. This upregulation may also be connected to decrease in GFAP seen in mice treated with GHK.

DNA Damage. As age increases so does the accumulation of unrepaired DNA damage affecting many aspects of the aging phenotype with potential as a unifying cause of aging (Schumacher, 2021). Females treated with GHK-Cu showed downregulation of genes associates with DNA repair. Males treated with GHK-Cu showed significant downregulation of genes associates with DNA repair. These results are unexpected as GHK-Cu was proposed to increase DNA repair. This was also expected given that ROS associated genes were downregulated, and ROS is a major source of DNA damage. While DNA repair genes were downregulated, females treated with GHK-cu showed significant upregulation of genes associated with the p53 pathway. Males treated with GHK-cu showed downregulation of genes associated with the p53 pathway. P53 is tumor suppressor protein that plays a role in cellular response to DNA damage and facilitates DNA repair by halting the cell cycle, allowing repair mechanisms to address damaged DNA and triggering cell death via senescence and apoptosis in cells with irreparable damage (Abuetabh, 2022). Even though female mice showed downregulation of DNA repair genes, it was not significant and there was a significant upregulation of p53 associated genes, so GHK-Cu could be having a positive effect on DNA repair pathways in female mice.

MTOR. There has been extensive research on the involvement of the mTOR pathway in regulating lifespan and aging. mTOR complex 1 (mTORC1) is a protein complex that regulates cell growth and metabolism in response to nutrients, growth factors and cellular energy conditions (Perluigi, 2015). As we age there is an increase in mTORC1 activity which leads to increased protein synthesis and altered mitochondrial function. There is overwhelming evidence that decreasing mTOR activity increases lifespan in model organisms such as mice that have been fed rapamycin or been calorically restricted (Querfurth, 2021). The P13 AKT MTOR pathway is a cellular signaling pathway that regulates cell growth, proliferation, survival and metabolism. Overactivation of this pathway in neurons can lead to neurogenerative diseases such as Alzheimer's Disease. Female mice treated with GHK-Cu showed significant downregulation of genes in both the mTORC1 pathway and P13 AKT MTOR pathway. Males treated with GHK-CU also showed downregulation, however, it was not statistically significant. These results suggest that treatment with GHK-Cu in females is regulating these pathways in a way that supports healthy brain aging. This is promising as it provides a way to decrease mTOR activity in a way that doesn't require changes in diet.

Hormone Response. As males age there is a decline in the endocrine system thus creating a decline in androgen levels such as testosterone and its derivatives. Like males, testosterone also decreases in females starting at about 30 years of age with production decreasing even more after menopause. One important role of testosterones is its neuroprotective effects against the harmful actions of ROS (Ketchem, 2023). It has also been shown to decrease beta amyloid toxicity in isolated hippocampal neurons and reduce the acclamation of tau tangles mainly via estrogen pathways. Both male and female mice treated with GHK-Cu showed significant up regulation of genes associated with androgen response. Female mice treated with GHK-Cu showed significant upregulation of genes associated with late estrogen response. Male mice showed non-

significant down regulation of genes associated with the late estrogen response. Given that aging leads to a decline in hormones such as testosterone resulting in decreased muscle mass and strength, these results suggest that GHK-Cu is having a positive effect. The upregulation of genes associated with the estrogen response shows a positive effect of GHK-Cu. These findings also provide support in using GHK-Cu to prevent or delay the progression from ARCD to AD, as increasing androgen response decreases the accumulation of the two proteins implicated in AD.

~~*EMT*~~ *Epithelial Mesenchymal Transition*. The epithelial mesenchymal transition (EMT) is a process where epithelial cells transform in epithelial cells. This process involves the downregulation of epithelial markers and the upregulation of mesenchymal markers. EMT is important during embryogenesis but can be reactivated for wound healing and tissue regeneration, however, pathological activation of EMT can adversely cause organ fibrosis as well as being implicated in cancer (Santos, 2019). This fibrosis comes from fibroblasts that have been proven to derive from epithelial cells that have undergone EMT. In the brain, certain areas have been found to have a high expression of genes associated with EMT. The most potent activator of EMT is TGFbeta, with enhanced signaling of TGFbeta strengthening the EMT process. P53 plays a role in negatively regulating EMT. Treated female mice showed significant upregulation of EMT genes. Treated males also showed upregulation of EMT genes. Given that treated female mice showed upregulation of the TGFbeta signaling pathway it makes sense that there was also upregulation of genes associated with EMT. These findings are unexpected as GHk-Cu was proposed to decrease levels of TGFbeta and would therefore decrease EMT.

Coagulation. Aging increases coagulation and the risk of thrombosis. There is also a link between coagulation factors and cognitive decline. In particular, overactivation of the coagulation system plays a role in dementias such as AD and vascular dementia. Female treated mice show a significant up regulation of coagulation genes while treated males show significant downregulation.

Conclusion

There is an increasing need to find therapeutics for age related diseases and conditions such as ARCD as the world population increases. GHK-Cu provides a promising gerotherapeutic for ARCD as well as other age-related diseases as it targets pathways of aging such as inflammation, DNA damage and mitochondrial dysfunction. This study demonstrated sex differences between female and male mice treated with GHK-Cu so further investigations are needed to understand the exact mechanisms by which these sex differences occur. Taken together the findings from this study provide rationale for continuing to research the potential of GHK-Cu as a therapeutic to delay or prevent the progression of ARCD.

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