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# Characterizing the Effect of Chronic Copper Exposure on Neuropathology and Induction of Gut Microbiome Dysbiosis in an APP Knock-In Model of Alzheimer's Disease

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Environmental Health Sciences

by

Janielle Stephanie Vidal

Dissertation Committee:
Associate Professor Masashi Kitazawa, Chair
Professor Stephen Bondy
Adjunct Professor Michael Kleinman

2022

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# DEDICATION

To

my parents Cecille and Harland, my siblings Bert and Cary, my best friend Nia, countless family members, friends, and well-wishers

in recognition of their patience, words of encouragement and sacrifices

To

All those who doubt themselves, who struggle with their mental health, who are persecuted, unrepresented, oppressed and those who face uphill battles at every turn

Just keep swimming

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# ACKNOWLEDGEMENTS

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To my friends across the US, in Jamaica, the UK, Canada, thank you for all your words of encouragement and plain persistence in maintaining our friendships across schedules and time zones. To my Bryn Mawr family, to my undergraduate mentor, Dr. Jason Schmink, who brought up the idea of graduate school to me, thank you.

Finally, to my mother and father, Cecille, and Harland, who have sacrificed everything for me to be here, I cannot form words that would express the gratitude I feel. To my second family, Nia, Carlene G, and Nicole, thank you for always being my home away from home. To my siblings, Cary and Englebert, thank you for all your love, support, and care packages. To my Texas family Carmen, Susan, Wayne, Michelle, thank you. To my New York family, Carlene M, Dane, Sasheka, Telisha, Ena, Kevin, Staci, thank you for all you have done. It took a village to raise this scientist and I am eternally grateful.

# VITA

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Lim, S. L., Rodriguez-Ortiz, C. J., Hsu, H.-W., Wu, J., Zumkehr, J., Kilian, J., **Vidal, J.,** Ayata, P., & Kitazawa, M. (2020). Chronic copper exposure directs microglia towards degenerative expression signatures in wild-type and J20 mouse model of Alzheimer's disease. *Journal of Trace Elements in Medicine and Biology*, *62*, 126578 https://doi.org/https://doi.org/10.1016/j.jtemb.2020.126578

Hsu, H. W., Rodriguez-Ortiz, C. J., Lim, S. L., Zumkehr, J., Kilian, J. G., **Vidal, J.,** & Kitazawa, M. (2019). Copper-Induced Upregulation of MicroRNAs Directs the Suppression of Endothelial LRP1 in Alzheimer's Disease Model. *Toxicological Sciences*, *170*(1), 144–156. https://doi.org/10.1093/toxsci/kfz084

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**Vidal, J.** and Lim, S. L.; Hsu, H-W; Killian, J.; Rodriguez-Ortiz, C.J.; Katagiri, H; Shiferaw, S; Nguyen, S; Kitazawa, M.; A Potent Lipoxin Analogue as a Potential Treatment for Alzheimer's Disease, *Publication in preparation*

**Vidal, J**.; Rodriguez-Ortiz, C.J.; Nguyen, S.; Katagiri, H.; Shiferaw, S.; Rodriguez-Ortiz, C.J.; Lim, S. L.; Hsu, H-W; Killian, J.; Saito,T; Saido, T.C; Kitazawa, M., Characterizing the Effect of Chronic Copper Exposure on Neuropathology and Induction of Gut Microbiome Dysbiosis in WT and an APP Knock-In Model of Alzheimer's Disease, *Publication in preparation*

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*Conversations with Neuroscientists, June 2021*
Depaul University, Neuroscience Program, Instructor: Dorothy Kozlowski, PhD

*Impact of Environmental Exposures on the Microbiome and Human Health, February 2021*
Virtual Meeting, National Institute of Environmental Health Sciences

**Vidal, J**.; Rodriguez-Ortiz, C.J.; Katagiri, H.; Shiferaw, S.; Rodriguez-Ortiz, C.J.; Lim, S. L.; Hsu, H-W; Killian, J.; Saito,T; Saido, T.C; Kitazawa, M., Exposure to an Environmentally Relevant Level of Copper in Drinking Water Alters the Microbiome in a Humanized APP Knock-in Mouse Model of Alzheimer's Disease (2020) *Annual UCI REMIND Symposium*

**Vidal, J**.; Rodriguez-Ortiz, C.J.; Katagiri, H.; Shiferaw, S.; Rodriguez-Ortiz, C.J.; Lim, S. L.; Hsu, H-W; Killian, J.; Saito,T; Saido, T.C; Kitazawa, M., Exposure to an Environmentally Relevant Level of Copper in Drinking Water Alters the Microbiome in a Humanized APP Knock-in Mouse Model of Alzheimer's Disease (2020) *Society of Toxicology Annual National Meeting*

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# ABSTRACT OF THE DISSERTATION

Characterizing the Effect of Chronic Copper Exposure on Neuropathology and Induction of Gut Microbiome Dysbiosis in an APP Knock-In Model of Alzheimer's Disease

by

Janielle Stephanie Vidal

Doctor of Philosophy in Environment Health Sciences

University of California, Irvine, 2022

Associate Professor Masashi Kitazawa, Chair

Chronic exposure to copper is a putative environmental risk factor for AD. Although copper is an essential metal, environmental exposure to its inorganic cupric form ($Cu^{2+}$) may exert toxicity. However, exact neurotoxic mechanisms of action of copper, and its contribution to AD neuropathology remain largely unelucidated. Copper is a natural antimicrobial agent, and here, we investigate its impact of copper exposure on the gut microbiome and integrity as well as effects on cognition and AD pathology. Recent studies implicate gut dysbiosis in the onset and progression of AD in both humans and animal models. We believe that chronic exposure to environmentally relevant dose of copper through drinking water will reduce richness and diversity of the gut microbiota in both WT and $hAPP^{NL\text{-}G\text{-}F}$ knock-in (APP-KI) mice. Thus, we hypothesize that this copper-induced dysbiosis may perturb host metabolism and inflammatory homeostasis to contribute to AD neuropathology. In this dissertation, male and female APP-KI and wildtype C57BL/6J mice were exposed to 1.3 ppm $CuCl_2$ in drinking water *ad-libitum* for 3(pilot study) and 9(chronic study) months. The present studies use a host of behavioral, immunohistochemical,

biochemical and microbial analyses to investigate the effect of copper on microbiota, amyloidosis, neuroinflammation and cognition in AD mice.

In our pilot study, we discovered that while there were no significant changes in AD neuropathology or inflammatory status in WT or APP-KI mice, we observed that WT mice microbiota were resilient to the effects of copper administration. However, beginning 1 month after initiation of exposure, APP-KI mice developed a significantly different microbial composition according to the Bray Curtis β-diversity. This was largely driven by a decrease in Firmicutes, and specifically in the genus *Allobaculum* and an increase in the phylum Bacteroidetes, specifically in the genus *S24-7*. These changes mirror some of the changes observed in studies in other AD mouse models and AD patients.

In our chronic study, spanning from after weaning and until the mice were 10 months, old, we discovered no significant changes in cognition and a mild change in the cytokine profile of AD mice, with a reduction of IL-4 in plasma, following copper administration. With this longer treatment paradigm, we did observe increased in amyloidosis, both dense core and diffuse plaques, with copper treatment. Further, along with evidence that plaque-associated microglia are of a diseased phenotype, the amount of these microglia was significantly increased with copper treatment indicating that copper perturbs neuroinflammation. In the gut microbiota, we observe unexpected results. Copper directs the developmental trajectory of WT mice but with time the microbiota of AD mice is not changed much. This may indicate differing windows of susceptibility to copper exposure between the genotypes of mice and between pilot study and the chronic study paradigms.

# Chapter 1

# Introduction

## 1.1 Significance

Alzheimer's disease (AD) is the principal cause of dementia affecting approximately 35 million worldwide (Alzheimer's Association, 2019, 2022; Brookmeyer et al., 2007; Prince et al., 2015; Scheltens et al., 2016). This number is expected to quadruple by 2050 (Brookmeyer et al., 2007) as the population worldwide ages and mortality from other diseases decreases (Alzheimer's Association, 2022; Prince et al., 2015; Qiu et al., 2009).

In the US, AD is the 6$^{th}$ leading cause of death as the mortality rate rose an alarming 145 percent between 2000 and 2022, while deaths from other major diseases, including heart disease, declined significantly (Alzheimer's Association, 2022). Moreover, in 2020, the COVID-19 pandemic contributed a 17% increase in Alzheimer's and other dementia related deaths. (Alzheimer's Association, 2022). In this era of the Anthropocene, where human activity is the driving force of changes in climate and environment, this is of special importance as pandemics are projected to become more prevalent (Allen et al., 2017; Jones et al., 2008; Rodó et al., 2021). The COVID-19 pandemic put a strain on health care systems in general and the care of older people with dementia given social distancing rules, supply chain issues and dealing with the change in lifestyle or routine for disease sufferers (Mok et al., 2020). Moreover, some patients suffering from COVID-19, approximately one-third, present with neurological symptoms. A recent study showed that SARS-CoV-2 infection was associated with oxidative stress, neuroinflammation and AD-like pathological changes (Reiken et al., 2022). In 2022, Alzheimer's Disease and other dementias related care will cost the US $321 billion and this cost is estimated to

rise to $1.1 trillion by 2050 without intervention (Alzheimer's Association, 2022). Developing countries will have the majority share of the aging population in the world, around 80%, posing major challenges to public health systems worldwide (Qiu et al., 2009; Shetty, 2012; UNDP, 2019). In a report from 2005, the worldwide cost of dementia was estimated at US$315.4 billion with the developed regions having 77% of the cost with only 46% of disease prevalence. This shows that there is a major cost burden and dependence on informal care, which overburdens caregivers and thus disease suffers themselves (Allegri et al., 2006; Clyburn et al., 2000), within less developed regions (Kalaria et al., 2008; Wimo et al., 2007).

Currently, despite billions of dollars in spending on research, there has not been a major breakthrough therapeutic to prevent or cure AD (Cummings et al., 2016; Kaeberlein, 2019; Scheltens et al., 2016). Therefore, the search for other more easily targeted, disease modifying targets are of utmost importance. In 2021, the FDA approved Biogen's Aduhelm (aducanumab) for the treatment of Alzheimer's Disease. This drug is a human recombinant anti-beta amyloid (Aβ) IgG1 monoclonal antibody. Aβ is one of the pathological hallmarks of Alzheimer's Disease and represents the first disease-modifying drug to be marketed for the treatment of AD (Cavazzoni, 2021). This decision has been fraught with controversy (K. Y. Liu & Howard, 2021). A reduction in Aβ may not be sufficient to modify disease etiology and this may especially true for symptomatic individuals (K. Y. Liu & Howard, 2021). The drug is also steeply priced at $56,000 per year, which does not include the cost of hospitalization, MRI and other indirect costs (Lovelace Jr., 2021). Shortly after Adulhelm's approval, the FDA granted breakthrough therapy designations to two other monoclonal antibody drugs, donanemab and lecanemab (Beasley, 2022; Biogen, 2021; Eli Lilly and Company, 2021; K. Y. Liu & Howard, 2021). It is very unclear if Medicare in US, the federal health insurance for persons 65 and older would cover Aduhelm or drugs like it in

the future. It may depend on data on the performance of these drugs from clinical trials, some of which may not be due for many years (Centers for Medicare & Medicaid Services, 2022; Nathan-Kazis, 2022).

### 1.2. Overview of Alzheimer's Disease and General Pathology

Development into AD is characterized by three hallmark stages (Figure 1.1) (Aisen et al., 2017). First, there is a preclinical stage, a period of mild cognitive impairment (MCI), followed by the final phase, dementia due to AD. In the preclinical stage, individuals have changes in the brain, cerebrospinal fluid (CSF) and in serum but have not outwardly displayed symptoms of the disease. These changes include Aβ and phosphorylated tau accumulation within the CSF (Sperling et al., 2011). Later, this individual will enter the MCI stage, which is defined by continued progression of pathologies that began in the preclinical stage, along with lapses in episodic memory and where synaptic dysfunction and changes in brain structure are detectable through brain imaging methods such as functional magnetic resonance imaging (fMRI) or positron emission tomography (Albert et al., 2011; Förstl & Kurz, 1999; Sperling et al., 2011). The individual retains most of their autonomy and symptoms might not even be recognizable to a stranger. Afterwards, the patient will progress into the dementia due to AD stage which is defined by a peaking of pathology developed over the years of preclinical and MCI stages. This then manifests into marked changes in cognition such as further erosion of memory, impaired speech and vision, wandering behavior and issues with problem solving (McKhann et al., 2011; Mucke & Selkoe, 2012). Wandering behaviors and loss of geographical awareness, hoarding, aggression, developing hallucinations, forgetting loved ones may develop (Förstl & Kurz, 1999). Eventually, patients may need support carrying out basic activities such as eating. Incontinence is not uncommon as motor disturbances continue to develop with severity of this disease (Förstl & Kurz, 1999). This leads to a loss of overall autonomy

(McKhann et al., 2011) and as such, AD patients require great care which can be emotionally, physically and financially taxing to caregivers (Alzheimer's Association, 2022; Ballard et al., 2011; Mucke & Selkoe, 2012; Reitz & Mayeux, 2014).

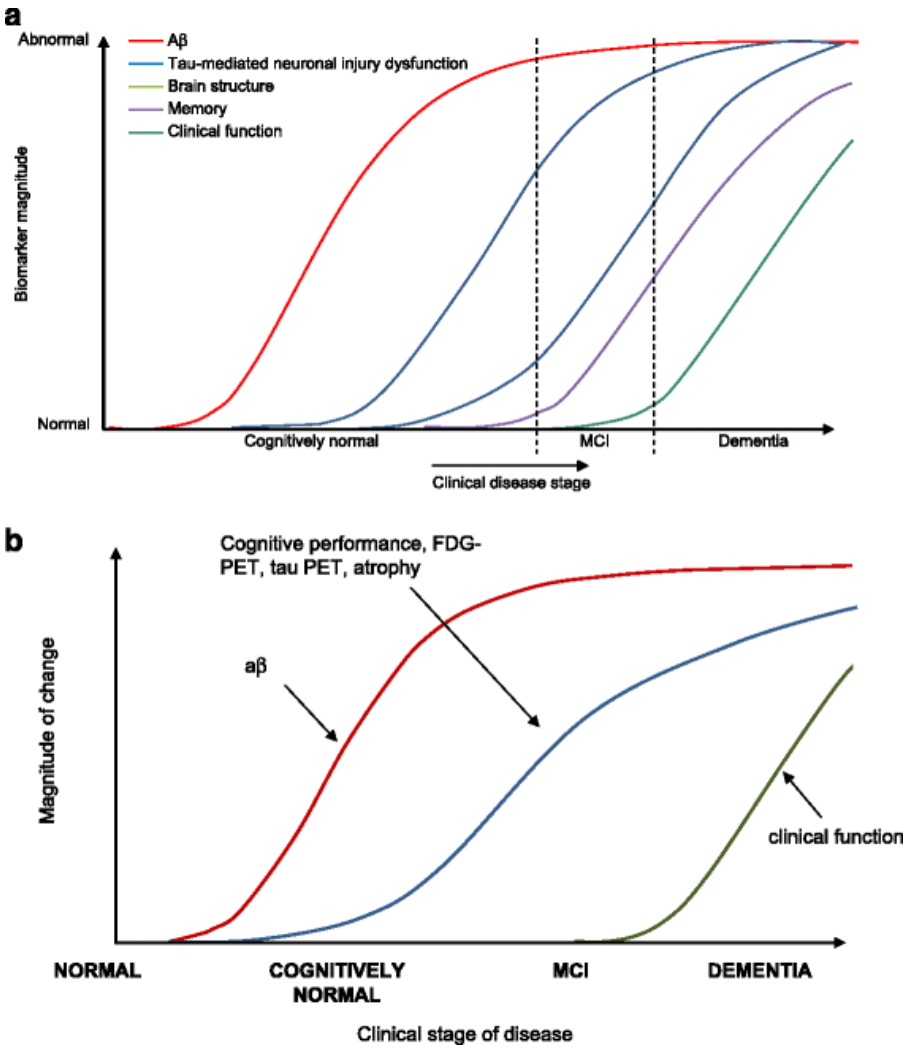


**Figure 1.1** Change in biomarkers over time. **a** Sequential change in measures of AD. **b** Modified graph showing that amyloid accumulation (measured as low CSF Aβ or elevated amyloid PET standard uptake value ratio) occurs first and functional decline occurs late in the continuum of AD (as before), but cognitive performance, FDG-PET, tau PET, and MRI atrophy change along a common, gradually steepening curve. *Aβ* amyloid beta, *FDG* flurodeoxyglucose, *PET* positron emission tomography, *MCI* mild cognitive impairment due to Alzheimer's disease

Reprinted from (Aisen et al., 2017) with permission under the terms of the Creative Commons Attribution 4.0

Pathologically, the presence of two hallmarks classically defines AD. These are extracellular amyloid beta (Aβ) aggregates forming diffuse and neuritic (senile) plaques and neurofibrillary tangles of misfolded, phosphorylated tau protein within the neuron (Mucke & Selkoe, 2012; Palop & Mucke, 2010; Selkoe, 2001). While Aβ and tau have been the central molecules triggering neuronal loss, recent emerging evidence indicates that neuroinflammation,

common to many other neurodegenerative diseases, contributes widely to the development of AD. Microglia and astrocytes are known to surround amyloid plaques. There is evidence of impaired clearance of Aβ (Mawuenyega et al., 2010), as microglia surrounding plaques are known to be less phagocytically active (Kitazawa et al., 2016), which contributes to the formation of the plaques and neuroinflammation. However, this aggregation of glia around plaques may be an attempt to encapsulate toxic Aβ, compact these species into dense core plaques to limit neuronal damage (Condello et al., 2015; Hansen et al., 2018). There is increased pro-inflammatory cytokine expression and infiltration of monocytes into the brain of AD patients and mouse models of the disease (Akiyama et al., 2000; Heneka et al., 2015; Heppner et al., 2015; Minter, Taylor, et al., 2016; Wyss-Coray & Mucke, 2002).

The progression of pathology to clinical manifestation of AD takes place over decades. There are several genetic risk factors, such as causative mutations and single nucleotide polymorphisms (Bertram et al., 2008; Bertram & Tanzi, 2012; Stocker et al., 2018) as well modifiable risk factors (Eid et al., 2019; Livingston et al., 2020) such as lifestyle (education, socioeconomic status, medical history) and environmental exposures to pesticides, metals and other xenobiotics (Gatz et al., 1997; Yegambaram et al., 2015) that mediate disease onset and progression. As the time between exposure to possible triggering agents and the years it takes for pathology to develop, the isolation of causative factors is difficult. Amyloidosis and neuroinflammation are pertinent to this work and will be discussed in further detail in the following sections of this introduction and dissertation.

## 1.3 Amyloid Precursor Protein Processing, the Production of Aβ and the Amyloid Cascade Hypothesis

The Amyloid Precursor Protein(APP) is encoded on chromosome 21, that contains the sequence for the neurotoxic amyloid beta peptides (St. George-Hyslop et al., 1987; Tanzi et al., 1987). It consists of a single transmembrane domain with a N-terminal region within the ectoplasm and cytoplasmic C-terminal region of a shorter length dubbed the AICD (C-terminal APP intracellular domain) (Jacobsen & Iverfeldt, 2009). It is synthesized in the endoplasmic reticulum and is transported through to the Golgi apparatus where it can be then transported to the cell surface via the trans-Golgi network derived secretory vesicles( Figure 1.3) (Y.-W. Zhang et al., 2011). APP can be alternatively spliced which can give rise to eight different isoforms, APP 770, 752, 751, 733, 714, 696, 695 and 667 (Sandbrink et al., 1994), which are expressed in a cell-specific manner (Haass et al., 2012; O'Brien & Wong, 2011). Three isoforms are most common; 695 which is predominantly expressed in the central nervous system, while 771 and 750 are expressed more peripherally(Figure 1.2) (O'Brien & Wong, 2011; Tanaka et al., 1989).

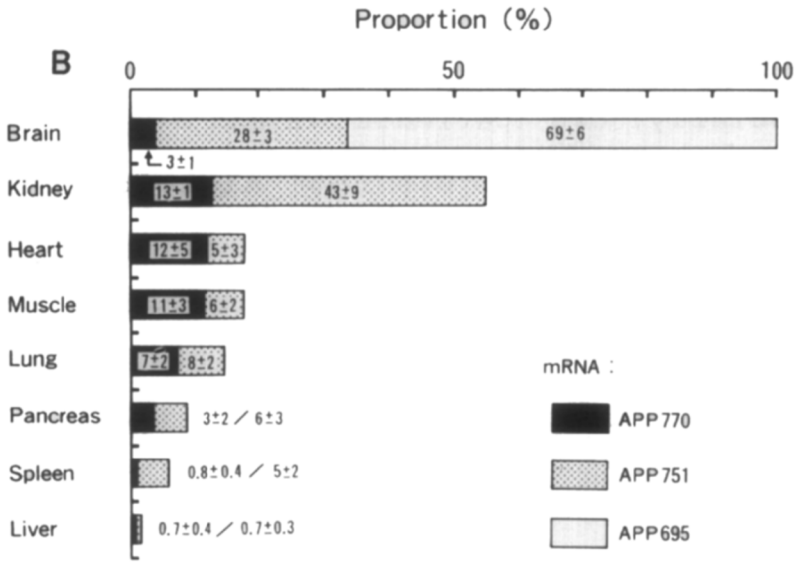


**Figure 1.2.** Analysis of APP mRNAs in eight tissues by ribonuclease protection assay. Figure represents relative proportions of APP mRNAs calculated from band densities of autoradiograms with correction for the number of cytosine residues in each fragment. Mean vales ± S.D of five controls are shown, taking the total content in the brain as 100%

*Reprinted from Biochemical and Biophysical Research Communications, Volume 165, No.3, 1989 Siego Tanaka et. al, Tissue-Specific Expression of Three types of β-protein Precursor mRNA: Enhancement of Protease Inhibitor-*

APP is subject to cleavage by α-, β-, and γ-secretases and depending on the sequence of

cleavage can give rise to different fragments that are thought to be neuroprotective/inert (non-amyloidogenic) or neurotoxic (amyloidogenic) (Chen et al., 2017; Haass et al., 2012; Haass & Selkoe, 1993; O'Brien & Wong, 2011; Y.-W. Zhang et al., 2011). The processing of APP and the functions of its various fragments has been widely studied but their main roles have not been fully elucidated (De Strooper & Annaert, 2000). Literature suggests that APP and it's fragments are involved in critical processes in the CNS like synaptic formation and function, neuronal development, cellular and neurite growth and cholesterol synthesis (De Strooper & Annaert, 2000; Nalivaeva & Turner, 2013; van der Kant & Goldstein, 2015).

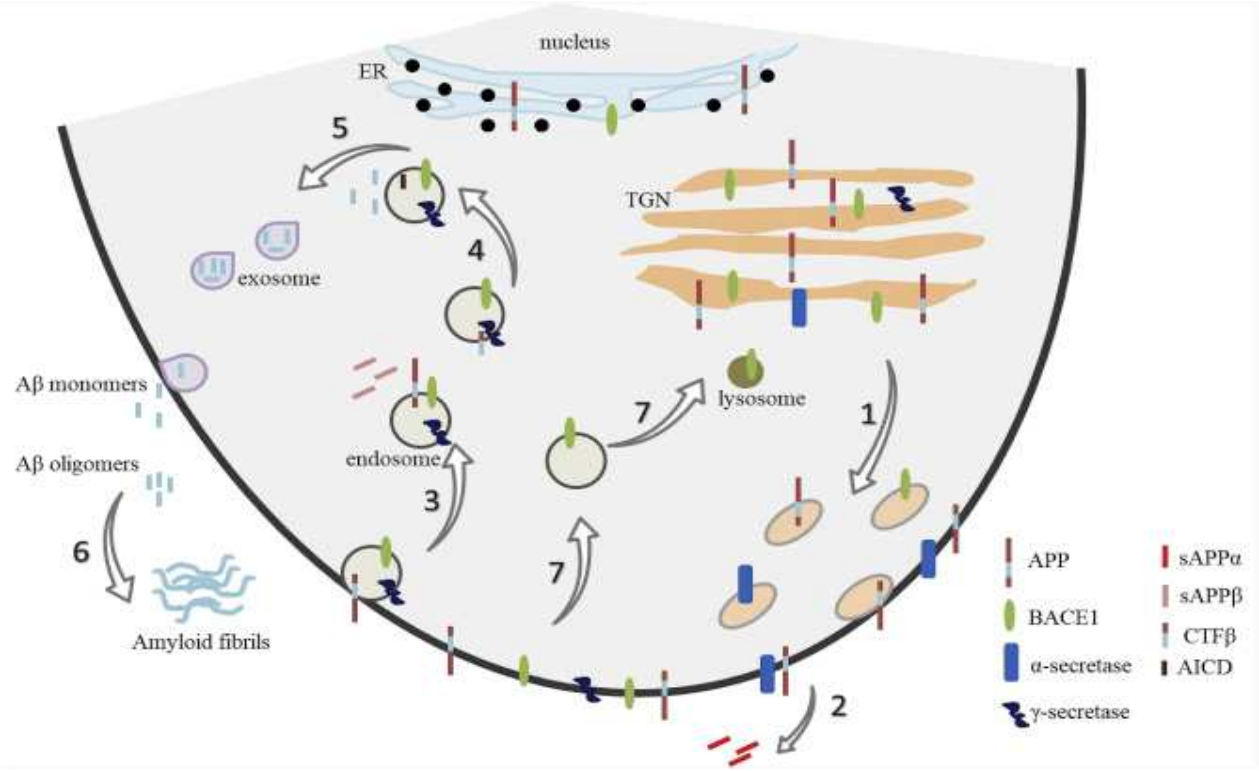


**Figure 1.3** Typical trafficking of APP and the canonical secretases. (1) Newly synthesized APP, BACE1, α- and γ-secretases are transported to the plasma membrane. (2) APP is cleaved on the plasma membrane by α-secretase, releasing sAPPα. (3) APP, BACE1 and γ-secretase are internalized into early endosomes from cell surface, where APP is firstly processed by BACE1. (4) sAPPβ is released into the lumen while CTFβ is cleaved by γ-secretase. (5) Aβ peptides are released outside of the cell in exosomes. (6) The accumulated Aβ peptides form amyloid fibrils. (7) BACE1 can also be retrograded into the TGN and lysosomes, favoring the non-amyloidogenic processing. *Reprinted from European Journal of Pharmacology, Volume 856, Yusksel, M & Tacal, O. Trafficking and proteolytic processing of amyloid precursor protein and secretases in Alzheimer's disease development: An up-to-date review. Copyright (2019), with permission from Elsevier*

In the non-amyloidogenic pathway, APP is first cleaved by α-secretase (ADAM10) in the extracellular domain which released the N-terminal fragment sAPPα into the extracellular space. APP can then be cleaved by the γ secretase complex which then produces the P3 fragment to the extracellular domain and AICD into the cytoplasm (Haass & Selkoe, 1993). In the amyloidogenic pathway, which occurs primarily in the endosomal membrane following internalization of the APP (Figure 1.1) (O'Brien & Wong, 2011; Yuksel & Tacal, 2019), Aβ monomers are released from the sequential cleavage of the APP by β and γ secretases (Haass et al., 2012; O'Brien & Wong, 2011). This activity also releases AICD. These Aβ are then released outside of the cell in exosomes. (Yuksel & Tacal, 2019; Yuyama & Igarashi, 2017) The Aβ peptide itself contains 28 residues of the extramembranous portion of the protein and 11-15 amino acid residues of the transmembranous portion. This liberated beta-amyloid protein is prone to fibrilization and oligomerization in the extracellular space. This aggregation of Aβ then forms senile plaques (Chen et al., 2017; Haass & Selkoe, 2007).

In the AD brain, the aggregation of amyloid beta precedes the formation of neurofibrillary tangles and may also play a role in their development. This theory has been popularized as the amyloid cascade hypothesis (J. A. Hardy & Higgins, 1992). Support of this hypothesis and for the relationship of APP processing in the pathogenesis of AD is further supported by the number of studies in human genetics. The first is evidence of this is through the discovery of many different missense mutations in the APP gene have been found through analyses of familial clusters of Alzheimer's disease (Goate et al., 1991; Karch & Goate, 2015; St. George-Hyslop et al., 1987). Most of these mutations, about 32 have been identified, exist near the cleavage site of γ-secretase (Bekris et al., 2010; Bertram & Tanzi, 2012). After the discovery of the first APP mutation, mutations in the presenilin-1 (PSEN1) and -2 (PSEN2) genes were also found to play a role in

familial AD (Bertram & Tanzi, 2012; Kumar-Singh et al., 2006). These two proteins form part of the γ-secretase complex and indicate that it's regulation or dysregulation is crucial to Aβ production and development of AD. These are thus considered then causative mutations in AD (Bertram & Tanzi, 2012). To further support APP's causative role in AD, evidence is gleaned from suffers of the most common form of Down's Syndrome, trisomy 21. In this disease, there is an additional copy of chromosome 21, in which the APP gene is located. This then confers a significantly high risk of AD (Head et al., 2012; Lott & Head, 2019). This is due to the overexpression of APP protein, which as mentioned earlier is located on chromosome 21 (St. George-Hyslop et al., 1987).

While in the pathogenesis of AD, amyloidosis tends to precede neurofibrillary tangles, brain atrophy and cognitive decline. In fact, literature has shown that Aβ oligomers can induce the hyperphosphorylation of tau, which comprises the neurofibrillary tangles (Bolmont et al., 2007; Jin et al., 2011; Pooler et al., 2015). Even though Aβ plaque formation precedes neurofibrillary tangle development, which then is associated with cognitive decline, deposits of Aβ often exist in the absence of clinical dementia (Aizenstein et al., 2008; Bennett et al., 2006; Knopman et al., 2003; Marchant et al., 2012). This then suggests that amyloidosis is not a sufficient cause of cognitive decline, but the downstream effects of this deposition may be (Jack et al., 2009; Marchant et al., 2012). This posits that AD is a complex disease and that single biomarker approaches/theories are not satisfactory alone to explain AD pathophysiology. This is discussed heavily within AD literature and the amyloid cascade hypothesis has been debated, reframed and expanded (Caselli et al., 2020; J. Hardy, 2009; Herrup, 2015; Levin et al., 2022). Despite, controversy, manipulation of the APP gene in models of the disease have been instrumental to furthering our understanding of the underlying mechanisms of the disease.

### 1.4 Neuroinflammation in Alzheimer's Disease

The canonical hallmarks of AD are the buildup of senile plaques in the extracellular space, and build-up of neurofibrillary tangles within the neuron, both of which are neurotoxic. As mentioned previously, neuroinflammation has burgeoned as a new area of interest within the AD research field. Aβ and NFT have been known to contributing to oxidative stress within the brain via generation of reactive oxygen species (ROS), and these protein aggregates can activate the complement pathway *in vitro* (Akiyama et al., 2000; Calsolaro & Edison, 2016; Wyss-Coray & Mucke, 2002). Moreover, several studies draw parallels between the inflammation in the periphery and what occurs in the brain; accumulation of insoluble deposits of proteins, chronic damage to tissue and activation of similar mechanisms that occur in localized peripheral inflammatory responses (Akiyama et al., 2000; Wyss-Coray & Mucke, 2002). In AD, there is an upregulation of complement defense proteins, like ApoJ which has been associated with senile plaques. Chemokines and cytokines that are considered proinflammatory such as interleukin (IL)-1β, IL-6, TNFα, IL-8 have all been upregulated in AD. Many of these cytokines can go on to influence other processes that contribute to cognitive decline and dysfunction observed in AD. For example, IL-1 can upregulate S100β in overactive astrocytes to further promote cytokine cycling and dystrophic neurite growth and/or increased acetylcholinesterase (AChE) activity in neurons which can then lead to cholinergic decline (Akiyama et al., 2000; Webers et al., 2020). Cytokines and chemokines might also interact with the proteins within these classical hallmarks of disease. For example, IL-1, IL-6 and TGF-β have been implicated in the modulation of APP synthesis. There can also be activation of transcriptional mechanisms as IL-1 and TNF-α pathways can activate NF-κB-inducing kinase. Further, the balance between pro-inflammatory and anti-inflammatory cytokines (such as IL-10, IL-4) may be disrupted. High proinflammatory cytokine and low anti-inflammatory

cytokines levels may then lead to an amplification cycle, promoting chronical inflammation and thus cytotoxicity (Akiyama et al., 2000; Heneka et al., 2015; Puig et al., 2012; Swardfager et al., 2010; Webers et al., 2020).

Astrocytes and microglia are the cell types primarily involved in and mediate the inflammatory response within the central nervous system (CNS) and produce a host of immune related proteins such as complement proteins, cytokines and chemokines (Calsolaro & Edison, 2016). Further, pathologically both astrocytes and microglia are found in proximity to and at least seem to be encircling or encapsulating senile plaques (Heneka et al., 2015). As microglia are the primary immune cells in the brain, this introduction is primarily focused on their state and activity. It is important to note though that there is contribution from peripheral myeloid cells to neuroinflammation (Akiyama et al., 2000; Webers et al., 2020).

*Microglia*

Microglia, the resident macrophages of represent about 10% of the total cell population of the brain. Not only are they the primary responders to inflammatory insult, they are deeply involved in the formation of new neurons and the reorganization/regeneration of neural networks (Kettenmann et al., 2011). In a resting state, these microglia have a ramified morphology, which is defined by increased branch complexity and long processes which offshoot from the cell body (Kettenmann et al., 2011). During neurodevelopment microglia participate in neuronal pruning to maintain/ form healthy neuronal circuits and the removal of normally occurring apoptotic neurons (Filipello et al., 2018; Song & Colonna, 2018).

After pathological injury, such as the parenchymal deposition of protein aggregates or dystrophic neurites, these microglia take on an ameboid morphology. These cells migrate, usually

to the site of injury, can proliferate and also have phagocytic capability (Kettenmann et al., 2011). It is also important to note that while astrocytes and microglia are prime participants in inflammation, that other cell types play a role as well. For example, brain endothelial cells have been observed to produce IL-1, interferon-gamma (IFNγ), and IL-6 while neurons have been immunoreactive for IL-6 and the three isoforms of transforming growth factor beta (TGF-β) (Akiyama et al., 2000; Wyss-Coray & Mucke, 2002).

Interest in characterizing the role for glia in AD can be traced to genome wide association studies (GWAS) (Andrews et al., 2020). These studies implicate a number of immune receptors such as TREM2, CLU, PICALM, CD33 as conferring additional risk of developing late-onset AD (Andrews et al., 2020; Harold et al., 2009; Hollingworth et al., 2011; Karch & Goate, 2015; Kunkle et al., 2019; Lambert et al., 2013; Naj et al., 2011). Further, pathologically both astrocytes and microglia are found in proximity to and at least seem to be encircling or encapsulating senile plaques (Heneka et al., 2015).

There has been growing interest in the field to identify the characteristics of these resting versus activated states of glia, particularly that of microglia. Through single-cell RNA sequencing, it has been found that there is a unique microglia type that is associated with AD and has been therefore termed disease-associated microglia (DAM) (Keren-Shaul et al., 2017; Olah et al., 2020). Homeostatic microglial transcriptomic profile include high expression of genes such P2ry12/P2ry13, Cx3cr1, and Tmem119, and are largely thought to be TGF-β signaling dependent (Butovsky et al., 2014). DAM transcriptomic profile signatures include an increase in several AD risk factor genes such as APOE, TREM2, CCL2, Ctsd, C3 (Keren-Shaul et al., 2017; Olah et al., 2020; Song & Colonna, 2018; J. Xu et al., 2021). The concept of DAM has been validated in several mouse models of AD such at the 5xFAD and APP/PS1 mice. DAM were localized to senile

plaque and were specifically detected in regions affected by AD but not others such as the cerebellum (Deczkowska et al., 2018; Song & Colonna, 2018). These studies together have highlighted that TREM2-signaling is important to the formation of DAM. Despite the discovery and determination of DAM, the term still describes transcriptionally distinct subsets of microglia. Their transcriptomic profile can depend upon location in brain region, sex, age and local pathology (Olah et al., 2020; Patel et al., 2022).

A recent study in mice also highlighted further DAM contribution to amyloidosis. Replicative senescence was observed in DAM and interrupting this proliferation led to reduced DAM and eventually reduced amyloidosis and synaptic damage (Hu et al., 2021). Since these DAM have been found to contribute to amyloidosis, some have studied microglial depletion *in vivo*. These studies have shown that plaque formation is dependent upon microglial activity (Spangenberg et al., 2019). Multiple studies have shown that sustained, early depletion of microglia is required to reduce amyloidosis (Dagher et al., 2015; Hu et al., 2021; Sosna et al., 2018; L. Zhan et al., 2019) and that these cells in later stages of the disease do not have an effect on plaque number or size nor clear Aβ from the brain (Spangenberg et al., 2016). The evidence suggests that they instead contribute to synaptic damage and neuronal loss (Gerrits et al., 2021; Spangenberg et al., 2016, 2019). This is most evident in a recent study using single nucleus RNA sequencing (SNuc-seq) on samples from AD donors, found that these DAM microglia are of two different subtypes depending on whether they are plaque associated or tau associated (Gerrits et al., 2021; Morabito et al., 2021) Plaque associated microglia, the AD-1 subtype, were the most genetically different from homeostatic microglia with 2000 differentially expressed genes with gene ontology associating these microglia with cell migration, phagocytosis and lipid localization. Their abundance was found to be correlated with Aβ load. The tau- associated AD-2 subtype arose

in response to phosphorylated tau bearing neurons with gene ontology indicating possible trophic functions such as synapse organization and axogenesis.

*Astrocytes*

Astrocytes are the most prevalent cell type in the CNS. The endfeet of astrocytic processed envelop both neurovasculature and neurons forming a neurovascular unit (Arranz & De Strooper, 2019). This system allows for regulation of the blood-brain barrier and helps regulate ion and metabolite homeostasis within the nervous tissue (Arranz & De Strooper, 2019; Sofroniew & Vinters, 2010; Verkhratsky et al., 2010). In addition to this, they assist with neurotransmitter homeostasis, particularly glutamate and participate in Aβ clearance (Arranz & De Strooper, 2019; Verkhratsky et al., 2010). Astrogliosis can be initiated through many processes but in AD, include signaling from neurons, activated microglia (Liddelow et al., 2017), and the Aβ peptide (Habib et al., 2020). The Aβ peptide can initiate calcium ion oscillations which can contribute to neuronal dysfunction. These activated astrocytes can then go on to participate in the neuroinflammation seen in microglia such as the release of proinflammatory cytokines, chemokines and factors as well contribute to oxidative stress. (Arranz & De Strooper, 2019; Verkhratsky et al., 2010). Astrocytes develop these distinct inflammatory states over time and lose many of their homeostatic functions such as phagocytic capabilities (Liddelow et al., 2017).

Usually, these reactive astrocytes in AD can be categorized as A1 or neurotoxic phenotype (Hasel et al., 2021). These A1 astrocytes have higher expression of proteins involved in the complement cascade, particularly C3, complement component 3. C3 is present in AD post-mortem brains and has been implicated in other neurodegenerative diseases (Arranz & De Strooper, 2019; Wu et al., 2019). Notably, A1 astrocytes are induced by activated microglia via secretion of IL-

1α, complement component C1q and TNF signals (Liddelow et al., 2017). Like in with microglia, these populations of reactive astrocytes exist within subtypes. Recent single cell studies have also shown at least 8 clusters of astrocytes. It seems that in disease states, these reactive astrocytes are interferon-gamma (IFN-γ) responsive potentially through STAT1-IGTP mediated signaling, and can express high levels of a metalloprotease *Timp1* and chemokine *Cxcl10* (Hasel et al., 2021). Astrocytes also have the highest expression of APOE in the nervous system and with the ε4 allele, studies observed impaired Aβ clearance and increased initiation of plaque seeding (Arranz & De Strooper, 2019).

A recent study employed longitudinal sNuc-seq to characterize astrocytes in the hippocampi of WT and 5xFAD AD model mice (Habib et al., 2020). Researchers discovered a disease-specific state in astrocytes, which they termed disease associated astrocytes (DAA). These DAA appeared early in AD mice and increasing with age and disease progression. Among upregulated genes in DAA were those related to Aβ accumulation and increased proteolytic processing of the APP which liberates Aβ peptide. Other upregulated genes were those involved in endocytosis, which can increase APP trafficking to endosomes promoting the amyloidogenic pathway and those involved in the complement cascade and aging. APOE and other genes involving amyloid metabolism and clearance were also increased. Additionally, DAA like cells were found in aged WT mice and in non-AD donor samples, implicating aging as part of astrocyte mediated disease pathology (Habib et al., 2020).

### 1.5 Risk Factors for Alzheimer's Disease

Advanced age is the greatest risk factor for AD. However, genetic factors play a significant role in both the early onset of the disease (EOAD), which occurs at less than 65 years of age, and in the late onset of the disease (LOAD) (Cacace et al., 2016; Tanzi, 2012). As mentioned before

in Section 1.3, the discovery of mutations in 3 genes were critical in understanding the development of AD. The genes encoding APP in which the Aβ sequence is located, and in PSEN1 and PSEN2 genes which affect the catalytic subunit of the γ-secretase complex, which cleaves APP to liberate the neurotoxic Aβ peptide (Cai et al., 2015; Kounnas et al., 2010; Tanzi, 2012), These genes have been crucial in our ability to study AD, and form the basis for creation of many mouse models of the disease. However, mutations in APP, PSEN1 and 2 only explain about 1% of all AD cases and we have not yet ascertained what constitutes the rest of the risk (Cacace et al., 2016; Tanzi, 2012). Polymorphisms in other genes have a role in LOAD. APOE ε4 specifically, confers risk of AD in a gene dose dependent manner (Corder et al., 2009; Strittmatter et al., 1993). Additionally, to further underscore the importance of inflammation in disease pathogenesis, genome-wide association studies (GWAS) have implicated many immune related genes in the pathology of AD (Braak & Braak, 1991; Kunkle et al., 2019). However, from large scale studies of monozygotic twins indicate that concordance of AD is not 100% and indicates that about 60% of AD is heritable (Gatz et al., 1997, 2005, 2006). GWAS, meta-analyses and evidence from basic science have estimated that 70% of AD risk is attributable to aging and genetics with APOE ε4 conferring the majority of the genetic risk (Ballard et al., 2011; Cacace et al., 2016; Hsu et al., 2018). This evidence implies that non-heritable, non-genetic factors interact with aging and host genetics leading to neurodegenerative phenotypes. It is imperative that we explore these factors as most therapeutic interventions as discussed in Section 1.1 of this introduction, have been focused on reduction of Aβ and sometimes tau protein, but these efforts have not been universally successful.

Various epidemiological studies and basic science studies, along with associated meta-analyses, have identified a number of modifiable risk factors for AD which can either be

detrimental or protective (Livingston et al., 2020; Mir et al., 2020). These include lifestyle factors such as family and medical history such as diabetes (Baglietto-Vargas et al., 2016) or cardiovascular disease (Santos et al., 2017), sleep (Bero & Tsai, 2016) obesity, education, bilingualism, smoking status, traumatic brain injury and many more (Eid et al., 2019; Livingston et al., 2020; Ou et al., 2021; W. Xu et al., 2015), occupational and environmental exposures to various toxicants and pollutants such as air pollution (Kilian & Kitazawa, 2018), heavy metals (Bakulski et al., 2020), pesticides and antimicrobials (Mir et al., 2020; Yegambaram et al., 2015). Other lifestyle factors have presented as protective such as social contact, dietary intervention, weight control, limiting excessive alcohol consumption, increasing exercise and practicing better sleep hygiene (Livingston et al., 2020).

Metal exposure is implicated in AD as epidemiological studies have correlated cognitive decline with changes in metal concentrations in brains or sera of patients (Huang et al., 2004; G. Liu et al., 2006; Lovell et al., 1998). Additionally, many heavy metals are known neurotoxicants and metal ion homeostasis is dysregulated in AD (Atwood et al., 2008; Bakulski et al., 2020; Yegambaram et al., 2015). These environmental/ lifestyle attributable factors can also interact with genetic factors (Farooqui & Farooqui, 2017; Moser & Pike, 2017; Qiu et al., 2009) to accelerate AD pathology and should therefore be investigated as they present as easier therapeutic targets, since they may be preventable/ modifiable (Gatz et al., 2006; X. X. Zhang et al., 2021).

### 1.6 Copper, an essential micronutrient and putative environmental risk factor for AD

Copper (Cu), an essential micronutrient was established as early as 1928, when copper deficiency led to anemia in rats and so is necessary for red blood cell production(Hart et al., 1928). A 70-kg adult human contains around 110mg of Cu with most of it being in being located in the liver, brain, blood bone marrow and skeletal muscle (Linder et al., 1998). This is likely due to

some of the functions copper serves as an enzymatic cofactor. As a transition metal capable of assuming different redox states, copper is incorporated into several enzymes. The most abundant of these being ceruloplasmin, involved in iron transport (Uriu-Adams & Keen, 2005). Copper is also a cofactor for cytochrome c, vital to aerobic respiration; anti-oxidant enzymes such superoxide dismutase, which rids the body of the superoxide radical and is thus critical in host defense against reactive oxygen species (ROS) (Deibel et al., 1996; Mason, 1979; Uriu-Adams & Keen, 2005). Further proof of essentiality is observed through observations from a copper deficiency. *In utero* deficiencies lead to cardiovascular, bone, immunologic and neurological abnormalities throughout lifespan (Huat et al., 2019; Madsen & Gitlin, 2007; Uriu-Adams & Keen, 2005). While a deficiency in copper is detrimental to health, like many other vitamins and cofactors, excess can be detrimental as well. This is really emphasized by the life-threatening disorders Menke's and Wilson's Diseases, which are caused by mutations in the P-type copper transporting ATP pumps, ATP7A and ATP7B respectively. Both diseases result in neuronal dysfunction and neuropsychological disorders, further underscoring the importance of copper homeostasis in the brain (Huat et al., 2019; Madsen & Gitlin, 2007).

Copper is also a redox active and when unbound in the aqueous $Cu^{2+}$ oxidation state, can partake in reactions which generate neurotoxic reactive oxygen species (ROS) and prevalent in AD brains(Deibel et al., 1996; Pham et al., 2013). APP has a $Cu^{2+}$ binding site and has a redox potential for the ion, which may play a role in redox cycling of between the $Cu^{2+}$ and $Cu^{+}$ states generating ROS (Huat et al., 2019; Multhaup et al., 1996). Studies have shown that Aβ can sequester copper, which may cause dyshomeostasis and potentiate plaque formation (Huat et al., 2019; Syme et al., 2004).

Numerous studies have found correlations with changes in labile, or free copper levels in

blood serum and brain parenchyma with cognitive decline and conversions to AD in humans. Pertinent examples include a study with AD patients and age-matched cognitively normal individuals, found that serum copper levels were significantly higher in AD patients with an increase of 1 μmol/L conferring 80% of the increased risk for the disease (R Squitti et al., 2002). Another study found that labile or free serum copper correlated with poorer cognitive status and increased CSF Aβ and phosphorylated tau. Moreover, CSF copper content was partially dependent on the free copper content in serum (R. Squitti et al., 2006). In a study of a pair of elderly monozygotic twins, serum copper and the levels of the ROS peroxide were 44% higher in the twin with greater cognitive decline and an Alzheimer's Disease diagnosis (Rosanna Squitti et al., 2004). In AD brain tissues, copper levels were significantly decreased in the amygdala and in the hippocampus (Deibel et al., 1996). These findings suggest copper dysregulation in the AD brain as copper levels are changed in various tissue types. A number of epidemiological studies have now correlated this labile, or free, copper serum levels with increased severity of cognitive decline, conversion from mild cognitive impairment (MCI) to AD and impaired clearance of Aβ evidenced by lower concentrations of the Aβ peptide in CSF (R. Squitti et al., 2003, 2005; R Squitti et al., 2002; Rosanna Squitti et al., 2004; Ventriglia et al., 2012).

In mice and other rodents, a number of studies have shown that exposure to copper increased cognitive impairment, increased Aβ plaque burden and tauopathy (Kitazawa et al., 2009), impaired phagocytosis of Aβ peptide by microglia (Hsu et al., 2019; Kitazawa et al., 2016; Singh et al., 2013) and the downregulation of a clearance associated protein low-density lipoprotein receptor-related protein 1(LRP1) (Hsu et al., 2019). Several studies show that even trace amounts of copper can have detrimental effects. These studies showed that even trace amounts of copper in drinking water, ten times less than the Environmental Protection Agency's

(EPA) limit, (Environmental Protection Agency, 1991) were associated with plaque burden and cognitive deficits (Hsu et al., 2019; Singh et al., 2013; Sparks & Schreurs, 2003). Recently, our lab has shown through Translating Ribosome Affinity Purification RNA sequencing (TRAP-seq) that copper induces dyshomeostasis in the microglia of J20 AD model mice prior to amyloidosis and potentiated inflammatory activation in the brain (Lim et al., 2020). Further, at 5 months of age copper-exposed WT animals displayed upregulated immune/inflammatory genes suggesting an immunomodulatory role of copper within the brain. Copper directs microglia towards the degenerative or DAM phenotype (reviewed in Section 1.4) After 3 months of exposure, homeostatic gene expression was reduced in both WT and J20 animals with a significant increase in the degenerative phenotype in J20 compared to WT. To further explore the role copper might have on microglia, Ingenuity Pathway Analysis (IPA) was conducted on microglia-specific genes. The incidence of immune responses increased with age in the WT mice, further highlighting an immunomodulatory role for copper (Tan et al., 2020; Zheng et al., 2010) beyond which we observe in conjunction with Aβ pathology (Lim et al., 2020). While much of the observations of copper dysregulation and disease has been in the brain, it is likely copper can induce change elsewhere in the peripheral system which may then play a role in disease etiology.

### 1.7 The Role of the Microbiome in Health and in Alzheimer's Disease

Microbes are present along the digestive tract from the stomach to the large intestine in mammals (Walter & Ley, 2011). While many other bodily sites have its own microbiota, that of the gut tends to be the most diverse (Marchesi et al., 2016; Shreiner et al., 2015). The gut microbiota is primarily made up of Firmicutes and Bacteroidetes, with smaller contributions from other phyla such as Proteobacteria, Verrumicrobia and Actinobacteria (Sommer & Bäckhed, 2013). In the human gut, about $10^{14}$ bacterial cells are present. Their combined genomes are about

two orders of magnitude larger than host cells, which then lends to a range of biochemical and metabolic functions which contribute impact our physiology in health and disease (Kau et al., 2011; Shreiner et al., 2015; Sommer & Bäckhed, 2013). Notably, have metabolic and immunologic functions which can directly or indirectly affect most of the physiologic functions in the host (Dalile et al., 2019; Morrison & Preston, 2016; Shreiner et al., 2015). Gut microbes have been found to help the development of lymphoid structures (e.g. mesenteric lymph nodes), modulate immune cell differentiation and immune mediators, carbohydrate and glucose metabolism, adiposity and neurobehavior (Cheung et al., 2019; Jiang et al., 2015; Larsen et al., 2010; Tilg et al., 2019; Valdes et al., 2018).

Carbohydrate fermentation is a fundamental activity of the gut microbiota. Conversion of indigestible, dietary carbohydrates into short-chain fatty acids (SCFA) such as butyrate, serves microbial cross-feeding and 10% of host energy requirements is derived from colonic fermentation (Marchesi et al., 2016). It is unsurprising then, that the microbiome has been found to be a key regulator in the development and maintenance of host immunity as SCFAs are ligands for free fatty acid receptors (FFARs) which are expressed on a number of cells types, including immune cells like microglia (Abdel-Haq et al., 2018; Kowalski & Mulak, 2019; Morrison & Preston, 2016; Ratajczak et al., 2019). The gut microbiota have therefore been found to be critical for development and maturation of the brain, immune, endocrine system (Belkaid & Hand, 2014; Erny et al., 2015; Fröhlich et al., 2016). Further, it has been shown that the gut microbiota synthesize or is at least involved in the metabolism/catabolism of several neurotransmitters such as dopamine, noradrenaline, serotonin, GABA, acetylcholine and histamine (Cox & Weiner, 2018; Strandwitz et al., 2019). As such, there is communication from the gut microbiota to the brain and this is thought to be bidirectional, with stress/inflammation/ dysfunction in either system affects the other

(Cox & Weiner, 2018; Powell et al., 2017; Valles-Colomer et al., 2019).

A healthy microbiome is resistant to pathogens and is anti-inflammatory. This depends on a healthy balance of microbial communities. This balance can be and is implicated to be disrupted by diet, medication (e.g. antibiotics) and environmental exposures (Belkaid & Hand, 2014; Fröhlich et al., 2016; Ratajczak et al., 2019). A dysbiotic (imbalanced, unhealthy) gut microbiome has been tightly connected to a number of number of metabolic, inflammatory and neurodegenerative disorders such as obesity (Hildebrandt et al., 2009; Turnbaugh et al., 2006), inflammatory bowel disease (Halfvarson et al., 2017; Manichanh et al., 2012), type 2 diabetes (Larsen et al., 2010; Qin et al., 2012), depression (Cheung et al., 2019; Jiang et al., 2015) and Parkinson's disease (Hill-Burns et al., 2017; Sampson et al., 2016).

The gut microbiome is indeed altered in Alzheimer's Disease patients. A recent study revealed that AD patients' microbiome had less richness, diversity and a distinct composition in comparison to asymptomatic, age- and sex-matched controls (Vogt et al., 2017). This was characterized by a decreased abundance of Firmicutes and Actinobacteria and an increased abundance in the gram-negative phylum, Bacteroidetes. This is a correlation which has been observed in other diseases such as type 2 diabetes and Parkinson's disease. Lipopolysaccharide (LPS), which is a large molecule usually found on the outer membrane of gram-negative bacteria, is thought to disrupt the integrity of the blood brain barrier and has been found to be colocalized with Aβ plaques (Miklossy, 2011; X. Zhan et al., 2016, 2018). A year later, a study in a Chinese cohort also found a difference in the gut microbiota in AD patients (Zhuang et al., 2018).

To give further evidence of the possible role of the microbiota in AD pathology, a recent study comparing wild-type (WT) and AD model mouse (APP/PS1), derived as littermates, revealed that a distinct gut microbiome develops with age in the transgenic mice (Harach et al.,

2017). In comparison to their WT littermates, APP/PS1 mice had significant reductions in Firmicutes, Verrucomicrobia, Proteobacteria and Actinobacteria with significant increases in Bacteroidetes and Tenericutes, a gram-positive phylum primarily made up of mucosal pathogens. The authors also revealed that germ-free APP/PS1 mice had greater levels of Aβ degrading enzymes, neprilysin degrading enzyme (NPE) and insulin degrading enzyme (IDE) and had decreased Aβ pathology in comparison to normal APP/PS1 mice, without any differences in APP expression. In another study, ablation of the gut microbiome in APP/PS1 mice using antibiotics reduced Aβ pathology, hinting at a direct role of microbial involvement in amyloidosis (Minter et al., 2017). Moreover, in a study using germ-free APP/PS1 mice, microglia isolated from these animals show a significantly altered inflammatory gene expression profiles of ramified microglia, which led to an attenuated, but not ablated immune response in response to an immune challenge (Minter, Zhang, et al., 2016).

### 1.8 Copper, gut microbiota, and the pathogenesis of AD

Exposure to copper in humans is primarily oral. Through diet, supplements, and drinking water, copper comes into direct access with the gut microbiome (Hsu et al., 2018). This is of some concern as copper is also a natural antimicrobial. It has demonstrated efficacy at eliminating pathogenic microorganisms such as *S. aureus* and *E. coli* and is incorporated into antimicrobial agents and surfaces today (Vincent & Engels-Deutsch, 2016).

Chronic copper exposure in drinking water can induce dysbiosis (Meng et al., 2018; Zhai et al., 2017; F. Zhang et al., 2017) in gut. It is possible that copper disrupts the beneficial balance of the commensal and mutualistic relationships in the gut in favor of the pathogenic. This may cause mucosal and epithelial layer thinning, infiltration of bacterial cells across the epithelium,

inhibition of dendritic cells and stimulation of inflammatory monocytes, culminating increased inflammation. In a study using Sprague Dawley rats and a dietary exposure to copper-containing chow, copper concentrated several-several fold in the ileum and the colon (F. Zhang et al., 2017), the sections of the intestine with the highest microbial loads (Walter & Ley, 2011). Importantly, this study showed that the copper can affect the composition of the gut microbiota and this change correlated with serum increase in the pro-inflammatory cytokine, TNF-α. There are several instances in the literature with similar findings of altered microbiota in mice and fish (Meng et al., 2018; Zhai et al., 2017; F. Zhang et al., 2017). In some of these studies, these changes are correlated with an increase in peripheral proinflammatory cytokines. Due to this reason and the significant role that neuroinflammation plays in AD, the connection between inflammation in the periphery and the brain in this study is investigated in this dissertation.

### 1.9 Mouse Models in AD and a Brief Overview the hAPP$^{NL-G-F}$ -KI (APP-KI) and the Triple Transgenic (3xTg-AD) mouse model

AD is a uniquely human disease. However, to be able to manipulate and investigate hypothesis about disease neuropathology, there is a need for *in vivo* modelling, as access to neural tissue is most often confided to post-mortem confirmation of disease and proper, permissible access to that tissue (Hall & Roberson, 2012; Tai et al., 2021). All the mutations that cause autosomal dominant AD alter, usually to increase, the production of Aβ, through APP processing. Since mouse Aβ does not tend to form fibrils and plaques like human Aβ, transgenic mice with human APP (hAPP) form some of the oldest and most widely used mouse models in AD research (Hall & Roberson, 2012). Normally, the transgene has been expressed from numerous promoters with thymocyte differentiation antigen (Thy-1) being a popular choice as it is neuron specific. These promoters drive different levels (often overexpression) and spatial patterns of expression of

Aβ and tau. To further investigate pathogenesis, many familial mutations are included on the transgene, for example K670N/M671L Swedish double mutation at the β-secretase cleavage site of hAPP protein (Hall & Roberson, 2012; Tai et al., 2021).

However, the genetic manipulation is not restricted to APP alone but expands to the presenilin cleavage proteins (PSEN1 and 2) and the MAPT gene (Tau) incorporating familial or risk mutations and single nucleotide polymorphisms as well (Hall & Roberson, 2012; Tai et al., 2021). The field incorporates the use of single and multi-transgenic models like the 3xTg-AD. The field is constantly striving to improve mouse models of the disease. As such, the field has been using recent genetic advances such as CRISPR/CAS9 (Paquet et al., 2016) to generate improved models of the disease, some to bypass disadvantages or molecular artefacts of transgenic models (Saito et al., 2014) and to better model LOAD (Baglietto-Vargas et al., 2016; Oblak et al., 2020). Most AD mouse models exhibit cognitive deficits and like in humans it is not proportional to Aβ plaque load but to the soluble Aβ species (LaFerla & Green, 2012).

*Triple Transgenic (3xTg-AD)*

This mouse model was developed with APP with the Swedish mutation and $tau_{P310L}$ transgenes cointegrated at the same genetic locus in a mouse line that had a knock in of the $PSEN1_{M146KV}$ mutation (Oddo et al., 2003) Overall, this mouse model develops age-related and progressive phenotypes that include amyloidosis, gliosis, neurofibrillary tangles as well as synaptic dysfunction (Sterniczuk et al., 2010) . In this mouse model, robust extracellular deposits are observed at about 12 months of age and has better penetrance in female mice(Belfiore et al., 2019).

*$hAPP^{NL-G-F}$ -KI (APP-KI)*

As mentioned before, many mouse models suffer from artificial phenotypes as they overexpress APP (and therefore Aβ and other APP fragments).This genetic manipulation allows for m Saito et. al, developed a knock in mouse model that harbors the Swedish, Beyreuther/Iberian and Arctic mutations (Saito et al., 2014). Together, these mutations increase the amount of toxic Aβ species present and enhances their oligomerization. Plaque pathology is detectable as early as 4 months and the cortex and hippocampus are saturated by 9 months (Masuda et al., 2016; Saito et al., 2014; Sakakibara et al., 2019). Robust gliosis and astrogliosis was detected at 9 months (Saito et al., 2014) but has been detected as early as 6 months (Masuda et al., 2016). This mouse model also displays abnormalities including declined spatial learning, increased impulsivity, loss of avoidance memory much like other transgenic models as early as 6 months but more robustly at 12 months (Masuda et al., 2016; Mehla et al., 2019)

**1.10 Dissertation Rationale**

Altogether, the evidence shows that there is a potential for copper to perturb the gut microbiome composition and exacerbate AD pathology. Most of the studies exploring copper's effect on the microbiome have done so using chow or drinking water with levels far exceeding the upper limit of 1.3 ppm for safe drinking water set by Environmental Protection Agency (Environmental Protection Agency, 1991). The studies investigating gut microbiota and its effect on AD, do so using germ-free or antibiotic ablation techniques in AD animals overexpressing the APP gene (Harach et al., 2017; Minter, Zhang, et al., 2016). It has been shown that host genetics can affect the composition of the gut microbiome (Goodrich et al., 2014) . This dissertation explores the temporal changes in the gut microbiome at the environmentally relevant level of 1.3

ppm of copper in wild-type, C57BL/6J and in the AD model, $APP^{NL-G-F}$-KI, while also examining the status of neuropathology and cognition. Examining the microbiome at various time points identifies copper-sensitive microbes and the ones that then proliferate in the dysbiotic gut. Functional implications of these changes such as peripheral inflammatory status, are determined through investigation of the literature on contributions of those microbes to host health. Amyloidosis, gliosis, and changes in cognition in water versus copper treated animals was also investigated to determine the contribution of the gut microbiome to disease etiology. Studies of the microbiota and its relationship to disease is a new and growing field of interest. These studies help to elucidate the role of the microbiota on Alzheimer's Disease outcome considering a chronic environmental exposure and identify critical populations of gut microbiota on host health for future investigations.

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# Chapter 2

## Characterizing the Effect of 3-month Copper Exposure on Neuropathology and on the Gut Microbiome in an APP Knock-In Model of Alzheimer's Disease

### 2.1 Introduction

Alzheimer's disease (AD) is the most common form of dementia amongst the elderly(Alzheimer's Association, 2022). It is a debilitating disease which at it most severe cause a complete loss of autonomy (Förstl & Kurz, 1999; Mehta & Schneider, 2021). While this is devastating for disease sufferers, there is large emotional and economic burden on caregivers as well (Allegri et al., 2006; Clyburn et al., 2000; Qiu et al., 2009). It is estimated that without a prevention or a cure, total annual payments for health, long-term and hospice care for suffers of Alzheimer's or other dementias is slated to increase from $321 billion to just under $1 trillion in 2050 in the US alone (Alzheimer's Association, 2022).

While aging and genetics are the primary risk factors of AD (Andrews et al., 2020; Bertram & Tanzi, 2012; Guerreiro et al., 2012), exposure to modifiable environmental factors (Cristóvão et al., 2016; Huang et al., 2004; Yegambaram et al., 2015), including toxicants (Kilian & Kitazawa, 2018), lifestyle, and medical conditions (Livingston et al., 2020), also greatly modulate the onset and progression of AD. Chronic exposure to copper is thought to be an environmental risk factor for AD (Bagheri et al., 2018). Recent epidemiological evidence has highlighted, in the elderly, that high serum levels of labile, or free, copper correlate with characteristics of AD such as pro-inflammatory activation, cortical thinning, and cognitive decline (R. Squitti et al., 2003, 2005; Rosanna Squitti, 2012). Although copper is in an essential metal, exposure, the inorganic cupric form ($Cu^{2+}$) from the environment may exert toxicity. Classically, $Cu^{2+}$ is known to seed amyloid

beta (Aβ) peptide and enhance its aggregation and toxicity (Lovell et al., 1998; Syme et al., 2004). We have demonstrated that copper exacerbates AD neuropathology and cognitive decline in several mouse models of AD. Our studies indicate that copper drives microglia to a more diseased associated phenotype(Lim et al., 2020), affect their phagocytic capability and clearance of amyloid beta from the brain(Kitazawa et al., 2016). Copper is also associated with cognitive deficits in both WT and AD model mice (Hsu et al., 2019).

Copper is a natural antimicrobial agent whose environmental exposure is predominantly through oral routes such as drinking water, food and through nutritional supplements (Morris et al., 2006). Therefore, before uptake via copper transporters in the gut, labile copper will interact with the gut microbiome. Chronic exposure to copper could eliminate potentially beneficial, copper-sensitive microorganisms in the gut, possibly reducing its diversity and richness with negative disease outcomes. The composition of gut microbiota is indeed distinct in AD patients in comparison with healthy age-matched individuals (Emery et al., 2017; Vogt et al., 2017). This observation is recapitulated in several mouse models of AD (Brandscheid et al., 2017; Chen et al., 2020; Harach et al., 2017; Kaur et al., 2021; Minter et al., 2016), with studies showing microbiota from AD mice promote Aβ pathology in the germ-free strain of the same AD model (Harach et al., 2017; Minter et al., 2016). Collectively, emerging findings implicate a pivotal role for the gut microbiota in AD pathology.

Recent studies demonstrate that oral $Cu^{2+}$ ingestion alters the gut microbiome, whose richness and diversity significantly modulate host immunity, metabolism, and neuronal activity (Meng et al., 2018; Zhai et al., 2017; Zhang et al., 2017). However, many studies that implicate copper in the gut microbiome tend use fairly high concentration of copper, some as high as 1000 ppm (Zhai et al., 2017), which is not environmentally relevant for the every day population.

Here, I conducted a pilot study to investigate a hypothesis that exposure to an environmentally relevant level of copper (1.3 ppm as set by the EPA(Environmental Protection Agency, 1991)) disrupts the gut microbiota leading to a more pathogenic composition to contribute to AD pathogenesis.

## 2.2 Materials and Methods

### 2.2.1 Animals

All mice used in this study were housed on a 12-hour light/12-hour dark cycle with the light cycle beginning at 6:30 am and concluding at 6:30 pm. All experimental protocols were performed during the light phase. All animal procedures were performed in accordance with National Institutes of Health and University of California guidelines and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine. Animals were fed water and food ad-libitum. The feed was the Teklad Global Soy Protein-Free Extruded Rodent Diet (Envigo 2020X, Madison, WI). 3 months-old male and female wild-type (n=18) and $APP^{NL-G-F}$-KI (n=21) mice were bred in house and were evenly randomly assigned to a treatment group.

### 2.2.2 Experimental Design

*Copper Chloride (CuCl2) Preparation and Administration*

1.3 ppm $CuCl_2$ (CAS # 7447-39-4, Sigma-Aldrich, Cat # 751944) was prepared fresh in ultrapure water every two weeks from a 100X stock solution stored at room temperature. The mice were exposed to either MilliQ water or 1.3ppm $CuCl_2$ in drinking water for 3 months and were supplemented with fresh water every two weeks at cage change.

*Fecal Sampling*

The fecal samples were collected before exposure and then monthly until sacrifice for WT mice. In the case of APP-KI mice, after consultation with the UCI Microbiome Initiative, sampling was more frequent in this cohort in the hope of capturing the earliest shifts, if any in the microbiome due to copper exposure. Fecal samples were then collected before exposure, weekly for the first month and then monthly until sacrifice. For fecal sampling, mice were placed in sterile cages without bedding. Approximately 5 pellets were collected into sterile 2 ml cryogenic tubes. Samples were then stored at -80$^0$C until DNA extraction for sequencing.

*Tissue Processing*

Upon sacrifice, the animals were euthanized with an overdose of pentobarbital. While anesthetized blood was collected from cardiac puncture and placed into Lithium Heparin-lined Microtainer tubes for later plasma extraction. The animals were then perfused with cold PBS, pH 7.4 and the brain extracted. One hemisphere was then fixed in 4% paraformaldehyde for 48 hours, cryopreserved in 30% sucrose then stored at -80°C for histopathology. The hippocampus and cortex were microdissected and snap frozen on dry ice for biochemical analyses.

*Immunofluorescence*

Cryopreserved brain hemispheres were sectioned at 40µM using the Leica Biosystems SM2010 R Sliding Microtome (Buffalo Grove, IL). The platform was kept at -80$^0$C by adry ice/ethanol mixture. Sections were stained for a variety of endpoints including GFAP (DakpMillipore, Burlington, MA), microglia (Iba-1, FUJIFILM Wako Chemicals USA Corporation, Richmond, VA , CD68, BioRad, Hercules,CA ) surrounding 82E1( IBL International, Morrisville, NC) and Thioflavin-S (Sigma- Aldrich, St. Louis, MO) positive plaques. Primary

antibodies were coupled with AlexaFluor antibodies(488, 555 or 633) raised in goat. Sections were counterstained with DAPI to identify cell nuclei. Images were captured using the EVOS FL Cell Imaging System. Analysis of the images were done on using ImageJ.

*Immunoblot and Enzyme Linked Immunoassay*

As mentioned before mice brains were dissected on ice, one hemisphere was microdissected and flash frozen for biochemical analysis; the other hemisphere was collected for immunohistochemical staining. Protein extracts were prepared by homogenizing frozen brain regions in T-PER extraction buffer (150 mg/ml, Thermo Fisher Scientific, cat# 78510), complemented with protease and phosphatase inhibitors (Thermo Scientific, cat# 78445), and ultracentrifuged at 100,000 x g for 1 hour at $4^0$C. The supernatant, the detergent soluble fraction, was removed and stored at -$80^0$C until protein concentration determination and use in assays. Protein concentration was determined from the supernatants using the Bradford assay following the manufacturer's protocol (Bio-Rad, Hercules, California). The precipitate was treated with 70% formic acid and ultracentrifuged at 100,000 x g for 1 hour at 4°C to digest and obtain the detergent insoluble protein fraction.

Protein concentration from the hippocampus in detergent-soluble fractions was measured using the Bradford protein assay. Standardized protein mass was separated by SDS- PAGE and transferred to Immobilon-FL PVDF membrane (EMD Millipore). Membranes were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) for 1h at room temperature before overnight with shaking at 4°C with primary antibody. The following antibodies diluted in Odyssey Blocking Buffer with 0.2% Tween 20: CT-20 (Calbiochem, catalog # 171610, 1:1000 dilution, GAPDH (Santa Cruz Biotechnology Inc., catalog # sc-25778, 1:5000 dilution). Membranes were then washed in TBS-0.02%Tween-20 (TBS-T), before incubated with corresponding IRDye secondary

antibodies (LI-COR Biosciences, 1:20000 dilution) diluted in Odyssey Blocking Buffer with 0.01%SDS, 0.02% Tween-20 for 1 hour at room temperature. Membranes were then washed in TBS-T and kept in TBS before being scanned using the Odyssey Imaging System (LI- COR Biosciences). Band intensities were quantified with Image Studio software (version 5.2, LI-COR Biosciences) and normalized to that of the GAPDH protein loading control.

Aβ38, Aβ40, and Aβ42 were quantified in both detergent- soluble hippocampal tissue using the V-PLEX Plus Aβ Peptide Panel 1 (6E10) Kit (Meso Scale Discovery). Aβ38, Aβ40, and Aβ42 peptide calibrators and protein fractions were loaded onto the MSD MULTI-SPOT® 96-Well 4-Spot plate after the addition of detection antibody solution. Plate was read on a SECTOR® Imager plate reader (MSD) after 2-h incubation, washed, and loaded with Read Buffer T. Oligomeric Aβ was quantified using the High Molecular Amyloid-β Oligomer ELISA Kit(Wako, cat #298-80101), a colorimetric 96-well assay which was read at 450 nm.

*16S ribosomal Ribonucleic Acid(rRNA) Amplicon Sequencing*

Approximately 100mg fecal matter from WT and APP-KI cohort was weighed and submitted to the UCI Microbiome Initiative for DNA isolation, sequencing, and taxonomic classification through their 2019 Pilot Project Award. DNA samples were isolated using the Zymobiomics DNA mini kit from Zymo Research. 16S rRNA amplicon PCR was performed targeting the V4 - V5 region using the EMP primers (515F (barcoded) and 926R) and the samples were then pooled into a library. The library was sequenced at the UC Irvine Genomics High-Throughput Facility using a MiSeq v3 chemistry with a PE300 sequencing length. Sequencing resulted in 17.1 M reads passing filtering step with an overall Q30>56.3%. The low-quality score resulted from a bad quality Read 2. Read 1 had Q30> 83.4%. Therefore, analysis was done only using the forward read (Read1). The raw sequences were imported into QIIME2. 11398918 single

end reads were binned into the designated barcodes. After an initial sample quality check and trimming (DADA2 in QIIME2) there were 7526370 single end reads. These reads were used for further analysis. From the sequences the first 5 bp were trimmed and the reads were truncated at 299 bp. All samples showed good number of reads, with the lowest being 7039 reads which was used as the rarefaction depth in analysis. The sequences were assigned a taxonomic classification using the May 2013 greengenes database), trained with the primer pairs that were used to amplify the 16S region.

*Statistical Analyses:* Analyses were conducted in R and Primer-E PRIMER6+ PERMANOVA software to calculate effect sizes, SIMPER and PERMANOVA analyses for microbiome data. All other data are presented as mean ± SEM, and statistical analyses were done using GraphPad Prism 7. For comparisons between three or more groups, one-way ANOVA, or two-way ANOVA (for two factors) with Fisher's LSD post hoc test was used to evaluate statistical significance, unless otherwise stated. Data with $p < 0.05$ was considered statistically significant.

## 2.3 Results

*Amyloid Pathology*

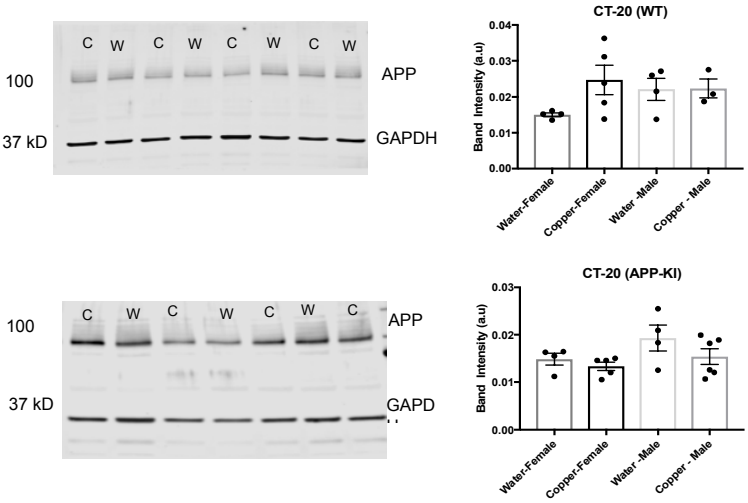



***Figure 2.1.*** *Full length APP expression through incubation with CT-20 antibody (EMD Millipore). Probe of smaller fragments CTF-$\gamma$. CTF-$\beta$ and CTF-$\alpha$ proved difficult and is therefore not shown here. Hippocampal lysates were used. One -Way ANOVA ($p > 0.05$) was conducted in GraphPad Prism 7 and graphs represent the mean ± SEM.*

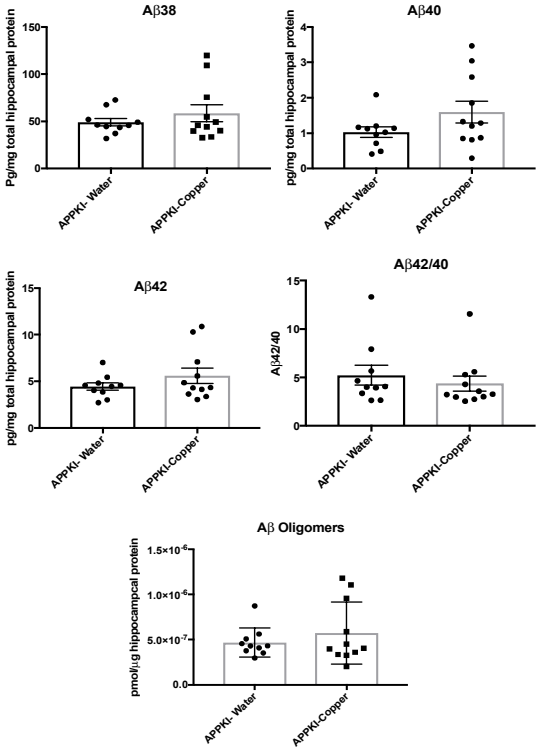



***Figure 2.2.*** *Aβ species as quantified by ELISA using the MSD V-Plex Aβ Peptide Panel 1(6E10) kit in the soluble protein fraction of hippocampal lysate(A-D) Aβ oligomer concentrations in the soluble fraction of hippocampal lysate. There was no statistical significance between treatment groups. Comparisons were performed in GraphPad Prism 7 with a Welch's T-test and the graphs represent the mean ± SEM*

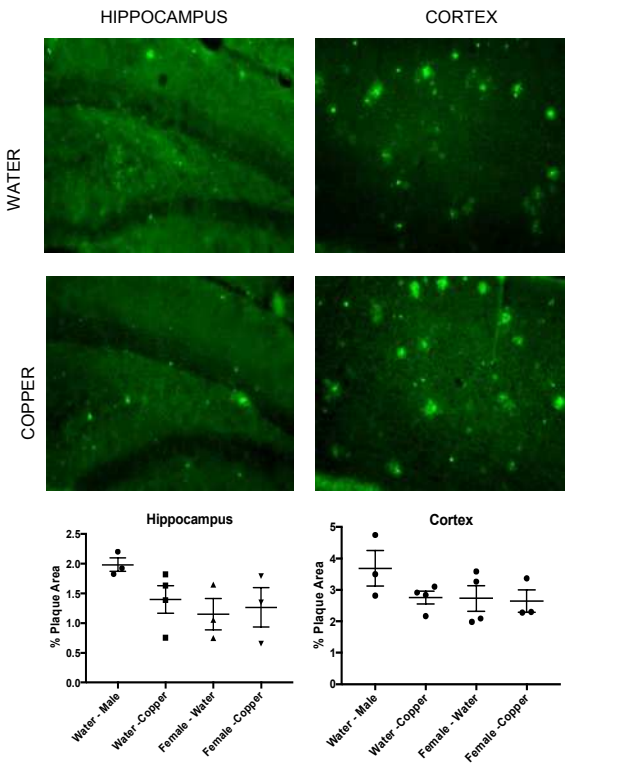
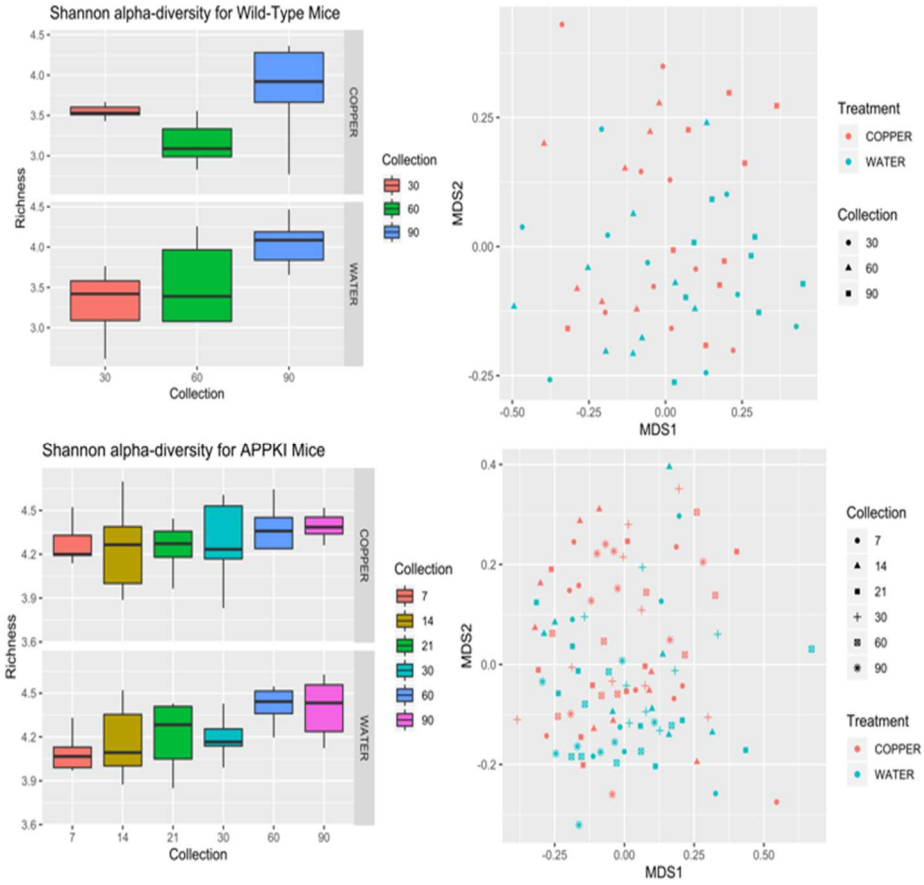



***Figure 2.3*** *Thioflavin S Positive Plaques in the hippocampus and cortex of APP-KI mice. Representative images were taken from male mice. Comparisons of the quantification of plaque area were performed in GraphPad Prism 7 using one-way ANOVA. Graphs represent the mean ± SEM*

Although, this mouse model has been characterized and shown to express physiological levels of APP(Saito et al., 2014), it was important to confirm this which was done by western blot (Figure 2.1). The differences were not significant between the groups and the genotypes. The bands from the lower molecular weight C-Terminal fragments of APP were not detected at the probe concentration of (1:2000), even with cutting the membrane and placing in a separate incubation container. It was then important investigate levels of Aβ in the soluble fractions from brain lysate. The hippocampal detergent soluble fraction was used in the MSD V-Plex Assay using the 6E10 antibody to detect Aβ 38, 40, and the more neurotoxic Aβ42 species. The soluble fraction was also used to detect oligomeric Aβ species since the Arctic mutation in the APP-KI model facilitates oligomerization of the peptide. (Figure 2.2). There were no significant differences in Aβ38,40 or

42 between treatment groups nor in the Aβ42/40 ratio, a marker for more severe AD pathophysiology. There were no changes in toxic Aβ oligomers between treatment groups either. 40 µM ventral sections (approx. bregma -3.16mm) sections of APP-KI mice were stained with Thioflavin-S to visualize Aβ aggregates. For analysis, 6 images (3 hippocampal and 3 cortical) taken at 10X were used to calculate plaque burden using ImageJ's Analyze Particles function. There were no significant differences observed between treatment groups. The cortex of these animals had greater cortical deposition of plaques than in the hippocampus (Figure 2.3).

*Changes to Gut Microbiota*




| WT PERMANOVA Result | |
|---|---|
| Collection Time Point | p-value of Treatment |
| 30 | 0.485 |
| 60 | 0.052 |
| 90 | 0.091 |
| Overall | 0.131 |

| APPKI PERMANOVA Result | |
|---|---|
| Collection Time Point | p-value of Treatment |
| 7 | 0.246 |
| 14 | 0.166 |
| 21 | 0.065 |
| 30 | 0.044 |
| 60 | 0.098 |
| 90 | 0.008 |
| Overall | 0.043 |

***Figure 2.4***. ***Left Panel*** – *Shannon alpha Diversity of WT and APP-KI mice by treatment groups over time. The species richness increases over time and is not significantly different between the groups.* ***Middle Panel*** – *NMDS plot of WT (top) and APP-KI (bottom) to depict dissimilarity of the samples with greater distance depicting greater dissimilarity.* ***Right*** - Tables of the PERMANOVAs at various time points in WT and APPKI and with time points considered showing that at 3 months of treatment had a significant effect on the microbiome of APP-KI mice.

The 0-time point collection was eliminated from analyses as it would be inaccurate for comparison as both sets of animals were on water supplied by the vivarium before sacrifice and then were shifted both microbiomes from its previous state. The conditions of this data point thus introduced another variable, and its exclusion is appropriate. A Tukey HSD test revealed that the copper treatment did not result in a significant difference in alpha diversity, a measure of species diversity within samples (Figure 2.4 Left Panel). However, in the Bray Curtis beta-diversity metric, which compares the similarity and dissimilarity of microbial composition in one sample compared to the other, the APP-KI group had a significant difference with copper exposure. While there is not a complete separation of sample points into distinct clusters in the NMDS (non -metric multi-dimensional) plot (Figure 2.4- Middle Panel), there is more separation of the samples by treatment in the APP-KI group in comparison to the WT group. A permutational multivariate analysis of variance (PERMANOVA) was done at each time point with treatment as a fixed effect and then another PERMANOVA of the data altogether with treatment, sampling time as fixed effects and the animal as a random effect (Figure 2.4 Right Panel) showing that there was a significant effect on the microbiome of APP-KI mice with a trend towards this effect in WT mice.

Since treatment had a significant effect on the APP-KI mice, it was the appropriate candidate to conduct downstream analyses to determine which species were contributing to the observed changes between treatment groups. This was done using SIMPER (similarity percentage analysis in PRIMER 6+) and the top ten taxa and their contributions were identified (Figure 2.4). The single most important genus in the change of composition was Allobaculum (Phylum: Firmicutes) which was decreased at the 3-month time-point and with every time point considered. Overall, there is a general reduction in Firmicutes (Figure 2.4 and 2.5) with copper treatment. The gut microbiome of WT and APP-KI vary tremendously, with APP-KI mice having greater richness

at the phylum level with two phyla that are not present in WT mice (Figure 2.6). This difference in genotype exists although these mice are of the same C57BL/6J background.

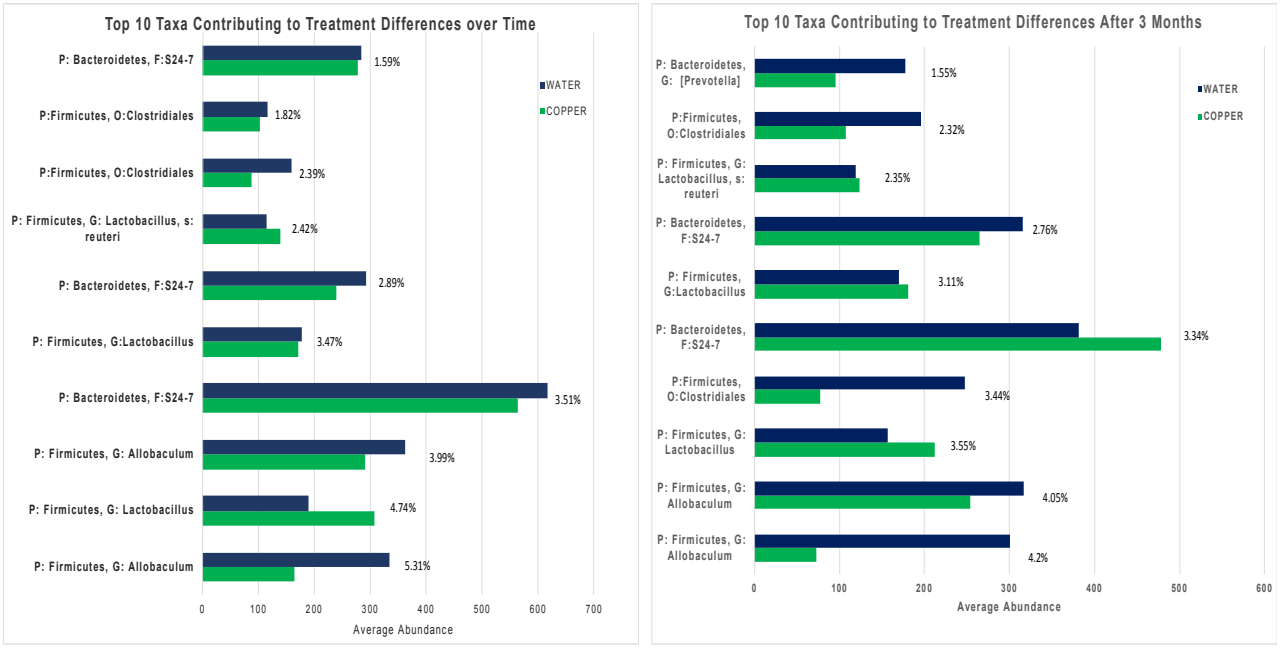



***Figure 2.5. The top 10 taxa contributing to treatment differences in APP-KI animals***. The contribution of each OTU towards the differences seen in the composition of the samples are listed beside each bar plot in percentages. **Left-** Top 10 taxa that contribute to treatment differences over the treatment period and includes time points at which the treatments were not significantly different from each other **Right-** Top 10 taxa contributing differences in APP-KI at the last timepoint, and most statistically significant time point ($p = 0.008$, PERMANOVA) in the study.

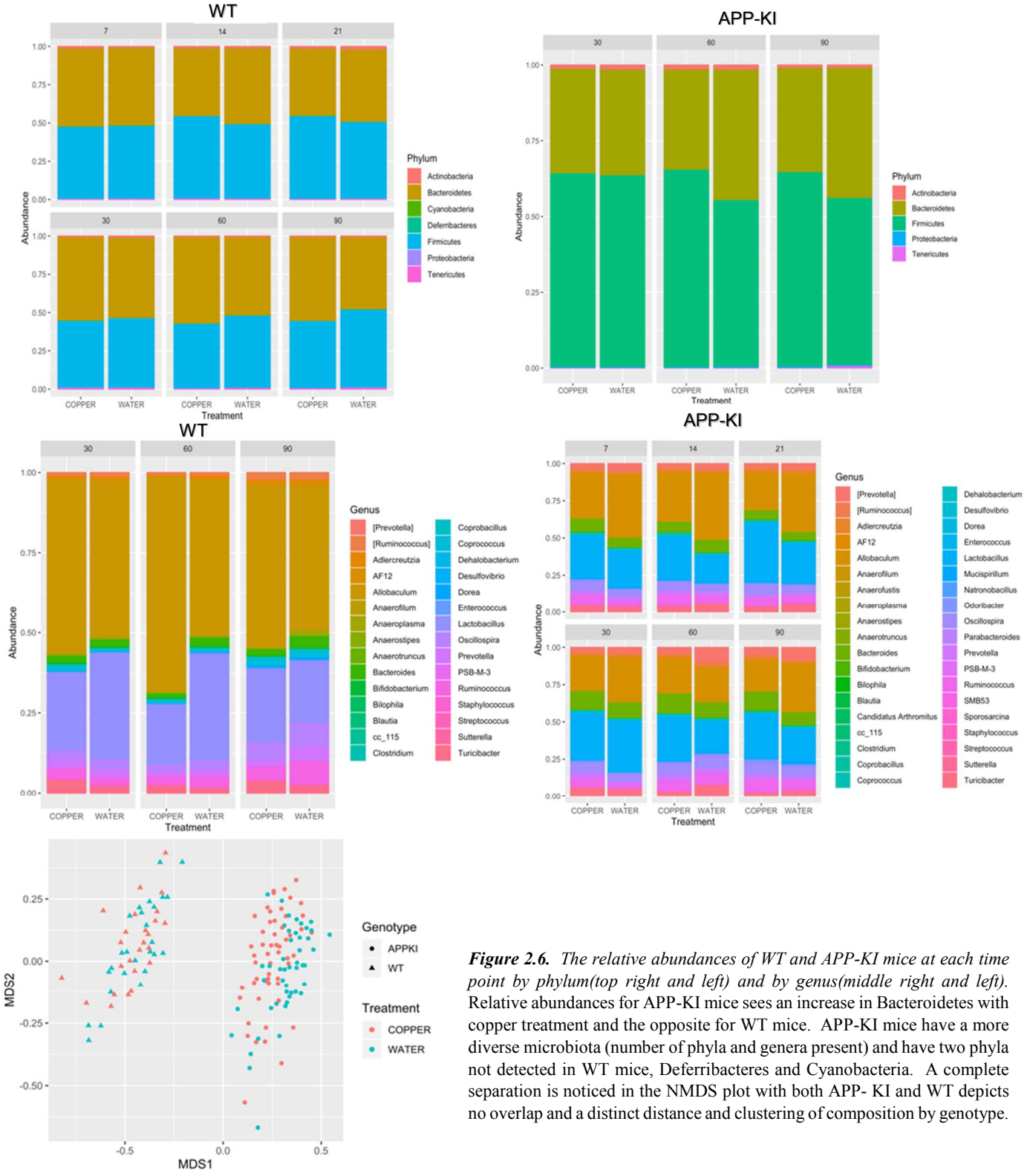



***Figure 2.6.*** *The relative abundances of WT and APP-KI mice at each time point by phylum(top right and left) and by genus(middle right and left).* Relative abundances for APP-KI mice sees an increase in Bacteroidetes with copper treatment and the opposite for WT mice. APP-KI mice have a more diverse microbiota (number of phyla and genera present) and have two phyla not detected in WT mice, Deferribacteres and Cyanobacteria. A complete separation is noticed in the NMDS plot with both APP- KI and WT depicts no overlap and a distinct distance and clustering of composition by genotype.

*Systemic Inflammation*

A customizable multiplexed MesoScale Discovery (MSD) U-PLEX Inflammation Panel Assay, which allows for investigation of ten pro and anti-inflammatory cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17A, MCP-1, TNF-α). Data from select cytokines are shown in Figure 2.7. Of all the cytokines investigated, none had any significant differences with treatment or between genotypes.

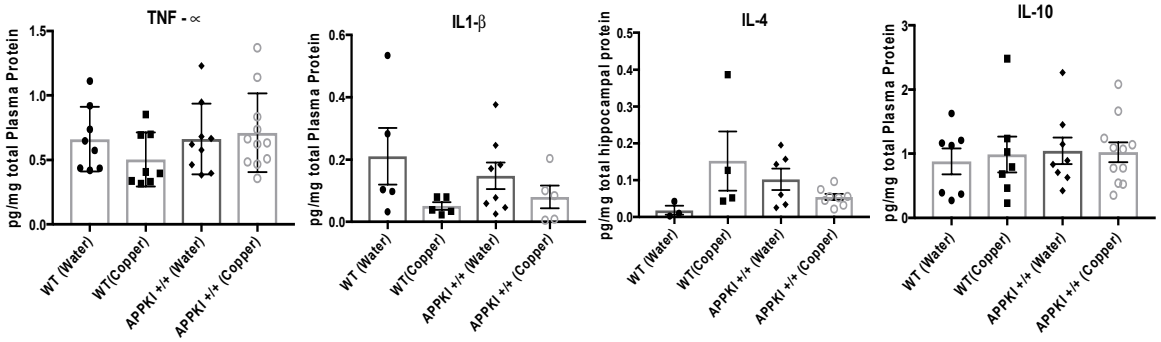



***Figure 2.7.*** Levels of select pro-inflammatory (TNF-α, IL-1β) and anti-inflammatory cytokines (IL-4) in plasma from the 10-cytokine panel from the MSD U-Plex Assay. The levels are not significantly different between the treatment groups nor across genotypes. A one-way ANOVA was performed in GraphPad Prism 7 with Tukey post-hoc test for multiple comparisons. Graphs represent the mean ±SEM.

## 2.4 Discussion

Here, we conducted an exploratory investigation into the microbiome the state of the amyloid pathology, systemic cytokine levels and changes with the microbiome following 3 months of copper exposure in drinking water to 3-month-old WT and APP-KI mice. First, I report that no significant changes occurred in amyloid deposition in the hippocampus nor in the cortex of APP-KI animals. Further, when I investigated the levels of Aβ 38, 40 and 42 as well as Aβ oligomers consisting of 9 monomers or more, there were no significant changes observed due to copper administration. Immunohistochemistry revealed that amyloid plaque deposition was unchanged in the APP-KI animals. This indicates that 3-month administration of 1.3 ppm of copper was not sufficient to alter amyloidosis in APP-KI animals. This is consistent with previous work in our lab

where changes to AD neuropathology was not seen until about 9 months of exposure in the J20 model of AD (Hsu et al., 2019).

However, changes were observed in the microbiome due to copper exposure especially in the APP-KI animals. Differences can be seen in the bacteria at the phylum level. Cyanobacteria are known known to be copper tolerant(Cervantes & Gutierrez-Corona, 1994) and neurotoxin producing(Pistollato et al., 2016), and Deferribacteres has a possible link to AD as it was found to be more abundant in APOE4 transgenic mice compared to APOE3.(Tran et al., 2019) Distinct differences were seen in WT and APP/PS1 littermates of heterozygous APP/PS1 mice(Harach et al., 2017) and host genetic influence on microbiome composition is still unclear. There have been reports of an association with the microbiome and host genetics(Bonder et al., 2016; Goodrich et al., 2014; Spor et al., 2011; Turpin et al., 2016) and some studies have even identified heritable taxa. Despite this, other studies have demonstrated that with time, the environment has a stronger effect in shaping microbiota than those inherited during birth (Rothschild et al., 2018).

When looking at the PERMANOVA to determine effect size on copper exposure on the microbiome, 1 month of exposure was enough to induce significant changes in the composition of gut bacteria in APP-KI animals, suggesting that the microbiome of the 3-month-old APP-KI mice are more susceptible to the effects of copper. Since this level was highly significant at the 3-month time point, this point was chosen to do the SIMPER test which identifies species that account for most of the change (**Figure 4**). A reduction in *Firmicutes*, one of the most dominant taxa in the gut microbiota, has been linked to better host inflammation and has been seen in other copper-related exposures(Zhai et al., 2017) and in human AD (Vogt et al., 2017). The genera implicated within the *Firmicutes* phylum in this is *Allobaculum.* This genera is largely thought to be anti-inflammatory, helps regulate host immunity and metabolism, and produces short chain fatty acids

(SCFA) thought to be beneficial for immunity but also but barrier integrity (Cox et al., 2014; Ma et al., 2020) This reduction of *Firmicutes* in accompanied by an increase in *Bacteroidetes*. Specifically, there is an increase in the family S24-7, for which the name *Muribaculaceae* has been proposed(Lagkouvardos et al., 2019). The function of the family is still not well described(Lagkouvardos et al., 2019) but increases in this phylum and family have been observed in aging, AD and other neurodegenerative disorders. (Brandscheid et al., 2017; Fang et al., 2020; Vogt et al., 2017; Zhuang et al., 2018). The genus *Lactobacillus* was increased at 3 months. Lactobacillus can produce acetylcholine and γ-aminobutyric acid both of which are neuroactive and production is thought to be positive for brain health (Pistollato et al., 2016). Lactobacillus reuteri is known to produce indole-3-aldehyde which is anti-inflammatory (Fung et al., 2017). This may be a potential resilience response of the gut microbiota in these APP-KI as other SCFA and good health-related genera such as Prevotella, a genus in which most species have long been associated with a plant-based diet and improved glucose handing(Kovatcheva-Datchary et al., 2015; Larsen et al., 2010; Ley, 2016), are decreasing with copper exposure. We did not observe any

Interestingly, the microbiome of WT mice seems resilient to the effects of copper, as seen by the lack of significant change through statistical analysis (PERMANOVA) and through NMDS analysis of the Bray-Curtis beta-diversity, in which the sample points are very much interspersed. However, identifying what microbes may be causing this resilience may prove difficult due to the large genotype effect in comparison to that of copper. This apparent genotype effect may be responsible for the difference in resilience and should be explored further. However, functional information like this would require the use of additional omics approaches such as shotgun metagenomics or coupling 16S rRNA sequencing with a fecal metabolomic profile. Shotgun

metagenomics, unlike 16S rRNA sequencing, looks at the entire genome of the microbes present and can then both assess taxonomic compositional and functional potential of microbes(Quince et al., 2017). Pairing 16S rRNA sequencing with metabolomics will look at the biological output of the microbiome through its metabolites, which then allows for analysis of the neuromodulatory or immunomodulatory effects of these products. Many studies in the literature have looked at this to investigate a host of variables such as antibiotics(Fröhlich et al., 2016), APOE status on the gut microbiome(Tran et al., 2019), gut microbial products to tie taxonomic composition with biological function within the context of the gut-brain axis.(Vogt et al., 2018). These methods do present an interesting target for future study within the context of copper exposure. If candidate bacteria are found, they can then be investigated through culture or by colonizing the gut of germ-free WT and AD model mice to further categorize *in vivo* function and contribution to brain health or disease.

These studies could have been bolstered by deeper analysis of the immune state of the brain through investigation of microglia and astrocytes and by performing cognitive tests. However, this was an exploratory study aimed at identifying if 1.3 ppm of copper supplementation influenced the microbiome, a hypothesis which these data suggest. This work was the basis for which the work described Chapter 3 was based, that does address some of these outcomes not assessed here.

**2.5 Acknowledgments**

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# Chapter 3

## Characterizing the Effect of 9-month Copper Exposure on Neuropathology and on the Gut Microbiome in an APP Knock-In Model of Alzheimer's Disease

### 3.1 Introduction

Numerous genome-wide association studies (GWAS) have implicated several risk gene loci and single nucleotide polymorphisms in the development of both early-onset or familial AD (EOAD) and late-onset or sporadic AD (LOAD) (Andrews et al., 2020; H. H. Chen et al., 2021; Harold et al., 2009; Jansen et al., 2019; Kunkle et al., 2019; Lambert et al., 2013; Ridge et al., 2016). Aberrant inflammation has now emerged as a pathological feature of AD as many of the susceptibility loci, such as *TREM2*, identified in these studies link to immunological pathways implicating a complex role for glia (Cheng-Hathaway et al., 2018; Harold et al., 2009; Karch & Goate, 2015; Kunkle et al., 2019; Naj et al., 2011).

While much of the focus in AD has been on genetic risk factors, the environment plays a role in the development of the disease as well and often interact with genetic factors to modify disease risk. The most studied of these gene x environmental risks include that between APOE ε4 status, which poses the highest risk within LOAD (Poirier et al., 1993), and modifiable risk factors such as education, diet and lifestyle habits such as alcohol consumption and smoking (Angelopoulou et al., 2021). There have also been studies linking APOE ε4 status with increased exposure to particulate matter in the air(Cacciottolo et al., 2017). This underscores the importance of investigating environmental factors on the development and progression of AD especially regarding inflammation, as the immune system acts as the interface between the host and xenobiotics.

As a potential dietary and environmental exposure factor, metal toxicity has long been implicated in AD (Bush, 2003; Das et al., 2021; G. Liu et al., 2006). Various studies have investigated the role of metals such as aluminum and cadmium as well as physiologically essential metals such as iron, copper and zinc as participants in AD etiology (Bakulski et al., 2020; Cristóvão et al., 2016; Everett et al., 2021; Huat et al., 2019; Li et al., 2015; L. Zhang et al., 2020). Labile or free copper exposure has been implicated in the development of AD (Hsu et al., 2018; Morris et al., 2006; R. Squitti et al., 2005; Rosanna Squitti, 2012a, 2012b; Rosanna Squitti et al., 2011, 2014). While evidence has shown that copper has redox potential and can act as seeding sites for senile plaque development (Everett et al., 2021; Sparks & Schreurs, 2003), there has been evidence that copper dysregulation/dyshomeostasis affect both astrocytes and microglia (Ashraf et al., 2019), directing them to a neurotoxic, disease associated state (Lim et al., 2020; Pal et al., 2021; Zheng et al., 2010). Copper exposure has been known to accelerate cellular senescence which can lead to metabolic and immune dysfunction (Ashraf et al., 2019; Masaldan et al., 2018; Matos et al., 2012). Work in in our lab has shown that copper exposure affect phagocytic activity of the microglia and LRP1-mediated clearance of amyloid(Storck et al., 2016) beta(Aβ) from the brain, which then allows for protein aggregation on plaque formation (Hsu et al., 2019; Kitazawa et al., 2016).

As copper exposure is primarily oral, it may influence the gut microbiome. The gut-microbiota-brain axis as emerged both as a major mediator of AD(Dinan & Cryan, 2017; Fröhlich et al., 2016; Saji et al., 2019; Vogt et al., 2017) and other neurological disorders such as Parkinson's Disease (PD) (Challis et al., 2020; Sampson et al., 2016) and Major Depressive Disorder(MDD) (Fung et al., 2017; Jiang et al., 2015). Recent studies demonstrate that oral copper ingestion alters the gut microbiome, whose richness and diversity significantly modulate host

immunity, metabolism, and neuronal activity (Meng et al., 2018; Zhai et al., 2017; F. Zhang et al., 2017). Moreover, many of these studies implicate microbial dysbiosis with altered systemic and/or brain inflammatory status (Abdel-Haq et al., 2018; Dodiya et al., 2019; Kowalski & Mulak, 2019; Minter et al., 2016; Thion et al., 2018). However, many studies that implicate copper in the gut microbiome tend use fairly high concentration of copper, some as high as 1000 ppm (Zhai et al., 2017) which is not environmentally relevant for the everyday population. Recently, we conducted a pilot study (see Chapter 2) to see if an environmentally relevant dose of 1.3 ppm of copper in drinking water would perturb gut microbiota. In this study, we found that the gut microbiota of 3-month AD (APP-KI) mice was more susceptible to the effects of copper than aged-matched WT (C57BL/6). There were no effects on the brain and systemic inflammation. However, to build on this work and to match previous experimental designs performed using a different AD model mouse, J20 mice (Hsu et al., 2019), we designed a study that exposed WT and APP-KI mice that from weaning for 9 months. We hypothesize this chronic exposure will exacerbate AD pathophysiology while inducing gut dysbiosis.

## 3.2 Materials and Methods

### 3.2.1 Animals

All mice used in this study were housed on a 12-hour light/12-hour dark cycle with the light cycle beginning at 6:30 am and concluding at 6:30pm. All experimental protocols were performed during the light phase. All animal procedures were performed in accordance with National Institutes of Health and University of California guidelines and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine. Animals were fed water and food ad-libitum. The feed was the Teklad Global Soy Protein-Free Extruded Rodent

Diet (Envigo 2020X, Madison, WI). 1 month-old male and female wild-type (n=27) and $APP^{NL-G-F}$-KI (n=30) mice were bred in house and were randomly assigned to a treatment group.

### 3.2.2 Experimental Design

*Copper Chloride (CuCl2) Preparation and Administration*

1.3 ppm $CuCl_2$ (CAS # 7447-39-4, Sigma-Aldrich, Cat # 751944) was prepared fresh in ultrapure water every two weeks from a 100X stock solution stored at room temperature. The mice were exposed to either MilliQ water or 1.3 $CuCl_2$ in drinking water for 3 months and were supplemented with fresh water every two weeks at cage change.

*Assessment of Spatial Memory Function*

All mice were subjected to cognitive evaluation in the object location memory (OLM) test, Object Recognition Memory (ORM) test and the Morris Water maze (MWM). The mice were allowed to rest for 4 days following each test.

*Object Location Memory and Object Recognition Memory Tasks*

OLM and ORM was performed as previously described (Figure 1) (Vogel-Ciernia & Wood, 2014).Before training, mice were handled 1–2 min for 3 days. They were subsequently habituated to the OLM (white, square arena with black paneling and Sani-Chips) or ORM arena (grey, circular arena with standard bedding) for 5 minutes, each day for 6 consecutive days in the absence of the experimental objects. During the training trial, acquisition, the mice were placed in the area with 2 identical objects; 100-ml beakers, 2.5-cm diameter, and 4-cm height filled with concrete (OLM) or circular spice tins or 2x2 square candle holders filled with concrete (ORM). The animals were allowed to explore these objects for 10 min. 24 hours later, animals' retention

was put to the test. During the test for OLM, one copy of the familiar object was placed in the same location as during the training trial, and one copy of the familiar object was placed in the different location to the previous day within the arena. During the test for ORM, one copy of the

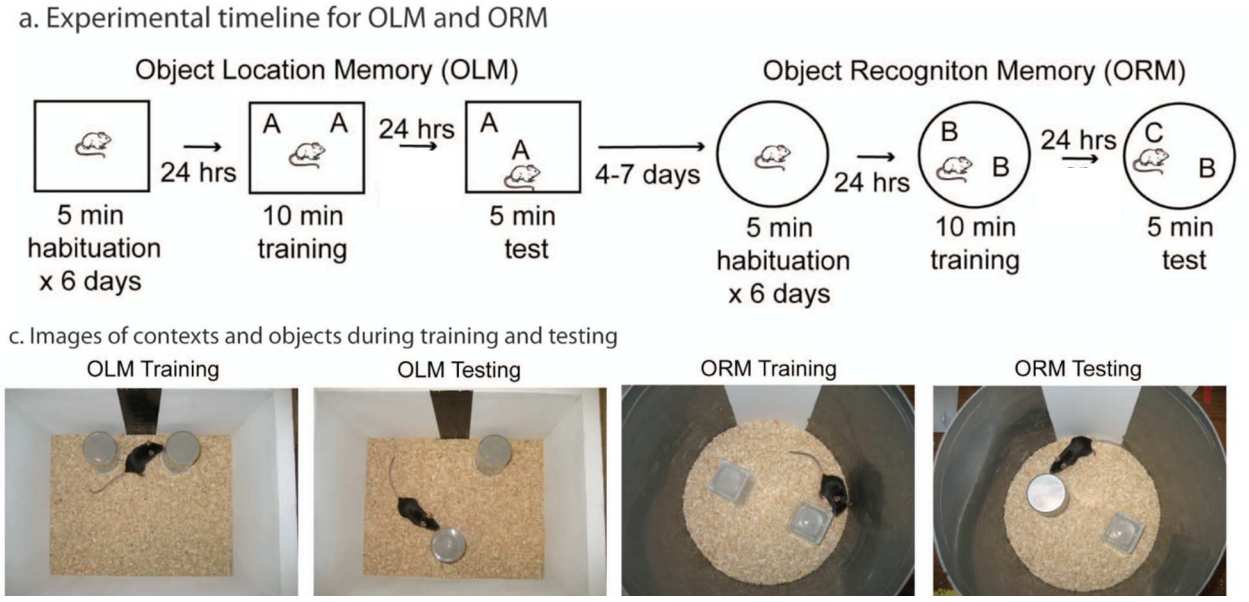


**Figure 3.1**. Adapted from (Vogel-Cierna & Wood, 2014) Object location and object recognition memory task design. (A) Experimental timeline for Object Location Memory (OLM) followed by Object Recognition Memory (ORM) in the same cohort of animals. (C) Images of the actual experimental setup for OLM and ORM. In the ORM experiment, half of the animals would be trained with two tins and the others with two candle holders. For the ORM test, the animals receive one of each object with the location of the novel object counterbalanced across groups.

familiar object was placed in the same location as the training trial and an object the animal had not seen before (novel object, was placed in the other location. The animals were then allowed to explore for 5 minutes. All training and testing trials were video recorded, and hand scored by individuals blind to animal treatments. Videos were analyzed for total exploration of objects in addition to the discrimination index (DI) [(time spent exploring object in new location time spent exploring object in familiar location)/ (total time exploring in both locations) *100]. Combinations and locations of objects were used in a balanced manner to reduce potential biases attributable to preference for a particular location.

*Morris Water Maze*

For the MWM task, a circular white tank, 128 cm diameter was filled with tap water maintained between $21^0$C–$24^0$C. The tank is divided into four quadrants with differing visual cues were placed by 3 of the quadrants. Mice were trained to swim and find a 4-inch diameter circular clear Plexiglas platform submerged about 1 cm beneath the surface of the water and invisible to the mice while swimming. A camera mounted directly above the pool monitors the mouse performance in the maze. On each training trial, mice were placed into the tank at 1 of 4 designated start points in a pseudorandom order. Mice were allowed to find and escape onto the platform. If mice failed to find the platform within 60s, they were manually guided to the platform and taught to remain on the platform for 30s to orient themselves using the visual cues. Each day, mice received 4 training sessions separated by intervals of 90 s under a warming lamp. The training period ended when the control group reached criterion (<25 s mean escape latency). The probe trial to test retention memory was assessed 24 h after the last training trial. In the probe trials, the platform was removed from the pool and the time to the platform area and the number of platform crosses were recorded. All trials were recorded and was useful subsequent analysis such as swim speed and time spent in target quadrants.

*Fecal Sampling*

The fecal samples were collected beginning at 3 months, at 6 months and then at 9 months before sacrifice. monthly until sacrifice for WT mice. For fecal sampling, mice were placed in sterile cages without bedding and allowed to pass several pellets. Approximately 5 pellets were collected into sterile 2 ml cryogenic tubes and flash frozen. Samples were then stored at -$80^0$C until DNA extraction for sequencing.

*16S ribosomal Ribonucleic Acid(rRNA) Amplicon Sequencing*

Samples from female WT and APP-KI mice were submitted to Zymo Research. There they were processed and analyzed with the ZymoBIOMICS® Targeted Sequencing Service (Zymo Research, Irvine, CA). DNA Extraction: One of three different DNA extraction kits was used depending on the sample type and sample volume. In most cases, the ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA) was used to extract DNA using an automated platform. In some cases, ZymoBIOMICS® DNA Miniprep Kit (Zymo Research, Irvine, CA) was used. For low biomass samples, such as skin swabs, the ZymoBIOMICS® DNA Microprep Kit (Zymo Research, Irvine, CA) was used as it permits for a lower elution volume, resulting in more concentrated DNA samples. Targeted Library Preparation: Bacterial 16S ribosomal RNA gene targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA). In most cases, the bacterial 16S primers amplified the V3-V4 region of the 16S rRNA gene. These primers were custom-designed by Zymo Research to provide the best coverage of the 16S gene, while maintaining high sensitivity.

The sequencing library was prepared using real-time PCR machines to control cycles and therefore limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA), then quantified with TapeStation®(Agilent Technologies, Santa Clara, CA) and Qubit® (Thermo Fisher Scientific, Waltham, WA). Control Samples: The ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, CA) was used as a positive control for each DNA extraction. The ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA) was used as a positive control for each targeted library preparation. Negative controls (i.e., blank

extraction control, blank library preparation control) were included to assess the level of bioburden carried by the wet-lab process. Sequencing: The final library was sequenced on Illumina® MiSeq™ with a v3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in. Bioinformatics Analysis: Unique amplicon sequences variants were inferred from raw reads using the DADA2 pipeline (Callahan et al., 2016). Potential sequencing errors and chimeric sequences were also removed with the Dada2 pipeline. Chimeric sequences were also removed with the DADA2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database, a 16S database that is internally designed and curated, as reference. Composition visualization, alpha-diversity, and beta-diversity analyses were performed with Qiime v.1.9.1 (Caporaso et al., 2010).Taxonomies that have significant abundance among different groups were identified by LEfSe (Segata et al., 2011)using default settings. Other analyses such as heatmaps, Taxa2ASV Decomposer, and PCoA plots were performed with internal scripts.

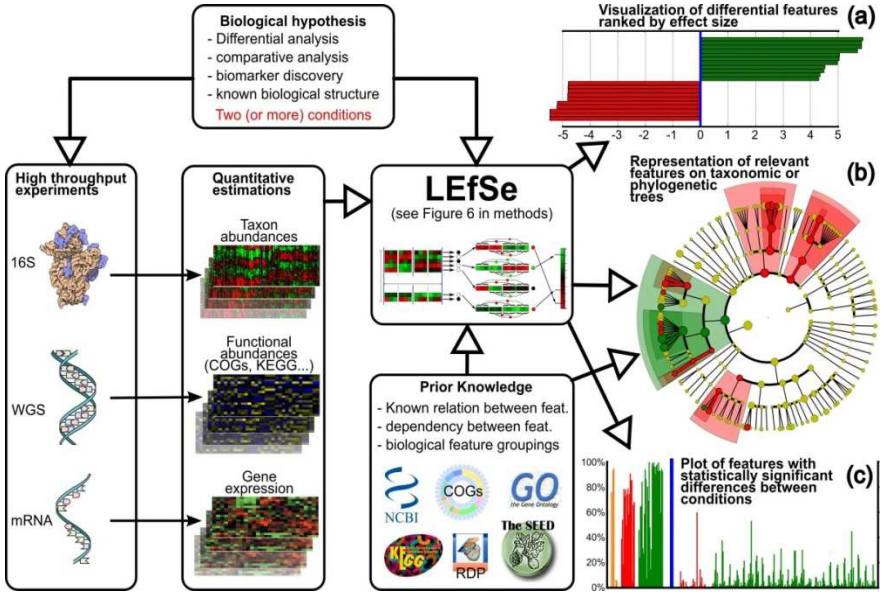


**Diagram A.** LEfSe determines the features (organisms, clades, operational taxonomic units, genes, or functions) most likely to explain differences between classes by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance

Adapted/Translated by permission from Springer Nature[Genome Biology] [Segata, N., Izard, J., Waldron, L. *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol* **12,** R60 (2011). https://doi.org/10.1186/gb-2011-12-6-r60

*Tissue Processing*

Upon sacrifice, the animals were euthanized with an overdose of pentobarbital. While anesthetized blood was collected from cardiac puncture and placed into Lithium Heparin-lined

Microtainer tubes for later plasma extraction. The animals were then perfused with cold PBS, pH 7.4 and the brain extracted. One hemisphere was then fixed in 4% paraformaldehyde for 48 hours, cryopreserved in 30% sucrose then stored at -80$^0$C for histopathology. The hippocampus and cortex were microdissected and snap frozen on dry ice for biochemical analyses.

*Immunofluorescence*

Cryopreserved brain hemispheres were sectioned at 40μM using the Leica Biosystems SM2010 R Sliding Microtome (Buffalo Grove, IL). The platform was kept at -80$^0$C by a dry ice/ethanol mixture. Sections were stained for a variety of endpoints including astrocytes: (a) GFAP (1:3000, Dako Millipore, Burlington, MA), C3(1:50, Hycult Biotech, Wayne, PA) microglia: (a), Iba-1, (1:500, FUJIFILM Wako Chemicals USA Corporation, Richmond, VA) (b) CD68 (1:50 BioRad, Hercules, CA ),(c) TMEM119(1:1000,Abcam, ab309064, Cambridge, UK), (d) Ferretin ( 1:1000, Sigma-Aldrich, St. Louis, MO), amyloid-beta plaques: McSA1(1:1000, Medimabs, Montreal, Quebec, Canada) and Thioflavin-S (0.1% in 50% Ethanol, Sigma- Aldrich, St. Louis, MO). Primary antibodies were coupled with AlexaFluor antibodies (488, 555 or 633) raised in goat. Sections were counterstained with DAPI to identify cell nuclei. Images were captured using the EVOS FL Cell Imaging System or the Keyence BZ-X800 All-in-one Fluorescence Microscope. Analysis of the images were done using Fiji ImageJ.

*Immunoblot and Enzyme Linked Immunoassays*

As mentioned before mice brains were dissected on ice, one hemisphere was microdissected and flash frozen for biochemical analysis; the other hemisphere was collected for immunohistochemical staining. Protein extracts were prepared by homogenizing frozen brain regions in T-PER extraction buffer (150 mg/ml, Thermo Fisher Scientific, cat# 78510),

complemented with protease and phosphatase inhibitors (Thermo Scientific, cat# 78445), and ultracentrifuged at 100,000 x g for 1 hour at $4^0$C. The supernatant, the detergent soluble fraction, was removed and stored at -$80^0$C until protein concentration determination and use in assays. Protein concentration was determined from the supernatants using the Bradford assay following the manufacturer's protocol (Bio-Rad, Hercules, California). The precipitate was treated with 70% formic acid and ultracentrifuged at 100,000 x g for 1 hour at $4^0$C to digest and obtain the detergent insoluble protein fraction.

Protein concentration from the hippocampus in detergent-soluble fractions was measured using the Bradford protein assay. Standardized protein mass was separated by SDS- PAGE and transferred to Immobilon-FL PVDF membrane (EMD Millipore). Membranes were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) for 1h at room temperature before overnight with shaking at 4 °C with primary antibody. The following antibodies diluted in Odyssey Blocking Buffer with 0.2% Tween 20: CT-20 (Calbiochem, catalog # 171610, 1:1000 dilution, GAPDH (Santa Cruz Biotechnology Inc., catalog # sc-25778, 1:5000 dilution). Membranes were then washed in TBS-0.02%Tween-20 (TBS-T), before incubated with corresponding IRDye secondary antibodies (LI-COR Biosciences, 1:20000 dilution) diluted in Odyssey Blocking Buffer with 0.01%SDS, 0.02%Tween-20 for 1 hour at room temperature. Membranes were then washed in TBS-T and kept in TBS before being scanned using the Odyssey Imaging System (LI- COR Biosciences). Band intensities were quantified with Image Studio software (version 5.2, LI-COR Biosciences) and normalized to that of the GAPDH protein loading control.

Aβ40 and Aβ42 were quantified in the formic acid - soluble hippocampal and cortical tissue using the V-PLEX Plus Aβ Peptide Panel 1 (6E10) Kit (Meso Scale Discovery). Aβ40 and Aβ42 peptide calibrators and protein fractions were loaded onto the MSD MULTI-SPOT® 96-

Well 4-Spot plate after the addition of detection antibody solution. Plate was read on a MESO QuickPlex SQ120 after 2-h incubation at room temperature on an orbital shaker set to 700rpm, washed, and loaded with Read Buffer T. Aβ40 and Aβ42 in the detergent-soluble fraction of hippocampal and cortical homogenates were quantified using Amyloid Human (1-40) ELISA kit (Wako, cat# 292-62301) and Amyloid Human (1-42) ELISA kit (Wako, cat# 298-6240). Briefly, 100μL of standards and samples were added to the plate and incubated in the refrigerator with plate seal overnight. HRP-conjugated detection antibody was added after washing for 1 hour. Then, the plate was incubated with TMB Solution for 30 minutes, then followed by stop solution. The absorbance of each well was then read at 450 nm with a microplate reader.

Cytokine (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO and TNFα) concentration in serum and brain lysates were determined using the V-Plex Proinflammatory Panel 1 Mouse Kit (K15048D). Briefly, cytokine calibrators and samples were loaded MSD MULTI-SPOT® 96-Well 10-Spot plate and left on an orbital shaker overnight at $4^0$C. The plate was then washed, incubated in detection antibody for 1 hour and washed again. Read Buffer T was added to the plates before being read on the MESO QuickPlex SQ120.

## 3.3 Results

*1.3ppm Copper exposure does not worsen cognition in WT nor APP-KI mice*

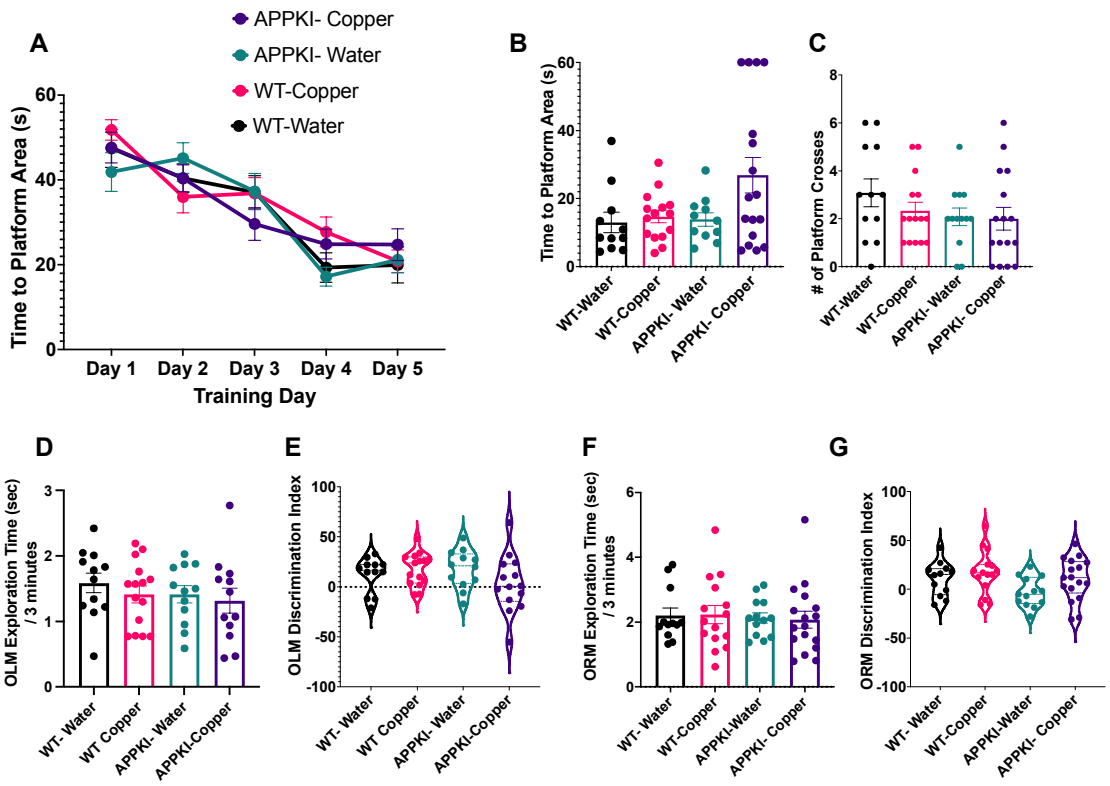


**Figure 3.2.** ***Performance of copper treated WT and APP-KI animals in the MWM, ORM and OLM memory tasks***. *A*) the average latency to the submerged platform for each treatment group in 4 trials per day per animal. B) latency to the platform area during the probe trial in which the submerged platform is removed. C) the number of platform crosses made by each animal during the probe trial D) the exploration time of the animals within the first 3 minutes of exploration in the OLM test phase. E) Discrimination Index during the OLM test phase when one object was moved from the position it was in the day before F) the exploration time of the animals within the first 3 minutes of exploration in the ORM test phase. G) Discrimination Index during the ORM test phase when one object was replaced with a novel object. Data are presented as mean ±SEM and significance was determined by ANOVA (2 Way RM ANOVA in **A,** Kruskal-Wallis in **B-G**) with alpha set to 0.05

Previously, we reported in the J20 mouse model that 9 months of 1.3 ppm of copper exposure exacerbated spatial memory (decreased discrimination index in OLM and higher test latency and lower platform crosses on MWM) in WT mice with no subsequent change in the J20 mice (Hsu et al., 2019). It was clear from that data that J20 mice had already exhibited impaired spatial memory of which copper did not further exacerbate. Here we notice a trend in that same direction, but it is

not significant (Figure 3.2 B-C). The animals all learned well and at similar rates which is like our previous report. However, on test day, the number of latency and platform crosses were not different. It is clear from the distribution of the data that copper animals tended to perform worse in the MWM test with slightly higher test latencies (Figure 3.2B) and lower platform crosses (Figure 3.2C) for both WT and MWM animals. In the OLM task from the distribution of the data, the copper-treated AD animals performed worst of all groups (Figure 3.2E). However, in the OLM task, while the APP-KI animals tended to perform worse than the WT, in both groups, the

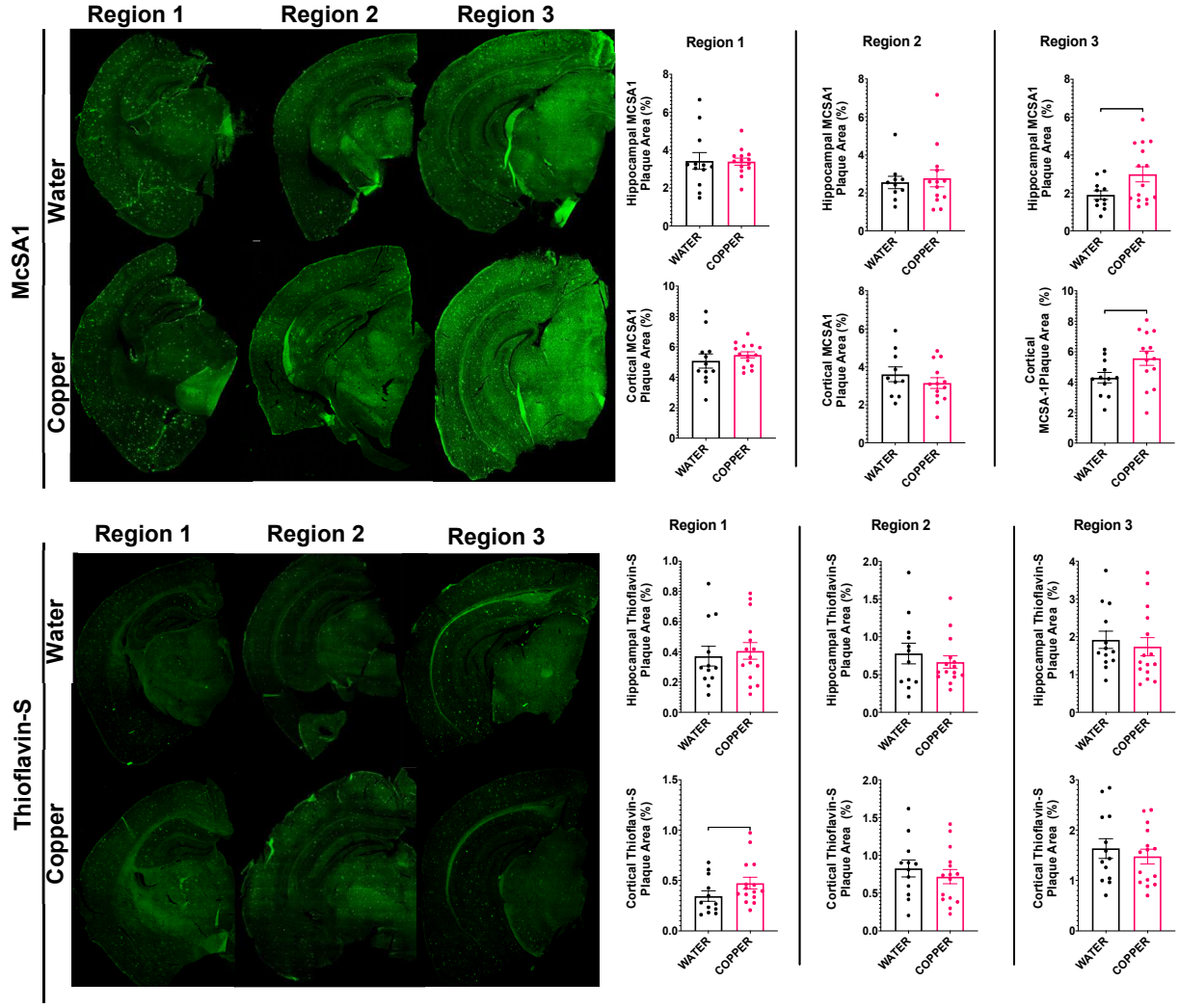


**Figure 3.3. Distribution of Diffuse and Dense Plaques across 3 brain regions in APP-KI mice**. Brain sections were stained with McSA1antibody and the chemical Thioflavin-S to detect diffuse and dense core plaques respectively. Representative images of each brain region captured at 20X and then stitched on the Keyence BZ-X800 is shown on the left of the figure. Quantification of the percent area covered by the plaques are to the right of the images. Data are presented as mean ±SEM and significance was determined by the Mann-Whitney nonparametric t-test with the $p<0.05$ denoted by an *

distribution of the data suggests, that while not significant, the copper animals performed slightly better (Figure 3.2G).

*1.3 ppm Copper Exposure modulates amyloidosis in the AD brain*

Homozygous $APP^{NL-G-F}$-KI mice have robust plaque pathology at beginning at 6 months of age (Masuda et al., 2016; Saito et al., 2014) and is evidenced by the McSA1 and Thioflavin-S staining (Figure 3.3). Treatment of the animals began around weaning before the onset of robust plaque pathology and it seems copper has had a mild effect on amyloid deposition which varies based on brain region. There are significantly higher area percent of the hippocampus and cortex covered by diffuse plaques in region 3, the posterior region, as evidenced by the McSA1 staining, where there is no difference in regions 1 & 2 which represent the more anterior sections of the brain. Interestingly, with the dense core plaques, detected by Thioflavin-S, the only difference seen is in the cortex of the anterior region. It should be noted that the dense core plaques increase in number/size from anterior to posterior due to the mean area increase from region 1 (around 0.5% on average to around 1.5% in region 3).

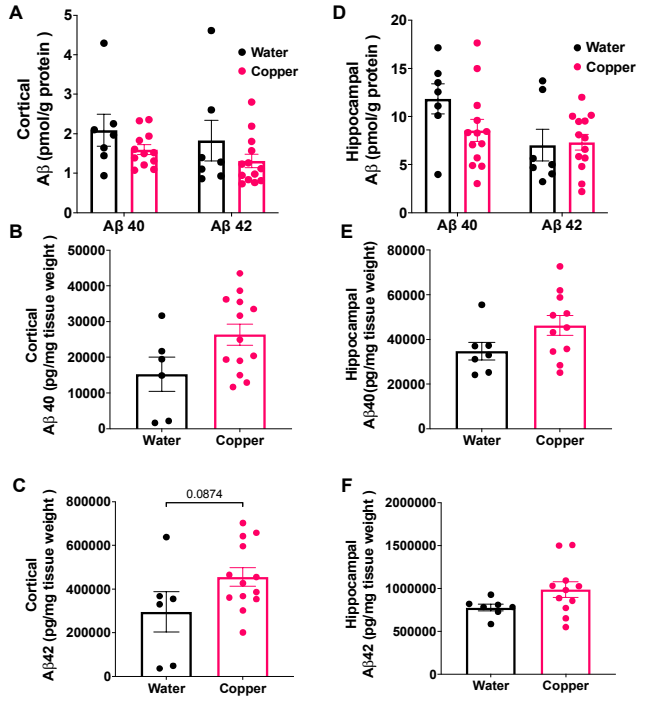


**Figure 3.4.** *Quantitation of Aβ levels in detergent and formic acid soluble fractions of hippocampal and cortical homogenates of APP-KI mice.* **A&D** represent the Aβ levels in the detergent soluble fractions**.** Aβ levels in the cortical **B-C** and hippocampal **D-F** acid fraction.

In both the cortical and hippocampal detergent soluble fraction there seems to be trend in a reduction in both Aβ species in the copper treated group but the means were not significantly different from each other (Figure 3.4 A&D). The opposite is seen in

the formic acid soluble fractions (Figure B-F) where while not significantly different, trends similarly to the immunohistochemistry data.

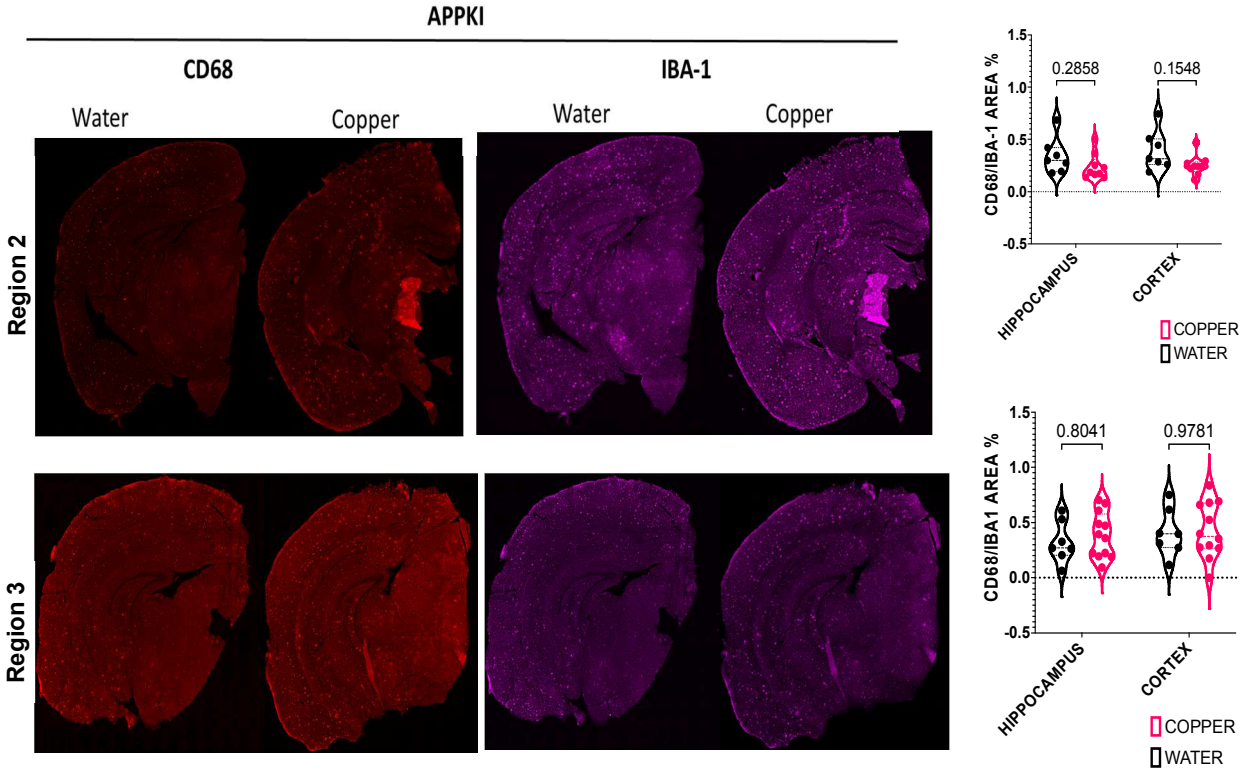


**Figure 3.5.** *Phagocytic Capabilities of Microglia in APP-KI Mice.*CD68, a marker for microglial autophagosomes was counterstained and normalized against the IBA-1 marker for activated microglia in two brain regions in AD mice. Marker coverage was determined using the Analyze Particles function in Fiji ImageJ following imaging and stitching at 20X on the Keyence BZ-X800. Quantification data is represented by the mean ± SEM and statistical analyses (Kruskal-Wallis ANOVA with Tukey's post-hoc test for multiple comparisons were performed in GraphPad Prism 9

*Copper exposure directs microglia to a more degenerative phenotype*

In our lab, we have shown that in the in the presence of cupric ($Cu^{2+}$) ion, microglia like BV2 cells have significantly reduced ability to phagocytose beads in the presence of neurotoxic Aβ42 fibrils (Kitazawa et al., 2016). However, in APP-KI mice in this study, no significant differenced were observed in the amount of phagocytic microglia in the hippocampus or cortex in two different brain regions (Figure 3.5). There is some region variability and a trend in the second region to reduced phagocytic capability in copper exposed animals, but no significant differences were

observed. Further, when we look at the homeostatic microglial marker, TMEM119,(Gerrits et al., 2021; Hasselmann & Blurton-Jones, 2020; Song & Colonna, 2018), there are significantly higher levels of TMEM119 positive cells in APP-KI animals in comparison to WT animals( Figure 3.6). This may just be due to higher recruitment of microglial recruitment due to increased inflammation

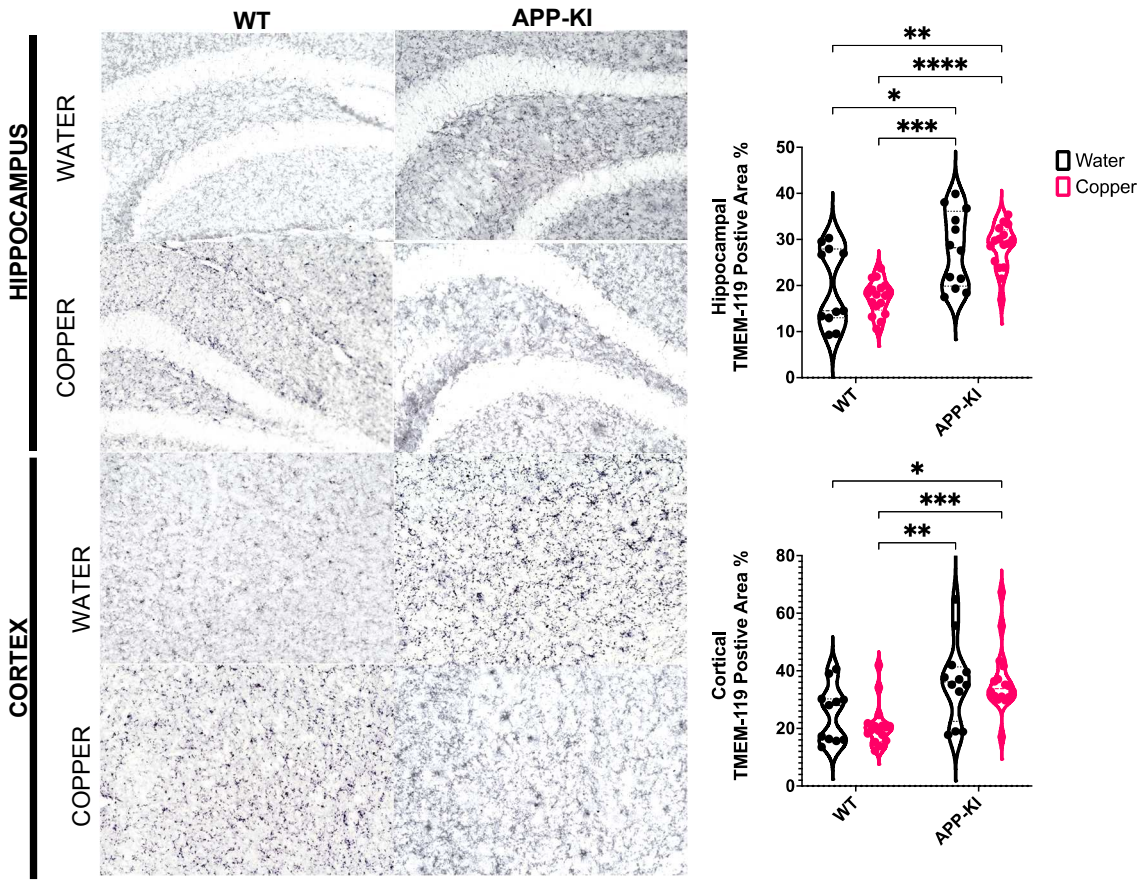


**Figure 3.6**. *Levels of homeostatic microglia determined by TMEM119+ cells in the brains of APP-KI and WT mice.* 4-6 images each from the hippocampus and the cortex were analyzed for each biological replicate in each treatment group. Images were taken of the entire brain at 10X in brightfield on the Keyence BZ-X800. Coverage of the microglia was determined using custom macro within Fiji ImageJ. Quantification data is represented by the mean ± SEM and statistical analyses (Kruskal-Wallis ANOVA with Tukey's post-hoc test for multiple comparisons were performed in GraphPad Prism 9. This analysis was carried out in region 1 brain sections *p<0.05, **p<0.005, ***p<0.0005, ***p<0.0001 denotes a significant result

in the AD mice as the increases observed (Figure 3.6) are due to genotype. Looking at the distribution of the data from both genotypes, there is a trend towards decreased TMEM119 levels in the copper treated group in comparison to the copper treated group. TMEM119 cells are

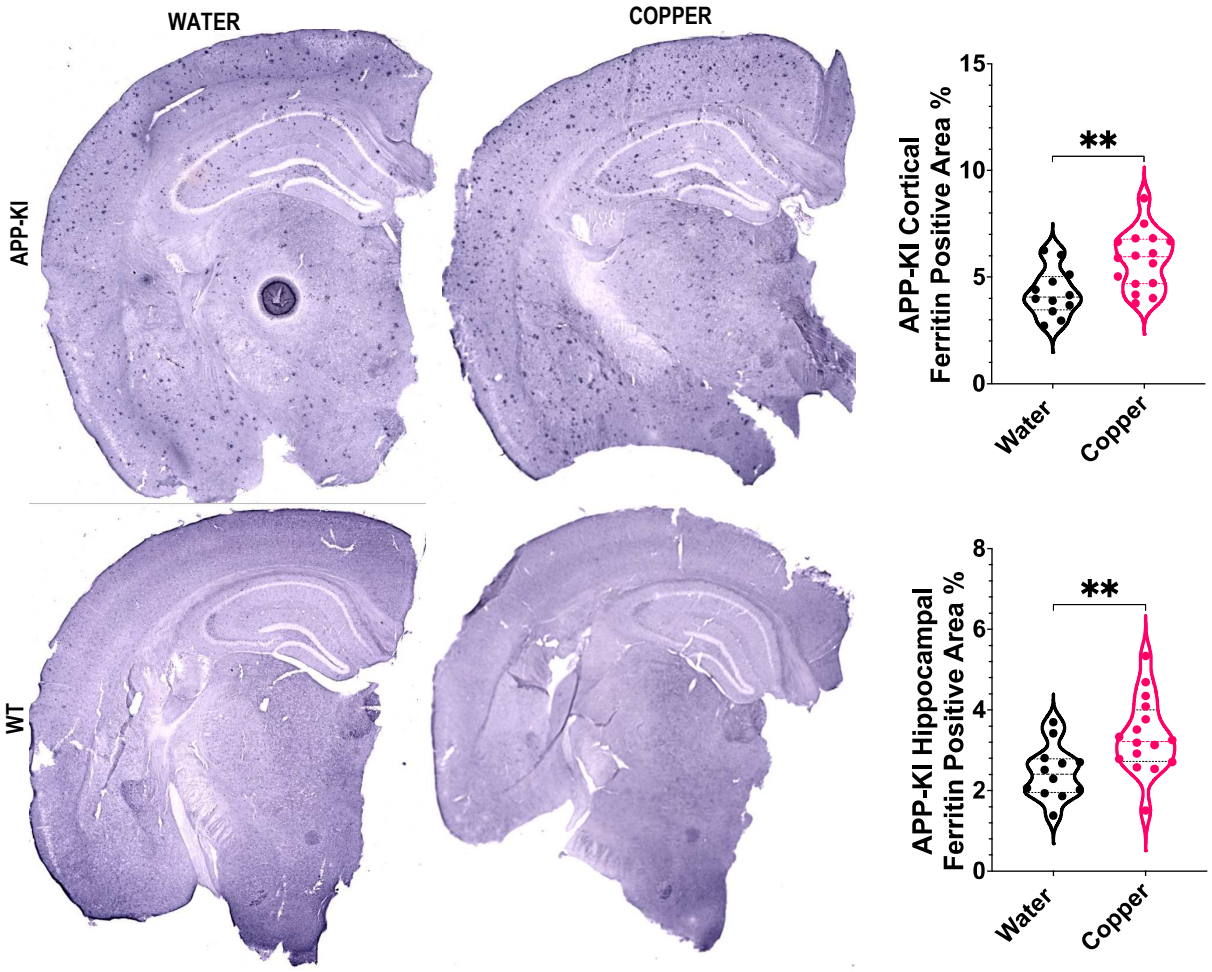


**Figure 3.7.** *Levels of degenerative microglia determined by ferritin cells in the brains of APP-KI and WT mice*. Images were taken of the entire brain at 10X in brightfield on the Keyence BZ-X800. Coverage of the microglia was determined using Fiji Image J Analyze Particles function. Quantification data (only done for APP-KI, WT is for visual comparison) is represented by the mean ± SEM and statistical analyses (Kruskal-Wallis ANOVA with Tukey's post-hoc test for multiple comparisons were performed in GraphPad Prism 9. This analysis was carried out in region 1 brain sections *p<0.05, **p<0.005, ***p<0.0005, ***p<0.0001 denotes a significant result

distributed throughout the brain in both WT and APP-KI animals rather than in aggregates (coinciding with Aβ plaques) as observed in IBA-1 staining.

Degenerative microglia or disease associated microglia (DAM) in AD have been found to have aberrant iron loading while having lower homeostatic gene expression. Therefore, the long-term iron storage protein ferritin, has emerged as a marker of DAM(Kenkhuis et al., 2021). We used this marker along with others to characterize the microglia. The most striking factor is that very little ferritin-positive staining was found within WT animals regardless of treatment (Figure

7). Within APP-KI animals we observe clusters of ferritin-positive cells that coincide with amyloidosis and IBA-1+ microglial cell positive clustering. Moreover, this ferritin-positive area is increased significantly with copper treatment in both the hippocampus and the cortex (Figure 3.7), indicating that plaque associated microglia are of a degenerative phenotype.

*Copper exposure does not increase the amount of neurotoxic astrocytes*

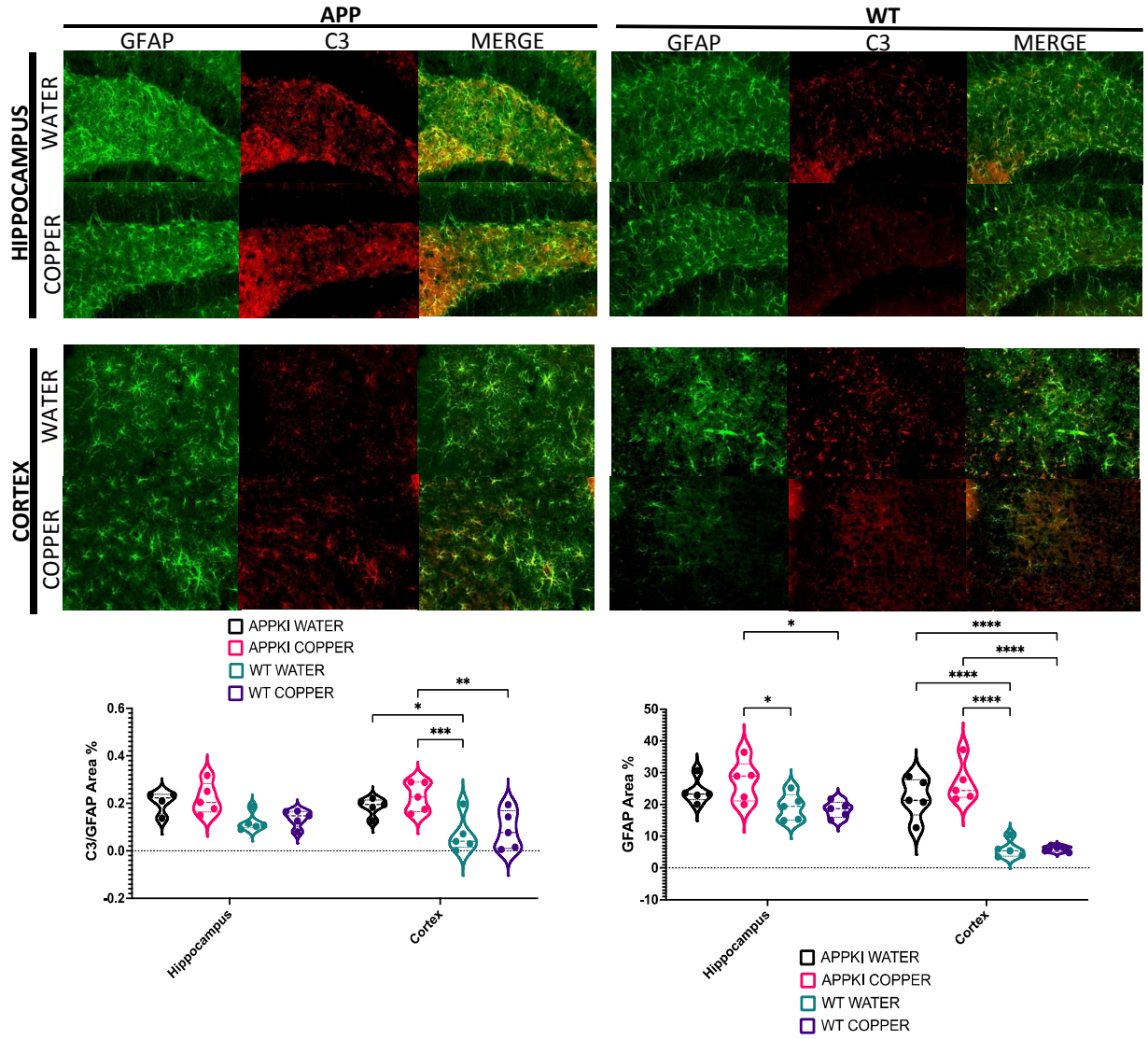


**Figure 3.8.** *Levels of neurotoxic astrocytes determined by C3+GFAP+ cells in the brains of APP-KI and WT mice*. Images were taken of the entire brain at 40X using the EVOS FL Cell Imaging System. Coverage of the astrocytes was determined using Fiji Image J Analyze Particles function. Quantification data (only done for APP-KI, WT is for visual comparison) is represented by the mean ± SEM and statistical analyses (Kruskal-Wallis ANOVA with Tukey's post-hoc test for multiple comparisons were performed in GraphPad Prism 9. This analysis was carried out in region 1 brain sections *p<0.05, **p<0.005, ***p<0.0005, ***p<0.0001 denotes a significant result.

Complement C3 is upregulated in the AD brain and contributes to neurodegeneration in mouse models of AD (Habib et al., 2020; Wu et al., 2019). C3 activation is a response to the classical pathway in the innate immune system being activated. In both models of tauopathy and amyloidosis, the classical complements to which C3 belongs, is upregulated in astrocytes the most (Wu et al., 2019). Therefore, we used C3 antibody to probe if copper induced this C3+ phenotype in GFAP+ astrocytes in both WT and APP-KI animals. We discovered that there were some C3 positive cells within WT animals. GFAP+ cells were mostly found in the hippocampus WT animals, while this was widespread in APP-KI. While in APP-Ki animals, it seems as if copper animals have slightly higher C3 levels based on the distribution of the data, this was not significantly different. When we investigate the levels of GFAP alone, statistical significance in GFAP+ cells are seen along genotype lines, with the highest level in the copper-treated APP-KI animals.

*Copper does not exacerbate proinflammatory cytokines in APP-KI mice and does not induce any inflammatory change in WT mice*

Marked changes in brain and peripheral cytokine profile has been noted in human AD and a variety of mouse models of the disease (Akiyama et al., 2000; Lim et al., 2015; Swardfager et al., 2010). Indeed, 250 ppm copper exposure increased levels of IL-1β and TNF-α were in 3xTg-AD mice. Here, in APP-KI mice exposed to 1.3 ppm copper in drinking water for 9 months, we observe a much milder inflammatory response. Much like the response with C3 (Figure 3.8) or with TMEM119 (Figure 3.6), significant differences in some proinflammatory cytokines such as IFNγ, IL-1β and TNF-α, were across genotypes, with WT animals having lower concentrations, most notably in hippocampal and cortical homogenates (Figure 3.9, Panel A). IL-4 is a known anti-inflammatory cytokine that has been known to attenuate AD pathogenesis (Kiyota et al.,

2010). In the periphery, this is the only cytokine that is decreased with copper exposure($p<0.05$) (Figure 3.9, Panel C).

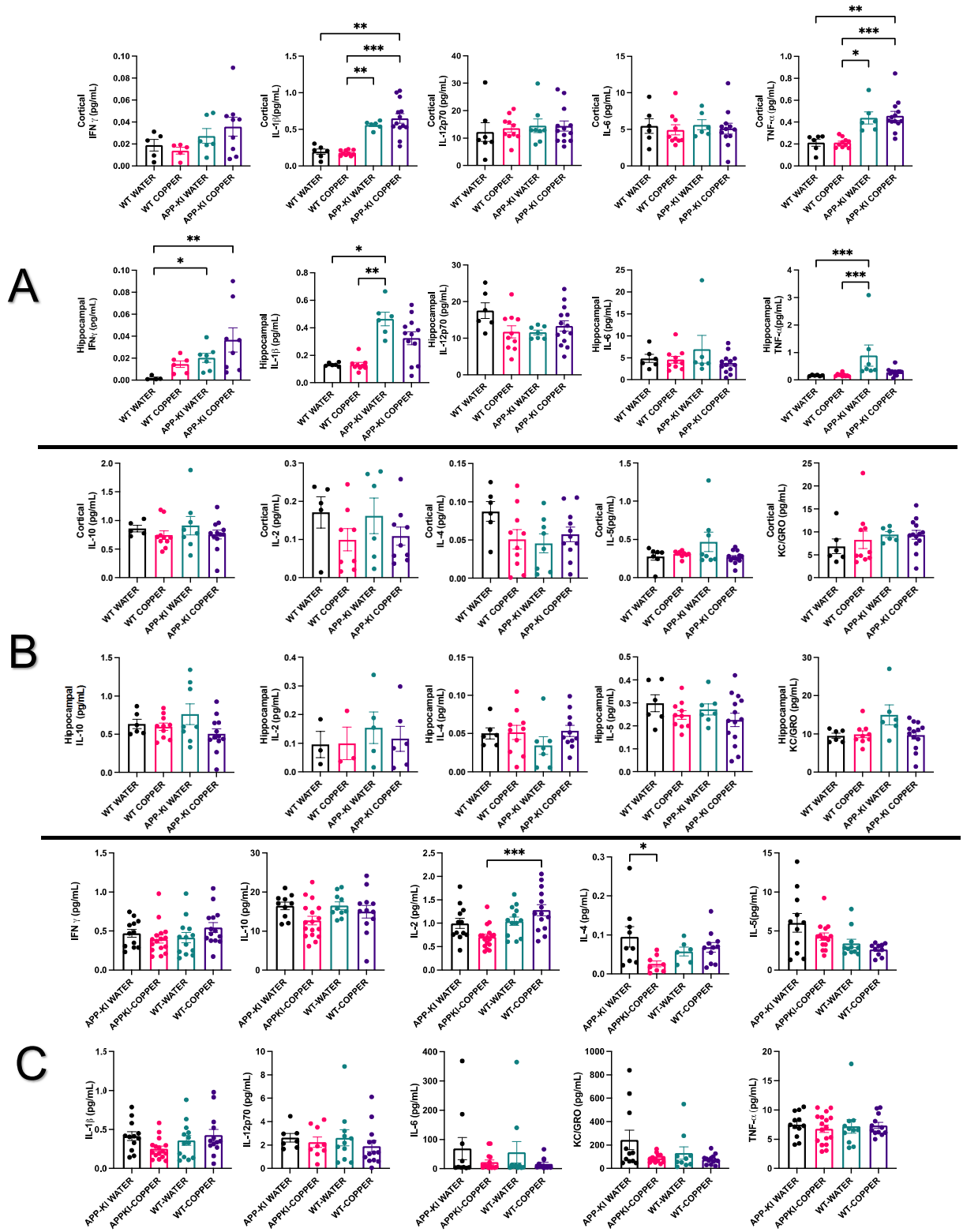


**Figure 3.9.** *Pro and anti-inflammatory cytokine/chemokine quantification in the cortex, hippocampus and plasma of APP-KI and WT copper- treated mice* Panel A and B represent cytokine levels from cortical hippocampal lysates stacked for comparison. Panel C represents cytokine data from 9-month plasma samples. Data are presented as mean ± SEM and significance was determined by Kruskal-Wallis ANOVA with *p<0.05, **p<0.005, ***p<0.0005, ***p<0.0001 denotes a significant result. Outliers were removed using the ROUT test.

*9 months of copper-exposure from weaning alters the microbiome in WT mice more so than in APP-KI animals*

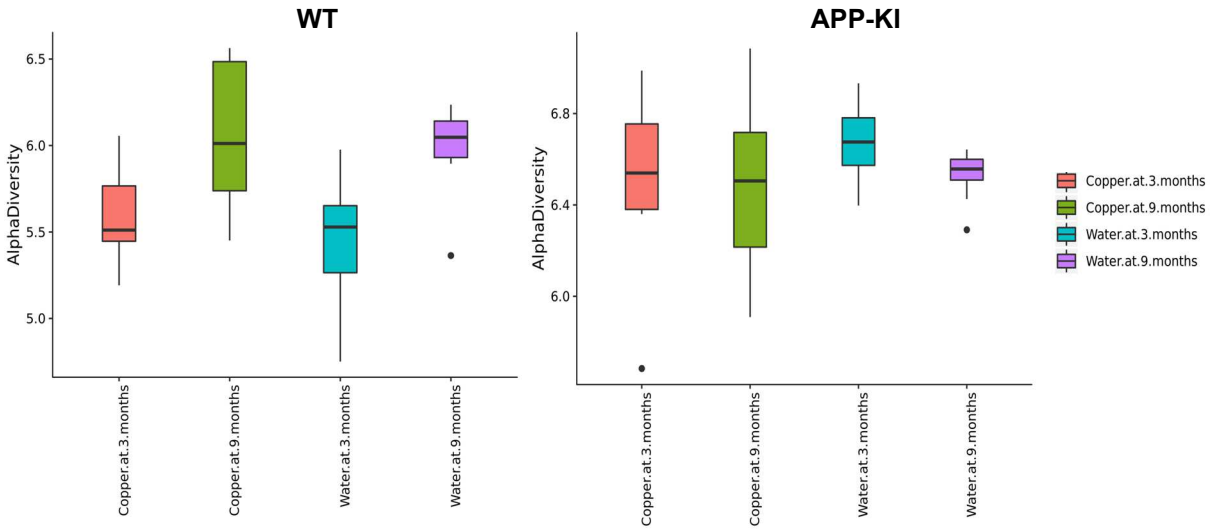


**Figure 3.10.** *The Shannon-alpha diversity in WT (right) and APP-KI (left) animals at 3 month and 9-month timepoints.* Overtime, the mean diversity in WT animals increases with age despite copper exposure, however, this does not happen for APP-KI animals. The diversity of APP-KI animals does not change much but remains consistently higher than that of WT

We previously reported in Chapter 2, that over a 3-month period, the alpha-diversity did not change much but was consistently higher in APP-KI mice in comparison to WT. Following 9 months exposure, this remains true (Figure 3.10). A gain in diversity with time is normally associated with a healthy microbiome (Lloyd-Price et al., 2016; McBurney et al., 2019), while just having more richness is not necessarily always associated with better health (Roager et al., 2016). Further, there seems to be a slight loss of species diversity in APP-KI animals at 9 months compared to the 3-month timepoint. Loss of diversity in this manner has been thought of as an indicator of disease(Lloyd-Price et al., 2016; McBurney et al., 2019).

When considering the beta-diversity, the differences in the abundances and therefore the composition of the microbiota, copper directs the trajectory of the microbiome in WT mice. The copper and water animals cluster together and is like the 3-month exposure in Chapter 2.

However, a subsequent 6-month exposure causes water and copper animals to cluster separately showing an effect of time and exposure on the microbiome (Figure 3.11, WT).

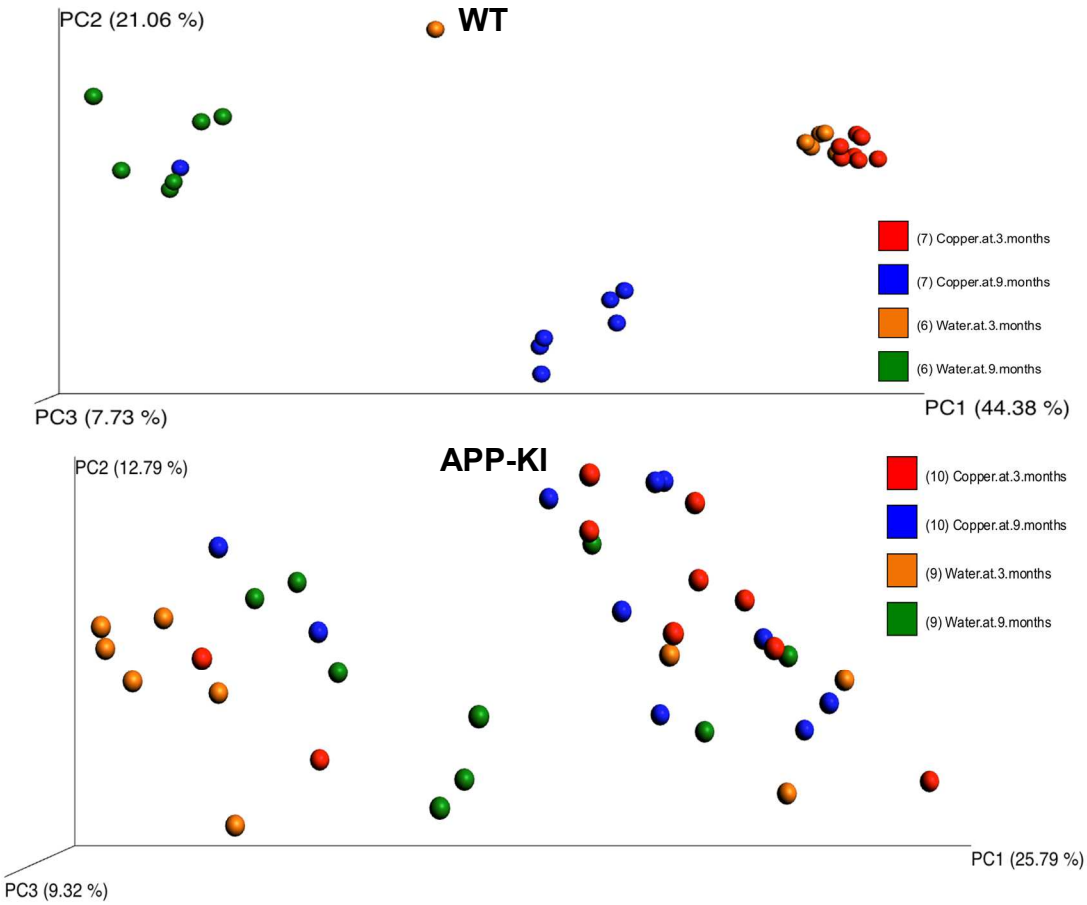


**Figure 3.11.** *The unweighted Unifrac distance for WT (top) and APP-KI (bottom) based on β-diversity.* The Unifrac distance is a qualitative phylogenetic measure of community diversity based on taxon abundance. Copper directs the trajectory of the microbiome in WT but less so in APP-KI animals.

The same cannot be said about the APP-KI animals. These animals tended to cluster by animal then by treatment with water and copper animals clustering separately with barely any drift due to time (Figure 3.11, APP-KI). This also explains the lack of change seen in the α-diversity (Figure 3.10).

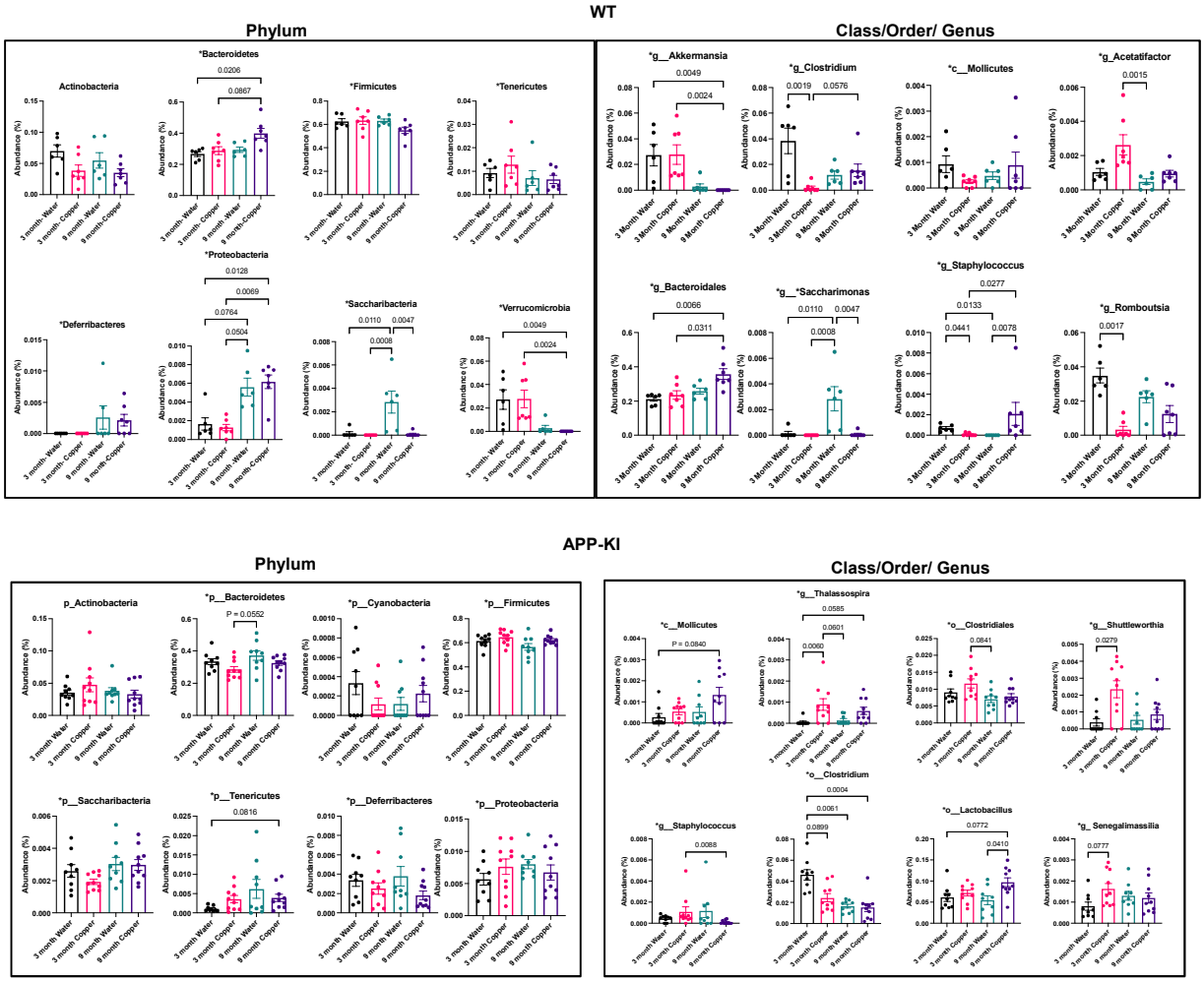


**Figure 3.12.** *The relative abundance within WT and APP-KI fecal samples at the phylum level (L2) and the level with the lowest classification before(L6) species level(L7)* Different phyla are detected in APP-KI mice compared to WT mice signaling a fundamental genotype difference between WT and APP-KI mice. While there were many other genera detected in the dataset, the above graphs for both WT and APP-KI represent genera of interest and/or significant changes were observed.

Investigating the abundance at the phylum level (Figure 3.12), we observe a shift in several phyla in WT mice. With copper exposure, we seen an increase in Bacteroidetes, one of the most abundant phyla which is also gram-negative, and this finding is previously reported in Chapter 2 but in APP-KI mice. Interestingly, that finding is not replicated in these APP-KI animals. There seems to be an age-related increase in Proteobacteria, and a decrease in Verrucomicrobia, another gram-negative phylum with age but not exposure. There is an age-related increase in *Saccharibacteria* in water-treated WT animals but not copper-treated animals.

This phylum represents ultra-small bacteria that are obligate parasites of larger bacteria and can therefore control microbial ecology within the gut microbiota (Bor et al., 2019).

On the L6 level, we observe a few changes in both WT and APP-KI, with more in the former. In WT, we see treatment related effects in the genera *Clostridium*, *Saccharimonas*, *Staphylococcus* and *Romboutsia*. Most *Clostridium* species are considered to have a commensal relationship with the host (Lopetuso et al., 2013) and it seems this may have been disturbed at 3 months with copper exposure. *Romboutsia* is a part of the class Clostria and is similar to the genera *Clostridium* (Gerritsen et al., 2014), which may explain the similarity in the changes in abundance in response to copper. *Staphylococcus* decreases with 3-month copper exposure but increases after 9 months of exposure. Many of the members of this genus are opportunistic pathogens, for example *S. aureus*, even though they are naturally members of many mucosal microbiota (skin, gut etc.) (Götz et al., 2006). In APP-KI animals, there is an increase in the class *Mollicutes*, which has been associated with obesity (Hildebrandt et al., 2009), with 9 months of copper exposure. *Shuttleworthia, Senegalimalssilia* and *Thalassospira* both increase with copper exposure in APP-KI mice but their role in gut microbiota remains unclear.

LEfSe analysis has identified features that are significantly different between WT (Table 1) and APP-KI (Table 2) treated groups. Much like what we observed from the abundances, Bacteroidetes are significantly increased in copper treated WT. This and other changes such as the increase in families such as *Rikenallaceae* have been in human AD and are more abundant in the copper-treated microbiota (Vogt et al., 2017). Interestingly, we see an opposite effect in APP-KI mice with an increase in some Bacteroidetes members in the water treated group and an increase in *Lactobacillus*, which belongs to the phylum Firmicutes in the copper treated group. *Ruminococcaceae* belong to a family less abundant in AD that is increased in the copper group.

Further, a species in *Roseburia*, another beneficial genus, is also more abundant in copper treated group (Bäuerl et al., 2018). However, another species of that same genus is more abundant in the water group. In water treated animals, there is still evidence of the presence of health associated microbes such as *B. acidifaciens,* which works to reduce inflammation through propionic acid production (Blander et al., 2017) and different species within the genus *Roseburia*.

| Top 10 Taxa for 9 month treated Water and Copper Treated WT Animals | | | |
|---|---|---|---|
| **Feature** | **Treatment Group Most Abundant** | **Effect Size** | **p-value** |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales | COPPER | 4.64145575 | 0.01012799 |
| k__Bacteria.p__Bacteroidetes | COPPER | 4.64145575 | 0.01012799 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia | COPPER | 4.64145575 | 0.01012799 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA | COPPER | 4.61713511 | 0.01515844 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA | COPPER | 4.61713511 | 0.01515844 |
| k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Allobaculum.s__sp36553 | COPPER | 4.47281495 | 0.0026998 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12804 | COPPER | 3.9739818 | 0.02154031 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12536_sp12753 | COPPER | 3.84539077 | 0.0026998 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae | COPPER | 3.81924519 | 0.03212457 |
| k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32630 | COPPER | 3.76025088 | 0.02046163 |
| | | | |
| k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Allobaculum.s__sp36552 | WATER | 4.55577792 | 0.0145743 |
| k__Bacteria.p__Firmicutes | WATER | 4.53485545 | 0.01515844 |
| k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32778 | WATER | 3.9976689 | 0.04249001 |
| k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32761 | WATER | 3.94578295 | 0.04550026 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae.g__Odoribacter.s__sp13176 | WATER | 3.62992884 | 0.01460509 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae.g__Odoribacter | WATER | 3.62992884 | 0.01460509 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae | WATER | 3.62992884 | 0.01460509 |
| k__Bacteria.p__Actinobacteria.c__Actinobacteria.o__Bifidobacteriales.f__Bifidobacteriaceae.g__Bifidobacterium.s__choerinum_pseudolongum | WATER | 3.44437392 | 0.03422009 |
| k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Allobaculum.s__sp36555 | WATER | 3.37417391 | 0.0145743 |
| k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32735 | WATER | 3.33339323 | 0.04249001 |

**Table 1**. *Top 10 Taxa for 9 Month Treated Water and Copper Treated WT Mice.* The result of LEfSE analysis on WT exposed for 9 months. The groups in which the taxa are most abundant is labelled. The effect size determined by linear discriminant analysis is given by the effect size and the p-value for the non-parametric analysis.

| Top 10 Taxa for 9 month treated Water and Copper Treated APP-KI Animals | | | |
|---|---|---|---|
| Feature | Treatment Group Most Abundant | Effect Size | p-value |
| k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales | COPPER | 4.292842247 | 0.017892268 |
| k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae.g__Lactobacillus | COPPER | 4.290679154 | 0.017892268 |
| k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae | COPPER | 4.290679154 | 0.017892268 |
| k__Bacteria.p__Firmicutes.c__Bacilli | COPPER | 4.273827258 | 0.017892268 |
| k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae.g__Lactobacillus.s__johnsonii | COPPER | 3.98660483 | 0.007050729 |
| k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae.g__Lactobacillus.s__reuteri_vaginalis | COPPER | 3.922587224 | 0.022243121 |
| k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp35310 | COPPER | 3.004903423 | 0.000366545 |
| k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33443_sp33760 | COPPER | 2.974893392 | 0.011225753 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12599 | COPPER | 2.959158937 | 0.001265334 |
| k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Roseburia.s__sp33112 | COPPER | 2.847944261 | 0.019052625 |
| | | | |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12656 | WATER | 3.8124962 | 0.002519103 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12473_sp12526 | WATER | 3.747015464 | 0.033762978 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__acidifaciens | WATER | 3.403546224 | 0.027486336 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12536_sp12753 | WATER | 3.246418842 | 0.001917723 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12520 | WATER | 3.232567816 | 0.007050729 |
| k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales | WATER | 2.958607513 | 0.030535797 |
| k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12641 | WATER | 2.838313518 | 0.017842075 |
| k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Roseburia.s__sp33143 | WATER | 2.837964505 | 0.008455784 |
| k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Staphylococcus | WATER | 2.826151278 | 0.046978626 |
| k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae | WATER | 2.824788459 | 0.046978626 |

**Table 2**. *Top 10 Taxa for 9 Month Treated Water and Copper Treated APP-KI Mice.* The result of LEfSE analysis on WT exposed for 9 months. The groups in which the taxa are most abundant is labelled. The effect size determined by linear discriminant analysis is given by the effect size and the p-value for the non-parametric analysis.

## 3.4 Discussion

In the current study, we expanded upon the pilot project conducted in Chapter 2 to assess changes to cognition, amyloidosis, gliosis, systemic cytokine profile and gut microbiota following chronic exposure to environmentally relevant copper through drinking water in both WT and a knock-in mouse model of AD. The exposure regimen was to expose these animals starting at 1 month of age until they were ten months of age. We have previously completed the same exposure study in a different mouse model (J20) and its wildtype counterpart (Kitazawa, Hsu papers). Interestingly, we did not observe the same (or similar) behavioral outcomes in the current cohorts

as we previously detected in WT and J20 mice(Hsu et al., 2019)`1, although the copper-exposed APP-KI mice tended to perform the worst on each behavioral task we performed. A study analyzing the progression of cognitive deficits age-dependently in $APP^{NL-G-F}$-KI mice showed that there was intra-study and intra-task variability before 12 months of age. It seems that by 12 months of age, there are more pronounced deficits are observed (Mehla et al., 2019).

Despite marginal changes in cognitive deficits, we observed some change in amyloid burden. Plaque deposition varied with brain region. 3 regions were chosen from anterior to posterior to cover to span much of the cortex and hippocampus. While the area covered by diffuse amyloid plaques remain relatively the same, between 4-6%, the area covered by dense core plaques increased from the anterior-most region to the posterior most region by about threefold. With copper exposure we see an increase in the diffuse plaques in the third, posterior region and within the dense-core plaques in first, anterior region. This is evidence that there is some modulation of senile plaque development by copper. Indeed, post-mortem examination of brain specimens from AD suffers have shown that senile plaques are enriched in copper (Everett et al., 2021; Lovell et al., 1998). In humans, where non-ceruloplasmin bound copper has been associated with decreased amyloid levels in CSF, indicating amyloid species are being retained in the brain (Bagheri et al., 2018; R. Squitti et al., 2006). In rodent models, we have seen increased plaque pathology following low and higher doses of copper (Kitazawa et al., 2009; Singh et al., 2013; Sparks & Schreurs, 2003).

Following amyloidosis, we conducted studies to determine if copper exacerbates neuroinflammation by modulating glial response. Indeed, when we use a ferritin antibody to detect improper iron-loading that is characteristic of DAM, we saw an increase in both the hippocampus and cortex in the anterior brain region of APP-KI copper-treated animals, without any subsequent

increase in the levels in WT mice. Indeed, the ferritin associated microglia resembles amyloid beta deposition which is surrounded by clusters of IBA-1 positive cells, indicating that only plaque associated microglia are ferritin-positive. This supports our recent findings transcriptomic studies that copper directs microglia to a degenerative phenotype through downregulation of homeostatic genes such as TMEM119 and P2RY12 (Lim et al., 2020). TMEM119 was not found to be significantly different via copper exposure. However, the clustering we observe with ferritin staining is noticeably absent in the TMEM119 cells, indicating that these cells expressed a lower level of the biomarker. We did not observe any changes to phagocytic ability via C68, autophagosome expression but we and others have shown that copper can impair microglial phagocytosis and subsequent clearance of Aβ (Kitazawa et al., 2016; Tan et al., 2020; Zheng et al., 2010). Many different phenotypes of disease associated microglia exist. Recent advances and an increasing number of studies incorporating RNA-seq and scRNA-seq methods have identified clusters of microglia that vary with age, sex and APOE ε4 status (Fixemer et al., 2022). It stands to reason that this could vary with other factors such toxicant exposure or biometal dyshomeostasis.

Much like microglia, copper dyshomeostasis can also affect astrocytes. As astrocytes are one of the key regulators of homeostasis in the brain connecting to neural cells as well as the vasculature, it is involved in uptake and export of a variety of substrates including copper through expression of the transporter ATP7A(Pal et al., 2021). As a mediator of uptake and export into and out of the brain, astrocytes could enter a disease associated state producing cytokines to perpetuate chronic neuroinflammation, like microglia, in response to copper dyshomeostasis. However, when we measured Complement C3, a marker for neurotoxic astrocytes, we did not observe any significant changes with copper exposure in WT nor APP-KI mice. There is an

increase across genotypes but there is a trend of higher C3 levels in the copper animals. Additionally, there is a very mild response noted in the cytokine profile of plasma and brain homogenates. Changes in cytokines such as TNFα and IL-1β are across genotypes with higher levels in APP-KI animals. Interestingly, IL-4 is decreased in APP-KI serum. IL-4 is an anti-inflammatory cytokine that has been shown to have reduced expression in AD patients and can stimulate microglia to clear Aβ *in vivo* (Su et al., 2016).

In addition to those pathological analyses in the brain, we included a new area of exploration to study the effects of copper exposure on the gut microbiota in this study. The gut microbiota in human AD is significantly different comparison to that in age matched controls in an American cohort (Vogt et al., 2017). In this study, there is a marked decrease in phylogenetic diversity, accompanied with a decrease in Firmicutes and an increase in Bacteroidetes. A study in a Chinese cohort investigated the differences between health controls, amnestic mild cognitive impairment sufferers and AD patients (P. Liu et al., 2019). In this study, they discovered a lower Firmicutes abundance in AD patients with higher levels of Proteobacteria. While they did not find any significant differences in Bacteroidetes between healthy controls and AD patients, in AD, Bacteroidetes was significantly correlated with scores on a cognitive impairment assessment. A variety of studies using AD model mice indicate that the gut microbiota influences amyloidosis, neuroinflammation and cognition in these animals. Many of the shifts described above have been recapitulated in several mouse models of the disease including the APP/PS1 (Bäuerl et al., 2018; Y. Chen et al., 2020; Dodiya et al., 2019), 5xFAD (Brandscheid et al., 2017; C. Chen et al., 2020), 3xTg-AD (Bello-Medina et al., 2021; Bonfili et al., 2017; Borsom et al., 2021) and in the mouse model used in this study, the $APP^{NL-G-F}$-KI (Kaur et al., 2021; Kundu et al., 2021; Sohrabi et al., 2021). Some of these effects on amyloidosis improve upon supplementation of probiotics (Bonfili

et al., 2017; Kaur et al., 2020; Kobayashi et al., 2017), indicating that there are certain key microbes that contribute to AD neuropathology and defining these may present a therapeutic target for disease management. Together, the investigation of the microbiota implicates the microbiome as a modifier for Alzheimer's Disease risk (Fang et al., 2020).

Early life gut microbiome is not stable and highly plastic, which could be affected by various endogenous and exogenous factors (Gritz & Bhandari, 2015; Sanidad et al., 2022; Sanidad & Zeng, 2020). The gut microbiota undergoes substantial changes from birth until weaning, where the microbiota is determined by lactation, from weaning to a "normal diet" and then in old age (Flint et al., 2012). Thus, it is important to investigate the microbiota along side effects seen in the brain as many studies have shown that the microbiota contribute to amyloidosis through use of antibiotic treated, specific pathogen free or germ free versions of popular AD mouse models (Brandscheid et al., 2017; Cattaneo et al., 2017; Y. Chen et al., 2020; Minter et al., 2016; Pistollato et al., 2016; Sochocka et al., 2019). Moreover, many studies have also shown the critical role the microbiome plays in the development of the peripheral and central nervous systems (Castillo-Ruiz et al., 2018; Minter et al., 2017; Thion et al., 2018). Indeed, one study showed the lack of response to an immune challenge and the presence of immature microglia in the without the presence of a complex microbiome (Thion et al., 2018).

One of the most fascinating findings in this study is that despite not affecting the acquisition of species richness with aging, copper exposure influenced a different microbiome trajectory in WT mice as evidenced by the clustering by age and exposure type β-diversity based Unifrac distances. We see an increase in the phyla Bacteroidetes and Proteobacteria and a decrease in Sachariabacteria and in many of the genera in these phyla. Many of these changes are observed in other studies in which investigators compare AD animals to WT, which is not done in this data set

as our focus was to investigate the effect of copper. Many of the changes we observe in the WT mice are characteristic of a dysbiotic gut as seen with APP-KI mice in the 3-month pilot project exposure paradigm. This differential effect in abundance in not as pronounced in APP-KI mice. Over the 9-month exposure period, copper seemed to not alter the gut microbiota of these APP-KI mice. It should be noted however, that the gut microbiota through alpha and beta diversity metrics did not vary much between the 3 month and 9-month period, which may indicate a dysbiotic gut composition at the start of the exposure. A healthy gut microbiota while it should be relatively stable over short periods of time, should change with aging (DAS & Nair, 2019; Flint et al., 2012; McBurney et al., 2019; Ratajczak et al., 2019).

Recently, studies have begun investigating the effects such changes may have on the brain. The C/EBPβ/AEP pathway, which can lead to elevated levels of Aβ and tau has been implicated in AD pathogenesis and activated/exacerbated by gut dysbiosis (C. Chen et al., 2020). Other studies, in trying to elucidate the meaning of these changes turn to metabolomics to measure bacterial metabolites as immunomodulators or neuroactive biomolecules such as BDNF (brain derived neurotrophic factor) or serotonin. The main class of molecules investigated are short chain fatty acids (SCFAs). These short chain fatty acids can bind a variety of receptors free-fatty acid and other g-coupled reports and thus can influence the brain through the immune, enteroendocrine, vagal and humoral pathways (Dalile et al., 2019). Many of the members of the phyla Firmicutes sand order *Clostridium* are short chain fatty acid producers. With copper exposure in WT and in Chapter 2, we saw a marked reduction of members of this phylum.

The results in Chapter 2 regarding microbiota are not replicated here and are the opposite to what was hypothesized based on those data. This could be due to several reasons. This study did not investigate the microbiota of all animals used in the study and investigated the microbiota

of female mice only. The reduction of sample size could affect the diversity in the study as fewer mouse cages were sampled, and cage effects can affect microbial analyses. The mice from one cage will be more similar in microbiota than another, as mice a coprophagic, consuming fecal matter (Ericsson et al., 2018). In future to control for this cage effect, animals should be spread out across as many cages as possible. The methods analytical pipelines used in this study were different from our previous study. For example, taxonomy was assigned in 2019 based on the GreenGenes database (McDonald et al., 2012) while this analysis done by Zymo Research in 2022 assigned using the SILVA database, the largest 16S database (Balvočiute & Huson, 2017; Quast et al., 2013). Further, analyses were done with PERMANOVA and SIMPER methods in the 2019 pilot study, while this current study utilized LEfSe which uses Kruskal-Wallis (KW) sum-rank test, Wilcoxon rank sum test, followed by a linear discriminant analysis.

Although we see an increase in amyloidosis and in degenerative microglia with copper exposure within this study, we did not observe concomitant exacerbation of cognitive performance in WT or APP-KI mice. Further, the changes we observed in gut microbiota of WT mice do not translate into inflammatory changes in the brain nor in the plasma. Conversely, we did not see a large change in the composition in the gut of APP-KI animals, not much alteration in brain and plasma cytokine profile but an exacerbation in AD pathology. It is unclear at this time what role the chronic exposure to 1.3ppm of copper had on the gut-brain axis. Further studies into bacterial metabolites with copper exposure and possibly exposing the mice for even longer (until at least 12 months of age), when there is expected robust cognitive impairment, may elucidate copper's role further.

## 3.5 References


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# Chapter 4

## Dissertation Summary, and Future Directions

### 4.1 Dissertation Summary and Contribution

*Dissertation Statement and Conclusion*

Exposure to 1.3 ppm of copper in drinking water induces dysbiosis APP-KI mice in a short-term exposure study in 3-month-old mice. In a chronic exposure spanning 9 months from weaning, 1.3 ppm copper exposure directs the trajectory of the gut microbiome in WT mice. We found no changes in systemic inflammation in the 3-month nor 9-month studies. However, in the 9-month exposure study, we saw evidence that copper exposure increases DAM in the cortex and hippocampus of AD mice. To our knowledge, this is one of the few studies incorporating the effects on the gut microbiome while exploring effects of trace amounts of a biometal on AD neuropathology.

Further, in investigating the effects of the contribution of neuroinflammation in AD, we explored the effects of a therapeutic candidate, ONO-451053 on resolving sustained chronic brain inflammation which we hypothesized would lead to decreased amyloidosis and improved cognition as we had seen in previous studies with $LXA_4$/ATL supplementation(Dunn et al., 2015; Medeiros et al., 2013). However, in the present study, we did not see improvement in amyloid plaque deposition, in the amyloid peptides in the detergent and formic acid soluble fractions of cortical and hippocampal homogenates.

Together, these studies indicate that the role of inflammation within AD is incredibly complex and additionally so when considering environmental factors or organ systems as variables. From the existence of subsets of microglia and astrocytes that are stimulated by different

cytokines and chemokines and thus present with different functional and transcriptomic phenotypes, targeting and even identifying specific mechanisms have been difficult. We have however shown in this study and previous work that 1.3ppm of copper can elicit pathological change to exacerbate AD pathology.

### 4.2 Future Directions

*Investigations into the Microbiome and the contribution of the microbiota-brain axis in APP-KI mice*

The current work is insufficient to address copper's influence on the microbiota-gut-brain axis within AD model mice. Further work in this mouse model is thus required to investigate or identify key bacterial species and their functional role within the microbiome with and without copper exposure. The immediate primary area of interest would be to conduct another study that sufficiently controls for variables such as breeding lineage, caging, sex effects(Ericsson et al., 2018; Hildebrand et al., 2013). It has been shown in both humans and animals that birth and lactation shapes microbiomes and offspring immunity(Sanidad et al., 2022; Sanidad & Zeng, 2020; Spor et al., 2011; Ubeda et al., 2012). This is called the maternal lineage effect. It has been shown that litters from sisters are even more alike in composition than non-related siblings. While this may be good for comparison within a study, it makes for poor correlation and reproducibility in other labs and other studies due to genetic drift. (Laukens et al., 2016). Although this data was not shown, when looking at individual samples in the 9-month copper exposure study, there was evidence of similar phylogeny within samples suggesting many of the bacteria colonizing the gut was inherited. With only a few parents, intra-litter variation may account for more compositional differences than exposure status, especially at low doses like in this study and this may present as an exposure effect. It is also well-characterized that there are more across strain variability than

within strain (Hildebrand et al., 2013; Laukens et al., 2016). Many studies choose to bypass this issue by deriving disease model animals alongside their WT background strain by using heterozygote parents (Brandscheid et al., 2017; Harach et al., 2017). Studies not using this model may opt for cross-fostering pups and or grouping animals of both genotype but similar treatment group (Laukens et al., 2016). Further, workflow in the microbiota can be influences by DNA extraction method, primer choice, sequencing methods, taxonomic classification and the tools used for datamining (J Pollock, 2018; Laukens et al., 2016; Nearing et al., 2022; Weiss et al., 2017). From studies like this candidate microbes can be identified to be used in culture experiments and gut repopulation studies with these microbes to investigate effects on the brain in the absence of copper. It would also be interesting to characterize the microbiome of APP-KI mice in a germ-free condition with and without copper exposure. This would give us a baseline indication of what neuropathological effects we can attribute to copper without interference of the microbiota.

A primary route of communication between the gut and the brain is via the vagus nerve (Dalile et al., 2019; Tremlett et al., 2017). Recently, Chen et. al discovered that vagotomies attenuate AD pathologies in the brains of 3xTg-AD mice following dextran sodium sulfate, which promotes gut leakage, and when Aβ/ Tau fibrils were introduced into the guy via colonic injection. Moreover, they found the fluorescent-tagged Aβ/ Tau fibrils in the brain (hippocampus and dorsal motor vagus nerve for the Aβ fibrils and hippocampus and locus coeruleus for or Tau) following injection into the colon, supporting that the fibrils do in fact spread from the gut to the brain(C. Chen et al., 2021), much like the case with α-synuclein in Parkinson's Disease (Challis et al., 2020; Kim et al., 2019). It would be interesting to do studies in a knock in mouse model such as the humanized $APP^{NL-G-F}$-KI utilized in this dissertation or the humanized APP-KI without any familial mutations (Baglietto-Vargas et al., 2021) or any of next generational models being

developed by the MODEL-AD consortium (Oblak et al., 2020) in the effort to produce more human translatable discoveries.

To further consider the role of immunity in gut microbiome dysbiosis and effects on the CNS, it is important to consider gut associated lymphoid tissue (GALT). GALT is a part of the mucosa-associated lymphoid tissues (MALT), which act as a defense between the host and the environment (Buettner & Lochner, 2016; Jiao et al., 2020; Mörbe et al., 2021). The GALT contains about 70-80% of the immune system and is largely considered to be the largest and most important immune organ (Sochocka et al., 2019). The gut microbiome consists of trillions of microorganisms that provide a plethora of benefits for host health. However, for continued host health, the microbiota must be contained. The immune cells in the GALT develop based on microbiota, thus maintaining tolerance to commensal microbes, recognizing pathogens, initiating the innate immune response and presenting antigens to activate and train the adaptive immune system (Jiao et al., 2020). Therefore, the integrity of the intestinal barrier – maintenance of mucosal barrier and tight junctions-and the function of the GALT should be investigated alongside circulating immune cells and cytokines (Ma et al., 2019). A major contributor to immune health and CNS health are the products formed by metabolic and catabolic reactions by gut microbiota. The most common of these are short chain fatty acids which act as fuel sources and help shape immunity. They also produce biogenic amines that can act as neurotransmitters and some neurotransmitters like serotonin are produced in the gut. Microbiota are crucial for the catabolism of amino acids, lipids, and dietary fiber (Oliphant & Allen-Vercoe, 2019).

*Quantification of Copper in the Gut, Plasma and Brain*

The studies of copper exposure in this dissertation could have been strengthened by tracking the relevant copper levels in exposed animals versus unexposed to characterize the extent

of copper dyshomeostasis within WT and the AD model. As the amounts of biometals and even the exposure concentrations are often trace amounts, especially in a rodent, the most appropriate method would be to use Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) for tissue homogenate (Ishihara et al., 2019; Li et al., 2015). However, the use of the laser ablation addition to this system has gained popularity. This allows for spatial resolution of copper accumulation as this may depend on the unique biology of the brain region (Deibel et al., 1996; Grochowski et al., 2019; Sussulini et al., 2017).

*Single cell RNA-Sequencing (scRNA-seq) of immune cells (glia and peripheral immune cells)*

1.3 ppm of copper exposure is the permissible limit in drinking water set by the US EPA. This is considered a trace amount and studies within this dissertation have shown that this may still induce deleterious effect. Moreover, we have seen that on a macroscale that this may result in mild changes or trends. As the brain has many different cell types and functionally distinct subpopulations, changes may be subtle or even masked by more traditional approaches such as western blotting and immunohistochemistry. When considering changes on a transcriptomic level, these may not even be detectable by traditional bulk RNA-sequencing (Johnson et al., 2020; Zeisel et al., 2015). scRNA-seq can thus be used to target network associations, for e.g., crosstalk between disease associated microglia and astrocytes, affected by exposures. These can then be targeted by therapeutic intervention (Wang et al., 2022; Xu et al., 2021). Multiple sequences in mouse models and humans can then be compared to each other to find correlations or trends to benefit to surveil the data for breakthroughs for future preventions or cures (Y. Chen & Colonna, 2021).

*Concluding Remarks*

Alzheimer's disease is a highly complex disease marked by heterogeneity and is likely due to a variety of pathophysiologic mechanisms (Mehta & Schneider, 2021). Currently, there are no treatments that completely cure or prevent disease progression. Due to the heterogeneity seen in humans, due to sex, age, genetics and even modifiable risk factors, further insights into the disease and the search for a therapeutic target is requires creative and multifaceted approaches. This body of work has explored the role of an environmental exposure, inorganic copper exposure and the effect on the brain and microbiota to expand beyond the idea that Alzheimer's Disease is purely neurological in origin. Many of the approaches used in this study or mentioned in this chapter produce large data sets. There are efforts within the field to consolidate these into publicly available databases, apply artificial intelligence to analyze the amount of data produced by different research institutions and different approaches to find common trends (Fabrizio et al., 2021; Jiang et al., 2020). These approaches should over time, answer many of the questions left by studies like the ones presented in this work.

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