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INVESTIGATING THE ROLE OF VASCULAR ACTIVATION IN ALZHEIMER'S DISEASE-RELATED NEUROINFLAMMATION

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INVESTIGATING THE ROLE OF VASCULAR ACTIVATION IN ALZHEIMER'S DISEASE-RELATED NEUROINFLAMMATION

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia, affecting 5.8 million people in the United States alone. Currently, there are no disease-modifying treatments for AD, and the cause of the disease is unclear. With the increasing number of cases and the lack of treatment options, AD is a growing public health crisis. As such, there is a push for investigation of novel pathological mediators to inform new therapeutic approaches.

Cardiovascular disease and associated cardiovascular risk factors (CVRFs) are linked to a significantly increased risk for developing AD, but the mechanisms whereby these risk factors contribute to the pathological processes in the AD brain have not been defined. Because CVRFs affect vascular function, a dysfunctional cerebrovasculature could be an important driver of CVRF-induced injury in the AD brain. Furthermore, the cerebrovasculature is both a source of, and target for, inflammatory mediators, indicating that a dysfunctional cerebrovasculature likely participates in the robust neuroinflammatory response found in the AD brain.

In particular, type II diabetes mellitus (T2D) is associated with a more than doubled risk for developing AD. Hyperglycemia-induced endothelial activation and related inflammatory processes are key pathological features in T2D. Here, we investigate vascular dysfunction and neuroinflammation in diabetes-like conditions *in vitro.* Results show that brain endothelial cells treated with hyperglycemia and hypoxia *in vitro* become injured and release inflammatory mediators. This endothelial injury activates a pro-inflammatory microglia phenotype in a co-culture system, characterized by significantly increased production of inflammatory cytokines and nitric oxide (NO), and increased expression of microglia activation markers. These microglia changes mimic those found in the AD brain, highlighting that endothelial damage and dysfunction may be a driver of neuroinflammatory activation in AD.

Mutations to the lipid transport protein apolipoprotein E (ApoE) have been identified as the strongest genetic risk factor for sporadic or late-onset AD, with the E4 allele representing an increased risk and the rare E2 having a reduced risk compared to the primary E3 form. While the risk is well documented, the mechanisms responsible for this risk are not well understood. We examined the effects of ApoE isoforms on markers related to vascular function, neuroinflammation, and neurotoxicity using *in vitro* and *in vivo* models*. In vitro,* the ApoE4 isoform induces pro-inflammatory activation of both microglia and astrocytes, as well as enhanced neurotoxicity. On the other hand, ApoE2 promotes neuronal survival through both direct and indirect actions on neurons. Using an *in vivo* mouse model, we show that ApoE4 promotes blood-brain barrier dysfunction and neuroinflammation, characterized by loss of tight junction proteins, and increased oxidative stress and pro-inflammatory cytokines. Additionally, our results indicate that the ApoE4 isoform promotes an inflammatory-oxidative state to a greater extent in female mice than in males.

Recent research indicates that components of the coagulation cascade may act as mediators of vascular dysfunction and inflammation in both cardiovascular disease and in AD. One such protein is the serine protease thrombin, which is elevated in the AD brain. Here, we present an extensive literature review, which posits that thrombin acts on endothelial cells of the blood-brain barrier, microglia, astrocytes, and neurons in a manner that promotes vascular activation, inflammation, and neurodegeneration. Additional evidence suggests that pharmacologically targeting thrombin may be an effective treatment strategy for targeting multiple points of AD pathology. We investigated thrombin's role as a pathological mediator and potential therapeutic target and show that treatment of a tau-based AD mouse model *in vivo* with the direct thrombin inhibitor dabigatran produced significantly reduced

oxidative stress and tau phosphorylation, and promoted cell survival and synaptic function.

Taken together, our results suggest vascular activation and related neuroinflammation as possible convergence points whereby CVRFs promote AD-related pathological processes. Furthermore, we highlight shared pathological mediators that may represent novel therapeutic targets for the treatment of multiple points of pathology in AD.

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DEDICATION

The following dissertation is dedicated to my grandparents, who have always been my inspiration in school and in life.

PREFACE

The following dissertation is presented in manuscript format organized into three major chapters. The publication status of each manuscript is as follows:

Chapter 1: Investigate the phenotypic changes induced in microglia following damage to the endothelial cells of the blood-brain barrier using an *in vitro* model of diabetes

Manuscript 1: High-glucose and hypoxia-mediated damage to human brain microvessel endothelial cells induces an altered, pro-inflammatory phenotype in BV-2 microglia in vitro. Submitted to Journal of Neuroinflammation, June 2020.

Chapter 2: Evaluate the neuroinflammatory role of apolipoprotein E4 in an *in vivo* mouse model and *in vitro* using human-derived brain glia and vascular cells

Manuscript 2: Effects of Apolipoprotein E isoforms on brain glial and neuronal cells *in vitro*. *In preparation.*

Manuscript 3: Sex-specific effects of Apolipoprotein E4 in the brain *in vivo*. *In preparation.*

Chapter 3: Explore the role of thrombin as a potential pathological mediator in Alzheimer's disease, and evaluate the efficacy of inhibiting thrombin activity as a therapeutic target in an Alzheimer's disease mouse model

Manuscript 4: Thrombin, a mediator of coagulation, inflammation, and neurotoxicity at the neurovascular interface: Implications for Alzheimer's disease. Published in Frontiers in Neuroscience, July 2020.

Manuscript 5: Direct thrombin inhibitor, dabigatran etexilate, reduces oxidative stress *in vivo* in a transgenic mouse model of Alzheimer's disease. Submitted to Journal of Alzheimer's Disease, May 2020.

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JUSTIFICATION OF RESEARCH

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by progressive loss of memory and other cognitive functions. In 2019, there were an estimated 5.8 million Americans with AD, costing approximately 290 billion in health care [1]. The causes of AD are unclear, but increased risk for development has been linked with a number of factors, including lifestyle, genetics, and environment [2]. The AD brain demonstrates profound neuron death, particularly in the hippocampus, as well as pathological protein aggregation in the form of amyloid plaques and neurofibrillary tangles, made up of aggregated amyloid-β (Aβ) and hyperphosphorylated tau, respectively [3]. The AD research field has largely focused on the amyloid hypothesis, postulating that disease progression is caused by pathological accumulation of Aβ oligomers and/or deposits of Aβ into plaques. The importance of this hypothesized role has recently been called into question following multiple clinical trial failures using agents that targeted Aβ generation, aggregation, or clearance [4-8].

Recent research has expanded our understanding of AD as a complex, multifactorial disorder with pathological roles implicated for neuroinflammation, vascular dysregulation, protein aggregation, mitochondrial dysfunction, and other non-amyloid based factors [7, 9-11]. The growing number of AD cases, along with the lack of successful treatment options, make AD a growing public health crisis. As such, the investigation of novel pathological mediators is needed in order to inform new therapeutic approaches that target multiple-disease related factors.

Cardiovascular disease and AD

Cardiovascular risk factors increase the risk for Alzheimer's disease

There is an ever-growing literature documenting the relationship between cardiovascular risk factors (CVRFs) and AD. The risk for developing AD significantly increases in the presence of a number of CVRFs, including hypertension, hypercholesterolemia, hyperhomocysteinemia, possession of an apolipoprotein E4 (ApoE4) allele, diabetes, and atherosclerosis [12-16]. CVRFs in early adulthood through midlife are associated with significantly worsened cognitive performance, including poor cognitive function, impaired executive function, and dementia later in life [17-19]. Furthermore, epidemiological evidence indicates a connection between midlife blood pressure and cholesterol levels and late-life AD [20]. Diabetes in particular is strongly linked to Alzheimer's, with type II diabetes mellitus (T2D) more than doubling the risk for developing AD [21-23].

Vascular dysfunction is present in cardiovascular disease and Alzheimer's

Vascular dysfunction occurs as the result of non-adaptive alterations in the phenotype of vascular endothelial cells in response to disease-related stimuli [24, 25]. Alterations in endothelial cell metabolism and imbalances in protein phosphorylation are characteristic of cardiovascular diseases, such as diabetes, hypertension, and atherosclerosis [25, 26]. Vascular dysfunction results in impaired endothelial nitric oxide synthase (eNOS) function, significantly increased oxidative stress and inflammation, abnormal angiogenesis, and impaired endothelial cell repair [24, 27, 28]. This dysfunction often includes endothelial activation, in which endothelial cells take on a highly synthetic, pro-inflammatory, and pro-coagulant phenotype.

Evidence indicates that vascular dysfunction is present in the pathogenesis of AD. Findings from both animal and human studies suggest that cerebrovascular dysfunction precedes neurodegenerative changes in AD [29]. Disruption of the blood-brain barrier (BBB) is also indicated in AD, with microhemorrhages detected in both

mild cognitive impairment (MCI) and AD patients, often co-localized to areas of amyloid plaque build-up [30, 31]. Moreover, BBB permeability is correlated with AD progression in both animal and human-based studies [32, 33]. Taken together, these findings indicate a role for vascular dysfunction in the pathological progression of AD.

Diabetes-related hyperglycemia as a mediator of vascular dysfunction and inflammation

T2D is associated with a substantially increased risk for developing AD, a relationship that is not fully understood [21-23]. Diabetes-related pathology is primarily the result of hyperglycemia-induced endothelial dysfunction throughout the body. There are several mechanisms suggested for vascular complications in response to hyperglycemia, with the primary factor implicated to be increased production of superoxide by the mitochondrial electron-transport chain [34-36]. Additionally, hyperglycemic conditions lead to significantly impaired eNOS function and NADPH oxidase (NOX) activation, producing high levels of oxidative stress [26].

Hyperglycemia in T2D is associated with BBB dysfunction, leading to decreased expression of tight junction proteins and increased secretion of inflammatory mediators. This further induces morphological changes to endothelial cells and significantly increases BBB permeability [37]. Diabetes-associated BBB dysfunction produces neuroinflammation, neuronal dysfunction, and protein aggregation [38]. Mouse models of diabetes exhibit significant BBB damage, memory deficits, alterations to protein expression by the cerebrovascular system, and increased activation of microglia [39, 40]. *In vitro,* high glucose treatment of a BBB model produced significantly decreased expression of tight junction proteins and increased NOX activity [41]. As previously mentioned, BBB dysfunction has been

found in Alzheimer's disease [29-31], indicating that the BBB may be one key area in investigating the relationship between diabetes and the risk for AD.

Hyperglycemia-related BBB damage and dysfunction leads to significant increases in markers of neuroinflammation, including increased reactive oxygen species (ROS) and inflammatory cytokines [37, 38, 42]. These neuroinflammatory effects may, in part, be consequences of endothelial cell-microglia cross talk. Microglia can induce disruptions in tight junctions, stimulate angiogenesis, and induce endothelial cell migration [43]. Less is known about endothelial cell effects on microglia, though endothelial cells are known to release a number of signaling molecules that influence microglia phenotype, including interleukin (IL)-1, IL-6, matrix metalloproteinases (MMPs), and thrombin [44-48]. Importantly, there are parallels found between inflammation in T2D and AD. Both processes exhibit common inflammatory mediators, particularly cytokines, including tumor necrosis factor-α (TNF-α) and IL-6. Additionally, the inflammatory response in both T2D and AD is largely driven by macrophages, in the periphery and brain, respectively [49].

Apolipoprotein E as a shared risk factor in cardiovascular disease and Alzheimer's disease

The most significant genetic risk factor for late-onset AD is an allele variant of apolipoprotein E (ApoE), a protein involved in cholesterol transport [50]. The ApoE gene has three polymorphic alleles in humans, E2, E3, and E4. The E4 variant is associated with cardiovascular disease in the periphery, particularly atherosclerosis, further highlighting the connection between cardiovascular disease and AD [51, 52]. Population based studies show that just one copy of the E4 allele is associated with an almost 50% increase in prevalence of AD and lower age of onset for the disease, while E2 appears to have protective effects, reducing the likelihood of developing AD

by approximately 40% [53]. Evidence for ApoE4's association with AD is supported by the findings that ApoE4 is related to impaired cognition in midlife and higher conversion of mild cognitive impairment (MCI) to AD [54-56]. Additionally, the presence of ApoE4 significantly increases the likelihood that cardiovascular disease and Alzheimer's will co-occur. For example, carrying the ApoE4 more than doubles the likelihood that individuals with T2D will develop AD or mixed dementia [52, 57, 58].

One mechanism whereby ApoE contributes to atherosclerosis and AD is through effects on vascular function. ApoE4 carriers exhibit altered cerebral blood flow (CBF) compared to ApoE3 starting in young adulthood and continuing through late adulthood, likely the result of impaired neurovascular coupling [51, 59]. ApoE4 carriers show greater decline in CBF during late adulthood [60]. ApoE4 individuals have an 8-10.5% reduction in cerebral glucose metabolism, starting in early adulthood [59, 61]. In mouse models, ApoE4 modulates cerebrovascular integrity and significantly increases BBB permeability via alterations in signaling at the neurovascular unit [62]. In an *in vitro* model of the BBB, ApoE4 was associated with significantly reduced tight junction integrity and increased barrier leakiness compared to ApoE3 [63].

Newer findings have presented a novel, alternative role for ApoE4 as a mediator of the brain's inflammatory response. ApoE4 is associated with changes in inflammatory markers in both *in vivo* and *in vitro* models of cardiovascular disease and AD [64]. ApoE signaling has been linked to the conversion of microglia to disease associated microglia (DAM) in the AD brain, and ApoE is a ligand for the triggering receptor expressed on myeloid cells 2 (TREM2) receptor [65, 66]. Microglia isolated from mice expressing human E4 show enhanced activation compared to E3,

including significantly increased production of TNF-α, IL-6, and reactive nitrogen species (RNS) [67, 68]. ApoE4 is also associated with increased secretion of inflammatory factors, such as IL-1β and NO, in both human and rat macrophages *in vitro* [69-71]. More recent research using mass spectrometry analysis of human brain tissue further supports a role for ApoE in modulating the expression of inflammation-related factors. In analyzing brain tissue expressing e2/e3, e3/e3, or e4/e4, researchers found that expression of proteins related to the regulation of inflammatory response was significantly correlated with ApoE genotype [72]. The study of ApoE4 in relation to vascular function and inflammation represents a shift from previous focus on ApoE4's lipid transport functions, including its role in metabolism and deposition of Aβ [64].

Thrombin as a pathological mediator of cardiovascular disease and Alzheimer's disease

A number of mediators implicated in the pathological progression of both cardiovascular disease and inflammation may also play a role in AD. One such factor is the serine protease thrombin, a coagulation protein that is elevated in both cardiovascular disease and AD [73, 74]. Thrombin levels are significantly elevated around atherosclerotic plaques, and increases in thrombin are correlated with atherosclerosis disease severity [75]. Thrombin activity is significantly elevated in T2D, and its activity is linked to endothelial activation and increased production of reactive oxygen species (ROS) [76, 77].

Thrombin has been implicated as a pathological mediator in Alzheimer's disease. Instances of traumatic brain injury (TBI), where neurons are exposed to high levels of thrombin, are associated with a 1.2 to 2-fold increased risk for AD [78, 79]. Brains of AD patients show increased thrombin post-mortem, as well as significantly

reduced levels of the thrombin inhibitor protease nexin-1 [74, 80, 81]. Thrombin expression is approximately doubled in AD patient-derived microvessels compared to controls, and brain endothelial cells isolated from AD patients synthesize their own thrombin *in vitro* [82, 83]. Animal models of AD exhibit elevated levels of thrombin *in vivo* [84]. Thrombin has also been associated with typical hallmarks of AD pathology. Rats treated with thrombin *in vivo* exhibit impaired reference and spatial memory [85]. Thrombin accumulates in neurofibrillary tangles and is related to rapid tau aggregation [86, 87]. Thrombin is implicated in altered processing and secretion of amyloid precursor protein (APP) [88, 89], and thrombin cleavage of ApoE4 results in a neurotoxic fragment [90].

Thrombin acts as a cell signaling mediator through its activation of protease activated receptors (PARs) [91, 92]. Thrombin signaling in the periphery has been linked to significantly increased pro-inflammatory cytokines and ROS production [73, 76, 93, 94]. PARs are expressed throughout the central nervous system (CNS), including by neurons, astrocytes, microglia, and neurons, and thrombin signaling has a number of effects on the brain [95, 96]. Thrombin has significant, deleterious effects on endothelial cells of the BBB, inducing increased leukocyte adhesion and decreased tight junction formation, as well as increased release of inflammatory factors and ROS [45, 82, 97-101]. Thrombin acts directly on both microglia and astrocytes to initiate a pro-inflammatory phenotype [46, 47, 102, 103] [85, 104-106]. High doses of thrombin are neurotoxic, indirectly through changes to the surrounding environment, and directly through alterations in the neuronal cell cycle [46, 102, 107-109].

Further evidence for the role of thrombin comes from the finding that fibrin, like thrombin, has been indicated as a mediator of vascular dysfunction and

neuroinflammation in neurodegenerative diseases, including AD [110, 111]. Fibrin is the result of thrombin cleavage of fibrinogen, and is therefore a product of increased thrombin activity [112]. Fibrin(ogen) has been shown to have proinflammatory effects in the CNS, including the activation of glia and disruption of the BBB [111]. Fibrinogen deposition was found in AD brains, indicative of BBB leakiness. Additionally, in both human AD and a rat model, fibrinogen was associated with areas of Aβ deposition, and enhanced the pro-inflammatory and vascular disrupting effects of $A\beta_{1-42}$ [113]. Fibrinogen activates microglia in a CD11b-dependent manner [114], and fibrinogen-CD11b binding was found to promote axonal degeneration, spine elimination, and cognitive impairment in a mouse model of AD [115].

Thrombin and fibrin are not the only components of the coagulation cascade found to act as pathological mediators in AD [116]. For example, the factor XII-initiated contact system is activated in AD patients and mice, and depletion of factor XII ameliorates brain pathology and cognitive impairment in an AD mouse model [117, 118]. Additionally, anti-coagulant components of the cascade, including activated protein C (APC), have been found to have anti-inflammatory and protective effects in AD models [119]. The finding that fibrin and other downstream components of the coagulation cascade act as disease mediators further highlights the potential role for thrombin in the development and progression of AD, through both direct and indirect effects in the brain.

Neuroinflammation in Alzheimer's disease

Neuroinflammation is a key component of disease pathology in a number of neurodegenerative diseases, including AD [3, 120, 121]. Pro-inflammatory cytokines and chemokines are significantly elevated in the brain and serum of AD patients compared to healthy controls, including cyclooxygenase-2 (COX-2), monocyte

chemoattractant protein-1 (MCP-1), TNF-α, IL-1β, and IL-6 [121-123]. Neuroinflammation appears early in disease time course, suggesting an important pathological role in the development of AD, and a potential target for early therapeutic intervention [121]. Additionally, a number of key AD risk genes are related to the function of the innate immune system, including complement receptor 1(CR1), CD33, and TREM2 [124-127].

Neuroinflammation in Alzheimer's is primarily driven by the glial cells of the brain, particularly microglia and astrocytes [128]. Activated microglia and astrocytes have been found localized around Aβ plaques in the AD brain [128-131]. Both cell types are responsible for the secretion of inflammatory cytokines and mediators, including NO and ROS, COX-2, TNF-α, IL-1β, and IL-6 [120, 128, 132-134]. While both astrocytes and microglia are involved in the inflammatory response, microglia are the primary drivers of inflammation in the brain [134].

Microglia in Alzheimer's disease

Microglia are the resident immune cells of the brain, and are key mediators of the neuroinflammatory response. These cells become activated in response to pathogen invasion or tissue damage and promote an inflammatory response. This inflammatory response is intended as an adaptive response, but chronic exposure to inflammatory mediators produced by microglia can activate apoptosis and necrosis, resulting in neurodegeneration [135]. Previously, microglia were recognized as having two activation states, the M1 pro-inflammatory phenotype, and the M2 "quiescent" phenotype. Newer evidence suggests this is an oversimplification, pointing instead to a range of microglia phenotypes that may be present during health and disease [120]. More recent literature has highlighted a "disease-associated microglia" (DAM) phenotype found around Aβ plaques in both human Alzheimer's and transgenic AD-

mouse brains [136]. This microglial phenotype has also been termed "neurodegenerative microglia" (MGnD) [65]. DAMs show downregulation of homeostatic microglia genes (ex. p2ry12/p2ry13, cx3cr1, tmem119) and upregulation of pro-inflammatory and AD-associated factors (ex. apoe, tyrobp, itgax, clec7a, trem2) [65, 136]. The conversion from homeostatic microglia to DAM is purported to occur in a two-step process: the first step is TREM2-independent and includes the down-regulation of microglia checkpoints, while the second is TREM2-dependent and causes the activation of lipid metabolism and phagocytic pathway genes [136]. Further study has indicated a possible subset of DAM that are pro-inflammatory [137, 138], but additional investigation is needed to fully understand the significance of DAM to AD pathology.

Dissertation Objective

The following dissertation proposal will explore new strategies to examine the role of cardiovascular disease-related risk factors and inflammation in Alzheimer-related pathology. Major outcomes of the proposed experiments will provide new insights into the relationship between cardiovascular disease, vascular activation, inflammation, and Alzheimer's pathology, as well as inform novel therapeutic strategies for the treatment of Alzheimer's.

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CHAPTER 1

MANUSCRIPT 1

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High-glucose and hypoxia-mediated damage to human brain microvessel endothelial cells induces an altered, pro-inflammatory phenotype in BV-2 microglia *in vitro*

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Abstract

A strong link between Type 2 Diabetes Mellitus (T2D) and Alzheimer's disease (AD) has been identified, but the reason behind this increased risk is not well understood. Hyperglycemia damages brain endothelial cells, and the cerebrovasculature is a key component of the brain's inflammatory response in disease. Neuroinflammation, primarily regulated by microglia, is a key aspect of AD pathology. The goal of this study was to assess the effect that human brain microvascular endothelial cell (HBMVEC) injury *in vitro* by stressors related to diabetes would have on the activation state of microglia. HBMVECs were treated with high-glucose with or without hypoxia. HBMVEC-conditioned media was then used to treat BV-2 microglia. Alterations in microglia phenotype were assessed through measurement of Nitric Oxide (NO) production, cytokine production, and changes in expression of known microglia markers. HBMVEC injury through glucose and hypoxia induced changes in BV-2 phenotype, including increased production of NO and TNFα, as well as altered expression of a number of homeostatic and "activated" microglia markers, including CD11c, CLEC7A, and P2Y12. Additionally, BV-2 exhibited altered phagocytic ability following treatment with HBVMEC-conditioned media. Together, these findings suggest that diabetes-like damage to the brain endothelial cells may help to initiate a neuroinflammatory response through activation of a "pro-inflammatory" phenotype in microglia.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive loss of cognitive function, leading to dementia and death. The staggering number of AD cases, 6 million currently in the US and projected to increase to over 13 million by 2050 [1], underlies the urgency to better understand and treat this devastating disorder. It has become clear in recent years that AD is a complex, multifactorial disorder where disparate processes likely converge to produce neuronal injury. Many studies have shown that cardiovascular risk factors (CVRFs), such as hypertension, atherosclerosis, and diabetes, are important risk factors for the development of AD [2-6]. Diabetes especially has been strongly linked with AD, as individuals with type 2 diabetes mellitus (T2D) have a greater than 50% increase in their risk for developing AD [7-9]. Though the mechanisms whereby diabetes contributes to pathological processes in the AD brain have not been delineated, in the periphery diabetes and other CVRFs are potent drivers of inflammation [10]. Because neuroinflammation is a consistent feature of AD, and other neurodegenerative diseases, inflammation could be a key target for the effects of diabetes in the brain.

Inflammation is, by definition, vascularized tissue response to injury, and as such, endothelial cells are key players in the development of inflammation in the brain [11]. Chronic inflammation is tightly linked to diseases associated with endothelial dysfunction. Sustained hyperglycemia, a pathognomonic feature of diabetes, leads to endothelial cell dysfunction and the activation of several pathways including stimulation of the polyol and glucosamine pathways, activation of protein kinase C, and formation of advanced glycation end products (AGEs) [12]. Furthermore, high levels of glucose in diabetes contribute to endothelial injury by increased expression of reactive oxygen species (ROS) and inflammatory proteins. The initiation of

vascular complications in diabetes is linked to mitochondrial superoxide overproduction in the endothelium [13-15].

Individuals with diabetes are at increased risk for a number of secondary CNS conditions, including stroke and cognitive dysfunction [16-18]. While diabetes negatively impacts multiple processes and cells in the brain, microvascular endothelial cells are the cell type at the blood-brain interface that are most directly exposed to the deleterious effects of high glucose levels. In this regard, numerous studies have documented diabetes-induced alterations of blood-brain barrier (BBB) function. Both *in vitro* models utilizing high-glucose conditions and *in vivo* experiments with diabetic animals have shown compromised BBB integrity. Significantly increased BBB permeability, as well as morphologic modifications of the microvasculature, including excess collagen deposition, accumulation of lipid peroxidation byproducts, and degeneration of endothelial cells, have been documented [18]. Glucose-induced increase in BBB permeability is mediated in part by partial loss of junctional proteins ZO-1, occludin, and claudin-5, and increased NADPH oxidase (NOX) activity [18-20]. Finally, gene expression profiling of isolated microvessels from diabetic animals shows increases in several inflammatory proteins, including tumor necrosis factor-α (TNFα) and matrix metalloproteinase (MMP)-9 [21].

There is robust elevation in inflammatory mediators in the AD cerebral microcirculation. AD brain endothelial cells express high levels of inflammatory adhesion molecules, such as monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and cationic antimicrobial protein 37 kDa (CAP37) [22]. Additionally, AD brain microvessels release significantly higher levels of nitric oxide (NO), thrombin, TNFα, transforming growth factor-β (TGF-β), interleukin (IL) IL-1β, IL-6, IL-8, and MMPs compared to age-matched controls [23-25]. In

regards to AD pathology, the cerebral microvasculature is also a participant in a cycle of events where inflammation precedes Aβ deposition, and Aβ in turn promotes release of inflammatory mediators. In this regard, exposure of brain endothelial cells to Aβ has been shown to evoke an array of pro-inflammatory responses. Cultured brain endothelial cells exposed to $A\beta_{1-40}$ exhibit increased expression of MCP-1, IL-1β, and IL-6 [22]. Therefore, the cerebromicrocirculation serves as both a source of, and a target for, inflammatory proteins.

The inflamed endothelium is a highly synthetic interface that produces numerous bioactive factors. We have previously shown that microvessels isolated from the AD brain are inflamed and secrete proteins that have direct neurotoxic effects on neurons [26]. It is likely that endothelial-derived mediators will also have critical effects on neighboring microglia, as many of the inflammatory proteins released by activated brain endothelial cells (such as IL-1β and thrombin) induce pro-inflammatory responses in microglia. Although the possible effects of injured brain endothelial cells on microglia have not been explored, microglia are part of the neurovascular unit (NVU). The NVU is defined as a metabolic unit consisting of brain endothelial cells, neurons, astrocytes, microglia, pericytes, vascular smooth muscle cells, and oligodendrocytes [27]. The functional interaction of NVU cells through bidirectional cell-cell signaling is critical for maintaining cerebral blood flow and regulating physiologic processes in the central nervous system (CNS). Microglia activated by treatment with LPS can induce alterations in brain endothelial cell morphology and function [28], but less is known about endothelial cell effects on microglia.

Microglia, the brain's resident immune cells, are key drivers of the neuroinflammatory response in AD and other neurodegenerative disorders [29, 30].

Once described as existing in a quiescent or resting state, microglia are dynamic cells that are constantly surveying their microenvironment for injury-inducing mediators [31]. Microglia were previously regarded as having either a pro-inflammatory (M1) or resting (M2) phenotype, but more recent research views microglial activation patterns as more complicated, with microglia assuming a diversity of phenotypes in response to noxious extracellular signal [32, 33]. Microglia play a pivotal role in the maintenance of brain homeostasis, but those homeostatic functions are lost in neurodegenerative disorders. Multiple groups have identified a disease associated microglia (DAM) or microglial neurodegenerative phenotype (MGnD), which is present in AD [34, 35]. These DAM show downregulation of homeostatic microglia genes, including p2ry12/p2ry13 and tmem119, and upregulation of pro-inflammatory and AD-associated factors, including apoe, clec7a, and cd11c. Additionally, proliferation and activation of microglia in the brain, concentrated around amyloid plaques, is a prominent feature of AD [36, 37]. Furthermore, a number of recently identified AD-risk genes are related to regulation of microglia and the innate immune system, including complement receptor 1 (CR1) and triggering receptor expressed on myeloid cells 2 (TREM2) [38-41]. These findings suggest that shifts in microglia function and phenotype are a key part of the pathological development of AD.

The goal of this study was to assess the effect that human brain microvascular endothelial cell (HBMVEC) injury *in vitro* by stressors related to diabetes would have on the activation state of microglia. HBMVECs exposed to high-glucose functions as an *in vitro* model of the hyperglycemia found in diabetes. Additionally, diabetes is related to a number of secondary complications, including significantly increased risk of cerebral ischemia, with some evidence suggesting that hyperglycemia-induced endothelial cell dysfunction may exacerbate damage in the event of hypoxic injury.

Therefore, hypoxia was implemented as an additional stressor to induce endothelial cell injury in this *in vitro* model. We hypothesize that diabetes-related endothelial cell injury will induce pro-inflammatory changes in the expression patterns of microglia.

Methods

Cell culture and other reagents

Human brain microvascular endothelial cells (HBMVECs- ACBRI 376) and complete medium with recombinant growth factors (4Z0-500) were purchased from Cell Systems (Kirkland, WA, USA). Murine immortalized microglia (BV-2) were kindly provided by Dr. Navindra Seeram (University of Rhode Island, Kingston, RI, USA). Low-glucose Dulbecco's Modified Eagle Medium (DMEM), cell culture grade D-glucose, antibiotic/antimycotic (Ab/Am), fetal bovine serum (FBS), and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Culture and treatment of HBMVECs and BV-2

HBMVECs were maintained in culture in complete medium in accordance with protocols described by Cell Systems. Cells were maintained at 37°C in 5% CO_2 in complete medium. Media was switched to low glucose (5mM) DMEM with 1% bovine serum albumin for treatments. HBMVECs were incubated with low glucose (5mM) or high glucose (30mM) treatment media for 6 hr. Cells were maintained in normoxia for the full 6 hr, or were exposed to hypoxic (1% O_2) conditions for the final 1 hr of treatment. Following treatment, conditioned media were collected and centrifuged briefly at 1,000 x g in sterile conditions to pellet debris, and used for subsequent treatment of BV-2.

BV2 were maintained at 37°C in 5% CO_2 in low glucose (5mM) DMEM with 10% FBS, 1% Ab/AM, and 250 nM L-glutamine added. HBMVEC-conditioned media was used to treat BV-2 for 24 hr. Control groups were treated with low glucose or high glucose DMEM + 1%BSA for 24 hr.

Cytotoxicity Assay

Cellular damage of HBMVECs was assessed by measuring lactate dehydrogenase (LDH) release in the conditioned medium. Following treatment, supernatant was transferred to a clear 96-well plate and total LDH was assessed using the cytotoxicity detection kit (Millipore Sigma, Burlington, MA, USA). Absorbance values were read at 490nm following incubation with supplied chromogenic dye and catalyst using a Synergy HTX multi-mode reader (Biotek Instruments, Winooski, VT, USA).

Detection of Reactive Oxygen Species (ROS)

The production of reactive oxygen species (ROS) in HBMVECs was determined by a fluorescent probe (DCF-DA) using a previously described method with modification (Ma et al., 2017). HBMVECs were seeded in a black-walled, clear-bottom 96-well plate at a density of 10,000 cells per well (100,000 cells per mL) and allowed to grow for 36 hr. Cells were then treated as previously described with high glucose with or without hypoxia for 6 hr in serum-free conditions. Following the 6-hr treatment, DCF-DA (10µM) was added to each well and incubated at 37°C in the dark for 25 min. Cells were washed 3 times with phosphate buffered saline (PBS). The fluorescence signal of each well was measured at 495nm (excitation) and 529nm (emission) using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Griess Assay

The production of nitric oxide species (NOS) was determined using the Griess reagent system. BV-2 were grown in clear 24-well plates at 100,000 cells/mL. Following treatment with HBMVEC-conditioned media for 24 hr, culture media was transferred to a clear 96-well plate and total NOS was assessed using the Griess

reagent kit (Promega, Fitchburg, WI, USA). Absorbance values were read at 535nm using a Synergy HTX multi-mode reader (Biotek Instruments, Winooski, VT, USA).

Western Blot

Following treatments, cells were rinsed in ice-cold PBS and lysed in 10mM Tris-HCl, 150mM NaCl buffer containing protease inhibitors. Lysates were mixed with 40% 4x Sample Buffer (4x Laemmli Sample Buffer (BioRad, Hercules, CA, USA) with 10% 2-mercaptoethonal) and heated to 100°C for 10 min. Samples were resolved by SDS-PAGE on 4-20% Novex gradient gels (Invitrogen, CA) transferred to nitrocellulose membranes (iBlot, Invitrogen, Carlsbad, CA, USA). Membranes were blocked in 5% BSA in Tris buffered saline (TBS) containing 0.05% Tween-20. Primary antibodies for this study include iNOS (Abcam, MA; 1:250), TREM2 (Novus Biologicals, CO, 1:500), Iba1 (GeneTex, CA, 1:1,000), CLEC7A (Novus Biolgoicals, CO, 1:500), P2RY12 (Novus Biologicals, CO, 1:500), CD11c (Novus Biologicals, CO, 1:250), and β-actin (Santa Cruz, CA; 1:10,000). Bound antibody was detected by InfraRed detectable secondary antibodies (LI-COR, NE; 1:10,000), using LI-COR Odyssey infrared scanner for imaging (LI-COR, Lincoln, NE, USA). Blots were analyzed using NIH ImageJ software and normalized against the housekeeper protein.

ELISA

IL-1β was measured from HBMVEC-conditioned media, and TNFα and IL-6 were measured from BV-2-conditioned media using Enzyme Linked Immunosorbent Assay (ELISA) from Biolegend (San Diego, CA, USA; Human IL-1β cat. #437004, Mouse TNFα cat. #430904, Mouse IL-6 cat. #431304). The protocol provided by the manufacturer was followed, without modifications. At the conclusion of the assay,

absorbance was detected at 570nm and 450nm using Synergy HTX multi-mode reader (Biotek Instruments, Winooski, VT, USA).

Gelatin Zymography

Gelatin zymography was performed as previously described (Milner et al., 2003) to assess the activity of MMP-9 in BV-2 supernatant following treatments. Briefly, samples were run on 10% Gelatin Zymogram Plus Protein Gels (Invitrogen, Carlsbad, CA, USA). Following electrophoresis, gels were incubated at room temperature with zymogram renaturing buffer (Novex, Carlsbad, CA, USA) for 30 min, and then incubated with zymogram developing buffer (Novex, Carlsbad, CA, USA) for 30 min. Gels were further incubated with fresh developing buffer overnight at 37°C. Gels were stained with Comassie Blue for 1 hr, and de-stained in diH_2O for 1 day. Images scanned on Epson Scanner (Epson, Long Beach, CA, USA) and analyzed using ImageJ.

Phagocytosis assay

Analysis of phagocytic activity of BV-2 was done by measuring uptake of fluorescently labeled beads. Protocol for the phagocytosis assay was derived from previously published works (Zhang et al., 2017; Cai et al., 2017; Lian et al., 2016; Lucin et al., 2013) with modifications. BV-2 cells were plated in 8-well chamber slides at a density of 10,000 cells per well in DMEM with 10% FBS and grown for 48 hr. Cells were treated as before with HBMVEC-conditioned media for 24 hr. Preopsonized latex beads of 1 um diameter (Sigma, cat. #L2778-1ML) were prepared in PBS with 50% FBS at 10% (v/v) for 1 hr at 37°C. Beads were then further diluted 1:100 in DMEM to make a 0.01% (v/v) beads and 0.05% (v/v) FBS solution.
At the conclusion of 24-hr treatment, BV-2 media was removed and 500 µL of bead solution was added to each well for 1 hr at 37°C. Beads were then removed and cells

were rinsed three times with cold PBS. Cells were fixed with 4% paraformaldehyde (PFA) in PBS (200 µL per well) for 4 min at room temperature. PFA was removed, cells were then blocked and permeabilized with 5% horse serum and 0.3% Triton X-100 in PBS (200 µL per well) for 30 min at room temperature. Cells were washed again with cold PBS three times.

To image, antifade mounting solution with DAPI (Invitrogen, cat. #S36964) was added, slide was covered with a cover slip, and sealed. Imaged beads at 575-610nm (red) and DAPI at 345-453nm (blue) using the EVOS® FL Auto Cell Imaging System (ThermoFisher Scientific, Waltham, MA, USA). ImageJ was used to quantify red/blue in all images.

Statistical Analysis

Data were analyzed for significance using one-way analysis of variance (ANOVA) and multiple comparisons carried out using the post-hoc Bonferroni test on GraphPad Prism (version 8.01). Data are represented as Mean +/- SEM. Groups contain n=6 unless otherwise specified.

Results

Injury of brain endothelial cells by exposure to high-glucose levels and hypoxia

Human brain microvascular endothelial cells (HBMVECs) were either treated with 30 mM glucose (6 hr), exposed to hypoxia (1 hr), or exposed to hypoxia plus high glucose (6 hr). LDH release in the supernatant was measured to estimate lethal cell injury. Treatment of cells with glucose evoked a small but not significant increase in LDH release. In contrast, hypoxia exposure significantly increased LDH release compared to untreated controls ($p<0.001$). The level of LDH detected in cells exposed to hypoxia plus high-glucose was higher than levels measured in response to hypoxia alone (Fig. 1A).

To explore indices of non-lethal injury the generation of ROS and release of the inflammatory mediator IL-1β were assessed. HBMVECs analyzed using the fluorescent probe DCF-DA to measure ROS showed that glucose treatment did not alter ROS production compared to control cells, while hypoxia exposure increased ROS levels relative to control, but the increase was not significant. HBMVECs exposed to both glucose and hypoxia showed significantly increased production of ROS compared to control ($p<0.001$), glucose ($p<0.001$), or hypoxia ($p<0.05$) (Fig. 1B). Measurement of IL-1β levels in supernatant by ELISA indicated that treatment with glucose increased IL-1β levels, but the increase was not significant ($p=0.11$), while hypoxia exposure did not affect IL-1β levels. Interestingly, the combination of glucose treatment and hypoxia exposure appeared to mitigate the increase evoked by glucose alone (Fig. 1C).

Injured brain endothelial cells increase reactive nitrogen species production in BV-2 cells

To determine whether injured endothelial cells can affect reactive nitrogen species (RNS) generation in microglia, the microglial cell line BV-2 was treated with HBMVEC-conditioned media for 24 hr. Conditioned media were collected following endothelial cell treatment with either 30 mM glucose (6 hr) (EC-G), exposed to hypoxia (1 hr) (EC-H), or exposed to hypoxia plus high glucose (6 hr) (EC-G+H). The Griess assay was used to assess nitric oxide (NO) production in the BV-2 supernatant (Fig. 2A) and iNOS levels in BV-2 lysates were analyzed by western blot (Fig. 2B).

BV-2 cells exposed to conditioned media from glucose-treated endothelial cell cultures (EC-G) showed a significant increase in both NO production ($p<0.001$) (Fig. 2A) and iNOS expression ($p<0.001$) (Fig. 2B) compared to BV-2 cells incubated with untreated endothelial cell-conditioned media. In contrast, treatment of BV-2 cells with conditioned media from hypoxia-exposed endothelial cells (EC-H) did not affect either NO production or iNOS levels (Figs 2A, 2B). Treatment of BV-2 cells with conditioned media from endothelial cells exposed to both glucose and hypoxia (EC-G+H) significantly increased NO production compared to BV-2 cells exposed to conditioned media from only glucose-treated endothelial cell cultures ($p<0.001$). There was no significant difference in BV-2 iNOS levels evoked by conditioned media from endothelial cells treated with glucose alone and media derived from endothelial cells exposed to both glucose and hypoxia (Fig. 2B).

Glucose-injured microvascular endothelial cells induce changes in inflammatory proteins released from BV-2 cells

The release of cytokines, TNFα and IL-6, by BV-2 cells treated with HBMVEC-conditioned media for 24 hr was assessed by ELISA. Conditioned media were collected following endothelial cells either treated with 30 mM glucose (6 hr) (EC-G),

exposed to hypoxia (1 hr) (EC-H), or exposed to hypoxia plus high glucose (6 h) (EC-G+H).

BV-2 cells that were treated with media from glucose-injured endothelial cells (EC-G) released a significantly ($p<0.001$) higher level of the pro-inflammatory cytokine TNFα compared to microglial cells exposed to control conditioned media (Fig. 3A). On its own, conditioned media from hypoxic-injured endothelial cells (EC-H) did not alter BV-2 production of TNFα. However, media from endothelial cells that were exposed to both glucose and hypoxia (EC-G+H) increased TNFα production in BV-2 to higher levels than elicited by media from glucose-only injured endothelial cells ($p<0.001$) (Fig 3A).

In contrast to the results obtained for TNFα, the data show that conditioned media from glucose-injured endothelial cells (EC-G) caused a significant ($p<0.001$) decrease in the release of IL-6, a complex cytokine with both pro- and anti-inflammatory activities (Fig. 2B). Conditioned media from hypoxic-injured endothelial cells (EC-H) did not affect BV-2 IL-6 levels. Also, media from endothelial cells exposed to both glucose and hypoxia (EC-G+H) showed a decrease in IL-6 release that was comparable to that observed for glucose (EC-G) (Fig. 3B).

Supernatant from BV-2 cells was collected following treatment with HBMVEC-conditioned media for 24 hr, as described above, and MMP-9 activity measured by gel zymography. Conditioned media from hypoxia-exposed endothelial cells (EC-H) did not affect MMP9 activity (Fig. 3B). There was a significant ($p<0.001$) increase in MMP9 activity, relative to controls, elicited media from glucose only (EC-G) and glucose plus hypoxia (EC-G+H) (Fig. 3C).

BV-2 treated with conditioned media from injured brain endothelial cells show altered expression of microglia activation markers

BV-2 cells were treated with HBMVEC-conditioned media for 24 hr, as described above, and western blot was used to determine changes in protein expression for several microglia activation- and disease-associated markers (DAMs). The expression of Iba1 was significantly ($p<0.01$) increased in in BV-2 cells treated with media from glucose plus hypoxia-injured endothelial cells (EC-G+H) compared to controls. Conditioned media from neither glucose alone nor hypoxic injury alone affected expression of Iba1 (Fig. 4A). Changes in the expression of BV-2 CD11c and CLEC7A in response to endothelial cell conditioned media were similar in pattern, differing slightly in magnitude. Conditioned media from glucose-injured endothelial cells (EC-G) significantly increased expression of CD11c ($p<0.01$) (Fig. 4B) and CLEC7A ($p<0.01$) (Fig. 4C) as did conditioned media from glucose plus hypoxia injury (EC-G+H) for both CD11c ($p<0.01$) and CLEC7A ($p<0.05$). Exposure of BV-2 cells to HBMVEC-conditioned media collected as described above did not affect expression of TREM2 (data not shown).

Western blot was also used to assess the expression of a homeostatic microglia marker, P2Y12. Expression of P2Y12 was significantly decreased when BV-2 cells were treated with media from glucose injury (EC-G) ($p<0.001$), hypoxic injury (EC-H) ($p<0.01$), or glucose plus hypoxic injury (EC-G+H) ($p<0.001$) compared to controls (Fig. 4D). Together, these changes indicate a shift towards a DAM phenotype in the BV-2 treated with conditioned media from damaged brain endothelial cells.

The phagocytic ability of BV-2 cells is altered in response to conditioned media from injured endothelial cells

BV-2 cells were treated with HBMVEC-conditioned media, as described above, and uptake of fluorescently labeled beads measured to assess phagocytic ability (Fig. 5A). Bead uptake by BV-2 cells treated with glucose injury media (EC-G) was significantly increased ($p<0.05$) compared to controls. In contrast, conditioned media from hypoxic injury (EC-H) did not significantly alter phagocytic ability of BV-2 cells. However, treatment of BV-2 with conditioned media from glucose plus hypoxia injury (EC-G+H) significantly ($p<0.001$) reduced the phagocytic response compared to glucose injury alone conditioned media (EC-G) (Fig. 5B).

Discussion

For the first time, we have characterized changes in microglia activation following damage to brain endothelial cells *in vitro*. HBMVECs were treated with high-glucose with and without hypoxia to assess injury to endothelial cells in diabetes-like hyperglycemic conditions. BV-2 microglia were subsequently treated with conditioned media from damaged HBMVECs, and analyzed for alterations in inflammatory phenotype. We found that endothelial cell damage by glucose and hypoxia produced pro-inflammatory alterations to microglia, including increased production of NO and TNFα, and increased MMP-9 activity. Additionally, endothelial cell damaged may induce a shift towards a disease-associated phenotype in microglia, with BV-2 showing increased expression of CD11c and CLEC7a following HBMVEC damage, and decreased expression of the homeostatic marker P2Y12.

The alterations found in this study hint at the phenotypic changes found in microglia following diabetes-related brain endothelial cell damage. Increases in NO and TNFα, like those observed in the EC-G and EC-G+H groups, are indicative of a pro-inflammatory microglia phenotype. Similar upregulations have been identified in neuroinflammatory disorders, including Alzheimer's disease [33]. MMP-9 activity has likewise been found to be increased in AD [42]. Past research focused on the M1 and M2 phenotypes of microglia and often used Iba1 as a marker to identify "activated" microglia [43]. Here, the expression of Iba1 was significantly elevated in microglia in the EC-G+H group. More recently, microglia research has recognized the complexity of microglia activation states, recognizing the previously mentioned DAM as one alternative phenotype. DAM exhibit reduction of homeostatic microglia genes and increased expression of pro-inflammatory and AD-associated genes [34, 35]. Our findings suggest that hyperglycemia-related endothelial cell damage induces the

conversion of microglial towards a DAM-like phenotype, indicated by significantly increased expression of CD11c and CLEC7A, as well as significantly decreased expression of P2RY12, in the EC-G and EC-G+H groups. However, this conclusion is hindered by the additional finding that there is no change in expression of TREM2 following HBMVEC injury. Past findings indicate that signaling through TREM2 may be responsible for the DAM phenotypic switch, suggesting that TREM2 expression changes may occur prior to those found in this study.

These findings also indicate that HBMVEC damage lead to alterations in phagocytic function in BV-2 microglia, with EC-G treated BV-2 exhibiting significantly increased fluorescent bead uptake, and a significant reduction found for the EC-G+H group. Phagocytosis is a key aspect of microglia function in the healthy brain, responsible for clearance of debris, apoptotic cells, and synaptic pruning [33]. In AD, phagocytic microglia can help to clear aggregated Aβ. However, there is still some debate as to whether microglia phagocytosis is beneficial or detrimental in disease [44]. Microglia phagocytosis is now thought to play a role in the conversion of microglia to DAM, specifically through the ApoE-TREM2 pathway [35]. TREM2 KO mice have impaired phagocytosis, related to increased Aβ plaque burden, reduced microglia localization to plaques, and increased neuritic dystrophy [33]. Other studies indicate that anti-inflammatory microglia highly express phagocytosis-associated genes [45]. Therefore, the increased expression of DAM markers along with reduced phagocytic ability identified in EC-G+H treated microglia indicates a likely AD-like, pro-inflammatory phenotype.

As indicated previously, endothelial cells may function as a key driver of the brain's inflammatory response, including in AD. Previous research has found that activated or damaged brain endothelial cells secrete a number of factors that have

been related to neuroinflammation and the activation of pro-inflammatory microglia, including thrombin, MMPs, and a number of cytokines [22, 46-49]. Our findings indicate a role for vascular-derived IL-1β and ROS in propagating the inflammatory cycle in the brain. Here, brain endothelial cells injured by glucose and glucose plus hypoxia exhibit an upregulation in IL-1β and ROS, respectively, and these increases were correlated with the up-regulation of pro-inflammatory markers in microglia. IL-1β acts as an amplifier of immune reactions, and can stimulate a number of downstream neuroinflammatory processes. In the periphery, IL-1β stimulated the differentiation of monocytes to M1-like macrophages [50]. *In vitro,* IL-1β enhanced microglia release of NO following LPS stimulation [51]. High levels of oxidative stress have been identified in the AD brain, and ROS function as mediators of injury and inflammation [52]. ROS-mediated signaling pathways are relevant for the induction of inflammatory signaling, including the stimulation of pro-inflammatory cytokines and NO [53]. TNFα, NO, and MMP9 are all produced in response to both oxidative stress and cytokine signaling [42, 52-54], highlighting the potential for these endothelial-derived mediators to activate a pro-inflammatory response like that found in our co-culture system. Of particular interest, both IL-1 and ROS activate NF-κβ-mediated pro-inflammatory signaling that stimulates the production on cytokines [52, 53, 55], providing one possible mechanism by which endothelial produced mediators may signal through. In some cases, including expression of iNOS and induction of phagocytosis, EC-G+H microglia showed reduced up-regulation in inflammatory mediators compared to EC-G. This finding suggests that there may also be mechanisms through which microglia are differentially activated in response to alternative inflammatory mediators, for example IL-1β and ROS.

It is interesting that hypoxic damage to endothelial cells alone did not show significant effects on microglia. Others have found exposure to high-glucose induced increased expression of pro-inflammatory cytokines in BV-2 and primary microglia, including TNFα and IL-6 [56-58]. Additionally, Zhang et al., 2015 found that exposure to high-glucose enhanced microglia response to LPS [59]. It is therefore plausible that the presence of high glucose levels exacerbates the response of microglia to endothelial cell damage, as indicated by the strong increases in pro-inflammatory mediators found in both glucose-associated groups in this study

This novel study is the first to look to characterize the effects of hyperglycemia-related brain endothelial cell injury on microglia activation and function. Though this simplified *in vitro* system contains some limitations, it does highlight an important connection between the cerebrovascular system and microglia in dictating the neuroinflammatory response. A strengthened understanding of how endothelial cell damage can alter microglia function and lead to an inflammatory response may help us to better characterize the neuroinflammation found in Alzheimer's and other neurodegenerative disorders. Additionally, greater knowledge of the mediators responsible for this endothelial cell-microglia crosstalk may help inform novel therapeutic targets and techniques for mitigating disease-related inflammation at its potential point of origin, the vasculature.

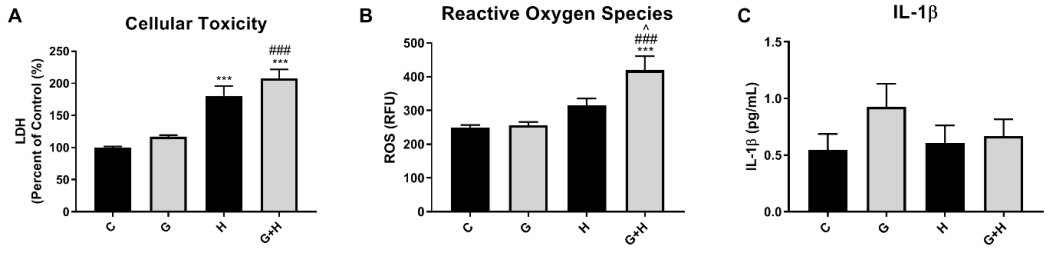

Figure 1 | HBMVEC injury following treatment with high glucose with and without hypoxia. Human brain microvascular endothelial cells (HBMVECs) were exposed to high-glucose, one-hour hypoxia, or a combination of both. Cells and supernatant were collected to assess measures of cellular injury: (A) lactate dehydrogenase (LDH) release as a measure of cell toxicity, (B) reactive oxygen species (ROS) production measured with DCF-DA, represented as relative fluorescence units (RFU), and (C) IL-1β secretion in the supernatant measured by ELISA. ***$p<0.001$ vs. Control, $^{###}p<0.001$ vs. Glucose, ^$p<0.05$ vs. Hypoxia.

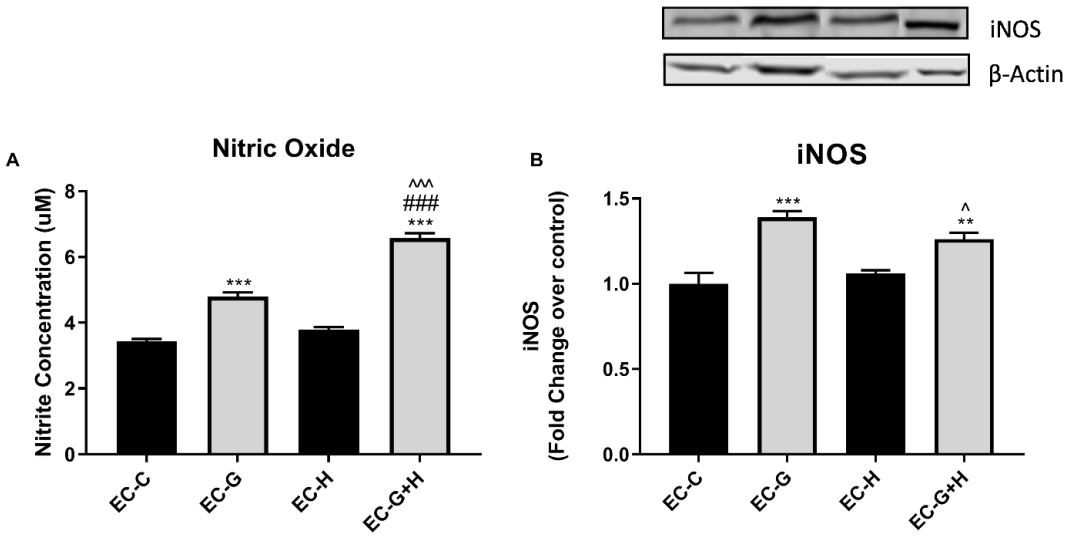

Figure 2 | HBMVEC injury induces increased nitric oxide production by BV-2 microglia. Conditioned media from injured human brain microvascular endothelial cells (HBMVECs) was used to treat BV-2 microglia for 24 hours. Supernatant and cell lysate were collected following treatments and used to measure (A) Nitric Oxide (NO) in the supernatant using Griess reagent and (B) expression of iNOS in the lysate by western blot. **p<0.01 vs. Control, ***p<0.001 vs. Control, $^{###}$p<0.001 vs. Glucose, ^p<0.05 vs. Hypoxia, ^^^p<0.001 vs. Hypoxia.

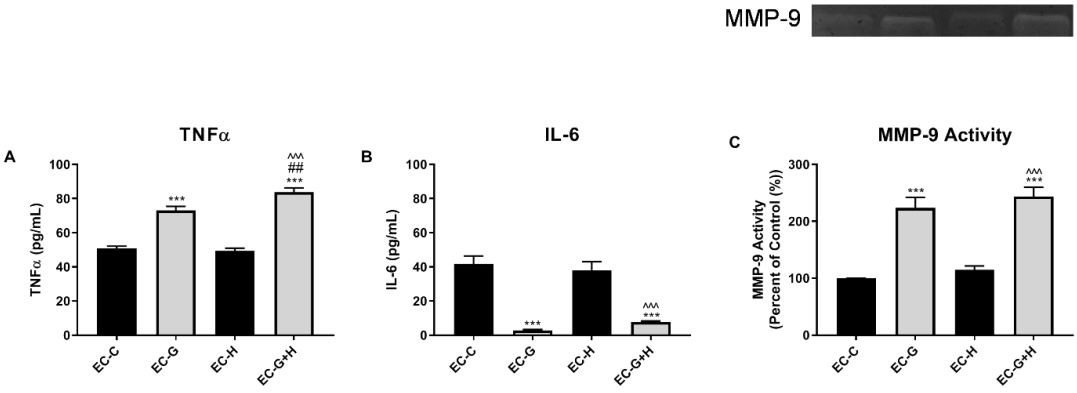

Figure 3 | HBMVEC injury induces increased production of inflammatory mediators by BV-2 microglia. Conditioned media from injured human brain microvascular endothelial cells (HBMVECs) was used to treat BV-2 microglia for 24 hours. Supernatant from BV-2 was collected following treatments. ELISA was used to measure secretion of (A) TNFα and (B) IL-6. (C) The enzymatic activity of MMP-9 was measured by gel zymography. ***p<0.001 vs. Control, $^{###}$p<0.001 vs. Glucose, ^^^p<0.001 vs. Hypoxia.

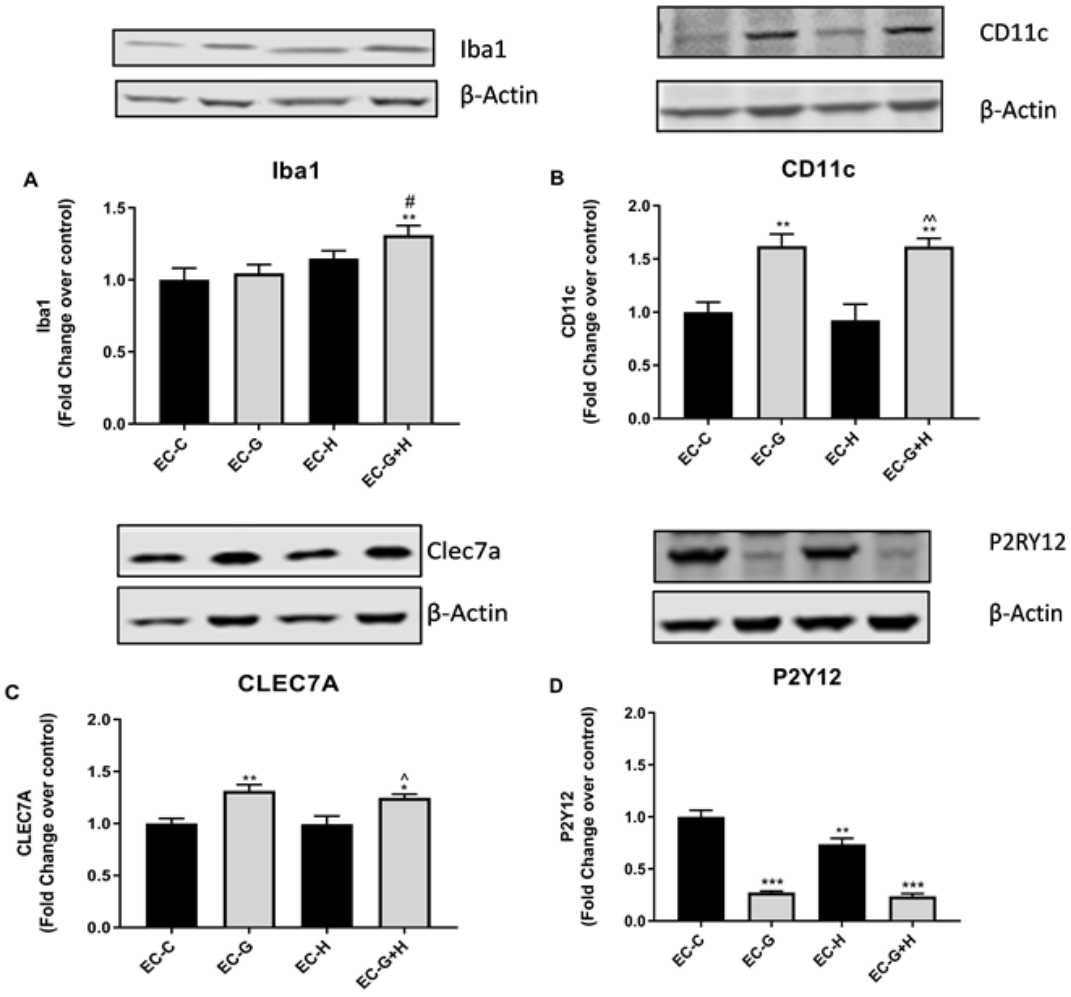

Figure 4 | BV-2 treated with conditioned media from injured HBMVEC show altered expression of microglia activation markers. Conditioned media from injured human brain microvascular endothelial cells (HBMVECs) was used to treat BV-2 microglia for 24 hours. Western blot was used to assess the expression of microglia markers, (A) Iba1, (B) CD11c, (C) CLEC7A, and (D) P2Y12. *p<0.05 vs. Control, **p<0.01 vs. Control, ***p<0.001 vs. Control, #p<0.05 vs. Glucose, ^p<0.05 vs. Hypoxia, ^^p<0.01 vs. Hypoxia.

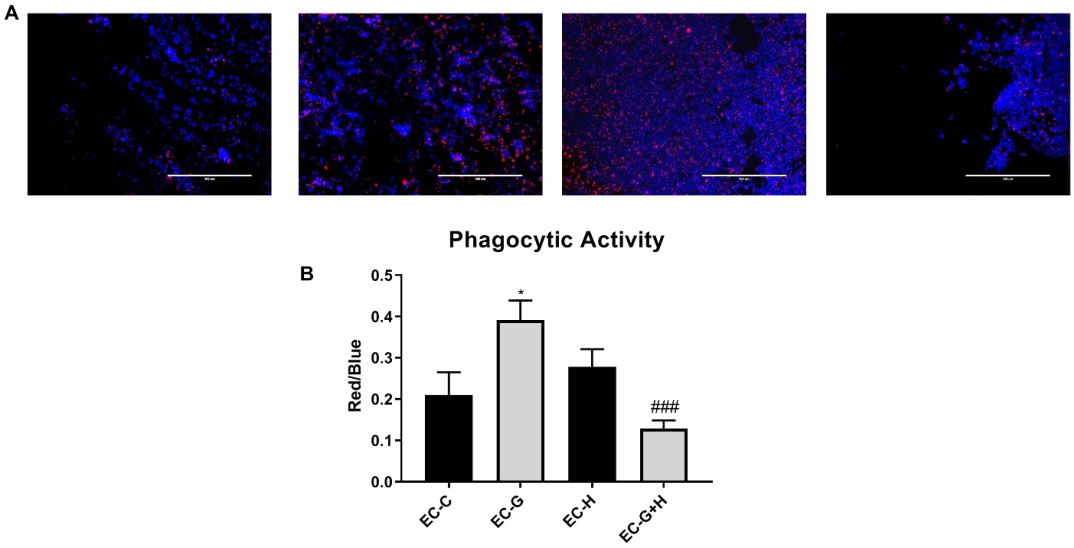

Figure 5 | BV-2 treated with conditioned media from injured HBMVEC show altered phagocytic ability. Conditioned media from injured human brain microvascular endothelial cells (HBMVECs) was used to treat BV-2 microglia for 24 hours. BV-2 were then incubated with fluorescently labeled beads for 1 hour. Cells were then imaged (EC-C, EC-G, EC-H, EC-G+H left to right) (A), and bead uptake per cell was quantified (B) as a measure of phagocytic activity. *$p<0.05$ vs. Control, $^{###}p<0.001$ vs. Glucose.

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CHAPTER 2

MANUSCRIPT 2

Manuscript in preparation

Effects of Apolipoprotein E isoforms on glial and neuronal cells *in vitro*

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Abstract

Mutations to the cholesterol transport protein apolipoprotein E (ApoE) have been identified as a major risk factor for the development of sporadic or late-onset Alzheimer's disease (AD), with the E4 allele representing an increased risk and the rare E2 allele having a reduced risk compared to the primary E3 form. The reasons behind the change in risk are not entirely understood, though ApoE4 has been connected to inflammation-related changes in both the brain and the periphery. It is our hypothesis that ApoE4 acts on astrocytes, microglia, and neurons to promote inflammation and neuronal injury. To test this hypothesis we compared the effects of ApoE isoforms *in vitro* on human astrocytes, a human immortalized microglia cell line (HMC3), and the human neuronal cell line SH-SY5Y. Cells were treated for 24 hours with or without recombinant ApoE2, ApoE3, or ApoE4 (20 nM) and inflammation, activation, and toxicity markers assessed. Our results indicated the expression of inflammatory cytokines IL-1β, TNFα, and IL-6 in human astrocytes was increased in response to all ApoE isoforms, with ApoE4 evoking the highest level of cytokine expression. ApoE4 increased inflammatory cytokines but not oxidative stress proteins in human astrocytes, as there was no measurable change in expression of either NOX4 or iNOS in astrocytes exposed to ApoE isoforms. In response to ApoE2 or ApoE3, microglial cells showed reduced levels of microglial activation markers TREM2 and Clec7a, while ApoE4 induced increased levels of both markers. We examined the effects of ApoE isoforms on neuronal viability and showed that ApoE2 promoted neuron survival through increased BDNF release from astrocytes, while ApoE4 inhibited BDNF release. In addition, ApoE4, acting directly on neurons, caused neuronal toxicity and reduced cell viability. Overall, these results suggest that ApoE4 acts on cells in the brain to promote inflammation and neuronal injury and that

the deleterious effects of ApoE4 on these cells may, in part, contribute to its role as a risk factor for AD.

Introduction

The most significant known genetic risk factor for late-onset Alzheimer's disease (AD) is an allele variant of apolipoprotein E (ApoE), a protein involved in lipid metabolism and cholesterol homeostasis [1]. The ApoE gene has three polymorphic alleles that occur in humans, E2, E3, and E4. These alleles occur at different frequencies, with E3 occurring most frequently at 65-70%, followed by E4 and E2 [1]. However, the frequency of the E4 allele is substantially increased from 13% to approximately 40% in AD patients compared to healthy counterparts [2]. Population studies have shown that the E4 allele is associated with a significantly elevated likelihood for developing Alzheimer's, with just one copy conferring a 4-fold increased risk for developing AD and an 8 year earlier average age of onset compared to individuals homozygous for E3. On the other hand, the E2 variant seems to have protective effects, with the risk for developing AD significantly lower in individuals carrying one E2 allele [1, 2]. Although the relationship between ApoE allele and AD has been well established, the mechanisms behind these risk differences have not been well characterized.

ApoE is primarily synthesized in the liver, but the brain synthesizes its own ApoE as well. In the brain, ApoE mRNA has been found in the cerebral cortex, hippocampus, cerebellum, and medulla [3]. ApoE is synthesized primarily by astrocytes, but it has also been found to be produced by oligodendrocytes, microglia, and neurons under stress conditions [1]. Due to its primary function as a lipid transport molecule, ApoE has largely been studied for its role in Aβ clearance in AD. ApoE4 impairs Aβ clearance compared to ApoE3, and increases amyloid formation in the brain [4]. Brain plaque load is the lowest in ApoE2 and the highest in ApoE4 individuals [2]. More recent research, however, is focusing on more diverse roles for

ApoE in the brain as a mediator of cerebrovascular function, neuroinflammation, synapse formation, and neurotoxicity [2].

A clear role for ApoE in the modulation of the innate immune system has been identified. Neuroinflammation is known to play a key role in the pathological progression of AD [5-7], and ApoE4 is associated with pro-inflammatory changes in both the central nervous system (CNS) and periphery [8]. Post-mortem ApoE4-expressing brain samples exhibit upregulation in proteins related to the regulation of inflammatory response compared to both ApoE3 and ApoE2-expressing tissue [9]. Specifically, a clear role for mediating microglia phenotype and function has been identified for ApoE. Microglia expressing human ApoE4 exhibit significantly increased production of pro-inflammatory mediators, including tumor necrosis factor-α (TNFα), interleukin (IL)-6, and nitric oxide (NO) [10-13]. More recent research using induced pluripotent stem cell (iPSC)-derived microglia expressing human ApoE found that ApoE4 microglia have impaired migration and reduced phagocytic function. These microglia also display enhanced cytokine production in response to LPS compared to ApoE3 expressing microglia [14]. Further support for the ability of ApoE to regulate neuroinflammatory processes comes from the finding that ApoE signaling via triggering receptor expressed on myeloid cells 2 (TREM2) regulates a switch in microglia towards a neurodegenerative phenotype. Targeting ApoE signaling has the ability to restore homeostatic function in microglia [15]. Therefore, ApoE modulation of the innate immune system, particularly through alterations in microglia phenotype, may play a key role in ApoE-related AD pathology.

Along with neuroinflammatory effects on glial cells in the brain, ApoE isoforms have also been shown to differentially modulate cerebrovascular function and blood-brain barrier (BBB) integrity, further contributing to its role as a modulator of

neuroinflammation and damage [2]. In humans, there is a relationship between ApoE4-mediated BBB breakdown and cognitive decline [16]. ApoE4 produces BBB breakdown *in vitro* via activation of cyclophilin A (CypA)-NF-κβ-matrix metalloproteinase-9 (MMP-9) signaling, but this same activation does not occur in ApoE2 or ApoE3. *In vivo*, astrocyte secreted ApoE3, but not ApoE4, suppressed the activation of CypA-NF-κβ-MMP9 signaling [17]. *In vitro,* endothelial cells that express ApoE4 have significantly increased production of inflammatory cytokines and enhanced thrombosis compared to cells expressing ApoE3 [18]. Together, these findings indicate that ApoE4 promotes vascular activation and vascular-based inflammation that likely contributes to the pro-inflammatory effects of ApoE4 in AD-related pathology.

ApoE isoforms may also regulate neuronal survival or toxicity, a key aspect of neurodegenerative pathological processes in AD. Directly, treatment of iPSC-derived neurons with recombinant ApoE2/3/4 stimulates various signal transduction cascades with different levels of potency. One such cascade is amyloid precursor protein (APP) synthesis and synapse formation, which is enhanced in ApoE4 treated neurons and reduced in ApoE2 treated compared to ApoE3 [19]. ApoE4 has been shown to promote tau phosphorylation and neurotoxicity [2], as well as impair synaptogenesis [3]. ApoE4 is more susceptible than other isoforms to proteolytic cleavage, and this cleavage fragment is neurotoxic [20]. Indirectly, ApoE may also influence synapse formation and neuron survival through astrocyte activity. ApoE3 enhances astrocyte phagocytosis of synapses, while ApoE4 decreases the same activity [21]. Astrocytes are also a primary producer of neurotrophic support, including brain derived neurotrophic factor (BDNF). BDNF is decreased in the serum and brain of AD patients compared to healthy controls [22, 23], and ApoE isoforms have been found

to modulate astrocyte production and secretion of BDNF [24]. Overall, these findings further suggest that ApoE isoform-specific effects on cells in the brain, including neurons and astrocytes, is responsible for the substantially increased ApoE4-related AD risk.

The goal of this study was to better understand how the ApoE isoforms (ApoE2/3/4) confer differential AD-related risk by assessing cell-specific ApoE-related neuroinflammatory and neurotoxic effects. It is our hypothesis that markers of inflammation and neurotoxicity are increased in response to ApoE4 compared to E2 and E3. Furthermore, we hypothesize that ApoE2 will reduce these same markers, highlighting its neuroprotective role. Human microglia, astrocytes, and neurons were treated with ApoE2/3/4 *in vitro*, and the effects of the ApoE isoforms on markers related to neuroinflammation and neurotoxicity were then assessed. The findings here help to better characterize the isoform and cell-specific effects of ApoE that may contribute to the development of AD and related inflammation and neuron death.

Methods

Cell culture and Other Reagents

Human immortalized microglia cell line (HMC3; CRL-3304) and Eagle's Minimum Essential Medium (EMEM; 30-2003) were purchased from ATCC (Manassas, VA, USA). Human primary astrocytes (#1800) and astrocyte medium (#1801) along with astrocyte growth supplements were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Human neuroblastoma cells (SH-SY5Y) were kindly provided by Dr. Navindra Seeram (University of Rhode Island, Kingston, RI, USA). Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12, Fetal Bovine Serum (FBS), Bovine Serum Albumin (BSA), poly-L-lysine, and recombinant human ApoE2 (rh-ApoE2), ApoE3 (rh-ApoE3), and ApoE4 (rh-ApoE4) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell Culture and Treatments

HMC3 were grown and maintained in EMEM with 10% FBS. Treatment media was serum free EMEM. Human primary astrocytes were plated on poly-L-lysine coated plates and maintained in astrocyte medium with growth supplements and 2% FBS, according to protocols provided by ScienCell. For treatments, media was replaced with serum-free DMEM. SH-SY5Y were grown and maintained in DMEM/F12 with 10% FBS, and treatments were done in serum-free DMEM/F12.

Rh-ApoE2/3/4 were dissolved in PBS pH 7.4 to make a stock at 6 μM. All cells were treated with cholesterol (100 μM) (control) with or without 20 nM rh-ApoE2/ApoE3/ApoE4 for 24 hr. ApoE and cholesterol were added separately to cultures.

Cell Viability

The viability of SH-SY5Y was assessed following treatments with rh-ApoE2/3/4. Cells were seeded in 96-well plates at 100,000 cells/mL, and were allowed to adhere for 48 hr. They were then treated with cholesterol with or without rh-ApoE2/3/4 for 24 hr. Cellular viability was determined as a percentage of control (cholesterol) by CellTiter 96 ® Aqueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI, USA), and read for absorbance at 490nm on the Synergy HTX multi-mode reader (Biotex Instruments, Winooski, VT, USA).

Cytotoxicity Assay

Cellular toxicity of SH-SY5Y following treatments was assessed by measuring lactate dehydrogenase (LDH) release in the conditioned media. Supernatant from cells was collected and transferred to a 96-well plate. Total LDH was assessed using a cytotoxicity detection kit (Millipore Sigma, Burlington, MA, USA). Samples were incubated with chromogenic dye and catalyst, then absorbance was read at 490nm using a Synergy HTX multi-mode reader (Biotek Instruments, Winooski, VT, USA).

Western Blot

Western blot was used to measure protein expression in all cell types following 24 hr treatment with rh-ApoE2/3/4. Cells were lysed in Tris-HCl buffer with protease inhibitors. Lysates were mixed with 40% 4x Laemmli Sample Buffer (BioRad, Hercules, CA, USA) with 10% 2-mercaptoethanol. Samples were run by SDS-PAGE on gradient gels (Invitrogen, Carlsbad, CA, USA) and transfer was done by dry blotting to Nitrocellulose Membranes (iBlot, Invitrogen, Carlsbad, CA, USA). All membranes were blocked in 5% BSA in Tris buffered saline (TBS) containing 0.05% Tween-20. Membranes were incubated with primary antibody overnight at 4°. Primary antibodies include IL-6 (Bioss, MA; 1:500), TNFα (Novus Biologicals, CO; 1:500), IL-1β (Bioss, MA; 1:1,000), iNOS (Abcam, MA; 1:250), NOX4 (Sigma, MA; 1:1,000),

TREM2 (Novus Biologicals, CO; 1:500), Clec7a (Novus Biologicals, CO; 1:500), BDNF (Abcam, MA; 1:1,000), and β-Actin (Santa Cruz, CA; 1:10,000). Bound antibody was detected by infrared secondary antibodies (Li-COR, NE; 1:10,000), and imaged on LiCor Odyssey (Li-COR Biosciences, Lincoln, NE, USA). Quantification of western blots was done in ImageJ, and values for each protein were normalized to β-Actin.

Statistical Analysis

All data were analyzed for significance using one-way analysis of variance (ANOVA) and multiple comparisons post-hoc were completed using Bonferroni test on GraphPad Prism (version 8.04; GraphPad, CA, USA). Data represented as percent of control (cholesterol), Mean± SEM, groups contain N=3 unless otherwise specified.

Results

ApoE4 increases inflammatory cytokines but not oxidative stress proteins in human astrocytes

ApoE4 is associated with increased inflammation in various model systems and disease states [8, 25, 26]. Recently, the regulation of cholesterol by ApoE4 in the brain has been shown to be cell-type specific [27]. While astrocytes have been widely studied for their role in ApoE4-related lipid homeostasis and glucose metabolism [28], less is known about their function in ApoE4-associated neuroinflammation. Here we explored the ability of ApoE isoforms to affect expression of pro-inflammatory cytokines in human astrocytes. Human astrocytes were treated with recombinant ApoE isoforms, ApoE2, ApoE3, or ApoE4 for 24 hr and cell lysate analyzed (Fig. 1).

Expression of inflammatory cytokines IL-1β (Fig. 1A), TNFα (Fig.1 B), and IL-6 (Fig. 1C) in human astrocytes was evaluated by western blot. Treatment of astrocytes with all three ApoE isoforms significantly ($p<0.001$) increased IL-1β expression compared to untreated control cells (Fig. 1A). However, ApoE4 evoked the highest level of IL-1β, which was significantly higher than that observed with either ApoE 2 ($p<0.01$) or ApoE 3 ($p<0.05$). ApoE2 and ApoE3 both appeared to elicit a small but not significant increase in TNFα. In contrast, ApoE4 significantly ($p<0.05$) increased TNFα compared to control as well as to both ApoE2 and ApoE3 (Fig. 1B). The ability of ApoE isoforms to affect IL-6 expression followed a pattern similar to that observed for IL-1β. Treatment of astrocytes with all three ApoE isoforms significantly ($p<0.001$) increased IL-6 expression compared to untreated control cells (Fig. 1C). However, ApoE4 evoked the highest level of IL-6, which was significantly ($p<0.05$) higher than that observed with either ApoE 2 or ApoE3.

Inflammation and oxidative stress are related processes and often respond similarly to external stimuli. To determine whether oxidative stress is affected by ApoE isoforms, human astrocytes were treated as described above and analyzed for the expression of the oxidative stress proteins NADPH oxidase 4 (NOX4) and inducible nitric oxide synthase (iNOS). The data showed that there was no measurable change in expression of either NOX4 or iNOS in response to ApoE2, ApoE3, or ApoE4 (Table 1).

ApoE4 enhances the inflammatory activation of microglia

Microglia exhibit a range of phenotypes and activation states in response to extracellular signals. We examined the effect of ApoE isoforms on the human microglial cell line HMC3. Cells were treated with recombinant ApoE isoforms, ApoE2, ApoE3, or ApoE4 for 24 hr and cell lysate analyzed. Western blot was used to measure the expression of cytokines and markers related to microglial activation (Fig. 2).

We examined the effect of ApoE isoforms on both the pro-inflammatory cytokine TNFα as well as on IL-6, a complex cytokine with both pro- and anti-inflammatory properties. It should be noted that the response/activity of HMC3 under control conditions (untreated) appears elevated. Although statistically significantly conclusions are difficult to draw, clear trends are evident. Exposure to ApoE2 or ApoE 3 lowers expression of TNFα while treatment with ApoE4 increases TNFα level (Fig. 2A). In contrast, neither ApoE2 nor ApoE3 affected IL-6 expression while ApoE4 significantly ($p<0.05$) decreased IL-6 expression compared to control (Fig. 2B). Microglia have been shown to take on an alternate activation state in AD, termed disease-associated microglia (DAM) [15]. DAMs are characterized by decreased expression of homeostatic markers and increased expression of a sub-set of

inflammatory indicators. HMC3 were treated as described above and western blot was used to assess the expression of markers related to microglia activation, TREM2 and CLEC7a (Figs. 2C, 2D). Similar to the pattern noted above, a high level of activation was observed in untreated (control) HMC3. Despite the elevated basal response of HMC3, the data showed that treatment with either ApoE2 or ApoE3 diminished expression of TREM2 and CLEC7a while ApoE4 exposure increased levels of both TREM2 (Fig. 2C) and CLEC7a (Fig. 2D). Expression of both TREM2 and CLEC7a was significantly ($p<0.05$) elevated in the ApoE4 treated group compared to ApoE3 (Fig. 2C-D).

ApoE2 promotes neuron survival while ApoE4 reduces neuronal viability

Studies have indicated reduced levels of the neurotrophic factor BDNF in AD patients compared to healthy controls [22, 23]. Astrocytes are a primary source of BDNF in the brain. We therefore sought to investigate the connection between ApoE isoform and BDNF expression levels. Human astrocytes were treated with recombinant ApoE isoforms, ApoE2, ApoE3, or ApoE4 for 24 hr, conditioned-media collected, and BDNF expression level evaluated by western blot (Fig. 3A). Treatment of astrocytes with ApoE2 significantly ($p<0.001$) increased the level of BDNF detected in the media (Fig. 3A). ApoE3 appeared to slightly increase BDNF, although to a significantly ($p <0.05$) lower extent than ApoE2. Exposure of astrocytes to ApoE4 had no effect on release of BDNF (Fig. 3A).

To further investigate the potential neuroprotective and neurotoxic effects of ApoE isoforms, we directly exposed the neuronal cell line SH-SY5Y to recombinant ApoE2, ApoE3, or ApoE4 for 24 hr and assessed cellular viability and toxicity. Cellular viability was significantly ($p<0.01$) increased in ApoE2 treated SH-SY5Y compared to control. Viability was significantly ($p<0.001$) decreased in ApoE4

compared to both ApoE2 and ApoE3 (Fig. 3B). SH-SY5Y cells exposed to ApoE isoforms were also analyzed for cellular toxicity. Neither ApoE2 nor ApoE3 had any effect on release of LDH. In contrast, ApoE4 treated cells display significantly ($p<0.05$) increased cytotoxicity compared to untreated controls, with an increase in LDH release of 14% ± 4.164 (Table 2). Together, these findings indicate that ApoE2 is neuroprotective through both increased BDNF and direct action on neurons. Additionally, ApoE4 is likely neurotoxic through both direct and indirect mechanisms.

Discussion

ApoE4 is the strongest known genetic risk factor for AD, while ApoE2 appears to be protective against AD. While the different levels of risk related to each isoform have been well established, the mechanism responsible for this relationship is still not entirely understood. Here, we investigated the cell type-specific effects of ApoE2/3/4 in the brain on markers related to key aspects of AD pathology neuroinflammation and neurotoxicity. Our results showed that ApoE acts in an isoform-specific manner to modulate pro-inflammatory activity of microglia and astrocytes, and affects neuron survival through both direct and indirect mechanisms.

We investigated the expression of pro-inflammatory cytokines in both microglia and astrocytes following treatment with ApoE isoforms. ApoE4 treatment increased the production of TNFα in relation to both ApoE2 and ApoE3, but significantly reduced the expression of IL-6 in microglia. While the pro-inflammatory activation of microglia by ApoE4 had been well-established, less is known about the ApoE-related inflammatory effects in astrocytes. Astrocytes, like microglia, are key regulators of the brain's inflammatory response, and have been implicated in AD-related inflammatory and pathological processes [29]. Astrocytes have been found to produce inflammatory cytokines, including IL-6 and TNFα, following traumatic injury and ischemia [30]. Here, we show that ApoE treatment significantly increases the production of pro-inflammatory cytokines IL-1β, IL-6, and TNFα in a dose-dependent manner, with ApoE2 representing the lowest cytokine expression and ApoE4 representing the highest. TNFα is a key mediator in neuropathology and has been found to promote excitotoxicity, synaptic loss, and exacerbated amyloidogenesis in AD. Additionally, there is evidence to suggest elevated serum levels of TNFα in AD [31, 32]. The production of TNFα is increased in both activated microglia and

astrocytes in response to injury [31], and here TNFα expression is increased by ApoE4 in both cell types. Similar to TNFα, IL-1β is a pleiotropic cytokine that amplifies immune reactions [33] and is elevated in the CSF of AD patients [34]. Both TNFα and IL-1 can induce increased production of IL-6 and other inflammatory mediators via NF-κβ activation [32]. Mice with astrocyte-targeted enhanced IL-6 production exhibit neurodegeneration and demyelination, along with decreased hippocampal long-term potentiation (LTP) and learning impairments. Microglia in these mice also have enhanced pro-inflammatory activation [35]. However, IL-6 signaling is largely context dependent, and has been shown to mediate both pro- and anti-inflammatory, protective processes [36], which may in part help to explain the reduction in IL-6 production by ApoE4-treated microglia. These findings highlight a previously underexplored role for ApoE in modulating the inflammatory state of astrocytes, and suggest that ApoE4 may influence AD pathology via increased production of inflammatory cytokines by both astrocytes and microglia.

The results of this study also reveal the ApoE-related differential activation of microglia. HMC3 microglia were treated with ApoE2/3/4 and markers related microglial activation state were assessed. DAM refers to a subset of microglia identified in the AD brain that have dysregulated expression of sensing, house-keeping, and host-defense genes [15, 37, 38]. DAM are largely localized around Aβ plaques and display elevation of lipid metabolism and phagocytic activity-related genes, as well as genes related to increased AD risk [15]. ApoE is known to play a key role in the phenotypic of switch of microglia through signaling at TREM2 [15]. Here, we further the idea that ApoE4 enhances activation of the DAM phenotype by showing that ApoE4 treatment increased the expression of both the receptor TREM2 and the downstream, DAM-related protein CLEC7a compared to ApoE3. Therefore,

ApoE4 may confer increased risk for AD through enhanced activation of DAM, and related, down-stream pro-inflammatory and disease progressing effects. An alternative explanation of these findings is that ApoE2 and ApoE3 act in a protective manner compared to the cholesterol control, suppressing expression of DAM-related markers, while ApoE4 represents a potential loss-of-function.

Finally, our findings highlight the idea that ApoE2 is neuroprotective while ApoE4 is neurotoxic through both direct and indirect mechanisms. Human neuroblastoma cells (SH-SY5Y) were treated with recombinant ApoE2/3/4 and cellular viability and toxicity were assessed. ApoE2 significantly increased cellular viability, while ApoE4 significantly decreased viability and increased toxicity. Synapse loss and progressive neuron death are core pathological hallmarks of AD, and these losses correlate with cognitive impairment [39]. Therefore, ApoE4 may help to promote AD pathological progression by enhancing neuron death, while ApoE2 protects against these same processes. As before, this may be a case of ApoE2 working in a protective manner against the cholesterol control, while ApoE4 represents a loss of this protective function. Additionally, human astrocytes were treated with recombinant ApoE2/3/4 and the secretion of the neurotrophic factor BDNF was assessed. Secretion of growth factors, including BDNF, is a major function of astrocytes [29]. BDNF has been found to be reduced in human AD, and a previous study found that ApoE isoforms modulated astrocyte production of specific BDNF isoforms [22-24]. Here, we show that ApoE2 significantly increased astrocyte secretion of mature BDNF. Peripheral BDNF has been found to protect against AD, with one standard deviation higher serum BDNF conferring a 33% decreased risk for AD [40]. BDNF enhances neurogenesis and neurotransmission, promotes synaptic growth and plasticity, and increases hippocampal LTP and related memory formation

[22]. Additionally, BDNF has been found to have anti-apoptotic and anti-oxidant functions in experimental models of neurodegenerative disease [41]. Taken together, these findings suggest that ApoE2 reduces risk for AD by acting in a neuroprotective manner, through both direct and indirect action at neurons. On the other hand, ApoE4 may further promote AD-related risk through its neurotoxic effects in the brain.

Overall, our findings highlight that ApoE acts as a multi-functional mediator in AD-related pathological processes. In an isoform-specific manner, ApoE regulates neuroinflammation and neurotoxicity through direct actions on microglia, astrocytes, and neurons. In general, ApoE4 promotes pro-inflammatory and neurotoxic processes, while ApoE2 acts in a neuroprotective manner, highlighting the differential risk associated with these ApoE isoforms in the development of AD. Additionally, these findings suggest that targeting ApoE-related signaling may be a potential therapeutic strategy for mitigating multiple AD-related pathological processes in individuals who carry the ApoE4 allele.

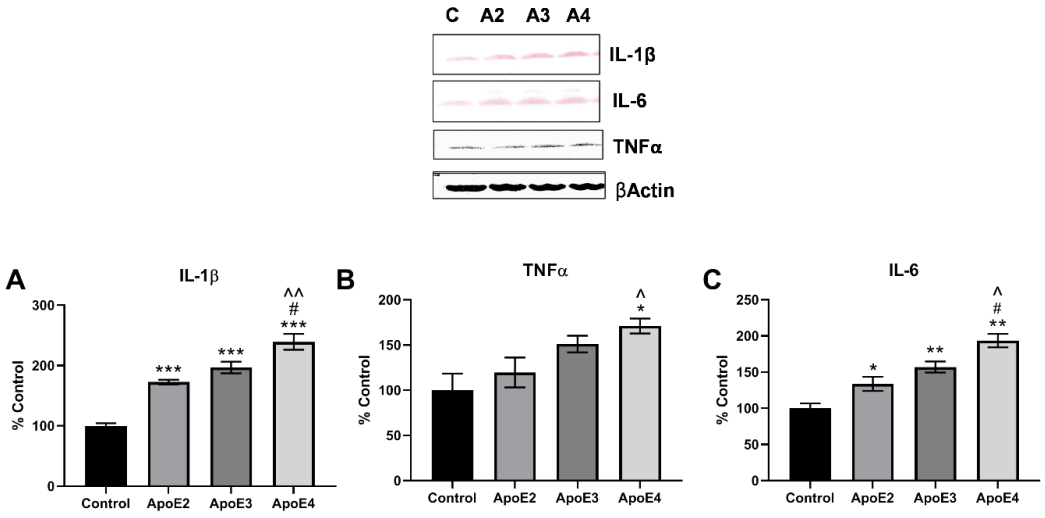

Figure 1 | *In vitro* expression of inflammatory cytokines by astrocytes treated with ApoE isoforms. Human primary astrocytes were grown *in vitro* and treated for 24 hr with 100 µM cholesterol (control) with or without recombinant ApoE2/3/4 (20 nM). Following treatment, cell lysate was collected and western blot was used to assess the expression of inflammation-related proteins: A) IL-1β, B) TNFα, and C) IL-6. N=3, *p<0.05 vs. Control, **p<0.01 vs. Control, ***p<0.001 vs. Control, $^{\#}$p<0.05 vs. ApoE3, $^{\wedge}$p<0.05 vs. ApoE2, $^{\wedge\wedge}$p<0.01 vs. ApoE2.

Protein Analyzed	Control	ApoE2	ApoE3	ApoE4	ANOVA result
NOX4	100 ± 6.32	90.17 ± 6.01	86.22 ± 3.67	99.45 ± 5.01	p=0.2554
iNOS	100 ± 10.03	101.95 ± 9.57	99.95 ± 6.26	89.91 ± 11.99	p=0.8139

Table 1 | Expression of oxidative stress markers by astrocytes treated with ApoE2/3/4 *in vitro*. Human primary astrocytes were grown *in vitro* and treated for 24 hr with 100 μM cholesterol (control) with or without recombinant ApoE2/3/4 (20 nM). Following treatment, cell lysate was collected and western blot was used to assess the expression of oxidative stress-related enzymes NOX4 and iNOS. Data are represented as percent of control Mean ± SEM, N=3. One-way ANOVA was used to test for statistical significant difference in means across groups.

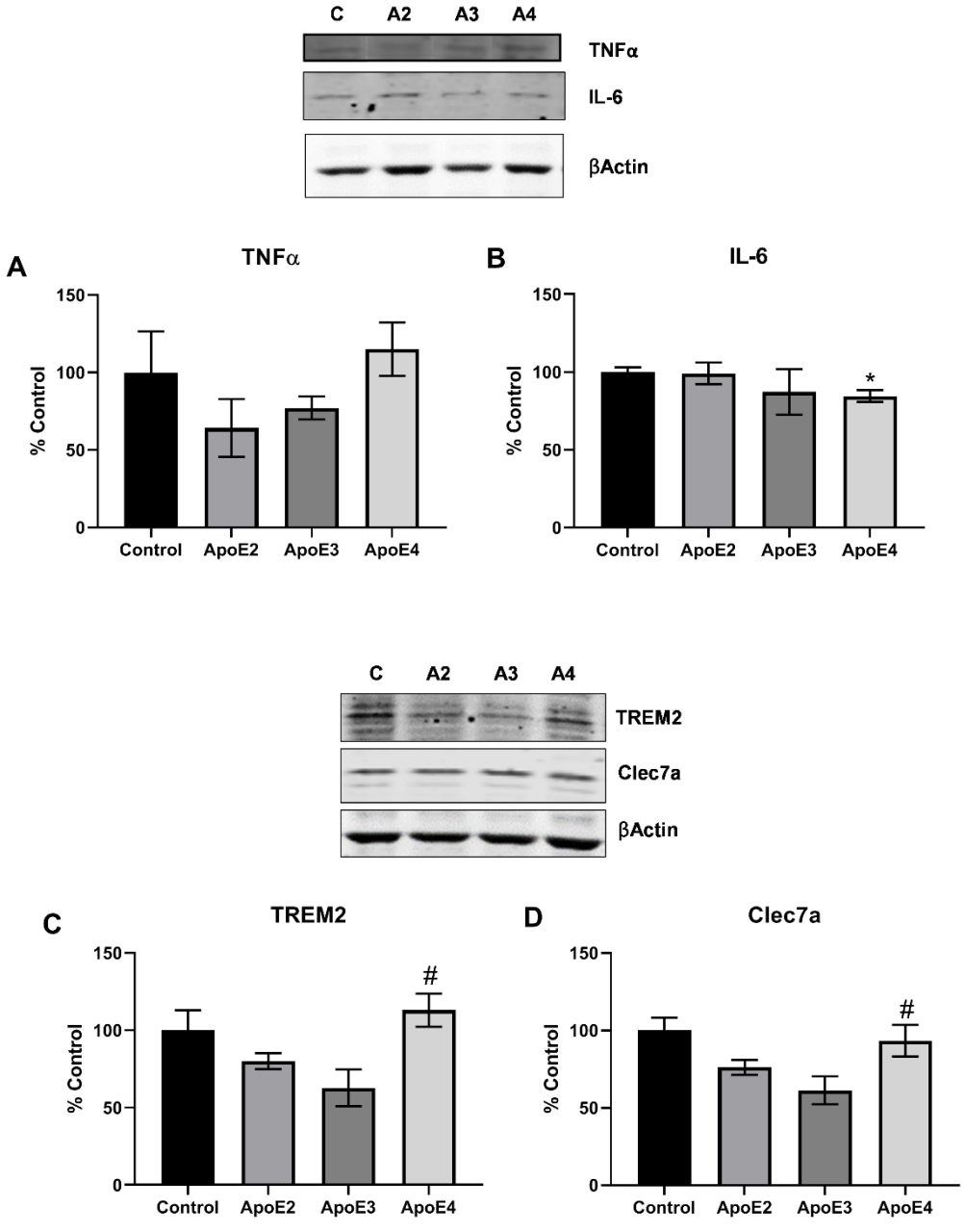

Figure 2 | *In vitro* expression of inflammatory cytokines and activation markers by microglia treated with ApoE isoforms. Human immortalized microglia cell line (HMC3) were grown *in vitro* and treated for 24 hr with 100 µM cholesterol (control) with or without recombinant ApoE2/3/4 (20 nM). Following treatment, cell lysate was collected and western blot was used to assess the expression of pro-inflammatory cytokines: A) TNFα and B) IL-6. Expression of markers related to disease-associated microglia were also evaluated: C) TREM2 and D) CLEC7a. N=3, *p<0.05 vs. Control, #p<0.05 vs. ApoE3.

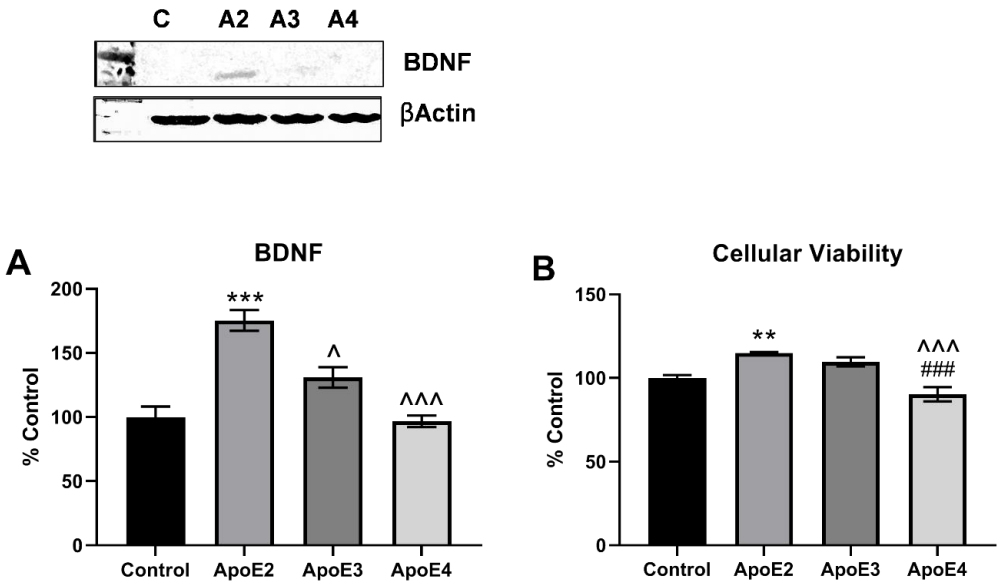

Figure 3 | Evaluation of neurotrophic factors and neuron survival following treatment with ApoE isoforms *in vitro.* Human primary astrocytes and human neuroblastoma cell line (SH-SY5Y) were grown *in vitro* and treated for 24 hr with 100 µM cholesterol (control) with or without recombinant ApoE2/3/4 (20 nM). A) Following treatment, astrocyte supernatant was collected and western blot was used to assess the expression of secreted BDNF. B) SH-SY5Y viability was measured by MTS. N=3-5, **p<0.01 vs. Control, ***p<0.001 vs. Control, $^{\#\#\#}$p<0.001 vs. ApoE3, $^{\wedge}$p<0.05 vs. ApoE2, $^{\wedge\wedge\wedge}$p<0.001 vs. ApoE2.

Cellular Toxicity (LDH)	ApoE2	ApoE3	ApoE4
Percent change vs. Control	+ 2.6 ± 3.05	+ 2.7 ± 4.16	+ 14 ± 4.16*

Table 2 | SH-SY5Y toxicity following treatment with ApoE2/3/4 *in vitro.* Human neuroblastoma cell line (SH-SY5Y) was grown *in vitro* and treated for 24 hr with 100 µM cholesterol (control) with or without recombinant ApoE2/3/4 (20 nM). Following treatment, cellular release of lactate dehydrogenase (LDH) was assessed as a measure of cellular toxicity. Data are represented as percent change versus control Mean ± SEM, N=3, *p<0.05 vs. Control.

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CHAPTER 2

MANUSCRIPT 3

Manuscript in preparation

Sex-specific effects of Apolipoprotein E4 on markers of vascular function and inflammation in the brain *in vivo*

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Abstract

Inheritance of the apolipoprotein E4 (ApoE4) is considered a major genetic risk factor for the development of sporadic or late-onset Alzheimer's disease (AD). The reasons behind the increase in AD risk are not entirely understood, though ApoE4 has been connected to inflammation and oxidative stress in both the brain and the periphery. Also, the prevalence of AD is significantly increased in females compared to males. Interestingly, the ApoE4-associated risk for AD is substantially higher for females than males, conferring an approximately 2-fold higher risk in females. It is our hypothesis that markers of vascular dysfunction, inflammation, and oxidative stress are increased in response to apolipoprotein E4, and that these changes occur to a greater extent in females. Male and female mice expressing human ApoE4 were analyzed for whole-brain protein expression by western blot at 10 months of age and compared to age-matched wild-type controls. Our data indicate that expression of the blood-brain barrier related junctional proteins claudin-5 and occludin are decreased in response to ApoE in a sex-specific manner, with claudin-5 decreased in females and occludin diminished in male ApoE4 mice. Mice expressing the E4 allele exhibited changes in expression of inflammation-related proteins (TNF-α, IL-6, IL-1β), and oxidative stress related proteins (iNOS, eNOS, and NOX4). The results indicated sex-related differences in the modulation of inflammatory/oxidant proteins. ApoE4 females displayed decreased eNOS expression, along with increases in iNOS, IL-1β, and TNF-α. Interestingly IL-6, a cytokine with pro- and anti-inflammatory properties, was increased in male ApoE4 but decreased in female ApoE4 mice. The effect of ApoE4 on expression of glial activation markers GFAP and Iba1 appears sex-specific, with female ApoE4 mice exhibiting higher GFAP and male ApoE4 mice demonstrating higher Iba1. Finally, expression of the neurotrophic factor BDNF is lower in female

wild-type mice compared to male wild-type. Overall, results indicate that the ApoE4 isoform promotes an inflammatory-oxidative state in female mice to a greater extent than in males. Further studies are needed to better understand the mechanism by which these sex-specific effects may play a role in determining AD-related risk.

Introduction

Cardiovascular risk factors (CVRFs) such as diabetes, hypoxia, and inheritance of the apolipoprotein E4 (ApoE4) allele are linked to the pathogenesis of Alzheimer's disease (AD), although the mechanisms whereby these risk factors lead to neurodegenerative injury and dementia have not been well defined [1-7]. In particular, ApoE4 is the most significant genetic risk factor for late-onset AD [8]. There are three polymorphic variants of ApoE, E2, E3, and E4. The E3 allele is the most common, while the worldwide frequency of E4 is only 13.7%. However, the frequency of E4 increases to approximately 40% in AD patients [9]. Additionally, the presence of the E4 allele leads to increased prevalence and lower age of onset of AD [10]. Further evidence for ApoE4's association with AD comes in the findings that ApoE4 is related to impaired cognition in midlife as well as an increased conversion of mild cognitive impairment (MCI) to AD [11-13]. In particular, ApoE4 acts synergistically with CVRFs to exacerbate cognitive decline in cardiovascular disease and AD [14]. For example, the Honolulu-Asia Aging Study found that the association between type II diabetes mellitus (T2D) and AD was stronger in the presence of ApoE4, particularly producing a higher number of neuritic plaques and neurofibrillary tangles [15].

The prevalence of AD is significantly increased in females compared to males [16]. The ApoE4-associated risk for AD is also substantially higher for females than males, conferring an approximately 2-fold higher risk in females [17, 18]. The mechanisms behind this gender-related effect have not been well studied. ApoE4 is associated with worsened cognitive decline in women, as well as a higher likelihood of conversion from MCI to AD [18]. This observation is further supported by the recent finding that women with 2 ApoE4 alleles had significantly poorer memory and global cognition than men with 2 ApoE4 alleles [19]. A similar relationship has been

identified in mouse models, with female mice expressing human E4 exhibiting increased pathology compared to males [20]. These differences include worsened cognitive impairment and decreased hippocampal presynaptic densities in females compared to their male counterparts [18].

The investigation into the relationship between ApoE and AD has largely focused on the role of ApoE as a lipid transport protein, including its role in metabolism and deposition of Aβ [21]. However, more recent study has investigated the role of ApoE4 in modulating vascular function and neuroinflammation. In the periphery the E4 variant is related to cardiovascular disease, particularly atherosclerosis [14, 15]. ApoE4 is also associated with altered cerebrovascular function. ApoE4 carriers exhibit altered cerebral blood flow (CBF) at a young age, and a greater decline in CBF during late adulthood [14, 22, 23]. Individuals with the E4 allele also have an approximately 10% reduction in cerebral glucose metabolism [22, 24]. The relationship between ApoE4 and cerebrovascular dysfunction has been furthered by findings in both *in vivo* and *in vitro* experimental models. ApoE4 increases blood-brain barrier (BBB) permeability in mouse models [25]. ApoE4 was also associated with significantly reduced tight junction integrity and increased barrier permeability *in vitro* [26]. Both animal and human studies suggest that cerebrovascular dysfunction is present early in the pathological progression of AD [27, 28]. More recent findings highlight the relationship between ApoE4-mediated BBB breakdown, particularly in the hippocampus, and subsequent cognitive decline [29]. This suggests that one such mechanism whereby ApoE4 confers AD-related risk is through alterations in cerebrovascular function and integrity.

ApoE4 is also being investigated for its role in modulating the innate immune system. ApoE4 is associated with inflammatory changes in both cardiovascular

disease and AD [30]. Analysis of human brain tissue shows that expression of proteins related to the regulation of inflammatory response is significantly correlated with ApoE genotype, with ApoE4 increasing the expression of inflammation-related mediators [31]. Both sex and ApoE status can modulate microglia phenotype [32]. Furthermore, microglia isolated from mice expressing human ApoE4 exhibit significantly increased production of pro-inflammatory cytokines and reactive nitrogen species (RNS) [33, 34]. Primary microglia expressing human ApoE4 display increased activation, including significantly increased secretion of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, along with induction of inducible nitric oxide synthase (iNOS) expression and activity [35]. ApoE4 is similarly related to significantly increased secretion of inflammatory factors *in vitro* in human and rat macrophages [36-38]. Neuroinflammation has been identified as a key aspect of AD pathology, and this neuroinflammatory response is primarily driven by microglia [39-41]. Therefore, differential regulation of inflammatory process via microglia activation may be another way in which ApoE4 confers elevated risk for AD.

Here, we sought to investigate the impact of ApoE4 on AD-related pathological processes in an animal model, with specific emphasis on markers related to cerebrovascular function and neuroinflammation. AD is believed to develop over a long time-course, with the cognitive impairment, protein aggregation, and neurodegeneration found towards the end of disease progress [42]. Therefore, mice expressing human ApoE4 were examined at 10 months of age, in order to explore a “middle-age” associated time point. Both male and female mice were included in the study in order to identify possible sex-specific changes in order to highlight possible mechanisms responsible for the sexual dimorphism found in ApoE4 and AD-

associated risk. We hypothesized that ApoE4 will exert sex-specific detrimental effects on vascular function, oxidative and nitrosative stress, and neuroinflammation.

Methods

Animals

Transgenic mice expressing human ApoE4 (Human APOE4 mouse: B6.129P2-Apoetm3(APOE*4)Mae N8, Model #1549-F, 1549-M) and wild-type controls (WT) (C57BL/6NTac, Model #B6-F, B6-M) were purchased from Taconic Biosciences, Inc. (Rensselaer, NY). Mice were maintained on normal chow (ENVIGO 2020X, Huntingdon, UK) with water available ad libitum. At 10 months of age, mice were deeply anesthetized with ketamine (80 mg/kg) and xylazine (10mg/kg) administered I.P. and euthanized. All animal procedures were performed in accordance with NIH “Guide for the Care and Use of Laboratory Animals” and University of Rhode Island Animal Care and Use Committee (IACUC) guidelines.

Western Blot

Whole brains were removed post-sacrifice and the cerebellum was detached. Tissues were homogenized by sonication (Branson SX150 Sonifier ®, Branson Ultrasonics, Danbury, CT) in phosphate buffered saline (PBS) with 1X protease inhibitors. Homogenates were mixed with equal parts 2x Sample Buffer (4x Laemmli sample buffer with 10% 2-mercaptoethanol, 50% diH_2O). Samples were then run on a 4-20% Gel (NOVEX by Invitrogen, Carlsbad, CA) in 1x SDS-running buffer. Proteins were transferred to nitrocellulose membranes using iBlot2 Gel Transfer Device (ThermoFisher Scientific, Waltham, MA). After transfer, membranes were blocked using 5% bovine serum albumin (BSA) in 0.05% tris buffered saline with tween (TBS-T) for 1 hr. Primary antibodies for this study include NOX4 (Sigma, MA; 1:1,000), iNOS (Abcam, MA; 1:250), eNOS (Abcam, MA; 1:500), GFAP (Abcam, MA; 1:10,000), Iba1 (GeneTex, CA; 1:1,000), TNF-α (Novus Biologicals, CO; 1:500), IL-6 (Bioss, MA; 1:500), IL-1β (Bioss, MA; 1:500), BDNF (Abcam, MA; 1:1,000), PSD-95

(Cell Signaling Technologies, MA; 1:500), Synaptophysin (Cell Signaling Technologies, 1:1,000), and β-Actin (SantaCruz, CA; 1:10,000). Bound antibody was detected with infrared secondary antibodies (LiCOR, NE; 1:10,000), which was imaged on a LiCor Odyssey (LI-COR Biosciences, Lincoln, NE). Quantification of western blots was done in ImageJ. Values for each protein were normalized to β-actin.

Statistical Analysis

All tests were performed in GraphPad Prism (GraphPad, CA). Data are represented as mean +/- standard error (SEM). Analysis was done by one-way analysis of variance (ANOVA) with Bonferroni multiple-comparisons follow-up. Statistical significance was determined at $p<0.05$, $n=3$ unless otherwise noted.

Results

ApoE4 reduces the expression of tight junction proteins in a sex-specific manner

Impaired BBB function leading to increased barrier permeability is associated with both AD and ApoE4 [29]. Tight junction proteins are crucial for the maintenance of BBB integrity, and reduced expression of junctional proteins, including claudin-5 and occludin, have been identified in AD models [43]. Here, we assessed the expression of claudin-5 and occludin in wild-type and ApoE4 mice as a measure of tight junction formation and BBB integrity.

Brains from 10-month-old male and female mice with ApoE4 or wild-type genotypes were collected, homogenized, and western blot was used to assess the expression of tight junction proteins. The expression of claudin-5 was significantly ($p<0.05$) increased in wild-type females compared to both wild-type and ApoE4 male mice. There was no difference in claudin-5 level between wild-type and ApoE4 males. In contrast, claudin-5 expression was lower in ApoE4 females compared to wild-type ($p=0.0897$) (Fig. 1A).

The expression of occludin was significantly ($p<0.05$) decreased in ApoE4 males compared to both wild-type males and ApoE4 females (Fig. 1B). There was no difference in occludin level between wild-type and ApoE4 females. These data suggest that ApoE4 reduces expression of tight junction proteins, thereby altering BBB integrity, in a sex-specific manner.

The effect of ApoE4 on markers related to oxidative stress differs between males and females

Oxidative stress is a key component of neuroinflammation in AD, marked by high amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The production of these mediators is driven by the activity of NADPH oxidase (NOX)

and nitric oxide synthase (NOS), respectively. NOS family consists of several isoforms that function differently under physiologic and pathologic conditions. Here we examined the expression of both inducible NOS (iNOS) and endothelial NOS (eNOS). Brains from 10-month-old male and female mice with ApoE4 or wild-type genotypes were collected, homogenized, and western blot was used to assess the expression of NOX4, iNOS and eNOS

There was no difference in the expression of NOX4 between male and female wild-type mice (Fig. 2A). The level of NOX expression in ApoE4 male was slightly, although not significantly, elevated compared to wild-type male. In contrast, female ApoE4 carriers showed decreased expression compared to wild-type mice. The expression of NOX4 was significantly ($p<0.05$) decreased in ApoE4 females compared to their male counterparts (Fig. 2A).

There appears to be a gender difference in iNOS between male and female wild-type mice, with females displaying significantly ($p<0.05$) higher levels of this enzyme. Unlike NOX, there was no difference in iNOS between wild-type and ApoE4 females. In male mice, iNOS was significantly ($p<0.05$) higher in ApoE4 animals compared to wild-type controls (Fig. 2B). eNOS expression was significantly ($p<0.05$) reduced in wild-type females compared to wild-type males. ApoE4 males also showed significantly ($p<0.01$) reduced expression of eNOS compared to wild-type males, while there was little difference in eNOS expressions between wild-type and ApoE female mice (Fig. 2C).

ApoE4 differentially affects gliosis in males and females

Another key component of the neuroinflammatory response found in AD is gliosis, the activation of glial species in the brain, particularly astrocytes and microglia. Glial fibrillary acidic protein (GFAP) gene activation and protein induction appear to play a critical role in astroglia cell activation (astrogliosis) following CNS injuries and neurodegeneration [44]. Similarly, ionized calcium binding adaptor protein1 (Iba1) has been used as an indicator of microgliosis [45]. Therefore, we investigated the expression of both GFAP and Iba1, as markers of astrocyte and microglia activation, respectively.

Brains from 10-month-old male and female mice with ApoE4 or wild-type genotypes were collected, homogenized, and western blot was used to assess the expression of GFAP and Iba1.There was no difference in GFAP expression between wild-type and ApoE4 males. In contrast there was a significant ($p<0.001$) increase in GFAP expression in ApoE females compared to either wild-type females or ApoE males (Fig. 3A).

The effect of ApoE4 on Iba1 expression appears to be sex-specific. The expression of Iba1 was significantly ($p<0.01$) increased in male ApoE4 compared to wild-type, while significantly ($p<0.05$) decreased in female ApoE4 compared to female wild-type animals. Additionally, there was a significant ($p<0.01$) difference in Iba1 expression between ApoE4 females and males (Fig. 3B). These results indicate that the inflammatory processes may differ for ApoE4 males versus females, with females being driven by astrocytes and males being driven by microglia.

ApoE4 exerts sex-specific effects on the expression of inflammatory cytokines

Experimental and clinical evidence has demonstrated increased synthesis of pro-inflammatory cytokines in the AD brain [46]. To further analyze the possible

inflammatory effects of ApoE4 we analyzed, by western blot, the expression of TNF-α, IL-1β, and IL-6 in brain homogenates prepared from 10-month-old male and female mice with ApoE4 or wild-type genotypes (Fig. 4).

Although there were no significant differences detected, expression of TNF-α trended down in male ApoE4 compared to wild-type and trended up in female ApoE compared to wild-type (Fig. 4A). The presence of the ApoE4 gene differentially affected expression of IL-1β based on sex. While ApoE4 males showed a slight decrease in IL-1β, relative to wild-type males, ApoE4 females showed a significant ($p<0.01$) increase in IL-1β compared to wild-type as well as ApoE4 males (Fig. 4B).There was no significant difference in the expression of IL-6 between wild-type males and females. There was a sex-based pattern of expression of IL-6 in the presence of the ApoE4 gene. IL-6 was significantly ($p<0.05$) increased in ApoE4 males compared to wild-type, while it was significantly ($p<0.05$) decreased in ApoE4 females compared to their wild-type controls. Additionally, the expression of IL-6 was significantly ($p<0.001$) decreased in ApoE4 females compared to their male ApoE4 counterparts (Fig. 4C).

ApoE4 affects BDNF expression but not synaptic markers

A key component of AD and other neurodegenerative disorders is neuronal cell death and the related loss of synaptic formation and function. To evaluate the ability of ApoE4 to indirectly affect neuronal viability, we evaluated expression of the neurotrophic protein brain-derived neurotrophic factor (BDNF) as well as two proteins (PSD-95 and synaptophysin) localized to the synapse that are related to synaptic formation.

Brains from 10-month-old male and female mice with ApoE4 or wild-type genotypes were collected, homogenized, and western blot was used to assess the

expression of BDNF, PSD-95, and synaptophysin. The expression of BDNF was lower in wild-type female mice compared to wild-type male mice. BDNF expression was reduced in male ApoE4 mice compared to wild-type ($p=0.0634$). There were no differences in the expression of BDNF between ApoE4 females and either wild-type females or male ApoE4 animals (Fig. 5). The expression of both PSD-95 and synaptophysin was comparable between male and females and was not significantly altered by ApoE4 expression (Table 1). These data suggest that the loss of BDNF expression in ApoE4 mice does not contribute to a loss in synaptic proteins at 10 months of age.

Discussion

The E4 allele of the lipid transport ApoE is the most significant genetic risk factor for late onset AD. While the exact mechanism behind the increased risk has not been identified, ApoE4 has been related to inflammation, vascular dysfunction, and neuron loss in AD [10]. There is sexual dimorphism in the risk for AD, and ApoE4 confers a greater risk in females than males [18]. Here, we investigated the effect of ApoE4 on markers related to vascular function, inflammation, and oxidative stress in both male and female mice. These data from 10-month-old wild-type and ApoE4 male and female mice (considered middle-age, 38-47 years human age equivalent) indicate a sex-dependent difference in ApoE4-related effects on vascular function and inflammation.

Cerebrovascular dysfunction and related BBB damage has been identified in AD pathology [27, 43]. Here, ApoE4 reduced the expression of TJ proteins *in vivo*. More specifically, ApoE4 was associated with decreased claudin-5 in females and decreased occludin in males, suggesting that ApoE4 modulate TJ formation and therefore barrier permeability in a sex-specific fashion.

Alterations in eNOS also likely indicate cerebrovascular dysfunction. The enzyme eNOS is thought to have a role in regulating vasodilation and BBB function. Here, eNOS showed significantly lower levels in wild-type female and both ApoE4 male and females compared to WT males. NO produced by eNOS is known to have vasoregulatory and neuroprotective functions [47-49]. Additionally, a significant negative correlation between eNOS capillary expression and neurofibrillary tangle and senile plaque burden has been reported in AD brains [50]. On the other hand, iNOS expression was upregulated in both wild-type and ApoE4 females. Unlike eNOS, iNOS activation is generally thought drive vascular activation, as well as

inflammatory processes in a number of cell types [51]. Deficiency of iNOS has been shown to protect AD-like mice from premature mortality, cerebral plaque formation, increased β-amyloid levels, protein tyrosine nitration, astrocytosis, and microgliosis [52]. In opposition to the iNOS findings, NOX4 expression levels were significantly lower in ApoE4 female compared to ApoE4 males. These findings may suggest that ApoE4-related dysfunction is largely driven by nitrosative processes rather than oxidative in females. Together, these data suggest ApoE4 promotes vascular dysfunction, nitrosative stress, and related inflammatory processes in females.

Similar sex-dependent differences were found in expression levels of proinflammatory cytokines. IL-6 expression was higher in ApoE4 males than in females, while IL-1β expression was significantly higher in females compared to males. At low concentrations, IL-1β increased GFAP expression in a dose-and time-dependent manner. Additionally, IL-1β activates NF-κβ and increases iNOS and pERK expression [53]. Here, we found that GFAP expression was also significantly higher in ApoE4 females, congruent with the IL-1β findings. These data indicate that IL-1β may drive the astrogliosis and nitrosative stress found in ApoE4 female mice. High levels of GFAP have previously been studied in association with AD pathology. Impaired spatial memory retention was found in female mice expressing ApoE4 compared to those expressing ApoE3 in astrocytes or lacking ApoE has been reported [54]. This highlights a potential role for GFAP in ApoE4 related cognitive dysfunction, and may indicate one such mechanism whereby ApoE4 exerts increased risk in females.

Similar to females, male ApoE4 mice exhibited reduced expression of eNOS compared to WT, indicating likely cerebrovascular dysfunction. However, male ApoE4 did not exhibit significant increases in iNOS, GFAP, or IL-1β. In contrast to ApoE4

females, ApoE4 male mice showed significantly increased expression of both Iba1 and IL-6 compared to WT males, indicating enhanced microglial activation. Microglia have an augmented inflammatory response in the aged brain, and IL-6 signaling throughout aging may enhance this response [55]. Transgenic mice that overexpress IL-6 in the brain show neurodegeneration, demyelination, and decreased hippocampal LTP. Additionally, these mice exhibit increased microglia number and a progressive microglia activation, related to neurodegeneration and learning impairment [56]. Moreover, IL-6 stimulated microglia increase production of complement C3, an inflammatory mediator implicated in neuron loss and synapse engulfment in the AD brain [56]. Finally, a mutation in the IL-6 surface receptor, related to increased IL-6 response genes in AD, has been associated with an earlier age of onset in AD patients with the ApoE4 allele [57]. Taken together, these findings suggest that enhanced IL-6/microglia signaling is a driver in ApoE4-related inflammatory changes and related memory impairments in males. The results of this study implicate sex-specific inflammatory processes in the brain's response to ApoE4.

A main pathological feature of Alzheimer's is neuron loss, indicated by substantial reduction in gray matter volume found in the AD brain [39]. AD pathology, including cognitive impairment, is the result of this neuron death and related synapse loss. Additionally, reduced levels of neurotrophic factors, including BDNF, have been identified in human AD [58, 59]. Compared with aged controls, BDNF expression levels were found decreased in hippocampus and frontal, parietal, and temporal cortex of AD post-mortem samples [58]. AD is believed to be a disease that develops over a long time-course, with neurodegeneration and related cognitive impairment belived to occur late in disease progress [42]. Though neuroinflammatory changes in ApoE4 mice were noticed, no signifcant change in expression levels of BDNF or

synaptic proteins PSD-95 and synaptophysin were observed. These data indicate there is little to no significant neurodegeneration in 10-month-old ApoE4 animals, highlighting the idea that vascular and inflammatory changes likely occur before neurodegeneration in the pathological progression of AD. Future studies may benefit from sampling animals at different time points, in order to develop an understanding of the longitudinal changes that occur as a result of ApoE4 in the brain.

In order to identify if cardiovascular risk factors injure the vasculature in ApoE4 mice triggering NOX and NOS which drive vascular activation and impaired cognition, additional future studies should investigate the role that these two mediators play in the vascular activation and inflammation found to be associated with ApoE4 *in vivo*. This may be done by investigating ApoE4-related pathology in iNOS and NOX knockout mouse models. Based on the results of this study, we would expect knockout of NOS and NOX to modulate the pro-inflammatory response to ApoE4 found in female and male mice, respectively. Overall, our findings highlight that ApoE4 exerts sex-specific effects on markers of inflammation and vascular dysfunction in males and females. In particular, female mice expressing human ApoE4 exhibited eNOS-mediated vascular dysfunction as well as pro-inflammatory pathology indicated by increases in iNOS, IL-1β, and GFAP. On the other hand, pro-inflammatory alterations in male ApoE4 mice were highlighted by increases in Iba1 and IL-6. Additionally, the changes in males were less robust than females. Altogether, these findings highlight possibly mechanisms whereby the ApoE4 allele increases the risk for AD, and shed light on how ApoE4 may differentially affect males and females.

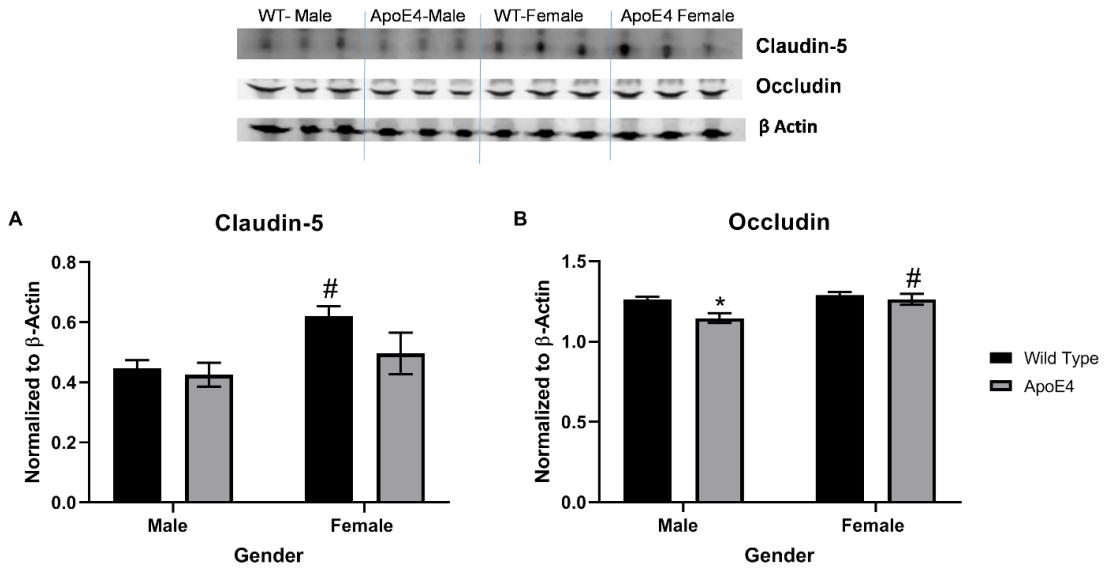

Figure 1 | *In vivo* expression of tight-junction proteins in ApoE4 vs. Wild Type mice. Brains from 10-month-old male and female mice with ApoE4 or Wild Type genotype were collected and homogenized. Western blot was used to assess the expression of tight junction proteins: A) Claudin-5 and B) Occludin. N=3, * denotes significance compared to the group of the same sex and opposite genotype (*$p<0.05$), $^{\#}$ denotes significance compared to the group from the opposite sex of the same genotype ($^{\#}p<0.05$).

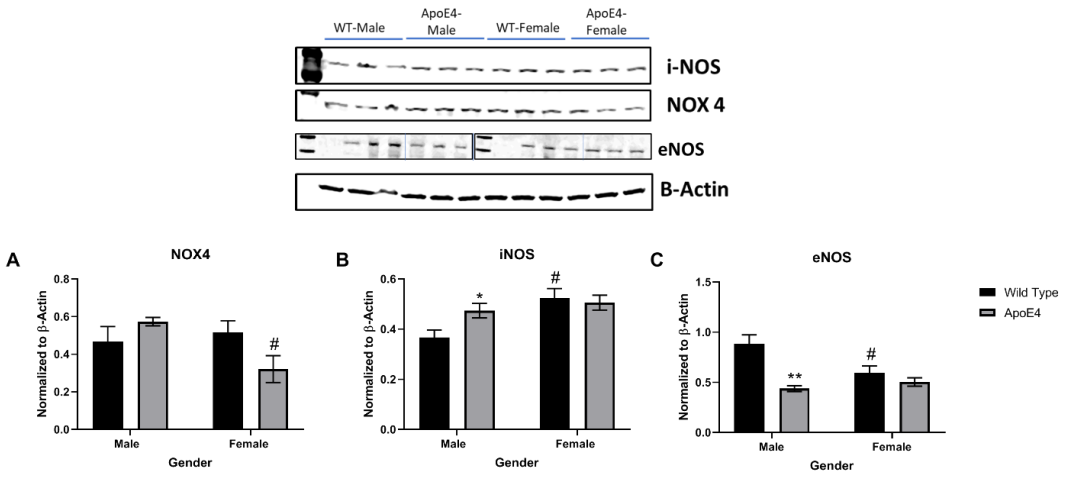

Figure 2 | *In vivo* expression of oxidative stress-related proteins in ApoE4 vs. Wild Type mice. Brains from 10-month-old male and female mice with ApoE4 or Wild Type genotype were collected and homogenized. Western blot was used to assess the expression of oxidative-stress related proteins: A) NOX4, B) iNOS, and C) eNOS. N=3, * denotes significance compared to the group of the same sex and opposite genotype (*p<0.05, **p<0.01), # denotes significance compared to the group from the opposite sex of the same genotype ($^{\#}$p<0.05).

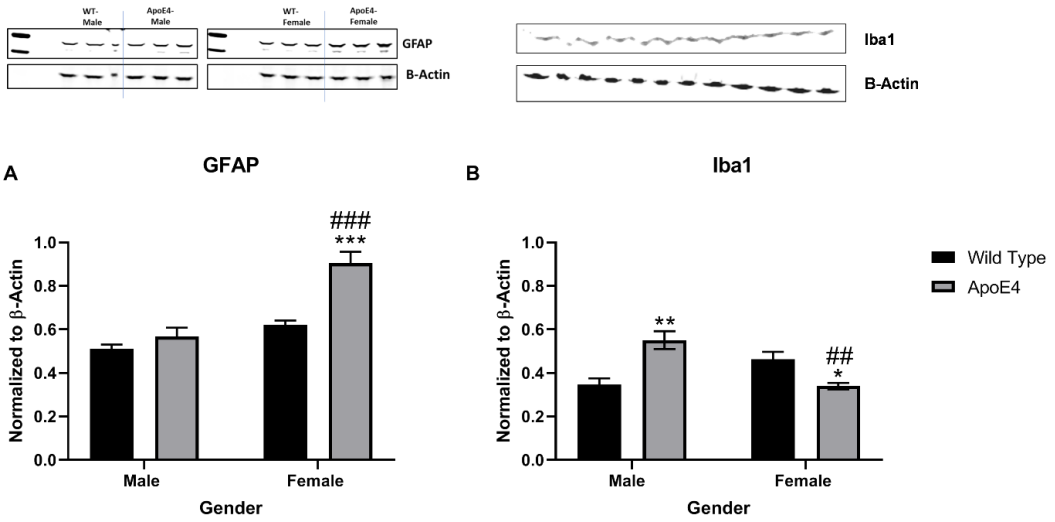

Figure 3 | Expression of gliosis markers *in vivo* in ApoE4 vs. Wild Type mice. Brains from 10-month-old male and female mice with the ApoE4 or Wild Type genotype were collected and homogenized. Western blot was used to assess the expression of markers of astrogliosis and microgliosis: A) GFAP and B) Iba1. N=3, * denotes significance compared to the group of the same sex and opposite genotype (***p<0.001), $^{\#}$ denotes significance compared to the group from the opposite sex of the same genotype ($^{\#}$p<0.001).

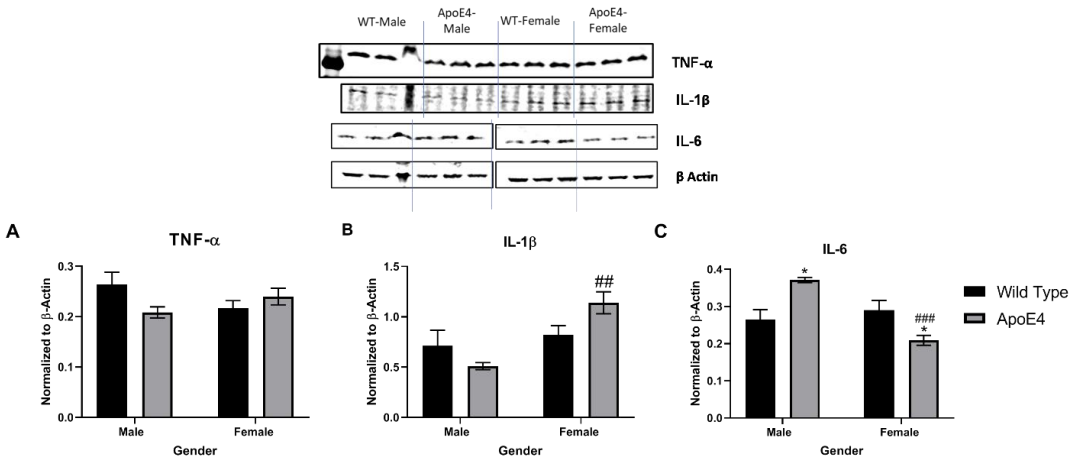

Figure 4 | Expression of inflammatory cytokines *in vivo* in ApoE4 vs. Wild Type mice. Brains from 10-month-old male and female mice with the ApoE4 or Wild Type genotype were collected and homogenized. Western blot was used to assess the expression of inflammatory cytokines: A) TNF-α, B) IL-1β, and C) IL-6. N=3, * denotes significance compared to the group of the same sex and opposite genotype (*$p<0.05$), # denotes significance compared to the group from the opposite sex of the same genotype (##$p<0.01$, ###$p<0.001$).

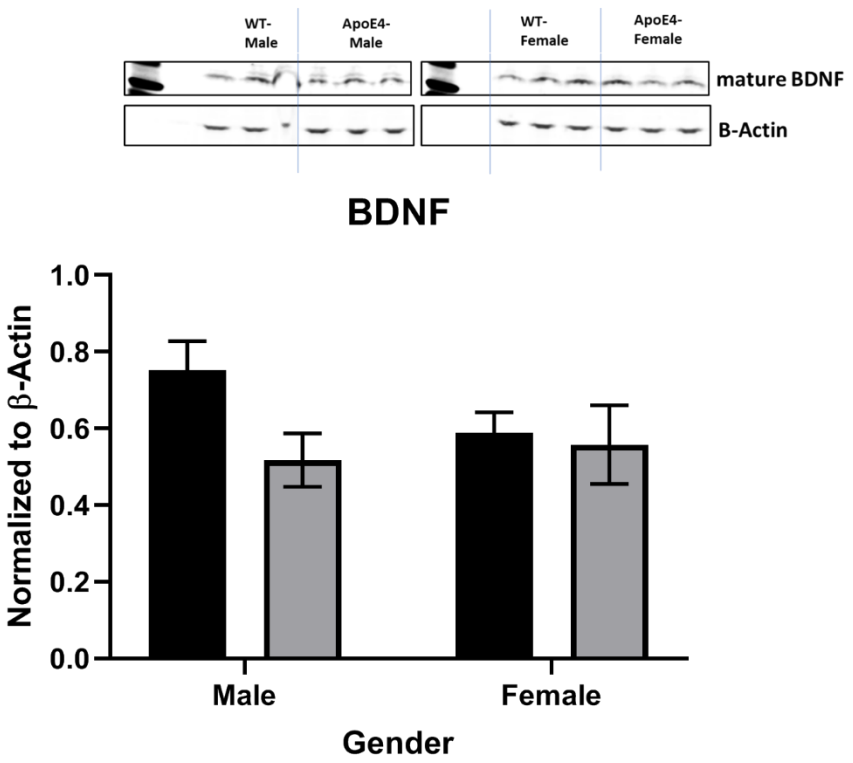

Figure 5 | Expression of BDNF *in vivo* in the brain of ApoE4 vs. Wild Type mice. Brains from 10-month-old male and female mice with the ApoE4 or Wild Type genotype were collected and homogenized. Western blot was used to assess the expression of the neurotrophic factor BDNF. N=3.

Table 1 | Expression of synaptic proteins in ApoE4 and Wild Type mice *in vivo*. Brains from 10-month-old male and female mice with the ApoE4 or Wild Type genotype were collected and homogenized. Western blot was used to assess the expression of the synaptic proteins PSD-95 and synaptophysin (SYP). Protein expression normalized to β-Actin, represented as Mean±SEM, N=3.

Evaluated protein	Wild-Type Male	ApoE4 Male	Wild-Type Female	ApoE4 Female	ANOVA result
PSD-95	0.8776±0.028	0.9061±0.089	0.9513±0.025	0.9285±0.048	p=0.7949
SYP	1.217±0.137	1.090±0.088	1.287±0.028	1.357±0.035	p=0.2191

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CHAPTER 3

MANUSCRIPT 4

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Thrombin, a mediator of coagulation, inflammation, and neurotoxicity at the neurovascular interface: Implications for Alzheimer's disease

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Abstract

The societal burden of Alzheimer's disease (AD) is staggering, with current estimates suggesting that 50 million people world-wide have AD. Identification of new therapeutic targets is a critical barrier to the development of disease-modifying therapies. A large body of data implicates vascular pathology and cardiovascular risk factors in the development of AD, indicating that there are likely shared pathological mediators. Inflammation plays a role in both cardiovascular disease and AD, and recent evidence has implicated elements of the coagulation system in the regulation of inflammation. In particular, the multifunctional serine protease thrombin has been found to act as a mediator of vascular dysfunction and inflammation in both the periphery and the central nervous system. In the periphery, thrombin contributes to the development of cardiovascular disease, including atherosclerosis and diabetes, by inducing endothelial dysfunction and related inflammation. In the brain, thrombin has been found to act on endothelial cells of the blood-brain barrier, microglia, astrocytes, and neurons in a manner that promotes vascular dysfunction, inflammation, and neurodegeneration. Thrombin is elevated in the AD brain, and thrombin signaling has been linked to both tau and amyloid beta, pathological hallmarks of the disease. In AD mice models, inhibiting thrombin preserves cognition and endothelial function and reduces neuroinflammation. Evidence linking atrial fibrillation with AD and dementia indicates that anticoagulant therapy may reduce the risk of dementia, with targeting thrombin shown to be particularly effective. It is time for "outside-the-box" thinking about how vascular risk factors, such as atherosclerosis and diabetes, as well as the coagulation and inflammatory pathways interact to promote increased AD risk. In this review, we present evidence that thrombin is a convergence point for AD risk factors and as such that thrombin-based therapeutics

could target multiple points of AD pathology, including neurodegeneration, vascular activation, and neuroinflammation. The urgent need for disease-modifying drugs in AD demands new thinking about disease pathogenesis and an exploration of novel drug targets, we propose that thrombin inhibition is an innovative tactic in the therapeutic battle against this devastating disease.

Introduction

The societal burden of Alzheimer's disease (AD) is staggering, with current estimates suggesting that 5.8 million Americans and 50 million people worldwide have AD ("2019 Alzheimer's disease facts and figures," 2019). Identification of new therapeutic targets is a critical barrier to our ability to develop a disease-modifying therapy for this devastating disease. A large body of data implicates vascular pathology and cardiovascular risk factors, such as atherosclerosis and diabetes, in the development of AD. Though the mechanisms whereby these risk factors contribute to pathological processes in the AD brain have not been defined fully, it has been suggested that inflammation plays a key role in both cardiovascular disease and AD (Grammas, 2011). It is clear that a better understanding of the relationship(s) among cardiovascular risk factors, inflammation, and neurodegeneration has the potential to reveal novel therapeutic targets in the battle against AD.

Recently, a number of laboratories have provided evidence that certain elements of the coagulation cascade may initiate and/or support inflammation in the brain (Davalos & Akassoglou, 2012; De Luca et al., 2017; Göbel et al., 2018). The proinflammatory properties of the protein fibrin have attracted particular attention (reviewed in (Cortes-Canteli et al., 2012; Petersen et al., 2018)). Fibrin increases expression of a number of inflammatory and oxidative mediators, activates glial cells, and disrupts the blood-brain barrier (BBB). Another potentially important protein related to both coagulation and inflammation is thrombin. Thrombin is widely appreciated for its contribution to fibrin formation and platelet aggregation in response to vascular injury. Importantly, thrombin is also a pleiotropic enzyme that is capable of triggering a large and diverse number of cellular events through receptor-mediated activation of protease-activated receptors (PARs) (Coughlin, 2005). Levels of both

thrombin and the thrombin receptor PAR-1 are elevated in AD (Krenzlin et al., 2016; Sokolova & Reiser, 2008) and thrombin expression is increased in brain microvessels collected from AD patients (Grammas et al., 2006). As we discuss below, thrombin can act in both a paracrine and autocrine manner to stimulate a noxious feed-forward cycle that likely contributes to neuroinflammation in the AD brain. The evidence summarized herein suggests that thrombin is a convergence point for AD risk factors and that thrombin-based therapeutics might target multiple points of AD pathology, including neurodegeneration, vascular activation, and neuroinflammation. We will present evidence that supports the hypothesis that thrombin could be a heretofore unexplored target for AD therapeutics.

Coagulation and inflammation: Functionally linked processes

Close integration and extensive crosstalk between coagulation and inflammation pathways are critical to the body's response to injury. Both processes utilize numerous bioactive mediators and cellular effectors that interact in a coordinated manner. Inflammatory proteins, such as cytokines, play a central role in the activation of coagulation. Meanwhile major coagulation factors, such as tissue factor, fibrinogen, and thrombin, are drivers of inflammation. Under normal physiological conditions these intertwined systems work in homeostatic balance, but dysregulation of this crosstalk likely contributes to cellular injury and disease pathogenesis.

Effect of cytokines on coagulation

Cytokines, particularly interleukin (IL)-6, tumor necrosis factor-α (TNF-α), and IL-1β, stimulate procoagulant effects both directly and indirectly. These proteins initiate the extrinsic coagulation pathway through up-regulation and activation of tissue factor (Nawroth & Stern, 1986; Witkowski et al., 2016). Blocking tissue factor greatly inhibits inflammation-induced thrombosis, and inhibition of IL-6 specifically blocks tissue factor-dependent thrombin generation (Levi et al., 1997). Cytokines, especially TNF-α, can also initiate inflammation-mediated platelet activation and clumping in the blood (Page et al., 2018). Inflammatory cytokines can inhibit the anticoagulant feedback pathways, resulting in increased thrombin and fibrin production (Levi & van der Poll, 2005). Both TNF-α and IL-1β can reduce activated protein C (APC) via down-regulation of thrombomodulin, an important cofactor for APC's anti-inflammatory and anti-coagulant activity (Nawroth & Stern, 1986).

Coagulation factors drive inflammation

Tissue factor, the main driver of the extrinsic coagulation pathway, can induce proinflammatory effects. This includes increases in the production of inflammatory cytokines, adhesion molecules, chemokines, and growth factors. These proinflammatory effects are largely mediated through tissue factor-activation of the protease thrombin (see below). Fibrin is the primary end-product of the coagulation system, but it also has proinflammatory characteristics. Fibrinogen and fibrin both have been shown to induce leukocyte migration, and directly modulate the inflammatory response of both leukocytes and endothelial cells. Fibrin induces the expression of several inflammatory cytokines and chemokines and increases the production of reactive oxygen species (ROS) (Jennewein et al., 2011). Similarly, fibrin has been shown to have extensive proinflammatory effects within the central nervous system (CNS), including activation of glia and disruption of blood-brain barrier (BBB) function (Petersen et al., 2018). Fibrinogen activates microglia in a CD11b-dependent manner; this activation is related to perivascular clustering, axonal degeneration, spine elimination, and cognitive impairment in animal models (Davalos et al., 2012; Merlini et al., 2019). Finally, the coagulation and immune systems are directly linked through activation of IL-1α by thrombin (Burzynski et al., 2019).

Thrombin is a key mediator of coagulation and inflammation via proteolytic and receptor-mediated mechanisms

The coagulation cascade consists of the intrinsic and extrinsic pathways, and thrombin is a key mediator in both. The extrinsic system is activated by tissue factor, which is found in the subendothelial surface and is only introduced to the blood following injury. Tissue factor in the blood complexes with factor VIIIa to initiate the cascade that will eventually lead to the formation of thrombin and the cleavage of fibrinogen to fibrin (Witkowski et al., 2016). The intrinsic system is activated by factor XII, which initiates a cascade that will lead to the production of thrombin via factors X and V (Palta et al., 2014). The activation of thrombin will also activate a positive feedback-loop which will continue to drive the generation of thrombin. Together, these systems work to increase the amount of active thrombin in the blood, which will in turn increase the insoluble fibrin available to form a clot at the site of damage.

As noted above, thrombin is a multifunctional protease that can initiate many cellular events through action at and activation of PARs. PARs are a unique class of G-protein-coupled receptors due to their unusual tethered-ligand mechanism of activation (Coughlin, 2000). Thrombin is responsible for enzymatic cleavage of the PAR N-terminus to expose a tethered ligand that intramolecularly activates the receptor. Activation of PARs by thrombin affects a multitude of functions throughout the body, including the regulation of platelet activation, cell adhesion, cell migration, angiogenesis, and inflammation (Coughlin, 2005; Kalz et al., 2014).

The vasculature is a critical nexus for the proinflammatory actions of thrombin

Much of thrombin's proinflammatory activity is likely due to its numerous effects on vascular endothelial cells. Thrombin acts on endothelial cells to stimulate synthesis and release of a large number of diverse bioactive proteins. Thrombin

stimulation of human umbilical vein endothelial cells (HUVECs) produces changes in gene expression related to inflammation, apoptosis, and matrix integrity (Okada et al., 2006). Thrombin-treated HUVECs exhibit increased expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and a related increase in monocyte adhesion (Kaplanski et al., 1998). In human aortic endothelial cells (HAECs), thrombin and high-glucose co-treatment produces increases in the expression of NADPH oxidase (NOX), inflammatory cytokines, and altered adhesion (Wang et al., 2014). Cultured endothelial cells exposed to thrombin release von Willebrand factor, express P-selectin at the plasma membrane, and produce chemokines thought to trigger the binding of platelets and leukocytes to endothelial surface (Collins et al., 1993). Thrombin can affect vessel diameter, alter endothelial cell shape, and increase permeability of the endothelial monolayer. Endothelial cells can both release thrombin and respond to this protein via functionally active thrombin receptors (PAR-1 and PAR-3). Thrombin causes endothelial activation and enhanced expression and/or release of many proinflammatory proteins including monocyte chemoattractant protein-1 (MCP-1), ICAM-1, IL-1, IL-6, and IL-8, which in turn can injure endothelial cells leading to increased release of thrombin (Colotta et al., 1994; Ishibashi et al., 2014; Marin et al., 2001; Miho et al., 2005; Okada et al., 2006; Rahman et al., 2002; X. Yin et al., 2010). Thus, thrombin can act in both a paracrine and autocrine manner stimulating a noxious feed-forward cycle.

Thrombin contributes to the development of Alzheimer's risk factors: atherosclerosis and diabetes

Given thrombin's effect on endothelial cells, it is not surprising that thrombin has been implicated in the pathogenesis of atherosclerosis. Thrombin has been widely studied for its role in the pathology of atherosclerosis, a progressive and chronic inflammatory vascular disorder (Jaberi et al., 2019; Kalz et al., 2014). A role for thrombin in the development of atherosclerosis is suggested by the observation that elevated levels of thrombin and the thrombin receptor PAR are found around atherosclerotic plaques, and that thrombin formation is correlated with disease severity in coronary atherosclerosis (Borissoff et al., 2012). Thrombin facilitates recruitment of circulating monocytes to atherosclerosis plaques by increasing expression of MCP-1, ICAM-1 and VCAM-1 (Colotta et al., 1994; Kalz et al., 2014; Minami et al., 2004). The importance of thrombin is further highlighted by the finding that thrombomodulin, which inhibits thrombin by binding to it, diminishes thrombin-induced endothelial cell dysfunction in atherosclerosis (Wei et al., 2011). Thrombin produces damage to endothelial cell barriers causing leakiness and associated leukocyte migration (Kalz et al., 2014). Thrombin inhibition has been shown to decrease ROS production, improve endothelial cell and barrier function, and attenuate atherosclerotic plaque formation (I. O. Lee et al., 2012).

Thrombin activity can influence onset, progression, and qualitative properties of atherosclerotic plaques. In atherosclerotic models, increases in thrombin have been found to be associated with increases in inflammation, angiogenesis, and cell proliferation (Kalz et al., 2014; ten Cate, 2012). Genetic reduction of thrombin in APOE-/- mice diminished atherosclerosis severity (Borissoff et al., 2013). Studies have further defined thrombin's role in atherosclerosis by finding reduced

atherosclerosis-related pathology in animal models treated with thrombin inhibitors (Palekar et al., 2016; Pingel et al., 2014; Preusch et al., 2015; Wei et al., 2011). On the other hand, genetic manipulations resulting in elevated thrombin levels produce exacerbated atherosclerosis-related pathology (Borissoff et al., 2013). These findings illustrate a causative role for thrombin in the development of atherosclerosis-related pathology and inflammation, largely through thrombin's endothelial cell-mediated effects.

Hyperglycemia, characteristic of Type 2 diabetes mellitus (T2DM), enhances thrombin generation and promotes a hypercoagulable state and high oxidative stress (Aoki et al., 1996; Chapman, 2013; Undas et al., 2008). Elevated thrombin activity has been linked with endothelial dysfunction in diabetes, including vascular inflammation and increased ROS production (Paneni et al., 2013). One mechanism whereby thrombin promotes diabetic oxidative stress is via calcium-mediated intracellular signaling pathways that regulate the transcription factor KLF14 and PLK1 kinase pathways, resulting in increased ROS production (Hao et al., 2018). Exogenous thrombin treatment has been shown to exacerbate pathology in experimental models of diabetes. In a mouse model of diabetes, thrombin treatment of pericytes result in increased barrier permeability, decreased expression of tight junction proteins, and increased expression of inflammatory cytokines (Machida et al., 2017). Induction of diabetes by streptozotocin (STZ) in mice increases the expression of PAR-1, PAR-3, and PAR-4 in the aorta. STZ-induced diabetic mice show impairment of endothelial function, while the administration of dabigatran etexilate, a direct thrombin inhibitor, significantly attenuates this endothelial dysfunction (Rahadian et al., 2020).

Patients with T2DM show increased blood thrombin levels correlated with the patient's level of albuminuria, an indicator of cardiovascular morbidity and mortality in patients with T2DM. This suggests that thrombin may play a role in the development of macrovascular disease (Ay et al., 2012). Thrombin-related pathways have also been implicated in diabetic microvascular injury and retinopathy. Samples taken from diabetic retinopathy patients show elevated expression of thrombin and PAR-1; similar trends are found in samples from a rat model of diabetes (Abu El-Asrar et al., 2016). Obesity promotes a chronic inflammatory and hypercoagulable state that drives cardiovascular disease and T2DM. Recent studies have suggested a link between the thrombin/fibrin(ogen) axis and obesity (Kopec et al., 2017). In a high fat animal model, treatment with the direct thrombin inhibitor dabigatran etexilate ameliorates the development of obesity and severity of associated sequelae (Kopec et al., 2014).

Thrombin causes vascular dysfunction and promotes inflammation in the brain.

Largely due to its proinflammatory effects on endothelial cells, thrombin has an important role in the pathology of various peripheral vascular diseases, including atherosclerosis and diabetes. Thrombin may similarly act as a pathological mediator in the central nervous system, through effects on the endothelial cells of the blood-brain barrier.

Effects of thrombin on the blood-brain barrier

Consistent with its ability to cause endothelial injury in the periphery, thrombin has been found to be involved in instances of damage and dysfunction to the brain endothelial cells of the BBB. Treatment of rat brain endothelial cells causes endothelial dysfunction characterized by increased production of ROS, nitric oxide (NO), inflammatory cytokines, and chemokines (Brailoiu et al., 2017). In human brain endothelial cells, thrombin treatment induces an inflammatory phenotype resulting in increased ICAM-1, VCAM-1 and increased mRNA expression for CXC chemokines (chemotactic for neutrophils) CXCL1 (GRO-alpha), CXCL2 (GRO-beta), CXCL8 (IL-8), and CXCL10 (IP-10) (Alabanza & Bynoe, 2012). Thrombin also increases F-actin stress fibers, disrupts tight junctions, and increases barrier permeability (Brailoiu et al., 2017). More specifically, thrombin has been found to alter barrier permeability by inducing microtubule disassembly (Birukova et al., 2004) and activating Src kinase (Liu et al., 2010). Treatment with a direct thrombin inhibitor reduces the ROS generation and expression of proinflammatory cytokines by cultured brain endothelial cells in response to hypoxia, indicating a mediating role for thrombin in the proinflammatory response of brain endothelial cells (Tripathy et al., 2013).

We have previously demonstrated that thrombin message is highly expressed in microvessels from AD brains but is not detectable in control vessels (X. Yin et al.,

2010) Similarly, Western blot analysis of microvessels shows that the thrombin protein is highly expressed in AD- but not control-derived microvessels (Grammas et al., 2006). Furthermore, injuring brain endothelial cells *in vitro* with oxidant stress (sodium nitroprusside) or an inflammatory cocktail (IL-1β, IL-6, TNF-α, lipopolysaccharide (LPS), interferon (IFN)-γ) results in thrombin release (Grammas et al., 2004). Since brain endothelial cells can both synthesize and respond to thrombin, it could function as an autocrine factor at the BBB. The importance of findings regarding the effects of thrombin on the cells of the BBB is two-fold. Directly, thrombin damage to the BBB increases permeability and may allow damaging substances, including thrombin and other inflammatory mediators from the blood, to enter the brain. Indirectly, these injured brain endothelial cells can produce their own thrombin into the brain, where it may have untoward effects on microglia, astrocytes and neurons (See Figure 1).

Neuroinflammatory effects of thrombin on microglia and astrocytes

The response of microglia to thrombin encompasses a number of processes that contribute to microglia activation and/or apoptosis. In the microglial cell line BV2, thrombin induces IL-1β release (C. Han et al., 2019). Thrombin has also been shown to stimulate a proinflammatory phenotype in microglia, characterized by increases in ROS, NO, and cytokine production (Huang et al., 2008; D. Y. Lee et al., 2006; Yang et al., 2015; Ye et al., 2017). PAR-1 activation was found to participate in activation of microglia, indicated by up-regulation of microglial CD40 and TNF-α production (Suo et al., 2002). Thrombin, via the TNF-α/TNFR-dependent pathway, downregulates expression of the mRNA species miR-181c, which in turn promotes nuclear factor (NF)-κB activity, and upregulates NF-κB target gene expression as well as increasing mixed lineage leukemia-1 (MLL1), a putative gene target for miR-181c (M. Yin et al.,

2017). BV2 cells treated with thrombin show an increase in ROS and the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome, a component of the innate immune system, which is associated with a wide range of human CNS disorders (Ye et al., 2017).

Astrocytes show a similar shift towards a pro-inflammatory phenotype in response to thrombin. In rat brain astrocytes, thrombin induces matrix metalloproteinase (MMP)-9 expression and promotes cell migration via activation of the c-Src/Jak2/PDGFR/PI3K/Akt/PKCδ pathway (Lin et al., 2013). Thrombin exposure can disrupt glutamate transport in astrocytes and modulate stellation, indicating an alteration in function (Cavanaugh et al., 1990; C. Piao et al., 2015). Thrombin treatment *in vivo* resulted in increased glial fibrillary acidic protein (GFAP) expression in the hippocampus, indicating a pro-inflammatory activation in astrocytes (Mhatre et al., 2004).

Thrombin an important mediator of neurotoxicity and neurodegenerative diseases

The multifunctional protease thrombin causes neuronal cell death both *in vitro* and *in vivo*. The neurotoxic effects of thrombin are orchestrated by multiple pathways. *In vitro*, thrombin-induced neurotoxicity involves activation of PAR-1, followed by RhoA activation and cell cycle re-entry. In neurons treated with thrombin, cyclin D1 and E (early G1 cyclins) and the cyclin dependent kinase, cdk4, are activated and that these events lead to upregulation of the pro-apoptotic protein Bim and apoptosis (Rao et al., 2007). Additionally, thrombin has been demonstrated to cause a rapid influx of calcium in neurons leading to neuronal cell death (Smirnova et al., 1998). Delivery of thrombin directly into the brain by intracerebral injection causes significant neuropathology, such as enlargement of cerebral ventricles, an increased number of TUNEL-positive cells, astrogliosis, and an increase in the immunoreactivity for phosphorylated neurofilament and apolipoprotein-E (ApoE) fragments, as well as cognitive impairments including deficits in reference memory and an increase in task latency (Mhatre et al., 2004). The large body of data indicating thrombin is an important mediator of neuroinflammation and neurotoxicity supports the idea that this protein is critically involved in pathological processes that contribute to the development of neurodegenerative diseases including multiple sclerosis (MS), amyolaterotrophic sclerosis (ALS), ischemia, traumatic brain injury (TBI), Parkinson's disease (PD) and AD.

Alterations in coagulation-related proteins have been indicated in motor-associated degenerative disorders. Both prothrombin and factor X are elevated in MS (Gobel et al., 2016). Proteomic analysis of chronic active MS lesions identified several dysregulated coagulation factors, highlighting a potential link between the coagulation

cascade and MS pathology (M. H. Han et al., 2008). In experimental autoimmune encephalomyelitis (EAE), an experimental model of MS, thrombin activity precedes onset of neurological signs, increases at disease peak, and correlates with fibrin deposition, microglial activation, demyelination, axonal damage, and clinical severity (Davalos et al., 2014). The potential pathological significance of coagulation factors in neurological disease is underscored by the finding that the diminution of fibrin, the end product of thrombin's proteolysis, either genetically or using anticoagulants, significantly reduces neurological signs, inflammation, and axonal damage in EAE (Davalos & Akassoglou, 2012). Additionally, thrombin has been linked to changes in interneuron calcium signaling and enhanced thrombospondin release in ALS (De Luca et al., 2017).

Thrombin has been extensively studied for its role in the response to ischemia (Matsuoka & Hamada, 2002). Thrombin was found to mediate neurovascular injury during ischemia (B. Chen et al., 2010), and increased thrombin activity was associated with subsequent neuronal damage in a model of acute focal ischemia (B. Chen et al., 2012). While high concentrations of thrombin seem to be neurotoxic, low concentrations of thrombin (.01 U/mL) were found to protect against neuronal death in cellular and animal models of ischemia (Striggow et al., 2000). Increases in thrombin have also been associated with traumatic brain injury (TBI). Alterations in BBB permeability have been found following TBI (Hay et al., 2015; Korn et al., 2005). In a model of TBI, BBB damage due to the injury was found to induce increases in thrombin (C. S. Piao et al., 2019).

A role for thrombin has also been identified in PD-related pathology. More specifically, thrombin-induced activation of microglia in the midbrain has been directly linked to dopaminergic neuron death (S. H. Choi et al., 2003). Thrombin receptor

PAR-1 is upregulated in the brains of patients with PD (Ishida et al., 2006; Sokolova & Reiser, 2008). Recently, our lab has identified that dabigatran treatment improved motor deficits and reduced markers of oxidative stress in a Drosophila melanogaster model of PD (unpublished findings).

A role for thrombin in PD is further indicated by the finding that treatment with a direct thrombin inhibitor is neuroprotective in a rotenone-induced PD rodent model (Kandil et al., 2018). Finally, mice deficient in PAR-1 are protected against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicity which causes PD-like syndrome (Hamill et al., 2007).

A case for thrombin as a driver of neurodegeneration and vascular activation in Alzheimer's disease

Thrombin is associated with typical hallmarks of AD-related pathology. Thrombin has been detected in senile plaques and in neurofibrillary tangles characteristic of this disease (Akiyama et al., 1992; Arai et al., 2006), and levels of both thrombin and the thrombin receptor PAR-1 are elevated in AD (Krenzlin et al., 2016; Sokolova & Reiser, 2008). Traumatic brain injury, a condition in which neurons are exposed to high thrombin levels, is associated with an increased incidence of AD (Mortimer et al., 1991; Nemetz et al., 1999). Thrombin may also contribute to ApoE-associated pathology in AD. Intracerebral administration of thrombin to rodents increases ApoE levels in the hippocampus and results in neuronal injury and cognitive deficits (Mhatre et al., 2006). The 22 kDa N-terminal thrombin-cleavage fragment of ApoE is highly neurotoxic and could contribute to ApoE-associated AD pathology (Tolar et al., 1997). Persistent thrombin signaling induces tau aggregation and related hippocampal degeneration (Arai et al., 2005; Suo et al., 2003). Thrombin induces secretion of amyloid precursor protein (APP) in endothelial cells *in vitro* (Ciallella et al., 1999) and may be involved in altered processing of APP into fragments that are found in amyloid plaques of AD brains (Chong et al., 1994; Ciallella et al., 1999; Igarashi et al., 1992). Aβ promotes thrombin generation through factor XII-mediated factor XI activation (Zamolodchikov et al., 2016). In this regard, depletion of factor XII ameliorates brain pathology and cognitive impairment in AD mice. Additionally, the factor XII-initiated contact system, of which thrombin is a key driver, is activated in AD patients and mice (Z. L. Chen et al., 2017; Zamolodchikov et al., 2015).

Vascular activation: A novel pathway in AD: A role for thrombin

Microvessels isolated from the brains of AD patients overexpress a diverse array of neurotoxic and inflammatory proteins (Grammas & Ovase, 2001; Grammas et al., 2006). Expression of these diverse mediators is consistent with the process of vascular activation and reflects the transition of endothelial cells from a quiescent to a highly synthetic phenotype. A similar pattern of vascular activation has been identified in transgenic AD animal models. Vascular activation in the AD brain has deleterious consequences for neuronal viability, as many vascular-derived factors are neurotoxic. The idea that vascular activation contributes to pathogenic events in the AD brain is strongly supported by pre-clinical studies in two AD mouse models where treatment with a vascular activation inhibitor reduced vascular-derived neuroinflammation and improved cognitive performance (Grammas et al., 2014).

Several lines of evidence support the idea that the cerebral vasculature is a convergence point for both the expression of thrombin as well as thrombin-mediated effects that contribute to neuroinflammation and neuronal injury in AD. While the majority of thrombin is produced in the liver, extrahepatic sources of locally generated thrombin, including in the brain, have been documented (Deschepper et al., 1991). Although not widely appreciated, evidence suggests the cerebrovasculature is an important source of thrombin in AD. Immunoreactivity for the major brain thrombin inhibitor, protease nexin-1, is found to be significantly decreased in AD brains, particularly around blood vessels, suggesting vascular release of thrombin (Vaughan et al., 1994). As previously stated, thrombin mRNA and protein are expressed in brain microvessels from AD patients but not detectable in brain microvessels isolated from age-matched control brains (Grammas et al., 2006; X. Yin et al., 2010). It is not surprising that thrombin is expressed in AD-derived brain microvessels, as *in vitro*

experiments with brain endothelial cells have shown that injuring these cells causes release of thrombin (Grammas et al., 2004). Because brain endothelial cells not only produce thrombin in response to injury but respond to thrombin with a robust inflammatory response, thrombin is likely a key mediator of cerebrovascular activation in AD.

The release of vascular-derived inflammatory proteins could stimulate/activate neighboring glial cells, both microglia and astrocytes, to release inflammatory proteins as well as noxious ROS and proteases. This noxious, neurotoxic cycle could be augmented by vascular-derived thrombin. In addition to thrombin's inflammatory effects on brain endothelia, thrombin could contribute to deleterious and self-perpetuating neuroinflammation via induction of proinflammatory cytokines including IL-1β, IL-6, TNF-α in microglia and astrocytes (M. S. Choi et al., 2008; S. H. Choi et al., 2003; Huang et al., 2008; D. Y. Lee et al., 2006). A recent study in the TgCRND8 AD mouse showed that long-term treatment with the thrombin inhibitor dabigatran preserved cognition, cerebral perfusion, and BBB function, and ameliorated neuroinflammation and amyloid deposition (Cortes-Canteli et al., 2019). Administration of dabigatran to transgenic AD mice diminishes ROS levels in brain and reduces cerebrovascular expression of inflammatory proteins, further supporting an important role for thrombin as a mediator of neuroinflammation in the AD brain (Tripathy et al., 2013).

Inhibiting thrombin: Implications for therapeutic intervention in AD.

Increasing evidence supports a chronic procoagulant state in AD, highlighting a possible pathogenic role for thrombin in this disease. A number of studies in both human populations and animal models utilizing anticoagulant therapies support the notion that abnormalities of coagulation may promote AD pathology. Treatment of transgenic AD mice with enoxaparin, a low molecular weight heparin, reduces plaques and Aβ accumulation (Bergamaschini et al., 2004) and improves spatial memory (Timmer et al., 2010). As previously stated, TgCRND8 AD mice on long-term dabigatran administration also showed significant improvement in AD pathology (Cortes-Canteli et al., 2019).

Earlier human studies showed improved cognition in dementia patients receiving the anticoagulant warfarin compared to untreated patients (Puccio et al., 2009). Results from these older studies in human patients hinted at a connection between dementia and coagulation abnormalities, but these data were not rigorously pursued. A more recent community-based study found that use of the thrombin inhibitor dabigatran was associated with a lower risk of new-onset dementia (Jacobs et al., 2016). An epidemiological study on atrial fibrillation (AF) patients shows increased thrombin generation and fibrin turnover in subjects with AF and dementia compared to those without dementia, and that long-term warfarin treatment appears to be protective against dementia (Barber et al., 2004; Madhavan et al., 2018).

The evidence supporting a direct connection between AF and dementia suggests the possibility that anticoagulant therapy for AF may reduce the incidence of dementia in this population, and results obtained in a number of recent population-based studies suggest that this is indeed the case (Ding et al., 2018; Field et al., 2019; Mongkhon et al., 2019; Silva et al., 2019). Interestingly, while some groups

have found that all anticoagulants provide a level of protection (e.g. (Friberg & Rosenqvist, 2018)), most studies report that the direct oral anticoagulants (DOACs) that target thrombin are particularly efficacious (N. Chen et al., 2018; Cheng et al., 2018; Jacobs et al., 2016). It is tempting to conclude that DOACs reduce the risk of AF-related dementia by decreasing the incidence of thrombotic and/or embolic events, but this scenario may be overly simplistic. While there is clear evidence that AF is associated with an increased risk of ischemic stroke (Glotzer & Ziegler, 2015), an ambitious meta-analysis of twenty-one studies found that the association between AF and dementia was not always dependent on the presence of clinical stroke (Kalantarian et al., 2013), raising questions regarding the mechanisms underlying this association (Chopard et al., 2018). Dietzel and collaborators have postulated that AF initiates and perpetuates an increase in systemic inflammation that may lead to dementia, noting recent evidence that AF is associated with increased levels of C-reactive protein (CRP), IL-2, IL-6, IL-8, TNF-α, and other inflammatory cytokines (Dietzel et al., 2018). It is probable that thrombin is a key mediator of this inflammatory state, as it is for the inflammation that accompanies AD dementia. Furthermore, the utility of DOACs in the reduction of AF-related dementia is at least partially due to the ability of these agents to inhibit thrombin's PAR-mediated inflammatory actions.

It is therefore possible to interrupt the inflammatory cascade that contributes to AD by utilizing DOACs to inhibit thrombin. There is as yet no direct evidence that this approach will be useful in humans, but an open-label study of a hirudin (natural antithrombin anticoagulant) compound in 84 patients with mild-to-moderate AD found that hirudin plus donepezil reduced the rate of cognitive decline compared to

donepezil alone, suggesting that direct thrombin inhibition may indeed be an effective strategy for treating this neurodegenerative disease (Li et al., 2012).

Although the use of any anticoagulant raises the possibility of unwanted bleeding, several studies have shown the safety and efficacy of dabigatran. Dabigatran does not interact with food, has minimal drug-drug interactions, and has a low risk of intracranial bleeding. A study documented that chronic use (over 30 months) of dabigatran in patients with an average age of 71.4±8.6 had minimal side effects, supporting the efficacy and safety of this drug in an elderly population (Jacobs et al., 2016). Although not without caveats, a body of data implicating thrombin in AD pathogenesis and the relative safety of dabigatran suggests a pilot clinical trial in AD patients is warranted.

Conclusion

The urgent need for disease-modifying drugs in AD demands new thinking about disease pathogenesis and an exploration of novel drug targets. A large, and growing, body of literature implicates vascular pathology and vascular risk factors in the development of AD, but the specific mechanisms whereby these factors contribute to injury in the AD brain have not yet been clearly defined. It is time for "outside-the-box" thinking about how vascular risk factors, such as atherosclerosis and diabetes, as well as the coagulation and inflammatory pathways interact to promote increased AD risk. The evidence summarized here suggests that thrombin is a convergence point for AD risk factors and as such that thrombin-based therapeutics may therefore target multiple points of AD pathology, including neurodegeneration, vascular activation and neuroinflammation. Next generation AD therapeutics should not focus on single-target drugs but rather employ a broader, combinatorial approach. We propose that thrombin inhibitors be considered as potential contributors to the dementia therapy pharmacopeia.

Conflict of Interest/Author Contributions/Funding

The authors declare that the research was conducted in the absence any personal, professional or financial relationships that could potentially be construed as a conflict of interest.

JI and PG contributed to the initial conception of the review. JI performed literature search and wrote the first draft of the review. PG contributed additional literature search and assisted in writing of subsequent drafts. WR wrote an individual section of the review, prepared the figure, and assisted with editing. All authors read and approved the final version before submission.

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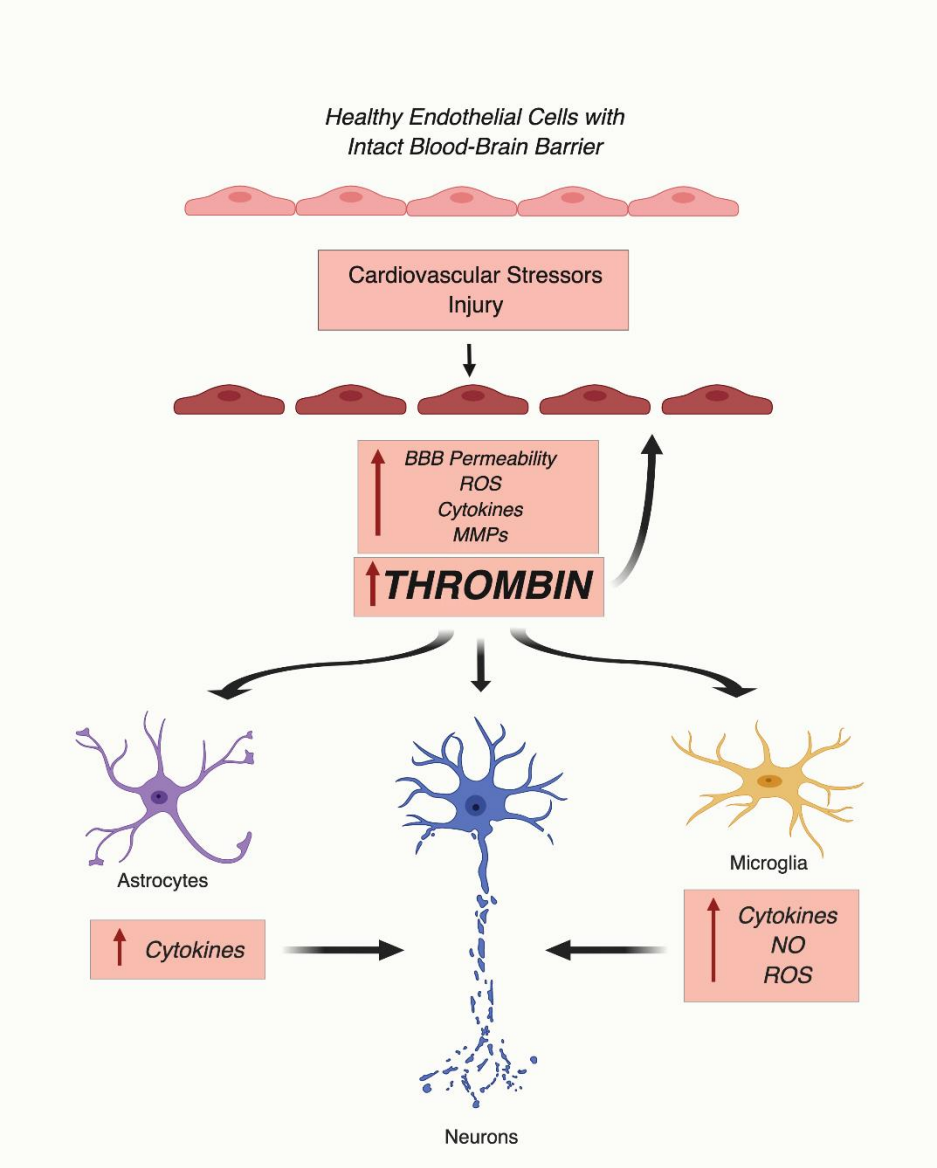
Figure 1. Hypothetical scheme for thrombin as a mediator of neuroinflammation and neurodegeneration



CHAPTER 3

MANUSCRIPT 5

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Direct thrombin inhibitor, dabigatran etexilate, reduces oxidative stress *in vivo* in a transgenic mouse model of Alzheimer's disease

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Abstract

Background. Proteins that regulate the coagulation cascade, including thrombin, are elevated in the brains of Alzheimer's disease (AD) patients. Studies using amyloid-based AD transgenic mice models have also implicated thrombin as a protein of interest. Although thrombin accumulation is co-localized with tau aggregation, the role of thrombin in tau-based animal models has not been explored.

Objective. The current study examined how inhibiting thrombin could alter oxidative stress, inflammation, and tau-related proteins in the Tg4510 mouse, a tau-based model of AD.

Methods. 15-month-old Tg4510 mice were treated with the direct thrombin inhibitor dabigatran etexilate or vehicle for 7 days. Brains were collected, and western blot and data-independent proteomics using mass spectrometry with SWATH-MS acquisition performed to evaluate proteins related to coagulation, oxidative stress, intracellular signaling, and tau-pathology.

Results. Dabigatran treatment reduced thrombin and thrombin-related proteins in Tg4510 mice. Dabigatran also reduced indicators of oxidative stress, including iNOS and NOX4, and increased antioxidant proteins, SOD1 and SOD2. Dabigatran also reduced phosphorylation of tau species (S396, S416). Additionally, dabigatran treatment increased expression of several signaling proteins related to cell survival and synaptic function.

Conclusion. Increasing evidence supports a chronic procoagulant state in AD, highlighting a possible pathogenic role for thrombin. Our data demonstrate that inhibiting thrombin produces alterations in the expression of proteins involved in oxidative stress, inflammation, and tau-related pathology. These results suggest that

thrombin-mediated signaling mechanisms in the brain affect multiple pathways relevant for AD and that thrombin inhibitors could play a role in a drug treatment regimen for AD.

Keywords Alzheimer's disease, Inflammation, Oxidative Stress, Thrombin

Introduction

Cardiovascular disease and cardiovascular risk factors are strongly associated with an increased risk of developing dementia, particularly Alzheimer's disease (AD). Hypertension, diabetes, atherosclerosis, and hypoxia are all linked to an increased risk of developing AD [1-5]. While the connection between cardiovascular risk factors and AD is well documented, the mechanisms whereby these risk factors predispose the brain to the development of AD have not been delineated. It is likely that one, or more, pathological mediators involved in the progression of cardiovascular diseases contributes to the pathology of AD. The multifunctional protein thrombin is implicated in the development of atherosclerosis and diabetes, and more recently suggested as a novel mediator in AD [6-10].

Thrombin is a pleiotropic enzyme that triggers a large and diverse number of cellular events through receptor-mediated activation of protease-activated receptors (PARs) [11]. Recently, several laboratories have provided evidence that proteins that regulate the coagulation cascade, including thrombin, also propagate oxidative stress and neuroinflammation, invariant features of neurodegenerative diseases [12-14]. Thrombin is a potent mediator of oxidative stress and inflammation in a variety of cell types and has been increasingly implicated in pathologic processes in the brain. In this regard, thrombin has been shown to increase NADPH-dependent superoxide anion and hydrogen peroxide production, as well as to injure neurons via microglial release of nitric oxide [15, 16].

Thrombin also appears to play a role in hypoxia-mediated increases in oxidative stress. Cerebral hypoperfusion, leading to hypoxia, has been implicated as an important underlying factor that promotes dementia [5, 17]. Exposure of brain endothelial cells to hypoxia *in vitro* causes an increase reactive oxygen species

(ROS) generation and pro-inflammatory gene expression [18, 19]. Treatment of cultured brain endothelial cells with the thrombin inhibitor dabigatran reduces hypoxia-mediated increase in ROS and inflammatory proteins, suggesting that thrombin may mediate the noxious downstream effects of hypoxic injury [20]. In a primarily amyloid-based AD mouse model (3xTgAD), we have previously shown that administration of the thrombin inhibitor dabigatran significantly decreases expression of ROS and inflammatory proteins in these mice [20].

Increasing evidence suggests that, in addition to oxidative stress, thrombin is linked to several pathological pathways that are relevant for AD. Thrombin is expressed in AD-derived brain microvessels but not in the microvasculature of vessels from control-derived brains [21]. The thrombin inhibitor protease nexin-1 is reduced in the perivascular space in AD, supporting the idea that vascular-derived thrombin is increased in this disease [22]. Levels of both thrombin and the thrombin receptor PAR-1 are elevated in AD [23, 24]. Thrombin is also associated with AD-related hallmarks in the brain, including amyloid and apolipoprotein E [25-29]. Thrombin can affect cellular secretion of amyloid-β precursor protein (AβPP) and could contribute to altered processing of AβPP into fragments that are found in amyloid plaques of AD brains [27, 28, 30]. Additionally, intracerebral administration of thrombin to rodents increases apolipoprotein E levels in the hippocampus and results in neuronal injury and cognitive deficits [31]. The 22 kDa N-terminal thrombin-cleavage fragment of apolipoprotein E is highly neurotoxic and could contribute to apolipoprotein E-associated AD pathology [32].

There is evidence that thrombin may also be relevant to tau-related processes. Thrombin accumulation has been identified in neurofibrillary tangles, and signaling through the thrombin receptor induces tau aggregation and related

hippocampal degeneration [25, 33]. What role thrombin may play as a pathological mediator in a tau-based AD model has not yet been explored. Currently, several tauopathy animal models are being used to study AD. One such model is the Tg4510, which overexpresses human tau with a P301L mutation at 13:1 versus murine tau [34]. These mice exhibit profound tau pathology and neuronal loss in the hippocampus and cortex, as well as cognitive deficits and metabolic changes. The pathology starts early at about 2 to 4 months of age and progresses with age [34, 35]. The pathological features, including tau hyperphosphorylation and synapse loss, are more pronounced in females than males [36]. Additionally, the Tg4510 mouse model displays blood vessel abnormalities accompanied by alterations in oxidative and inflammatory markers [37].

Data linking thrombin to oxidative and inflammatory stress as well as AD-related pathology suggest that thrombin could be a target for therapeutic intervention in this disease. Our previous demonstration in the 3xTgAD mouse that inhibiting thrombin reduces oxidative stress and inflammatory markers supports this idea. The objective of the current study is to investigate the potential therapeutic benefits of inhibiting thrombin in a tau-based animal model of AD. Here we explore the effects of short-term treatment with direct thrombin inhibitor, dabigatran etexilate, in aged Tg4510 mice. It is our hypothesis that inhibiting thrombin will reduce oxidative stress and inflammation-related indicators corresponding to an overall reduction in tau-related dysfunction in the brain.

Materials and Methods

Animals and Treatment

Female transgenic Tg4510 AD mice (PF/CamKII-tTA Tg 129 x tetO-MAPT(P301L) Tg (TG2510 TG/TG)) overexpressing human mutant tau (P301L) and background matched controls (PF/CamKII-tTA Tg 129 x tetO-MAPT(P301L) Tg-3 (TG2510 WT/WT)) were a kind gift from MindImmune Therapeutics, Inc. originally obtained from Charles River (Wilmington, MA, USA). Mice were maintained on normal chow (ENVIGO 2020X, Huntingdon, UK) with water available ad libitum. At 15 months of age, mice were treated via oral gavage with vehicle (2.5% DMSO, 2.5% koliphor EL, 90% diH2O) or dabigatran etexilate (100 mg/kg in uniform suspension, Cayman Pharm, Ann Arbor, MI) daily for 7 days. After 7 days, mice were deeply anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) administered I.P. and euthanized. All animal procedures were performed in accordance with NIH “Guide for the Care and Use of Laboratory Animals” and University of Rhode Island Institutional Animal Care and Use Committee (IACUC) guidelines.

Western blot

Whole brains minus the cerebellum, were homogenized by sonication (Branson SX150 Sonifier ®, Branson Ultrasonics, Danbury, CT) in 1X phosphate buffered saline (PBS) with protease inhibitors. Samples containing 25 μg total protein were resolved in 4-20% poly-acrylamide gel and transferred to nitrocellulose membrane. Membranes were blocked using antibody-specific concentration of bovine serum albumin (BSA) or Milk in Tris-buffered saline + Tween (TBS-T). Primary antibodies for this study include thrombin (Abcam, MA; 1:500), fibrin (Santa Cruz, CA; 1:250), NOX4 (Sigma, MA; 1:1,000), iNOS (Abcam, MA; 1:250), SOD1 (Abcam, MA; 1:1,000), SOD2 (Abcam, MA; 1:2,000), GFAP (Abcam, MA; 1:10,000), Iba1

(GeneTex, CA; 1:1,000), and β-Actin (Santa Cruz, CA; 1:10,000). Tau and p-tau species were detected using phosphor-tau family antibody sampler kit (Cell Signaling, MA; 1:500). Bound antibody was detected with infrared secondary antibodies (Li-COR Biosciences, Lincoln, NE). Membranes were imaged using LiCor Odyssey (LI-COR Biosciences, Lincoln, NE), and quantification was done in ImageJ. Values for each protein were normalized to β-actin loading control on the same blot.

Pressure cycling technology based protein digestion

Whole brain homogenates were further digested using pressure cycling technology (PCT) for LC-MS/MS SWATH acquisition following the method by Jamwal et al., 2017 with slight modifications [38]. Each sample, containing 500 μg of protein, was spiked with 2 ng of BSA. Samples were then incubated with dithiothreitol (100 mM) at 90°C for 15 min, with shaking. Iodoacetamide (200 mM) was added and samples were incubated at room temp in the dark for 30 min. Protein was then precipitated by using the ice-cold chloroform, methanol and water method (1:2:1) followed by centrifugation at 13,400 xG for 5 min at 10°C. The protein pellet was rinsed with methanol and resuspended in 3% w/v sodium deoxycholate (DOC) in 50 mM ammonium bicarbonate. Samples were placed in MicroTubes (Pressure BioSciences Inc, South Easton, MA) with trypsin at a 1:20 ratio of trypsin:protein. Digestion was performed at 55°C for 75 cycles (50 sec at 35kpsi, 10 sec at ambient pressure) in a Barocycler NEP2320-45k (Pressure BioSciences Inc). A second digestion was performed by adding fresh trypsin at the same ratio and running the barocycler for an additional 60 cycles. Samples were then transferred to microcentrifuge tubes where digestion was stopped, and DOC was precipitated by the addition of formic acid in acetonitrile at a final percentage of 0.5%. Samples were centrifuged and supernatant was collected for analysis.

Data independent proteomics using Mass Spectrometry with SWATH-MS Acquisition

Mass spectrometry was performed as previously described with minor modifications [38]. Samples were analyzed on a SCIEX TripleTOF® 5600 mass spectrometer using a DuoSpray™ ion source (SCIEX, Framingham, MA) coupled to an Acquity HClass UHPLC system (Waters Corp., Milford, MA). Separation was achieved on an Acquity UPLC Peptide BEH C18 column (2.1 x 150 mm, 300 Å, 1.7 µm) with an Acquity VanGuard pre-column (2.1 x 5 mm, 300 Å, 1.7 µm). The column temperature was set to 50˚C and the autosampler was set to 10˚C. A linear gradient was used with a flow rate of 100 µL/min for 90 min. Mobile phase A consisted of 99.9% acetonitrile and 0.1% formic acid. Mobile phase B consisted of 99.9% water and 0.1% formic acid. The gradient was as follows: 98% A from 0 to 5 min, 98% to 75% from 5 to 55 min, 75% to 50% A from 55 to 60 min, 50% to 20% from 60 to 70 min. Mobile phase A was held at 20% from 70 to 75 min and returned to 98% A at 80 min. The column was held at 98% A for 10 min to equilibrate prior to the next sample. A mixture of trypsin-digested β-galactosidase peptides was used between every 8 samples to calibrate masses and monitor the TOF detector.

Positive ionization mode was used for data dependent acquisition. The mass spectrometer parameters are as follows: gas 1, gas 2, curtain gas, temperature and ion spray voltage floating were 55 psi, 60 psi, 25 psi, 450˚C, 5500 V, Respectively. Declustering potential was 10, collision energy 10 and collision energy spread 15. For data acquisition, a maximum of 50 candidate ions were monitored for each survey scan. All ions had a charge state from 2 to 4. A range of *m/z* 300-1250 was used for exclusion criteria and all ions that had an intensity greater than 25 cps were chosen for MS/MS analysis. The temperature was set at 450˚C and the total cycle time was 3.5 sec with a mass tolerance of 50 mDa during the first 0.75 sec survey scan.

For SWATH analysis, all parameters were the same as above except for the following: Seventy SWATH windows per cycle were collected over *m/z* 400-1100 with each window size being *m/z* 10 and TOF masses were collected from *m/z* 300 to 1500.

Mass Spectrometry Data Analysis

LC-MS/MS SWATH data were used to generate spectral libraries through ProteinPilot (SCIEX, Framingham, MA). Proteins of interest were identified and investigated. FASTA files were downloaded from UniProt [39] and imported into Skyline (MacCoss Lab, University of Washington). In Skyline, at least 3 transitions were selected per peptide, and at least 3 peptides per protein were chosen. Once data were analyzed, the MPPreport (MacCoss Lab, University of Washington) was generated and exported to excel. In excel, transitions were averaged, and the sum of each peptide was calculated to yield the total area under the curve representative of each protein. These were then standardized to internal standard, BSA.

Statistical analysis

Data from each experiment are expressed as mean +/- standard error (SEM), unless otherwise indicated. All tests were performed in GraphPad prism. Analysis was done by unpaired, one-tail t-test. Statistical significance was determined at $p<0.05$. Significance for all tests was defined as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Results

Dabigatran treatment reduces expression of coagulation-related proteins in Tg4510 mouse brain

The effect of short-term (one week) treatment of Tg4510 mice with dabigatran on the expression of coagulation-related proteins, prothrombin and fibrin, was evaluated by western blot (Fig. 1A). Levels of both prothrombin and fibrin were significantly ($p<0.01$, $p<0.001$, respectively) elevated in Tg4510 mice compared to levels expressed by wild-type mice (Fig. 1B). Dabigatran treatment of Tg4510 mice significantly ($p<0.05$) reduced prothrombin level by 46.7% compared to levels observed in untreated Tg4510 mice (Fig. 1B). Similarly, inhibiting thrombin with dabigatran significantly ($p<0.05$) reduced fibrin expression in the Tg4510 animals to essentially the level demonstrated by wild-type mice (Fig. 1C).

Expression of oxidative stress-related proteins in Tg4510 mouse brains is reduced by dabigatran

Inducible nitric oxide synthase (iNOS) and NADPH oxidase 4 (NOX4) are enzymes involved in the generation of oxidative stress by catalyzing the production of NOS and ROS. The expression of both enzymes was evaluated by western blot (Fig. 2A). The expression of iNOS was significantly ($p<0.01$) elevated in Tg4510 mice compared to wild-type, and treatment of Tg4510 mice significantly ($p<0.05$) reduced expression by 17.4% (Fig. 2B). Dabigatran treatment also significantly ($p<0.01$) reduced NOX4 expression by 24.7% compared to untreated Tg4510 mice (Fig. 2C).

Dabigatran treatment increases expression of antioxidant proteins in Tg4510 mouse brains

To more fully determine the oxidative environment in the Tg4510 brain the expression of antioxidant-related proteins, superoxide dismutase 1 (SOD1) and

superoxide dismutase 2 (SOD2) was evaluated by western blot (Fig. 3A).The expression of both antioxidant proteins did not significantly differ between Tg4510 and wild-type mice. However, Tg4510 mice treated with dabigatran showed significantly increased expression of both SOD1 (34.2%, $p<0.01$) (Fig. 3B) and SOD2 (20.7%, $p<0.05$) (Fig. 3C).

Dabigatran treatment does not alter expression of astrocytic or microglial activation-associated proteins

Glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (IBA1) are markers of activated astrocytes and microglia, respectively. The expression of these markers in brain homogenates was evaluated by western blot (Fig. 4A). Both GFAP and IBA1 were significantly ($p<0.001$) elevated in Tg4510 as compared to wild type-mice (Fig. 4). Treatment with dabigatran did not alter GFAP expression in Tg4510 mice (Fig. 4B). In contrast, dabigatran did lower Iba1 expression, but this reduction was not significant (Fig. 4C).

Treatment of Tg4510 mice with dabigatran diminishes levels of phosphorylated tau species

Total tau and phosphorylated forms of tau S396 and S416 were evaluated by western blot (Fig. 5). Tg4510 mice expressed 75.9% more total tau than wild-type mice ($p<0.001$). Treatment of Tg4510 mice with dabigatran lowered total tau (22.1%) compared to untreated Tg4510 mice, but this reduction was not significant (Fig. 5A). The expression of both phosphorylated tau species S396 and S416 were significantly ($p<0.001$) elevated in Tg4510 mice compared to levels in wild-type mice. Treatment of Tg4510 mice with dabigatran significantly ($p<0.05$) lowered levels of both S396 (Fig. 5B) and S416 (Fig. 5C).

Proteomic expression differences between wild-type mice and Tg4510 mice are detected by LC-MS/MS SWATH analysis

LC-MS/MS SWATH analysis was undertaken based on proteins relevant for AD-related pathological processes as well as thrombin/dabigatran-related mechanisms including proteins involved in the coagulation cascade, AD, inflammation and cell signaling. Protein sequences were obtained from the UniProt database and results from LC-MS/MS with SWATH acquisition analyzed in Skyline, an open source software for targeted proteomics (Table 1).

Significant differences in protein expression between wild-type and Tg4510 mice were identified and visualized with a volcano plot (Fig. 6A). Specific proteins within relevant functional categories that display significant differences between wild-type mice and Tg4510 mice are shown in Figure 6B-C. Within the coagulation cascade classification, significant differences were detected in FIBB and THRB (Fig. 6B). Differences were also seen in proteins related to inflammation (ICAM5, GFAP) and cellular signaling (RACK1) (Fig. 6B). AD-related proteins that demonstrated significant differences between wild-type mice and Tg4510 animals were ApoE, BACE1, TAU (Human), TAU (Mouse), TAU (total), G6PD1, HPCA, KCC2A, and VIME (Fig. 6C).

Treatment of Tg4510 mice with dabigatran causes differences in protein expression as delineated by volcano and box plots

Significant differences in AD, coagulation, inflammation, and signaling-related protein expression between Tg4510 mice and Tg4510 mice that were treated with dabigatran were identified and visualized with a volcano plot (Fig. 7A). Dabigatran treatment significantly increased proteins related to both inflammation and coagulation, including ITB2, ANT3, and COX2 (Fig. 7B). Dabigatran treatment also

significantly increased ($p<0.05$) the levels in a number of cell signaling-related proteins, including DYN1, MP2K2, MP2K1, MK08, and KPCB (Fig. 7B) as well as AD-related ANS1B, NCKP1, SV2B, and SYGP1 (Fig. 7C).

Discussion

Increasing evidence has shown that thrombin, and thrombin-related proteins of the coagulation cascade such as fibrin, are elevated in the brains of AD patients [22, 25-29, 40-42]. Furthermore, studies using amyloid-based AD transgenic mice models have also implicated thrombin as a protein of interest [20]. Although thrombin accumulation is co-localized with tau aggregation [25, 26], thrombin's potential role in tau-based animal models has not been explored. In the current study we examined how inhibiting thrombin could alter tau-related pathologic processes.

Our data show that efficacy of treatment with a direct thrombin inhibitor, dabigatran, significantly affects several AD-related pathological processes in the tau-based Tg4510 mouse. As expected, the drug treatment reduces thrombin and proteins increased by thrombin activity (prothrombin, fibrin) in Tg4510 mice. Additionally, mass spec analysis found that dabigatran significantly increased levels of antithrombin III (ANT3). Antithrombin binds to thrombin and reduces its catalytic activity [43]. These findings indicate that dabigatran treatment effectively decreases thrombin levels and activity. Dabigatran treatment also produced alterations in the expression of proteins involved in oxidative stress, inflammation, and tau-related pathology. These results suggest that thrombin-mediated signaling mechanisms in the brain affect multiple pathways relevant for AD.

Thrombin's pro-inflammatory effects throughout the body, including in the brain, are largely mediated through the signaling of its receptors, protease-activated receptors (PARs) 1, 3, and 4. PARs represent a unique family of G-protein coupled receptors that are activated by a self-ligand [11]. PARs have been found in a number of cell types in the periphery and the brain, including endothelial cells, neurons, astrocytes, and microglia [24]. When cleaved and activated by thrombin, PAR1

initiates a variety of intracellular signaling. Activation of PAR1 activates the mitogen-activated protein kinase (MAPK) pathway, among others, which can lead to cell growth, proliferation, or migration [44].

Activation of PAR1 can trigger MAPK signaling cascade, an interactive network of sequentially activated protein kinases that modulates a diverse array of cellular responses driving both physiologic (growth, survival) and pathologic (inflammation, apoptosis) processes [44, 45]. A large literature implicates dysfunctional MAPK in neurodegenerative diseases, especially AD. p38 MAPK has been shown to increase transcription of inflammatory genes and play a role in phosphorylation of tau [46, 47]. In this current study LC-MS/MS SWATH analysis revealed that the MAPK-related proteins MP2K1, MP2K2, and MK08 were significantly elevated in the brains of Tg4510 mice receiving dabigatran treatment, compared to untreated Tg4510 mice. In contrast to p38MAPK, MP2K1 and MP2K2, also known as MEK1 and MEK2, are kinases responsible for the activation of the ERK signaling pathway; ERK-mediated signaling is generally thought to regulate mechanisms of cell growth, differentiation, and survival [45, 48, 49]. There is also some evidence that this kinase pathway may protect against amyloid-β (Aβ)-induced toxicity [49, 50]. This finding suggest alternative, protective cleavage and activation of PAR1 in response to thrombin inhibition, possibly by activated protein C (APC), or another anti-coagulant mediator.

Treatment of Tg4510 mice with dabigatran also increased expression of MK08 (JNK1). The JNK subfamily is encoded by three separate but related genes: jnk1, jnk2, and jnk3, giving rise to at least ten distinct splice variants [51]. The JNKs appear to be involved in both neuronal degeneration and regeneration [51]. Although JNK has been implicated in accelerating the formation of neurotoxic Aβ and neurofibrillary

tangles, it is also hypothesized to play a beneficial role in autophagy [52]. In the current study, it is interesting to note that dabigatran also increases dynamin-1 (DYN1). This protein has been shown to be essential for synaptic vesicle recycling and, hence, for memory formation and information processing [53]. Published studies have shown that Aβ oligomers decrease dynamin-1 and generate toxic tau fragments in cultured hippocampal neurons [54]. It is unclear how to interpret the increase in both MK08 and DYN1 caused by dabigatran, but the relationship of both of these to autophagy and organelle recycling suggests these pathways should be further explored in light of the literature showing that autophagosome-lysosomal degradation is impaired and AD [55]. Finally, dabigatran treatment caused an increase in the level of PKC-β (KPCB). This kinase has been implicated as critical enzyme in learning and memory function as decreased levels of endogenous PKC-β have been associated with impaired spatial learning and memory [56]. This elevation of PKC-β is consistent with the increase we show in DYN1, a protein also functionally linked to information processing, and suggests that future long-term studies probing the cognitive effects of dabigatran in this AD model are warranted. It should be noted that a recent study in the TgCRND8 transgenic AD mice showed that treatment with dabigatran for one year improved spatial memory deficits [57].

Thrombin activation of PARs contributes to oxidative stress and inflammation in both the periphery and brain. Oxidative stress resulting from generation of high levels of ROS in AD is consistently associated with neuron injury, Aβ accumulation, and tau phosphorylation [46, 58]. While a meditator of several physiologic functions, nitric oxide (NO) is also mediator of oxidative stress in the brain when continuously produced [46]. NO interacts with ROS leading to the formation of reactive nitrogen species (RNS), and targets vital organelles, such as mitochondria,

ultimately causing cellular death. Our laboratory and others have documented an increase in NO and iNOS in AD models [59, 60]. Here we show that iNOS is elevated in the Tg4510 mice and that treatment with dabigatran significantly reduces the expression of iNOS. Another source of noxious ROS in the brain is NADPH oxidase (NOX). NOX are a family of enzymes that directly regulate ROS production [61]. There is growing evidence that the isoforms NOX1, NOX2, and NOX4 are upregulated in a variety of neurodegenerative disease. Although it is unclear why NOX4 levels were comparable between wild-type mice and Tg4510, dabigatran was still able to lower NOX4 expression in Tg4510 mice. These findings likely indicate an overall reduction in oxidative stress in the Tg4510 mice, but follow-up examination of enzyme activity would be necessary to confirm a reduction in pathogenic activity.

The SOD enzyme is responsible for reducing oxidative stress by converting superoxide radicals to hydrogen peroxide and oxygen [62]. Our data show that expression of both SOD1 and SOD2 is comparable between expressed wild-type and Tg4510 mice. Diminished levels of these enzymes are often associated with instances of ROS-mediated cellular damage [63]. Interestingly, high levels of SOD are also observed in instances of oxidative stress as metabolic disturbances lead to upregulation of cellular antioxidant capacity to maintain ROS levels below a toxic threshold [64]. Increasing endogenous SOD levels and/or activity has been a target of antioxidant intervention strategies using compounds like curcumin [65]. Here we show that dabigatran can significantly increase SOD1, and SOD2 levels in the tau-mouse model. It is possible that dabigatran not only reduces oxidant stress by directly reducing the direct actions of thrombin, but also by increasing SOD.

The role of dabigatran as an anti-inflammatory drug in this animal model is unclear. Both GFAP and IBA1, inflammatory proteins and markers of cellular

activation for astrocytes and microglia, respectively, were significantly (p<0.001) elevated in Tg4510 as compared to wild type-mice. Treatment with dabigatran did not significantly affect their expression. This is congruent with another study that found no significant difference in GFAP or IBA1 following dabigatran treatment in Tg2576 [66], but it is in contrast to other studies that reported treatment of the 5xFAD mouse with dabigatran diminished expression of both GFAP and IBA1 [67]. This divergence in results may in part reflect length of treatment, as dabigatran dosing was much longer (4 weeks) in the 5xFAD mice compared to our short-term 7-day timeframe. Also, the animals in that study were young (8-week-old) and our mice were in late-stage (15 months old) disease state. Another observation we document herein is that COX-2 is elevated in response to dabigatran. Although COX-2 is primarily identified as a noxious mediator of neuroinflammation, failure of clinical trials with selective COX-2 inhibitors and NSAIDs suggest that the involvement of COX-2 in AD pathology is more complex [68]. In that regard, COX-2 expression in AD neurons is associated with aberrant expression of cell cycle proteins which could suggest involvement of COX-2 in regenerative pathways [68]. Future studies are needed to further the role of COX-2 in neuronal viability.

Total tau and related phosphorylated (S396, S416) tau species were significantly increased in transgenic mice compared to wild type, as was expected. Dabigatran treatment slightly reduced expression of total tau and significantly decreased S396, S416. S396 phosphorylation is found early in the disease course of AD [69, 70] and is related to destabilization of microtubules [71, 72]. S396 is also linked with abnormal truncation of the tau protein, indicating altered functionality [69]. Phosphorylation at S416 by CamKII is largely found within the neuronal soma, rather than localized to microtubules, and has been found to be associated with the

promotion of AD-related cell death [73, 74]. Together, our findings indicate that dabigatran etexilate treatment may reduce AD-related tau dysfunction through altered phosphorylation, particularly decreased phosphorylation at S396 and S416.

To further explore AD-related pathology, we performed LC-MS/MS SWATH analysis on a variety of previously identified AD and tau-related proteins. Here, ANS1B, NCKP1, SV2B and SYGP were significantly increased with dabigatran treatment, compared to Tg-Vehicle. In a previous report analyzing tau pathology in human AD brains, these proteins were found to be significantly downregulated in late-stage AD [75]. ANS1B (Ankyrin repeat and sterile alpha motif domain-containing protein 1B) is a scaffold protein localized to the post-synaptic density. It has an identified role in facilitating long-term potentiation (LTP). Additionally, ANS1B has been found to regulate endothelial barrier function and permeability; ANS1B knockdown produced significantly increased endothelial barrier permeability *in vitro* [76]. A significant increase in ANS1B may therefore suggest improved endothelial barrier function following dabigatran treatment. Alterations in blood-brain barrier (BBB) function and permeability have been identified in AD pathology [77-79], further highlighting the possible therapeutic benefits of dabigatran treatment. Significant increases in SV2B (synaptic vesicle glycoprotein 2B) and SYGP1 (Ras/RTP GTPase activating protein), further highlight the possible protective benefits of dabigatran treatment. SV2B is a mediator of synaptic vesicle transport and exocytosis, and an increase in SV2B expression has previously been tied to behavioral improvements in mice following environmental enrichment [80]. SYGP1 regulates the trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors to the cell membrane, and therefore plays a critical role in regulating neuronal plasticity, synaptic function, and cognition [81, 82]. Together, these findings suggest that

dabigatran treatment may increase neuronal function at the synapse and promote improved plasticity and learning potential.

Dabigatran may impact oxidative and inflammatory processes by mechanisms other than direct inhibition of thrombin-receptor-mediated pathways. Thrombin activity drives the formation of fibrin, and fibrin has been identified as a potential pathological mediator in AD [83]. Higher levels of fibrin have been identified in the AD brain compared to healthy controls [42], and fibrin accumulation in AD, similar to thrombin, has been linked with increases in inflammation and oxidative stress, as well as alterations in both amyloid and tau pathology [83]. Our findings showed a decrease in fibrin expression as a result of dabigatran treatment. It is likely that the beneficial effects of dabigatran treatment, including decreases in oxidative stress-related and inflammatory proteins, are attributable to both decreased fibrin accumulation as well as reduced thrombin receptor-mediated activity.

Thrombin inhibition may also indirectly promote anti-oxidative and anti-inflammatory processes through increased activity of endogenous anti-coagulants. Anti-coagulant proteins, such as activated protein C (APC), act in opposition to thrombin [84]. For example, activation of PAR1 by thrombin induces proinflammatory signaling responses whereas the activation of the same receptor by APC elicits anti-inflammatory responses in cultured human umbilical vein endothelial cells (HUVECs) [85, 86]. Several studies have shown anti-inflammatory effects of APC-like ligands mediated through PAR signaling [87]. Just as reduced thrombin activity may result in reduced fibrin accumulation and therefore reduced inflammation, reduced levels and activity of thrombin following dabigatran treatment may allow for APC and other anti-coagulant proteins to bind to PARs and activate anti-inflammatory signaling processes.

Increasing evidence supports a chronic procoagulant state in AD, highlighting a possible pathogenic role for thrombin in this disease. Studies in both animal models and human populations utilizing anticoagulant therapies support the notion that mediators of the coagulation cascade may promote AD pathology. A recent study showed that treatment of TgCRND8 transgenic AD mice with dabigatran improved spatial memory deficits, reduced neuroinflammation, and amyloid plaque formation [57]. An open-label study of a hirudin (natural antithrombin anticoagulant) compound in 84 patients with mild-to-moderate AD found that hirudin plus donepezil reduced the rate of cognitive decline compared to donepezil alone, suggesting that direct thrombin inhibition may indeed be an effective strategy for treating this neurodegenerative disease [88]. Indirect thrombin inhibitors, such as warfarin, exert anti-inflammatory and neuroprotective effects in models of AD [89]. However, direct thrombin inhibition with dabigatran may be a safer treatment option, with a reduced risk of intracerebral hemorrhage [90]. In a longitudinal, community-based study use of dabigatran was associated with a lower risk of new-onset dementia compared to warfarin [91]. Taken together these studies demonstrate that targeting thrombin could be beneficial in AD and that thrombin inhibitors could play a role in a drug treatment regimen for AD.

Acknowledgements/Conflicts of Interest

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The authors have no conflict of interest to report.

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Table 1. List of proteins and corresponding abbreviations. Proteins of interest were identified, classified, and categorized based on previous literature. Proteins were obtained from UniProt and data obtained by LC-MS/MS with SWATH acquisition were analyzed in Skyline.

Pathway	Abbreviation	Full Protein Name
Alzheimer's Disease	A4	Amyloid-beta A4 protein (APP)
	ACTN2	Alpha-actinin-2
	ANS1B	Ankyrin repeat and sterile alpha motif domain-containing protein 1B (Amyloid-beta protein intracellular domain-associated protein 1)
	APOE	Apolipoprotein E (Apo-E)
	BACE1	Beta-secretase 1
	BACE2	Beta-secretase 2
	G3BP2	Ras GTPase-activating protein-binding protein 2
	G6PD1	Glucose-6-phosphate 1-dehydrogenase X
	GNAZ	Guanine nucleotide-binding protein G(z) subunit alpha
	HPCA	Neuron-specific calcium-binding protein hippocalcin
	KCC2A	Calcium/calmodulin-dependent protein kinase type II subunit alpha
	NAC2	Sodium/calcium exchanger 2
	NCKP1	Nck-associated protein 1
	PACN1	Protein kinase C and casein kinase substrate in neurons protein 1 (Syndapin-1)
	Q8CFX3	Protocadherin 1
	SV2B	Synaptic vesicle glycoprotein 2B
	SYGP1	Ras/Rap GTPase-activating protein SynGAP
	TAU (Human)	Microtubule-associated protein tau
	TAU (Mouse)	Microtubule-associated protein tau
	TAU (Total)	Microtubule-associated protein tau
	TBB3	Tubulin beta-3 chain
	TLN1	Talin-1
	TTBK2	Tau-tubulin kinase 2
	VIME	Vimentin
Cellular Signaling	CDK5	Cyclin-dependent-like kinase 5
	DYN1	Dynamin-1
	MK01	Mitogen-activated protein kinase 1
	MK03	Mitogen-activated protein kinase 3
	MK08	Mitogen-activated protein kinase 8
	MK09	Mitogen-activated protein kinase 9
	MP2K1	Dual specificity mitogen-activated protein kinase kinase 1
	MP2K2	Dual specificity mitogen-activated protein kinase kinase 2
	KPCA	Protein kinase C alpha type
	KPCB	Protein kinase C beta type
	KPCD	Protein kinase C delta type
	RACK1	Receptor of activated protein C kinase 1
	ROCK1	Rho-associated protein kinase 1
	ROCK2	Rho-associated protein kinase 2
Coagulation Cascade	ANT3	Antithrimbin-III
	FA5	Coagulation factor V (Activated protein C cofactor)
	FA8	Coagulation factor VIII (Procoagulant component)
	FA10	Coagulation factor X
	FIBA	Fibrinogen alpha chain
	FIBB	Fibrinogen beta chain
	ITAL	Integrin alpha-L
	ITAM	Integrin alpha-M
	ITAV	Integrin alpha-V
	ITB2	Integrin beta-2
	ITB3	Integrin beta-3
	THRB	Thrombin
Inflammation Related	COX2	Cytochrome c oxidase subunit 2
	DLG4	Disks large homolog 4
	GFAP	Glial fibrillary acidic protein (GFAP)
	ICAM1	Intercellular adhesion molecule 1
	ICAM5	Intercellular adhesion molecule 5
	MILK2	MICAL-like protein 2
	NFKB1	Nuclear factor NF-kappa-B p105 subunit
	NOS3	Nitric oxide synthase, endothelial
	PARK7	Protein/nucleic acid deglycase DJ-1
	PTPRC	Receptor-type tyrosine-protein phosphatase C
	TLR4	Toll-like receptor 4
	VCAM1	Vascular cell adhesion protein 1

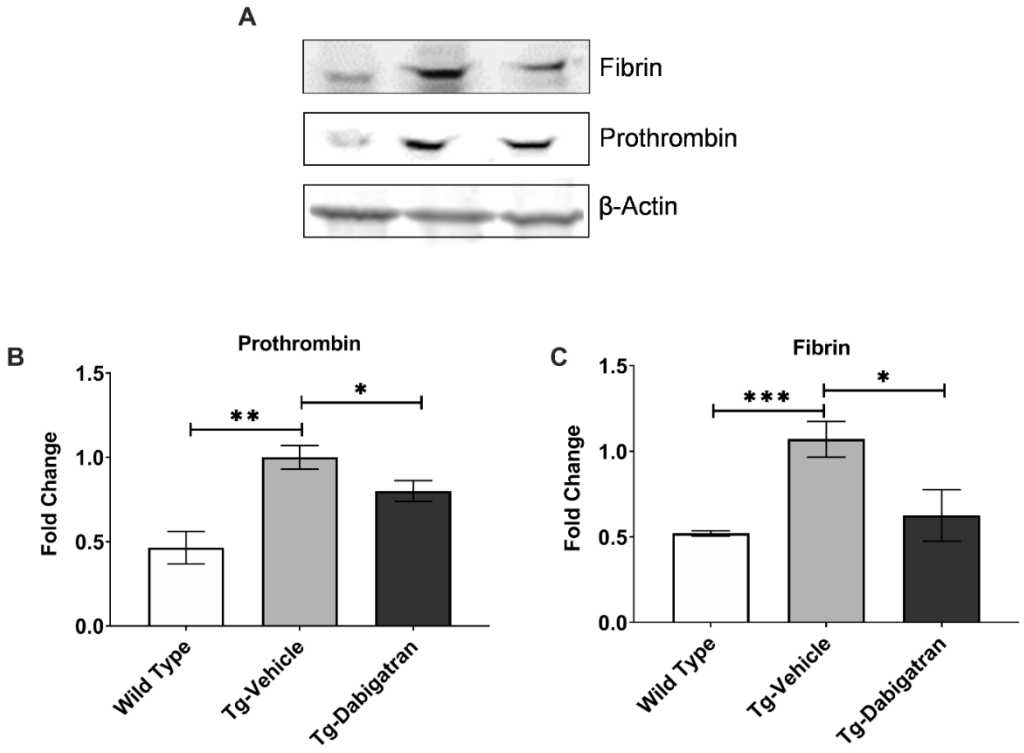

Figure 1. Dabigatran treatment reduces expression of coagulation-associated proteins in Tg4510 mouse brain. Brain homogenates from wild-type mice, Tg4510 mice (Tg-Vehicle), and Tg4510 mice treated with dabigatran (Tg-Dabigatran) for one week were analyzed by western blot (A) for prothrombin (B) and fibrin (C). Data are expressed as mean ± SEM (n=4-6) and significance determined by t-test, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$.

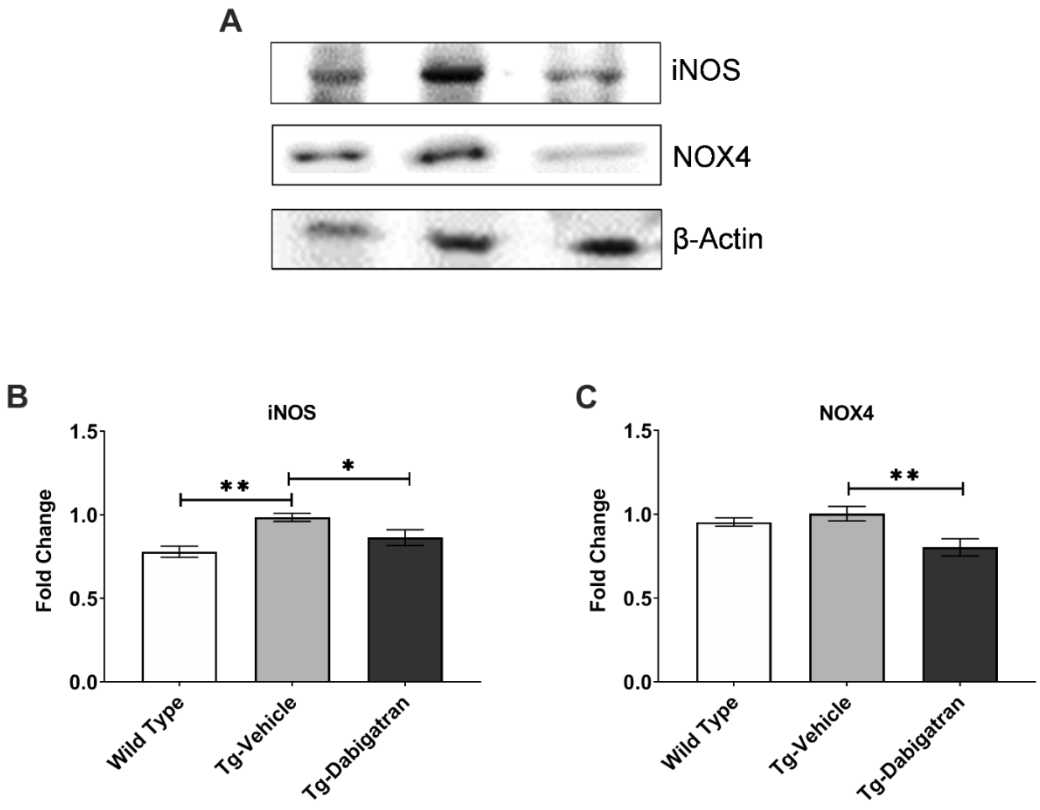

Figure 2. Expression of oxidative stress-related proteins in Tg4510 mouse brains is reduced by dabigatran. Brain homogenates from wild-type mice, Tg4510 mice (Tg-Vehicle), and Tg4510 mice treated with dabigatran (Tg-Dabigatran) for one week were analyzed by western blot (A) for iNOS (B) and NOX4 (C). Data are expressed as mean ± SEM (n=4-6) and significance determined by t-test, *$p \leq 0.05$, **$p < 0.01$.

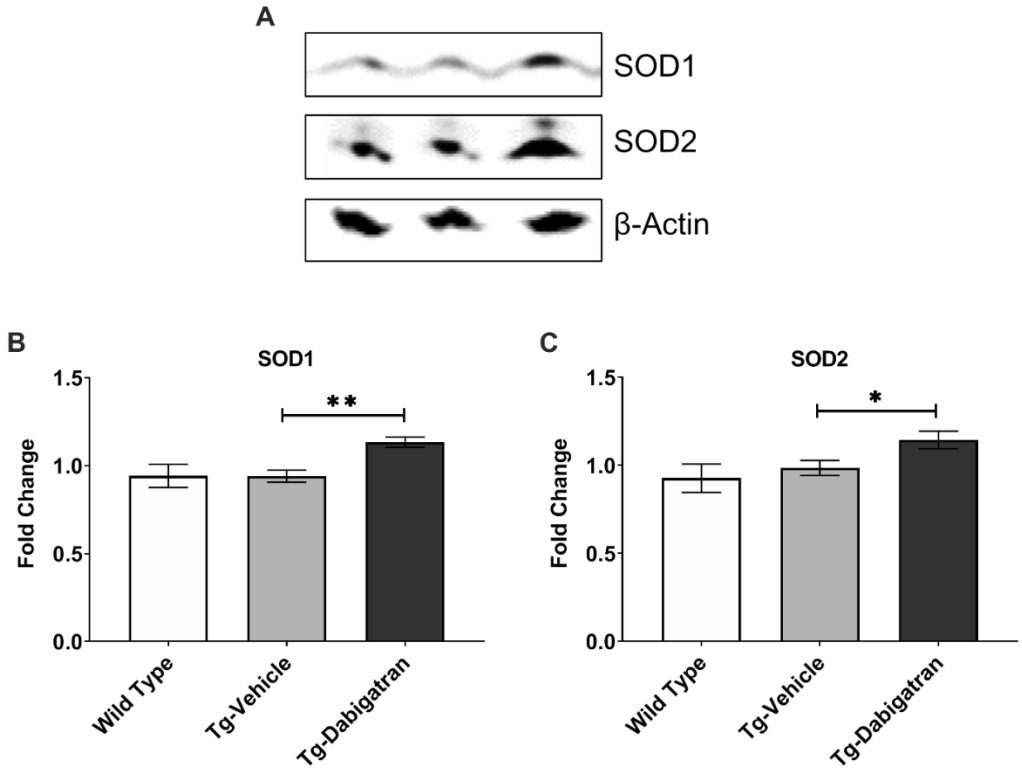

Figure 3. Dabigatran treatment increases expression of antioxidant proteins in Tg4510 mouse brains. Brain homogenates from wild-type mice, Tg4510 mice (Tg-Vehicle), and Tg4510 mice treated with dabigatran (Tg-Dabigatran) for one week were analyzed by western blot (A) for SOD1 (B) and SOD2 (C). Data are expressed as mean ± SEM (n=4-6) and significance determined by t-test, *$p \leq 0.05$, **$p < 0.001$.

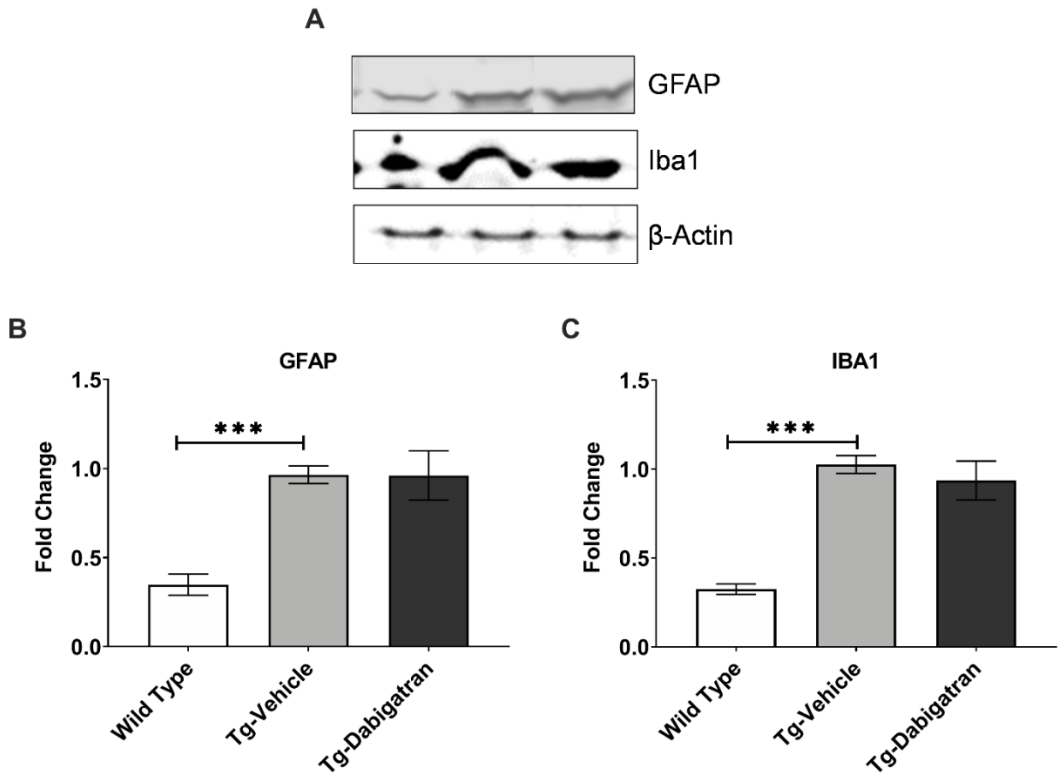

Figure 4. Dabigatran treatment does not alter expression of astrocyte or microglial activation-associated proteins. Brain homogenates from wild-type mice, Tg4510 mice (Tg-Vehicle), and Tg4510 mice treated with dabigatran (Tg-Dabigatran) for one week were analyzed by western blot (A) for GFAP (B) and IBA1 (C). Data are expressed as mean ± SEM (n=4-6) and significance determined by t-test, ***$p < 0.001$.

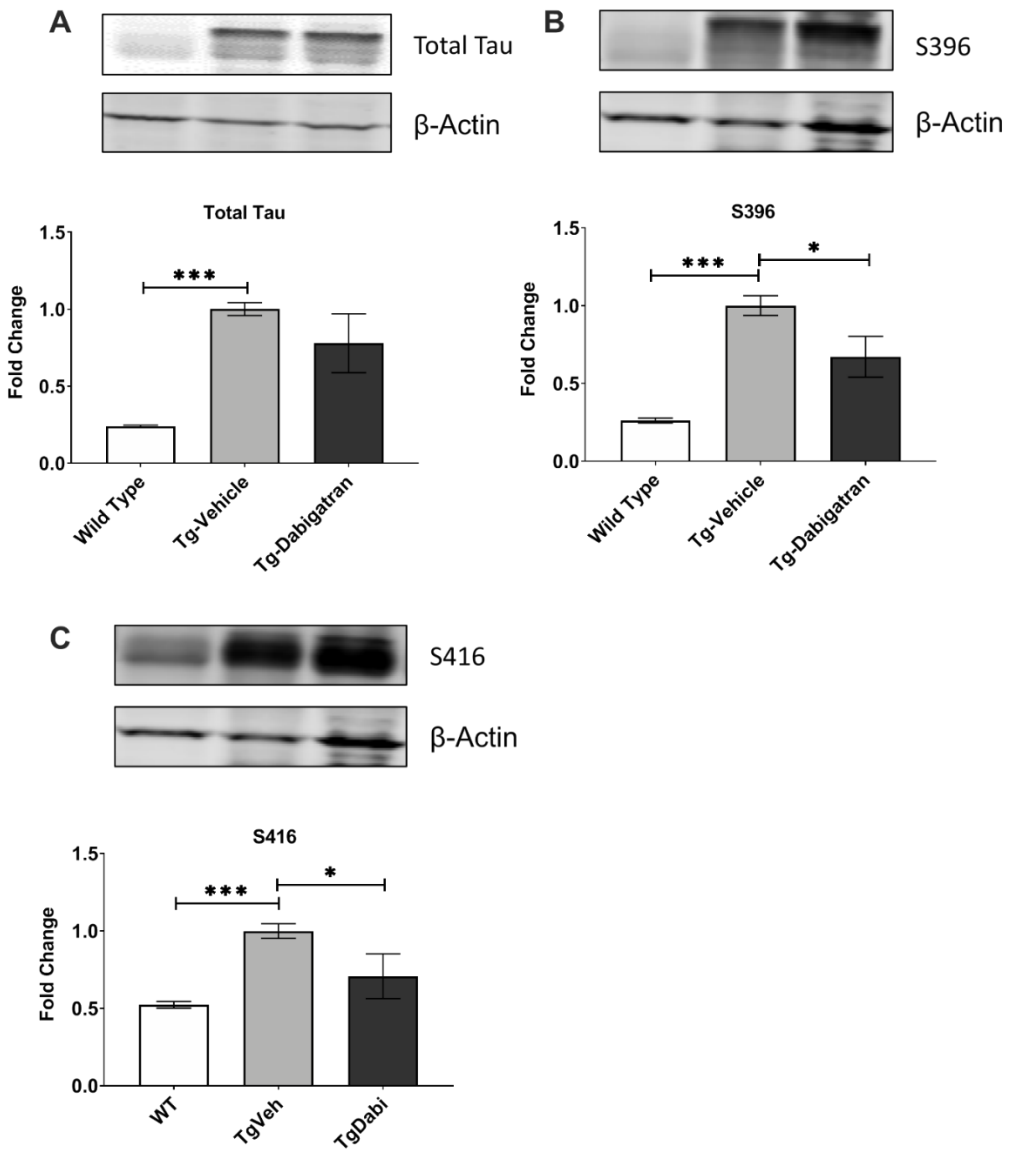

Figure 5. Treatment of Tg4510 mice with dabigatran diminishes levels of phosphorylated tau species. Brain homogenates from wild-type mice, Tg4510 mice (Tg-Vehicle), and Tg4510 mice treated with dabigatran (Tg-Dabigatran) for one week were analyzed by western blot for total tau (A) tau S396 (B) and tau S416 (C). Data are expressed as mean ± SEM (n=4-6) and significance determined by t-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

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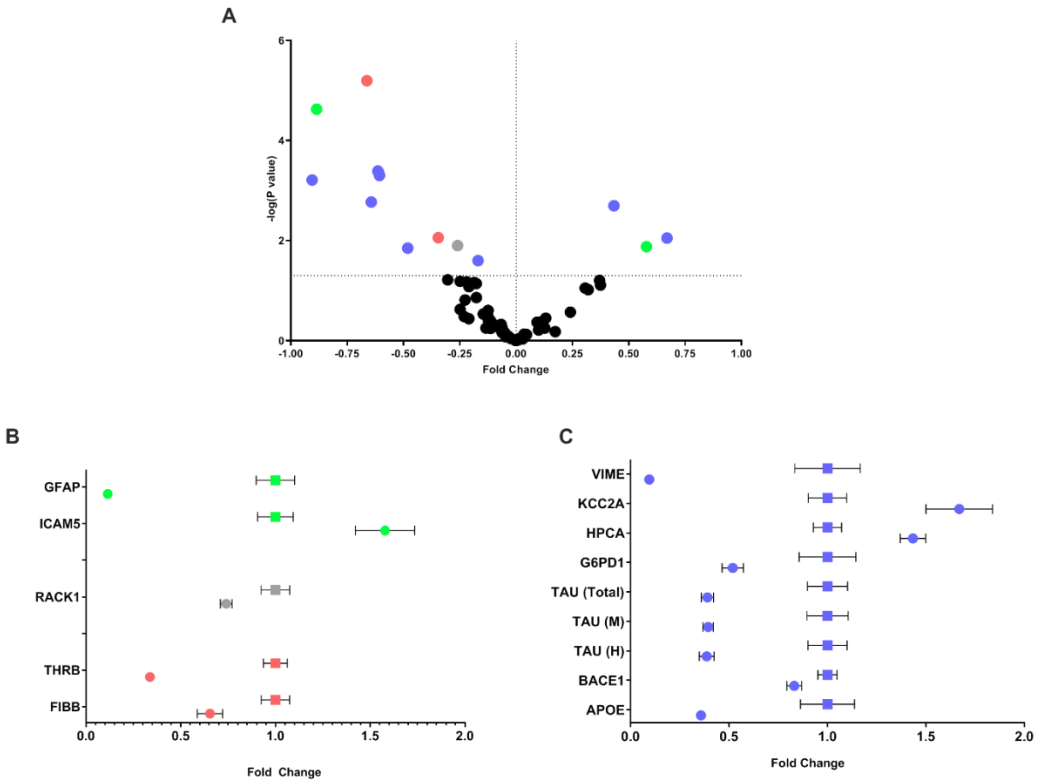

Figure 6. Proteomic expression differences between wild-type mice and Tg4510 mice are detected by LC-MS/MS SWATH analysis. Volcano plot of data sets from brain homogenates of wild-type mice and Tg4510 mice (Tg-Vehicle) is shown in panel A. Data points are color-coded into functional categories as follows: Alzheimer's Disease (blue), coagulation cascade (pink), inflammation-related (green), and cell signaling (grey). Non-significant differences are denoted in black (A). Proteins related to inflammation, cell signaling, and the coagulation cascade that show significant expression differences between wild-type (○) and Tg-Vehicle (□) are shown in B. Alzheimer's disease-related proteins with significant expression differences between wild-type and Tg-Vehicle in are shown in C. Data points are expressed as mean +/- SEM, and statistical differences determined by multiple t-tests, $p \leq 0.05$ (n=5).

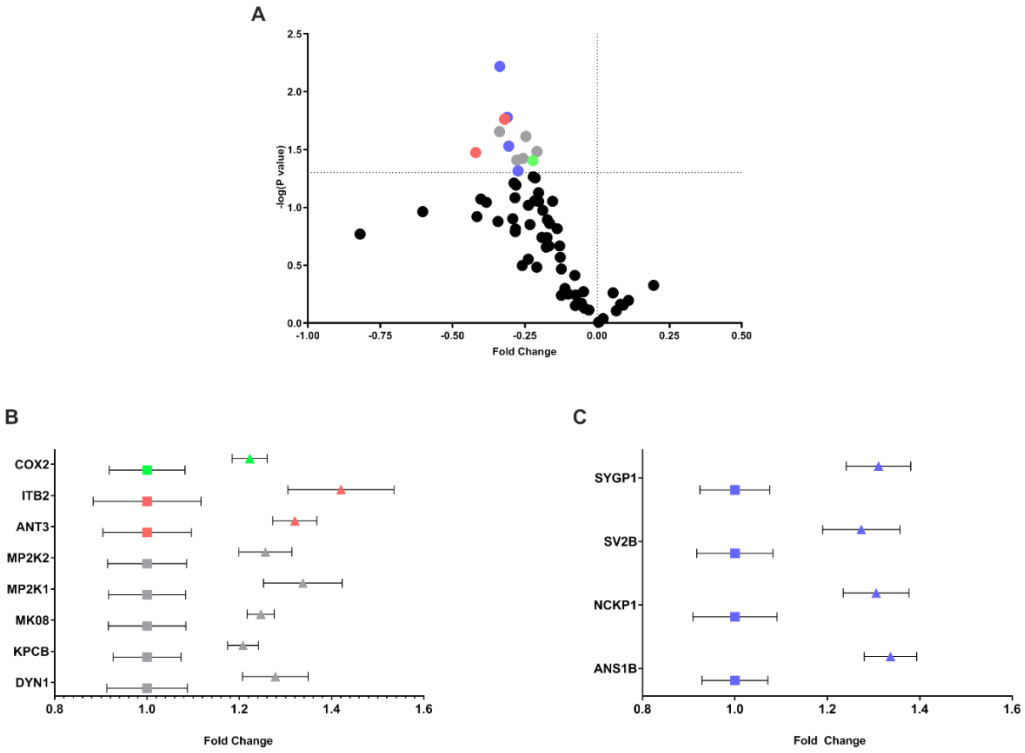

Figure 7. Treatment of Tg4510 mice with dabigatran causes differences in protein expression. Volcano plot of data sets from brain homogenates of Tg4510 mice (Tg-Vehicle) and Tg4510 mice treated with dabigatran (Tg-Dabigatran) is shown in panel A. Data points are color-coded into functional categories as follows: Alzheimer's Disease (blue), coagulation cascade (pink), inflammation-related (green), and cell signaling (grey). Non-significant differences are denoted in black (A). Functional categories of proteins that show significant expression differences between Tg-Vehicle (□) and Tg-Dabigatran (Δ) are shown as follows: coagulation cascade, inflammation-related, and cell signaling (B) and Alzheimer's disease-related (C). Data points are expressed as mean +/- SEM, and statistical differences determined by multiple t-tests, $p \leq 0.05$ (n=5).

CONCLUDING REMARKS

Alzheimer's disease (AD) is the most common form of dementia, and represents a growing health crisis. There is a preponderance of literature highlighting the relationship between cardiovascular disease and increased risk for developing AD, but the mechanism responsible for this risk is not well understood. Here, we explore the relationship between cardiovascular risk factors and AD pathology, highlighting possible shared pathological processes and mediators.

Type II diabetes mellitus (T2D) more than doubles the risk for AD, and one area of possible convergence is endothelial activation and endothelial-based inflammation. Here, diabetes-like hyperglycemic conditions injure brain endothelial cells. In turn, this hyperglycemia-mediated endothelial cell injury induces activation of a pro-inflammatory microglia phenotype like that found in the AD brain. These results highlight the pro-inflammatory nature of cerebrovascular dysfunction, and a possible role for endothelial cells in the neuroinflammatory response found in AD.

The E4 allele of the lipid transport protein apolipoprotein E4 (ApoE4) is the strongest known genetic risk factor for AD. In chapter two, we explore the mechanisms behind ApoE-associated increased AD risk. ApoE4 produced increased neuroinflammation and neurotoxicity *in vitro*, while ApoE2 was found to be neuroprotective. Additionally, ApoE4 produced vascular dysfunction and related inflammation in the brain *in vivo*, and these changes appeared to be heightened in females compared to males.

Finally, chapter three highlights the role for the coagulation protein thrombin as a shared pathological mediator in cardiovascular disease and AD. An extensive literature review implicates thrombin as a driver of vascular activation and inflammation in the brain. The role for thrombin as a pathological mediator in AD was

supported by the finding that pharmacological inhibition of thrombin with the direct thrombin inhibitor dabigatran significantly reduced oxidative stress and alleviated AD-related pathology in a tau-based animal model of AD. These changes included reduced iNOS and NOX4 expression, reduced tau phosphorylation, and increased neurogenic signaling processes.

Altogether, this body of work highlights vascular dysfunction and related neuroinflammation as convergence points for cardiovascular disease and AD-related pathology, and emphasizes the role of shared pathological mediators, including ApoE and thrombin. Importantly, these findings give insight into the possible long-developing mechanisms responsible for the elevated AD risk associated with cardiovascular disease and related risk factors, and offers new potential therapeutic targets for treating multiple aspects of pathological development in AD.

APPENDIX

Scholarly work resulting from graduate studies

Publications

Johnson SL**., Iannucci, J.,** Seeram, NP., Grammas, P. (2020) Inhibiting thrombin improves motor function and decreases oxidative stress in the LRRK2 transgenic *Drosophila melanogaster* model of Parkinson's disease. *Biochem Biophys Res Commun*. doi: 10.1016/j.bbrc.2020.04.068.

Iannucci, J., Renehan, W., Grammas, P. (2020) Thrombin, a mediator of coagulation, inflammation, and neurotoxicity at the neurovascular interface: Implications for Alzheimer's disease. *Frontiers in Neuroscience*. https://doi.org/10.3389/fnins.2020.00762

Iannucci, J., Johnson, S., Majchrzak, M., Barlock, BJ., Akhlaghi, F., Seeram, NP., Sen, A., Grammas, P. Direct thrombin inhibitor, dabigatran etexilate, reduces oxidative stress *in vivo* in a transgenic mouse model of Alzheimer's disease. *Submitted, Journal of Alzheimer's Disease,* May 2020.

Vittal Rao, H., Bihaqi, SW., **Iannucci, J.,** Sen, A., Grammas, P. Thrombin signaling contributes to hyperglycemia-induced injury of human brain microvascular endothelial cells. *Submitted, Journal of Alzheimer's Disease,* May 2020.

Iannucci, J., Vittal Rao, H., Grammas, P. High-glucose and hypoxia-mediated damage to human brain microvessel endothelial cells induces an altered, pro-inflammatory phenotype in BV-2 microglia *in vitro. Submitted, Journal of Neuroinflammation,* June 2020.

Iannucci, J., Sen, A., Grammas, P. Effects of apolipoprotein E isoforms on glial and neuronal cells *in vitro. Manuscript in preparation,* July 2020.

Iannucci, J., Sen, A., Majchrzak, M., Grammas, P. Sex-specific effects of apolipoprotein E4 on markers of vascular function and inflammation in the brain *in vivo. Manuscript in preparation,* July 2020.

Poster Presentations

Iannucci, J., Grammas, P. *Protective Effects of Probucol against H2O2-induced Stress on Brain Endothelial Cells.* RI Alzheimer's Disease Research Conference, Warwick, RI, April 21, 2017.

Iannucci, J., Vittal Rao, H., Grammas, P., *Thrombin modulates the pro-inflammatory response of BV-2 microglia to high glucose and oxidative stress.* Alzheimer's Drug Discovery Foundation's 19th International Conference on Alzheimer's Drug Discovery, Jersey City, NJ, September 2018.

Iannucci, J., Vittal Rao, H., Grammas, P. *Glucose enhances endothelial cell-mediated activation of pro-inflammatory phenotype in BV-2 microglia in vitro*, Society for Neuroscience, San Diego, CA, November 2018.

Vittal Rao, H., **Iannucci, J.,** Renehan, W., Grammas, P. *Hyperglycemia and thrombin induced cellular injury, activation of MMP-2 and mitochondrial fission protein Drp1 in cultured primary human brain microvascular endothelial cells.* Society for Neuroscience, San Diego, CA, November 2018.

Iannucci, J., Majchrzak, M., Sen, A., Renehan, W., Grammas, P. *Treatment with the direct thrombin inhibitor dabigatran etexilate reduces oxidative stress in vivo in a mouse model of Alzheimer's disease.* Alzheimer's Association International Conference, Los Angeles, CA, July 2019.

Iannucci, J., Sen, A., Majchrzak, M., Renehan, W., Grammas, P. *Neuroinflammatory Effects of Apolipoprotein E4.* Society for Neuroscience, Chicago, IL, October 2019.