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The effects of airborne ultra-fine particulate exposure on cognition and neuropathology in an
amyloid 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

Jason Kilian

Dissertation Committee:
Associate Professor Masashi Kitazawa, PhD (Chair)
Associate Professor Kim Green, PhD
Professor Stephen Bondy
Adjunct Professor Michael T. Kleinman, PhD

2020

Contents

List of Tables

Chapter 1

List of Figures

tissue were also unaffected by the exposure. (E) No differences in insoluble $A\beta_{40}$ or $A\beta_{42}$ levels were found in the cortex or hippocampus by ELISA. Bars represent mean values, with error bars expressed as standard error of the mean (N = 6 animals per group for immunofluorescent staining and quantification, and N = 4 animals per group for immunoblot, V-PLEX, and qPCR. Scale bars = 100 μm). * denotes p value < 0.05, ** denotes p < 0.01…………………………47

Figure 11: Glial markers GFAP and Iba1 do not increase with exposure to UF PM in 12 month old $App^{NL-G-F/+}$-KI mice. (A) Representative images of microglial marker Iba1 (red), CD68 (purple), and Aβ (green) staining from the parietal cortex (top)of wild-type (Wt) and $App^{NL-G-F/+}$-KI (APP) mice exposed to filtered air or ultra-fine PM and quantification of the mean fluorescent intensity of Iba1 staining for the cortex and hippocampus from all mice and total count of Iba1 and CD68 positive microglia in $App^{NL-G-F/+}$-KI mice (bottom). Exposure to UF PM does not increase the levels of Iba1 or Iba1+/CD68+ microglia in the brain in either group. (B) Representative images of astrocytic marker GFAP (red) and Aβ (green) staining from the parietal cortex (top) and quantification of the mean fluorescent intensity of GFAP staining for the cortex and hippocampus (bottom). UF PM exposure does not change the levels of GFAP observed in either group. (C) mRNA levels of cytokines *Il6* and *Il1β* normalized to *Gapdh* mRNA. Neither *Il1β* or *Il6* mRNA levels from cortical tissue were significantly affected by the exposure status or genotype. Bars represent mean values, with error bars as standard error of the mean (N = 6 animals per group for immunofluorescent staining and quantification, and N = 4 animals per group for immunoblot and qPCR. Scale bars = 100 μm)…………………………………………49

Figure 12: Tight junction markers do not decrease in wild-type or $App^{NL-G-F/+}$-KI mice exposure to UF PM (A) Representative images of ZO-1 staining of cerebral vasculature from the cortex in wild-type (Wt) and $App^{NL-G-F/+}$-KI (APP) mice exposed to filtered air or UF PM (left) and quantification of vascular width (right). No differences are seen across treatment or genotype. (B) Representative western blot of albumin in cortical protein extract. Differences between groups are not significant. (C) Representative western blot of tight junction marker Claudin-5 and (D) intercellular junction marker CD31 from vascular enriched protein samples. Neither genotype nor exposure status shows significant differences. Bars represent mean values, with error bars expressed as standard error of the mean (N = 4-5 animals per group for all assays. Scale bars = 100μm)…………………………………………………………………..52

Figure 13: Levels of astrocytic glutamate transporter GLT-1 decrease in the hippocampus of mice exposed to UF PM (A) Representative western blots of GLT-1 protein levels relative to GFAP in the cortex (left) and hippocampus (right) from 6 month old $App^{NL-G-F/+}$-KI mice exposed to either filtered air or UF PM, with quantification of western blot band intensity (below). GLT-1 levels decrease in the hippocampus, but not cortex, of UF PM exposed animals. FA- Filtered air UF- UF PM. (B) Representative western blots of GLT-1 protein levels relative to GFAP in the cortex (left) and hippocampus (right) from 12 month old wild-type (Wt) or $App^{NL-G-F/+}$-KI (APP) mice exposed to either filtered air or UF PM with quantitation (below). GLT-1 is decreased in UF PM exposed animals compared to wild-type air exposed animals, but not within the $App^{NL-G-F/+}$-KI group. Bars represent mean values, with error bars expressed as standard error of the mean (N = 8 animals per group). * denotes p<0.05……………………...………54

Chapter 3

Chapter 4

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<div align="center">

Vita
JASON G. KILIAN

</div>

Education

PhD in Environmental Health Sciences toxicology emphasis   Spring 2020
UC Irvine School of Medicine

PhD in Quantitative and Systems Biology   Program Transfer 2016
UC Merced

BS in Genetics   2010
University of California, Davis

Research Experience

1/2016-Present	Environmental Health Sciences Program, UC Irvine SoM. Graduate Researcher. Design experiments for testing Alzheimer's Disease model mice with exposure to air pollution. Support ongoing study of AD model mice exposure to environmental copper.
08/2013-12/2015	Quantitative and Systems Biology Program, UC Merced Graduate Researcher. Design and execute experiments in *Drosophila melanogaster* Alzheimer's Disease model. Support mouse model and cell culture AD research performed in the lab.

Teaching Experience

01/2017-03/2017	School of Biological Sciences, UC Irvine Teaching assistant for Evolution and Ecology lecture. Responsible for creating course material, leading discussion sections, and assigning discussion grade.
08/2014-05/2015	School of Natural Sciences, UC Merced Teaching Assistant for Microbiology laboratory course. Responsible for leading students in experiments in basic microbiology techniques and grading lab reports and tests.
01/2014-05/2014	School of Natural Sciences, UC Merced Teaching Assistant for Microbiology lecture. Responsible for leading discussion sections, creating activities, and all grading.
08/2013-12/2013	School of Natural Sciences, UC Merced Teaching Assistant for Introduction to Cell Biology. Aid professor with class load including grading and quiz creation and lead small

discussion and laboratory sections.

Publications

Hsu HW, Rodriguez-Ortiz CJ, Lim SL, Zumkehr J, Kilian JG, 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.

Kilian, J, and Kitazawa, M. 2018. "The emerging risk of exposure to air pollution on cognitive decline and Alzheimer's disease – Evidence from epidemiological and animal studies." *Biomedical Journal*. 41(3) 141-162.

Kilian, J, Hsu HW, Mata K, Wolf FW, Kitazawa M. 2017. "Astrocyte transport of glutamate and neuronal activity reciprocally modulate tau pathology in Drosophila." *Neuroscience* 348 191-200. 10.1016/j.neuroscience.2017.02.011

Zumkehr J, Rodriguez-Ortiz CJ, Cheng D, Kieu Z, Wai T, Hawkins C, Kilian J, Lim SL, Medeiros R, Kitazawa M. 2015. "Ceftriaxone ameliorates tau pathology and cognitive decline via restoration of glial glutamate transporter in a mouse model of Alzheimer's disease." *Neurobiology of Aging*. 36(7) 2260-2271.

Conference Presentations

Kilian, J, Herman DA, Johnson R, Vidal J, Dalal H, Rodriguez-Ortiz CJ, Renusch SR, Kleinman MT, Kitazawa M. 2018. Exposure to Ultra-fine Particulate Matter Impairs Memory in APP Knock-in Mice without overt changes in Amyloid Pathology. Poster. Society of Toxicology annual meeting. Baltimore, Maryland.

Kilian, J, Herman DA, Johnson R, Hasen I, Ting A, Renusch SR, Kleinman MT, Kitazawa M. 2018. Chronic exposure to ultra-fine particulate matter exacerbates cognitive decline in the APP-KI mouse model of Alzheimer's disease. Poster. UCI ReMIND. Irvine, California.

Professional Memberships

-   Society of Toxicology

Technical Skills

Protein, DNA, and RNA assays in animal tissue and cell culture including RT-PCR, immunoblot, ELISA, immunostaining, gel electrophoresis, and plasmid cloning
Process mouse and fly tissue samples for protein, RNA, DNA
Behavioral tests in *Drosophila melanogaster* and mice

Abstract

The effects of airborne ultra-fine particulate exposure on cognition and neuropathology in an amyloid model of Alzheimer's disease

Jason G. Kilian

Doctor of Philosophy in Environmental Health Sciences

University of California, Irvine

2020

Associate Professor Masashi Kitazawa, Chair

Alzheimer's disease (AD) is the leading cause of dementia among the elderly and sixth leading cause of death in the US. No effective therapeutic intervention is currently available to treat, cure or halt this devastating disease. The prevalence of AD is expected to rise rapidly in coming years and is predicted to overwhelm our socioeconomic reserves. Thus, unveiling critical pathogenic mechanisms of the disease and risk factors that modulate the disease progression and onset is an urgent matter to slow this upswell. While aging and genetics are unequivocal risk factors for AD, there is a growing evidence supporting the pivotal role of environmental or modifiable factors in contributing the onset of AD. Among them, air pollution has recently been highlighted as a prominent culprit accelerating cognitive decline and increasing a risk for AD. However, little work has been done to elucidate its underlying molecular mechanisms by which exposure to air pollution contributes to the pathological development of AD. The purpose of this dissertation is to investigate the neurotoxic effects of airborne particulates in a humanized amyloid mouse model of AD. Previous reports indicate that PM exposure in non-AD model mice increases inflammatory markers such as cytokines, glial activation, and oxidative stress in the CNS and may increase expression of native mouse amyloid or alter its processing. Thus, we **hypothesize that exposure to ultra-fine particulates exacerbates memory impairment by increasing neuroinflammation and amyloid β burden in a humanized amyloid mouse model**

of AD. To test this, we exposure wild-type and $App^{NL-G-F/+}$-KI mice to concentrated ultra-fine particulate matter both *in utero* and at adult ages. We show exposure dependent decreases in memory behavior tasks, as well as increases in Aβ plaque burden in the $App^{NL-G-F/+}$-KI model. However, memory impairment occurs independently of plaque burden increase, and only in the *in utero* exposure group were neuroinflammatory markers increased. In the adult exposure, we instead saw a decrease in the astrocytic glutamate transporter GLT-1. Previous work has shown a link between glutamate transporter loss and tau pathology, indicating a potential amyloid independent pathway linking particulate exposure and AD pathology. This research provides evidence of Aβ plaque burden increase dependent only on particulate exposure, as well as evidence that exposure during development impacts AD neuropathology in adult mice.

Chapter 1: Introduction

1.1 Dissertation Statement

Exposure to airborne ultra-fine particulate matter exacerbates memory impairment and neuropathology in an Alzheimer's disease mouse model, indicating potential mechanisms linking particulate matter exposure to Alzheimer's risk in humans.

1.2 Significance

Alzheimer's disease (AD) is the most common form of dementia among the elderly, with an estimated number of patients of over 5.8 million in the U.S. and 35 million worldwide (Alzheimer's Association, 2018, 2020). Death by AD and other dementias is about 1.6 million in 2015, which is doubled from 2000 and it is currently ranked at the sixth leading cause of death worldwide (WHO, 2017). These numbers will be doubled or even tripled as the aged population rapidly increases in next few decades (Alzheimer's Association, 2020), and such rapid increase in the number of AD cases will create major socioeconomic burdens among us unless effective therapeutic interventions to slow, halt, or cure this devastating disease are developed. In lieu of conquering the disease, the most expedient and actionable course to curb the predicted rise in AD cases is to identify and eliminate environmental factors that increase risk of the disease. Advancing age is the single greatest risk factor for AD, and genetic predispositions have also contributed significant risks to the disease onset. Although these two risk factors have been extensively studied to better understand the pathogenesis of AD, they are intrinsic and not essentially modifiable in terms of delaying the onset and progression of the disease. In addition, aging and genetic risk factors do not fully explain the cause of every AD case, as large cohorts of

homozygote and heterozygote twin studies reveal key involvement of additional modifiable risk factors in AD etiology (Brusco et al., 1998; Gatz et al., 2006; McGeer et al., 1996; Sullivan et al., 2012). Those include, but are not limited to, lifestyle, disease history, educational background, dietary habits, and exposure to environmental and occupational hazards (Campdelacreu, 2014; Yegambaram et al., 2015). Environmental risk factors, such as metals and toxic contaminations in drinking water, exposure to agricultural chemicals, and air pollution, impact on large bodies of the population and influence the risk of AD. Chronic exposure to airborne environmental factors has been shown to increase the risk for developing AD through epidemiological studies and in animal models (Cacciottolo et al., 2017; Jung et al., 2015; Oudin et al., 2016). Further work is required to determine the extent of the association between air pollution and AD pathology, as well as the mechanisms by which it occurs.

1.3 Alzheimer's Disease: Background and Pathology

AD cases are broadly classified based on age of onset as either familial or sporadic. Sporadic AD represents the great majority of diagnosed cases, accounting for over 95% of AD cases (NIA, 2016). Sporadic AD is typically diagnosed at the age of 65 or older. While there is no single cause of sporadic AD, age is the largest risk factor, with risk doubling for every five years past age 65. Familial AD accounts for the remaining cases. As the name implies, familial AD is caused by genetic factors- namely mutations in amyloid precursor protein (APP) or proteins involved in processing it. Familial is also known as young onset AD, as symptoms commonly manifest in the patient's 40s or 50s. Clinical manifestations of the two forms are highly similar outside of age of onset.

Clinical diagnosis of AD in patients relies on neuropsychological assessments and cerebral imaging. The most common assessment tool is the Mini-Mental State Examination (MMSE),

which is a short 30-point questionnaire testing cognitive impairment (Folstein et al., 1975). Lower scores indicating increasing cognitive impairment, measured as mild, moderate, or severe. Magnetic resonance imaging (MRI) and positron emission tomography (PET) scans are the most common imaging techniques used to detect pathology in living patients; MRIs detect atrophy and volume change while PET scans can detect activity alterations. Typically, brain abnormalities are first observed in the entorhinal cortex, with the amygdala, hippocampus, and parahippocampus following.

While work is ongoing to determine accurate biomarkers for definitive diagnosis of AD, currently full diagnosis is confirmed only with post-mortem histopathological study. The main pathological hallmarks of AD are the presence of senile plaques composed of amyloid β (Aβ), neurofibrillary tangles (NFTs) composed of the hyper-phosphorylated microtubule associate protein tau, and neuronal and synaptic loss. While all of these makers are considered hallmarks for AD pathology, synaptic loss best correlates with cognitive decline. However, familial AD occurs entirely due to mutations affecting APP processing that produces Aβ or aggregation capacity of Aβ. Thus, abnormal production and buildup of Aβ are typically considered the primary initiating factors in AD pathology. The exact molecular processes linking Aβ to the downstream pathology of AD and cognitive decline are still under investigation.

1.4 Amyloid Precursor Protein and Amyloid-β

The *APP* gene is located on chromosome 21 and consists of 18 exons. Alternative splicing of *APP* creates many isoforms, but the three primary isoforms are 695, 751, or 770 amino acids in length (U. C. Müller & Deller, 2017; Walsh et al., 2007). All of these isoforms are type 1 transmembrane proteins, with the 695 amino acid isoform as the most prevalent in neurons and the brain (Nalivaeva & Turner, 2013). Secretase proteins cleave APP in either the

amyloidogenic or non-amyloidogenic pathways, depending on which secretases are involved. For the non-amyloidogenic pathway α-secretases cleave APP at the extracellular domain and γ-secretase complex cleaves in the cell membrane domain, producing sAPPα, the amyloid precursor intercellular domain (AICD), and the P3 fragment (Chow et al., 2010). The amyloidogenic pathway creates Aβ peptides, which in turn aggregate and eventually form the senile plaques found in AD patients (Glenner & Wong, 1984). APP is cleaved by β-secretase and γ-secretase, into sAPPβ, the AICD, and the Aβ peptides (Chow et al., 2010; García-González et al., 2019). The Aβ peptides have a propensity to aggregate into oligomeric forms, which can be highly toxic (Haass & Selkoe, 2007). While the peptides can vary from 38-43 amino acids in length, the $Aβ_{1-42}$ and $Aβ_{1-40}$ species are the most associated with AD. $Aβ_{1-40}$ is the more common peptide under normal physiological conditions, but $Aβ_{1-42}$ has higher propensity to aggregate, having a disproportionate representation in senile plaques, and has been shown to be the more toxic peptide (Marina et al., 2003; Lennart Mucke & Selkoe, 2012; Murphy & Levine, 2010).

The normal physiological functions of APP and its derivatives are still not completely understood. Reviews of available evidence suggest that APP is involved in synapse formation, synaptic transport, CNS metal homeostasis, and hormone regulation (U. C. Müller & Deller, 2017; Nalivaeva & Turner, 2013; Tang, 2019). The sAPPα and sAPPβ fragments contain binding domains for heparin, copper, and other factors (U. C. Müller & Deller, 2017). These fragments also exhibit neuroprotective effects and are important for long-term potentiation (LTP) as well as neurite outgrowth, respectively (Chasseigneaux et al., 2011; Xiong et al., 2017). AICD functions as a transcription regulator for genes related to proteins implicated in Aβ degradation and Ca^{2+} homeostasis (K. Chang & Suh, 2010; Leissring et al., 2002; T. Müller et

al., 2008). The Aβ peptide itself exhibits antimicrobial properties (Brothers et al., 2018), which

may help protect the CNS. It also stimulates LTP and synaptic plasticity (Morley et al., 2010;

Puzzo et al., 2011), and exhibits neuroprotective effects such as limiting excitotoxicity at normal

physiological concentrations (Pearson & Peers, 2006; Soucek et al., 2003; Yu et al., 2006),

indicating a varied and robust role in the brain under normal physiological function.

As mentioned previously, amyloidogenic APP processing sufficiently explains the pathogenesis

of familial AD, which in turn strongly argues for Aβ as a key initiating factor in sporadic AD.

The genetic factors that cause familial AD are either mutations in *APP* itself or in γ-secretase

component genes *PSEN1* and *PSEN2* leading to increased Aβ production or higher ratios of the

more pathogenic $Aβ_{1-42}$ compared to other forms (Finckh et al., 2005; Johnston et al., 1994;

Mann et al., 1996; Mullan et al., 1992; Murrell et al., 1991). Additionally, duplication of *APP*

either directly or through an additional copy of chromosome 21 as seen in Down syndrome

significantly increase risk of AD and speed the onset of the disease (Lautarescu et al., 2017;

Sleegers et al., 2006; Wisniewski et al., 1985).

While Aβ plaques are considered a primary hallmark of AD pathology, they are found even in

cognitively normal patients and plaque load does not correlate strongly with the strength of

cognitive impairment (Dickson et al., 1992). On the other hand, in many animal models memory

impairment is seen before plaque accumulation (Götz et al., 2018). However, various Aβ

oligomers have been shown in animal models to disrupt cognition and memory, impair synaptic

plasticity, and inhibit synaptic transmitter release, as well as exhibiting toxicity in cell culture

(Cleary et al., 2005; He et al., 2019; Klein, 2013; Shankar et al., 2008). Aβ dimers have also

induce hyperphosphorylation of tau, which leads to aggregation into the NFTs seen in AD (Jin et

al., 2011). Clinically, in patients increasing Aβ oligomer levels in the CNS correlate with

advancing levels of cognitive decline, indicating a more direct role in impairing cognitive

function than plaques (Jongbloed et al., 2015; Tomic et al., 2009). However, the exact role of

Aβ peptides and plaques in AD pathogenesis remains unknown.

1.4.1 Tau Protein in AD Pathology

Along with the Aβ plaques, the other historical marker of AD is the presence of NFTs composed

of hyperphosphorylated tau protein. There are six isoforms of tau in humans, described by the

number of N-terminal inserts- from zero to two- and whether the protein has three or four

microtubule binding domains (Y. Wang & Mandelkow, 2016). The primary function of the

protein in the CNS is to stabilize axonal microtubules, though it is also implicated in protein

translation and memory formation regulation (C. Li & Götz, 2017; Y. Wang & Mandelkow,

2016). However, the protein is vulnerable to hyperphosphorylation on threonine and serine

residues by calcium-dependent kinases which causes dissociated from microtubules and

aggregation into insoluble filaments (Cowan et al., 2010; Martin et al., 2013; Y. Wang &

Mandelkow, 2016). These filaments further aggregate into the NFTs observed in AD and other

diseases (Wolfe, 2012). In AD patients, tau pathology correlates more strongly with degenerated

brain regions than Aβ, indicating that tau pathology may be more directly upstream of

neurodegenerative outcomes (C. Li & Götz, 2017; Wolfe, 2012).

In animal models, tau has been shown to induce neurodegeneration and impair neurogenesis

(Ballatore et al., 2007; Chatterjee et al., 2009; Komura et al., 2015; Wittmann et al., 2001). In

AD models, Aβ species are known to induce tau hyperphosphorylation (Götz et al., 2000; Jin et

al., 2011; Oddo et al., 2006), and in turn tau is a mediator of some aspects of Aβ induced

toxicity, including hippocampal LTP impairment (Chapuis et al., 2013; L. M. Ittner et al., 2010;

Roberson et al., 2007; Shipton et al., 2011). Interestingly, tau pathology can itself induce further tau pathology in a positive feedback loop. This can occur either by inducing neuronal excitation, likely by impairing glutamate clearance (Dabir et al., 2006; Hunsberger et al., 2015; J. W. Wu et al., 2016), or through a prion like mechanism where hyperphosphorylated tau causes normal tau to dissociate from microtubules and convert to pathological tau (Alonso et al., 1996; J. W. Wu et al., 2013). These mechanisms also allow the propagation of tau pathology along linked neuronal pathways (De Calignon et al., 2012; J. W. Wu et al., 2016). Impairment of glutamate clearance also been shown to strongly contribute to neuronal loss and cognitive deficits in AD models itself (Dabir et al., 2006; Masliah et al., 1996; Mookherjee et al., 2011), and rescue of glutamate transport can ameliorate these effects (Takahashi et al., 2015; Zumkehr et al., 2015).

1.4.2 Inflammation

While Aβ oligomer accumulation is viewed as the major upstream event in AD pathogenesis, there is increasing evidence for a critical role of inflammation response in the progression of AD- particularly in the sporadic cases that constitute the majority of patients (Stephenson et al., 2018; Xu et al., 2016). Specifically, genome risk studies identify multiple risk genes for sporadic AD that are expressed highly by the brain's resident macrophage cells, microglia (Hansen et al., 2018; Hemonnot et al., 2019; Stephenson et al., 2018). Microglia are capable of recognizing and digesting Aβ to clear it from the CNS under normal conditions (Cho et al., 2014; Y. Liu et al., 2005; Paresce et al., 1996). However, similar to the rest of the body chronic immune activation can exhibit negative effects in the CNS (Hansen et al., 2018; Stephenson et al., 2018). Relevant to AD pathology progression, chronic inflammation has been shown to mediate pathology in AD models. Microglia impact synaptic loss in Aβ pathology, and are required for amyloid induced production of radical oxygen species (Coraci, 2002; Hong, 2016).

While they are critical for Aβ clearance under normal circumstances, in the disease state microglia contribute to seeding amyloid aggregation (Venegas, 2017). Inflammation induced by LPS increases tau hyperphosphorylation (Lee, 2010) and removal of microglia inhibits tau propagation, indicating the importance of microglia in tau pathology (Asai et al., 2015). Studies using colony-stimulating factor 1 receptor inhibitors, shown to remove the great majority of microglia in the adult mouse brain (Elmore et al., 2014), show rescue of memory function and neuronal loss without altering amyloid pathology (Dagher et al., 2015; Spangenberg et al., 2016). Overall, these studies argue for a complex and critical role of microglia and inflammation in AD pathogenesis. Thus, other insults leading to neuroinflammation are likely to exacerbate AD pathology and risk.

1.5 Alzheimer's Disease and Particulate Matter

1.5.1 Particulate Matter: Sources and Composition

Exposure to unhealthy levels of polluted air is a worldwide problem, particularly in heavily urbanized areas in developing or developed countries where the ambient levels of air pollution can be over 10 times more concentrated than recommended health guidelines suggest (WHO, 2016). Major constituents of air pollution are PM, nitrogen oxide species (NOx), sulfur oxide species (SOx), carbon monoxide, ozone, hydrocarbons, volatile organic compounds (VOCs), metals, and other inorganic chemicals. While pollutants from natural sources, such as volcanic activities, wildfires, dust, and coastal aerosols, are difficult to reduce, those released by human activities can be more reasonably curbed if found to adversely impact health to a sufficient degree. The major sources of human contribution are traffic- and industrial-related combustion of fossil fuels, mining, agricultural activities, and burning fossil and biomass fuels for cooking and heating (Craig et al., 2008; Karagulian et al., 2015; Mazzei et al., 2008; Simoneit et al.,

2004). PM found in the atmosphere is either generated directly from these sources as primary

PM or a result of complex photochemical reactions of NOx, SOx, and ammonia released from

motor vehicles, industrial combustion, and agricultural activities, respectively, as secondary PM

(Valavandis et al., 2008). This gas-to-particle chemical conversion occurs within water droplets

and aerosols in the atmosphere and produces ammonium nitrate as a nucleation step for PM

formation. The ammonium nitrate core eventually grows and ages together with other

constituents to form mature PM (K. M. Zhang et al., 2005). In certain regions, ammonium

nitrate could take up over 50% of all chemical mass in PM (Kelly et al., 2013).

PM, also referred to as aerosol when present as a mixture in the air, includes a wide variety of

microscopic liquid or solid matter in the atmosphere. Particulate contaminants can include

biological elements such as pollen, bacteria, viruses, and spores, and suspended non-biological

solids such as dust and smoke. Exact composition varies considerably based on its size, location,

weather, the season, time of day, and a multitude of other factors. The major components of

airborne particulates worldwide are sulfates, nitrates, ammonium, chlorides, elemental and

organic carbon, biological materials, and minerals and dust (Harrison & Yin, 2000; Valavandis et

al., 2008). The US EPA primarily divides PM into an "ultrafine" designation for PM <100 nm

($PM_{0.1}$), a "fine" fraction of PM of diameter 2.5 microns or less ($PM_{2.5}$), and a "coarse" fraction

of PM between 2.5 and 10 microns (PM_{10}) (US EPA, 2004). While most components of PM can

be found in all size fractions, the smaller PM fractions generally contain higher amounts of toxic

metals, black carbon, gases, and other products of combustion while the coarse fraction contains

more organic debris, road dust, dust from mechanical processes, and other resuspended materials

(Geller et al., 2002; Sardar et al., 2005) (Table 1).

Table 1: Particulate Matter Size Fractions

Size Fraction	Designation	Diameter range (µm)	Major Constituents	Minor Constituents
Coarse PM	PM_{10}	2.5 - 10	Metals, inorganic ions	Organic matter
Fine PM	$PM_{2.5}$	0.1 - 2.5	Inorganic ions	Metals, organic matter
Ultra-Fine PM	$PM_{0.1}$	≤ 0.1	Organic matter	Metals, inorganic ions

Table 1 shows the three common size fractions of PM as designated by the US EPA. Major (>25%) and minor constituents (<25%) are estimated by particle constituent mass from USC studies (Geller et al., 2002; Sardar et al., 2005); note that composition of PM can vary considerably with time and location, and that this is only a general estimate of the components of each size fraction.

 The outlined size classifications are commonly used at least in part as the size and weight of the particles plays a major role in determining inhalability and particle deposition in the respiratory tract. While larger particles tend to deposit in and affect the upper respiratory tract, it is largely $PM_{2.5}$ and ultrafine PM that deposit in the lungs (Brauer et al., 2001; Churg & Brauer, 2000). Once in the lung, the soluble components of fine PM are taken in by cells in the respiratory system (Bermudez et al., 2004; A. K. Müller et al., 2004; Stearns et al., 2001) and enter the blood stream (Kreyling et al., 2002; Nemmar et al., 2002)(Fig. 1). PM uptake to the lung cells and blood may act as a shuttle for other chemicals on the surface of the PM to penetrate to these areas (Seaton et al., 1995). PM has additionally been shown to impair the function of macrophages in the lung (Lundborg et al., 2001). While making up only a small percent of total PM by weight, ultrafine PM accounts for a majority of the total particle number and available surface area of PM (M. Chang et al., 2001; S. Kim et al., 2001; K. M. Zhang et al., 2005). In part due to these traits, which increase reaction surface and ability to carry other agents, ultrafine

PM is generally considered the most toxic form of PM (S. Kim et al., 2001; Valavandis et al., 2008). The ultrafine fraction causes increased oxidative stress and mitochondrial damage over larger sizes in macrophages and epithelial cells (N. Li et al., 2003). Evidence suggests that the ultrafine PM can directly infiltrate to the brain through olfactory nerves, and potentially penetrate to the CNS via systemic uptake (Block & Calderón-Garcidueñas, 2009; González-Maciel et al., 2017). Once in the CNS, PM may lead to inflammation response and oxidative damage, similar to what is seen in macrophages and the lungs (Calderón-Garcidueñas, Solt, et al., 2008; Kleinman et al., 2008). Vehicle exhaust is one of the most significant human contributions to $PM_{2.5}$ and $PM_{0.1}$ levels (Schauer et al., 1996; Viana et al., 2008). As PM tends to aggregate and increase in size over time (Zhu et al., 2002), sources of $PM_{2.5}$ to which people are immediately exposed, such as vehicle exhaust in traffic, have a higher impact on human exposure than more distant sources.
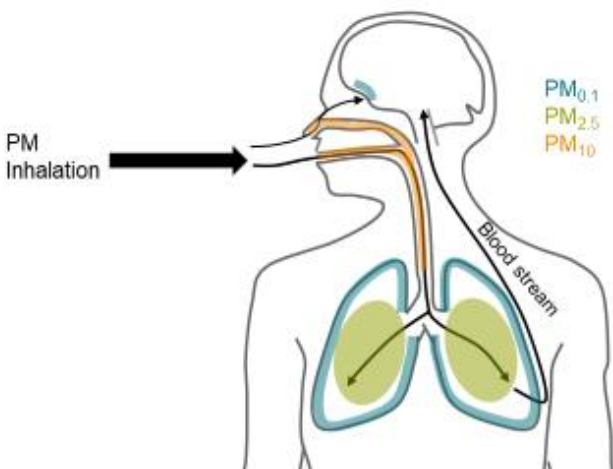
Figure 1: A depiction of primary deposition areas of particulate matter (PM) in the body and potential routes to affect the CNS. Larger particles (PM_{10}, orange) are trapped in the upper respiratory tract, while the fine ($PM_{2.5}$, green) and ultra-fine ($PM_{0.1}$, blue) fractions can penetrate deeply into the lung tissues. $PM_{0.1}$ deposit in the alveoli and can cross into the interstitium and blood, where they may cause systemic effects. $PM_{0.1}$ can also directly cross the olfactory epithelium into the CNS

In the U.S., it is estimated that over 43 million people live in areas where the air concentration of $PM_{2.5}$ exceeds the EPA's 24 hour ambient air quality standard limit of 35 μg/m^3, and 20 million people in the US live with exposure levels higher than the EPA long-term exposure standard of 12 μg/m^3 year round average (ALA, 2018). These areas include major metropolitan regions such as the Los Angeles basin, which had 2015 average $PM_{2.5}$ of 12.4 μg/m^3, with 24 hour averages up to 70 μg/m^3 (SCAQMD, 2016). Worldwide, the WHO estimates that 92% of the population lives in areas where WHO air quality guidelines (10 μg/m^3 year average, 25 μg/m^3 24 hour average) are not met (WHO, 2016). WHO attributed an estimated 3 million premature deaths to ambient air pollution in 2012. PM levels in developing countries are still rising (van Donkelaar et al., 2014), leaving many people at risk of being exposed to unhealthy levels. In highly polluted areas such as Delhi, India, and Xingtai, China, experienced annual average $PM_{2.5}$ level of over 120 μg/m^3. Together, these data indicate the importance of exploring and understanding of the health ramifications of PM exposure.

1.5.2 Epidemiological Studies of PM and General Air Pollution Neuronal Disease Risk

It has been well-documented that exposure to polluted air and particulates is associated with cardiopulmonary mortality and morbidity since major incidents during the 20th century, such as the 1952 London Great Smog event (Khafaie et al., 2016; Pope & Dockery, 2006). It is only in the 21st century that epidemiological studies have begun to uncover a correlation between air pollution and accelerated intellectual and cognitive decline, including AD.

The adverse effect of polluted air on cognition can be caused by exposure as early as *in utero*. Exposure to polycyclic aromatic hydrocarbons (PAHs), a component of the PM organic carbon fraction, during pregnancy correlates with reduced Bayley scale of infant development (BSID-II) scores in the children at 3 years old and reduced verbal and full IQ at 5 years (Perera et al., 2006,

2009), and decreased non-verbal IQ scores at 5 years of age and verbal IQ at 7 years of age (Edwards et al., 2010; Jedrychowski et al., 2015). These studies suggest that PAH exposure during development is associated with delayed impairment of performance during childhood, as in all cases negative cognitive effects were not observed at earlier time points.

In addition to PAH, traffic associated gases are also commonly associated with decreased cognitive ability of children if exposed *in utero* (Guxens et al., 2014; Lertxundi et al., 2015; Porta et al., 2016). NO_2 exposure, $PM_{2.5}$ exposure, and traffic intensity, but not PM_{10}, benzene, or reduced distance to roadways during pregnancy is correlated with reduced IQ performance in children (Lertxundi et al., 2015; Porta et al., 2016). A meta-analysis of six other European studies examining the effects of $PM_{2.5}$, PM_{10}, and NO_2 exposure during pregnancy on children 1 to 6 years old found the only significant association to be between NO_2 levels and psychomotor development deficit (Guxens et al., 2014). On the other hand, exposure to high levels of SO_2 and non-methane hydrocarbons, but not NO_2 and other pollutants, during 2nd or 3rd trimester pregnancy are found to be associated with reduced motor skills in infants at 6 and 18 months of age in Taiwan (C.-C. Lin et al., 2014). Overall, these studies show that exposures to certain constituents of polluted air during pregnancy likely exhibit adverse effect on cognitive performance of infants. However, the inconsistency between studies of whether a given active constituent causes impaired cognitive functions requires further investigation. Differences in study populations, the concentrations of pollutants seen in the studies, and times of exposure and endpoint testing may account for many the discrepancies. Another interesting possibility raised is that the overall mixture of air pollutants, depending on the constituents and concentrations, may elicit a complex and novel toxicity in the body.

Adverse effect of air pollution on cognition is not limited to *in utero* exposures. Increasing levels of NO_2 are associated with decreased gross motor skills at 5 years and memory span in 9 to 11 year old schoolchildren (Freire et al., 2010; van Kempen et al., 2012). Levels of black carbons are inversely associated with various intellectual performances including vocabulary, composite intelligence, and visual skills of learning and memory in children (Chiu et al., 2013; Suglia et al., 2008). Measuring performance of children living in areas with very high levels of air pollution, versus rural areas with relatively little pollution indicates that children in polluted areas are behind age normalized levels of multiple intelligence subscales, including full scale IQ and vocabulary, and have increased risk of poor psychomotor stability, motor coordination, and response time tests (Calderón-Garcidueñas et al., 2011; Calderón-Garcidueñas, Mora-Tiscareño, et al., 2008; S. Wang et al., 2009). Data using the Project Viva cohort in the U.S. indicated decreased non-verbal IQ in 8 year olds whose mothers lived in residences nearer to major roadways at the time of birth to child age 6 as compared to children of mothers who lived further from major roadways (Harris et al., 2015). These studies provide strong evidence that air pollution exposures, especially those related to PM levels such as black carbon and roadway distance, can negatively impact cognition during youth. The findings of reduced memory ability show that exposure to air pollutants can impact brain functions also affected by AD, and potentially has neurotoxic effect in the regions associated with those functions. Long term studies following from childhood to senescence are highly challenging, but it is interesting to consider that early life PM exposure extends its adverse effect in later life and triggers neurodegenerative diseases like AD.

Elderly adults who are exposed to polluted air also experience cognitive impairment. In China, Mexico and the U.S., elderly residents over 65 years old who live in areas with high air pollution

generally performed significantly worse on a mini-mental state examination (MMSE), one of common cognitive tests to assess dementia, than those living in cleaner areas (Sánchez-Rodríguez et al., 2006; Wellenius et al., 2012; Zeng et al., 2010). Black carbon, and $PM_{2.5}$ are particularly associated with poor performance on MMSE among the elderly (Gatto et al., 2014; Power et al., 2011). It is estimated that every 10 μg/m^3 annual average increase to black carbon exposure is equivalent to an extra two years of cognitive decline by aging (Power et al., 2011). A strong association between increased rate of errors in tests of working memory and orientation was observed in adults age 55 or older exposed to high levels of $PM_{2.5}$ (J. A. Ailshire & Clarke, 2014), and with reduced episodic memory as compared to the group with low $PM_{2.5}$ exposure (J. A. Ailshire & Crimmins, 2014; Younan et al., 2020). Elderly women exposed to high $PM_{2.5-10}$ or $PM_{2.5}$ levels for up to 14 years, as estimated by modeling of US EPA environmental data, have greater decline in global cognitive function than those in the lowest exposure level (Weuve et al., 2012). $PM_{2.5}$ exposure was also linked to increase in cognitive disability, as determined by the World Health Organization Disability Assessment Schedule, in a study of populations from lower income countries (H. Lin et al., 2017). Other population-based studies identify $PM_{2.5}$ and PM_{10} levels associating with reduced memory scores in multiple tests (J. Ailshire et al., 2017; Tonne et al., 2014). Interestingly, $PM_{2.5}$ and cognitive decline are much better correlated when neighborhood stressor factors, such as empty lots and abandoned buildings, are added (J. Ailshire et al., 2017). This study raises the important point that a combination of environmental factors may synergize in modulating the effects and must be considered when determining the impact of PM on cognition. It is also of note that a study focusing on younger adults, mean age approximately 37, found no significant association between PM_{10} exposure levels and reduced cognition (J.-C. Chen & Schwartz, 2009). This could indicate that PM exposure is a more

significant risk factor in vulnerable populations, such as the elderly or children, than it is in healthy adults, though additional research in this age group is required to be sure they are not vulnerable.

Recent works demonstrate a correlation between airborne particulate matter exposure and risk for neurological disease outcomes, including AD and vascular dementia (VaD). A European study cohort of elderly women (<75 years) in Germany reveals that living within 50 meters of a busy roadway and PM exposure are correlated with lower executive function, olfactory function, and reduced cognitive performance relevant to AD by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropsychological test battery (Ranft et al., 2009; Schikowski et al., 2015). A similar trend is observed in a Canadian cohort of ages 55-85, showing a significant adjusted hazard ratio (HR) for incident dementia at 1.07 for those living within 50 meters of the nearest major roadway compared to those living at a further distance (H. Chen, Kwong, Copes, Hystad, et al., 2017; H. Chen, Kwong, Copes, Tu, et al., 2017). $PM_{2.5}$ exposure specifically is significantly associated with increased overall mild cognitive impairment (MCI) incidence and incidence of amnestic MCI in Germany (Tzivian et al., 2015), as well as AD, Parkinson's disease, and VaD risk in the U.S. and Taiwan (Jung et al., 2015; Kioumourtzoglou et al., 2016; Y.-C. Wu et al., 2015). Together, these studies show consistent evidence that AD risk is increased with exposure to higher levels of PM. As VaD and general dementia are also shown to be linked to PM exposure, it remains an open question whether AD risk is elevated more than other forms of dementia.

Genetic predisposition may play an important role in air pollution dementia risk. The *APOE* gene remains the strongest known genetic risk factor, and a few studies have examined whether air pollutant exposure risk is modulated by *APOE* allele status. Elderly women living in areas

with high PM$_{2.5}$ concentrations, greater than the EPA recommended long term exposure limit of 12 µg/m^3, are found to have increased risk of dementia which is exacerbated by *APOE ε4* status, with increasing risk when exposed to high levels PM in carriers of one copy of the allele and the highest risk with two copies (Cacciottolo et al., 2017). Similarly, traffic pollutant exposure was linked to impaired visuospatial function, but only in *APOE ε4* allele carriers (Schikowski et al., 2015). In a younger population, children with *APOE ε4* carrier status and in higher air pollution areas showed greatly increased risk of cognitive impairment compared to either factor alone (Calderón-Garcidueñas et al., 2016). The ability of *APOE* status to modulate risk of dementia and impaired cognition strongly suggests a gene-environment interaction in determining the likelihood of developing dementia.

1.5.3   Animal Studies of the effects of PM in the CNS

Growing bodies of epidemiological studies have unveiled pathological link between PM exposure and AD related cognitive decline. Underlying cellular and molecular mechanisms are being investigated using *in vivo* and *in vitro* models to provide powerful insight into understanding the etiopathogenesis of AD. In relatively the same environmental conditions as humans, dogs living in Mexico City with high levels of ambient air pollution develop white matter lesions, damage to the blood-brain barrier, degenerating neurons, oxidative damage, glial activation and neuroinflammation, as well as diffused Aβ plaques and neurofibrillary tangles, two key pathological hallmarks of AD, while dogs living in rural and less polluted areas of Mexico do not (Calderon-Garciduenas et al., 2003; Calderón-Garcidueñas, Mora-Tiscareño, et al., 2008). These studies suggest that PM adversely impacts neuroanatomical and neuropathological changes in the brain, leading to the development of AD-like pathology via

multifactorial mechanisms, including neurodegeneration, altered glial cell levels, amyloid processing, and immune response. However, as in the similarly structured epidemiological studies, the lack of tight control on the exposure paradigm leaves considerable room to question which components of air pollution are critical in these processes.

In the laboratory environment and under controlled exposure settings, different rodent models can exhibit various degrees of neuropathological signs and cognitive decline following PM exposure. These variances may be in part due to different strain background, varying PM concentrations and constituents, or exposure paradigm differences; however, these *in vivo* studies still provide significant insights into the neurotoxic effects of PM exposure and may allow us to decipher underlying mechanisms linking to the development of dementia and AD. High levels of ultrafine PM for short durations (2-6 weeks) in mice elicit significant increases in pro-inflammatory cytokines interleukin-1α (IL-1α) and tumor necrosis factor-α (TNFα), glial responses, activation of NF-κB and AP-1 transcriptional factors in brain tissue, and glutathione redox imbalance (Campbell et al., 2005; Kleinman et al., 2008; Park et al., 2020). Increased brain inflammation, measured by IL-1α, IL-1β, TNFα, heme oxygenase-1 (HO-1), glial fibrillary acidic protein (GFAP), CD14, or CD68 mRNA, is also observed in C57BL/6 mice and Wistar rats exposed to nanoscale PM, ultra-fine PM, or $PM_{2.5}$ after 6 to 10 weeks of exposure (Cheng et al., 2016; Guerra et al., 2013; Morgan et al., 2011). Long term exposure (30-39 weeks) to concentrated $PM_{2.5}$ in young mice induces similar changes in inflammatory responses, as measured by TNFα, IL-10, IL-13, eotaxins, and HO1 mRNA or protein in the brain (Bhatt et al., 2015; Fonken et al., 2011). Ex vivo hippocampal slices from mice treated with PM had buildup of Aβ and NMDA receptor mediated neurotoxicity, and media taken from PM treated glial cells impaired neurite outgrowth (Morgan et al., 2011). These data strengthen the hypothesis that

immune activation may be a critical pathway for air pollutants to affect the CNS and establish a role for glial cells and oxidative damage in the brain in response to PM exposure.

Exposed mice develop multiple AD related pathologies and changes in the CNS. Long term PM exposure leads to a significant loss of dendritic spine density and dendrite length in the CA1 region of the hippocampus, which correlates with impaired cognitive outcomes (Fonken et al., 2011), and increases β-secretase (BACE) expression (Bhatt et al., 2015). Upregulation of BACE is suspected to promote amyloidogenic pathway of APP processing and increase the production of Aβ in the exposed mice. Exposure to very concentrated (1 mg/m^3) PM$_{2.5}$ or nickel nano-particles can increase the Aβ$_{42}$ and tau load in the CNS (S. H. Kim et al., 2012; Levesque et al., 2011), as did a lower level exposure to ultrafine PM (Park et al., 2020). Levels of hyperphosphorylated tau also increase in mice exposed to PM (Calderón-Garcidueñas et al., 2018, 2020). These findings bridge the association between chronic exposure to PM and inflammation and the development of AD-like neuropathology coupled with cognitive decline, demonstrating that PM and other inhaled exposures can lead to neuronal loss similar to what is seen in AD and other neurodegenerative diseases in animal models over longer exposures.

Although there is strong epidemiological evidence that developmental and early life PM exposures can have significant effects on cognitive function, there are currently limited animal model studies examining pre-natal and post-natal during development exposures. Rats exposed to PM$_{2.5}$ for 24 hours per day either during gestation, post-natal until testing and sacrifice at 5 months of age, or both, showed changes in oxidative damage indicated by MDA, SOD, and tGSH protein levels, and reduced short-term discriminative memory and habituation, but only with both exposures (Zanchi et al., 2010). This suggests early life exposure as a possible potentiating factor to effects of exposure later in life. Mice exposed to concentrated ambient

ultra-fine particulates either during post-natal days 4-7 and 10-13, post-natal days 56-60, or both showed deficiencies in the novel object recognition (NOR) and Fixed-interval schedule control performance tasks at 10 weeks of age (Allen et al., 2013; Allen, Liu, Pelkowski, et al., 2014; Allen, Liu, Weston, et al., 2014). Levels of multiple neurotransmitters, cytokines, and GFAP were also altered with exposures, though there was considerable variance in which exposure groups saw significant changes and large variance between sexes. As the tissues were not harvested until 9 months, it is difficult to directly compare the protein changes in the brain with behavior performance. Mice exposed to $PM_{2.5}$ during gestation exhibited decreased spatial memory in the cross maze task and higher anxiety as assessed by a light-dark box, as well as changes in COX2 and synaptophysin levels (Kulas et al., 2018). In all, these studies suggest that exposure during development causes both immediate and long-term molecular changes in the CNS that need to be further investigated.

Studies of PM exposure effects in AD model animals are limited but indicate PM's role in exacerbating AD pathology. 5xFAD mice with either the ε3 or ε4 allele of *APOE* exposed to ultra-fine ambient concentrated PM from the Los Angeles, CA area (Cacciottolo et al., 2017). Cerebral cortex sections showed significant increases in Aβ plaques, thioflavin S staining, and Aβ oligomers, but only with the ε4 allele. Decreases in the AMPA receptor subunit GluR1 and CA1 neurite density were also observed but were not dependent on *APOE* allele status. These findings demonstrate pathological changes that may explain the link between *APOE ε4* carrier status and increased risk for cognitive impairment seen in epidemiological studies (Cacciottolo et al., 2017; Calderón-Garcidueñas et al., 2016; Schikowski et al., 2015). 12 month old triple transgenic mice (3xTg-AD) exposed to ultrafine PM had decreased performance in spatial learning and memory tasks, but the decrease was not significantly different from what is

observed in wild type animals (Jew et al., 2019); Aβ pathology was not assessed. Ex vivo hippocampal tissue slices from 3xTg-AD mice cultured with PM show increased Aβ load, glial activation, and PARP-1 activation (Jang et al., 2018). Inhibition of PARP-1 reduced the effects, suggesting the involvement of DNA repair pathways in PM mediated AD exacerbation. Since exacerbation of Aβ load has also been shown in wild type mice as described above (Bhatt et al., 2015; S. H. Kim et al., 2012; Levesque et al., 2011), determining whether PM acts through any AD specific pathways in addition to inflammation response, ROS generation, and PARP-1 is of interest.

1.5.4   Human Studies of PM Effects on the Central Nervous System

Studies in humans primarily consist of studies in younger age group performed by comparing Mexico City residents to those living in less polluted areas of Mexico. MRI scans showed increased white-matter hyperintensity in Mexico City children and young adults compared to controls, and minor decreases in bilateral and parietal temporal lobe white matter volume, while necropsy tissue shows white matter lesions and disruption of the blood-brain barrier based on staining of tight junctions (Calderón-Garcidueñas et al., 2011; Calderón-Garcidueñas, Mora-Tiscareño, et al., 2008; Calderón-Garcidueñas, Solt, et al., 2008). Blood, urine, and necropsy tissue sample from children and adults showed increases in multiple cytokines, inflammatory response markers, and oxidative stress markers, including COX2, IL-1β, IL-12, NF-κB, CD14, TNFα, and down regulation of prion-related protein PrPC in multiple brain tissues for those living in Mexico City (Calderón-Garcidueñas et al., 2004; Calderón-Garcidueñas, Mora-Tiscareño, et al., 2008). Children and young adults living in the high pollution area exhibited greater amounts of $A\beta_{42}$ immunoreactivity, Aβ diffuse plaques, and hyperphosphorylated tau pre-tangles in the olfactory bulb, hippocampus, and cortical neurons than subjects in low

21

pollution areas (Calderón-Garcidueas, Kavanaugh, et al., 2012; Calderón-Garcidueñas et al., 2004, 2020; Calderón-Garcidueñas, Solt, et al., 2008). Critically, subjects homozygous for *APOE ε4* had more Aβ plaque pathology, Aβ$_{42}$ immunoreactivity, and hyperphosphorylated tau, and performed worse in olfaction tests, than those with the ε3 allele (Calderón-Garcidueas, Kavanaugh, et al., 2012; Calderón-Garcidueñas, Solt, et al., 2008). A separate group performed a MRI study of children ages 8-12 and found a correlation between higher levels of PM$_{2.5}$ elemental carbon and NO$_2$ with indications of slower maturation (Pujol et al., 2016). In older patients, MRI scans showed increasing similarity to patterns seen in AD patients with increasing PM$_{2.5}$ exposure (Younan et al., 2020). These studies indicate that the animal model and *in vitro* findings that PM exposure can affect amyloid processing and inflammation response are also present in humans and help validate further use of those models in further investigations. They also demonstrate overt neurotoxicity and vascular damage in the brain similar to what the group observed in canines, suggesting a potential mechanism for cognitive impairment due to pollution exposure in humans. However, as the majority of this work has been performed by one group in the same area and populations, additional studies with expanded scope may be required for extrapolation to the population at large. These findings further strengthen the epidemiological and animal model evidence that environmental exposure may particularly exacerbate pathology in tandem with AD risk genes, emphasizing the importance of studying these interactions.

Summarizing the current information on PM effects in the CNS, studies *in vitro* and *in vivo*, both animal model and human, show PM exposure involvement in inflammatory pathways, oxidative stress, and amyloidogenesis, as well as negative effect on cognition and behaviors in animals, providing potential mechanisms by which PM exposure can increase AD risk (Fig. 2). Additional research is required to determine which of these pathways, if any, are the major

factors in increasing AD risk, and whether the risk association between PM exposure and AD is specific to AD, or largely caused by general insult.
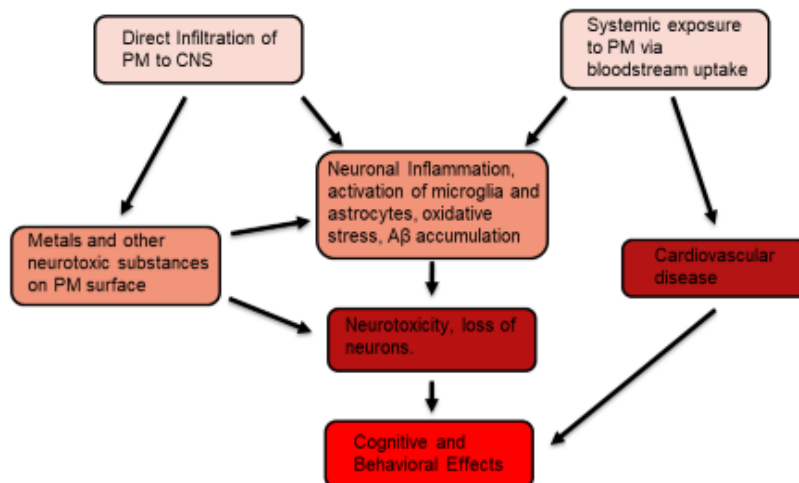
Figure 2: Proposed pathways by which PM exposure leads to neurotoxicity and cognitive deficits. Direct infiltration of PM into the brain can provide a pathway for metals and other neurotoxic chemicals to accumulate in neural tissues, and potentially provide a reactive surface. Systemic effects from PM infiltrating the blood via the alveoli include cardiovascular disease, which can lead to impaired cognition and promote AD pathology. Both pathways potentially contribute to the inflammatory, glial, and amyloid pathology responses observed in animal models and human studies. The cascade from these responses to neurotoxicity and cognitive loss is well documented, and consistent with results showing neuronal toxicity and behavioral effects observed with PM exposure.

1.6 Hypothesis

Exposure to the ultrafine fraction of airborne particulate matter exacerbates memory loss by increasing inflammation and Aβ pathology build up in the humanized APP animal model of AD.

1.7 Dissertation Contribution

❖ Adult and *in utero* exposure to ultrafine particulate matter impairs memory in adult wild-type and $App^{NL-G-F/+}$ mice.

❖ UF PM exposure at adult age in mice:

➢ Increases amyloid plaque burden, but only after plaque pathology onset in the AD model

➢ Does not increase astrocyte or microglia activation

➢ Decreases total protein levels of the astrocytic glutamate transporter GLT-1 in both wild-type and the AD model

➢ Memory impairment in the adult AD model due to UF PM exposure occurs without changes in glia activation and before effects on amyloid pathology.

➢ Glutamate transport disruption is implicated as a potential mechanism

❖ UF PM exposure *in utero* effects in adult mice:

➢ Increases astrocyte response when paired with the $App^{NL-G-F/+}$ genotype

➢ Increases Iba1+/CD68+ microglia only in the wild-type

➢ Increases amyloid plaque build up in the AD model

➢ Early life exposure to UF PM can impact AD like pathology at later life in mice

❖ In a *Drosophila* tauopathy model:

➢ increase or loss of GLT-1 homolog dEaat1 directionally modulates lifespan loss and geotaxic response.

➢ Direct neuronal excitation increases tau propagation.

1.8 Conclusion

Exposure to airborne ultra-fine particulates exacerbates memory loss and AD like pathology in $App^{NL-G-F/+}$-KI mouse model of AD. However, adult and *in utero* exposure may impact adult neuropathology through different pathways. Glutamate transport may be impacted, which suggests a connection to tau pathology, requiring further study.

Chapter 2: Exposure to ultra-fine particulate matter impairs memory function and exacerbates amyloid pathology in the *App^{NL-G-F}* knock-in mouse model

2.1 Abstract:

Exposure to traffic-related air pollution consisting of particulate matter (PM), nitrogen oxides, and volatile organic compounds, is associated with cognitive decline and memory impairment in wide spectrum of age groups. Among elderly, growing evidence suggests that PM contributes to accelerated onset of Alzheimer's disease (AD) and other forms of dementia. Here, we examined the impact of PM exposure on cognition and neuropathology in wild-type mice and a knock-in mouse model of AD (*App$^{NL-G-F/+}$*-KI). Three-month exposure to concentrated ultrafine PM from ambient air resulted in a marked impairment of memory tasks in young, pre-pathological *App$^{NL-G-F/+}$*-KI mice without measurable changes in amyloid-β (Aβ) pathology, synaptic degeneration, and neuroinflammation. The same duration of exposure in older mice, both wild-type and *App$^{NL-G-F/+}$*-KI, showed similar decline in memory tasks without overt changes in synaptic proteins or inflammation. However, Aβ plaque burden was significantly increased in the *App$^{NL-G-F/+}$*-KI mice exposed to ultrafine PM. Both age groups also exhibit a decrease in the astrocytic glutamate transporter, which may suggest future experiments to determine the mechanisms behind the reduction in memory performance with ultrafine PM exposure.

2.2 Introduction:

Generation and dispersion of fine and ultrafine particulate matter (PM) from automobiles and other anthropogenic activities constitutes a major part of air pollution (Karagulian et al., 2015; Mazzei et al., 2008), which in turn contributed substantially to the global public health concern and disease burden (Cohen et al., 2017). While exposure to PM and other toxic constituents in

polluted air are well known to increase morbidity and mortality from cardiovascular and cardiopulmonary diseases (Khafaie et al., 2016; Pope & Dockery, 2006), growing evidence from multiple epidemiological studies worldwide unveils a strong association between exposure to air pollution and accelerated cognitive decline and memory impairment across a wide spectrum of ages, from infants to the elderly (Freire et al., 2010; Guxens et al., 2014; Jedrychowski et al., 2015; X. Zhang et al., 2018). Among elderly populations, exposure to traffic-related pollution, which consists primarily of fine and ultrafine PM, nitrous oxides, carbon monoxide and dioxide, hydrocarbons, and volatile organic compounds (Westerdahl et al., 2009; Zavala et al., 2013), significantly increases the risk of dementia including Alzheimer's disease (AD) and other neurological disorders (Cacciottolo et al., 2017; H. Chen, Kwong, Copes, Hystad, et al., 2017; Jung et al., 2015; Oudin et al., 2016). However, the precise neurotoxic mechanisms of PM and other constituents leading to cognitive decline and potential neurodegeneration remain largely unknown.

In animal models, ultrafine PM (UF PM, aerodynamic diameter ≤ 0.1 μm) can infiltrate the central nervous system (CNS) via the olfactory epithelium (Block & Calderón-Garcidueñas, 2009; González-Maciel et al., 2017), and its chronic exposure recapitulates certain aspects of cognitive decline, synaptic loss, and/or buildup of amyloid-β (Aβ) species (Bhatt et al., 2015; Cacciottolo et al., 2017; Durga et al., 2015; Fonken et al., 2011). These studies suggest that PM perturbs inflammatory homeostasis by increasing levels of the inflammatory cytokines, such as interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α) (Fonken et al., 2011; Guerra et al., 2013). Total Aβ load (Bhatt et al., 2015; Cacciottolo et al., 2017) and pathogenic Aβ$_{42}$ (Durga et al., 2015) are also increased due to PM exposure, suggesting a possible connection between PM and AD. Age-related inflammatory dyshomeostasis and perturbed microglia

functions associated with increased cytokine levels- similar to what is seen with PM exposure-have been linked to pathological buildup of Aβ and loss of synapses and neuronal integrity (Heppner et al., 2015; W. Y. Wang et al., 2015). It remains unclear if the pathways leading to Aβ buildup and increased inflammatory cytokines operate independently in relation to PM exposure. Here, we hypothesized that chronic exposure to concentrated fine and ultrafine PM from ambient air accelerates cognitive decline through aberrant neuroinflammation and buildup of AD-like neuropathology in the brain. We tested this hypothesis using a recently generated *App* knock-in mouse model of AD (App^{NL-G-F}-KI) (Saito et al., 2014). We examined the effect of PM exposure in young, pre-pathological, mice as well as in older mice in order to determine whether early-life exposure substantially accelerates age-related pathological changes in the brain of these mice.

2.3 Methods:

2.3.1 Animals

All experiments were performed in accordance with the Institutional Animal Care and Use Committee at University of California. Mice were housed on a 12 hour light-dark cycle with feed and water ad libitum. Humanized APP with the Swedish, Arctic, and Iberian mutations (App^{NL-G-F}-KI) mice in the C57BL/6 background (Saito et al., 2014) were obtained from the RIKEN Institute (Japan) and maintained as heterozygous $App^{NL-G-F/+}$-KI by crossing with C57BL/6J mice. $App^{NL-G-F/+}$-KI animals were either 3 or 9 months of age at the start of exposure start and animal were sacrificed at 6 or 12 months of age, respectively. C57BL/6J male and female mice were obtained from Jackson Laboratory (Bar Harbor, ME). In total 60 $App^{NL-G-F/+}$-KI mice and 36 C57BL/6J mice were used in this experiment. 24 $APP^{NL-G-F/+}$-KI animals, 12 male and 12 female, were used at the 6 month time point. 20 $App^{NL-G-F/+}$-KI mice and 20

C57BL/6J mice, divided evenly at 10 male and 10 female mice per group, were used for the 12 month time point.

2.3.2 Exposure Paradigm

Ambient particles in the area around the UC Irvine area in Orange County, California with particle diameters smaller than 180 nm (quasi-ultra-fine particles) were concentrated using a versatile aerosol concentration and enrichment system (VACES) as previously described (S. Kim et al., 2001). The VACES consists of size selective inlets, saturator/chiller modules that supersaturate the aerosol with water vapor causing ultrafine particles to grow to a size that can be inertially separated using a virtual impactor, and a diffusion drier module that removes excess water vapor and returns the aerosol to a size distribution similar to the ambient air. The system can enrich the concentration of particles in the 0.03 - 2.0 µm size range by a factor of up to of 30x ambient, depending on output flow rate. The VACES system is located adjacent to a major roadway in Irvine, CA, and exposure occurs over morning commute hours to emphasize motor vehicle associated PM. Starting at 3 or 9 months of age, $App^{NL-G-F/+}$-KI mice were exposed to either air filtered to remove particulate matter or concentrated UF PM. Animals were exposed 4 days per week (Tuesday – Friday), for 5 hours per day, for 12 weeks, from 07:30-12:30 local time, which captured the period of maximum PM concentration during the day. Our concentrated PM exposure regimen was still within the range of environmentally-relevant concentrations in those highly polluted areas (Moreno et al., 2008). The 4 days/wk exposure paradigm reflects typical air pollution episodes (Kleinman et al. 2005, Kleinman et al. 2007). Animals were sacrificed immediately following exposure end for tissue collection. Particulate mass and particle counts were obtained using a DustTrak aerosol monitor with DustTrak Pro

software (TSI) and a Condensation Particle Counter with Aerosol Instrument Manager software (TSI).

2.3.3 Cognitive Assessments

Animals in the were tested in Object Location Memory (OLM) and Object Recognition Memory (ORM) tasks. OLM was performed 3 weeks from exposure end. ORM was run following a 1 week break period after OLM testing. These tasks were performed based on a previously described protocol (Vogel-Ciernia & Wood, 2014). For both tests, animals were habituated to the test arena for 6 days, 5 minutes per day, and then exposed to two identical objects for 10 minutes for training. Different base objects were used for ORM and OLM acquisition. Testing occurred 24 hours following training. In the OLM task one of the two objects was moved to a new location, while for ORM one of the objects was replaced with a novel object. Replaced or moved objects alternated between mice. Mice were allowed to explore during the test for 5 minutes. Both test and training exploration were recorded, with the video used to score animal performance. Total time spent exploring each object- determined as time with the animal's nose within 1 cm of the object and pointing directly at the object- was recorded. Time exploring each of the two objects was summed to obtain total exploration time. Animals showing a strong preference for exploring one object over the other during the acquisition phase were removed from the final analysis pool. The discrimination index was calculated as the difference between time spent exploring the novel object or location and time spent exploring the familiar object or location expressed as a percentage of the total time spent exploring during the test phase.

2.3.4 Protein Extraction and Western Blot Analysis

Half brain cortical tissue and hippocampi were homogenized in T-PER buffer with protease and phosphatase inhibitor cocktails (Thermo Fisher). Protein extract was then centrifuged at 100,000

x g for 1 hour at 4°C and the supernatant was taken as the detergent soluble fraction. The pellet was resuspended in 88% formic acid and centrifuged again at 100,000 x g for 1 hour at 4°C and the supernatant from this step was taken as the formic acid soluble fraction. For the vascular enriched protein samples, half brains were homogenized in sucrose buffer (0.32M sucrose, 3mM HEPES, Fisher) using a glass Dounce homogenizer. Samples were centrifuged at 1,000 x g for 10 minutes at 4°C. This process was repeated for the resultant pellet. The pellet was then re-homogenized and centrifuged at 100 x g for 30 seconds with the supernatant kept, and this process repeated once. The supernatant fractions were pooled and spun for 2 minutes at 200 x g, and this final pellet was resuspended in 0.1% BSA (ThermoFisher) as the vascular enriched protein fraction. Protein concentration was determined by the Bradford protein assay. Protein samples were run on Bio-Rad Mini-PROTEAN® TGX™ gels (Bio-Rad) at 150V and transferred to Immobilon®-FL PVDF membranes (Millipore). After 1 hour blocking in Li-Cor Odyssey® Blocking Buffer in TBS (Li-Cor Biosciences), membranes were immunoblotted with the following antibodies overnight at 4°C: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1,000, Santa Cruz Biotechnology), tubulin (1:25,000 Abcam), post synaptic density protein 95 kDa (PSD95, 1:1,000, Cell Signaling Technology), synaptophysin (1:1,000, Cell Signaling Technology), glial fibrillary acidic protein (GFAP, 1:1000, DAKO), Iba1 (1:1,000, Abcam), GLT-1 (1:500, a gift from Dr. Jeffery Rothstein at Johns Hopkins University), amyloid precursor protein, c-terminal (751-770) (CT-20, 1:1000, EMDMillipore), CD31 (1:1,000, Abcam), or Claudin-5 (1:500, Thermo Fisher). Membranes were then washed, incubated for 1 hour at room temperature with secondary antibodies Goat anti Rabbit or Goat anti Mouse IRDye® 680 and 800 (1:20000 Li-Cir Biosciences), washed again and read. Blots were read using the Li-Cor Odyssey system and Image Studio software version 5 (Li-Cor Biosciences) to obtain band signal intensity.

Signal is expressed relative to tubulin or GAPDH levels, which were used for protein loading control, before statistical analysis. GLT-1 levels were expressed relative to GFAP levels to account for potential changes due to astrocyte activation or loss.

2.3.5 Immunofluorescent Staining

Frozen brain hemispheres sectioned into 40μm slices coronally using a microtome and stored in phosphate buffered saline with 0.05% sodium azide. Sections were mounted on standard glass microscope slides (Fisher) before staining. For antibody staining sections were permeabilized with 0.1% triton-x 100 in tris buffered saline (TBS) for 15 minutes and blocked with 3% bovine serum albumin (Fisher), 5% normal goat serum (Vector), and 0.1% triton-x 100 in TBS for one hour. For Aβ plaque staining sections were treated with 88% formic acid for 7 minutes before other treatments. After pre-treatment and blocking, the sections were incubated with primary antibodies against ZO-1 (1:100, Thermo Fisher), PSD95 (1:1,000, Cell Signaling Technology), synaptophysin (1:1,000, Cell Signaling Technology), GFAP (1:1000, DAKO), Iba1 (1:1,000, Abcam), CD68 (1:1000, Bio-Rad) Lectin (1:500, Sigma Aldrich) or anti amyloid 82E1 (1:1000, Immuno-Biological Laboratories) overnight at 4°C. Sections were washed with TBS and treated for 1 hour at room temperature the following day in 3% BSA, 5% normal goat serum, and 0.1% triton-x 100 in TBS with secondary antibodies conjugated with Alexa Fluor 488, 555, or 633 (Fisher). For Thioflavin S staining sectioned were rehydrated with ethanol at 100%, 95%, 70%, and 50% and then treated with Thioflavin S (Sigma) in 50% ethanol for 10 minutes. Slides were mounted with Fluoromount-G (Fisher). Images were taken with either a Leica TCS SPE confocal microscope for Aβ plaque staining and GFAP and IBA1 staining. Plaque burden was assessed as the percent of total measured area occupied by amyloid beta plaques from the cortex

and hippocampus CA1 region. Vascular width was determined by the average of 5 measurements across the vessel.   ImageJ version 1.52 was used to analyze images.

2.3.6 Real-Time Polymerase Chain Reaction

RNA was isolated from *App^{NL-G-F/+}*-KI mouse hippocampal and cortical brain tissue in TRI Reagent and extracted using the Direct-Zol RNA MiniPrep kit (ZYMO Research Corp) used according to manufacturer instructions. RNA concentration in the extract was quantified using a NanoDrop Lite (ThermoFisher). 1 μg of total RNA was used in a single cycle reverse transcriptase reaction of 5 minutes at 25°C, 20 minutes at 46°C, 1 minute at 95°C to make cDNA using iScript reaction mix and reverse transcriptase (Bio-Rad). 2 μL of cDNA were used per reaction using iTaq Universal SYBR Green Supermix (Bio-Rad) for detection of *Il1β*, *Il6*, and *App*. Expression levels were normalized with *Gapdh* mRNA levels. Primer sequences are as follows: *Il1β* forward 5'- TGGACCTTCCAGGATGAGGACA-3' reverse 5'-GTTCATCTCGGAGCCTGTAGTG-3' (Origene NM_008361), *Il6* forward 5'-TACCACTTCACAAGTCGGAGGC-3' reverse 5'-CTGCAAGTGCATCATCGTTGTTC-3' (Origene NM_031168), *Gapdh* forward 5'-AACTTTGGCATTGTGGAAGG-3' reverse 5'-ACACATTGGGGGTAGGAACA-3' (Origene NM_010277), *App* 5'-TCCGTGTGATCTACGAGCGCAT-3' reverse 5'-GCCAAGACATCGTCGGAGTAGT-3' (Origene NM_007471). The PCR cycle parameters are listed: denaturing step (95°C for 15 seconds), annealing step (60°C for 60 seconds), and extension step (72°C for 30 seconds). Bio-Rad CFX Manager software version 3.1 was used to determine Cq values. ΔCq values were determined as determined by the difference between the gene of interest Cq and *Gapdh* Cq. ΔCq values between filtered air exposed and UF PM exposed animals were compared to obtain relative expression values, with the filtered air group set to 1.

2.3.6 Aβ Quantitation

Detergent soluble and formic acid soluble $Aβ_{1-40}$ and $Aβ_{1-42}$ levels were measured using the V-PLEX Aβ Peptide Panel with 6E10 capture antibody used as recommended by the manufacturer and read on a MESO QuickPlex SQ 120 (Meso Scale Discovery) for the 6 month group. For the 9 and 12 month groups Aβ levels were quantified using Human βAmyloid (1-40) ELISA kit and Human βAmyloid (1-42) ELISA kit (Wako). Protein sample extracts were obtained and quantified as described under Protein Extraction and Western Blots. 100 μL of protein was loaded per well for each sample. Bradford protein assay concentration readings were used to adjust for total protein loaded per sample.

2.3.7 Statistical Analysis

Immunoblots were quantified using Image Studio Software version 5. Immunofluorescent images were quantified using ImageJ version 1.52. All other data were analyzed using Microsoft Excel (Microsoft Office 365 ProPlus) or Prism version 3 (GraphPad Software). Statistics were carried out using unpaired t-test or two-way analysis of variance with Tukey's post hoc test. p values ≤ 0.05 were considered significant. Sex differences were generally not observed, and data are presented as mixed groups.

2.4 Results:

Initial experiments were performed in the 3 month old $App^{NL-G-F/+}$-KI exposed to concentrated UF PM for 3 months. To test our hypothesis, we examined cognitive decline with the ORM and OLM tasks and assessed synaptic density, glial neuroinflammation, and Aβ levels. As all of these have previously been linked to both PM exposure (Bhatt et al., 2015; Cacciottolo et al., 2017; Kleinman et al., 2008; Morgan et al., 2011) and AD (Cleary et al., 2005; Shankar et al.,

2008; Stephenson et al., 2018), they are likely pathways to connect UF PM exposure to AD. At 6 months the $App^{NL-G-F/+}$-KI model is at a pre to early stage in Aβ pathology (Saito et al., 2014), ideally making determination of acceleration of Aβ pathology clearer compared to animals with already advanced pathology as ascertained by Cacciottolo et al (Cacciottolo et al., 2017).

2.4.1 Concentration of ambient particulates

Quasi-ultrafine PM was concentrated 7-fold by particle count and 5-fold by weight over the ambient levels over the course of the experiment (Fig. 1A, B). Average particulate concentration during the experiment for the UF PM exposed animals was 131 μg/m^3, which was still environmentally relevant to highly polluted areas, such as Mexico City (Moreno et al., 2008). In addition, considering human heterogeneity of sensitivity to exposure, animal-to-human extrapolation, and the differences in exposure durations in humans (lifetime) and animal models (less than a lifetime), exposing mice to higher concentrations than average human exposure is still translatable (Rees & Hattis, 2004).
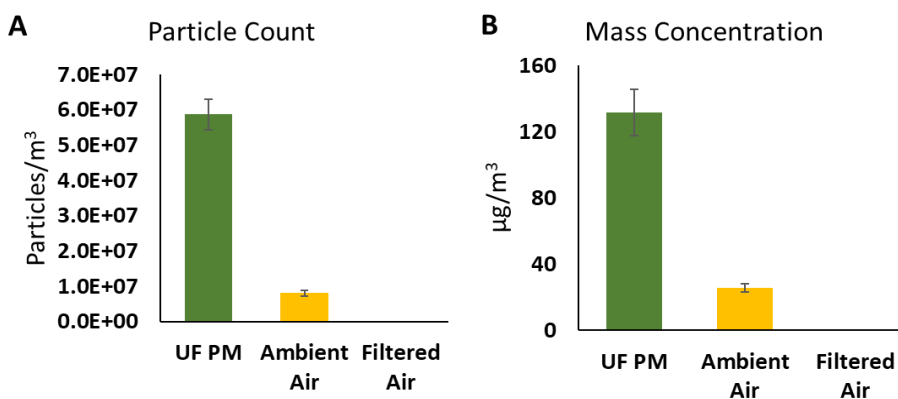
Figure 1: Exposure particle concentration.
(A) Average particle count per cubic meter during the exposure period of $App^{NL-G-F/+}$-KI mice. (B) Average particle mass concentration during exposure for concentrated quasi-ultrafine PM (UF PM), total ambient air, and filtered air using the VACES system. Bars represent mean values, with error bars expressed as standard error of the mean.

2.4.2 UF PM exposure impairs cognition in young $App^{NL-G-F/+}$-KI mice

Beginning after week 9 of exposure, 6 month old $App^{NL-G-F/+}$-KI mice were tested with the OLM and ORM behavior tasks (Vogel-Ciernia & Wood, 2014). In the younger animals PM-exposed $App^{NL-G-F/+}$-KI mice significantly performed worse in both tasks as compared to $App^{NL-G-F/+}$-KI mice exposed to filtered air (Fig. 2). The discrimination index was 20.3% less in OLM ($p<0.05$) (Fig. 2A) and 8.9% less in ORM ($p<0.05$) (Fig. 2B).
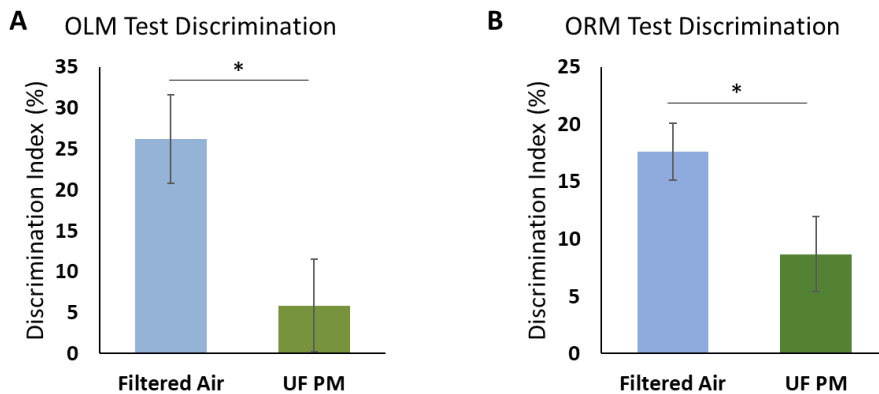
Figure 2: UF PM exposure reduces memory task performance in young $APP^{NL-G-F/+}$-KI mice.
Object location memory (OLM) and object recognition memory (ORM) task results for $App^{NL-G-F/+}$-KI mice and synaptic marker quantification. Discrimination index is the difference between time spent exploring the novel object or object in novel place and the familiar as a percentage of the total time. (A) Discrimination index for OLM test. Quasi ultrafine particulate matter (UF PM) exposed mice exposed mice showed significantly decreased ability to discriminate the novel object location compared to those exposed to filtered air. (B) Discrimination index for ORM. UF PM exposed mice showed decreased ability to discriminate the novel object compared to animals exposed to filtered air. Bars represent mean values, with error bars expressed as standard error of the mean (N =10 animals per group). * denotes p-value < 0.05.

After sacrifice at 6 months of age, cortical and hippocampal tissues were analyzed for synaptic markers to determine if synaptic loss due to UF PM exposure could explain the observed memory performance reductions. Despite the significant decrease in memory performance levels of the synaptic proteins PSD95 or synaptophysin did not vary with PM exposure status, indicating that the observed cognitive loss is not resultant from gross synaptic loss (Fig. 3). Neither protein varied due to UF PM exposure using immunofluorescent quantification of the cortex and CA1 region of the hippocampus (Fig. 3A, 3B) or by western blot protein quantification of whole cortex or hippocampus homogenate (Fig. 3C, 3D).
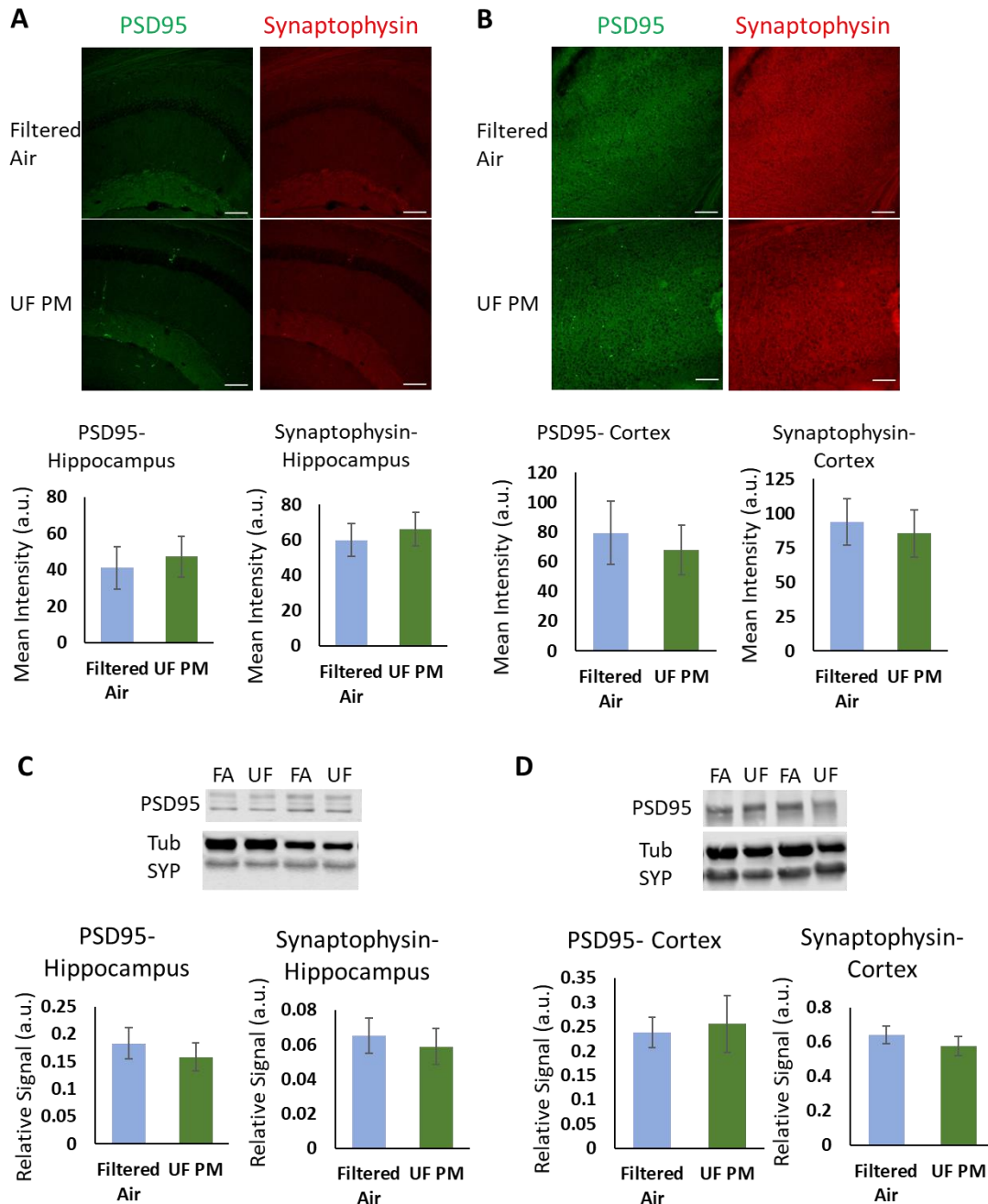
Figure 3: UF PM exposure does not induce changes in synaptic markers in young $APP^{NL-G-F/+}$-KI mice. Quantification of synaptic markers PSD95 and synaptophysin by immunofluorescence and western blot. (A) Representative images of PSD95 (green) and synaptophysin (red) immunofluorescent staining and fluorescent intensity quantification for the CA1 region of the hippocampus. (B) Representative images of PSD95 (green) and synaptophysin (red) immunofluorescent staining taken from the parietal and somatosensory cortex and fluorescent intensity quantification from the whole cortex. (C) Representative western blot of synaptophysin and PSD95 from hippocampal tissue samples with band intensity relative to tubulin. FA- Filtered Air, UF- UF PM, Tub- Tubulin. (D) Representative western blot of synaptophysin and PSD95 from cortical tissue with band intensity relative to tubulin. Bars represent mean values, with error bars expressed as standard error of the mean (N = 8 animals per group for immunostaining and N = 10 animals per group for immunoblot. Scale bars = 100 μm). * denotes p value < 0.05.

2.4.3 UF PM exposure does not affect Amyloid β burden or glial inflammation in young $App^{NL-G-F/+}$-KI mice

Heterozygous $App^{NL-G-F/+}$-KI mice at 6 months do not develop extensive plaque pathology (Saito et al., 2014), and exposure to concentrated PM did not significantly accelerate the buildup of plaques or Aβ peptides (Fig. 4). Only a few Aβ plaques were present in the cortex (Fig. 4A), and nearly no plaques were observed in the hippocampus (Fig. 4B). Virtually no dense core plaques were detected in either group by Thioflavin S staining (data not shown). The steady-state levels of full-length APP protein did not significantly increase with exposure, and App mRNA levels in the cortex and hippocampus were unaffected by the PM exposure (Fig. 4C, 4D). Similarly, no significant difference in soluble $Aβ_{42}$ peptide levels was detected between exposed and unexposed animals (Fig. 4E); $Aβ_{40}$ peptides and insoluble $Aβ_{42}$ peptides were not detectable (data not shown).

As previous studies in non-Alzheimer's model animals have shown altered central nervous system (CNS) inflammation with PM exposure (Allen, Liu, Pelkowski, et al., 2014; Campbell et al., 2005; Guerra et al., 2013; Kleinman et al., 2008), we also investigated whether UF PM exposure increased markers of inflammation in the CNS in the current model (Fig. 5). No clear upregulation of either Iba1 positive microglia or GFAP positive astrocytes was evident with exposure to PM in either the cortex of hippocampus (Fig. 5A, 5B). mRNA levels of the cytokines interleukin-6 (*Il6*) and interleukin 1β (*Il1β*) similarly did not significantly vary with PM exposure status (Fig 5C).
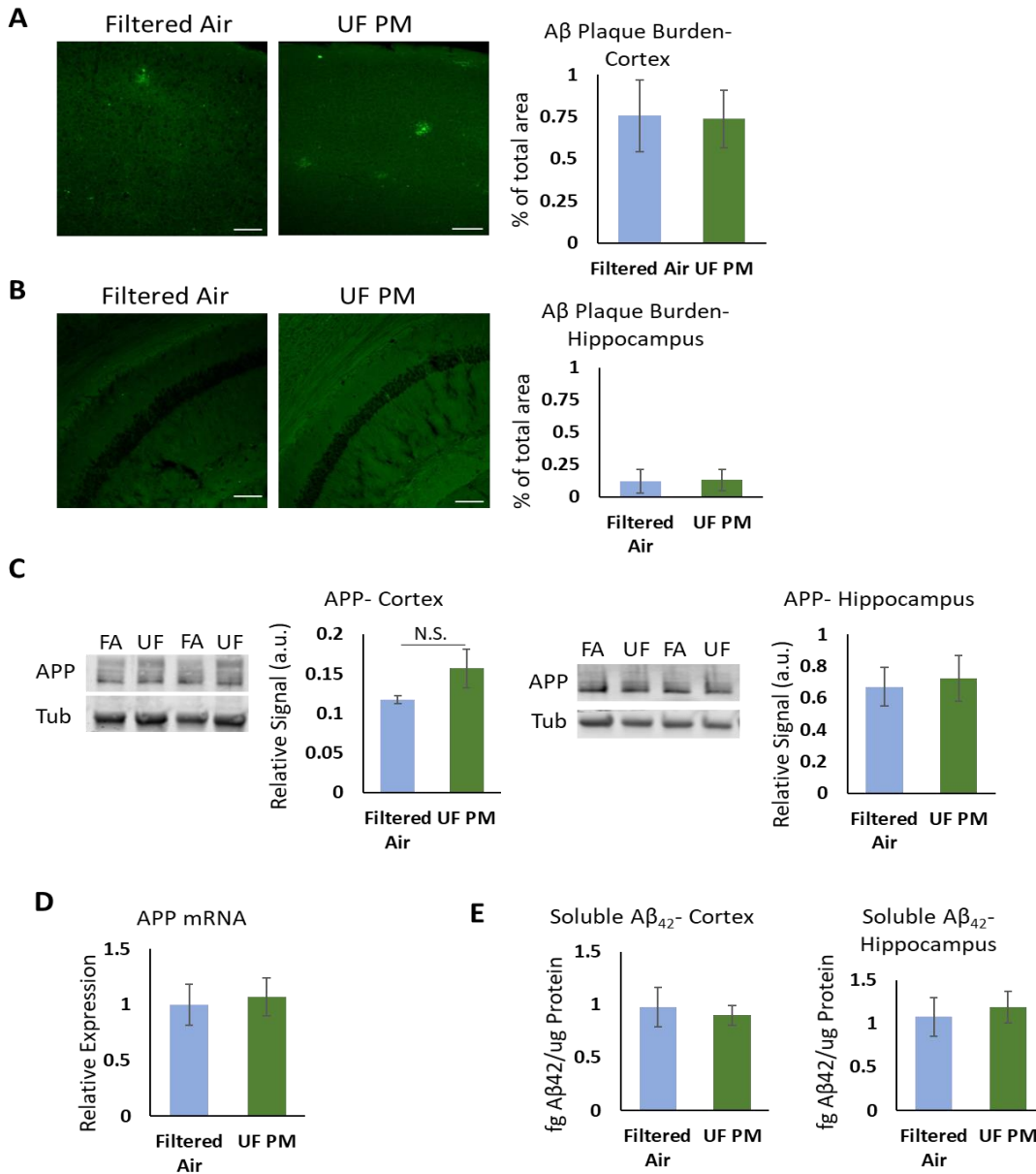
Figure 4: Aβ deposition and protein load is unaffected by UF PM exposure in the 6 month old $App^{NL-G-F/+}$-KI mouse model

Brain sections were stained with 82E1 antibody to detect Aβ plaques in $App^{NL-G-F/+}$-KI mice. (A) Representative images of cortical Aβ plaque burden from the parietal cortex in $App^{NL-G-F/+}$-KI mice exposed to filtered air or UF PM (left), and quantification of plaque burden expressed as percentage of the total area measured occupied by plaques (right). (B) Representative images of hippocampal Aβ plaque burden in $App^{NL-G-F/+}$-KI mice exposed to filtered air or UF PM from the hippocampal CA1 region (left), and quantification of plaque burden expressed as percentage of the total area measured occupied by plaques (right). In neither region was total plaque load increased. (C) Steady-state levels of full-length APP in tissue homogenates extracted from cortical and hippocampal tissues were not significantly affected by the PM exposure. FA- Filtered Air, UF- UF PM, Tub- Tubulin. (D) mRNA levels of *App* normalized to *Gapdh* mRNA. *App* mRNA levels from cortical tissue were also unaffected by the exposure. (E) No differences in soluble $A\beta_{42}$ levels were found in the brain. Bars represent mean values, with error bars expressed as standard error of the mean (N = 8 animals per group for immunofluorescent staining and quantification, and N = 6 animals per group for immunoblot, V-PLEX, and qPCR. Scale bars = 100 μm).
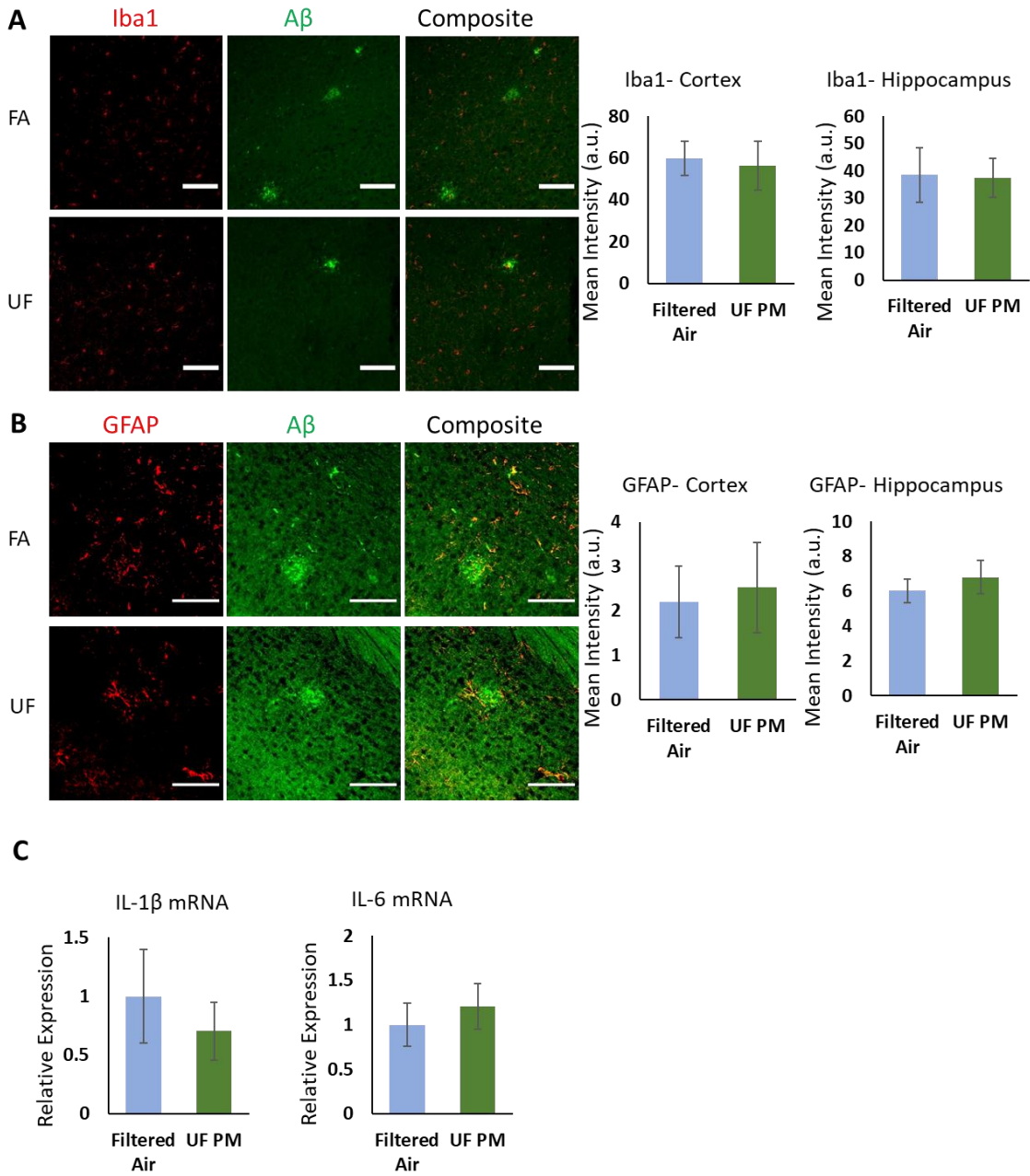
Figure 5: Glia markers GFAP and Iba1 do not increase with exposure to UF PM in 6 month old $App^{NL-G-F/+}$-KI mice

(A) Representative images of microglial marker Iba1 (red) and Aβ (green) staining taken from the parietal cortex (top) and CA1 region of the hippocampus (bottom), as well as quantification of the mean fluorescent intensity of Iba1 staining for the cortex and hippocampus. Exposure to UF PM does not increase the levels of Iba1 in the brain. FA- Filtered Air, UF- UF PM. (B) Representative images of astrocytic marker GFAP (red) and Aβ (green) staining taken from the parietal cortex (top) and CA1 region of the hippocampus (bottom), as well as quantification of the mean fluorescent intensity of GFAP staining for the cortex and hippocampus. UF PM exposure does not change the levels of GFAP observed. (C) mRNA levels of cytokines *Il6* and *Il1β* normalized to *Gapdh* mRNA. Neither *Il1β* or *Il6* mRNA levels from cortical tissue were significantly affected by the exposure status. (N = 8 animals per group for immunofluorescent staining and quantification, and N = 6 animals per group for immunoblot and qPCR. Scale bars = 100 μm).

2.4.4 UF PM exposure in aged $App^{NL-G-F/+}$-KI and wild-type mice

As UF PM exposure induced little change in the pre-to-early pathology 6 month old $App^{NL-G-F/+}$-KI model, we next sought to examine whether UF PM exposure exacerbates Aβ pathology, inflammation, and other markers of neurodegeneration in $App^{NL-G-F/+}$-KI mice at a more advanced aged with greater existing Aβ pathology. 9 month old $App^{NL-G-F/+}$-KI mice were exposed to concentrated UF PM for 3 months to determine whether inflammation and Aβ pathology are affected in aged animals with established pathology. Behavior results in the younger group in absence of altered Aβ pathology indicate some functional change without Aβ perturbation, so wild-type animals of the same background and age were included in this second cohort to better investigate Aβ independent effects of UF PM. Given the lack of effect seen in the younger cohort in inflammation or Aβ, we additionally expanded our scope to include other targets linked to AD progression, PM exposure, and memory function. Previous reports indicating damage to the blood brain barrier (BBB) in association with air pollution exposure (Calderón-Garcidueñas, Solt, et al., 2008; MohanKumar et al., 2008) and in AD pathology (Sweeney et al., 2018; Van De Haar et al., 2016) lead us to assess tight junction makers in the brain. We also investigated the reelin positive neuronal subset in the entorhinal cortex, as they have been shown to be involved in pyramidal neuron synaptogenesis and memory function (Chameau et al., 2009; Stranahan et al., 2011) and have an affinity for early Aβ accumulation and may be vulnerable before general Aβ pathology changes (Kobro-Flatmoen et al., 2016). Finally, based on reports showing glutamate receptor dyshomeostasis with PM exposure (Cacciottolo et al., 2017; Morgan et al., 2011) we examined levels of the glutamate transport protein GLT-1, which has known roles in AD progression, long term potentiation, and memory

ability (Katagiri et al., 2001; Kobayashi et al., 2018; Meeker et al., 2015; Mookherjee et al., 2011).

2.4.5 Concentration of ambient particles in the 12 month old animal cohort

UF PM levels were concentrated 7-fold by count and 9-fold by weight on average over the course of this exposure (Fig. 6). The concentrated particle count was approximately 124 million particles per cubic meter (Fig. 6A), while the concentration by mass was at a similar level as in the younger cohort at 126 µg per cubic meter of air (Fig. 6B). The increase in total particle count compared to the younger cohort exposure while maintaining a similar concentration by mass indicates a smaller average PM size in this second exposure.
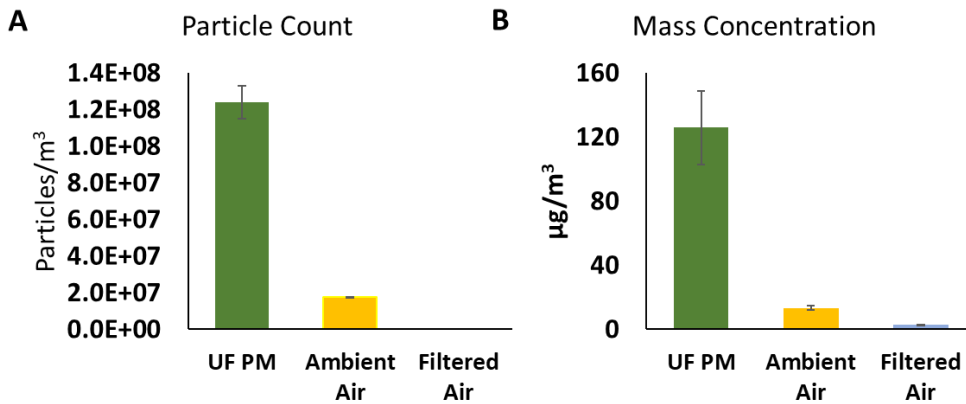
Figure 6: Aged exposure particle concentration.
(A) Average particle count per cubic meter during the exposure period of $App^{NL-G-F/+}$-KI and wild-type mice. (B) Average particle mass concentration during exposure for concentrated quasi-ultrafine PM (UF PM), total ambient air, and filtered air using the VACES system. Bars represent mean values, with error bars expressed as standard error of the mean.

2.4.6 UF PM exposure impairs cognition in 12 month old wild- type and $App^{NL-G-F/+}$-KI mice

In the 12 month old group, memory performance was decreased both by $App^{NL-G-F/+}$ genotype and by UF PM exposure (Fig. 7). For OLM only the performance difference between the air exposed wild-type and UF PM exposed $App^{NL-G-F/+}$-KI mice was significant (14.3% decrease in discrimination index, p<0.01) (Fig. 7A). In the ORM task object discrimination decreased by

41

11% (p<0.05) with UF PM exposure within the wild-type group, 12% (p<0.05) when comparing air exposed wild-type animals to air exposed $App^{NL\text{-}G\text{-}F/+}$-KI animals, and the largest decrease, 22.6% (p<0.001), was observed comparing the air wild type group to the UF PM exposed $App^{NL\text{-}G\text{-}F/+}$-KI group (Fig. 7B); the difference between within the $App^{NL\text{-}G\text{-}F/+}$-KI mice with UF PM exposure did not reach significance. These results indicate an additive effect of UF PM exposure and amyloid genotype on cognitive impairment.
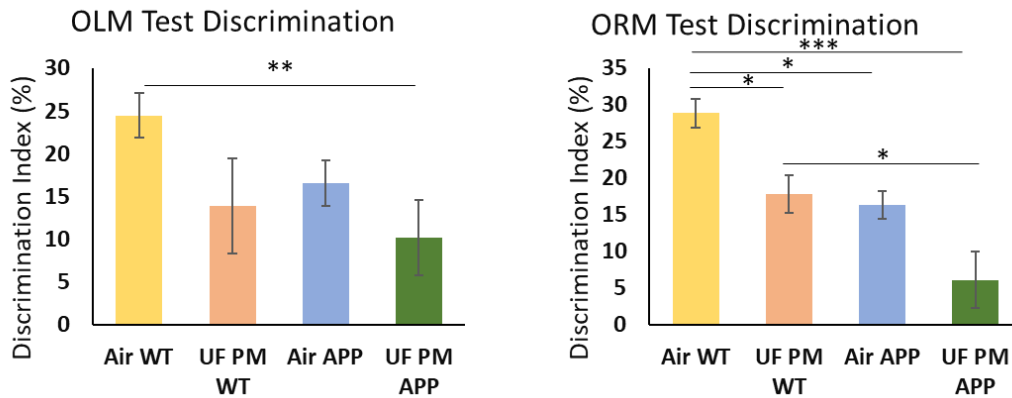
Figure 7: UF PM exposure impairs memory in aged wild-type and $App^{NL\text{-}G\text{-}F/+}$-KI mice.
Object location memory (OLM) and object recognition memory (ORM) task results for $App^{NL\text{-}G\text{-}F/+}$-KI (APP) and wild-type (Wt) mice. Discrimination index is the difference between time spent exploring the novel object or object in novel place and the familiar as a percentage of the total time. (A) Discrimination index for OLM test. Quasi ultrafine particulate matter (UF PM) exposed mice exposed mice showed significantly decreased ability to discriminate the novel object location compared to those exposed to filtered air. (B) Discrimination index for ORM. UF PM exposed mice showed decreased ability to discriminate the novel object compared to animals exposed to filtered air. (N = 10-12 animals per group. Scale bars = 100 μm). * denotes p value < 0.05, ** denotes p < 0.01, and *** denotes p < 0.001.

As in the younger group, there were no detectable changes in synaptic density markers synaptophysin and PSD95 in either the hippocampus or cortex either between genotypes or due to UF PM exposure status by immunofluorescent quantification or western blot analysis (Fig. 8A-D).

A

PSD95

Synapto-
physin

| | Wt Air | Wt UF PM | APP Air | APP UF PM |

PSD95- Cortex

Mean Intensity (a.u.)

Wt Air | Wt UF PM | APP Air | APP UF PM

Synaptophysin- Cortex

Mean Intensity (a.u.)

Wt Air | Wt UF PM | APP Air | APP UF PM

B

PSD95

Synapto-
physin

| | Wt Air | Wt UF PM | APP Air | APP UF PM |

PSD95- Hippocampus

Mean Intensity (a.u.)

Wt Air | Wt UF PM | APP Air | APP UF PM

Synaptophysin- Hippocampus

Mean Intensity (a.u.)

Wt Air | Wt UF PM | APP Air | APP UF PM

Figure 8: Synaptic markers do not change with UF PM exposure in aged $App^{NL-G-F/+}$-KI and wild-type mice. (A) Representative images of PSD95 (green) and synaptophysin (red) immunofluorescent staining and fluorescent intensity quantification from the parietal and somatosensory cortex and fluorescent intensity quantification from the whole cortex in aged $App^{NL-G-F/+}$-KI (APP) and wild-type (Wt) mice. (B) Representative images of PSD95 (green) and synaptophysin (red) immunofluorescent staining taken from CA1 region of the hippocampus and quantification. (C) Representative western blot of synaptophysin and PSD95 from cortical tissue samples with band intensity relative to tubulin. UF- UF PM. (D) Representative western blot of synaptophysin and PSD95 from hippocampal tissue with band intensity relative to tubulin. Bars represent mean values, with error bars expressed as standard error of the mean (N = 4 animals per group for immunostaining and quantification and N = 4-6 animals per group for immunoblot. Scale bars = 100 μm).

The reelin positive neuron subpopulation in the entorhinal cortex was also assessed to determine if UF PM exposure may be specifically toxic to certain subsets of neurons that may account for observed cognitive deficits. However, the number of reelin positive neurons in the entorhinal cortex did not vary significantly with UF PM exposure or genotype (Fig. 9), indicating that loss of the reelin positive neuronal subset is not responsible for the cognitive impairment seen.
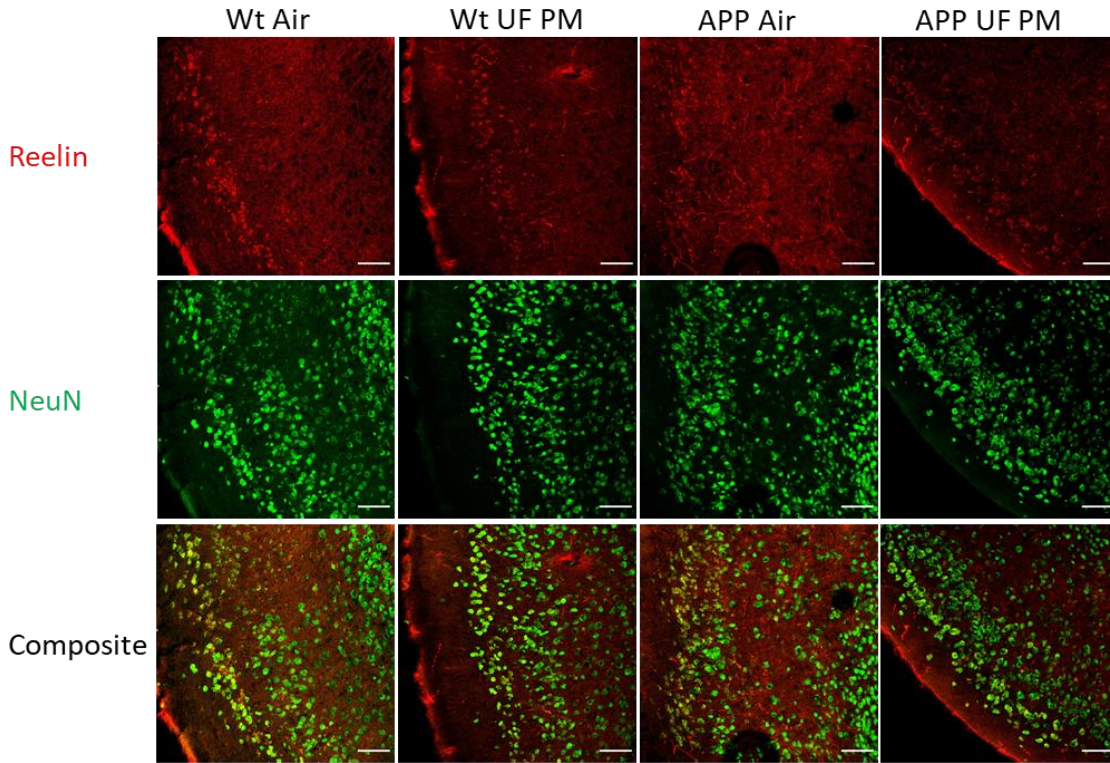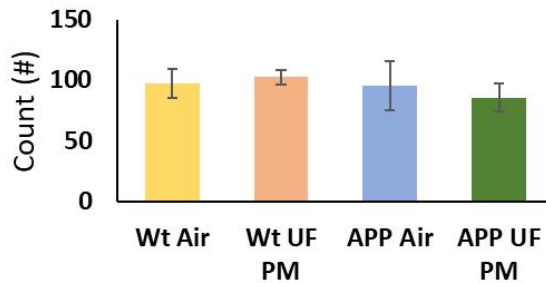
Figure 9: Entorhinal cortex reelin positive neurons are not reduced by UF PM.
Representative images of reelin (red), NeuN (green), and merged staining in the entorhinal cortex and reelin positive neuron count in 12 month old wild-type (Wt) and $App^{NL-G-F/+}$-KI (APP) exposed to filtered air or quasi ultrafine PM. Bars represent mean values, with error bars expressed as standard error of the mean (N = 10 animals per group for behavior tests, N = 8 animals per group for immunostaining and quantification, and N = 10 animals per group for immunoblot. Scale bars = 100 μm).

2.4.6 UF PM exposure increases Aβ plaque load in $App^{NL\text{-}G\text{-}F/+}$-KI mice with established pathology

In the 12 month group plaque pathology is more advanced in the heterozygous animals (Fig. 10). Plaque load as detected by 82E1 was increased in the cortex of $App^{NL\text{-}G\text{-}F/+}$-KI mice exposed to UF PM as compared to the air group, with a percent of area covered by plaques at 2.2% versus 1.4% (p<0.01)(Fig. 10A). In the hippocampus, 82E1 plaques did not increase with exposure. UF PM exposure also increased Thioflavin S stained dense core plaque load in the cortex (0.48% compared to 0.83% of total area, p<0.05) and hippocampus (0.26% compared to 0.59%, p<0.05) (Fig. 10B). Despite increases in plaque load, as in the younger group no significant differences were seen in APP protein levels or App mRNA (Fig. 10C, 10D). Due to concerns about the efficacy of the binding antibody used in the MSD V-Plex kit used in the younger group, Aβ peptide levels in this group were measured using WAKO ELISA kits. Aβ peptide levels were not detectable in the soluble fraction (data not shown) and were not altered with PM exposure status in in the detergent insoluble fraction (Fig. 10E).
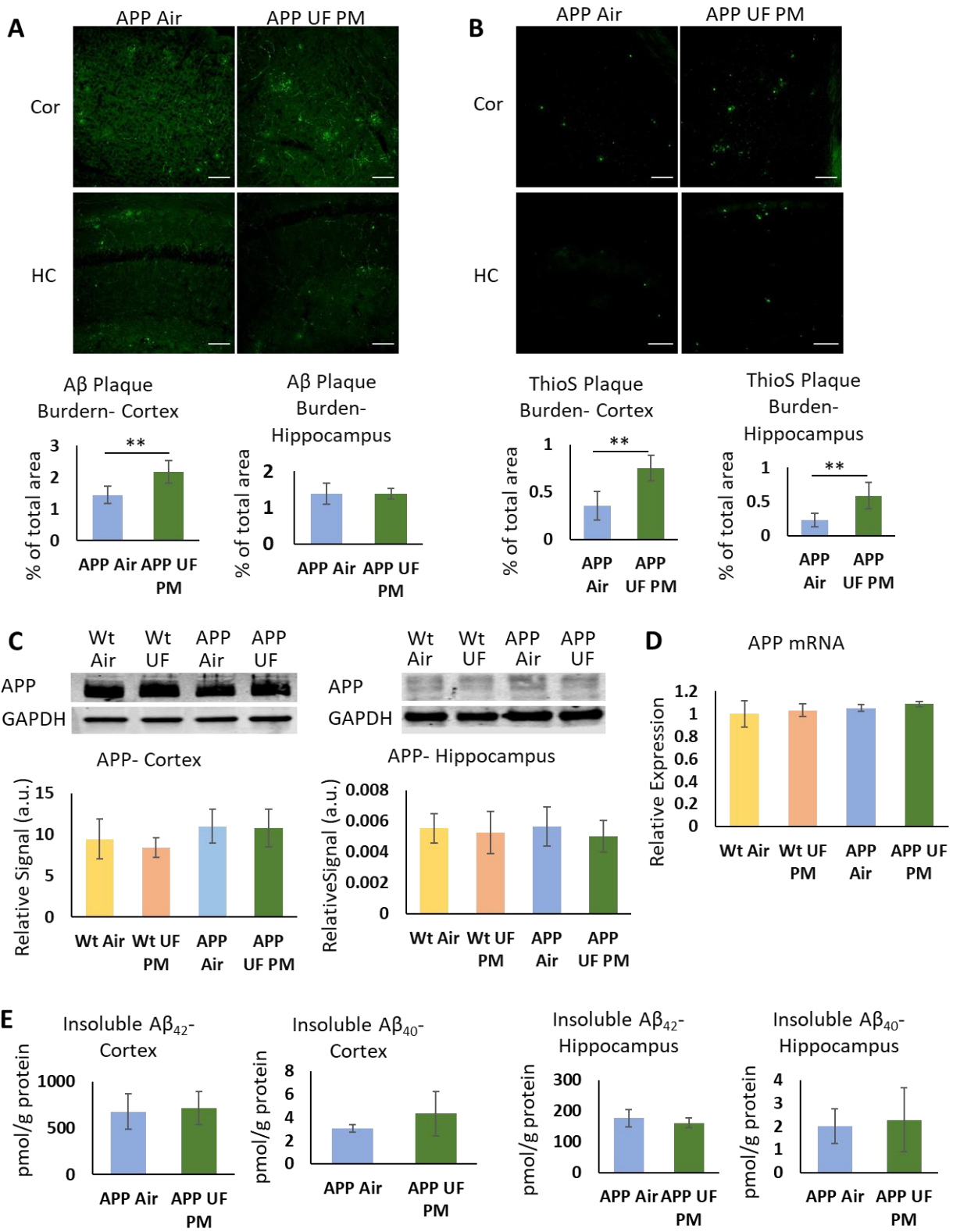
Figure 10: Aβ plaque burden is increased by UF PM exposure in the 12 month old $App^{NL-G-F/+}$-KI mouse model

Brain sections were stained with 82E1 antibody and ThioflavinS to detect diffuse and dense core Aβ plaques in 12 month old $App^{NL-G-F/+}$-KI (APP) mice. (A) Representative images of Aβ plaque burden detected by 82E1 from the parietal cortex (Cor) and CA1 region of the hippocampus (HC) in $App^{NL-G-F/+}$-KI mice exposed to filtered air or UF PM (top), and quantification of plaque burden expressed as percentage of the total area measured occupied by plaques (bottom). Area % covered by plaques increases with UF PM exposure in the cortex but not hippocampus. (B) Representative images of Aβ dense core plaque burden from the parietal cortex (Cor) and CA1 region of the hippocampus (HC) (top) and quantification of plaque burden (bottom). Dense core plaque burden in the cortex and hippocampus also increases with UF PM exposure. (C) Steady-state levels of full length APP in tissue homogenates extracted from cortical and hippocampal tissues were not significantly affected by the PM exposure. UF- UF PM. (D) mRNA levels of *App* normalized to *Gapdh* mRNA. *App* mRNA levels from cortical tissue were also unaffected by the exposure. (E) No differences in insoluble $Aβ_{40}$ or $Aβ_{42}$ levels were found in the cortex or hippocampus by ELISA. Bars represent mean values, with error bars expressed as standard error of the mean (N = 6 animals per group for immunofluorescent staining and quantification, and N = 4 animals per group for immunoblot, V-PLEX, and qPCR. Scale bars = 100 μm). * denotes p value < 0.05, ** denotes p < 0.01.

No increase in glia markers Iba1 or GFAP was detected with UF PM exposure in the 12 month old group (Fig. 11). Iba1 positive microglia non-significantly trended toward a decrease with exposure and Iba1 levels did not change with genotype (Fig. 11A). Microglia co-expressing IBA1 and CD68, which indicates a reactive state (Muhleisen et al., 1995), were not observed in the younger cohort or the wild-type animals in this older cohort (data not shown). However, in the $App^{NL-G-F/+}$-KI mice microglia expressing both Iba1 and CD68 were observed in areas around amyloid plaques; the number of observed microglia expressing Iba1 and CD68 was not significantly different between the UF PM and air exposed groups by count (Fig. 11A). GFAP levels increased in the App^{NL-G-F}-KI animals as compared to wild-type but did not change with UF PM exposure status (Fig. 11B). *Il1β* and *Il6* mRNA levels were not different between the exposure groups within each age group (Fig. 11C). These results indicate that amyloid pathology and glial inflammation in the CNS are not responsible for the cognitive decline observed in either the 6 month or 12 month groups. While increased amyloid plaque load may contribute some to the decreased memory performance seen in the older App^{NL-G-F}-KI animals, it cannot explain the deficits in the younger group or the wild-type animals in the older group.
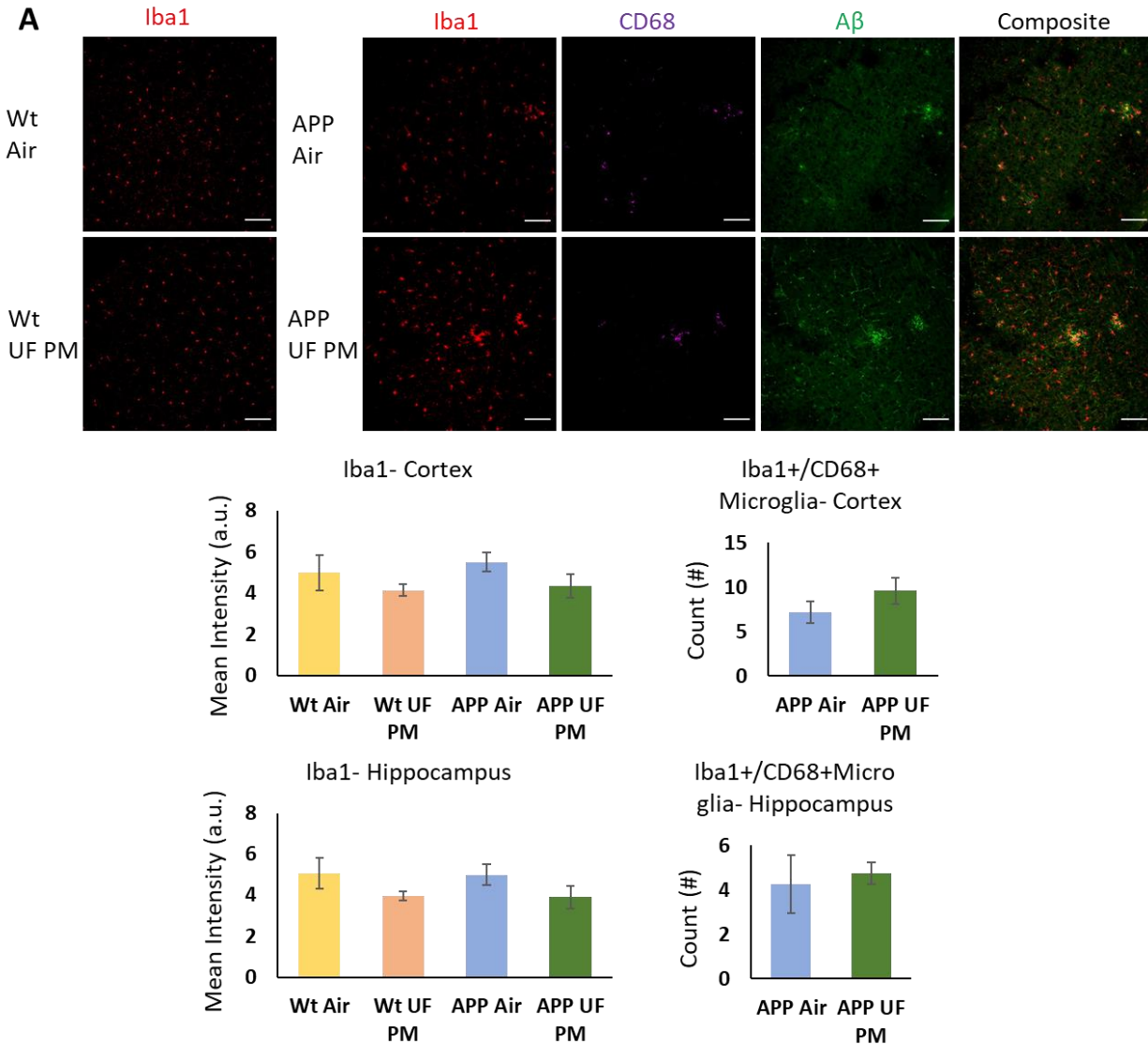
Figure 11: Glia markers GFAP and Iba1 do not increase with exposure to UF PM in 12 month old *App^{NL-G-F/+}*-KI mice

(A) Representative images of microglial marker Iba1 (red), CD68 (purple), and Aβ (green) staining from the parietal cortex (top)of wild-type (Wt) and *App^{NL-G-F/+}*-KI (APP) mice exposed to filtered air or ultra-fine PM and quantification of the mean fluorescent intensity of Iba1 staining for the cortex and hippocampus from all mice and total count of Iba1 and CD68 positive microglia in *App^{NL-G-F/+}*-KI mice (bottom). Exposure to UF PM does not increase the levels of Iba1 or Iba1+/CD68+ microglia in the brain in either group. (B) Representative images of astrocytic marker GFAP (red) and Aβ (green) staining from the parietal cortex (top) and quantification of the mean fluorescent intensity of GFAP staining for the cortex and hippocampus (bottom). UF PM exposure does not change the levels of GFAP observed in either group. (C) mRNA levels of cytokines *Il6* and *Il1β* normalized to *Gapdh* mRNA. Neither *Il1β* or *Il6* mRNA levels from cortical tissue were significantly affected by the exposure status or genotype. Bars represent mean values, with error bars as standard error of the mean (N = 6 animals per group for immunofluorescent staining and quantification, and N = 4 animals per group for immunoblot and qPCR. Scale bars = 100 μm).

49

B

GFAP | GFAP | Aβ | Composite

Wt Air

Wt UF PM

APP Air

APP UF PM

GFAP- Cortex

GFAP- Hippocampus

C

IL-6 mRNA

IL-1β mRNA

2.4.7 UF PM exposure does not impact tight junction markers in the blood-brain barrier in 12 month $App^{NL-G-F/+}$-KI or wild-type mice

We next sought to investigate whether the BBB integrity was impacted by the current exposure in the 12 month old wild-type and App^{NL-G-F}-KI group (Fig. 12). Using tight junction marker ZO-1, cerebral vasculature width was assessed with immunofluorescent staining (Fig. 12A). No differences were seen in the width of the blood vessels due to either exposure status or genotype. We also assessed total albumin protein levels in the cortex by western blot, as a leaky BBB could cause a build up of albumin in brain tissue, but again saw no significant changes between the groups (Fig. 12B). Finally, using a vascular enriched whole brain protein fraction, we assessed the levels of two other tight junction proteins, Claudin-5 and CD31 (Fig. 12C, 12D). Once more there were no differences between either treatment status or genotype. Overall, we observed no indications of BBB disruption with exposure to UF PM.
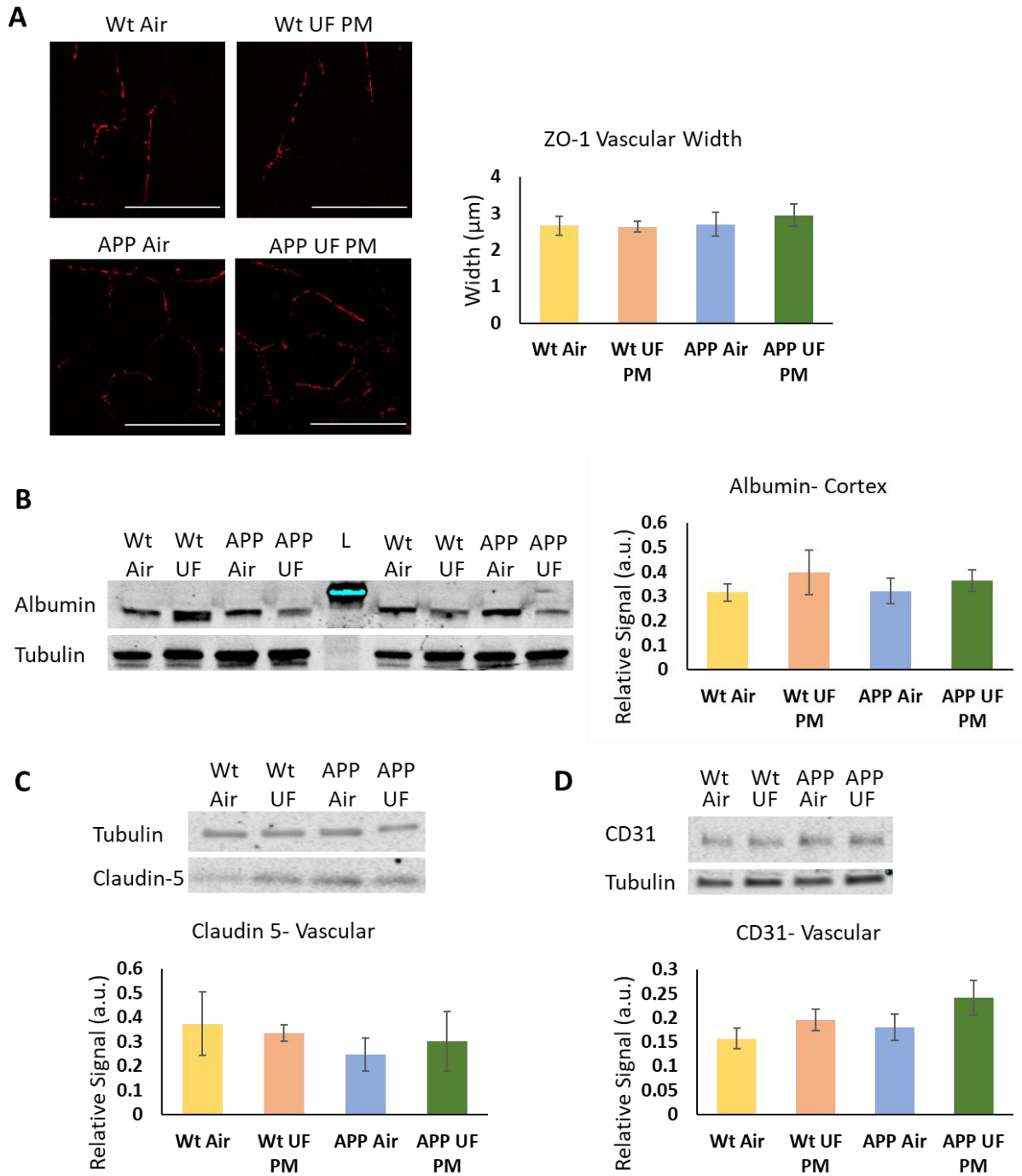
Figure 22: Tight junction markers do not decrease in wild-type or *App*[NL-G-F/+]-KI mice exposure to UF PM (A) Representative images of ZO-1 staining of cerebral vasculature from the cortex in wild-type (Wt) and *App*[NL-G-F/+]-KI (APP) mice exposed to filtered air or UF PM (left) and quantification of vascular width (right). No differences are seen across treatment or genotype. (B) Representative western blot of albumin in cortical protein extract. Differences between groups are not significant. (C) Representative western blot of tight junction marker Claudin-5 and (D) intercellular junction marker CD31 from vascular enriched protein samples. Neither genotype nor exposure status shows significant differences. Bars represent mean values, with error bars expressed as standard error of the mean (N = 4-5 animals per group for all assays. Scale bars = 100μm).

2.4.8 UF PM exposure reduces total protein level of GLT-1 in both wild-type and App^{NL-G-}

$^{F/+}$-KI mice

Glutamatergic neuron dysfunction has been shown with nano-scale particulate matter *in vitro* (Morgan et al., 2011) and is suggested in a rodent model of AD (Cacciottolo et al., 2017). We sought to determine if the astrocytic glutamate transporter 1 (GLT-1), which is implicated strongly in AD pathology (Kobayashi et al., 2018; Mookherjee et al., 2011; Zumkehr et al., 2015), is also affected by exposure to airborne particulates. Using western blot total protein assays of GLT-1 levels relative to levels of the astrocytic marker GFAP, we found that GLT-1 is decreased in the hippocampus of mice exposed to UF PM, which we also confirmed in the younger exposure cohort (Fig. 13). In the 6 month group, a 41% decrease of GLT-1 is observed in the hippocampus of UF PM exposed *App^{NL-G-F}*-KI animals compared to the filtered air exposed group p<0.05, (Fig. 13A). This change is not evident in the cortex of those animals. In the 12 month old group wild-type and *App^{NL-G-F}*-KI UF PM exposed animals again show decreased levels of GLT-1 in the hippocampus as compared to the wild-type air exposed group, 44% and 47% respectively, p<0.05 for both (Fig. 13B). Within the *APP^{NL-G-F}*-KI group there is a smaller non-significant decrease, as the *App$^{NL-G-F/+}$*-KI air group itself shows a non-significant decrease compared to the wild-type air group. These results indicate that region specific GLT-1 loss is associated with UF PM exposure. Whether GLT-1 loss is involved in the pathway leading to cognitive decline in these animals is unknown.

Figure 13: Levels of astrocytic glutamate transporter GLT-1 decrease in the hippocampus of mice exposed to UF PM

(A) Representative western blots of GLT-1 protein levels relative to GFAP in the cortex (left) and hippocampus (right) from 6 month old $App^{NL-G-F/+}$-KI mice exposed to either filtered air or UF PM, with quantification of western blot band intensity (below). GLT-1 levels decrease in the hippocampus, but not cortex, of UF PM exposed animals. FA- Filtered air UF- UF PM. (B) Representative western blots of GLT-1 protein levels relative to GFAP in the cortex (left) and hippocampus (right) from 12 month old wild-type (Wt) or $App^{NL-G-F/+}$-KI (APP) mice exposed to either filtered air or UF PM with quantitation (below). GLT-1 is decreased in UF PM exposed animals compared to wild-type air exposed animals, but not within the $App^{NL-G-F/+}$-KI group. Bars represent mean values, with error bars expressed as standard error of the mean (N = 8 animals per group). * denotes p<0.05.

54

2.5 Discussion:

We report that chronic exposure to concentrated UF PM was associated with decreased performance in spatial and object recognition memory tasks in both a young $App^{NL-G-F/+}$-KI mouse model and older wild-type and $App^{NL-G-F/+}$-KI mice, which recapitulates the adverse impact of PM in cognition observed at multiple ages in humans (Harris et al., 2015; Jedrychowski et al., 2015; H. Lin et al., 2017; X. Zhang et al., 2018). We also show that 12-week PM exposure exacerbated AD-related neuropathology, particularly Aβ burden, in older heterozygous $App^{NL-G-F/+}$-KI mice, while no significant change was observed in younger $App^{NL-G-F/+}$-KI mice with pre-to-early plaque pathology. These changes in memory tasks and Aβ plaque load with UF PM exposure are not accompanied by gross loss of synaptic proteins, increases in glial inflammatory markers, or BBB integrity. However, we observe a decrease in the protein levels of the astrocytic glutamate transporter GLT-1 in both $App^{NL-G-F/+}$-KI and wild-type animals exposed to UF PM, which has not been previously reported.

Our current findings on increased Aβ plaque load in $App^{NL-G-F/+}$-KI mice following UF PM exposure are not only consistent with previous reports in different mouse models (Bhatt et al., 2015; Cacciottolo et al., 2017; Durga et al., 2015), but also adding new knowledge that UF PM exposure exacerbates plaque development even in the brain with physiological expression of APP, and that advancing age and pathology may be more susceptible to such change. However, the absence of increased Aβ pathology with exposure to concentrated PM in the 6 month old group, as well as the impairment of behavior in the wild-type animals in the aged group, suggests that the mechanisms by which PM exposure impairs cognitive function is not primarily reliant on Aβ pathology. Additionally, while both inflammation and the loss of BBB integrity are associated with PM exposure in other models (Block & Calderón-Garcidueñas, 2009; Calderón-

Garcidueñas, Solt, et al., 2008; Fonken et al., 2011; Kleinman et al., 2008), we do not see

changes in those areas in the current model. Differences in exposure paradigms as well as the

composition of exposure may account for the discrepancy. Determination of the precise

mechanisms leading to cognitive decline in this model may also provide insight into whether the

changes in inflammation and BBB integrity seen elsewhere would potentially be caused by the

same pathway, or if they are separate pathologies.

GLT-1 loss is well associated with neurodegenerative outcomes (Colangelo et al., 2014; Pajarillo

et al., 2019). The observed loss of GLT-1 in the hippocampus with UF PM exposure here

indicates glutamate transport disruption as a possible pathway linking to the cognitive deficits

observed. Previous PM exposure studies mice show changes in the glutamate receptor subunit

GRIA1 with exposure *in vivo* (Cacciottolo et al., 2017; Morgan et al., 2011) as well as *in vitro*

effects on neurite outgrowth and NMDA toxicity (Morgan et al., 2011). Together with our

finding of decreased GLT-1, these results form a basis to further investigate the role of glutamate

dyshomeostasis in PM induced cognitive decline. Glutamate excitotoxicity (Lewerenz & Maher,

2015) of particular neuronal subsets may in part account for our observed memory task decline,

and bears inquiry. As glutamate transport dyshomeostasis and excitotoxicity are known to be

involved in tau pathology progression, both by increasing phosphorylation and encouraging tau

propagation (Dabir et al., 2006; Hunsberger et al., 2015; Kilian et al., 2017; J. W. Wu et al.,

2016), the loss of GLT-1 here indicates a possible mechanism linking tau pathology and air

pollution exposure. Numerous reports have shown an increase in total tau as well as

phosphorylated tau in humans, dogs, and mice exposed to air pollution (Calderón-Garcidueas,

Mora-Tiscareño, et al., 2012; Calderón-Garcidueñas et al., 2018, 2020; Levesque et al., 2011;

Park et al., 2020), providing a clear connection between exposure and tau pathology. Further

study in a human tau transgenic model may provide insight into possible non-amyloid based mechanisms linking air pollution exposure and AD risk, as well as potential involvement in other tauopathies.

The current study, however, has several shortfalls limiting the ability to fully ascertain the mechanisms at play connecting UF PM, cognitive decline, and amyloid pathology. As mentioned previously, despite strong existing evidence showing neuroinflammation and Aβ changes with PM exposures, we found no evidence of either in the younger animal cohort, and only Aβ plaque accumulation in the older $App^{NL-G-F/+}$-KI but not wild-type animals, providing no clear mechanism for the memory task impairment seen in these groups. The finding of GLT-1 loss in the hippocampus suggests possible mechanisms, but without additional evidence of LTP impairment, synapse dysfunction, or neuronal damage, it does not fully explain the changes either. While the younger cohort was intended as a pilot group to study Aβ changes in an AD model and was thus performed with only $App^{NL-G-F/+}$-KI animals, the lack of wild-type animals there limits our ability to determine whether the cognitive changes observed are at all related to the $App^{NL-G-F/+}$-KI genotype or were entirely independent. Similar findings in the older cohort, as well as a lack of change in any observed amyloid markers, suggests an amyloid independent pathway. It is, however, feasible that even the low levels of Aβ present interacted with PM toxicity. In both the ORM task and GLT-1 protein assays in the older cohort we observe a decrease with UF PM exposure in the wild-type group, but not the $App^{NL-G-F/+}$-KI. In both cases, the $App^{NL-G-F/+}$-KI and wild-type UF PM groups have similar reported values, while the $App^{NL-G-F/+}$-KI air group shows a non-significant decrease compared to the wild-type air group. It may be that UF PM exposure toxicity and amyloid toxicity act in the same pathway in such a way that the additional insult has limited impact, but that cannot be determined from the current data.

Finally, the particle number in the UF PM exposure in the older study is nearly twice the value as in the younger cohort study but the mass concentrations are relatively close, which suggests a smaller average particle size in the older cohort exposure. As PM is typically considered more toxic and better able to infiltrate the body the smaller the size (S. Kim et al., 2001; Valavandis et al., 2008), it is unknown to what degree this influenced the changes observed in Aβ plaques in $App^{NL-G-F/+}$-KI animals with exposure in the older cohort compared to the younger cohort. Investigation of potential metal accumulation in the brain may provide additional insights into the pathways by which cognitive impairment occurs and the degree of PM infiltration to the CNS. PM is highly heterogeneous and consists of various compounds and metals (Hand et al., 2012; Kleeman et al., 2000; Perrone et al., 2013). Exposure to nickel through inhalation has been shown to increase Aβ load in wild-type mice (S. H. Kim et al., 2012). PM associated metals appear to deposit in multiple areas of the body following exposure (Q. Li et al., 2015) and it is known that the smaller PM size fractions can both infiltrate to the blood (Kreyling et al., 2002; Nemmar et al., 2002) and to the CNS directly via the olfactory nerves (Block & Calderón-Garcidueñas, 2009; González-Maciel et al., 2017). Manganese exposure has been linked to impaired glutamate transport (Erikson et al., 2002; Hazell & Norenberg, 1997), including loss of GLT-1 protein (Mutkus et al., 2005) as observed here, and leads to dopaminergic neuronal loss (Pajarillo et al., 2018) which could potentially explain the observed cognitive deficits. However, whether manganese or any other metals accumulate in the brain with PM exposure in the current model remains purely speculative and requires investigation.

In summary, we find reduction of memory function in both wild-type and the $App^{NL-G-F/+}$-KI mouse model with exposure to concentrated UF PM. This is accompanied by a reduction of the astrocytic glutamate transporter GLT-1, but not changes in glial inflammatory markers. Aβ

plaque burden is increased in aged animals but is not accelerated at a pre-pathological age, indicating that PM exposure is sufficient to increase amyloid burden, but also that cognitive decline due to PM exposure cannot be only due to amyloid pathology. Further investigation into the mechanisms linking PM exposure to memory impairment, particularly the extent of glutamate transport disruption and whether it plays a causative role in this impairment, in the model is required.

Chapter 3: *In Utero* exposure to ultra-fine particulate matter impairs cognition and increases neuroinflammation in aged mice and increases Amyloid β plaque load in AD model mice.

3.1 Abstract:

Accelerated cognitive decline and increased risk for Alzheimer's disease (AD) have recently emerged as a direct consequences of chronic exposure to airborne particulate matter (PM) in elderly populations, and its exposure during the prenatal period has been associated with decreased intellectual performance in children and young adults. However, little is known about the neurotoxic impact of early-life exposure to PM on cognition and late-life neurological disorders, despite the fact that life-long exposure to PM is inevitable particularly for individuals residing highly polluted areas. Here, we examined the impact of *in utero* PM exposure on cognition and neuropathology in adult wild-type mice and the amyloid precursor protein knock-in ($App^{NL-G-F/+}$-KI) model of AD. Two-week exposure to concentrated quasi-ultrafine PM (UF PM) collected from ambient air resulted in decreased performance in the object location memory task in both groups as compared to animals exposed to filtered air, while the object recognition task was only decreased when comparing the wild-type air exposed group to the App^{NL-G-F}-KI UF PM group. Markers of glial neuroinflammatory response, astrocytic GFAP and microglia expressing Iba1 and CD68, were also increased with UF PM exposure. Exposure to UF PM also increased amyloid-β plaque load pathology in the App^{NL-G-F}-KI animals. Overall, these results show that developmental exposure to UF PM can affect cognition, neuroinflammation, and AD pathology progression in adult animals in a murine model. The molecular mechanisms altered during the exposure period leading to these changes remain to be elucidated.

3.2 Introduction:

There is substantial and increasing evidence that exposure to air pollution is linked negatively to cognitive function in a wide range of ages. Particulate matter (PM), a heterogenous mixture of small solids and liquids suspended in the air, compromises a significant portion of traffic related air pollution and has been specifically linked to some of these outcomes in exposures as early as *in utero*. Exposure to polycyclic aromatic hydrocarbons (PAHs), a component of PM, during pregnancy correlates with reduced verbal and non-verbal IQ in adolescence (Edwards et al., 2010; Jedrychowski et al., 2015; Perera et al., 2006, 2009). More generally, exposure to fine particulate matter ($PM_{2.5}$) is correlated with reduced IQ performance in children as well (Lertxundi et al., 2015; Porta et al., 2016). In the elderly exposure to PM is associated with reduced working and episodic memory (J. A. Ailshire & Clarke, 2014; J. A. Ailshire & Crimmins, 2014; Younan et al., 2020) as well as significantly increased risk of dementias, including Alzheimer's disease (AD) (Cacciottolo et al., 2017; H. Chen, Kwong, Copes, Hystad, et al., 2017; Jung et al., 2015; Oudin et al., 2016). While there is significant evidence of a link between PM exposure and accelerated cognitive decline in humans, the molecular mechanisms driving this are still incompletely understood. Additionally, there is limited research investigating whether the cognitive changes and presumed underlying molecular changes with developmental PM exposure persist into adulthood and affect the risk of developing AD and other dementias in later life.

Experiments in rodent models exposed to ultra-fine PM (UF PM, a diameter of 100 nm or less) have demonstrated cognitive decline, synaptic loss, and inflammation in the CNS (Bhatt et al., 2015; Durga et al., 2015; Fonken et al., 2011; Guerra et al., 2013). UF PM exposure in adult animals increases levels of inflammatory cytokines and glia activation in wild type animals (Bhatt et al., 2015; Fonken et al., 2011; Guerra et al., 2013; Kleinman et al., 2008), and in an AD

model increases amyloid-β (Aβ) levels (Cacciottolo et al., 2017), suggesting possible

mechanisms by which PM exposure may impact cognitive function. Studies of the effects of PM

exposure during development in adult animal models are limited, but available reports indicate

that such an exposure can alter memory function and neuroimmune state in adult mice (Allen et

al., 2013; Allen, Liu, Pelkowski, et al., 2014; Allen, Liu, Weston, et al., 2014; Kulas et al., 2018;

Zanchi et al., 2010). Currently no studies have examined whether exposure to PM during

development in AD model animals can influence AD pathology progression later in life. Here,

we hypothesized that exposure to concentrated ultrafine PM from ambient air during

development accelerates cognitive decline later in adult mice through aberrant

neuroinflammation and/or accelerated buildup of AD-like neuropathology in the brain. To test

this hypothesis, we designed an *in utero* exposure using pregnant wild-type females mated with

either wild-type males or homozygous *App* knock-in ($App^{NL-G-F/NL-G-F}$-KI) male mice (Saito et al.,

2014), which carried wild-type or heterozygous $App^{NL-G-F/+}$-KI offspring, respectively. The use

of wild-type females also allowed us to eliminate maternal genetic variances that may impact on

the development of fetus. We aged offspring until 12 months of age and examined the effect of

in utero PM exposure in order to determine whether developmental exposure substantially

accelerates age-related cognitive and pathological changes in these mice.

3.3 Methods:

3.3.1 Animals

All experiments were performed in accordance with the Institutional Animal Care and Use

Committee at University of California. Mice were housed on a 12 hour light-dark cycle with

feed and water ad libitum. Humanized APP with the Swedish, Arctic, and Iberian mutations

($App^{NL-G-F/+}$-KI) mice in the C57BL/6 background (Saito et al., 2014) were obtained from the

RIKEN Institute (Japan). C57BL/6J male and female mice were obtained from Jackson Laboratory (Bar Harbor, ME). A total of 16 female C57BL/6J mice were bred to either male C57BL/6J or $App^{NL-G-F/NL-G-F}$-KI mice and exposed to concentrated particulates during pregnancy. Of 80 pups 11 were wild-type air exposed, 19 were wild-type UF PM exposed, 23 were $App^{NL-G-F/+}$-KI air exposed, and 27 were $App^{NL-G-F/+}$-KI UF PM exposed.

3.3.2 Exposure Paradigm

Ambient particulates in the area around the UC Irvine campus in Orange County, California with particle diameters smaller than 180 nm (quasi-ultra-fine particles) were concentrated using a versatile aerosol concentration and enrichment system (VACES) as previously described (S. Kim et al., 2001). The VACES is composed of size selective inlets, saturator and chiller modules that supersaturate the aerosol with water vapors to grow them to a size that can be inertially separated using a virtual impactor, and a diffusion drier module that removes excess water vapor and returns the aerosol to a size distribution similar to the ambient air. The system can enrich the concentration of particles in the 0.03 - 2.0 μm size range by a factor of 30x ambient, depending on output flow rate. The VACES system is located adjacent to a major roadway in Irvine, CA, and exposure occurs over morning commute hours to emphasize motor vehicle associated PM. Upon observation of a vaginal plug, female wild type mice bred with either $App^{NL-G-F/NL-G-F}$-KI or wild-type male mice were transferred to the facility housing the VACES to begin exposure. Presumed pregnant dams were exposed to either air filtered to remove particulate matter or concentrated UF PM. Animals were exposed 4 days per week (Tuesday – Friday), for 5 hours per day, for approximately 2.5 weeks, from 07:30-12:30 local time, which captures the period of maximum PM concentration during the day. Average particulate concentration during the experiment for the UF PM exposed pregnant mothers was 206 μg/m³, or 42.9 μg/m³ per day if

weighted for 24 hours, and 0 μg/m^3 for the filtered air exposed mothers. The UF PM exposure is within values that are seen in highly polluted areas (WHO, 2016), and accounting for heterogeneity of sensitivity to exposure in humans as well as animal-to-human extrapolation exposing mice to is translatable to potential human exposure during pregnancy (Rees & Hattis, 2004). The 4 days/wk exposure paradigm reflects typical air pollution episodes (Kleinman et al. 2005, Kleinman et al. 2007). Pups were allowed to age under normal condition with no additional air pollution exposure until 12 months of age. At 12 months, the pups were sacrificed for tissue collection. Particulate mass and particle counts were obtained using a DustTrak aerosol monitor with DustTrak Pro software (TSI) and a Condensation Particle Counter with Aerosol Instrument Manager software (TSI).

3.3.2 Cognitive Assessments

Animals in the were tested in Object Location Memory (OLM) and Object Recognition Memory (ORM) tasks. OLM was performed 3 weeks from sacrifice, at approximately 11.5 months of age. ORM was run following a 1 week break period after OLM testing. These tasks were performed based on a previously described protocol (Vogel-Ciernia & Wood, 2014). Animals were habituated to the test arena for 6 days, 5 minutes per day, and then exposed to two identical objects for 10 minutes for training. Different base objects were used for ORM and OLM acquisition. Testing occurred 24 hours following training. In the OLM task one of the two objects was moved to a novel location, while for ORM one of the objects was replaced with a novel object. Objects replaced or moved were alternated between mice. Mice were allowed to explore during the acquisition for 10 minutes and the test for 5 minutes. Both test and training exploration were recorded, with the video used to score performance. Total time spent exploring each object- set as the time with the animal's nose within 1 cm of the object and pointing directly

at the object- was recorded. Time exploring each of the two objects was summed to obtain total exploration time. Animals showing a strong preference for exploring one object over the other during the acquisition phase were removed from the final analysis pool. The discrimination index was calculated as the difference between time spent exploring the novel object or location and time spent exploring the familiar object or location expressed as a percentage of the total time spent exploring during the test phase.

3.3.3 Protein Extraction and Western Blot Analysis

Half brain cortical tissue and hippocampi were homogenized in T-PER buffer with protease and phosphatase inhibitor cocktails (Thermo Fisher). Protein extract was then centrifuged at 100,000 x g for 1 hour at 4°C and the supernatant was taken as the detergent soluble fraction. The pellet was resuspended in 88% formic acid and centrifuged again at 100,000 x g for 1 hour at 4°C and the supernatant from this step was taken as the formic acid soluble fraction. Protein concentration was determined by the Bradford protein assay. Protein samples were run on Bio-Rad Mini-PROTEAN® TGX^{TM} gels (Bio-Rad) at 150V and transferred to Immobilon®-FL PVDF membranes (Millipore). After 1 hour blocking in Li-Cor Odyssey® Blocking Buffer in TBS (Li-Cor Biosciences), membranes were immunoblotted with the following antibodies overnight at 4°C: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1,000, Santa Cruz Biotechnology), tubulin (1:25,000 Abcam), post synaptic density protein 95 kDa (PSD95, 1:1,000, Cell Signaling Technology), synaptophysin (1:1,000, Cell Signaling Technology), glial fibrillary acidic protein (GFAP, 1:1000, DAKO), Iba1 (1:1,000, Abcam), GLT1 (1:500, a gift from Dr. Jeffery Rothstein at Johns Hopkins University), or amyloid precursor protein, c-terminal (751-770) (CT-20, 1:1000, EMDMillipore). Membranes were then washed, incubated for 1 hour at room temperature with secondary antibodies Goat anti Rabbit or Goat anti Mouse

IRDye® 680 and 800 (1:20000 Li-Cir Biosciences), washed again and read. Blots were read

using the Li-Cor Odyssey system and Image Studio software version 5 (Li-Cor Biosciences) to

obtain band signal intensity. Signal is expressed relative to tubulin or GAPDH levels, which

were used for protein loading control, before statistical analysis. GLT1 levels were additionally

expressed relative to GFAP levels to account for potential changes due to astrocyte activation or

loss.

3.3.4 Immunofluorescent Staining

Frozen brain hemispheres sectioned into 40μm slices coronally using a microtome and stored in

phosphate buffered saline with 0.05% sodium azide. Sections were mounted on standard glass

microscope slides (Fisher) before staining. For antibody staining sections were permeabilized

with 0.1% triton-x 100 in tris buffered saline (TBS) for 15 minutes and blocked with 3% bovine

serum albumin (Fisher), 5% normal goat serum (Vector), and 0.1% triton-x 100 in TBS for one

hour. For Aβ plaque staining sections were treated with 88% formic acid for 7 minutes before

other treatments. After pre-treatment and blocking, the sections were incubated with primary

antibodies against PSD95 (1:1,000, Cell Signaling Technology), synaptophysin (1:1,000, Cell

Signaling Technology), GFAP (1:1000, DAKO), Iba1 (1:1,000, Abcam), CD68 (1:1000, Bio-

Rad), or anti amyloid 82E1 (1:1000, Immuno-Biological Laboratories) overnight at 4°C.

Sections were washed with TBS and treated for 1 hour at room temperature the following day in

3% BSA, 5% normal goat serum, and 0.1% triton-x 100 in TBS with secondary antibodies

conjugated with Alexa Fluor 488, 555, or 633 (Fisher). For Thioflavin S staining sectioned were

rehydrated with ethanol at 100%, 95%, 70%, and 50% and then treated with Thioflavin S

(Sigma) in 50% ethanol for 10 minutes. Slides were mounted with Fluoromount-G (Fisher).

Images were taken with a Leica TCS SPE confocal microscope for Aβ plaque staining and

GFAP and Iba1 staining. Plaque burden was assessed as the percent of total measured area

occupied by amyloid beta plaques from the cortex and hippocampus CA1 region. Vascular

width was determined by the average of 5 measurements across the vessel.   ImageJ version 1.52

was used to analyze images.

3.3.5 Aβ Quantitation

Detergent soluble and formic acid soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels were measured using the

Human βAmyloid (1-40) ELISA kit and Human βAmyloid (1-42) ELISA kit (Wako). Protein

sample extracts were obtained and quantified as described under Protein Extraction and Western

Blots. 100 μL of protein was loaded per well for each sample. Bradford protein assay

concentration readings were used to adjust for total protein loaded per sample.

3.3.6 Statistical Analysis

Immunoblots were quantified using Image Studio Software version 5. Immunofluorescent

images were quantified using ImageJ version 1.52. All other data were analyzed using Microsoft

Excel (Microsoft Office 365 ProPlus) or Prism version 3 (GraphPad Software). Statistics were

carried out using unpaired t-test or two-way analysis of variance with Tukey's post hoc test. p

values ≤ 0.05 were considered significant. Sex differences were generally not observed, and data

are presented as mixed groups.

3.4 Results:

3.4.1 UF PM exposure in utero impairs cognition in 12 month old wild-type and $App^{NL-G-F/+}$-KI mice

Beginning at 11.5 months the $App^{NL-G-F/+}$-KI and wild-type mice born from the exposed mothers

were tested for memory function with object recognition memory (ORM) and object location

memory (OLM) tasks following established protocols (Vogel-Ciernia & Wood, 2014) (Fig. 1).

In the spatial memory OLM task no genotype effect was detected but UF PM exposure significantly decreased performance in both the wild-type and $App^{NL-G-F/+}$-KI groups (Fig. 1A). In the wild-type group the discrimination index was 29.1% for air exposed animals versus 13.4% for the UF PM exposed group (p<0.01), and 24.1% compared to 16.2% respectively in the $App^{NL-G-F/+}$-KI group (p<0.05). For the ORM task the wild-type air exposed group performed best at a 25.9% discrimination index, with the wild-type UF PM exposed and $App^{NL-G-F/+}$-KI air exposed mice both at 21.4%, and the $App^{NL-G-F/+}$-KI UF PM group the worst at 16.3% (Fig.1B). Only the wild-type air to UF PM $App^{NL-G-F/+}$-KI comparison was significantly different (p<0.05). Together these results indicate that exposure to UF PM during development can impact memory function in adult mice, and that the $App^{NL-G-F/+}$-KI genotype and *in utero* UF PM exposure may have additive effects on memory impairment.
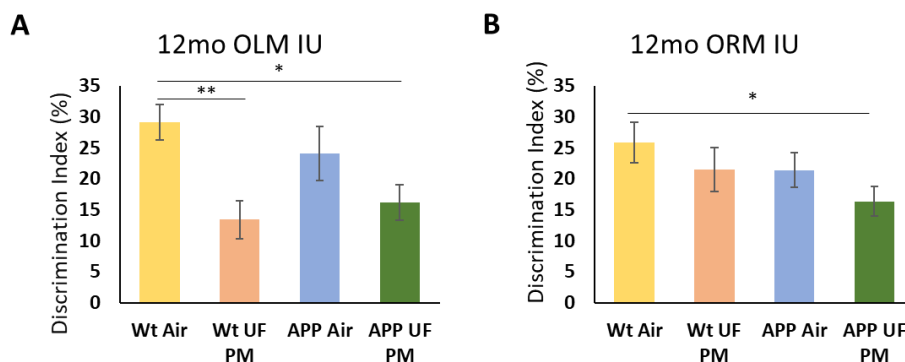
Figure 1: UF PM exposure *in utero* impairs memory in aged wild-type and $App^{NL-G-F/+}$-KI mice.
Object location memory (OLM) and object recognition memory (ORM) task results for wild-type (Wt) and $App^{NL-G-F/+}$-KI (APP) mice. Discrimination index is the difference between time spent exploring the novel object or object in novel place and the familiar as a percentage of the total time. (A) Discrimination index for the OLM task. UF PM exposed mice of either genotype mice showed significantly decreased ability to discriminate the novel object location compared to wild-type mice exposed to filtered air. (B) Discrimination index for ORM. $App^{NL-G-F/+}$-KI UF PM exposed mice showed decreased ability to discriminate the novel object compared to wild-type animals exposed to filtered air. (N = 8-12 animals per group. Scale bars = 100 μm). * denotes p value < 0.05 and ** denotes p < 0.01.

3.4.2 In utero UF PM exposure decreases synaptic marker PSD95 and increases glia

inflammation in combination with the $App^{NL-G-F/+}$-KI genotype

A previous report examining *in utero* fine PM exposure on adult mice found increases in both synaptic marker synaptophysin and astrocytic marker GFAP in the exposed group (Kulas et al., 2018). We first examined synaptic density markers PSD95 and synaptophysin (SYP) in the cortex and hippocampus and found that PSD95 decreased by 30% in the cortex and 35% in the hippocampus comparing the wild-type air exposed group to the $App^{NL-G-F/+}$-KI UF PM group ($p<0.05$ for both, Fig. 2A, B). However, we failed to detect a significant difference in PSD95 between filtered air and UF PM exposed groups within the same genotype. There was no significant difference in synaptophysin in any of the groups tested in either area (Fig. 2A, B).

PM exposure has been associated with increased inflammatory reactions in the CNS (Calderón-Garcidueñas, Mora-Tiscareño, et al., 2008; Calderón-Garcidueñas, Solt, et al., 2008; Kleinman et al., 2008; Kulas et al., 2018). While we did not observe inflammatory reactions with UF PM exposure of adults in the $App^{NL-G-F/+}$-KI (Chapter 2), development may be a time of particular vulnerability. $PM_{2.5}$ exposure in pregnant rats causes cognitive deficits in the pups which are ameliorated by administration of anti-inflammatory compounds reducing microglia and oxidative stress (Tseng et al., 2019). An exposure paradigm similar to the current experiment in developing mice has also shown an increase in astrocyte presence in adult mice (Kulas et al., 2018), which play important roles in both inflammation response and synaptic signaling function.

A

	Wt Air	Wt UF PM	APP Air	APP UF PM
PSD95				
SYP				

PSD95- Cortex

Synaptophysin- Cortex

B

	Wt Air	Wt UF PM	APP Air	APP UF PM
PSD95				
SYP				

PSD95- Hippocampus

Synaptophysin- Hippocampus

70
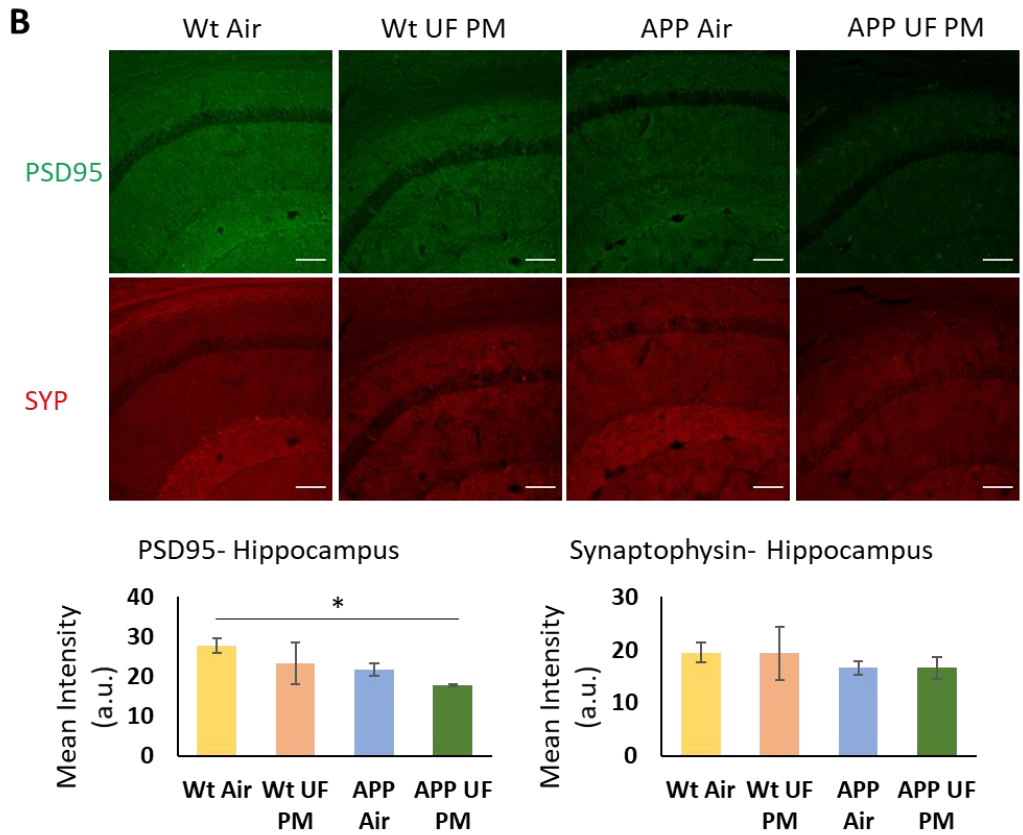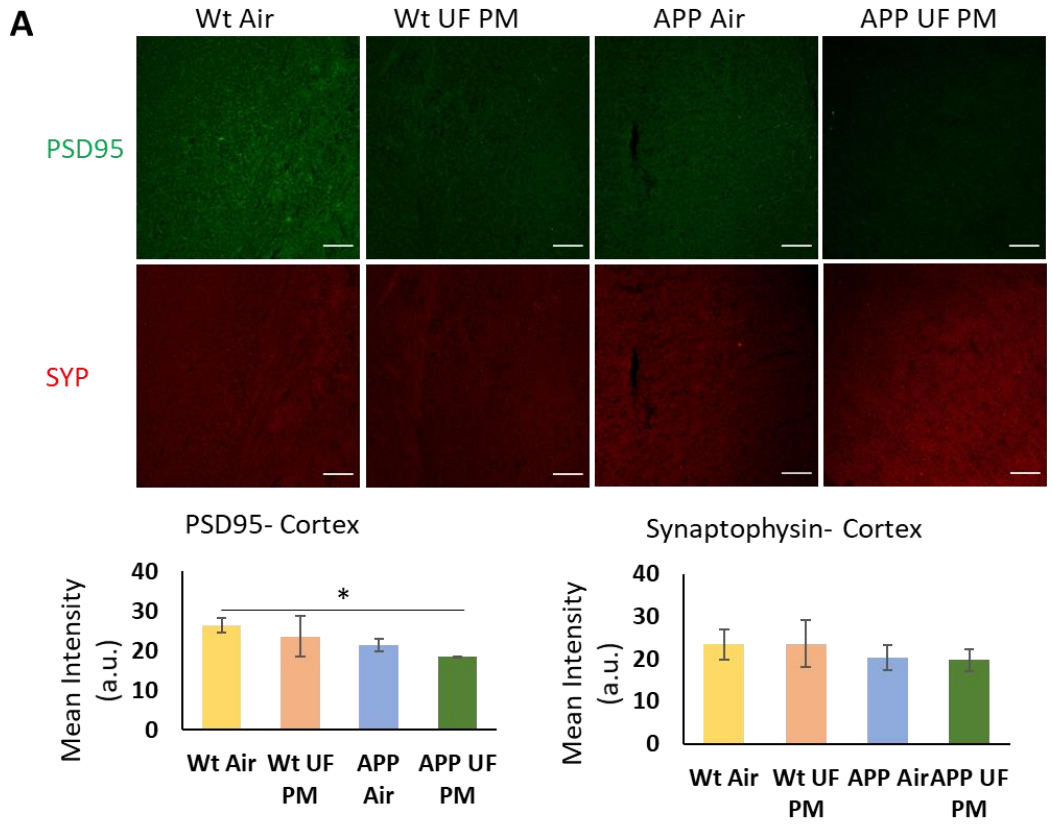
Figure 2: Synaptic marker PSD95 decreases in 12 month old mice with joint *in utero* UF PM exposure $App^{NL\text{-}G\text{-}F/+}$-KI genotype. (A) Representative images of PSD95 (green) and synaptophysin (red) immunofluorescent staining taken from the parietal cortex with fluorescent intensity quantification from the whole cortex. PSD95 is decreased in the $App^{NL\text{-}G\text{-}F/+}$-KI (APP) animals exposed to UF PM *in utero* as compared to wild-type (Wt) animals exposed to filtered air. (B) Representative images of PSD95 (green) and synaptophysin (red) immunofluorescent staining and fluorescent intensity quantification for the CA1 region of the hippocampus. As in the cortex, PSD95 is decreased in the $App^{NL\text{-}G\text{-}F/+}$-KI animals exposed to UF PM *in utero* as compared to wild-type animals exposed to filtered air. (C) Representative western blot of synaptophysin and PSD95 from cortical tissue samples with band intensity relative to tubulin. (D) Representative western blot of synaptophysin and PSD95 from hippocampal tissue with band intensity relative to tubulin. Bars represent mean values, with error bars expressed as standard error of the mean (N = 4 animals per group. Scale bars = 100 μm). * denotes p value < 0.05.

We examined neuroinflammation by detecting GFAP+ astrocytes, Iba1+ microglia, and CD68 as a marker of autophagosomes to quantify the phagocytic capacity of microglia in the brain (Fig. 3). Total Iba1 signal was not significantly changed by genotype or exposure status in the cortex or hippocampus, however the count of reactive microglia, described here as micro glia with co-expression of CD68 and Iba1, was increased comparing the wild-type air exposed animals to the other three groups in the cortex (Fig. 3A). In the cortex the number of reactive microglia increased by 31% comparing exposure groups within the wild-type genotype (p<0.01), 36% comparing air exposed wild-type to the $App^{NL\text{-}G\text{-}F/+}$-KI group (p<0.01), and 27% comparing the wild-type air group to the $App^{NL\text{-}G\text{-}F/+}$-KI UF PM group (p<0.05); there were no changes in reactive microglia count in the hippocampus. Total GFAP signal was increased 74% comparing the wild-type air exposed group to the UF PM exposed $App^{NL\text{-}G\text{-}F/+}$-KI animals in the cortex (p<0.05) and showed non-significant trends to increase comparing exposure status within genotype in the cortex (Fig. 3B). In the hippocampus there were no significant changes in GFAP signal. We also examined the total protein level of the astrocytic glutamate transporter GLT-1 in the hippocampus of these mice, as it was shown to be decreased with UF PM exposure in adult mice (Chapter 2 Fig. 13). However, in this study hippocampal GLT-1 levels were not significantly decreased with UF PM exposure (Fig. 3C). Both the PSD95 and GFAP results indicate that an additive effect may exist between UF PM exposure *in utero* and $App^{NL\text{-}G\text{-}F/+}$-KI genotype, supporting the result of the behavior data from the ORM task.

A

Iba1   CD68   Composite

Wt Air

Wt UF PM

Iba1   CD68   Aβ   Composite

APP Air

APP UF PM

Iba1- Cortex

Iba1+/CD68+ Microglia-Cortex

Iba1- Hippocampus

Iba1+/CD68+ Microglia-Hippocampus
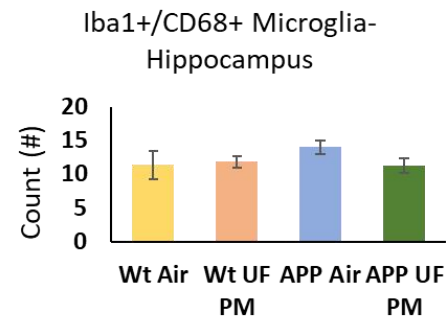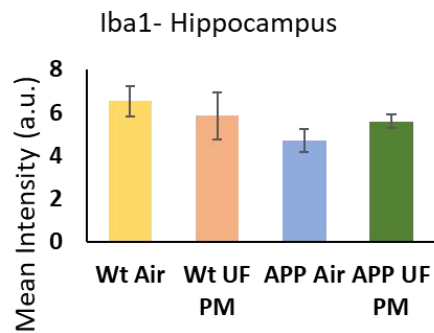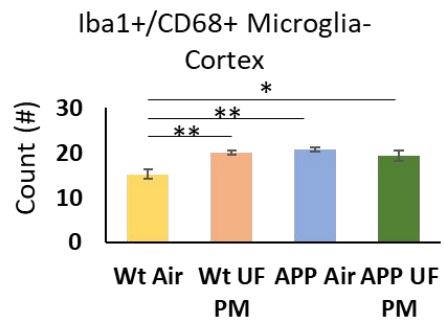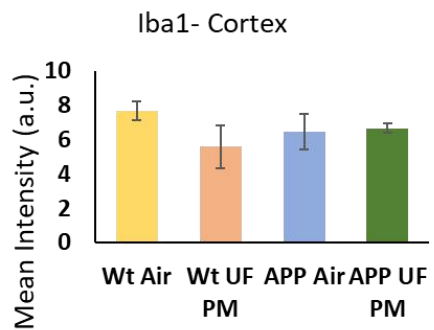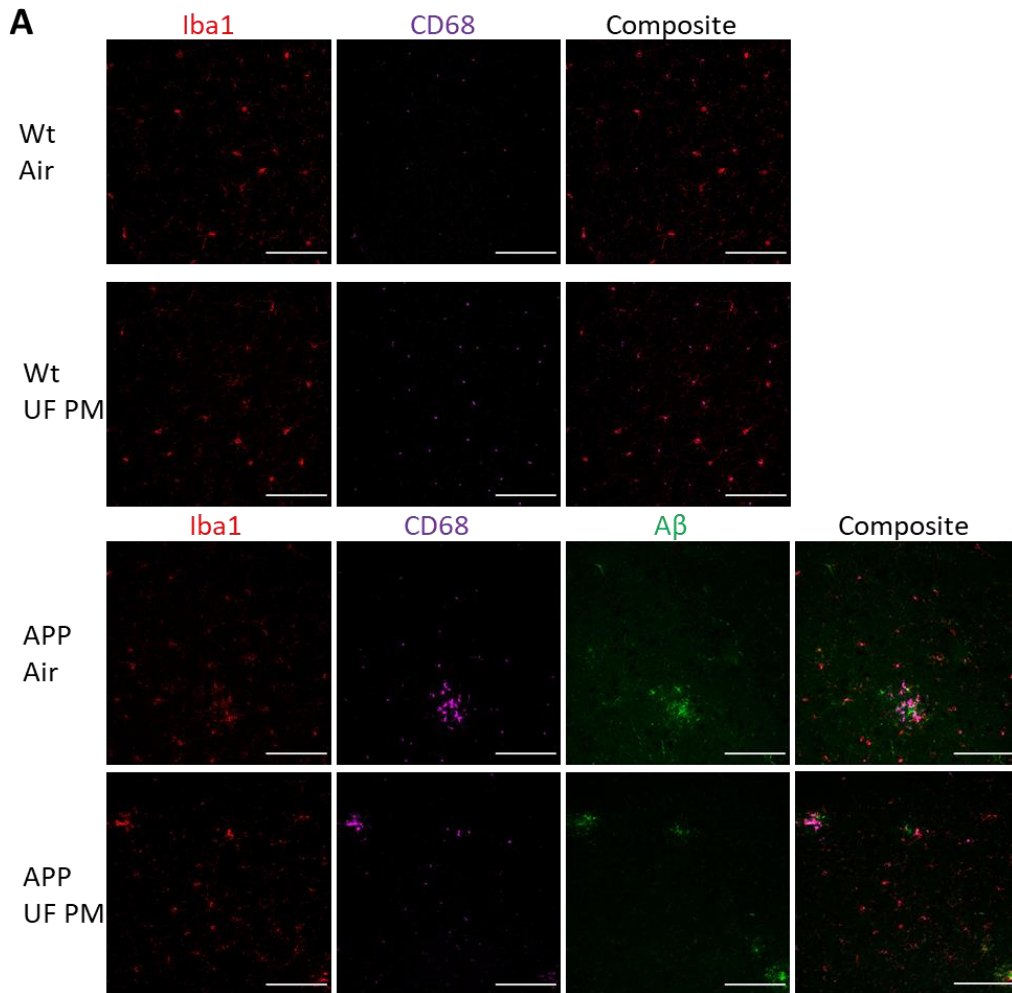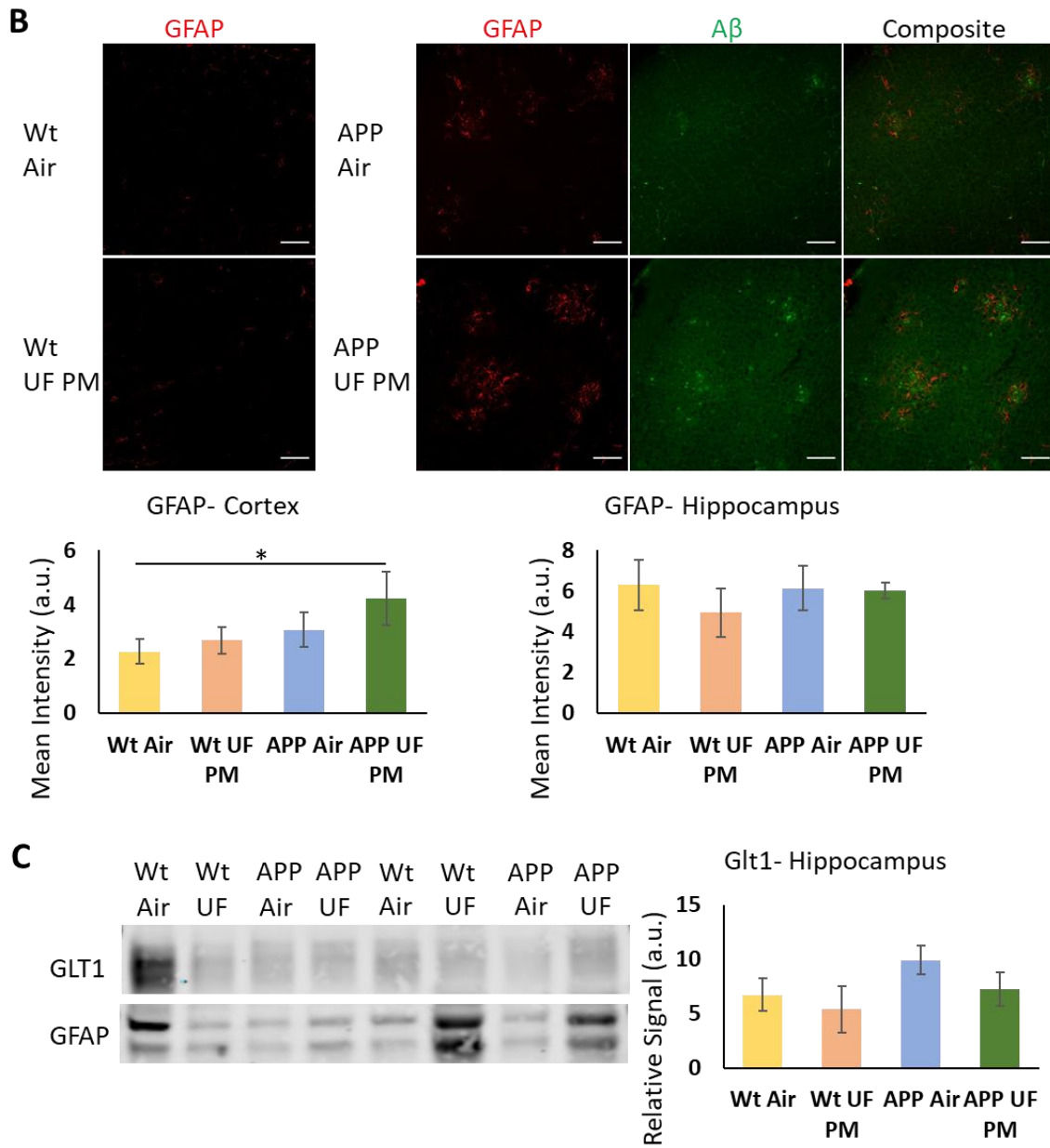
Figure 3: Glial marker GFAP increases with *in utero* exposure to UF PM in 12 month old *App*[NL-G-F/+]-KI mice. Iba1+/CD68+ microglia increase with *in utero* exposure to UF PM in 12 month old wild-type mice.
(A) Representative images of microglial marker Iba1 (red), CD68 (purple), and Aβ (green) staining taken from the parietal cortex of wild-type (Wt) and *App*[NL-G-F/+]-KI (APP) mice exposed to filtered air or UF PM, as well as quantification of the mean fluorescent intensity of Iba1 staining and total count of Iba1 and CD68 positive microglia in *App*[NL-G-F/+]-KI mice from the cortex and hippocampus. Exposure to UF PM increases the number of Iba1+/CD68+ microglia in the cortex of wild-type animals, but not in *App*[NL-G-F/+]-KI animals. (B) Representative images of astrocytic marker GFAP (red) and Aβ (green) staining taken from the parietal cortex as well as quantification of the mean fluorescent intensity of GFAP staining for the cortex and hippocampus. UF PM exposure combined with *App*[NL-G-F/+]-KI genotype increases the levels of GFAP compared to wild-type animals exposed to filtered air. (C) Representative western blot of GLT-1 in the hippocampus and quantification. No changes are seen in GLT-1 levels. (N = 4-6 animals per group for immunofluorescent staining and quantification, and N = 6 animals per group for immunoblot. Scale bars = 100 μm). * denotes p value < 0.05 and ** denotes p < 0.01.

73

3.4.3 UF PM exposure in utero increased amyloid β plaque load in adult $App^{NL-G-F/+}$-KI mice

We additionally sought to determine whether UF PM exposure *in utero* might affect amyloid pathology in the $App^{NL-G-F/+}$-KI AD model animals, which does not occur in the heterozygous model until 6-7 months of age (Saito et al., 2014) and is thus significantly removed temporally from the exposure. We found that *in utero* UF PM exposure increases the total amyloid plaque load in the cortex of 12 month $App^{NL-G-F/+}$-KI mice, but otherwise does not increase amyloid burden (Fig. 4). The total area occupied by Aβ plaques as detected by 82E1 antibody increased 90% in UF PM exposed $App^{NL-G-F/+}$-KI mice as compared to the filtered air exposed group (p<0.05), but was did not significantly increase in the hippocampus (Fig. 4A). Dense core plaque area, as detected by Thioflavin-S, did not increase in either the cortex or hippocampus (Fig. 4B). Total APP protein detected by western blot was also not significantly affected by UF PM exposure in either the wild-type or $App^{NL-G-F/+}$-KI groups (Fig. 4C). Finally, there were no changes in the detergent insoluble $Aβ_{1-40}$ or $Aβ_{1-42}$ peptide levels with UF PM in the cortex exposure either (Fig. 4D), while hippocampal insoluble and the detergent soluble fraction were below detection limits (data not shown). Together these data indicate that the changes due to *in utero* UF PM exposure may increase Aβ aggregation, but do not affect total production of APP protein or peptides.

A

APP Air | APP UF PM

Cor

HC

Aβ Plaque Burden- Cortex

Aβ Plaque Burden- Hippocampus

B

APP Air | APP UF PM

Cor

HC

ThioS Plaque Burden- Cortex

ThioS Plaque Burden- Hippocampus

C

APP- Cortex

Wt Air | Wt UF | APP Air | APP UF
APP
Tubulin

APP- Hippocampus

Wt Air | Wt UF | APP Air | APP UF
APP
Tubulin

D

Insoluble Aβ$_{42}$- Cortex

Insoluble Aβ$_{40}$- Cortex
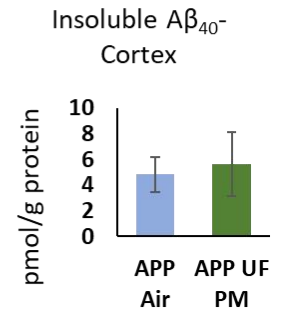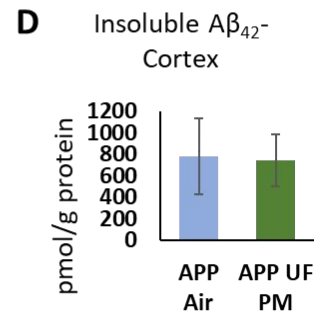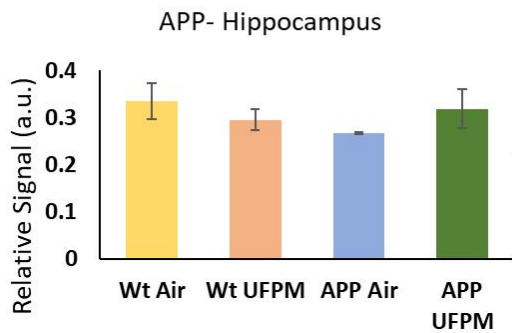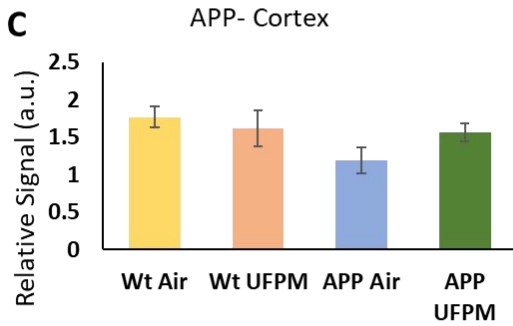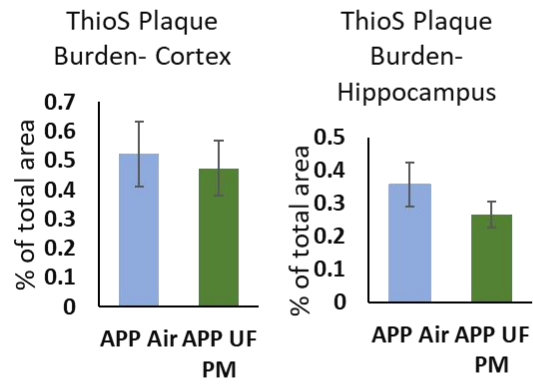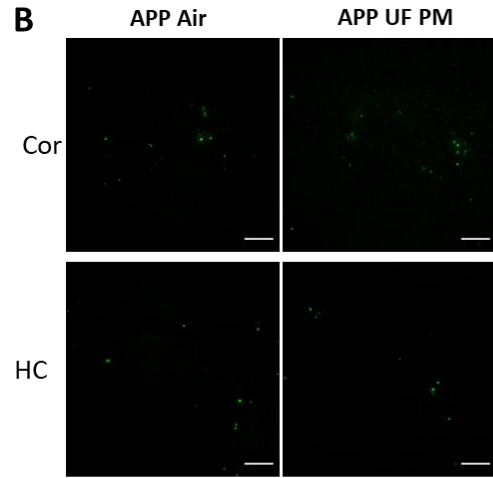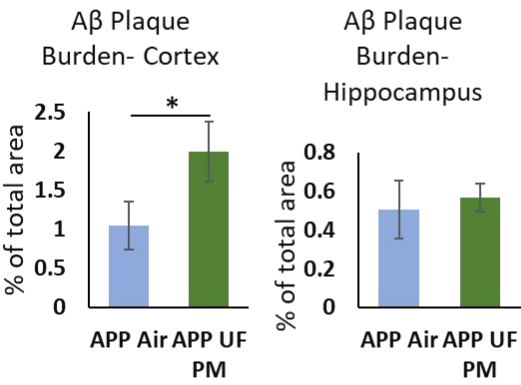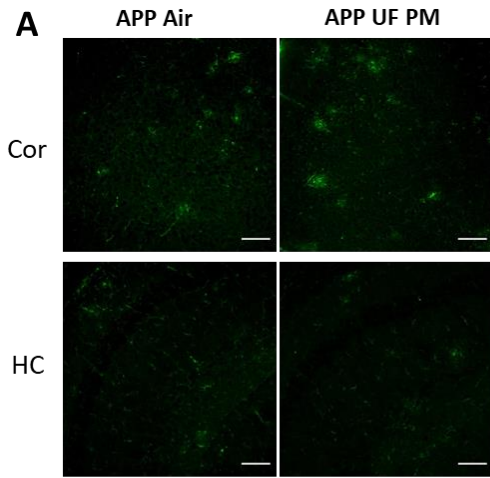
Figure 4: Aβ plaque burden is increased in the cortex by *in utero* UF PM exposure in the 12 month old $App^{NL-G-F/+}$-KI mice

Brain sections were stained with 82E1 antibody and Thioflavin-S to detect diffuse and dense core Aβ plaques in 12 month old $App^{NL-G-F/+}$-KI mice. (A) Representative images and quantification of total area of Aβ plaque burden detected by 82E1 from the parietal cortex (Cor, top) and CA1 region of the hippocampus (HC, bottom) in $App^{NL-G-F/+}$-KI (APP) mice exposed to filtered air or UF PM. Area % covered by plaques increases with UF PM exposure in the cortex, but not hippocampus. (B) Representative images of cortical and hippocampal Aβ dense core plaque burden from the parietal cortex (Cor, top) and CA1 region of the hippocampus (HC, bottom) and quantification of plaque burden for both. Dense core plaque burden does not increase with UF PM exposure. (C) Representative western blots and quantification. Steady-state levels of full length APP in tissue homogenates extracted from cortical and hippocampal tissues were not significantly affected by PM exposure *in utero*. (D) Quantification of Aβ peptides. No differences in insoluble $Aβ_{40}$ or $Aβ_{42}$ levels in $App^{NL-G-F/+}$-KI mice were found in the brain by ELISA. Bars represent mean values, with error bars expressed as standard error of the mean (N = 6 animals per group for immunofluorescent staining and quantification, and N = 4 animals per group for immunoblot, V-PLEX, and qPCR. Scale bars = 100 μm). * denotes p value < 0.05.

3.5 Discussion:

This is the first report examining the long-term adverse effects of *in utero* exposure to quasi ultrafine particulate matter on adult neuropathology and behavior in a knock-in mouse model of AD. We find that *in utero* exposed mice impair spatial memory and object recognition memory, and that synaptic proteins are decreased while markers of glial inflammation are increased. In both behavior and neuropathological changes, we find the greatest effect with joint UF PM exposure and $App^{NL-G-F/+}$-KI genotype compared to either alone. However, it remains unanswered whether this effect is due to exacerbation of the AD pathology by UF PM, or simply an additive effect of two independent insults. We additionally report that *in utero* exposure can increase Aβ plaque build-up in adult AD model mice. This result is of interest as Aβ pathology does not start in the $App^{NL-G-F/+}$-KI model until 6-7 months of age, indicating the PM exposure during development can impact processes that do not even begin until adult life. Whether this is mediated by the abnormal inflammatory activation remains to be determined. Regardless, this result suggests that early life exposure to UF PM in AD model mice affects AD outcomes in later life.

Comparing to the other known work examining *in utero* exposure on CNS changes in adult wild-type mice (Kulas et al., 2018), there are several notable differences. Kulas et al found no change in the synaptic marker PSD95 and an increase in synaptophysin when examining FVB mice. Conversely, we find no change in synaptophysin and a decrease in PSD95, but only when comparing wild-type C57BL/6J animals exposed to filtered to $App^{NL-G-F/+}$-KI mice exposed to UF PM. In both studies astrocyte marker GFAP is increased, but again only with both $App^{NL-G-F/+}$-KI genotype and UF PM exposure together in the current study. In both studies no changes where seen in total Iba1. While Iba1 and CD68 co-expressing microglia were found to be increased here with UF PM exposure in both the wild-type and $App^{NL-G-F/+}$-KI groups, they were not assessed in the Kulas et al paper. Memory impairment is associated with exposure both here and in Kulas et al, though working memory was assessed in that study compared to spatial and recognition memory in the present study. As the Kulas et al study did not use an AD model, they were unable to assess amyloid plaque pathology, but both here and in that study mouse APP protein levels were unaffected by exposure. The discrepancies between the two studies may be accounted for in the different models used or differences in exposure length, severity, and particulates. The Kulas et al study had a lower average concentration by weight at 46.7 $\mu g/m^3$ as compared to 206 $\mu g/m^3$ here, but with a longer exposure period of 30 hours per week versus 20 hours per week here. Additionally, we use UF PM of diameter less than 180 nm, while they used the fine PM fraction- PM of diameter 2.5 μm or less. Finally, the composition of airborne particulates is highly variable (Harrison & Yin, 2000; Valavandis et al., 2008), and differences in composition may account for some of the differences seen. Overall, however, these studies agree that *in utero* exposure to particulate matter can impair cognitive function and increase

inflammation in adult mice and together form a strong foundation to encourage further examination the effects of such exposure of neuropathology in disease models.

The current study is intended as a pilot to determine whether *in utero* exposure to airborne particulates can impact neuropathology that develops later in life rather than a comprehensive examination of molecular pathways. There are several limitations regarding determination of the mechanisms by which this neuropathological impact occurs. Most critically, we do not have data from the pregnant dams and the fetuses being exposed to particulates at the time of exposure. Additional research at this critical time point is required to understand which pathways are being altered and how such changes might persist into adult mice. Whether these changes are occurring by direct PM influence in the fetus or indirectly by effects on the mother, or both, is also unknown currently. Examination of the model at multiple time points is required to determine if the inflammatory changes seen are persistent starting at exposure, or only occur in aged animals. Similarly, behavioral testing at multiple ages may reveal whether the *in utero* exposure only potentiates memory deficits later in life, or if the animals are impaired at young ages as is seen in human exposure (Lertxundi et al., 2015; Porta et al., 2016). Finally, the health of the mice outside of the CNS was not assessed. Other works have indicated that *in utero* exposure can have effects in the cardio-pulmonary system and may induce high blood pressure in adult mice (Morales-Rubio et al., 2019; Rychlik et al., 2019; Tanwar et al., 2018; Ye et al., 2018), which in turn potentially affects memory ability and AD pathology (Cifuentes et al., 2015; Tucsek et al., 2017). Thus, with the current findings we are unable to determine whether the effects seen are the primary driving factors or simply derivative from potential systemic effects in the body.

Despite these shortcomings, this study provides preliminary evidence for further investigation into the effects of exposure to particulates during development on later life neuropathological outcomes in the mouse model of AD. We observe behavior deficits concurrent with changes in synaptic proteins, glial response, and Aβ build up in 12 month old adult mice with *in utero* UF PM exposure. Further study to determine the causative molecular pathways of the memory deficits in the current model, whether other dementia animal models exhibit the same vulnerability to early-life PM exposure, and whether this relationship translates to humans will help determine the contribution of *in utero* PM exposure to dementia risk later in life.

Chapter 4: Astrocyte transport of glutamate and neuronal activity reciprocally modulate tau pathology in *Drosophila*

4.1 Preamble

This work was completed at University of California, Merced, in the Quantitative and Systems Biology program and published in *Neuroscience*, vol. 348 April 2017 as Kilian et al "Astrocyte transport of glutamate and neuronal activity reciprocally modulate tau pathology in *Drosophila"* prior to transfer to University of California, Irvine and the Environmental Health Sciences program. As such, this work does not directly continue the work in air pollution and mouse models of AD presented in chapters 2 and 3. However, the nature and findings of this work focusing on glutamate transport connect directly to loss of GLT-1 seen in the adult exposure model (Chapter 2), and inform future directions I suggest in Chapter 5. Thus, I feel this work has value to be presented in the current thesis.

4.2 Abstract

Glutamate homeostasis disruption is increasingly implicated in Alzheimer's disease (AD) progression and cognitive decline. Particularly, the loss of the major astrocytic glutamate transporter, GLT-1, accelerates cognitive decline and AD pathology accumulation. Previously, we demonstrated that a restoration of GLT-1 ameliorated a buildup of tau pathology and rescued cognition in a mouse model of AD. Abnormal buildup of the microtubule associated protein tau is a major pathological hallmark of Alzheimer's disease and various tauopathies. The mechanisms by which pathological tau accumulates and spreads throughout the brain remain largely unknown. We hypothesized that aberrant extracellular glutamate and abnormal neuronal excitatory activities promoted tau pathology. In the present study, we investigated genetic interactions between tau and the GLT-1 homolog dEaat1 in *Drosophila melanogaster*.

Neuronal-specific overexpression of human wildtype tau markedly shortened lifespan and impaired motor behavior. RNAi depletion of dEaat1 in astrocytes worsened these phenotypes, whereas overexpression of dEaat1 improved them. However, the synaptic neuropil appeared unaffected, and we failed to detect any major neuronal loss with tau overexpression in combination with dEaat1 depletion. To mimic glutamate-induced aberrant excitatory input in neurons, repeated depolarization of neurons via transgenic TrpA1 was applied to the adult *Drosophila* optic nerves, and we examined the change of tau deposits. Repeated depolarization significantly increased the accumulation of tau in these neurons. We propose that increased neuronal excitatory activity exacerbates tau-mediated neuronal toxicity and behavioral deficits.

4.3 Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease associated with dementia (Reitz et al., 2011). The main pathological hallmarks of AD are the formation of amyloid plaques and neurofibrillary tangles (NFTs), respectively composed of amyloid beta (Aβ) peptides and hyperphosphorylated microtubule associated protein tau. Mutations that cause familial AD are either in the amyloid precursor protein gene or in genes of proteins critical for APP processing, supporting the hypothesis that Aβ buildup is the major upstream factor in familial AD pathogenesis (De Strooper et al., 1998; Reitz et al., 2011; Scheuner et al., 1996). Abnormal buildup of Aβ alone may not be sufficient to cause AD dementia, however, suggesting that other pathological changes, such as inflammation, tauopathy, and synaptic dysfunction are essential to development of AD (Hämäläinen et al., 2007; Jacob et al., 2007; Jay et al., 2015; Kimura et al., 2013; Roberson et al., 2007; Shipton et al., 2011; Vom Berg et al., 2012). Disruption of glutamate homeostasis is implicated in the development of AD pathology and cognitive decline (Masliah et al., 1996; Meeker et al., 2015; Mookherjee et al., 2011). Glutamate

is the main excitatory neurotransmitter of the central nervous system. Its clearance from the

extracellular space and its recycling is regulated by five glutamate transporters in humans.

Excitatory amino acid transporter 2 (EAAT2), expressed in astrocytes, is responsible for roughly

90% of glutamate uptake in the synaptic cleft (Danbolt et al., 1992; Tanaka et al., 1997).

Reduced *EAAT2* expression in AD patients and transient hyperactivation of neurons in the

hippocampus and medial temporal lobe suggest functional impairment of glutamate clearance in

synapses (Bookheimer et al., 2000; Hämäläinen et al., 2007; Jacob et al., 2007; S. Li et al.,

1997). In addition, decreased activity of EAAT2 correlates with neuronal and synaptic loss in

AD (Masliah et al., 1996). Impairment of synaptic glutamate uptake may lead to sustained

excitatory neuronal activities and abnormal cellular signaling through extrasynaptic NMDA

receptors in neurons (Parsons & Raymond, 2014). These findings suggest glutamate-based

excitotoxicity as a potential cellular mechanism promoting AD neuropathology. In a mouse

model of AD decreased expression of GLT-1 (the mouse homolog of EAAT2) accelerates the

onset of cognitive deficits, increases pathological Aβ, and alters brain metabolic pathways

(Meeker et al., 2015; Mookherjee et al., 2011). Pharmacological upregulation of GLT-1 rescues

AD pathology, reducing amyloid plaques, pathological tau accumulation, synaptic loss, and

cognitive deficiency (Takahashi et al., 2015; Zumkehr et al., 2015). The connection between

GLT-1 loss and tau may be self-reinforcing, as astrocytic expression of human tau in a mouse

model reduces GLT-1 function and GLT-1 levels, and vesicular glutamate transport is altered in

human tau expressing mice (Dabir et al., 2006; Hunsberger et al., 2015). Additionally, non-AD

tauopathies also exhibit neurodegeneration and dementia in humans without Aβ buildup

(Ballatore et al., 2007; Wolfe, 2012), linking synaptic dysfunction and tau pathology as Aβ

independent pathways leading to dementia in AD. The complete underlying molecular

mechanisms by which abnormal synaptic function and tau pathology in AD remain incompletely understood.

To better understand the molecular link between the glutamate transporter and tau pathology we used *Drosophila melanogaster* as a model organism. *Drosophila* provides an alternative to the mouse model for genetic disease studies. *Drosophila* is widely used to identify the molecular basis of neurodegenerative diseases, including AD and tauopathies (Lu & Vogel, 2009; McGurk et al., 2015; Prüssing et al., 2013). Particularly, *Drosophila* has been used to investigate AD-related mechanisms of tau pathology exacerbation, including the role of the GSK-3β pathway in tau phosphorylation, the negative effects of phosphorylated tau on normal tau, and the AD risk gene *BIN1*'s effect in modulating tau pathology (Chapuis et al., 2013; Chatterjee et al., 2009; Cowan et al., 2010; Jackson et al., 2002). *Drosophila* expresses two glutamate transporters, the human EAAT2 homolog dEaat1, and dEaat2, with dEaat1 being the only high affinity glutamate transporter in *Drosophila* (Besson et al., 2000, 2011). Here, we decreased and increased the astrocytic expression of dEaat1 and examined behavioral and pathological impacts on *Drosophila* expressing human wildtype 2N4R tau. Our results provide evidence for a role of astrocytic glutamate transport reduction and glutamate-induced excitatory signal in the overall phenotypes and the progression of tau pathology.

4.4 Materials and Methods:

4.4.1 Fly Stocks: Fly stocks were maintained on cornmeal, molasses, and agar food at 25°C and 70% humidity. cDNA from the 2N4R human tau isoform was subcloned into a modified P{13XLexAop2-IVS-myr::GFP} plasmid (Addgene) (Pfeiffer et al., 2010). Microinjection of the *lexAop-tau^{wt}* 2N4R was carried out by BestGene Inc. (Chino Hills, CA). *UAS-dEaat RNAi* flies have been previously described (Rival et al., 2004). *alrm-Gal4* flies were obtained from

83

Marc Freeman at the University of Massachusetts Medical School (Doherty et al., 2009). Other transgenic flies were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN): *UAS-tau^{wt}* 1.13 (51362), *nsyb-LexA* (56166), *GMR-Gal4* (1104), *UAS-dEaat1* (8202), and *UAS-dTrpA1* (26263).

4.4.2 Lifespan Analysis: Groups of ten recently eclosed adult male flies were placed in a standard food vial without yeast and kept at 25°C. The flies were transferred to a new vial three times a week without anesthesia, and the number of surviving flies were counted. Each genotype was tested with total n >= 100.

4.4.3 Negative Geotaxis Climbing Assay: To assess motor ability and geotaxis response a climbing assay as described by Ali *et al.* (2011) was used. Ten male flies were placed into food vials via CO_2 anesthesia and allowed to recover for at least 24 hours. A simple apparatus was prepared consisting of two empty polystyrene vials. One vial was marked 8 cm from the bottom using a black marker. Flies were transferred to one of the vials without anesthesia, and the two vials were taped together open end to open end and placed upright. The flies were allowed five minutes to acclimate. Flies were tapped to the bottom of the marked tube, and both the number of flies that reach the 8cm mark within 10 seconds and the total amount of time, in seconds, that was required for half of the flies to cross the 8 cm mark was recorded. This process was performed a total of ten times per cohort, with one minute between trials. All flies were tested three days after eclosion.

4.4.4 Immunohistochemistry: Brains from adult flies 10-12 days after eclosion were dissected in PBT (Phosphate buffered saline + 0.05% Tween-20) and fixed in 2% paraformaldehyde in PBT at 4°C for 16 hours (J. S. Wu & Luo, 2006). After washing in PBT and blocking in PBT plus 0.5% BSA and 5% normal goat serum, primary then secondary antibodies were incubated

with the fixed brains for 24 hours, with PBT washes in between incubations. Brains were

mounted in Vectashield (Vector Laboratories). Images were obtained on a Nikon Eclipse Ti

C1® confocal microscope using the 40x oil immersion objective or Leica TCS SPE confocal

microscope using the 10x objective. Images were analyzed with Image J software version 1.47.

Antibodies used were rabbit anti-human tau (Dako) 1:200, mouse PHF-1 (phosphorylated tau at

S396/S404; from Dr. Peter Davies, Albert Einstein College of Medicine) 1:1000, rabbit anti-

tyrosine hydroxylase (T8700, Sigma) 1:100, and mouse anti-discs large (4F3, Developmental

Studies Hybridoma Bank at University of Iowa) 1:100.

4.4.5 Immunoblot: For sarkosyl insoluble protein extraction 200 heads from adult flies 1 and 12

days after eclosion were homogenized in 15 mM NaCl, 25 mM Tris-HCl at pH 7.4, 1mM EGTA,

1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, and 1x protease inhibitor cocktail (complete™

Roche). The homogenate was quickly centrifuged to remove debris, and then centrifuged for 1

hour at 100,000 x g. The supernatant was saved as the soluble fraction. The pellet was re-

suspended in 10% sucrose, 0.8 M NaCl, 10 mM Tris-HCl at pH 7.4, 1 mM EGTA, 1 mM

Na_3VO_4, 50 mM NaF, and 1x protease inhibitor cocktail, and centrifuged for 30 minutes at

15,000 x g. Sarkosyl was added to 1% and incubated at 37°C for 1 hour, then centrifuged for 2

hours at 100,000 x g. The sarkosyl insoluble pellet was resuspended in 15 µL homogenization

buffer. Soluble protein fraction concentration was quantified by the Bradford protein assay (Bio-

Rad). Antibodies used were mouse AT8 (phosphorylated tau at S202/T205, MN1020,

ThermoFisher) 1:2000, mouse CP13 (phosphorylated at S202/T205; from Dr. Peter Davies,

Albert Einstein College of Medicine), 1:1000, rabbit anti-human tau antibody (Dako) 1:1000,

mouse anti-discs large (4F3, Developmental Studies Hybridoma Bank at University of Iowa)

1:100, mouse anti-alpha-tubulin 1:25000 (T6074, Sigma), and mouse PHF-1 1:1000. Sarkosyl

insoluble fraction extraction was performed as described (Colodner & MB., 2010; Goedert et al., 1992). Band intensities were analyzed with an Odyssey image station and Image Studio software v2.1.10 (Licor).

4.4.6 Eye Morphology: The temperature sensitive cation channel TrpA1 was used to stimulate *Drosophila* optic neurons under the glass multiple reporter (GMR-Gal4) driver. All flies were temperature treated to activate TrpA1 at 31°C for 2 hours/day for five consecutive days. Images were captured with a Nikon SMB-2Z dissecting microscope and digital camera. Images were taken from adult male flies 20-22 days after eclosion. Immunohistochemistry was done on flies aged 20-22 days after eclosion.

4.4.7 Statistical Analysis: Lifespan data were analyzed using the log-rank test, with $p < 0.05$ considered significant. One way ANOVA with Tukey post hoc testing for more than 2 groups and unpaired *t*-test for 2 groups were used to test for significance for negative geotaxis and immunofluorescent intensity data, and $p < 0.05$ was considered to be significant. The chi squared goodness of fit test was used to determine normality.

4.5 Results

4.5.1 dEaat1 expression modulates lifespan and behavioral phenotypes Drosophila
   overexpressing human tau

Flies expressing the longest human tau isoform, 2N4R, were used to assess the effects of human tau in the *Drosophila* brain. The 2N4R isoform contains all terminal inserts and microtubule binding repeats present in the six isoforms of tau, ensuring that domain-specific interactions can be observed. Human wildtype 2N4R tau (*LexAop-htau*) was overexpressed in all neurons using LexA/LexAop binary expression system with *nsyb-LexA*. *nsyb>tau* flies showed reduced life-

span compared to control (*nsyb>+*) flies (Figure 1A), consistent with previous reports for the 0N4R human tau isoform (Wittmann et al., 2001). Deficiency of dEaat1 by RNAi in astrocytes has previously been achieved using the GAL4/UAS binary expression system with the *alrm-Gal4* astrocyte-specific driver (*alrm>Eaat1.IR*), and these flies exhibited reduced lifespan, as previously reported (Rival et al., 2004). Combined neuronal tau overexpression and astrocytic dEaat1 depletion (*alrm>Eaat1.IR;nsyb>tau*) resulted in further reduced survival than expression of either transgene alone. Conversely, overexpression of *dEaat1* in astrocytes (*alrm>Eaat1*) did not affect lifespan (Figure 1A). Importantly, *dEaat1* overexpression in human tau expressing flies (*alrm>Eaat1;nsyb>tau*) significantly rescued lifespan. These data demonstrate that the functional consequences of neuronal tau depends on glutamate homeostasis by astrocytes.

Neuronal tau expression decreases motor function in *Drosophila* (Ali et al., 2011). We assessed performance in a startle-induced negative geotaxis climbing assay to determine if loss of dEaat1 exacerbates this phenotype. *nsyb>tau* flies showed reduced climbing (Figure 1B), as previously reported (Ali et al., 2011). *alrm>Eaat1.IR* flies exhibited a seizure-like response reaction to the initial mechanical impulse, as documented (Rival et al., 2004), which greatly reduced climbing immediately after the impulse compared to *nsyb>+* animals. The *alrm>Eaat1.IR;nsyb>tau* flies were indistinguishable from *alrm>Eaat1.IR* flies.

Due to the seizure-like reaction that interfered with the immediate climbing response in *alrm>Eaat1.IR* flies, we also measured negative geotaxis following a prolonged recovery (Figure 1C). All experimental genotypes, with the exception of *alrm>Eaat1*, showed a reduction of climbing compared to *nsyb>+*. While *alrm>Eaat1.IR;nsyb>tau* flies exhibited reduced climbing as compared to *alrm>Eaat1.IR* flies, *alrm>Eaat1.IR;nsyb>tau* flies did not reduce climbing compared with *nsyb>tau* flies. Interestingly, increased geotaxis was observed in

alrm>Eaat1;nsyb>tau flies as compared to *nsyb>tau* flies. While decreased dEaat1 did not exacerbate the impairment of negative geotaxis, increased dEaat1 partially rescued geotaxis response in human tau expressing flies. Together with the lifespan analysis, this indicates that dEaat1 moderates tau pathology.
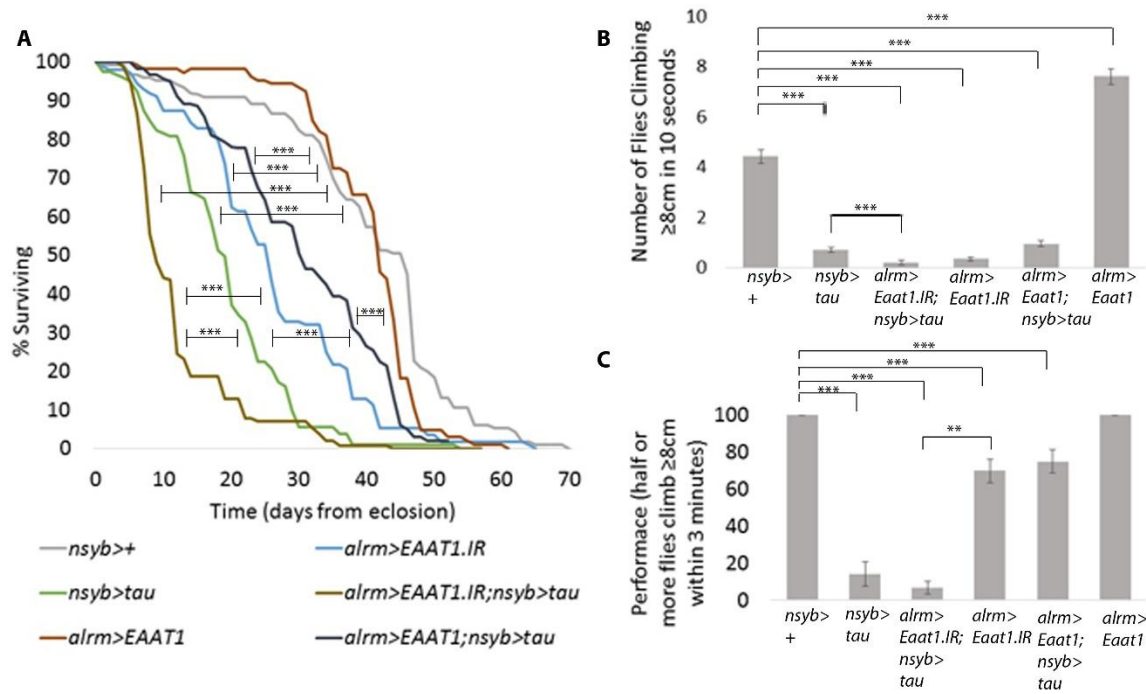
Figure 1: RNAi decreased dEaat1 worsens and overexpressed dEaat1 partially rescues tau phenotypes. (**A**) Survival curve of *nsyb>+*(nSyb-LexA/+), *alrm>Eaat1.IR* (*UAS-Eaat1.dsRNA/Y;alrm-Gal4/+*), *nSyb>tau* (*nSyb-LexA/+;LexAop-htau/+*), *alrm>Eaat1.IR;nsyb>tau* (*UAS-Eaat1.dsRNA/Y;nSyb-LexA/alrm-Gal4;LexAop-htau/+*) flies, *alrm>Eaat1* (*alrm-Gal4/+;UAS-Eaat1/+*), and *alrm>Eaat1;nsyb;tau* (*nsyb-LexA/alrm-Gal4;LexAop-htau/UAS-Eaat1*) flies. By log rank test *alrm>Eaat1.IR* (p<0.001), *nSyb>tau* (p<0.001), *alrm>Eaat1.IR;nsyb>tau* (p<0.001), and *alrm>Eaat1;nsyb;tau* (p<0.001) show reduced lifespan compared to the control. *alrm>Eaat1.IR;nsyb>tau* flies have reduced lifespan as compared to both *alrm>Eaat1* (p<0.001) and *nsyb>tau* (p<0.001). *alrm>Eaat1;nsyb>tau* flies show increased lifespan compared to *nsyb>tau,* but reduced compared to *alrm>Eaat1*. n ≥ 100 flies per genotype. (**B**) Climbing response, determined by the number of flies that climb ≥ 8 cm in 10 seconds after mechanical impulse. Flies 3 days after eclosion were used to minimize age dependent performance decreases. *alrm>Eaat1.IR* (p<0.001), *nSyb>tau* (p<0.001), *alrm>Eaat1.IR;nsyb>tau* (p<0.001), and *alrm>Eaat1;nsyb;tau* (p<0.001) exhibited reduced response compared to *nsyb>+*, while *alrm>Eaat1* showed increased response (p<0.001). *alrm>Eaat1.IR;nsyb>tau* flies perform worse than *nsyb>tau* flies (p<0.01). n>= 5 independent biological replicates, with 10 flies per replicate. (**C**) Geotaxic response of control and test flies, expressed as a percentage of trials where 50% or more of the flies climbed ≥ 8 cm by 180 seconds. n>=5. Each bar is the mean ± the standard error of the mean. ** p≤0.01, *** p≤0.001.

4.5.2 Expression of hTau or loss of Eaat1 does not significantly alter overall synaptic or

neuronal loss in Drosophila brain

Extensive neuronal loss has previously been reported in human tau overexpressing *Drosophila* models (Jackson et al., 2002; Wittmann et al., 2001). We attempted to detect any synaptic and neuronal loss in our *Drosophila* models as a pathological outcome using various techniques. However, quantification of the post-synaptic density marker DLG1 detected no significant differences comparing *nsyb>+* to either *nsyb>tau*, *alrm>Eaat1.IR*, or *alrm>Eaat1.IR;nsyb>tau* fly brains in whole brains imaging (Figure 2A and B) or protein quantification (Figure 2C and 2D). Similarly, TUNEL or active caspase-3 staining did not show significant difference among these groups (data not shown). As small scale neuronal loss may be difficult to detect in whole brain assays, we next attempted to stain a well-defined subpopulation of neurons. Dopaminergic neurons in the dopaminergic PPL1 cluster were significantly reduced in *nsyb>tau* flies compared to *nsyb>+* control group (Figure 2E and F). The loss of dEaat1 in tau flies (*alrm>Eaat1.IR;nsyb>tau*) further reduced the number of dopaminergic neurons in the PPL1 cluster (Figure 2E and F). The overexpression of *dEaat1* in tau flies (*alrm>Eaat1;nsyb>tau*) significantly rescued the number of dopaminergic neurons from (*alrm>Eaat1.IR;nsyb>tau* group but not *nsyb>tau* group (Figure 2E and F).
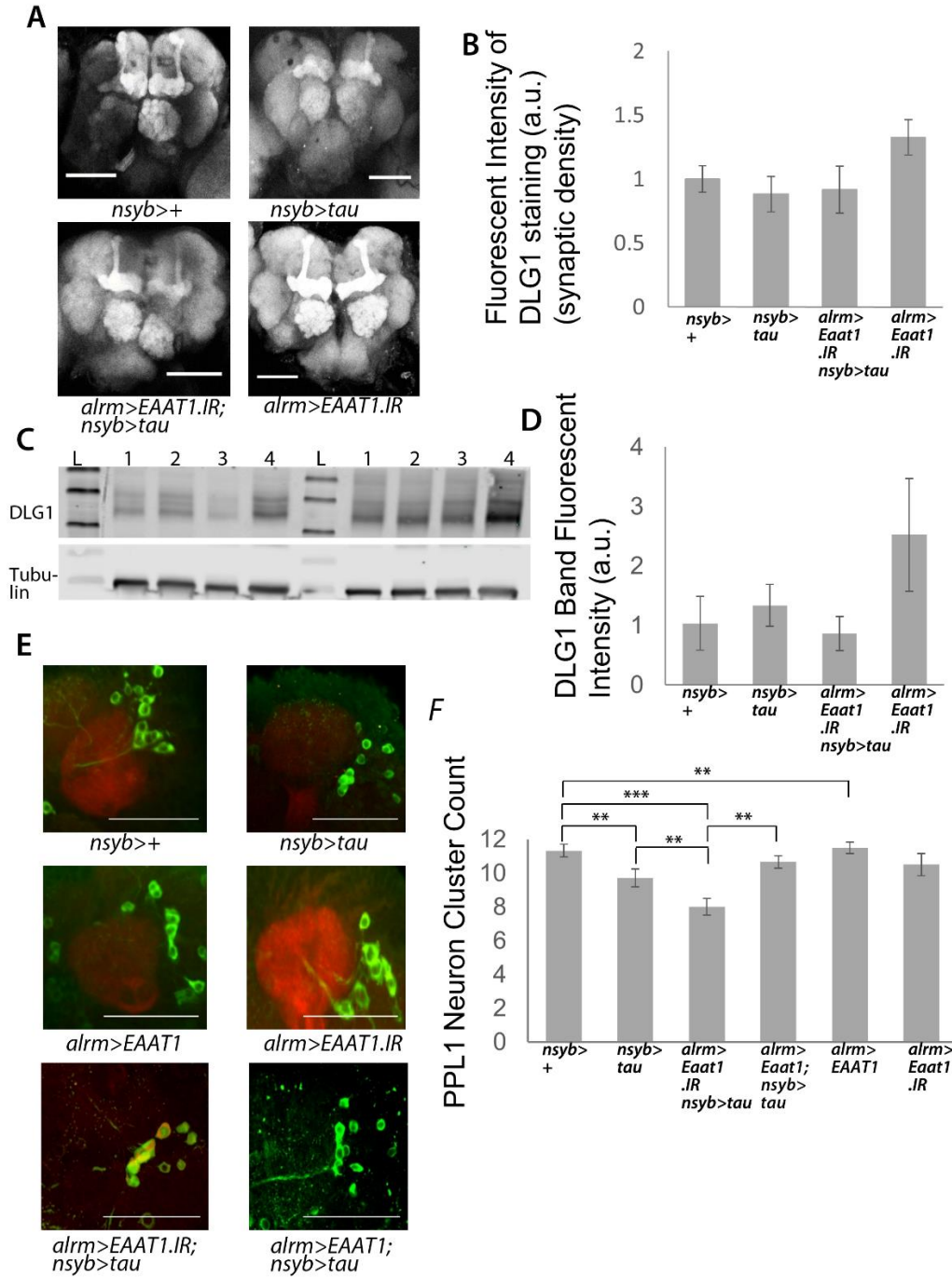
Figure 2: Human Tau expression shows no general neuronal loss but does induce reduction in a limited neuronal subset. (**A**) Representative images of adult fly brains immunostained for the post-synaptic protein DLG1 for *nsyb>+*, *nsyb>tau*, *alrm>Eaat1.IR;nsyb>tau*, and *alrm>Eaat1.IR* flies. (**B**) Fluorescent quantification of total synaptic density in the central brain and protocerebrum structures by DLG1 staining of adult flies 12 days after eclosion. No differences were found between experimental genotypes. n = 5. (**C**) Representative western blot bands for DLG1 from whole brain homogenate with tubulin loading control. L- molecular weight ladder 1- *nsyb>+* 2- *nsyb>tau* 3- *alrm>Eaat1.IR;nsyb>tau* 4- *alrm>Eaat1.IR*. (**D**) Densiometric quantification of DLG1 western blot band intensity normalized to tubulin loading control. n = 4. (**E**) PPL1 dopamine neuron cell bodies detected with anti-tyrosine hydroxylase (green) and anti-DLG1 (red). (**F**) Number of PPL1 cell bodies in adult flies 12 days after eclosion. n=4-9. Each bar is the mean ± the standard error of the mean. ** p≤0.01, *** p≤0.001. Scale bars 100 μm.

4.5.3 Changes in dEaat1 glutamate transporter expression do not change the accumulation of insoluble tau

Quantification of total and phosphorylated tau levels was performed by western blot using both the soluble fraction and sarkosyl insoluble fraction from adult flies approximately 12 days after eclosion. There was no detectable shift in the amount of total tau present in *nsyb>tau*, *alrm>Eaat1.IR;nsyb>tau*, or *alrm>Eaat1;nsyb>tau* fly heads in either total protein extraction or the sarkosyl insoluble protein fraction (Figure 3A). Similarly, no change in the relative burden of phosphorylated tau species was detected (Figure 3B). This suggests that the observed pathology is not dependent on tau levels.
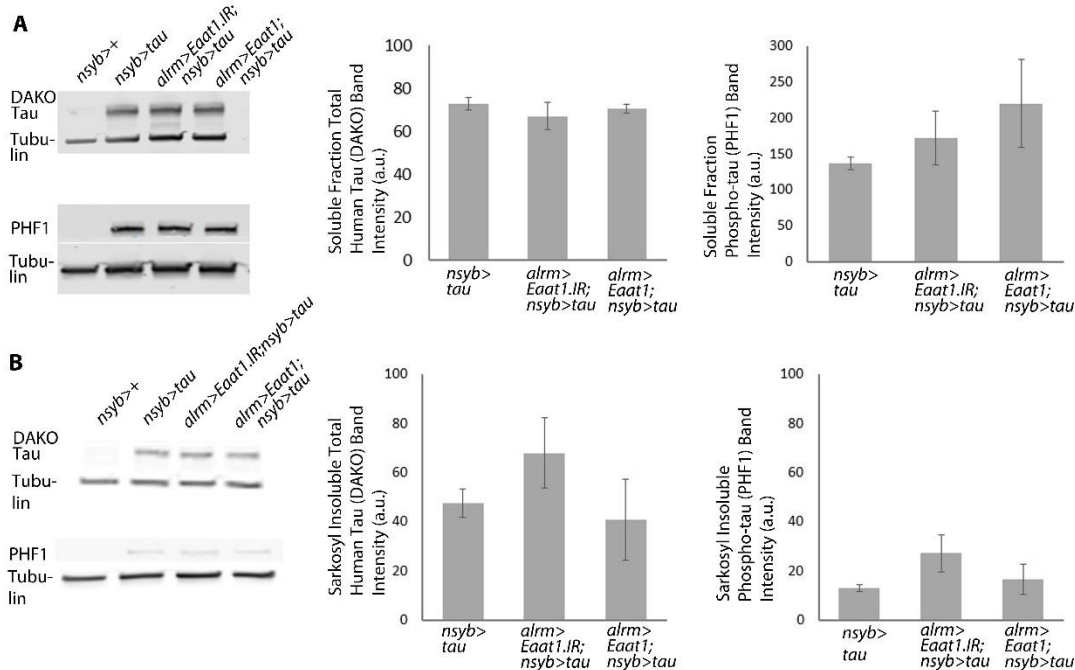
Figure 3: Dysregulation of astrocytic dEaat1 does not affect human tau accumulation. Protein samples taken from flies 10-12 days after eclosion, matching with the 50% surival point for the most severe phenotype (*alrm>Eaat.IR;nsyb>tau*). (A) Representative western blot bands for soluble fraction human total (DAKO antibody) tau and phospho-tau (PHF1 antibody), with tubulin antibody for load control, of *nsyb>+*, *nsyb>tau,* *alrm>Eaat1.IR;nsyb>tau,* and *alrm>Eaat1;nsyb>tau* whole brain homogenate protein samples. Quanification of band intensity normalized to tubulin loading control. No difference between genotypes was found using two-way ANOVA. (B) Representative western blot bands for sarkosyl insoluble fraction total tau (DAKO antibody) and phospho-tau (PHF1 antibody) and quanification by band intensity. No difference between genotypes was found using two-way ANOVA. n=3-4. Each bar is the mean ± the standard error of the mean.

4.5.4 Repeated overstimulation of neurons in the Drosophila eye increases tau load

To test if increased neuronal activity promotes tau pathology, we co-expressed the temperature-sensitive cation channel, TrpA1, with human wildtype tau in neurons in the fly eye. Human tau expression causes reduced eye size, a rough eye surface, and degeneration of the optic lobes (Jackson et al., 2002; Wittmann et al., 2001). Using GMR-Gal4, we generated flies expressing human tau (*GMR>tau*), TrpA1 (*GMR>TrpA1*), and the combination (*GMR>TrpA1,tau*). These flies were repeatedly exposed to TrpA1 activation conditions. At 3 weeks after eclosion, treated *GMR>tau* and *GMR>TrpA1,tau* showed a reduction in eye size and a rough eye phenotype compared to *GMR>TrpA1* flies; there was no difference between *GMR>tau* and *GMR>TrpA1,tau* (Figure 4A). Repeated neuronal activation lead to a marked increase of tau positive cells in the optic lobe (Figure 4B, 4C). Importantly, there was no difference in tau accumulation between *GMR>tau* and *GMR>TrpA1,tau* control flies without temperature activation of TrpA1 (Figure 4D). Temperature activated *GMR>TrpA1,tau* flies exhibited increased levels of total tau compared to the *GMR>TrpA1* flies, but not *GMR>tau* flies (Figure 4E). The accumulation of phosphorylated tau levels were also significantly increased with temperature activation of TrpA1 in *GMR>TrpA1,tau* flies as compared to *GMR>tau* flies (Figure 4F). Together, these results suggest that tau accumulation is increased by increased neuronal activity.
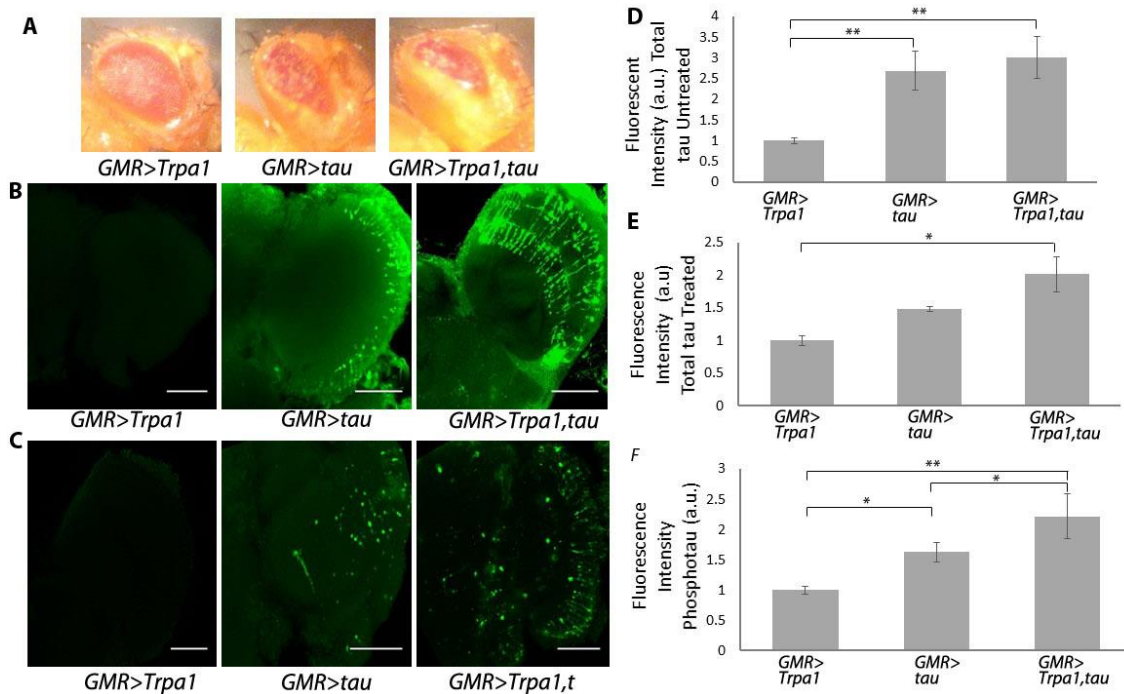
Figure 4: Chronic activation of neurons in the eye increased tau accumulation. (A) Brightfield images of the *Drosophila* eye of *GMR>TrpA1* (*GMR-Gal4/+;UAS-TrpA1/+*), *GMR>tau* (*GMR-Gal4/UAS-Tau^{wt}1.13*), and *GMR>TrpA1,tau* (*GMR-Gal4/UAS-Tau^{wt}1.13;UAS-TrpA1/+*) genotypes. *GMR>tau* and *GMR>TrpA1,tau* show roughness and reduced eye size. (B) Representative staining of *Drosophila* optic lobe neurons for human total tau of *GMR>TrpA1*, *GMR>tau*, and *GMR>TrpA1,tau* genotypes. (C) Representative staining of Drosophila optical lobe neurons for p-tau. (D) Fluorescence intensity quantification of tau immunofluorescence in the optic lobe in non-temperature treated *GMR>TrpA1*, *GMR>tau*, and *GMR>TrpA1,tau* flies. (E) Fluorescence intensity quantification of total human tau based immunofluorescence. (F) Fluorescence intensity quantification with p-tau antibody of optic lobes. N= 4-5. Each bar is the mean ± the standard error of the mean. * p≤0.05, ** p≤0.01. Scale bars 50 µm.

4.6 Discussion

We report that glutamate transporter dEaat1 levels in a *Drosophila* model of tauopathy moderate reduction in lifespan and geotaxis. However, we failed to detect any significant pathological changes, including overall synaptic or neuronal loss and buildup of tau in the brain. Although our models showed a lack of definitive association between phenotypes and the CNS pathology, the importance of tau and its neurotoxicity has been reported in several Drosophila models (Jackson et al., 2002; Wittmann et al., 2001) with shortened lifespan following the introduction of human tau gene, similar to our current finding. In this study, we also report that changes in

neuronal excitation frequency by temperature-sensitive TrpA increase tau load, suggesting a potential avenue for the link between the loss of dEaat1, glutamate-induced excitotoxicty, and the buildup of tau in neurons.

The discrepancy between results in our dEaat1 models and TrpA1 model may be in part due to technical limitations to detect human tau in these flies. We failed to detect tau in our newly generated human wildtype overexpressing fly brains by immunostaining using various tau antibodies in the LexA-LexAop driver system (data not shown). As a result, we were unable to examine the effect of dEaat1 on the localization or intensity of tau. Drosophila tau protein dTau, a homolog of human tau, is potentially a complicating factor in interpreting results. However, it has been found that tau protein toxicity is mainly mediated by soluble phosphorylated human tau even when dTau is present in the *Drosophila* model (Feuillette et al., 2010), suggesting that the effects of dTau on pathology in this model are minimal. While increased levels of phosphorylated tau or insoluble tau are widely accepted pathological phenomena, mislocalization of tau in synapses and somatodendritic compartments equally impacts neuropathological and phenotypic alterations (L. M. Ittner et al., 2010). Migration and propagation of tau in neurons when glutamate transporter activity is reduced in astrocytes needs to be further investigated, and adverse effects of tau and dEaat1 dysregulation must be considered regarding exacerbation of pathological phenotypes. The ability to rescue tau phenotypes by overexpression of dEaat1, along with results from our group using ceftriaxone in a mouse model of AD (Zumkehr et al., 2015), strongly implies that glutamate transport deficiency plays a pivotal role in tau pathology. Investigation of vesicular glutamate transport, which has been shown to be increased in conjunction with reduced GLT-1 levels in human tau expressing mice (Hunsberger et al., 2015),

in this model may elucidate the interaction between tau pathology and glutamate transport observed.

Recent findings clearly demonstrate the ability of tau to spread between anatomically connected neurons (De Calignon et al., 2012; L. Liu et al., 2012; Martin et al., 2013; J. W. Wu et al., 2013, 2016). While the underlying mechanisms are currently unknown, the release of tau may depend on neuronal activity (Pooler et al., 2013), exosomes (Asai et al., 2015), or both. These new findings help to better understand the development and progression of tau pathology in AD and tauopathies. Our data support that neuronal over stimulation promotes tau burden, suggesting the possibility that initial tau pathology leads to over-excitation, which in turn leads to propagation of tau pathology to proximal neurons. In all, it is suggested that initial tau pathology triggers glutamate dysregulation, which is then involved in the spread and continuation of further tau pathology by inducing neuronal over stimulation. However, direct excitation by TrpA1 channel induction may provide a stronger stimulus than glutamate based over-excitation, and further study is needed to confirm that reduction of glutamate clearance induces neuronal activity in a manner similar to TrpA1 induction in *Drosophila*. Additionally, the acute treatment regimen used here may poorly reflect chronic over-excitation potentially found with glutamate transporter malfunction. Further study using spatially limited neuronal tau expression may determine if the increase in tau load and tau spread seen by TrpaA1 activation can be replicated using the glutamate transporter knockdown model, as tau spread may be masked under a pan neuronal expression pattern as used here. Extensive neuronal and synaptic loss is a well-documented characteristics in AD (Scheff et al., 2006; Terry et al., 1991), and is also observed in mouse models (L Mucke et al., 2000; Oddo et al., 2003). In our model, we were unable to detect wide scale loss of neurons or a major change in the synaptic neuropil concurrent with tau expression

95

or reduced dEAAT1 expression. As the time point chosen for analysis coincides with the 50%

lethality point of *alrm>Eaat1.IR;nsyb>tau* flies, this indicates that general synaptic loss may not

be a primary cause of early lethality. We considered the possibility that smaller scale neuronal

loss, not detectable by general synaptic density assays, accounts for some phenotypic change.

Loss of neurons in the PPL1 cluster does occur in *nsyb>tau* flies, and is exacerbated in

alrm>Eaat1.IR;nsyb>tau flies, indicating the possibility of low levels of neuronal toxicity.

Interestingly, the PPL1 cluster is dopaminergic, and not directly affected by dEaat1 loss.

Although Drosophila uses cholinergic neurotransmission as excitatory input more than

glutamatergic neurotransmission in the CNS, a number of glutamatergic neurons are distributed

throughout the *Drosophila* brain (Daniels et al., 2008); dysregulation of glutamatergic input by

modulating the glutamate transporter appears to exhibit global effects in the brain. Thus, it is

likely that changes in neuronal function and activity in connected neurons account for the

toxicity seen in the PPL1 cluster.

We propose that tau pathology is moderated by glutamate transporter activity. Further, this

likely occurs by glutamate based neuronal over-excitation, which leads to spread and

propagation of tau between neurons. This matches with our findings that dEaat1 expression

affects the phenotypic effects of human tau in *Drosophila*, and that excitation of neurons leads to

increased tau burden. Further studies are needed to confirm that these effects are due to changes

in neuronal activity resulting from glutamate over-excitation, and to determine the effects of

glutamate transporter knockdown on tau spread and propagation in the brain. Our study supports

our earlier finding that glutamate transporter activity affects tau pathology, and therefore the

possibility that Eaat2 may be an effective target for disease-modifying treatment of tauopathies.

Chapter 5: Discussion, future projects, and conclusion

There is increasing recognition of air pollutants, such as particulate matter (PM), nitric oxide species (NOx), sulfur dioxide, ozone, and toxic hydrocarbons such as poly aromatic hydrocarbons and benzene , as neurotoxic agents leading to intellectual and cognitive impairment in individuals with wide range of ages (J. A. Ailshire & Crimmins, 2014; Gatto et al., 2014; Lertxundi et al., 2015; Perera et al., 2009; Porta et al., 2016; Power et al., 2011; Rocha et al., 2020). Among elderly population, exposure to elevated levels of air pollutants markedly increases the risk for dementia and Alzheimer's disease (AD) (Oudin et al., 2016; Ranft et al., 2009; Tzivian et al., 2016). While there are a number of existing reports examining the neurotoxic effects of these constituents in the central nervous system (CNS), the underlying molecular mechanisms linking to neuronal damage and cognitive decline remain incompletely understood. Particularly, the body of work examining how chronic exposure to PM and other constituents interacts with disease models of AD and other dementias is currently limited. Existing data in non-AD model animals has shown a correlation between PM exposure and an altered immune state including increases in inflammatory cytokines such as the interleukins IL-1α, IL-1β, and IL-6, and tumor necrosis factor α, changes in glial activation markers glial fibrillary acid protein (GFAP), Iba1, and CD68, and NF-κB activation in the CNS (Bhatt et al., 2015; Calderón-Garcidueñas, Solt, et al., 2008; Campbell et al., 2005; Cheng et al., 2016; Guerra et al., 2013; Kleinman et al., 2008; Morgan et al., 2011; Park et al., 2020). Additionally, PM exposures in non-AD models have shown some ability to induce build-up of amyloid precursor protein (APP) and amyloid-β (Aβ) peptides (Bhatt et al., 2015; S. H. Kim et al., 2012; Levesque et al., 2011). More recently, neurotoxic effects of PM exposure have been tested in mouse models of AD (Cacciottolo et al., 2017; Jew et al., 2019). These studies find compelling gene

and environment interactions of PM exposure with the APOE4 genotype on Aβ pathology and cognition in 5xFAD mice (Cacciottolo et al., 2017) and cognitive decline following a short, 2-week exposure in 3xTg-AD mice (Jew et al., 2019). However, impediments to comprehensively understand the neurotoxic effects of PM in these animals include lacking of evaluation of AD neuropathology (Jew et al., 2019) and limited effect of PM exposure without the additional APOE risk genes (Cacciottolo et al., 2017). Thus, I hypothesized that exposure to ultra-fine particulate matter (UF PM) would exacerbate AD pathology and cognitive impairment in a mouse model of AD by increasing glial inflammation in the brain and accelerating Aβ buildup.

To test my hypothesis, we exposed mice of the heterozygous $App^{NL-G-F/+}$-KI (Saito et al., 2014) model of AD to concentrated UF PM as adults (Chapter 2) or *in utero* (Chapter 3). In all groups UF PM exposure showed decreased performance in the object location and recognition memory tasks, indicating some ability of PM to impact CNS function. While in certain cases this impairment was only seen when comparing wild-type animals exposed to filtered air only to animals with both the $App^{NL-G-F/+}$-KI genotype and UF PM exposure, implying some additive effect between the two, it was also observed comparing exposure status within the wild-type groups. In the adult exposure no changes were observed in microglia or astrocytes in either the 6 month or 12 month group. Aβ plaque burden, a hallmark for AD pathology, was increased. However, this increase was only seen in the 12 month old $App^{NL-G-F/+}$-KI mice with established Aβ pathology (Saito et al., 2014). Together these data showed that, while UF PM exposure may increase behavioral deficits in combination with an AD genotype, our initial hypothesis that glial inflammation and Aβ were the critical molecular pathways for this link is not supported in the model. While additional assays of synaptic proteins and the integrity of the blood brain barrier tight junctions also showed no changes with exposure, there was a non-genotype specific effect

of UF PM exposure on the astrocytic glutamate transporter GLT-1 at both age points in the adult exposure. While these data alone are not enough to prove a mechanism or that GLT-1 is in the causal pathway for behavior deficits, they do suggest a relation between glutamate dyshomeostasis and UF PM exposure. Linking back to AD risk, glutamate disruption and excitotoxicity are well connected to AD pathology (Dabir et al., 2006; Masliah et al., 1996; Mookherjee et al., 2011). Specifically, tau pathology and propagation is affected by glutamate related excitotoxicity (Dabir et al., 2006; Hunsberger et al., 2015; J. W. Wu et al., 2016), potentially linking UF PM mechanisms to previous work we have done examining excitotoxicity and tau in a *Drosophila* model of tauopathy (Chapter 4). In these experiments we showed that genetic loss or increase of the *Drosophila* GLT-1 homolog dEaat1 modulates lifespan and geotaxic response in a human tau expressing model. We also demonstrated that over excitation by heat mediated depolarization events increased the propagation of tau. These results may inform future studies of the effects of PM in AD models.

Interestingly, in the *in utero* exposure experiment adult $App^{NL-G-F/+}$-KI and wild-type animals exposed to UF PM during development did display changes in glial inflammation and Aβ plaque burden. Additionally, there is evidence that the synaptic marker PSD95 may be reduced, which was not apparent in the adult exposure model. Lastly, there was not a significant drop in GLT-1 in the hippocampus as seen in the adult animals. As in the adult exposure, in certain comparisons there were only statistical differences when looking at the wild-type air exposed animals versus the $App^{NL-G-F/+}$-KI UF PM exposed group, implying additive effects of genotype and exposure on pathological outcomes. Otherwise, these data establish two main points: that there is a link between developmental exposure to UF PM and Aβ pathology in later life in the $App^{NL-G-F/+}$-KI model and that the mechanisms by which *in utero* PM exposure affect the CNS

may differ from those of adult PM exposure in this model. While this experiment did establish the link between *in utero* exposure and Aβ pathology, it was not designed to and does not prove a mechanism for this link. It is tempting to suggest the observed changes in reactive microglia and astrocytes lead to the increased Aβ accumulation and behavior deficits, but investigation in younger animals may indicate otherwise as in the adult exposure model.

5.1 Future research in the *App^{NL-G-F}*-KI model of AD

As mentioned, the current work is insufficient to prove mechanisms connecting UF PM exposure in the mouse model to observed behavioral deficits. Thus, further work in the model guided by the findings here is required to thoroughly investigate the molecular pathways involved. The immediate primary area of interest will be to determine the extent of glutamate transport disruption, the effects thereof, and whether this is directly due to PM exposure or a derivative effect from some other, yet unobserved, neurobiological or systemic changes. For the last, *in vitro* investigation using primary astrocyte and neuron co-culture, as has been performed by the lab previously (Zumkehr et al., 2018), and direct application of collected UF PM to the media will indicate whether PM has a direct effect on GLT-1 and the tripartite synapse system. Regarding glutamate transport, changes in proteins levels of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits GluR1 and GluR2 have been associated with PM exposure outcomes previously (Cacciottolo et al., 2017; Morgan et al., 2011), suggesting that the changes may not be limited to GLT-1. The N-methyl-D-aspartate (NMDA) receptor is also well associated with AD and neurodegeneration (Danysz & Parsons, 2012; R. Wang & Reddy, 2017) and *in vitro* work has indicated an increase in NDMA toxicity in neuronal culture when nano-scale PM is also applied (Morgan et al., 2011), providing another avenue to explore connecting the effects of PM in AD progression. To connect the memory behavior deficits in the

UF PM exposed groups, measurement of LTP in *ex vivo* hippocampal slices should indicate whether the changes in glutamate transport are impairing this function, which is critical for memory. As LTP has been shown to be highly dependent on GLT-1 mediate glutamate uptake (Katagiri et al., 2001; Pita-Almenar et al., 2012), impairment is expected with loss of GLT-1. Finally, pharmacological upregulation of GLT-1 has been previously shown to ameliorate AD pathology (Takahashi et al., 2015; Zumkehr et al., 2015), and a similar experiment in the current model may provide further insight into whether the behavior effects observed are dependent on GLT-1 loss.

5.2 Tau models of AD and PM

Multiple reports indicate that exposure to air pollutants increases hyper-phosphorylation of tau in animal models (Calderón-Garcidueñas et al., 2018, 2020; S. H. Kim et al., 2012; Levesque et al., 2011) and in humans (Calderón-Garcidueas, Kavanaugh, et al., 2012; Calderón-Garcidueñas et al., 2020). As tau and tau phosphorylation are highly involved in the pathology of AD, these results suggest tau interactions as another potential pathway to link PM exposure to AD risk. The phosphorylation and propagation of tau are affected by glutamate transport disruption driven neuronal over-excitation (Hunsberger et al., 2015; J. W. Wu et al., 2016), providing an additional link between the current finding of a loss of GLT-1 with UF PM exposure in mice. Further, it is possible that PM driven tau hyperphosphorylation can feed back into glutamate excitotoxicity. Hyperphosphorylated tau aggregates into neurofibrillary tangles (NFTs), which mislocalize to the cell bodies and dendrites (Holtzman et al., 2016; A. Ittner & Ittner, 2018). At the dendrites, they facilitate Fyn mediated phosphorylation of the NMDA receptor subunit GluN2B, leaving the post synapse vulnerable to over stimulation (L. M. Ittner et al., 2010; Miyamoto et al., 2017). However, to date there is no report examining the effects of PM exposure in a humanized tau

animal model of AD. Recently, a humanized tau (hTau) knock in model also expressing the App^{NL-G-F}-KI genotype has been created (Saito et al., 2019), providing a strong option to explore the effects of PM exposure on tau and in turn tau pathology as another potential pathway linking exposure to AD, while still maintaining the characterized effects in the $App^{NL-G-F/+}$-KI model here. Given the suggestive links between UF PM and tau pathology progression we would anticipate that the effects of UF PM exposure would be magnified in the model expressing both $App^{NL-G-F/+}$-KI and hTau.

5.3 Analysis of PM associated metals in the brain

UF PM potentially acts as a carrier for other potentially toxic constituents such as metals and organic compounds to infiltrate the body (Seaton et al., 1995). Due to its small size it can bypass blood brain barrier defenses via the olfactory nerves, demonstrated in animal models and humans (Block & Calderón-Garcidueñas, 2009; González-Maciel et al., 2017). Alternatively, it can enter the bloodstream directly through the lungs after inhalation and exert toxicity in the CNS or systemically (Kreyling et al., 2002; Nemmar et al., 2002), presenting unique risk compared to the exposure through oral ingestion. However, little work has been done to identify the extent to which PM related metals accumulate in the CNS after PM exposure. Such work will provide a foundation to indicate whether metal toxicity may be playing a role in the CNS effects of PM exposure. The extent of build-up of PM associated metals in the brain may help suggest whether direct infiltration of PM is a major exposure pathway as compared to systemic effects in the blood. Similarly, in the *in utero* exposure model, analysis of metal build up may help determine whether PM and PM related components are directly affecting the fetus, or acting primarily on the mother.

5.4 Conclusion

This dissertation shows that exposure to UF PM both *in utero* and in adult mice causes memory deficits in wild-type and $App^{NL-G-F/+}$-KI mouse model of AD, as well as increases in Aβ plaque load. In the *in utero* model, we demonstrate that glial inflammation and Aβ pathology at adult age are increased with exposure during development, suggesting potential pathways by which PM exposure influences behavior and Aβ burden consistent with our hypothesis (Chapter 3). In the adult exposure model, however, no changes are seen in examined inflammation markers and Aβ increase does not appear to be required for PM induced cognitive deficit, but levels of the astrocytic glutamate transporter GLT-1 are decreased (Chapter 2). This result links to previous work in a *Drosophila* model showing that expression of the *Drosophila* homolog of GLT-1 and direct excitation of neurons can increase tau pathology (Chapter 4), establishing a potential non-Aβ driven link between PM exposure and AD. In all, our findings suggest that PM exposure does induce cognitive decline and increase AD like pathology in an AD model mouse, but that glial inflammation and Aβ build up are not the primary pathways by which this occurs. Further studies both in the $App^{NL-G-F/+}$-KI model and in tau models of neuronal disease are required to fully understand the mechanisms linking PM exposure and AD risk outcomes.

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Appendix A- Abbreviations

Aβ- Amyloid β

AD- Alzheimer's Disease

AICD- Amyloid precursor protein intracellular/cytoplasmic C-terminal domain

Alrm- astrocytic leucine-rich repeat molecule

AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA- Analysis of variance

APOE- Apolipoprotein E

APP- Amyloid precursor protein. Alternative: shorthand in figures for $App^{NL-G-F/+}$-KI genotype

BBB- Blood brain barrier

BSA- Bovine serum albumin

CD31, CD68- Cluster of differentiation 31, cluster of differentiation 68

CNS- Central nervous system

Cor- Cortex

COX2- Cyclooxygenase 2

Dlg1- discs large 1

Eaat- excitatory amino acid transporter

F- *APP* Iberian mutation I716F

FA- Filtered air

G- APP Arctic mutation E693G

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

GFAP- Glial fibrillary acid protein

GLT- glutamate transporter

GluR1, GlurR2- Glutamate receptor 1, Glutamate receptor 2

GMR- Glass multiple reporter

GS3Kβ- Glycogen synthase kinase 3 beta

HC- Hippocampus

Htau- Human tau

Iba1- Ionized calcium binding adaptor molecule 1

Il6, Il1β- interleukin 6, interleukin 1β

IR- Interferring RNA

KI- Knock in

LTP- Long term potentiation

MAPT- Microtubule associated protein tau

MRI- Magnetic resonance imaging

NF-κB- Nuclear factor kappa-light-chain-enhancer of activated B cells

NFT- Neurofibrillary tangle

NIA- National Institute on Aging

NIEHS- National Institute of Environmental Health Sciences

NIH- National Institute of Health

NL- *APP* Swedish mutation KM670/671NL

NMDA- N-methyl-D-aspartate

NO_x^- Nitrogen oxides

nSyB- Neuronal synaptobrevin

OLM- Object location memory

ORM- Object recognition memory

PAH- Poly aromatic hydrocarbon

PBS- Phosphoate buffered saline

PBT- Phostphate buffered saline + 0.5% tween

PCR- Polymerase chain reaction

PET- positron emission tomography

PHF- Paired helical filament

PM-Particulate matter

PSD95- Post synaptic density protein 95

SYP- Synaptophysin

TBS- Tris buffered saline

TNF- Tumor necrosis factor

Trpa1- Transient receptor potential cation channel, subfamily A, member 1

Tub- Tubulin

TUNEL- terminal deoxynucleotidyl transferase dUTP nick end labeling

UF- UF PM (see below)

UF PM- Ultrafine particulate matter

VACES- Versatile aerosol concentration and enrichment system

ZO- Zonula occludens