2018

VASCULAR COGNITIVE IMPAIRMENT AND DEMENTIA: THE IMPORTANCE OF MIXED PATHOLOGIES FROM MOUSE MODELS TO HUMANS

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Digital Object Identifier: https://doi.org/10.13023/etd.2018.408

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VASCULAR COGNITIVE IMPAIRMENT AND DEMENTIA: THE IMPORTANCE OF MIXED PATHOLOGIES FROM MOUSE MODELS TO HUMANS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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2018

ABSTRACT OF DISSERTATION

VASCULAR COGNITIVE IMPAIRMENT AND DEMENTIA: THE IMPORTANCE OF MIXED PATHOLOGIES FROM MOUSE MODELS TO HUMANS

Age-related neurologic disease is a significant and growing burden on our society. Although the largest share of research effort has typically been devoted to the common neurodegenerative illnesses (such as Alzheimer's disease, or AD), the reality is that nearly all cases of neurodegenerative disease possess elements of mixed pathology. Vascular contributions to cognitive impairment and dementia (VCID) is a complex form of dementia, combining aspects of vascular disease and other forms of dementia, such as Alzheimer's disease. This pathology is heterogeneous and can include cerebral amyloid angiopathy (CAA), hemorrhages, white matter infarcts, and changes to the neurovascular unit. Given the heterogeneous nature of VCID, we hypothesized that we could further elucidate mechanisms that drive dementia in VCID by examining pathology in mouse models and use this data to guide the study of human autopsy cases. Using a mouse model of VCID, we identified NHE1, a sodium hydrogen exchanger that was upregulated in these mice, as a possible candidate for a factor involved in cerebrovascular disease in humans. We saw a significant age effect of NHE1 in cases with Down syndrome (DS), leading us to further examine cerebrovascular pathology in individuals with DS. People with DS are at a high risk of developing cognitive impairment and dementia after the age of 50. In fact, virtually all adults with DS develop the neuropathology for an AD (beta-amyloid (Aß) senile plaques and tau neurofibrillary tangles) diagnosis by the age of 40 due to a triplication of chromosome 21. We found that these individuals develop CAA and microhemorrhages as a function of age, and that these rates are as severe as sporadic AD, despite an age difference of ~30 years. We also found that individuals with DS have different microglial morphologies than controls or individuals with AD. This data indicates that people with DS develop significant cerebrovascular and AD pathology, indicative of VCID. Overall, we found that mixed pathologies, specifically VCID, is an important contributor to the development of dementia and should be studied further to better understand how this pathology drives cognitive impairment. Further, it is clear that mouse models map imperfectly onto complex human diseases, and that significant work remains to be done towards achieving an adequate model of VCID.

KEYWORDS: VCID, Vascular contributions to cognitive impairment and dementia, Alzheimer's disease, Down syndrome, NHE1, cerebrovascular

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August 15, 2018
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VASCULAR COGNITIVE IMPAIRMENT AND DEMENTIA: THE IMPORTANCE OF MIXED PATHOLOGIES FROM MOUSE MODELS TO HUMANS

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For my Zadie, Leonard Helman, and my Memaw, Marian Graybeal, who would be proud to see their granddaughter become a Doctor…but not a "Doctor Doctor".

Acknowledgements

This work would not have been possible without the support, guidance, and mentorship of my P.I., Dr. Paul Murphy. Paul, I will forever be indebted to your generous support of both my research and career. You have taught me how to think critically about my work and have always believed in me, even when I doubted myself. I will forever cherish our engaging, educational, and occasionally challenging conversations about science, politics, and whatever Netflix show we're both currently watching. I hope they continue once I've left Lexington.

I would also like to thank my committee members: Drs. Elizabeth Head, Donna Wilcock, Becky Dutch, and Harry Levine. It has been a pleasure discussing my research and working with a team of scientists whom I admire so much. I would like to particularly acknowledge Dr. Elizabeth Head, who has served as so much more than a committee member to me. This work would not have been possible without your guidance and I thank you for treating me as part of your laboratory. Perhaps someday we will actually get around to watching the Matrix, but if not, at least we'll have enjoyed some really delicious food together. Additionally, I would like to thank Dr. Chris Norris for serving as my outside examiner.

To the members of the Murphy and Head labs, past and present, none of this would have been possible without your assistance, patience, and friendship. Particularly Tina, Dana, Tom, Kat, Theresa, and my "work wife" Katie-thank you for everything over the years.

I would like to thank my parents, Reed and Ellen Helman, and my sister, Rachel Helman, for their endless love and support. Thank you for keeping me company on many a walk home, always encouraging me through the hard times, and for giving me the tools and support to make graduate school a possibility. I love you and am eternally grateful to you all.

This dissertation is dedicated to my Memaw and my Zadie, two of my grandparents who passed away last year. They were my biggest cheerleaders and I am so thankful for the time I had with them, particularly over the last few years. To my extended family-my aunts and uncles, cousins, second cousins, and more-the past few years have brought a lot of love, loss, and challenges and I am so lucky to have such a wonderful family to help each other through it.

In theory, graduate school fosters intellectual and professional growth. However, it can feel competitive, socially and professionally exclusionary, and personally and politically stunting (Linz and Smyth 2017). To my feminist coven, both here at the University of Kentucky and around the country, your friendship, laughter, rage, brilliance, and love have gotten me through the last 5 years. I would not be the woman I am today without you all. Stay willful. Stay monstrous.

TABLE OF CONTENTS

Acknowledgements iii
List of Tables ix
List of Figures x
CHAPTER 1: Introduction 1
1.1 What is AD? 2
1.2 What is VCID? 4
1.3 - The Neurovascular Unit and Cell Culture Models 6
1.4 - Animal Models of Altered Blood Flow 8
1.5 - Animal Models of Small Vessel Disease 11
1.6 - Animal Models of CAA 13
1.7 - Animal Models of Mixed Dementia: Interacting Disease States 16
1.8 - Conclusions 22
Chapter 2: Sodium Hydrogen Exchanger 1 (NHE1) Increases in Response to Injury and in a Mouse Model of Alzheimer's Disease with Vascular Complications 26
2.1 - Introduction 26
2.2 – Materials And Methods 27
2.2.1 - Mice 27
2.2.2 - Microarray 28
2.2.3 - Quantitative RT-PCR 29
2.2.4 - Western Blot Analysis 29
2.2.5 - Mouse NHE-1 ELISA 30

2.2.6 - Immunohistochemistry 31

2.2.7 - Spot Blot Analysis 31

2.2.8 - Cell Culture 31

2.2.9 - ELISA on Human Cases 32

2.3 - Results 33

2.3.1 - Microarray and Quantitative RT-PCR 33

2.3.2 - NHE1 Expression in Mice 34

2.3.3 - NHE1 and Amyloid Beta 35

2.3.4 - NHE1 and Tau 36

2.3.5 - NHE1 Expression in Human Cases 37

2.4 - Discussion 38

Chapter 3: Microbleeds and Cerebral Amyloid Angiopathy in the Brains of People with Down Syndrome with Alzheimer's Disease 52

3.1 - Introduction 52

3.1.1 - Genes Associated with AD on Chromosome 21 52

3.1.2 - AD Pathology in DS 59

3.1.3 - Future Directions 70

3.2 – Materials and Methods 71

3.2.1 - Tissue Samples 71

3.2.2 - Immunohistochemical Methods 72

3.2.3 - Prussian blue staining 73

3.2.4 - Image analysis for CAA staining 73

3.2.5 - Image analysis for Prussian blue staining 73

3.2.6 - Statistical Analysis 73

3.3 - Results 74

3.3.1 - CAA Pathology 74

3.3.2 - Microhemorrhages 75

3.3.3 - Correlation between CAA and MB 76

3.4 - Discussion 76

Chapter 4: Microglial Phenotypes in the Occipital and Posterior Cingulate Cortex in the Brains of People with Down syndrome with Alzheimer's Disease 92

4.1 - Introduction 92

4.2 - Methods 93

4.2.1 - Human Tissue Samples 93

4.2.2 - Immunohistochemistry 93

4.2.3 - Image Analysis 94

4.2.4 - Statistics 95

4.3 - Results 95

4.3.1 - DS vs Controls 95

4.3.2 - AD vs Controls 98

4.3 - Discussion 98

Chapter 5: Conclusions and Future Directions 115

5.1 – NHE1 Discussion 115

5.1.1 - Summary 115

5.1.2 - Future Directions 116

5.1.3 - Conclusions 117

5.2. – Cerebrovascular Pathology in Individuals with Down Syndrome 118

5.2.1 – Summary 118

5.2.2 – Future Directions 119

5.2.3 – Conclusions 124

5.3 – Microglia Morphology in DS Discussion 124

5.3.1 – Summary 124

5.3.2 – Future Directions 125

5.3.3 – Conclusions 126

5.4 - Conclusions 126

Appendix 1: List of Abbreviations 129

References 132

Vita 170

List of Tables

Table 2.1: A Microarray Identified Several Possible Targets..... 42

Table 2.2: NHE1 was measured in frozen tissue from humans with different neurodegenerative diseases. 44

Table 3.1: Ongoing Clinical Trials for Aging adults with Down syndrome (queried from ClinTrials.Gov). 80

Table 3.2: Case characteristics for FC tissue obtained from brain banks (N=59). 81

Table 3.3: Case characteristics for OCC tissue obtained from brain banks (N=63)..... 82

Table 4.1: Case characteristics for PCC tissue obtained from brain banks (n=60). 104

Table 4.2: Case characteristics for OCC tissue obtained from brain banks (n=63)..... 106

List of Figures

Figure 1.1. AD senile plaques and neurofibrillary tangles in DS. 24

Figure 1.2: Elements of VCID Pathology 25

Figure 2.1: Gene Expression by RT-PCR in db/AD Mice (Taqman). 45

Figure 2.2: NHE1 levels are increased in the db/AD animals. 46

Figure 2.3: Aβ levels do not change when NHE1 is inhibited. 47

Figure 2.4: NHE1 increases along with pTau in the db/AD mice and after head injury in db/AD mice. 48

Figure 2.5: NHE1 is not associated with markers of cerebrovascular pathology in human cases with dementia, but decreases with age in individuals with DS. 50

Figure 3.1: AD pathology is present in the brains of elderly individuals with DS. 83

Figure 3.2: Individuals with DSAD have CAA develop microhemorrhages. 84

Figure 3.3: Hypothetical sequence of neuropathological events as a function of age in DS. 85

Figure 3.4: Scoring of CAA. 86

Figure 3.5: DSAD and AD individuals have more severe CAA than age-matched controls. 87

Figure 3.6: Microbleeds increase with age in the FC and OCC. 89

Figure 3.7: Microbleeds increase with age in individuals with Down syndrome. 90

Figure 4.1: Different microglia morphologies were evaluated in the PCC and OCC. ... 108

Figure 4.2: Fewer ramified microglia are present in the GM and more hypertrophic microglia are in the white matter of DSAD cases. 109

Figure 4.3: DSAD cases have significantly more dystrophic and amoeboid microglia than age-matched controls. 111

Figure 4.4: DSAD cases have different microglia phenotypes than age-matched controls. 113

Figure 5.1: T2*-weighted images detect hemorrhages in individuals with DS. 128

Chapter 1: Introduction

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Helman, A. M. and M. P. Murphy (2016). "Vascular cognitive impairment: Modeling a critical neurologic disease in vitro and in vivo." Biochim Biophys Acta 1862(5): 975-982.

Age-related dementias are rapidly becoming one of the largest public health problems of our time. As baby boomers age - with more access to healthcare than ever before - Americans face a demographically larger population of elderly individuals. Alzheimer's disease (AD) is the seventh leading cause of death in the United States, with numbers rising each year. In the absence of effective therapeutics, the population affected by AD is projected to triple, from 5.1 million today to 13.8 million people by 2050, with an estimated healthcare cost of more than $1 trillion (Alzheimer's Association 2015). Despite considerable efforts, the mechanism behind neurodegeneration remains unknown, driving a need to better understand not only AD, but all forms of dementia.

The first detailed description of dementia in the literature was at the end of the 19th century, as biological and medical knowledge was expanding (Beach 1987). It was originally thought that arteriosclerosis and chronic cerebral ischemia caused dementia (Hershey, Modic et al. 1986). This view changed with the discovery that infarcts, not chronic ischemia, were causing what eventually became known as multi-infarct dementia (Bowler 2007). This evolved into the term vascular dementia, as multiple other pathological features of the disease became known, such as white matter hyperintensities,

single infarcts, hemorrhages, and others. However, vascular dementia became overshadowed by the discovery of AD in 1898.

1.1 What is AD?

AD, the most common form of dementia in the elderly, affects 5.7 million people in the USA (Alzheimer's Association 2018) and is currently the 6th leading cause of death in the USA. It is anticipated that by 2050, the number affected by AD will increase to 14 million. The risk of AD rises with age such that 2% of people 71-79 have AD, 18% of those 80-89 years of age may be affected and 28% of people over 90 years have AD (Plassman, Langa et al. 2007). Dementia is a term used to describe changes in cognition that affect daily function. There are many causes of dementia (i.e. – the pathology that causes the dementia can vary) including vascular dementia, frontotemporal dementia, Lewy body disease, and AD (Plassman, Langa et al. 2007). Currently, there is discussion of modifying the criteria for a diagnosis of AD to primarily depend on the pathological accumulation of senile plaques and neurofibrillary tangles (NFT) in the brain measured by cerebrospinal fluid (CSF) protein levels and/or neuroimaging using positron emission tomography (PET) but not clinical criteria (Jack, Bennett et al. 2018). AD is confirmed at autopsy; neuropathological evidence of AD requires the presence of NFT, senile plaques, and neuron loss (Jack, Bennett et al. 2018).

NFTs are made up of abnormally hyperphosphorylated tau protein. Tau protein is a normal protein used by neurons to support their structure, form a cytoskeleton and assist in the transport of proteins and organelles within the cell body and axons (Gao, Wang et al. 2018). When tau becomes abnormally hyperphosphorylated, it forms paired helical

filaments that disrupt normal neuronal function (Gendron and Petrucelli 2009). These filaments fill the neuron and form NFT and neuropil threads (Figure 1.1A).

Senile plaques are deposits of a toxic protein, beta-amyloid (Aβ), which aggregate outside and between neurons (Selkoe 2001) (Figure 1.1B). The Aβ protein can be of various lengths but typically is 40-42 amino acids long, and is cut from a longer beta-amyloid precursor protein (APP – will be discussed in more detail shortly). The gene for APP is on chromosome 21, which leads to overexpression in people with full trisomy 21 and is thought to be the underlying mechanism for early onset AD in DS (Prasher, Farrer et al. 1998, Doran, Keator et al. 2017). Senile plaques can vary in size and appearance. Typically plaques first form as diffuse fibrils that can be large and amorphous (Figure 1.1C). Senile plaques can also take on a more compact form with some having a dense core and associated NFT pathology; these are called neuritic plaques or cored plaques (Figure 1.1D). These smaller, denser plaques are typically observed in more severe stages of AD in DS. When sufficient numbers of NFTs and senile plaques are observed affecting the hippocampus, underlying entorhinal cortex and neocortex, a diagnosis of AD can be made.

It is helpful for research purposes and for diagnosis to capture how extensive AD pathology is in the brain to allow comparison with the severity of dementia a person exhibits prior to death. This helps researchers test hypotheses about what types of brain pathology might underlie specific cognitive deficits in DS with age. The most commonly used scoring system, Braak staging, describes the extent and location of NFT and senile plaques (Braak and Braak 1991), with higher "stages" being associated with more severe pathology. NFT pathology is scored on a scale of I-VI and senile plaques are scored as A,

B or C. Thal and colleagues have expanded the staging system for describing the extent and severity of senile plaques to 5 categories (Phases 1-5)(Thal, Rub et al. 2002). Thus, a typical report from a neuropathologist will include information regarding the final diagnosis as well as the extent of senile plaques and NFTs.

1.2 What is VCID?

Currently, AD is the most common form of dementia, followed closely by vascular dementia. Vascular dementia, when it is thought of as a somewhat distinct entity, accounts for about 20% of all age-related dementias. A diagnosis of vascular dementia is commonly associated with certain risk factors such as obesity, hypertension, cardiac disease, and type 2 diabetes mellitus (T2DM). Over 40 million Americans aged 70 years or older have at least one of these risk factors, yet we know relatively little about how these factors contribute to cognitive decline (Roger, Go et al. 2011). Recent debate has centered on the role of cerebrovascular disease in dementia, both as a primary cause of cognitive impairment, and also as a contributing factor to dementia in combination with other pathologies. This has led to the adoption of a range of new terminologies in the field of dementia research, one of which is the umbrella term of vascular contributions to cognitive impairment and dementia (VCID).

VCID is ambiguous in that it can describe any clinical cognitive disorder of cerebrovascular origin. VCID therefore does not denote a specific disease, but rather a heterogeneous disease state under the larger umbrella of cerebrovascular disease (Hachinski, Iadecola et al. 2006, Jiwa, Garrard et al. 2010). Past definitions of VCID (which has also been called VCI) used multi-infarct dementia or vascular dementia constructs to define a tentative diagnostic threshold (Gorelick, Scuteri et al. 2011). Recent

definitions have expanded to cover a continuum of the interactions, from “pure” AD pathology all the way to “pure” vascular dementia (O'Brien, Wiseman et al. 2002) (Figure 1.2).

The wide umbrella of definitions combined with the multiple dimensions of vascular injury leaves a large amount of ambiguity for what does and does not constitute VCID. For example, there is a controversy in the field over which types of vascular lesions contribute to cognitive impairment, including large cortical infarcts, lacunar infarcts, subcortical white matter disease, subcortical infarcts, or any combination of these (Gorelick, Scuteri et al. 2011). This is further complicated by the presence of AD pathology, which is thought to lead to dementia more quickly in the presence of certain types of strokes (Snowdon, Greiner et al. 1997). In fact, it is very rare for an aged subject to not have any AD or cerebrovascular pathology. The two main pathological hallmarks of AD, amyloid plaques and neurofibrillary tangles, are present with overlapping cerebrovascular lesions in up to 50% of dementia cases (Jellinger 2013). However, the balance between the pathology of these diseases may be the determining factor for displaying clinical symptoms (Petrovitch, Ross et al. 2005).

The inherent heterogeneity of VCID makes it difficult to develop representative models. VCID is not a complication of AD nor simply a form of stroke, but may encompass these etiologies as well as others. At our current level of knowledge, VCID is the best term we have to represent how vascular issues contribute to dementia. However, as the field expands, the term VCID may become too vague and could evolve into more specific definitions of particular disease states. This introduction will give an overview of the strengths and weaknesses of current models of VCID, including an overdue discussion on

models of mixed dementias. While there is no current model that encompasses all aspects of VCID, there are ways to examine aspects of the disease separately, or in combination with different facets of neuropathology. These approaches encompass a range of strategies, from cell culture systems to a number of animal models with varying degrees of complexity.

1.3 - The Neurovascular Unit and Cell Culture Models

Within the past few years, we have gained a larger understanding of the synergistic roles of the cell types encompassing the blood brain barrier (BBB) (Iadecola 2010, Quaegebeur, Lange et al. 2011). This interaction, known as the neurovascular unit, provides an entirely different framework for examining how cerebrovascular disease contributes to cognitive impairment.

The neurovascular unit is composed of endothelial cells, myocytes, neurons and their processes, astrocytes, perivascular cells, and other supporting cells (microglia and oligodendroglia) (del Zoppo 2010, Popa-Wagner, Buga et al. 2013). These cells work together to coordinate cerebral blood flow and exchange across the BBB. A functioning neurovascular unit is important for mediating blood flow in order to meet the metabolic demands of the brain (Iadecola 2010). Astrocytes, which line the outer walls of cerebral microvessels, are responsible for regulating blood flow to an area of high activity in the brain (Lo and Rosenberg 2009). If there is insufficient blood flow to an area of metabolic demand, a cascade of rapid responses to the hypoxia stimulates angiogenesis, resulting in increased blood flow to the area of need (Moeller, Cao et al. 2004). Most models of the neurovascular unit use *in vitro* tissue culture with rodent cells to better understand all of the interacting components. However, there is a general lack of microvascular models

using human cells.

Many *in vitro* BBB models rely on using endothelial cells, as they are the principal cellular component of the BBB. Primary endothelial cells isolated from rat, pig, or cow (Franke, Galla et al. 2000, Kido, Tamai et al. 2002), or human endothelial cell lines that are not of cerebral origin, such as human umbilical vein endothelial cells (HUVECs) are often used for BBB studies (Gomez-Gaviro, Scott et al. 2012). However, there is a large amount of heterogeneity within endothelial cells from different vascular origins which should be taken into consideration when using these cells as a model of the BBB (O'Donnell, Mille-Baker et al. 2000). To examine interacting cell types, endothelial cells are grown alongside astrocytes, pericytes, or a combination of the two using a co-culture system (Dehouck, Meresse et al. 1990, Nakagawa, Deli et al. 2007). Co-culture systems have high transendothelial electrical resistance and low permeability coefficients, indicating the presence of a tight barrier similar to the BBB (Fricker, Nobmann et al. 2002, Kido, Tamai et al. 2002, Freese, Reinhardt et al. 2014). However, these systems do not examine all aspects of the neurovascular unit simultaneously and are therefore better for understanding the role of a specific factor rather than the interacting cell types that work together to coordinate blood flow.

One of the newer BBB models uses synthetic microvessels for an *in vitro* model of the microvasculature. This model involves growing endothelial cells in collagen channels to form a microstructure. The endothelial cells form continuous junctions between cells and eventually form complex adherence junctions accompanied by slight re-structuring of channels (Lopez and Zheng 2013). Additionally, the collagen matrix can be remodeled to promote cell growth and angiogenesis. Vessel wall conditions and blood flow can be

mimicked by seeding the endothelial cells in the matrix with pericytes and platelets (Zheng, Chen et al. 2014). Synthetic microvessels are currently limited to growth within a single plane, but three dimensional models using 3D printing of carbohydrate-glass lattices may be able to solve this problem. This 3D structure encourages endothelial growth in all directions and is a promising model of the microvasculature (Miller, Stevens et al. 2012).

Information on the microvasculature has often lagged behind the wealth of information on large vessels despite the growing knowledge on its contribution to disease. *In vitro* models of the microvasculature are new and exciting tools to study its role in disease states such as VCID. Co-culture systems have taught us a lot about the BBB, from permeability studies of drugs (Booth and Kim 2014, Roda, Nion et al. 2014) to how Aβ crosses the BBB (Candela, Saint-Pol et al. 2015). Little has been published thus far on the applications of synthetic microvessels, as it is a relatively new technique but the potential applications of the model are broad and range from better understanding the BBB, to having a more clear understanding the roles of individual cell types in the neurovascular unit, and eventually understanding the specific role of the microvasculature in certain diseases. However, these models are limited by the fact that they do not involve a physiological system to study the complex interactions of VCID. Therefore, while tissue culture models are useful tools, animal models are needed for studying the interacting players of VCID.

1.4 - Animal Models of Altered Blood Flow

Cerebrovascular changes alter the macro and microvasculature, leading to both structural and functional brain damage. The development of new neuroimaging techniques has revolutionized our ability to examine these cerebrovascular changes (Petrovitch, Ross

et al. 2005). One of the most important neuropathological markers of cognitive decline due to cerebrovascular dysfunction is cerebral infarcts (Gorelick, Scuteri et al. 2011). There is a strong association between increased number of macroscopic infarcts and increased likelihood of dementia, but the relationship is not a simple one, and there is currently no defined volume or number necessary for a diagnosis of VCID (White, Small et al. 2005). One of the reasons for this is that infarct severity has varied effects on cognition, depending on the individual. Infarct location may determine the impact on dementia, with infarcts in regions such as the thalamus, angular gyrus, and basal ganglia more likely to lead to dementia (Vinters, Ellis et al. 2000, Jellinger 2008). In other words, a single strategically placed infarct can be just as cognitively devastating as many smaller ones scattered throughout the brain.

Animal models have helped us understand how infarcts contribute to VCID. Chronic cerebral hypoperfusion (CCH) surgery is a good way to study infarcts in rodent models. CCH is one of the major causes of vascular - related dementia and is a result of various diseases, such as obstructive sleep apnea, congestive heart failure, and cardiac arrhythmias, that cause reduced blood flow to the brain (Meyer, Rauch et al. 2000, Roman 2002). CCH typically develops as a result of vascular lesions caused by artery stenosis or occlusion, cerebral hemodynamic changes such as prolonged hypotension and reduced cardiac output, or by a change in blood viscosity, commonly associated with hyperlipidemia or elevated homocysteine levels (Zhao and Gong 2015). Over time, these changes can decrease blood flow to the brain, causing increased neuroinflammation and oxidative stress, neuronal energy failure, and white matter lesions, all of which lead to cognitive impairment.

One of the more common CCH surgeries performed is the occlusion of the bilateral common carotid arteries (CCA). In rats, both the left and right CCAs are occluded, causing hippocampal and neuronal damage, striatal infarcts, white matter lesions, increased neuroinflammation, increased oxidative stress, and BBB disruption (Nanri and Watanabe 1999, Soria, Tudela et al. 2013). Additionally, these rats perform poorly on several tests of cognition, suggesting cognitive impairment. However, this model is strictly feasible in animals with a complete circle of Willis (this excludes mice), which allows for continued, decreased blood flow to the brain via the basilar artery (Farkas, Luiten et al. 2007). Additionally, there is a high amount of variability in the number of infarcts the rats develop and amount of hippocampal damage among animals from different vendors (Marosi, Rakos et al. 2006, Soria, Tudela et al. 2013). Similar to the bilateral CCA occlusion model, the four vessel occlusion (4VO) model involves blockages of both vertebral arteries in addition to the CCAs. These animals have a low incidence of seizures and develop predictable ischemic neuronal damage (Pulsinelli and Brierley 1979). However, many of the same drawbacks from the bilateral CCA model are present in the 4VO, with high variability among species and differences in CCA occlusion times reported in the literature (Pulsinelli and Brierley 1979, Neto, Paganelli et al. 2005).

Bilateral CCA stenosis (BCAS) may be a more disease-relevant variant of the CCH models, as there is simply a reduction of blood flow rather than a total occlusion. BCAS is done by placing micro-coils consecutively around the CCAs, causing around an 80% decrease in cerebral blood flow (Shibata, Ohtani et al. 2004). BCAS works well in mice, causing a decrease in brain metabolism, increased neuroinflammation, and cognitive impairments such as decreased working and reference memory (Nishio, Ihara et al. 2010).

However, due to the small size of the coils, BCAS is a technically challenging procedure and very few labs have been successful in performing the surgery. Therefore, despite its efficacy as a rodent model to study CCH, there is currently little published literature on the technique.

1.5 - Animal Models of Small Vessel Disease

Small vessel disease (SVD) causes nearly a fourth of all ischemic strokes and is a leading cause of vascular dementia. People with SVD often have cerebral amyloid angiopathy (CAA) and display deficits in information processing and motor function (Kalaria 2012). These cognitive impairments are often due to cerebral white matter lesions and subcortical lacunar infarcts (Wardlaw, Allerhand et al. 2014). Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a form of SVD and the most common hereditary cause of vascular dementia (Chabriat, Joutel et al. 2009). CADASIL causes progressive white matter degeneration and ischemic strokes and can be exacerbated by vascular risk factors, such as high cholesterol, smoking, and hypertension (Singhal, Bevan et al. 2004). Nearly all CADASIL cases are caused by mutations in *Notch homolog 3* (*NOTCH3*). NOTCH3 is required for the maturation and function of small vessels and is primarily found in vascular smooth muscle cells (Domenga, Fardoux et al. 2004). The mutations in *NOTCH3* cause accumulation of granular osmiophilic material (GOM) and a NOTCH3 ectodomain on vascular smooth muscle cell membranes. These vascular smooth muscle cells eventually die, causing enlarged perivascular spaces. This in turn causes stenosis of penetrating arteries, leading to strokes and white matter degeneration (Okeda, Arima et al. 2002, Miao, Paloneva et al. 2004).

We have learned a lot about the role of NOTCH3 in cerebrovascular disease from *NOTCH3* knockout mice. These mice are viable and develop impaired cerebrovascular reactivity, reduced myogenic tone, and structural arterial defects (Domenga, Fardoux et al. 2004, Belin de Chantemele, Retailleau et al. 2008). Interestingly, *NOTCH3* knockout mice have more than two-fold larger infarcts when compared to controls after middle cerebral artery occlusion. Restoring *NOTCH3* expression using a ROSA *NOTCH3* mouse crossed to an appropriate Cre line (*SM22-Cre*) restored the stroke phenotype by reducing infarct volume (Arboleda-Velasquez, Zhou et al. 2008). However, *NOTCH3* knockouts do not develop CADASIL pathology such as white matter degeneration and lacunar strokes (Domenga, Fardoux et al. 2004). Additionally, these mice do not develop GOM or NOTCH3 accumulation. Interestingly, a knock-in mouse model using the *C455R* mutation from a large Colombian CADASIL family causes a CADASIL phenotype with more severe stroke pathology than the *NOTCH3* knockout mice (Arboleda-Velasquez, Manent et al. 2011). This tells us that loss-of-function *NOTCH3* mutations do not solely cause CADASIL, but may play a larger role in stroke pathology.

CADASIL transgenics, such as the *R90C* mouse, express a human *NOTCH3* mutation which causes early CADASIL onset. These mice show age-associated vascular smooth muscle cell loss, as seen in humans, and accumulation of the NOTCH3 ectodomain occurs around 10 months (Ruchoux, Domenga et al. 2003). Additionally, R90C mice develop diffuse white matter degeneration and subcortical infarcts in the basal ganglia and white matter (Utku, Celik et al. 2002). However, these mice display vascular smooth muscle cell changes prior to any NOTCH3 accumulation, suggesting that NOTCH3 accumulation triggers but does not cause vascular dysfunction (Ruchoux, Domenga et al.

2003, Lacombe, Oligo et al. 2005). Although these models are helpful in understanding how vascular dysfunction occurs in people with CADASIL, and may have some role in elucidating broader mechanisms involved in other SVDs, they are somewhat limited in scope. This is a common problem with mouse models based on rare familial mutations, a point which we will return to below.

1.6 - Animal Models of CAA

CAA is an important contributor to age-related cognitive decline. The main hallmark of CAA is the buildup of Aβ deposits in the penetrating arterioles and capillaries of the leptomeninges and cortex. APP gets cleaved into Aβ peptides of differing length, with senile plaques primarily composed of Aβ42 and cerebrovascular Aβ mainly consisting of Aβ40 (Prelli, Castano et al. 1988, Suzuki, Iwatsubo et al. 1994). When neurons release Aβ, it is thought that Aβ42 sticks together and aggregates, while Aβ40 is flushed out of the brain via interstitial fluid drainage pathways (Weller, Massey et al. 1998). While further discussion of Aβ is outside the scope of this review, there are many outstanding reviews of Aβ production and clearance (Sun, Bromley-Brits et al. 2012, Zhang and Song 2013, Muresan and Ladescu Muresan 2015, Tarasoff-Conway, Carare et al. 2015). Over time, Aβ intravessel accumulation can lead to necrosis, perivascular leakage of red blood cells, and eventually intracerebral hemorrhages and microbleeds (Mandybur 1986, Vonsattel, Myers et al. 1991, Cordonnier and van der Flier 2011). Additionally, CAA contributes to cognitive decline and is the most common vascular pathology associated with AD, present in up to 90% of AD cases (Vinters 1987, Jellinger 2002). CAA is most commonly seen as an underlying cause of intracerebral hemorrhages, but studies show that it also plays a major role in age-related cognitive decline, even when subsequent AD pathology is not

present (2001). However, this mechanism is not well understood. CAA has been studied in several model systems over the years, and there are many excellent animal models of this disease pathology.

Canines provide a unique resource for studying aging and dementia. Dogs show age-associated cognitive decline with many similarities to humans. Canines accumulate Aβ in both plaques and the cerebral vasculature and develop neurodegeneration from oxidative stress, much like humans. Additionally, they are a good model for studying possible therapeutics for dementia, as they share similar pharmacokinetic and pharmacodynamic profiles with humans. One of the largest advantages to using a canine model is that, unlike most animal models, they often share a common environment and diet with humans (Head 2013). In 1956, Anton von Braunmuhl first observed that canines develop CAA (Von Braunmuhl 1956) and several studies have since confirmed this finding (Cummings, Su et al. 1993, Borras, Ferrer et al. 1999). Cognitive dysfunction and incidence of intracerebral hemorrhage correlates strongly with severity of CAA in both canines and humans (Uchida, Nakayama et al. 1991, Colle, Hauw et al. 2000). Furthermore, amyloid deposits in canines are primarily found in the intracellular spaces of the tunica media, similar to human CAA (Yamaguchi, Yamazaki et al. 1992, Walker 1997). Though canines are good models of CAA, there is considerable individual variability in the extent of pathology. Canines develop CAA by about the age of 13, but the severity of CAA varies largely, much like humans. Therefore, it is important to have large groups of subjects when using canine models (Wegiel, Wisniewski et al. 1995, Walker 1997).

Cerebrovascular β-amyloidosis is also commonly found in non-human primates (NHPs), particularly in rhesus and squirrel monkeys. Rhesus monkeys commonly develop

amyloid deposits in the neural parenchyma at around 25 years old (Uno and Walker 1993) with some developing moderate CAA. This variability is similar to human CAA, though it is probably true that rhesus monkeys develop sporadic CAA more frequently than humans (Walker 1997). Squirrel monkeys, on the other hand, develop CAA by age 15. These monkeys more reliably develop CAA than rhesus monkeys, but unlike humans, the CAA is usually found in capillaries. NHPs are physiologically relevant models of human disease, as we are closely related and they mimic complex behaviors seen in humans. However, along with this close relation comes increased ethical consideration for the care and use of NHPs (Coleman 2011), requiring additional levels of scrutiny and justification for approval to ensure that their use is necessary, beneficial, and humane. Additionally, NHPs are costly to breed and house, particularly in aging studies, where animals require housing for nearly their entire lifetime (Capitanio and Emborg 2008).

There are several transgenic mouse lines that are valid models of CAA (for a comprehensive review, see (Klohs, Rudin et al. 2014)). Transgenic mice with artificial promoters to drive APP overexpression commonly show CAA pathology, with vascular Aβ deposition developing at different ages depending on the mutation. Transgenic mouse models of CAA have taught us a lot about the role of Aβ in the progression of CAA. For example, *APPDutch* mice, which bears an *APP E693Q* mutation causing CAA, strokes, and dementia, and *APP23xAPPDutch* mice, which have an *APP KM670/6771NL* mutation causing a 7-fold overexpression of mutant human APP, both have a high Aβ40/42 ratio and develop severe CAA, indicating that Aβ40 is the form most found deposited in the vasculature (Herzig, Winkler et al. 2004).

One of the most common CAA mouse models is the *Tg-SwDI* mouse, which has *the APP KM670/671NL Swedish* mutation, the *APP E693Q Dutch* mutation, and the *APP D694N Iowa* mutation, and develops extensive amyloid deposition in the cerebrovasculature. These mice start displaying CAA at around 6 months and this pathology increases with age, eventually causing oxidative stress, neuroinflammation, activated astrocytes and microglia, and impairments in learning and memory (Fan, Xu et al. 2007, Xu, Grande et al. 2007). However, the *Tg-SwDI* mice largely display pathology in the microvessels, which is rarely the case in humans with sporadic CAA.

Animal models of CAA are excellent models to study VCID. These studies give us a better understanding of how Aβ in the vasculature contributes to cognitive impairment and cerebrovascular disease. Further, the larger animal models, such as canines and NHPs, allow us to study the disease in mammals more closely related to humans than rodents. These animals not only share more complex physiological systems, but also have similar lifestyles to humans. This interaction sheds some light into how environmental factors contribute to CAA and VCID. However, these large animal models cannot easily undergo genetic modification, and require increased ethical and financial concerns.

1.7 - Animal Models of Mixed Dementia: Interacting Disease States

Mixed dementia describes the comorbidity of two or more dementias, the most common being the overlap of AD and vascular dementia (Kammoun, Gold et al. 2000). There are several risk factors that contribute to this mixed disease state, such as obesity, hypertension, and T2DM (Gorelick, Scuteri et al. 2011). Over 40 million Americans aged 70 years or older have at least one of these risk factors, yet we know relatively little about how these factors contribute to cognitive decline (Roger, Go et al. 2011).

Cognitive impairment strongly correlates with obesity and T2DM in both rodents and humans. This risk is exacerbated with the presence of AD, forming a unique type of dementia with vascular pathology, small strokes and AD related neuropathology. Interestingly, people with this disease state often have lower plaque and tangle counts. It is thought that the presence of vascular pathology in these cases (mainly subcortical and/or lacunar infarcts) lowers the threshold of AD pathology required for development of dementia (Snowdon, Greiner et al. 1997, Schneider, Boyle et al. 2007). The presence of diabetes, therefore, does not change the amount of AD pathology, but rather increases cerebrovascular pathology leading to dementia (Ahtiluoto, Polvikoski et al. 2010, Nelson, Head et al. 2011).

One of the main models for studying these interacting disease states is through treatment with streptozotocin (STZ), a pancreatic islet toxin. STZ damages pancreatic β cells, causing hypoinsulinemia and hyperglycemia (Lenzen 2008). However, STZ is mostly used as a model for type 1 diabetes and does not address the issue of obesity (Salkovic-Petrisic, Knezovic et al. 2013). Transgenic mice are a common tool for studying diabetes, but are limited in scope. When *ob/ob* mice (which are leptin deficient) are crossed with *APP23* mice (which overexpress *APP KM670/6771NL* under a Thy1 promoter), the mice show early cognitive deficits (2-3 months) independent of amyloid pathology (Takeda, Sato et al. 2010). While the oldest animals (12 months) did not show any plaque pathology, a small number (n=3) showed significant levels of Aβ in the blood vessels. It is important to note, however, that in a separate study in *CRND8* mice (which contain both the APP double *Swedish* mutation and the *Indiana* mutation), short-term leptin administration caused a reduction in Aβ deposition and improvements in cognitive function

and it is unclear how to reconcile these results with the *ob/ob* cross study (Greco, Bryan et al. 2010). The db/AD mouse, a cross between the obese and diabetic *db/db* mouse and the *APP Swedish x PSEN1 L1660* knock-in mouse model of AD, is one such model of a mixed dementia state (Niedowicz, Reeves et al. 2014). These mice are diabetic, develop amyloid deposits with increasing age, have ischemic strokes and increased neuroinflammation, and display profound cognitive impairments at a much younger age (12 months) than the *APP Swedish x PSEN1 L1660* knock-in mice alone. However, these mice show no signs of CAA or hypertension, which is unlikely in a human with mixed dementia, although this may also suggest that CAA and hypertension are not necessary for strokes to occur in an aging brain with AD pathology.

Neuroinflammation is thought to contribute largely to AD progression and cognitive decline. There is an established link between activated microglia and AD (Colton and Wilcock 2010). This is further complicated by the presence of proinflammatory cytokines, which are known to contribute to neuronal loss (McGeer and McGeer 1998). Increased inflammation is thought to accelerate cognitive decline and is often used as a hallmark of neurodegeneration. A/T transgenic mice, a cross between an APP overproducing mouse (*APP Swedish, Indiana*) and the constitutively-active *TGF-β1* mouse (*TGF* mice, line T64), is a mouse line that combines AD and cerebrovascular pathology. These mice have increased cerebral and cerebrovascular Aβ deposition, reduced neurovascular and neurometabolic coupling, astrocyte activation, and display cognitive impairment by decreased water maze performance (Papadopoulos, Rosa-Neto et al. 2013). However, these mice show delays in cognitive decline compared to the APP overexpressing mice alone, indicating that TGF-β may play some sort of neuroprotective

role. Additionally, these mice develop cerebrovascular pathology that is unique to the increased activity of TGF-β and the mechanism behind this is not fully understood (Ongali, Nicolakakis et al. 2010).

It is now widely accepted that there is a link between high fat diets and cognitive decline in the elderly population. The Rotterdam study, a population-based cohort recruited to study diseases in the elderly, showed a strong link between dementia with a vascular component and total and saturated fat levels, also confirmed in rodent models (Kalmijn, Launer et al. 1997). Mice fed high fat diets have expected metabolic issues in addition to high oxidative stress, impaired cognition, increased inflammation, and decreased BDNF levels (Morrison, Pistell et al. 2010, Pistell, Morrison et al. 2010). Rats fed diets high in saturated fats and sugar showed cognitive deficits accompanied by increased BBB permeability (Davidson, Monnot et al. 2012).

Studies show changes in the cerebrovasculature of animals fed a high fat diet (Freeman, Haley-Zitlin et al. 2014). However, there is a discrepancy in the field for the percentage of lard used in a high fat diet. The typical western diet consists of 40% lard, but studies have shown that cerebrovascular changes only occur when a 60% lard diet is used (Morrison, Pistell et al. 2010, Pistell, Morrison et al. 2010). Additionally, these models are independent of amyloid pathology and only account for a specific lifestyle risk of dementia. While there is a large amount of literature on obesity and diabetes in the context of high fat diets, there is little available on the effect of high fat diets on brain aging (Uranga, Bruce-Keller et al. 2010). The literature contains conflicting reports on whether high fat diets actually promote or accelerate brain aging and there are currently no comprehensive studies on what metabolic parameters promote brain aging.

Homocysteine (Hcy) is a methionine-derived amino acid that is linked with cardiovascular disease. Methionine synthase maintains normal Hcy levels and uses vitamin B12 and folate as cofactors to remethylate Hcy back to methionine (Ansari, Mahta et al. 2014). Elevated levels of Hcy, known as hyperhomocysteinemia, are strongly associated with cardiovascular and various neurologic diseases (Farkas, Keskitalo et al. 2013). Studies suggest that these elevated levels are toxic to endothelial cells and cause other disruptions, such as platelet adhesion, suppression of heparin sulfate expression, and several others (Selhub 1999). Dietary intake of methionine, folate, and vitamin B12 determine levels of Hcy, so hyperhomocysteinemia is modifiable by diet (Huang, Chang et al. 2003). Deficiencies in folate and vitamin B12 are known to be a cause of stroke and data shows that dietary folate fortification reduced levels of stroke in the United States and Canada (Yang, Botto et al. 2006).

Mice that are put on a hyperhomocysteinemic diet (folate and B12 deficient with excess methionine) show cognitive decline, high microhemorrhage counts, increased neuroinflammation, and elevated matrix metalloproteinase levels, indicative of BBB breakdown (Sudduth, Powell et al. 2013). However, B-vitamin deficiencies can cause cognitive impairment, so it is unclear whether the B-vitamin deficiency or the hyperhomocysteinemia itself causes the cognitive decline shown in these animals (Moore, Mander et al. 2012). Additionally, although hyperhomocysteinemia is a known risk factor of stroke (Kelly and Furie 2002) and correlates strongly with AD (Seshadri, Beiser et al. 2002, Ravaglia, Forti et al. 2005), this is an independent risk factor for disease and by no means represents the majority of VCID cases. Further, hyperhomocysteinemia is toxic to

neurons (Parsons, Waring et al. 1998, Sachdev 2005), which may argue that rodents put on the diet show cognitive decline from toxicity effects and not from VCID.

Although risk of developing both hypertension and dementia increases with age, hypertension is a major risk factor for dementia independent of age. The Honolulu Asia Aging Study examined 3703 men starting midlife and followed up with them for the next 26 years (Launer, Ross et al. 2000). This study showed a strong correlation between middle aged men with untreated hypertension and both AD and vascular dementia. Several other longitudinal studies show correlations between high blood pressure and dementia (Kuller, Lopez et al. 2003, Qiu, von Strauss et al. 2003, Li, Rhew et al. 2007). It is thought that chronic high blood pressure causes vessel wall thickening and reduction in microvessel diameter (Kennelly, Lawlor et al. 2009). Additionally, plaques in the larger cerebral arteries can rupture, causing complete blockage of arteries and infarcts in the surrounding tissue (Swales 1994).

The most popular model for studying hypertension is the stroke prone spontaneously hypertensive rat (SHRSP). These rats are normal at birth and develop high blood pressure as they age. This eventually leads to ischemic lesions in the cortex and basal ganglia (Yamori, Horie et al. 1976). Additionally, these hypertensive rats perform poorly on learning and memory tests and worsen post-stroke. Vessel occlusion surgery in SHRSP has been shown to cause an even more exaggerated vascular phenotype, with white matter lesions, hardening of vessel walls, BBB breakdown, and increased neuroinflammation (Fredriksson, Auer et al. 1985, Henning, Warach et al. 2010) It is very important to carefully observe SHRSP, as they often develop paralysis due to the ischemic strokes which is easily misinterpreted as muscle weakness or cognitive decline (Venkat, Chopp et

al.). Overall, these rats are important for studying the influence of hypertension on vascular pathology, a known risk factor of VCID in humans.

1.8 - Conclusions

As people in the developing world are living longer, our aging population is increasing. Given that cognitive impairment is a common condition in the elderly, the incident rates of dementia will increase drastically within the next 50 years. Understanding of the common causes of dementia, such as AD and VCID, has come a long way in the last 10 years. However, there is still a great deal that we do not know about different types of cognitive impairments. This review has focused on VCID and the current models that we have for understanding this heterogeneous disease state.

Currently, there are no definitive guidelines for diagnosing VCID. While there are several recommendations for physicians, there is a general lack of consistency in stroke counts and type, location of vascular injury, along with several other thresholds to determine if VCID is present. The molecular and cellular basis for how lifestyle factors influence vascular injury, particularly in white matter, remains unknown. Understanding how risk factors influence disease would be helpful for developing potential therapeutics to treat different aspects of VCID. We have a general understanding of the roles that hypoperfusion, the neurovascular unit, and inflammation play in cerebrovascular injury in animal models. Yet this understanding has not yet led to any viable therapeutic targets. As we develop better models of VCID, we will have a more complete understanding of the disease state and the best way to treat it.

While we have several useful animal models to model certain aspects of VCID, none of them are able to fully model VCID, which encompasses several spectrums of pathological markers. This gap in the literature stalls the development of therapeutics and hinders our understanding of VCID. However, the broad definition of VCID will likely mean that there will never be an all-encompassing model of the disease state. Current and future VCID models will likely tackle different aspects independently, resulting in slow pathways to VCID treatments..

The goal of this dissertation is to examine cerebrovascular contributions to aging in an animal model of VCID. We hypothesize that by investigating gene expression patterns in animal models of VCID, we be able to better understand the factors involved in VCID and eventually create more specific definitions and models for each individual disease state. This would create an opportunity for the development of therapeutics and treatments for VCID in an aging population.

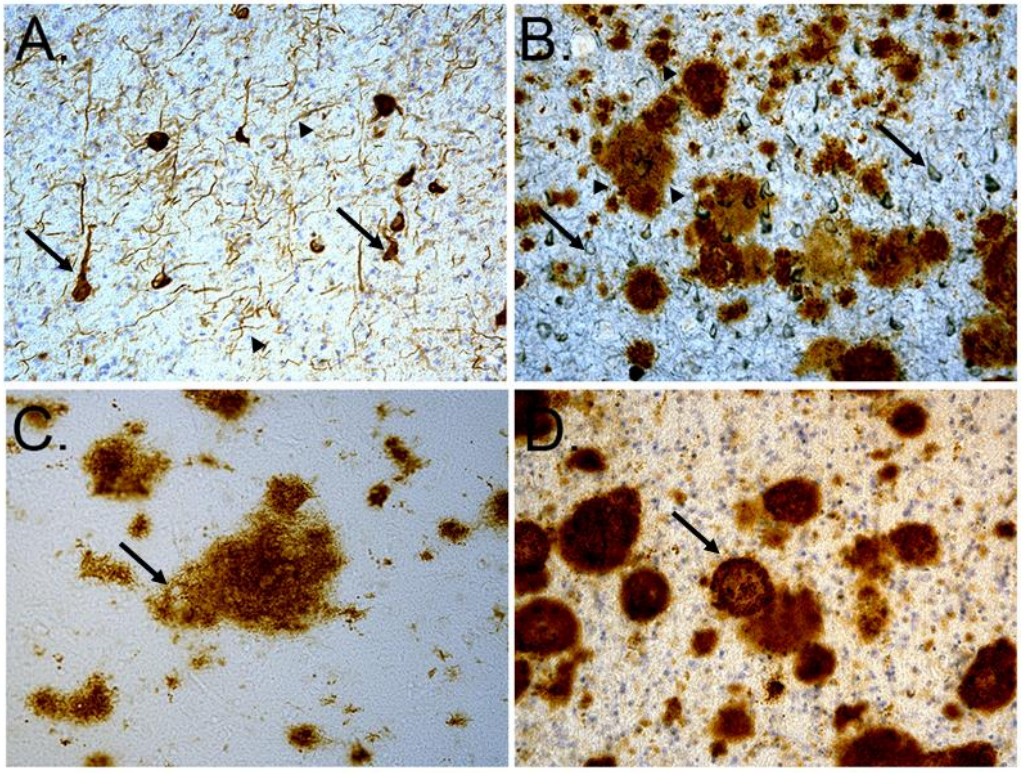

Figure 1.1. AD senile plaques and neurofibrillary tangles in DS.

Neurofibrillary tangles (arrows – PHF-1 immunostaining) and neuropil threads (arrowheads) can be observed in the frontal cortex of a 46-year old female with DS **(A)**. Aβ plaques (brown deposits – immunohistochemistry using an anti-Aβ42 antibody-arrowheads) are present in the space between neurons and neurofibrillary tangle bearing neurons (blue using anti-PHF-1 immunohistochemistry– arrows) in the frontal cortex of a 46-year old female with DS **(B)**. Diffuse plaques are large amorphous deposits of Aβ that can show "holes" where intact neurons can be found in a 48-year old female with DS (arrow – immunohistochemistry with anti-Aβ42 antibody)**(C)**. Compact or dense core

(arrow) Aβ plaques are observed in the frontal cortex of a 54-year old female with DS (anti-Aβ42 antibody) **(D)**.

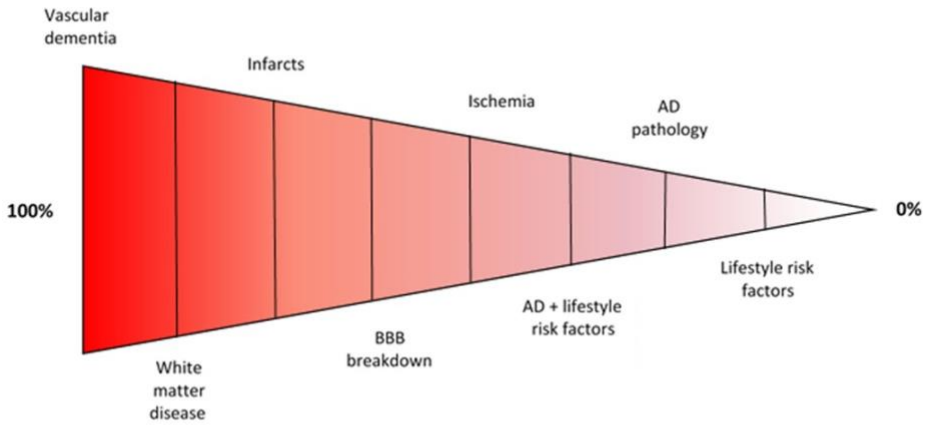

Figure 1.2: Elements of VCID Pathology

A large majority of cases of dementia can be attributed to AD, cerebrovascular pathology, or a combination of the two. Although the relative importance and placement of different aspects of pathology along this continuum are debatable, it is clear that cases of both pure AD and vascular dementia are relatively rare, and that most cases of dementia will display elements of both.

Chapter 2: Sodium Hydrogen Exchanger 1 (NHE1) Increases in Response to Injury and in a Mouse Model of Alzheimer's Disease with Vascular Complications

2.1 - Introduction

While the AD field has largely focused on plaque and tangle pathology over the years, there is a growing interest in the impact of cerebrovascular pathology on Alzheimer's disease, known as vascular contributions to cognitive impairment and dementia, or VCID (Gorelick, Scuteri et al. 2011). Our lab developed a novel animal model of VCID, which are morbidly obese, glucose intolerant, insulin resistant, and develop amyloid and tau pathology (Niedowicz, Reeves et al. 2014, Platt, Beckett et al. 2016). These mice were derived following the observation made by our lab (Niedowicz, Studzinski et al. 2013) and others (Fewlass, Noboa et al. 2004, Lieb, Beiser et al. 2009) that leptin signaling may be connected to the development of late-life dementia. For instance, it is possible that the well-known link between obesity, T2DM, and dementia (Luchsinger and Gustafson 2009) is connected to this phenomenon. We created this mouse line, which we call db/AD, by crossing the diabetic *db/db* mice (Chen, Charlat et al. 1996), which have a deficient leptin receptor, and the $APP^{\Delta NL/\Delta NL} \times PS1^{P264L/P264L}$ knock-in model of AD (Reaume, Howland et al. 1996, Siman, Reaume et al. 2000, Murphy, Beckett et al. 2007, Niedowicz, Studzinski et al. 2013). The db/AD mice also display profound cognitive impairment and develop cerebrovascular pathology, including aneurysms, and microhemorrhages (Niedowicz, Reeves et al. 2014). We hypothesize that the cerebrovascular pathology is the primary driver of cognitive impairment in these mice.

As mentioned in the overall introduction, animal models of VCID are uncommon, and have substantial limitations. Nonetheless, we reasoned that we could use such a model to elucidate the underlying mechanism(s) that might connect some of these processes. We identified the *SLC9A1* gene (which encodes the Na^+/H^+ exchanger, NHE1 (Hendus-Altenburger, Kragelund et al. 2014)), as a possible candidate molecule, and one that has legitimate therapeutic potential. NHE1 is the most abundant isoform of the NHE family in the central nervous system (Ma and Haddad 1997, Douglas, Schmitt et al. 2001) and is crucial in maintaining intracellular pH. NHE1 is a transmembrane, cell surface protein regulated by sensing the internal environment and is activated by various stimuli, such as acidification of the cell, osmotic shrinkage, growth factors, hypoxia, and mechanical stress (Luo and Sun 2007). While the main function of NHE1 is as a pH regulator, NHE1 has several other functions, including maintaining cell volume and cell motility (Valles, Bocanegra et al. 2015). NHE1 has been primarily explored as a target in myocardial infarction (Karmazyn 2013), although there has been a recent body of research exploring its role in ischemic damage, including that from stroke (Leng, Shi et al. 2014, Uria-Avellanal and Robertson 2014).

2.2 – Materials And Methods

2.2.1 - Mice

All animal work was approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC), and was performed in accordance with PHS guidelines. All procedures were performed under conditions designed to minimize pain and distress. The University of Kentucky is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved institution, and follows the current

version of the Guide for the Care and Use of Laboratory Animals (8th Edition), as adopted by the Office of Laboratory Animal Welfare (OLAW). Animals were maintained in a temperature controlled facility, under standard 12:12 light:dark conditions. A subset of animals, including C57BL/6 mice and db/AD and WT mice, obtained from Adam Bachstetter, were subjected to a single closed head injury (CHI), as described (Webster, Van Eldik et al. 2015, Bachstetter, Zhou et al. 2016). Briefly, mice were anesthetized with 5% isoflurane and isoflourane was continuously delivered during surgey via nosecone. A midline craniotomy was performed via trephination midway between bregma and lambda. An injury hub with a modified Luer-Lock hob (BD Biosciences) was affixed using cyanoacrylate gel and dental acrylic (Hygenic Corp., Akron, OH). Mice were recovered for 12-14 hours and re-anesthetized with isoflurane. The injury hub was attached to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). The pendulum was released onto the fluid filled piston, resulting in an injury of moderate severity (1.2±0.05 atm). Sham mice were subjected to the exact same experimental design described above, except the pendulum was not released onto the piston.

2.2.2 - Microarray

Frozen hemi-brains from young WT (n=2 M/ 3 F; age=3 months), db (n=3 M / 2 F; age=3 months), AD (n=1 M / 4 F; age=3 months), and db/AD mice (n=3 M / 2 F; age= 3 months) were homogenized in 1 mL of TRIzol Reagent (Invitrogen) followed by phenol/chloroform extraction and ethanol precipitation, as per the manufacturer's instructions. RNeasy cleanup columns (Qiagen, Valencia, CA) were run for each sample. RNA was quantified using the Biospec nano spectrophotometer and 100 ng of RNA in 5

ul RNase-free water were sent to the Microarray Core Facility at the University of Kentucky, where RNA was run on Affymetric Mouse Gene 2.0 array cards. Data was corrected for FDR using Hochberg's step-up procedure and genes were associated with annotated information on the Patrick Genomics Suite Program.

2.2.3 - Quantitative RT-PCR

We used quantitative RT-PCR to verify some of the gene changes we saw in the microarray. We used RNA from young WT (n=2 M / 3 F; age=3 months), db (n=3 M / 2 F; age=3 months), AD (n=1 M / 4 F; age=3 months), and db/AD mice (n=3 M / 2 F; age= 3 months) and older WT (n=4 M / 4 F; age=12 months), db (n=2 M / 2 F; age=12 months), AD (n=2 M / 2 F; age=12 months), and db/AD mice (n=6 M / 7 F; age=12 months). RNA was converted to cDNA using the cDNA High Capacity Kit (ThermoFisher) according to the manufacturer's instructions. Taqman probes (ThermoFisher) were used for six gene targets: *Serpina3n, Slc38a6, Cntnap2, CYP4X1, Slc9a1, Chga*. RT-PCR was performed using the Fast TaqMan Gene Expression assay (ThermoFisher). In each well of a 96-well plate, 0.5 µl cDNA (100 ng, based on the RNA concentrations) was diluted with 6.5 µl RNase-free water. One microliter of the appropriate gene probe was added along with 10 µl of Fast Taqman to each well. Target amplification was performed using the ViiA7 (Applied Biosystems). All genes were normalized to 18s rRNA, and the fold change was determined using the -ΔΔCt method (Livak and Schmittgen 2001).

2.2.4 - Western Blot Analysis

Frozen hemibrains were homogenized using a PowerMax Advanced Homogenizing System 200 (VWR, Batavia, IL) in RIPA buffer supplemented with protease inhibitor cocktail with EDTA (PIC; Amresco, Solon, OH). Whole tissue

homogenate was centrifuged at 200 *x g* for 15 minutes to pellet insoluble material, followed by an additional spin at 20,8-00 *x g* for 30 minutes at 4 ℃. The protein concentrations of the supernatants were determined by bicinchoninic acid assay (Pierce, Rockford, IL). Twenty µg of protein was loaded onto 4-12% Bis-Tris Criteron gels (Bio-Rad; Hercules, CA) and separated via SDS-PAGE in MOPS running buffer (Bio-Rad; Hercules, CA). The gels were then transferred to a 0.2 µm nitrocellulose membrane (BioRad; Hercules, CA) and blocked overnight in PBS with 1% bovine serum albumin and 2% BlockAce (AbD Serotec, Raleigh, NC). Blots were probed with mouse NHE-1 antibody (BD Transduction Laboratores, 1:1000), NHE1 (Abcam, 1:1000), Actin (AC15, Sigma, 1:500), β-tubulin (Abcam, 1:1000), and pTau (AT8, Pierce, 1:500) and then probed with HRP-conjugated goat anti-mouse secondary antibody (ThermoFisher, 1:15,000). Membranes were then incubated with SuperSignal West Dura chemiluminescent substrate (Pierce) and exposed to film. Films were developed and densitometric analysis was performed using Image J software (NIH, www.imagej.net), using actin and tubulin as covariates.

2.2.5 - Mouse NHE-1 ELISA

NHE-1 levels in mice were measured quantitatively by sandwich ELISA using a commercially available kit (Cloud-Clone Corp.; Houston, TX) following manufacturer's instructions. RIPA extracts (see above for extraction protocol) from young (2-4 months) and older (9-14 months) WT (n=3 M / 9 F young, n=7 M / 4 F old), db (n=5 M / 8 F young, n=4 M / 4 F old), AD (n=7 M / 9 F young, n=4 M / 4 F old), and db/AD (n=8 M / 7 F young, n=4 M / 4 F old) mice were diluted 1:1000 to load 13 mg/mL in each well. Absorbance was measured at 450 nm using a multiwall plate reader (BioTek, Winooski, VT).

2.2.6 - Immunohistochemistry

Formalin fixed db/AD mice hemibrains, WT mice hemibrains, and frontal cortex (FC) tissue from human AD cases were sectioned at 50 microns on a Vibrating Blade Microtome (Leica Biosciences; Buffalo Grove, IL) and stored in PBS with 0.05% NaN_3. Immunohistochemistry was performed using NHE-1 (BD Transduction Laboratories, 1:5000), 6E10 (Sigma-Aldrich, 1:1000) to stain for Aβ, and PHF-1 (courtesy of Dr. Peter Davies, 1:100) to stain for NFTs/tau pathology, with 3,3'diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA). Slides were coverslipped with DPX Mountant (Sigma-Aldrich).

2.2.7 - Spot Blot Analysis

RIPA extracts (see above for extraction protocol) from young (2-4 months) and old (9-14 months) WT (n=3 M / 9 F young, n=8 M / 6 F old), db (n= 5 M / 9 F young, n=6 M / 4 F old), AD (n=7 M / 9 F young, n=5 M / 5 F old), and db/AD (n=8 M / 7 F young, n=4 M / 4 F old) mice were diluted in PBS to 10 µg/mL. 100 µl of extract was loaded for a total of 1 µg of protein per spot on spot blot apparatus. Protein was vacuumed onto a nitrocellulose membrane and blocked in PBS with 1% bovine serum albumin and 2% BlockAce (AbD Serotec, Raleigh, NC) overnight. Spot blots were probed for NHE-1 (BD Transduction Laboratories, 1:1000), AT8 [26-29] for pSer202/pThr205 tau (Pierce, Rockland, Illinois, 1:1000), β-tubulin (Abcam, 1:1000) and then with HRP-conjugated rabbit anti-mouse (Rockland, 1:15,000) and donkey anti-rabbit (1:15,000), respectively.

2.2.8 - Cell Culture

Chinese hamster ovary (CHO) cells stably transfected with human βAPP 695WT, CHO2B7 (Murphy, Uljon et al. 2000, Haugabook, Yager et al. 2001) (Mayo Clinic) were

cultured in Ham's F-12 medium (Life Technologies, Inc) with 10% fetal bovine serum, 1% penicillin/streptomycin, and maintained with 200µg/mL Zeocin (Invitrogen, Carlsbad, CA). H4 neuroglioma cells over-expressing Swedish mutant APP (APPΔNL695), H4 15x APP (Kukar, Murphy et al. 2005) (Mayo Clinic), were cultured in OptiMEM (Invitrogen, Carlsbad, CA) with 10 % fetal bovine serum, 1% penicillin/streptomycin, and maintained with Hydromycin (Life Technologies, Inc). Both cell types were treated with the NHE1 inhibitor Amiloride (Sigma-Aldrich) and Chloroquine (Sigma-Aldrich), which disrupts lysosomal pH, as a positive control (Chu, Tran et al. 1998). We treated cells with 10 µM Amiloride, 1 µM Amiloride, and 10 µg Chloroquine, and ddH_20 (as a negative control) for 24 hours. Separately, we tested the effect of a more specific inhibitor of NHE1, Cariporide (Sigma-Aldrich)(Masereel, Pochet et al. 2003), along with Amiloride and Chloroquine, on the CHO2B7 cells to evaluate if one drug altered Aβ levels more than the other. We treated the CHO2B7 cells with 1, 10, and 100 µM of Amiloride, 0.1, 1, and 10 µM of Cariporide, and 10mg µg of Chloroquine. After treatment, conditioned media was collected, and EDTA was added to a final concentration of 5mM to inhibit Aβ degradation (Beckett, Niedowicz et al. 2010). Secreted Aβ40, Aβ42, and total Aβ was measured by ELISA, as previously described (Kukar, Murphy et al. 2005, McGowan, Pickford et al. 2005).

2.2.9 - ELISA on Human Cases

We obtained frozen tissue samples with different types of neurodegenerative pathology from the superior and middle temporal gyri (SMTG) and the FC. SMTG cases were kindly provided by the University of Kentucky Alzheimer's Disease Center and FC cases were provided by Dr. Elizabeth Head. Tissue was homogenized using a PowerMax Advanced Homogenizing System 200 (VWR, Batavia, IL) in RIPA buffer supplemented

with protease inhibitor cocktail with EDTA (PIC; Amresco, Solon, OH). Whole tissue homogenate was centrifuged at 200 *x g* for 15 minutes to pellet insoluble material, followed by an additional spin at 20,8000 *x g* for 30 minutes at 4 ℃. Samples were diluted 1:1000 to load 13 mg/mL in each well of a commercially available kit (Cloud-Clone Corp.; Houston, TX) following manufacturer's instructions. We also measured $A\beta_{40}$ and $A\beta_{42}$ in these cases using an ELISA, as previously published (Beckett, Niedowicz et al. 2010). Briefly, monoclonal antibody Ab9 (human sequence Aβ1-16) was used for capture. Antigen detection was performed using biotinylated antibodies 13.1.1 (for $A\beta_{40}$) and 12F4 for $A\beta_{42}$, followed by Neurtravid-HRP (Pierce Biotechnologies, Rockford, IL). Absorbance for all ELISAs were read at 450 nm using a multiwell plate reader (BioTek, Winooski, VT).

2.3 - Results

2.3.1 - Microarray and Quantitative RT-PCR

To measure gene expression changes in our db/AD mice, we performed an Affymetrix 2.0 microarray, which measures changes in more than 35,000 transcripts. We identified over 200 genes that were significantly different across genotypes, but did not find any major differences in pathways using the Patrick Genomics Suite Program. However, using the annotated information in the software package, we identified a subset of genes that we found particularly interesting, given their role in neurodegeneration and vascular abnormalities (Table 2.1). We performed qRT-PCR follow-up on a subset of these: *Serpina3n, Slc38a6, Cntnap2, CYP4X1, Slc9a1, and Chga*. We chose these six because of their novelty, therapeutic potential, and connection to cerebrovascular pathology, which may provide explanation to the pathology and cognitive decline we

examine in our db/AD mice. We used tissue from both 3 month and 12 month old mice, to evaluate potential age differences in the selected genes (Figure 2.1). We ran a multivariate ANOVA for age, gender, AD genotype (animals with APPxPS1 knock-in), and db genotype (leptin receptor-deficient mice) for all genes tested. We found a significant overall effect of AD on *Serpina3n* expression ($F(1,42)=9.983$, $p<0.004$) (Figure 2.1A). We also found a significant AD interaction in *Slc38a6* expression ($F(1,42)=11.662$, $p<0.002$), along with an overall difference in expression driven by age ($F(1,42)=21.484$, $p<0.001$) (Figure 2.1B). While we didn't find any significant genotype differences in *Chga* expression, we did see an overall effect of age ($F(1,42)=78.481$, $p<0.001$), showing a significant increase in *Chga* expression in the older animals (Figure 2.1C). For *Cntnap2* expression, we saw an overall effect of age ($F(1,42=40.378$, $p<0.001$), with an increase in expression occurring with age (Figure 2.1D). We saw an overall significant decrease in *CYP4X1* expression with age ($F(1,42)=34.571$, $p<0.001$) and a significant db by AD by age interaction ($F(1,42)=4.772$, $p<0.04$) (Figure 2.1E). While many genes were potentially interesting, we chose to focus on *SLC9A1* (encoding the Na^+/H^+ exchanger, NHE1 (Hendus-Altenburger, Kragelund et al. 2014)). The qRT-PCR f showed an increase in *SLC9A1* expression attributed to both the db ($F(1,42)=5.280$, $p<0.03$) and AD ($F(1,42)=21.564$, $p<0.001$) genotypes, as well as a db by AD by age interaction ($F(1,42)=19.294$, $p<0.0001$) (Figure 2.1F).

2.3.2 - NHE1 Expression in Mice

To determine whether NHE1 changed at the protein level, we ran an ELISA on a large group of younger and older mice (n=89), including all genotypes. We ran an ANOVA examining the interactions between age and animals with an AD genotype

(animals with APPxPS1 knock-in) and db genotype (leptin receptor-deficient mice). We found a significant age effect ($F(1,87)=9.154$, $p<0.003$)(Figure 2.2), with NHE1 levels decreasing with age. We did not find any significant genotype differences. However, when we ran an ANOVA to examine differences between all four genotypes on only the old mice, we saw a modest, yet significant ($F(3,34)=2.849$ $p<0.05$), increase in NHE1 protein levels in the db/AD mice compared to wild type mice. We did not find any significant group differences in the younger mice ($F(3,54)=0.654$, $p=0.584$). These data suggest that genotype differences may either develop or become more pronounced with increasing age.

2.3.3 - NHE1 and Amyloid Beta

As a regulator of intracellular pH (Chesler 2003), we chose to focus on the potential impact of NHE1 on APP processing. β-Site APP-cleaving enzyme (BACE), is the rate-limiting enzyme involved in Aβ production (Vassar, Kovacs et al. 2009). BACE is a pH sensitive enzyme, operating optimally at an acidic pH. Therefore, BACE is primarily found in acidic compartments, such as endosomes and *trans*-Golgi. Although we initially thought that this mechanism was unlikely given the cellular localization of NHE1, a previous publication found that Amiloride, an inexpensive potassium-sparing diuretic and NHE1 antagonist, is a potent inhibitor of Aβ (Wang, Ho et al. 2007). We hypothesized that NHE1 may plays a role in cognitive impairment through APP processing by modulating AD-related enzymes, β-secretase and γ-secretase. However, we did not see any significant differences in the levels of $A\beta_{42}$, $A\beta_{40}$, nor total Aβ with either concentration of Amiloride used in either cell line (Figure 2.3A). Interestingly, we also did not see any significant differences in Aβ with the Chloroquine treated cells, indicating

that Chloroquine does not work as a positive control in these cells lines. We did not find any significant differences in the levels of $A\beta_{42}$ with either the Amiloride (Chi-square=4.834, p=0.184) (Figure 2.3B) or Cariporide (Chi-square=0.567, p=0.904) (Figure 2.3C) treatment on the CHO2B7 cells.

We followed this up with immunohistochemistry (IHC) with an NHE1 antibody and a double label with NHE1 and Aβ in human AD tissue. The NHE1 antibody showed labeling of both the cerebrovasculature and neurons. The no primary control did not show any positive labeling and our western blots probed with this antibody were clean, indicating specificity of our NHE1 antibody (e.g. Figure 2.2A). The co-label showed some co-localization of NHE1 with Aβ in some CAA affected vessels (Figure 2.3D), although this was inconsistently observed.

2.3.4 - NHE1 and Tau

In the db/AD mice, we consistently observe a modest, but significant increase in tau phosphorylation at several epitopes (Niedowicz et al, 2014; Platt et al, 2016). Additionally, when mice are injected with adeno-associated virus (AAV) mutant tau^{P301L}, we detect a significant increase in tau pathology (Platt, Beckett et al. 2016). Given that NHE1 is tethered to the cytoskeleton and has a major role in regulating cell shape (Denker, Huang et al. 2000, Hendus-Altenburger, Kragelund et al. 2014, Valles, Bocanegra et al. 2015), we hypothesized that might be an underlying connection between an increase in NHE1 and the microtubule associated protein, tau. We performed a spot blot in a large sample of our mice to test this hypothesis, and found a strong positive correlation between NHE1 and phosphorylated tau (R^2=0.27, p<0.0001) (Figure 2.4A).

Additionally, a double label IHC shows neurofibrillary tangles in some NHE1-positive neurons, although there did not appear to be an obvious pattern (Figure 2.4B).

Interestingly, NHE1 has been reported to be unregulated following traumatic axonal injury (Yang, Xie et al. 2006), a well-known and accepted basis for the development of tau pathology in both humans and in animal models (McKee, Stein et al. 2015, Ojo, Mouzon et al. 2016). We performed two studies to address this, using head trauma mice courtesy of Adam Bachstetter. WT mice that had been subjected to a single CHI, and allowed to survive out to two months, showed significant increases in NHE1 ($F(1,12)=100.888$, $p<0.001$) and pTau ($F(1,12)=12.201$, $p<0.01$) expression (Figure 2.4C). Next, in an attempt to better understand the time course of these changes and to observe any genotype differences, we evaluated a set of WT versus db/AD mice at just one day post-injury. We ran an ANOVA with β-tubulin as a covariate and observed an increase in NHE1 expression following head injury in both the WT and db/AD mice ($F(1,19)=5.139$, $p=0.039$) (Figure 2.4D-E). Further, the db/AD mice had consistently higher levels of NHE1 compared to WT mice, both before and after injury ($F(1,10)=7.608$, $p=0.015$).

2.3.5 - NHE1 Expression in Human Cases

In order to understand how NHE1 relates to neurodegenerative diseases in humans, we ran a large number of human cases from the SMTG and FC regions (Table 2.2). We were able to correlate NHE1 levels with several measures of pathology that were taken at autopsy in the SMTG cases. In both regions, we were able to correlate NHE1 levels to Aβ levels.

In the SMTG, we did not find any significant group differences in NHE1 levels (Table 2.2). We also did not find a significant correlation between NHE1 and total infarcts (p=0.681) (Figure 2.5A), CAA (p=0.189) (Figure 5B), arteriosclerosis (p=0.629), microinfarcts (p=0.684) (Figure 2.5C), nor mini mental score exam (MMSE) to measure cognition (p=0.834) (Figure 2.5D). We did find a marginally significant correlation between NHE1 and $A\beta_{42}$ levels (p=0.048), but did not find a significant correlation with $A\beta_{40}$ (p=0.572) (Figure 2.5E).

We did not find any significant group differences in NHE1 levels in the FC cases. We also did not find any significant correlation between NHE1 and $A\beta_{40}$ or $A\beta_{42}$ in the control nor DS cases. However, we did find that NHE1 decreases significantly with age in individuals with DS (p=0.033) (Figure 2.5F).

2.4 - Discussion

We have shown through various methodologies that NHE1 expression, both at the mRNA and protein level, increases in our db/AD mice. This increase is modest, but consistently significant. We found this to be a novel target to follow, given that NHE1 is thought to be a mediator of post-ischemic damage, and might therefore be related to the development of CVD in both this mouse line, and in humans.

Neuron health depends on efficient H^+ efflux mechanisms, as intracellular acidosis impacts neuron excitability. Therefore, over-stimulation of NHE1 has been implicated as a major cause of cell death following an ischemic event. Under non-ischemic conditions, NHE1 opens as the H^+ concentration increases in the cytosol, allowing Na^+ to flow into the cell, reversing the acidity of the cell. However, when there is a reduction in blood flow (such as during ischemia), oxygen levels are not sufficient to

maintain ATP levels. As ATP stores are depleted, lactate, pyruvate, and protons accumulate within the cell, causing hyperacidfication of the cells. This causes NHE1 to become over-activated and Na^+ to rapidly rushing into the cell. The high concentration of Na^+ then causes a reversal of Na^+ / Ca^{+2} exchange, leading to excess Ca^{+2} buildup in the cell. The excess Ca^{+2} accumulation causes a cascade of cell-damaging actions that eventually results in cell death (Siesjo 1992).

Not surprisingly, both the genetic (Luo, Chen et al. 2005, Wang, Luo et al. 2008) and pharmacologic (Hwang, Yoo et al. 2008) reduction of NHE1 has been shown to be neuroprotective following ischemic stroke. Additionally, an NHE1 inhibitor, Amiloride, is a potassium-sparing diuretic used in the management of hypertension in congestive heart failure since 1967. Amiloride is on the "*WHO list of Essential Medicines*", and is both widely available and inexpensive. Therefore, if NHE1 really is implicated in neurologic disease, the implications for the treatment of dementia in both the developed and developing world are incredibly significant.

Initially, we hypothesized that this increase in NHE1 is due to increases in Aβ, since NHE1 is regulates intracellular pH (Chesler 2003) and Aβ production is linked to pH through BACE (Vassar, Kovacs et al. 2009). However, the cell culture experiments did not show a significant change in Aβ production when cells were treated with two different types of NHE1 inhibitors (Figure 2.3A-C). Additionally, we only saw a marginally significant correlation between $A\beta_{42}$ and NHE1 in the SMTG region in human cases, but did not find any correlation with $A\beta_{40}$ or any significant correlations in the frontal cortex (Figure 2.5E). Therefore, the weight of the available evidence indicates that

there is no reason to believe that NHE1 is related to amyloid pathology, or Aβ production.

Despite not finding a connecting with Aβ, we have gathered some significant evidence to indicate a possible link between levels of tau phosphorylation and NHE1. We consistently see NHE1 and phosphorylated tau levels highly correlated in our db/AD mice (Figure 2.4A). This is further supported by evidence showing an increase in both NHE1 and phosphotau following head injury in the db/AD animals (Figure 2.4E). It is possible that NHE1 and phosphotau increase together when there is a change in cell size or shape due to injury. This change in membrane curvature causes NHE1 to interact with the cytoskeleton. Because tau's primary function is to serve as a microtubule stabilizing protein, NHE1 and tau are likely coordinated through this cytoskeletal interaction. However, at this time we are unsure of the significance of a coordinated increase in NHE1 and tau phosphorylation. Our immunohistochemistry data shows that there are tau neurons that are NHE1 positive, but this is not a consistent feature in human AD tissue (Figure 2.4B). However, it is possible that because we are only looking at a small section of cortex, that we are missing a possible interaction. It is also possible that what we are seeing in the db/AD and WT mice is different from what we observe in the human cases. For instance, the increase in phosphotau in the mice is not the same form of tau that is found in NFTs in human brain, which represents a different pool of pathologic (insoluble) tau.

The role of NHE1 in VCID, if any, remains unknown. We did not see any correlation between NHE1 levels and markers of cerebrovascular pathology in our human cases. However, it is possible that there is a localized upregulation in NHE1 following

injury, which perhaps is getting lost in our tissue homogenate. A review of several human trials involving NHE1 inhibitors to treat cardiac ischemia and recovery shows disappointing outcomes (Avkiran, Cook et al. 2008). However, it is unclear if this is because the inhibitors are not being given at the proper time (during ischemia or reperfusion) or if NHE1 inhibitors are not as effective in humans as they have been in animal models (Murphy and Allen 2009). A recent study in canines found that an NHE1 inhibitor did not reduce infarct size, nor protect against ischemia-reperfusion injury (Kingma 2018), so it is possible that NHE1 inhibitors are not a viable treatment option for ischemia-reperfusion in larger mammalian systems, like canines and humans. However, our own studies are largely correlative. Experiments in either NHE1 knockout mice, or through the administration of an NHE1 inhibitor to db/AD mice, will be necessary in order to determine if there may yet be a viable connection between NHE1 and neuropathology.

However, as we attempted to extend these studies into human cases, it became abundantly clear that there was no established form of VCID pathology that we should be using in order to evaluate the relationship between NHE1 and cerebrovascular disease. One of the possibilities we considered when evaluating our large amount of negative data in the human cases was simply whether or not this might be because we did not know what, exactly, it was that we were looking for. One of the only modestly significant pathology related effects was the small decrease in NHE1 in the DS cases with age (Figure 2.5F). Since individuals with DS develop a marked amount of CVD with age, we therefore wondered if we could further investigate these cases to establish which forms of vascular neuropathology might be better predictors of VCID.

Table 2.1: A Microarray Identified Several Possible Targets
We identified a subset of genes from our microarray that we found particularly interesting given their role in neurodegeneration and vascular pathology.

Gene Symbol	p-value (genotype) *corrected for FDR using Hochberg's step-up	Function
Serpina3n	0.0000	Serine protease inhibitor (Dickson and Alper 1974) highly expressed in the brain. Serpina3n has been found in amyloid plaques(Abraham, Selkoe et al. 1988) and has been associated with the progression of AD(Kamboh, Sanghera et al. 1995), likely because levels increase during an inflammatory response (Abraham 2001).
Slc38a6	0.0000	Selective expression in excitatory neurons with high expression in synapses-function unknown (Bagchi, Baomar et al. 2014).
Nova1	0.0006	Encodes a neuron-specific RNA-binding protein that regulates alternative splicing (Buckanovich, Yang et al. 1996, Lewis, Chen et al. 1999, Ule, Stefani et al. 2006). Knockout studies provide evidence that Nova1 is important for synapse formation and function and in neuronal migration (Jensen, Dredge et al. 2000, Huang, Shi et al. 2005, Ruggiu, Herbst et al. 2009, Yano, Hayakawa-Yano et al. 2010). Nova1 is upregulated in neurons after ischemia, and may place a role in neural repair after an ischemic event (Li, Sun et al. 2013).
ECSIT	0.0009	An adapter protein of the Toll-like and IL-1 receptor signaling pathway (Kopp, Medzhitov et al. 1999). ECSIT has been hypothesized to play a role in AD by integrating oxidative stress, inflammation, and mitochondrial dysfunction (Kopp, Medzhitov et al. 1999).
Tbc1d23	0.0014	General inhibitor of innate immunity signaling, strongly inhibiting multiple TLR and dectin-signaling pathways (De Arras, Yang et al. 2012).
Cntnap2	0.0027	Encodes a neuronal transmembrane protein member of the neurexin superfamily involved in neuron-glia interactions and clustering of K+ channels in myelinated axons (Poliak, Gollan et al. 1999). Knockout mice have defects in the migration of cortical projection neurons and a reduction in the number of GABAergic interneurons, as well as accompanying neurophysiological alterations (Rodenas-Cuadrado, Ho et al. 2014, Scott, Sanchez-Aguilera et al. 2017).
CYP4X1	0.0042	Expressed in the cytoplasm of neurons in the cerebellum and in the vascular endothelium (Bylund, Zhang et al. 2002). In humans, it metabolizes arachidonic acid to form epoxyeicosatrieinoic acids, which are released from astrocytes, neurons, and vascular

		endothelial cells to act as potent vasodilators, increasing blood flow (Carver, Lourim et al. 2014).
Slc9a1	0.0059	Slc9a1 encodes a Na+/H+ exchanger, known as NHE-1 that regulates intracellular pH, Na+ concentration, and cell volume (Wakabayashi, Shigekawa et al. 1997). NHE-1 knockout mice have hypotrophy of vascular smooth muscle cells, reduced artery tension and lower blood pressure (Boedtkjer, Damkier et al. 2012). NHE-1 protein is abundantly expressed in activated microglia and astrocytes and inhibition reduced microglial proinflammatory activation following ischemia (Wakabayashi, Hisamitsu et al. 2013).
Chga	0.0081	Chga encodes the protein chromogranin A (CgA), a neurosecretory acidic glycoprotein with many functions, including regulation of metabolism, innate immunity, cardiovascular system, vascular contractility, and endothelial barrier function (Helle, Corti et al. 2007, Loh, Cheng et al. 2012). CgA has been found in senile plaques of AD (Yasuhara, Kawamata et al. 1994, Rangon, Haïk et al. 2003, Lechner, Adlassnig et al. 2004) and can strongly activate microglia to induce the production and secretion of IL-1β (Kayo, Jun et al. 2010, Sun, Wu et al. 2012)

Table 2.2: NHE1 was measured in frozen tissue from humans with different neurodegenerative diseases.
NHE1 levels were measured via ELISA in human samples from the SMTG and FC regions.

NHE1 values are reported as mean±SEM.

Group	Brain Region	Number of Cases	Average Age (years)	Average PMI (hours)	NHE1 (AU)
Controls	SMTG	9	84.33	2.75	32.04±12.21
Preclinical AD (PCAD)	SMTG	10	85.60	2.57	15.88±5.31
Mild Cognitive Impairment (MCI)	SMTG	7	89.00	2.81	19.94±7.24
Frontotemporal Dementia (FTD)	SMTG	6	61.00	4.83	15.79±4.68
AD	SMTG	10	83.40	3.08	26.54±11.47
AD	FC	11	80.91	8.55	35.05±10.39
Down Syndrome (DS)	FC	9	17.11	18.00	44.21±12.71
Down Syndrome with AD (DSAD)	FC	35	51.66	8.33	26.91±3.63
Young Controls (YC)	FC	24	19.58	17.66	27.44±4.77
Middle-Aged Controls (MC)	FC	21	51.33	11.91	25.44±5.84
Old Controls (OC)	FC	8	81.25	3.44	42.93±6.77

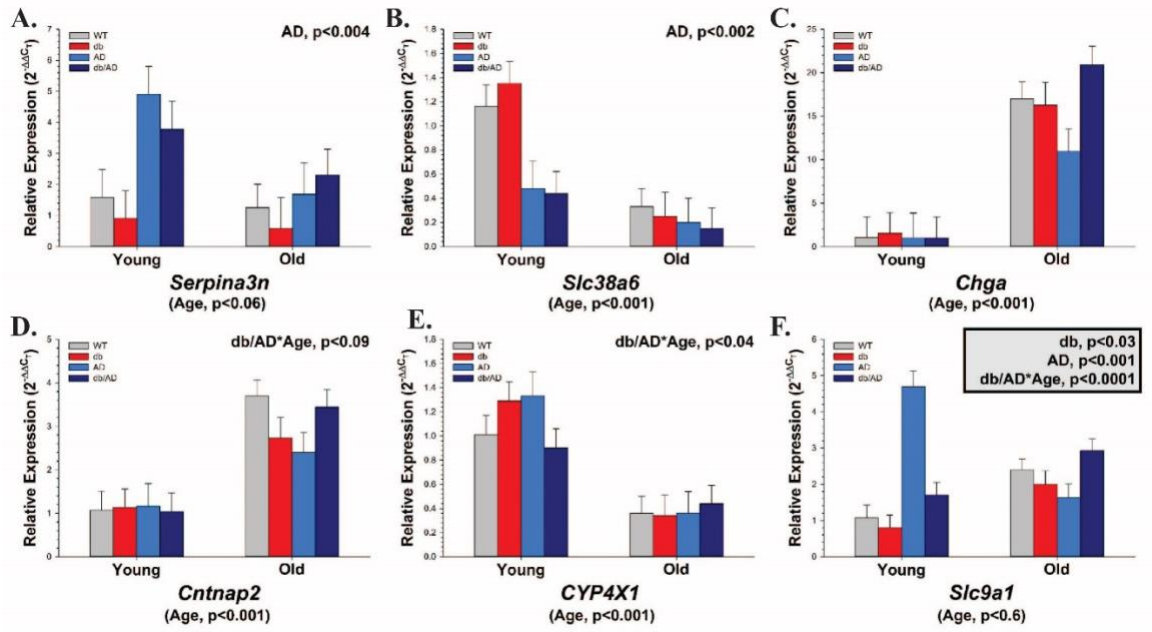

Figure 2.1: Gene Expression by RT-PCR in db/AD Mice (Taqman).
Chosen based on possible involvement in vascular disease; *Polr1a*, *Ppia*, and *Prl30* were used as housekeeping genes. **(A)** *Serpina3n*, a serine protease inhibitor with high brain expression; **(B) Slc38a6**, a gene that is highly expressed in excitatory neurons with a relatively unknown function; **(C)** *Chga*, an acidic glycoprotein involved in neuroinflammation; (D) Cntnap2, a transmembrane protein involved in neuron-glia interaction; **(E)** *CYP4X1*, a cytoplasmic enzyme involved in arachidonic acid metabolism, and linked to regulating cerebral blood flow; **(F)** *Slc9a1* (encoding NHE1), Na^+/H^+ exchanger involved in ischemia and neurodegeneration. NHE1 was the most promising, showing significant differences in diabetes, AD, and a db/AD by age interaction.

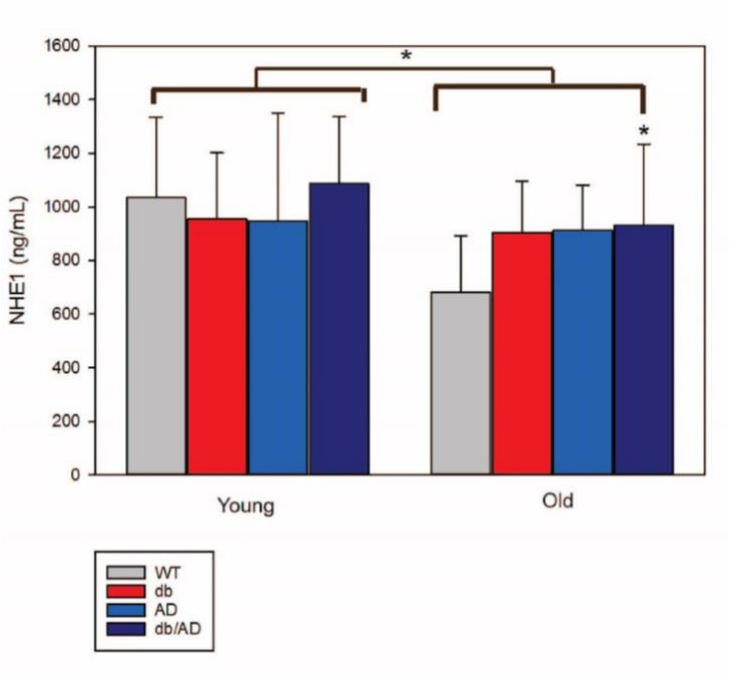

Figure 2.2: NHE1 levels are increased in the db/AD animals.
We ran an ELISA using a large sample size of younger and older mice (n=89) and saw a significant age effect ($p<0.003$), with NHE1 levels decreasing with age. We also saw a significant increase in NHE1 in the db/AD mice in our older mice ($p<0.05$).

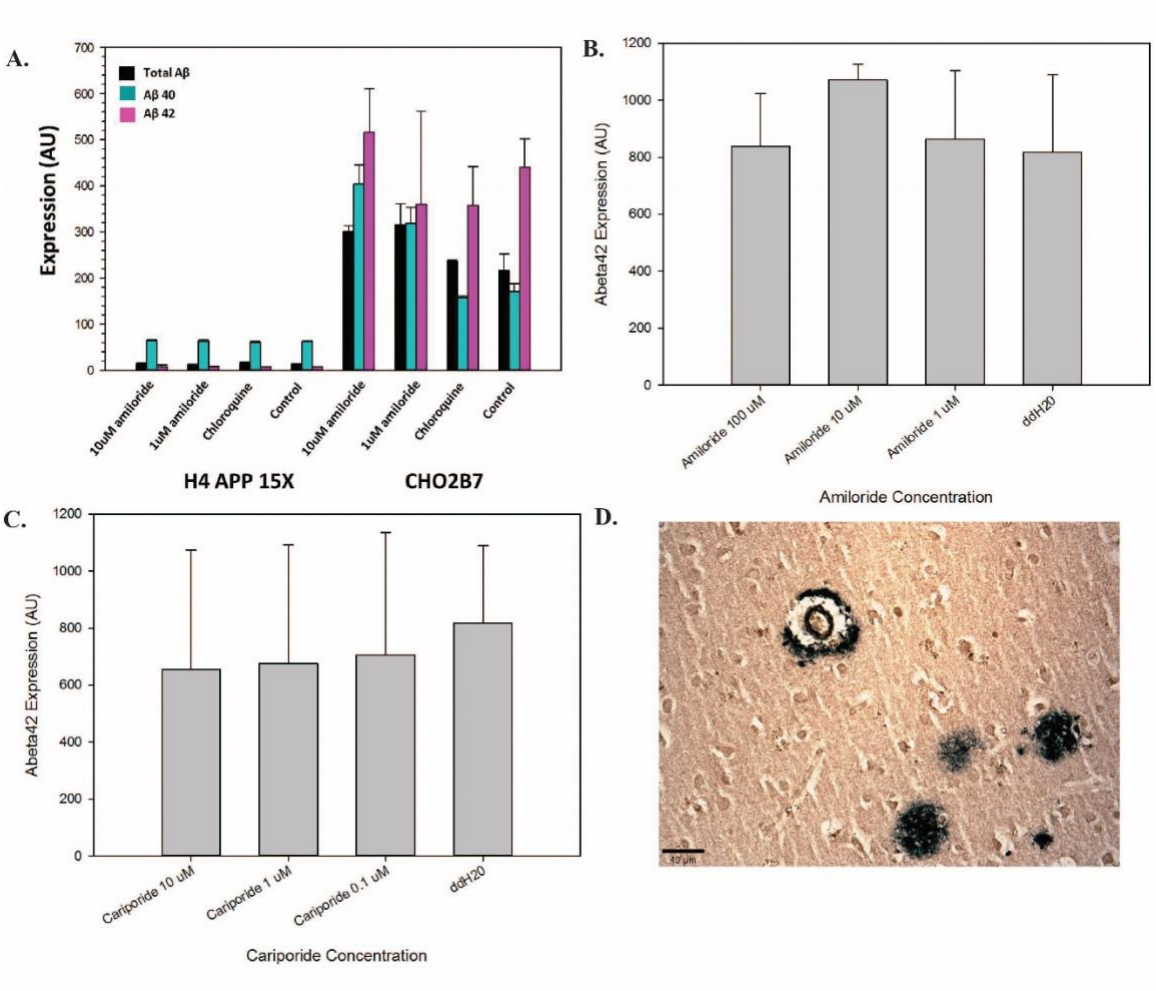

Figure 2.3: Aβ levels do not change when NHE1 is inhibited.
(A) We treated H4 APP 15x cells and CHO2B7 cells with two different concentrations of the NHE1 inhibitor, Amiloride, and measured Aβ levels with an ELISA. We used chloroquine as a positive control and ddH20 as a negative control. We did not find any significant differences in Aβ after treatment with Amiloride. We followed this up with a higher concentration of amiloride **(B)** and a more potent NHE1 inhibitor, Cariporide **(C)**, in CHO2B7 cells and did not find any significant differences in Aβ. **(D)** We also did a double label IHC in human AD tissue for Aβ (6E10-blue) and NHE1 (brown), which shows some co-localization in some CAA affected vessels.

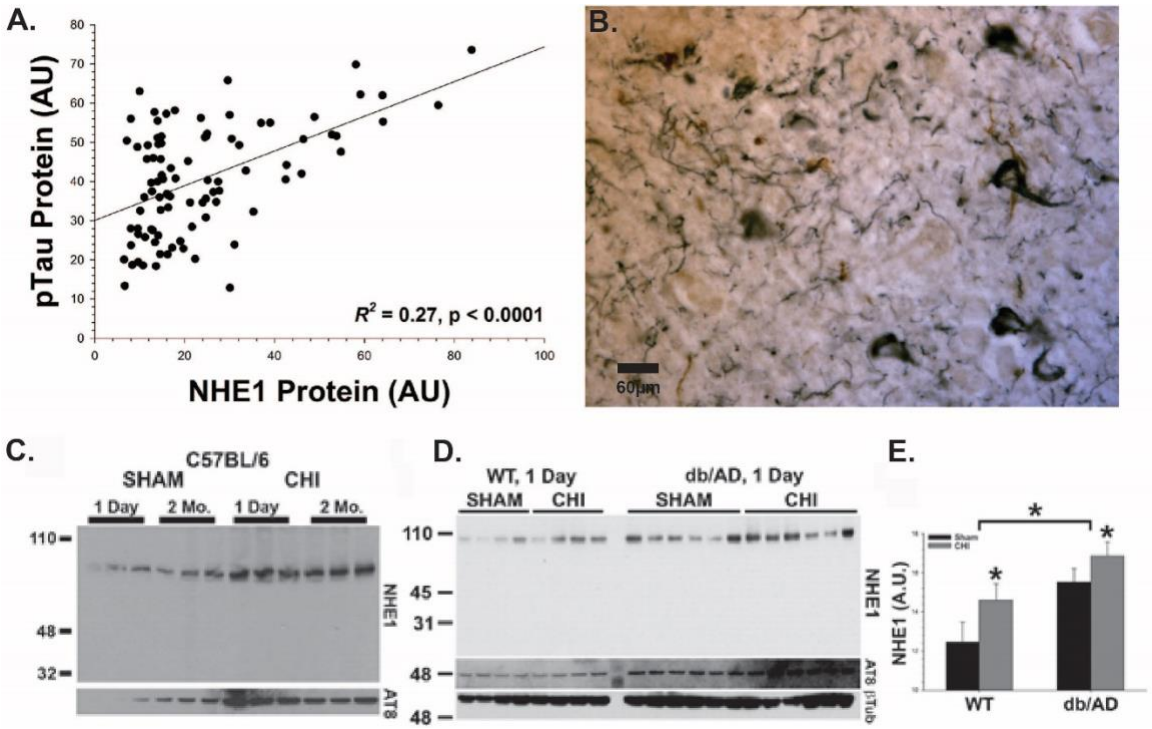

Figure 2.4: NHE1 increases along with pTau in the db/AD mice and after head injury in db/AD mice.

Slot blot densitometry (N = 91, run in triplicate, standardized to βTubulin) from db/AD line (mixed genotypes) probed for NHE1 (BD Biosciences) and pTau (AT8, Pierce), showing a correlation between NHE1 and pTau. ***(B)*** NHE1 (Brown) and NFT (Blue/Black; PHF1, provided by Peter Davies) co-localization (arrows) in human AD tissue. **(C)** Normal C57BL/6 mice were subjected to a single CHI and allowed to survive for up to 2 months; NHE1 ($p<0.01$) and pTau ($p<0.05$) were both increased; CHI causes NFT pathology in both humans and in animal models. **(D)** WT and diabetic mice were subjected to CHI, and evaluated one day later for NHE1 (Abcam) and pTau (AT8); βTubulin (Abcam) is shown as a loading control. **(E)** As expected from our earlier data, NHE1 expression was higher in diabetic mice as compared to WT controls; even at one day post-injury, NHE1 levels were increased, consistent with its role in both swelling and cellular injury. We saw higher pTau in diabetic mice, and an increase following CHI.

This suggests that NHE1 and pTau are connected, and that NHE1 activation may be an early event in the development of cytoskeletal pathology. * = $p<0.05$

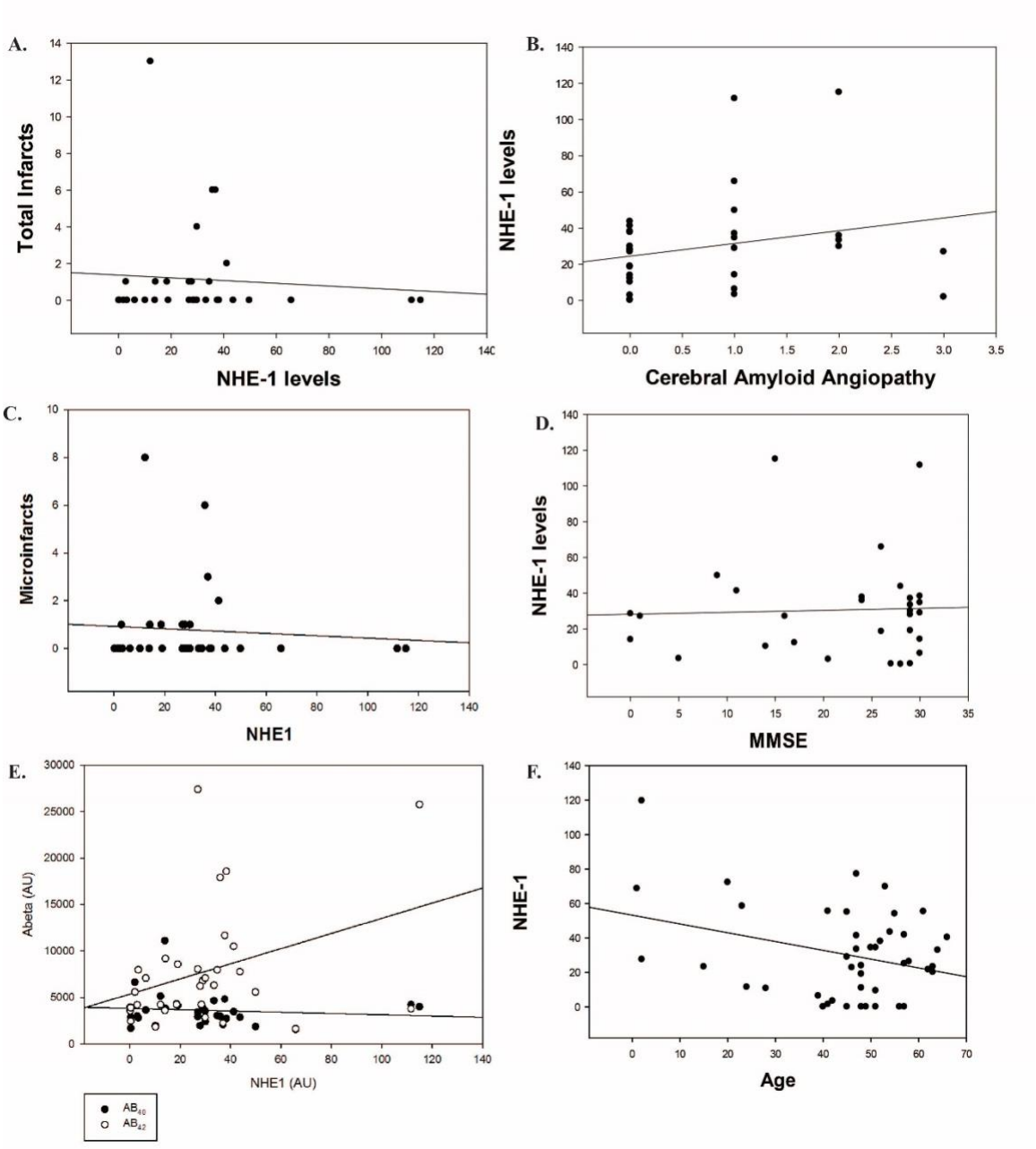

Figure 2.5: NHE1 is not associated with markers of cerebrovascular pathology in human cases with dementia, but decreases with age in individuals with DS.
In the SMTG region, we did not find any significant correlation between NHE1 and **(A)** total infarcts (p=0.681), **(B)** CAA (p=0.189), **(C)** microinfarcts (p=0.684), or **(D)** MMSE (p=0.834). **(E)** In the FC, we found a marginally significant correlation between NHE1 and $A\beta_{42}$ (p=0.048), but did not find a significant correlation with $A\beta_{40}$. **(F)** We did found

that NHE1 significantly decreases with age in the FC of individuals with DS ($p=0.033$), mirroring what we saw in our db/AD animals.

Chapter 3: Microbleeds and Cerebral Amyloid Angiopathy in the Brains of People with Down Syndrome with Alzheimer's Disease

3.1 - Introduction

Down syndrome (DS) is a genetic disorder whereby an individual has an extra full or partial copy of chromosome 21 (Lejeune, Gautier et al. 1959). DS is one of the most common causes of intellectual disability with 5,429 annual DS births (Centers for Disease and Prevention 2006) and an estimated 250,000 individuals with DS in the USA (World Health Organization 2018). As improvements in medical technology continue, the life expectancy of people with DS continues to increase. In 1929, a diagnosis of DS was associated with a life expectancy of around 9 years. However, people with DS are now living longer lives, with an average life span of about 60 years of age (Bittles and Glasson 2004). As with the general population, the risk of developing health-related problems increases as people with DS get older (Bayen, Possin et al. 2018). In particular, people with DS are at a high risk of developing cognitive impairment and dementia associated with AD after the age of 50 years (Zigman, Schupf et al. 1996, Zigman 2013).

3.1.1 - Genes Associated with AD on Chromosome 21

There are a number of genes on chromosome 21 that are thought to play a role in the development of AD in DS. Although we will not provide an exhaustive discussion of all possible genes, those genes that have been studied in DS and have strong links to AD are highlighted.

3.1.1.1 - Aβ and DS: APP and BACE2

There are several genes associated with an increased risk of AD development located on chromosome 21. The most well-studied of these is APP, which is the precursor to the Aβ peptide, implicated as a possible cause of AD (Hardy 2006). In AD, mutations in APP and one of its processing enzymes, γ-secretase, discussed below, can cause an accumulation of Aβ similar to that seen in DS. Mutations in the catalytic component of the γ-secretase complex, known as presenilin, and discussed thoroughly in Strooper et al. (2012) (De Strooper, Iwatsubo et al. 2012), is one of the main causes of familial AD (Levy-Lahad, Wasco et al. 1995, Rogaev, Sherrington et al. 1995, Sherrington, Rogaev et al. 1995, 1995). These genetic mutations are the driving force behind familial forms of AD, which often have a much earlier age of onset than sporadic AD (Levy-Lahad, Wasco et al. 1995, Rogaev, Sherrington et al. 1995, Sherrington, Rogaev et al. 1995). In cases of familial AD and in DS, Aβ accumulates throughout affected individuals' lifetimes, which causes AD pathology to develop earlier than in the sporadic AD population. However, in DS the accumulation of Aβ is caused by an overexpression of APP rather than a mutation in the gene itself.

There are numerous ways that APP is processed in neurons, but the crucial processing step for development of AD occurs at the cell surface. It is here that APP is either proteolyzed by enzymes, preventing the production of the Aβ fragment (Sisodia 1992), or is cleaved to form Aβ. The amyloidogenic pathway of APP involves an initial cleavage by β-secretase, or BACE1. This is followed by an additional cleavage by γ-secretase, generating an amyloid precursor protein intracellular domain (AICD) fragment along with Aβ. In DS, the overexpression of APP at the cell surface causes an increase in

levels of Aβ, which overwhelms clearance mechanisms, leading to accumulation of Aβ in the brain (Mann and Esiri 1989).

The initial cleavage by BACE1 is thought to be the rate-limiting step in Aβ production. In sporadic AD, both BACE1 levels and enzymatic activity increase in regions where Aβ deposition occurs (Fukumoto, Cheung et al. 2002, Yang, Lindholm et al. 2003). BACE1 activity increases with age in DS along with increased production of β-C terminal fragments of APP (Nistor, Don et al. 2007). Relevant to DS is BACE2, a protein homologous to BACE1, which is located on chromosome 21. BACE2 can cleave APP (Farzan 2000) and studies have shown that BACE1 and BACE2 levels are highly correlated in the brain (Holler, Webb et al. 2012). Despite this, the function of BACE2 in the development of dementia remains elusive. Studies show that despite an increase in BACE2 mRNA levels in DS (Holler, Webb et al. 2012), post-transcriptional modifications either cause an increase in degradation of BACE2 protein or block translation to prevent overexpression of BACE2 protein (Barbiero 2003, Cheon, Dierssen et al. 2008). Additionally, several groups have reported that BACE2 cleaves in the middle of the Aβ domain, causing a reduction in the amount of Aβ produced following cleavage (Fluhrer, Capell et al. 2002, Sun, He et al. 2006). Therefore, it is unlikely that BACE2 contributes to Aβ deposition in DS, although further mechanistic studies on BACE2 and APP are needed. Indeed, evidence from partial trisomy cases suggest that overexpression of APP is critical to the development of AD neuropathology in DS (Prasher, Farrer et al. 1998, Doran, Keator et al. 2017).

3.1.1.2 - Tau and DS: DYRK1A and RCAN

Studies in partial trisomy 21 cases suggest that there is a critical region on chromosome 21 that when triplicated is sufficient for developing DS features (Olson, Roper et al. 2007, Korbel, Tirosh-Wagner et al. 2009). This region, known as the Down syndrome critical region (DSCR, 21q22.1-22.3), contains more than 30 presumed genes including the dual-specificity tyrosine-(Y)-regulated kinase 1A gene (Dyrk1A) and regulator of calcineurin 1 (RCAN1) (Rahmani, Blouin et al. 1989, Korenberg, Kawashima et al. 1990, Guimera, Casas et al. 1996, Shindoh, Kudoh et al. 1996, Song, Sternberg et al. 1996, Jung, Park et al. 2011). Dyrk1A is a highly conserved serine-threonine kinase that regulates a wide variety of neurodevelopmental processes (Tejedor and Hammerle 2011). Dyrk1A is expressed in neurons across an individual's lifetime, including fetal development, suggesting that it plays an important role in neuron development, maturation, and aging (Wegiel, Kuchna et al. 2004). RCAN1 is an endogenous regulator of calcineurin (Caln), a calcium and calmodulin dependent serine/threonine protein phosphatase (Rusnak and Mertz 2000). Overexpression of RCAN1 in cell and animal models results in inhibition of pathways that are controlled by the transcription factor nuclear factor of activated T-cells (NFAT) (Davies, Ermak et al. 2007) and knockdown or overexpression of the *Drosophila melanogaster* RCAN1 homolog causes severe intellectual disability, indicating that RCAN1 plays a role in the learning deficits seen in DS (Chang, Shi et al. 2003).

In DS, Dyrk1A is expressed at 1.5-fold higher protein and activity levels in the brain (Guimera, Casas et al. 1999, Ferrer, Barrachina et al. 2005, Kimura, Kamino et al. 2007). This overexpression and increased activity has significant implications for brain development in individuals with DS, including dysregulation in the differentiation of

neuronal progenitor cells (Tejedor and Hammerle 2011). Dyrk1A acts in synergy with another gene on chromosome 21, RCAN1, to impact some phenotypic characteristics of DS, including the development of AD pathology (Jung, Park et al. 2011). Dyrka1A interacts and phosphorylates RCAN1, which primes RCAN1 to inhibit Caln, leading to inhibition of NFAT, a nuclear transcription factor that is crucial for mammalian development (Arron, Winslow et al. 2006). Inhibition by Caln causes NFAT to leave the cytoplasm, inactivating its transcriptional activity (Gwack, Sharma et al. 2006). This, in turn, results in learning and cognitive deficits and motor skill impairments that are often associated with individuals with DS, as exemplified by both DYRK1A overexpressing mice and NFAT knockout mice (Altafaj, Dierssen et al. 2001, Martinez de Lagran, Altafaj et al. 2004, Ahn, Jeong et al. 2006, Arron, Winslow et al. 2006, Altafaj, Ortiz-Abalia et al. 2008).

Dyrk1A also plays a key role in aging in people with DS through phosphorylation of tau. The role of tau in AD is discussed further below, but briefly, the hyperphosphorylation of tau is a major contributor to neurofibrillary pathology that may lead to neurodegeneration and dementia (Gong, Liu et al. 2006). Dyrk1A phosphorylates tau at Thr212 and several other sites, which promotes tau to be further phosphorylated by GSK-3β (Liu, Liang et al. 2008). This leads to a hyperphosphorylated form of tau, which causes the loss of tau's biological function and stimulates the formation of neurotoxic paired helical filaments indicative of neurofibrillary tangles.

Additionally, studies show that overexpression of Dyrk1A regulates alternative splicing of endogenous tau exon 10. This alternative splicing of exon 10 leads to two different tau isoforms: one with three microtubule-binding repeats (3R) and one with four

microtubule binding repeats (4R) (Goedert, Spillantini et al. 1989). In a non-diseased adult brain, there are equal expression levels of 3R-tau and 4R-tau. However, imbalances in the 3R:4R ratio are associated with several tauopathies, including frontotemporal dementia with Parkinsonism, frontotemporal lobar degeneration, progressive supranuclear palsy, and cortico-basal degeneration (Ishizawa, Ko et al. 2002). In T65Dn mice, a common mouse model of DS, Dyrk1A overexpression causes dysregulation of tau exon 10 splicing, causing an increase in 3R tau and a decrease in 4R tau, possibly contributing to the cognitive decline seen in individuals with DS (Wegiel, Kaczmarski et al. 2011).

3.1.1.3 - Inflammation and DS: S100β

S100β is a small astrocyte-derived protein implicated in the growth of neurons and proliferation and differentiation of glia (Selinfreund, Barger et al. 1990). This gene for this cytokine is located on chromosome 21 and is elevated throughout the lives of individuals with DS (Royston, McKenzie et al. 1999), with twice the number of astrocytes expressing S100β in DS patients at all ages compared to controls. While S100β is vital for growth and development of the central nervous system, elevated levels have significant consequences. Overexpression of S100β causes abnormal growth of neuronal processes (Kligman and Marshak 1985, Reeves, Yao et al. 1994) and is commonly found in astrocytes associated with Aβ plaques (Griffin 1989, Mrak, Sheng et al. 1996). Transgenic mice that overexpress S100β have increased dendritic density in the hippocampus when young, but density of the dendrites was drastically lower at one year of age (Whitaker-Azmitia, Wingate et al. 1997). Similarly, human fetuses with DS have initially normal dendritic morphology, but dendritic development becomes stunted in infants with DS (Takashima, Becker et al. 1981, Takashima, Iida et al. 1994). In a stem cell model of DS, S100β staining is more extensive

than controls and astrocytes exhibited an activated morphology, with more branching and thicker branches (Chen, Jiang et al. 2014). S100β causes astroglial dysfunction and oxidative stress in induced pluripotent stem cells (iPSCs) derived from DS patients (Chen, Jiang et al. 2014). This decrease in dendrites corresponds well with learning and memory deficits, showing that S100β may play a significant role in the progression of cognitive decline in aging individuals with DS.

In addition to S100β, there are several other genes on chromosome 21 associated with inflammation including: CXADR, ADAMTS1, ADAMTS5, TIAM1, IFNAR1, IFNAR2, IFNGR2, RIPK4, CBS and PRMT2 (Wilcock and Griffin 2013). In a review of the potential role of these genes in the development of AD in people with DS, Wilcock and Griffin (2013) suggest that cytokines may drive some of the AD pathogenesis. Further, the triplication of inflammatory genes overexpressed due to trisomy 21 may lead to a complex immune profile in DS that can lead to accelerated AD (Wilcock 2012). Indeed, as will be discussed later, there appears to be a neuroinflammatory profile that is unique to DS individuals as they age.

3.1.1.4 - Other Genes Associated with AD in DS

Although we have focused on only a few genes thought to be important for AD in DS, there are other researchers who have discussed genes associated with oxidative stress and mitochondrial dysfunction (Lott, Head et al. 2006, Pagano and Castello 2012), compensatory genes (Head, Lott et al. 2007) and additional genes that may underlie cognitive deficits at younger ages (Sturgeon, Le et al. 2012) all present on chromosome 21. It is clear that there are multiple possible mechanisms and pathways that either singly or in combination may lead to early onset AD in people with DS.

3.1.2 - AD Pathology in DS

As mentioned in the introduction, people with DS develop AD neuropathology at an earlier age than that observed in sporadic AD. The key features of AD in DS, senile plaques, neurofibrillary tangles and neuron loss will be described. There is also a comprehensive recent review of the neuropathological hallmarks of AD in DS (Head, Lott et al. 2016).

3.1.2.1 - Aβ and Senile Plaques

Due to the triplication of APP, individuals with DS produce Aβ throughout the course of their lives. Similar to sporadic AD, $A\beta_{42}$ is the most prevalent form of Aβ and appears in the cortex before $A\beta_{40}$ (Iwatsubo, Mann et al. 1995). In fact, the first marker of AD pathology in individuals with DS is the presence of senile plaques, which usually develop over the age of 30 years (Mann and Esiri 1989) (Figure 3.1A-B), although some diffuse pathology is found in younger individuals. In an autopsy study of 29 people with DS ranging in age from 3 to 73 years old, $A\beta_{42}$ appeared earliest at age 12 years in temporal cortex and was present in about half of cases under the age of 30 years (Lemere, Blusztajn et al. 1996). Interestingly, no $A\beta_{40}$ was found in subjects under 29 years old. The precise pattern of Aβ deposition is not fully understood, although there is evidence in people with DS that Aβ accumulates in clusters, starting in the superficial layers of the entorhinal and frontal cortices. As an individual ages and the disease progresses, Aβ then spreads into deeper cortical layers (Azizeh, Head et al. 2000). Another study found that Aβ appears as early as 8 years in the hippocampal and parahippocampal region, which then causes

seeding and progressive Aβ deposition with age (Lemere, Blusztajn et al. 1996, Leverenz and Raskind 1998).

With the exciting development of positron emission tomography using ligands that bind to Aβ *in vivo*, more has been learned about the age of onset and clinical correlations of Aβ in DS. The first of these ligands, Pittsburgh Compound B (PiB), was a breakthrough in the AD field (Cohen and Klunk 2014). Studies using PiB binding show a similar Aβ deposition pattern as that observed at autopsy. PiB binding, representing Aβ accumulation, becomes evident around age 40 years (notably much later than seen in immunohistochemistry studies), usually beginning in the striatum (Handen, Cohen et al. 2012, Hartley, Handen et al. 2014, Lao, Betthauser et al. 2016, Lao, Handen et al. 2017). Following PIB binding in the striatum, PiB binding begins to appear in the rostral prefrontal-cingulo-parietal regions, then caudal frontal, rostral temporal, primary sensorimotor and occipital, and finally mediotemporal regions and the rest of the basal ganglia (Annus, Wilson et al. 2016). Interestingly, this initial pattern of striatal PiB binding replicates what is seen in familial AD (Koivunen, Verkkoniemi et al. 2008), but once Aβ pathology is present, it follows similar patterns of deposition to sporadic AD (Klunk, Engler et al. 2004). PiB binding is observed in people with DS who are not demented, suggesting a preclinical phase of the disease (Hartley, Handen et al. 2014, Lao, Betthauser et al. 2016). Autopsy studies of PiB binding reveal an affinity for both senile plaques and for CAA (LeVine, Spielmann et al. 2017). Additionally, peripheral changes in plasma Aβ may reflect the presence of plaques in AD (Mayeux, Honig et al. 2003). Individuals with sporadic AD have significantly higher Aβ42, not Aβ40, levels in plasma during early stages of AD, but these levels decline thereafter as amyloid plaque pathology increases (Mayeux,

Honig et al. 2003). In DS, both Aβ40 and Aβ42 are elevated in the plasma (Mehta 1998), but these levels are even higher in those individuals who develop dementia (Cavani, Tamaoka et al. 2000, Schupf, Patel et al. 2001)

3.1.2.2 - Post translationally modified Aβ

Full-length Aβ peptides are more commonly associated with disease, but heterogeneity in the N-terminus of Aβ plaques has been known since the initial purification of an amyloid plaque core (Masters 1985). Post-translational modifications result in truncated forms of Aβ that are commonly found in both AD and DS. The most common APP cleavage site for Aβ species is Asp 1, although 13 other N-terminal starting points have been described (Haass, Schlossmacher et al. 1992, Busciglio, Gabuzda et al. 1993, Haass, Hung et al. 1994). It is thought that the most toxic form of Aβ is pyroglutamate 3, which seeds oligomerization and further deposition of Aβ (Piccini, Russo et al. 2005, Schlenzig, Manhart et al. 2009, Nussbaum, Schilling et al. 2012). Pyroglutamate 3 Aβ is formed by an initial truncation of the first two amino acids of Aβ, followed by a cyclization of glutamate residues 3 or 11 by glutaminyl cyclase (Saido 1995, Lemere, Blusztajn et al. 1996, Schilling, Hoffmann et al. 2004). At this time, it is unknown whether other N-terminal Aβ species have any pathological relevance, although it has been hypothesized that these differences could play a role in AD pathogenesis through destabilization of the cytoskeleton in neuronal processes (Larner 1999). In DS, Aβ peptides starting at pyroglutamate 3 or aspartate 1 have been detected as early as age 29, but not in younger cases. Once these isoforms were present, pyroglutamate 3 was detected in much larger quantities than aspartate 1, a pattern than appeared to increase with age (Saido 1995, Lemere, Blusztajn et al. 1996). In addition to pyroglutamate modifications, racemized,

isomerized and oxidized Aβ are also found in DS brain (Fonseca, Head et al. 1999, Azizeh, Head et al. 2000, Head, Garzon-Rodriguez et al. 2001) and increase in age, providing insights into the earliest sites of deposition.

3.1.2.3 - Neurofibrillary Tangles

Similar to sporadic AD, individuals with DS develop NFTs in addition to Aβ deposition (Burger and Vogel 1973)(Figure 3.1C-D). NFTs accumulate in a similar pattern to that seen in AD, with pathology appearing after plaques and the majority of NFT deposition occurring in the hippocampus, entorhinal cortex, and neocortex (Hof, Bouras et al. 1995, Hyman 1995). Given that NFTs develop after the deposition of Aβ suggests that Aβ may drive this pathology (Oddo, Caccamo et al. 2008). NFTs are composed primarily of the hyperphosphorylated microtubule-associated tau protein, which forms bundles of paired helical filaments and straight filaments in diseased neurons (Selkoe 1991, Azizeh, Head et al. 2000). The presence of hyperphosphorylated tau is thought to cause NFTs to form, which eventually leads to neuronal death.

Several studies suggest that the *DYRK1A* gene, located on chromosome 21, discussed earlier in this chapter, plays a major role in driving NFT pathogenesis in DS (Ryoo, Jeong et al. 2007, Wegiel, Dowjat et al. 2008, Wegiel, Kaczmarski et al. 2011). DYRK1A phosphorylates tau, which "primes" tau for further phosphorylation by making it a better substrate for GSK3β (Liu, Liang et al. 2008). As described previously, DYRK1A regulates alternative splicing of tau, causing an increase in the ratio of 3R:4R tau, which is known to cause neurodegeneration, memory deficits, and anxiety (Wegiel, Kaczmarski et al. 2011, Yin, Jin et al. 2017). Another possible cause for the early presence of NFTs in DS is the overexpression of RCAN1, which is located on chromosome 21. RCAN1 levels are

already higher in individuals with AD and levels increase in the brain with normal aging. RCAN1 has been shown to both inhibit and activate Caln expression (Rothermel, Vega et al. 2000, Liu, Busby et al. 2009), although its role appears to be inhibitory in AD. RCAN1 inhibits Caln phosphatase activity, increasing GSK3β levels, which enhances tau phosphorylation (Cardenas, Ardiles et al. 2012, Wong, Levenga et al. 2015). Interestingly, both RCAN1 and DYRK1A are upregulated by $A\beta_{42}$ and it is therefore likely that all three of these overexpressed genes interact in some way to contribute to neurodegeneration (Kimura, Kamino et al. 2007, Lloret, Badia et al. 2011).

3.1.2.4 - Braak Staging of AD neuropathology in DS

Assessment of AD neuropathology at autopsy involves protocols that characterize the severity and distribution of senile plaque and NFT pathology. The most commonly used scoring system, Braak staging, describes the extent and location of Aβ and NFT at autopsy (Braak and Braak 1991). NFT pathology is scored on a scale of I-VI. As an aside, new developments in the neuroimaging field include tau ligands for PET that will allow researchers to determine Braak stage NFTs *in vivo*(Schwarz, Yu et al. 2016). Recently, Scholl et al. published their research on *in vivo* Braak staging using a PET ligand with high affinity for paired-helical filaments, or insoluble fibers composed of hyperphosphorylated tau (Scholl, Lockhart et al. 2016). Although this is a still ongoing study, this paper showed that patterns of tau PET tracer retention corresponded well with Braak staging. This provides a promising future for evaluating AD severity *in vivo*, including individuals with DS. There is currently only one on-going clinical trial, according to ClinicalTrials.gov, using tau PET imaging in individuals with DS and AD. This study, out of St. Joseph's Hospital and Medical Center in Phoenix, AZ, is intended to examine progression of AD

pathology in aging individuals with DS. Another pilot study recently completed out of the University of California, San Diego, examined tau PET as part of an AD in DS biomarker study (Rafii et al., AAIC meeting, July 2017, Abstract#F4-02-02). There is one additional study located at NIA that examined tau PET imaging in DS participants, but the status of this trial is unknown and there has been no update posted since 2009.

As important as Braak staging of Aβ and NFTS is in evaluating disease severity and progression, Braak staging has not been systematically conducted in brains from people with DS. This makes post-mortem evaluation difficult, as pathology may develop and progresses differently in individuals with DS compared to sporadic AD. Therefore, it is our belief that this represents a significant gap of knowledge in the field of aging in DS and needs to be addressed. However, in vivo imaging of Aβ and tau will significantly advance our understanding of AD progression and clinical correlations in people with DS.

3.1.2.5 - Neuron Loss

Aging and disease associated neuronal loss in DS occurs in a similar pattern to that of AD. Magnetic resonance imaging (MRI) studies in DS have consistently reported significant atrophy with age (Teipel and Hampel 2006). As individuals with DS age, neuronal loss occurs in the temporal cortex, hippocampus, and entorhinal cortex (Ball and Nuttall 1980, Hyman 1995). One particular area with a large amount of neuron loss both in AD and DS is the locus coeruleus (LC). The LC is a small nucleus in the pons, containing neurons with extensive projections, including the spinal cord, cerebellum, hypothalamus, the thalamic relay nuclei, the amygdala, the basal telencephalon, and the cortex. Neuron loss in this region follows an identical pattern to that of AD, with the largest burden of cell

loss located rostrally in LC, with full topography of LC cell loss described in Marcyniuk et. al, 1986 (Marcyniuk, Mann et al. 1986).

The nucleus basalis of Meynert (nbM) is another common area of neuron loss in both AD and DS. Individuals with DS have lower neuronal counts in the nbM than controls across all ages, as individuals with DS and AD having even lower counts than subjects with sporadic AD (Casanova, Walker et al. 1985). This may be because individuals with DS have fewer neurons to begin with, therefore they have less "neuronal reserve" to lose. However, brain weight has is not statistically significantly correlated to number of neurons in the nbM. Therefore, it is likely that there is a reduction in the number of nbM neurons in individuals with DS, and that this burden impacts elderly individuals with DS more severely.

3.1.2.6 - Cerebrovascular Pathology

VCID is widely considered the second most common form of dementia after AD and VCID is estimated to be co-morbid in as many as 40-50% of AD cases(Bowler, Munoz et al. 1998, Kammoun, Gold et al. 2000, Langa, Foster et al. 2004). The most obvious hallmark of VCID is stroke, but the majority of people with VCID have much more subtle pathophysiologies including multiple small strokes, chronic cerebral hypoperfusion, cerebrovascular occlusions, cerebral microhemorrhages, and CAA (Levine and Langa 2011, Wilcock, Schmitt et al. 2016). Vascular factors likely lower the age of onset of dementia and accelerate the progression of neurodegenerative diseases, but it is still unknown how AD and VCID mechanisms interact.

Although VCID is a major contributing factor to dementia, particularly in sporadic AD, it is relatively unexplored in DS. Individuals with DS represent a unique group to study the cerebrovascular features of aging and AD in a setting of more limited systemic vascular risk factors. In a previous study of adults with DS ranging from 40-66 years, there was an absence of atheroma and DS individuals had lower blood pressure than age matched controls (Murdoch 1977). Lower blood pressure is consistently found in children (Rodrigues, Coelho et al. 2011) and younger adults with DS (Draheim, McCubbin et al. 2002, Draheim, Geijer et al. 2010). Additionally, unlike the general population, blood pressure does not increase with age in DS. The lower risk of atheroma in people with DS compared to controls has been reported in other studies as well (Brattstrom 1987, Yla-Herttuala 1989).

As discussed previously, individuals with DS develop significant AD pathology, including NFTs and Aβ plaques. In addition to the formation of amyloid plaques, people with DS often have a buildup of amyloid in medium to smaller size arteries, known as CAA (Reijmer, van Veluw et al. 2016). CAA causes changes to the microvasculature and is a major risk factor for both micro and macrohemorrhages (Vinters 1987). CAA in DS is consistently reported in the literature (Belza and Urich 1986, Donahue and Steinfeld 1998, Naito, Sekijima et al. 2008, Mendel, Bertrand et al. 2010)(Figure 3.1B, Figure 3.2B), and a recently published study shows that individuals with DS have more severe CAA observed by MRI than people with both sporadic and autosomal dominant AD (Carmona-Iragui, Balasa et al. 2017). Carriers of the APOE ε4 allele are known to be at a higher risk for CAA in the general population and in individuals with AD (Greenberg, Briggs et al. 1996, Rannikmae, Samarasekera et al. 2013). However, there does not appear to be a similar risk

in individuals with DS (Carmona-Iragui, Balasa et al. 2017). It has also been recently reported that there is a 38.5% frequency for lobar microbleeds in symptomatic DS and a 15.4% frequency for intracerebral hemorrhage. This is higher than the number of microbleeds found in late-onset AD, which ranged from 20%-30% (Carmona-Iragui, Balasa et al. 2017). We have also observed microhemorrhages in aged DS brain that may correspond to CAA (Figure 3.2C-D). Thus, adults with DS represent an important cohort to study cerebrovascular co-morbidities because of their unique characteristics: atheroma-free model and lower blood pressure but with significant CAA.

Another important contributor to cerebrovascular pathology in DS is Moyamoya disease. Moyamoya disease is a chronic cerebrovascular occlusion disorder, where arties in the basal ganglia become blocked, often causing strokes and re-occurring transient ischemic attacks (Suzuki and Takaku 1969). Children with DS are thought to be predisposed to develop Moyamoya disease, and there is a 26-fold greater prevalence of DS in children with co-existing Moyamoya disease than prevalence of DS among all births (Kainth, Chaudhry et al. 2013). The connection between Moyamoya disease and aging in DS is currently unknown, although it has been suggested that genes on chromosome 21 dealing with arterial physiology may play a role (Cramer, Robertson et al. 1996). Thus, the contribution of vascular factors to AD pathogenesis in DS is an area that requires further attention and resources.

3.1.2.7 - White Matter Degeneration

Cerebrovascular neuropathology may lead to abnormalities in white matter of the brain (Back, Kroenke et al. 2011, Chao, Decarli et al. 2013, Erten-Lyons, Woltjer et al. 2013). In addition to AD and the cerebrovascular pathology discussed above, there is evidence that individuals with DS have increased white matter degeneration. Neuroanatomical MRI studies show that adults with DS have lower white matter volumes than non-DS controls, as measured by voxel-based morphometry (White, Alkire et al. 2003). Two recent publications have examined white matter integrity using diffusion tensor imaging (DTI) in individuals with DS. DTI measures the diffusion of water molecules in neural tissue to give a microstructural read on the integrity of the white matter (Basser, Pajevic et al. 2000). Both of these imaging studies found that individuals with DS have impaired white matter tract integrity compared to age-matched non-DS controls even in younger individuals (35 years) (Powell, Caban-Holt et al. 2014, Fenoll, Pujol et al. 2017). Additionally, one of these groups found that white matter integrity losses were more exaggerated in those DS individuals with dementia, compared to non-demented persons with DS (Powell, Caban-Holt et al. 2014). There was no significant difference in white matter degeneration with age in the more recent study of nondemented individuals with DS (Fenoll, Pujol et al. 2017). This suggests that early changes in white matter integrity in individuals with DS may be due in part to developmental differences and that these changes become more severe with the development of AD neuropathology.

Interestingly, both DTI studies showed white matter impairments in the frontal cortex, indicating that this region may be vulnerable to structural changes as AD develops in DS. This is consistent with a prior structural MRI study, which showed lower frontal volume with age in individuals with DS (Teipel, Alexander et al. 2004). To our knowledge,

however, there have been no longitudinal studies to describe decline in white matter integrity with aging or with the development of dementia in DS and this represents a gap in our knowledge.

3.1.2.8 - Neuroinflammation

There have been two recent reviews that describe the role of neuroinflammation in DS (Wilcock 2012, Wilcock and Griffin 2013). As mentioned briefly, there are several genes on chromosome 21 that are associated with immune system function. In autopsy studies of DS brains, neuroinflammation appears in association with AD neuropathology. For example, C1q, the first protein in the classical compliment pathway, a cascade pathway leading to inflammation, is increased in DS brains with AD neuropathology (Stoltzner 2000, Head, Azizeh et al. 2001). In addition, IL-1β, a key pro-inflammatory cytokine, is increased in DS brain along with increased S100β (Griffin 1989). Microglial cells, key mediators of inflammation in the brain, show interesting morphological changes in DS suggesting degeneration of these cells in association with increase NFT accumulation (Xue and Streit 2011).

In a study by Wilcock and colleagues of DS autopsy cases with a range of ages, RNA levels of several key neuroinflammatory proteins were quantified and revealed a distinct neuroinflammatory profile in the DS brain with AD neuropathology (Wilcock, Hurban et al. 2015). CD86 and FCGR1β were unique increased only in the DS with AD cases, indicating that there may be immune complexes forming in the brains of individuals with DS that can activate microglial cells and toll-like receptors. Increased CD86 and FCGR1β RNA expression has been observed only rarely in sporadic AD. The implications of this study may indicate a novel treatment target for clinical trials in AD in DS.

It is interesting to note that not only central nervous system inflammation may be important for DS but also systemic inflammation. For example, periodontal disease is a frequent peripheral inflammatory condition in people with DS that may also contribute to the development of AD, and may be a modifiable risk factor for this vulnerable cohort (Kamer, Fortea et al. 2016).

3.1.3 - Future Directions

Our understanding of factors involved with AD progression in DS is growing rapidly, and has been significantly accelerated by PET imaging using AD neuropathology-specific ligands. There are fewer autopsy studies in this cohort primarily due to challenges in acquiring tissue and particularly tissue from clinically characterized people. It will be a benefit to better understanding how genes on chromosome 21 contribute to the development of dementia, particularly those involved in neuroinflammation and cerebrovascular disease. As more molecular pathways are identified that are pathologically activated (or suppressed) in DS, we can identify novel targets for interventions. Given the unique and strong age-dependency of AD neuropathology in DS, we also have an exciting opportunity to prevent AD in DS once we identify the pathways that are critical at different age epochs. Indeed, Figure 3.3 shows a summary of our current understanding of various biomarker changes in DS as a function of age. There are currently few clinical trials for AD in DS (Table 3.1)(Ballard, Mobley et al. 2016) emphasizing the need to continue to enroll volunteers into longitudinal aging studies that involve clinical measures, neuroimaging, plasma or cerebrospinal fluid biomarkers and autopsy.

In this study, we hypothesize that individuals with DS will have more MBs relative to sporadic AD and controls and that CAA and MBs are linked in two regions of interest:

the FC and the occipital cortex (OCC). To this end, we initially examined the FC because this region is associated with the earliest signs of dementia in DS as manifested by changes in personality, behavior, and communication (Ball, Holland et al. 2008). An additional rationale for focusing on the frontal lobe is reinforced by our published data showing that adults with DS have decreased white matter integrity and a reduced number of tracts in the FC, all associated with poorer cognition (Powell, Caban-Holt et al. 2014). We also examined the pathology in the OCC because it is a common location of CAA in AD (Jellinger 2002, Nelson, Pious et al. 2013).

3.2 – Materials and Methods

The goal of this study was to quantify the extent of CAA and MBs in an autopsy series. Fixed brain tissue was examined in individuals with DS prior to the development of AD neuropathology as compared to individuals with DS and documented AD neuropathology and brain tissue samples from patients with sporadic AD.

3.2.1 - Tissue Samples

We obtained FC specimens from several sources including the University of California, Irvine, Alzheimer's Disease Research Center, the NIH NeuroBioBank, and the University of Kentucky Alzheimer's Disease Center. We obtained all autopsy tissue from the OCC from the NIH NeuroBioBank. Human tissue collection and handling adhered to the University of Kentucky and/or University of California, Irvine Institutional Review Board guidelines.

Six autopsy groups were included in the study: young controls (YC; age matched to young DS group; OCC: n=10; FC: n=6), middle-aged controls (MC; age matched to

DSAD group; OCC: n=10; FC: n=12), old controls (OC; age-matched to sporadic AD group; OCC: n=6 FC: n=11), DS (OCC: n=11 FC: n=11), DSAD (OCC: n=14 FC: n=9), and sporadic AD (also assessed clinically as being demented) (OCC: n=12 FC: n=10). Since individuals with DSAD come to autopsy at younger ages than those with sporadic AD, we were not able to match for age between these two groups. All control cases were selected to match for post mortem interval (PMI) to the DS, DSAD and AD cases (Tables 3.2 and 3.3). Our groups contained both males and females, but due to the limited availability of cases, we were not able to match for sex across groups. Although the majority of cases from UCI were clinically assessed as being demented, we do not have clinical data for the remaining cases. Thus, the relationship between CAA, MBs and dementia/cognitive status could not be evaluated systematically.

3.2.2 - Immunohistochemical Methods

Fixed tissue was sectioned on a vibratome (Leica Biosystems, Buffalo Grove, IL) at 50 μm. Sequential sections were collected and stored in PBS with 0.02% NaN_3 until used. CAA was visualized by immunohistochemistry for $A\beta_{1\text{-}40}$ (Invitrogen, Camarillo, CA, 1:5000) as described previously(Sarsoza, Saing et al. 2009). Briefly, free-floating sections were pretreated with 90% formic acid for 4 min and then incubated overnight with the primary antibody, incubated with anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA). The signal was amplified and visualized with an avidin-biotin complex peroxidase kit (Vector Laboratories, Burlingame, CA), and 3,3' diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). Sections were mounted on glass slides and coverslipped with Depex mounting media.

3.2.3 - Prussian blue staining

Tissue sections adjacent to the section stained for CAA were selected for Prussian blue staining to visualize MBs as described in previous studies (Wilcock, Rojiani et al. 2004).

3.2.4 - Image analysis for CAA staining

For CAA assessment, we expanded on a previously established method in order to categorize CAA in thick tissue sections (Biffi and Greenberg 2011, Boyle, Yu et al. 2015). For both the FC and OCC, meningeal and parenchymal vessels were scored on a scale from 0 to 4, where 0=no deposition, 1=scattered segmental deposition of amyloid, 2=circumferential deposition in up to 10 vessels, 3=widespread, strong, circumferential deposition in up to 75% of vessels (may include dyshoric changes) 4= deposition in over 75% of region (includes dyshoric changes) (Figure 3.4).

3.2.5 - Image analysis for Prussian blue staining

Ten 6 x 6 micron boxes were drawn in the white and grey matter of images captured using the Aperio ImageScope (v11.1.2.752) software. Positive Prussian blue labeling 2 cell diameters away or less from a blood vessel was counted as a MB, as described in previously published literature (Wilcock, Rojiani et al. 2004, Wilcock, Rojiani et al. 2004, Davis, Giannini et al. 2017). MB counts were totaled across all boxes for each case and averaged across groups.

3.2.6 - Statistical Analysis

Overall group differences in CAA scores were assessed with Fisher's Exact test, while group differences in frequency of MBs was assessed with the Wilcoxon Rank-Sum

test. Five hypotheses were tested: (1) CAA scores are more severe in DS and DSAD vs. control, (2) CAA scores are more severe in DSAD vs. AD, (3) MBs are more frequent in DS and DSAD vs. control, (4) MBs are more frequent in DSAD vs. AD, and (5) severity of CAA is correlated with MB frequency. In general, hypotheses (1) and (2) were tested using ordinal logistic regression, hypotheses (3) and (4) were tested using the Wilcoxon Rank-Sum test, and hypothesis (5) was tested using a binomial negative regression. Since groups were age-matched, analyses were not adjusted for age, except for analyses for hypothesis (5), which was not based on the age-matched groups. CAA was specified as a categorical variable in these analyses. Model fit was assessed based on Deviance/DF; results close to 1.00 were taken to support the adequacy of the negative binomial distribution to model the MB counts. Where sparse or empty cells prevented the ordinal regression model from converging, the CAA score was dichotomized into none vs. any, and exact binary logistic regression was used. For the ordinal regression models, the proportional odds assumption was assessed with the Score test. Statistical significance was set at 0.05. To test hypotheses regarding the association between the extent of MBs and age in DS and in controls, we used a Spearman rank correlation test.

3.3 - Results

3.3.1 - CAA Pathology

In the FC, distribution of the CAA score differed across groups ($p<.0001$) (Figure 3.5E). Using an unadjusted ordinal regression model, DS (DS and DSAD) autopsy cases overall were 11.5 times more likely to have more severe CAA (95% CI 2.0-64.9, $p=0.006$) than specimens from YC and MC, respectively. Similarly, DSAD cases were also 4.5 times more likely (95% CI 0.8-26.5 $p=0.097$) to have more severe CAA than sporadic AD cases.

Likewise, in the OCC, the 6 groups showed significant differences in CAA scores ($p<.0001$) (Figure 3.5F). Since all YC and MC cases had an OCC CAA score of 0, exact binary logistic regression was used to estimate the odds of any CAA in DS overall (DS and DSAD) vs. control (YC and MC); DS cases showed 51 times the odds of any CAA vs. control (lower bound of the 95% CI = 9.7, $p<0.0001$). DSAD cases had a 1.8 times the odds of more severe CAA score than sporadic AD, but this result was not significant (95% CI 0.45, 7.4, $p=0.4$).

3.3.2 - Microhemorrhages

Using the FC section adjacent to that used for CAA quantification, Prussian blue was used to identify MBs. The frequency of MBs varied across groups ($p<0.0001$) (Figure 3.6G). A Wilcoxon Rank-Sum test revealed a significantly higher number of MBs in the FC of DS (DS, DSAD) cases relative to controls (YC, MC) ($p=0.03$) (Figure 3.6G) . However, the number of MBs was similar in DSAD cases relative to sporadic AD ($p=0.82$, Wilcoxon rank test). In the OCC, there were significantly more MBs in DS (DS, DSAD) compared to their age-matched controls ($p=0.02$) (Figure 3.6H). However, we did not find a significant difference in the number of MBs in the DSAD group compared to the AD group ($p=0.43$), similar to the CAA outcomes.

In the FC, there was a significant increase in MBs in DS, and the individual variability of older controls showing MBs increased after 80 years of age (Figure 3.7A). MB in the OCC increased as a function of age in DS cases (Spearman $r=0.83$ $p<.0005$) and also in the control cases (Spearman $r=0.44$ $p=0.02$) (Figure 3.7B).

3.3.3 - Correlation between CAA and MB

In the OCC, the presence of CAA was associated with higher MB counts ($p=0.0004$), however cases with CAA had similar MB counts regardless of the level of CAA. For example, cases with no CAA had an average of 4.5±1.0 MBs, while cases with a CAA level 1 had mean MB = 31.5±13.8 and CAA level 4 had mean MB = 20.0±10.1. This association persisted after adjustment for age and sex ($p<0.0001$). In the FC, this association was characterized by a quasi-linear dose-response relationship ($p=0.0044$), such that predicted mean MBs were lowest in the absence of CAA (2.57±0.94) and increased monotonically until CAA level 3 (mean MB=58.0±85.5). MBs for level 4 CAA were the same as level 3 (56.3±67.8). This association also persisted after adjustment for age and sex ($p<0.0001$).

3.4 - Discussion

CAA was more severe in people with DS (combined DS and DSAD) relative to their aged matched controls, both in the OCC and FC. Further, we found that all individuals with DS, regardless of age, have more severe CAA scores than controls, confirming and extending our previous report (Head, Phelan et al. 2017). Our data indicates that individuals with DSAD have CAA scores that are equally severe in the FC and OC as those in older individuals with sporadic AD, despite an average age difference of 27 years. These results appear to differ from a recent publication on CAA in DS, which indicates that individuals with DSAD have more severe CAA scores than individuals with sporadic AD (Head, Phelan et al. 2017) but this is likely due to quantification techniques. In the current study, only 2 brain regions were considered whereas in the previous study, multiple brain regions were included in the analysis, thus our estimates are likely to be more conservative.

To our knowledge, there have been no systematic studies of the extent of MB in DS as a function of age and AD. Based on our data from the Prussian blue stain, we observed MBs in DSAD cases with a similar frequency with AD cases in both FC and OCC. In both regions, DS cases overall (DS and DSAD) had significantly more frequent MBs than similarly aged controls. MBs are likely driven by the increased severity in CAA scores in both regions as more severe CAA was associated with higher MB counts.

When we plotted the number of MBs in all individuals with DS (DS and DSAD) against age, we found that in both the FC and OCC, the number of MBs increased significantly with age. However, MBs appear earlier in the OCC of DS individuals (during their 30s), than in the FC (during their 40s). This indicates that the OCC is perhaps more vulnerable to CAA and MBs at earlier ages than the FC.

Additionally, we found that our control cases develop MBs with advancing age. This indicates that the DSAD and AD groups having similar numbers of MBs and CAA levels despite a 27 year age difference is worth examining further. The CAA frequency in our DSAD group and AD groups indicate that with the added age difference, the DSAD individuals would likely have much more cerebrovascular pathology. This is something that needs to be taken into consideration, as individuals with DS are now living longer lives.

Our data on autopsy cerebral MBs confirms and extends those of recently published imaging studies, showing that cerebral MBs are common in DSAD using neuroimaging approaches than previously reported (Carmona-Iragui, Balasa et al. 2017). The increase of cerebrovascular burden is notable in DS because this accumulation of pathology is usually

thought to occur in the presence of risk factors from which people with DS are protected. Therefore, the increase in cerebrovascular pathology is independent of hypertension, atherosclerosis, and hyperhomocysteinemia, suggesting CAA may be the underlying cause. It remains unclear exactly how this cerebrovascular pathology contributes to cognitive impairment in individuals with DS. However, the presence of MBs is known to cause impaired executive functioning and contribute to mild cognitive impairment through white matter damage in individuals without DS (Van der Flier and Cordonnier 2012, Akoudad, de Groot et al. 2013).

There are several possible additional consequences of the presence of MBs in DS. Extensive CAA is associated with microhemorrhages and strokes in the general population (Arvanitakis, Capuano et al. 2017, Banerjee, Carare et al. 2017). Intracerebral hemorrhages driven by CAA have been reported in families with APP duplication(Cabrejo, Guyant-Marechal et al. 2006, Rovelet-Lecrux, Hannequin et al. 2006). However, stroke is relatively rare in DS, suggesting possible protective factors in the DS brain (Buss, Fisher et al. 2016). Further, CAA may affect blood vessel function and can lead to impaired cerebrovascular regulation (Grinberg, Korczyn et al. 2012), which in turn would lead to reduced blood flow. Reduced blood flow could impair perivascular clearance of Aβ and additional accumulation of Aβ (Banerjee, Carare et al. 2017). Given that we observe a dramatic rise in MBs after age 30 or 40 years in DS, and that AD neuropathology typically accelerates during this period of the lifespan, we speculate that cerebrovascular pathology contributes to AD pathogenesis (Head, Lott et al. 2016). Serum proteins can leak into the brain parenchyma as a consequence of MBs in DS. Indeed, in an autopsy study by Wilcock and colleagues, the neuroinflammatory phenotype of the DSAD brain reflects that of immune

complexes forming in the brain that can lead to inflammation (Wilcock, Hurban et al. 2015). Thus, CAA and associated MBs in DS may have consequences for brain function in the absence of overt infarcts or strokes.

Moving forward, our data suggests that we need to strongly consider cerebrovascular pathologies when studying adults with DS. It is particularly important as we think about designing clinical trials in this population, especially with all of the anti-Aβ immunotherapy trials that are ongoing or have already concluded (van Dyck 2017). We also need to further examine how cerebrovascular pathologies, such as MBs and CAA, contribute to the development of dementia in individuals with DS. MRI studies will likely play a key role in helping us understand this connection and the clinical significance of these pathologies (Haller, Vernooij et al. 2018).

Table 3.1: Ongoing Clinical Trials for Aging adults with Down syndrome (queried from ClinTrials.Gov).

Study Title	Purpose of Study	Cohort
A Phase Ib Multi-Center, Double-Blind, Randomized, Placebo-Controlled Dose Escalation Study of the Safety, Tolerability and Immunogenicity of ACI-24 in Adults With Down Syndrome	To examine the safety, tolerability, and immunogenicity of the ACI-24 vaccine	Adults with DS, ages 25-45
Nicotinic Treatment of Age-Related Cognitive Decline in Down Syndrome: An Open Label Pilot Trial	To ascertain the safety and tolerabilty of nicotine in DS patients and look for evidence of enhancements in cognitive function.	DS adults with mild cognitive impairment
Phase II Multicenter 16-Week Randomized Double Blind Placebo-Controlled Evaluation of the Efficacy, Tolerability and Safety of Memantine Hydrochloride on Enhancing the Cognitive Abilities of Adolescents and Young Adults With Down Syndrome	To learn if Memantine Hydrochloride can help adolescents and young adults with Down syndrome	Children and Adults with DS, ages 15-32

Table 3.2: Case characteristics for FC tissue obtained from brain banks (N=59).

Characteristic	YC (n=6)	MC (n=12)	OC (n=11)	DS (n=11)	DSAD (n=9)	AD (n=10)
Age at death, y	17.5 (16.1)	52.7 (10.4)	84.5 (5.0)	20.1 (18.7)	53.9 (7.4)	80.6 (8.2)
Male/Female (n/n)	2/4	8/4	4/7	6/5	4/5	8/2
Post Mortem Interval (PMI), h	14.0 (7.4)	14.6 (7.1)	3.9 (3.1)	18.7 (9.3)	8. (7.4)	5.8 (2.9)
Microhemorrhage counts	0	3.3 (6.2)	2.5 (4.0)	2.7 (8.4)	38.4 (45.5)	33.0 (49.1)
CAA Score (n)						
0 – No deposition	6	10	9	9	0	1
1 – Scattered, segmental	0	2	1	1	2	4
2 – Circumferential, ≤ 10 vessels	0	0	1	1	3	4
3 – Widespread, ≤ 75% vessels	0	0	0	0	2	0
4 – Over 75%	0	0	0	0	2	1

Table 3.3: Case characteristics for OCC tissue obtained from brain banks (N=63)

Characteristic	YC (n=10)	MC (n=10)	OC (n=6)	DS (n=11)	DSAD (n=14)	AD (n=12)
Age at death, y	17.2 (12.4)	53.5 (6.7)	78.7 (2.2)	19.9 (14.8)	53.3 (4.5)	79.7 (1.6)
Male/Female (n/n)	8/2	6/4	3/3	8/3	4/10	6/6
Post Mortem Interval (PMI), h	21.5 (3.9)	16.2 (6.8)	9.0 (6.5)	19.7 (6.0)	9.8 (8.6)	7.9 (8.6)
Microhemorrhage counts	1.8 (2.2)	2.9 (3.2)	13.0 (11.0)	2.8 (5.8)	24.1 (22.3)	28.3 (19.3)
CAA Score (n)						
0 – No deposition	10	10	5	8	0	1
1 – Scattered, segmental	0	0	1	3	1	4
2 – Circumferential, ≤ 10 vessels	0	0	0	0	3	1
3 – Widespread, ≤ 75% vessels	0	0	0	0	7	2
4 – Over 75%	0	0	0	0	3	4

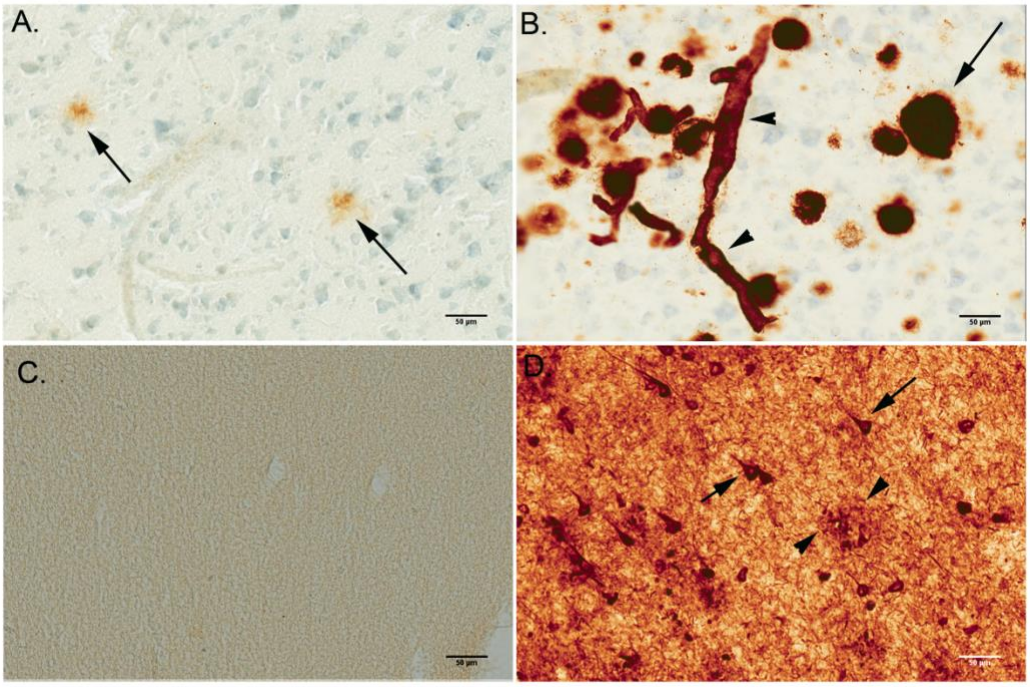

Figure 3.1: AD pathology is present in the brains of elderly individuals with DS. Immunoreactivity for Aβ (6E10) in the posterior cingulate cortex of **(A)** a young individual with DS (age=39) with diffuse amyloid plaques (arrows) and **(B)** an individual with DSAD (age=51) with amyloid plaques (arrows) and CAA (arrowheads). Tau pathology (AT8) also increases with age, as shown in the posterior cingulate cortex of **(C)** a young individual with DS (age=25) and **(D)** an individual with DSAD (age=51) with neuronal AT8 labeling (arrows) and neuropil AT8 labeling (arrowheads).

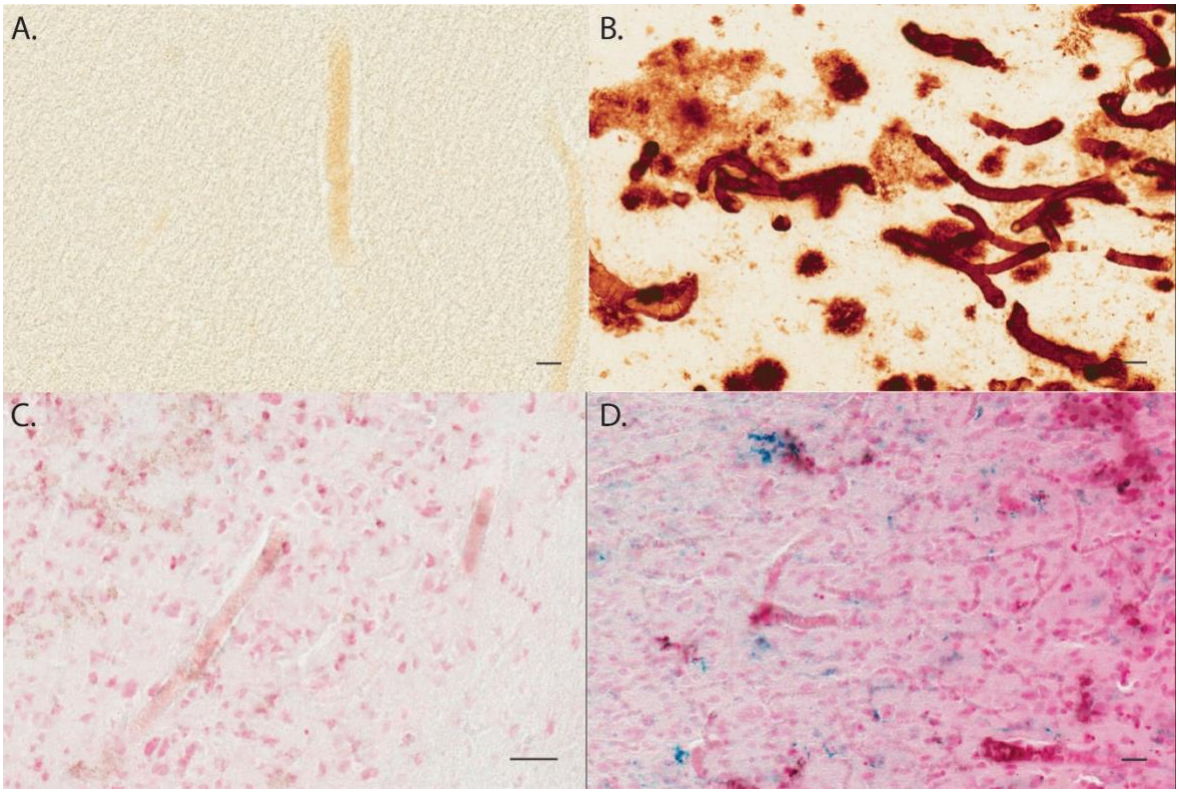

Figure 3.2: Individuals with DSAD have CAA develop microhemorrhages.
$A\beta_{1\text{-}40}$ stain shows that CAA pathology increases in the occipital cortex from a **(A)** young individual with DS (age=25) to **(B)** a person with DSAD (age=51). This increase in CAA correlates with an increase in microhemorrhages, shown with Prussian blue staining, from **(C)** a young subject with DS (age=39) to **(D)** an older subject with DSAD (age=57).

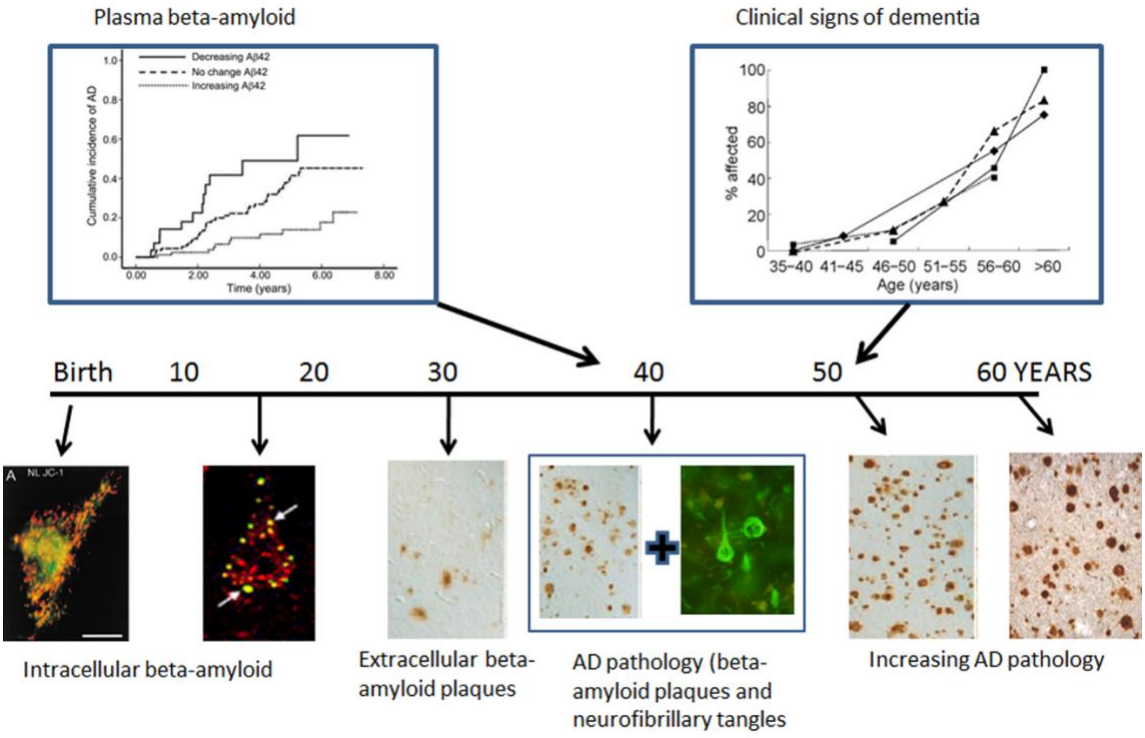

Figure 3.3: Hypothetical sequence of neuropathological events as a function of age in DS.

Reprinted from European Journal of Neurodegenerative Diseases, Vol. 1, number 3, Head, E., Powell, D., Gold, B.T., Schmitt, F.A., Alzheimer's Disease in Down syndrome, 353-363, 2012, with permission from Biolife.

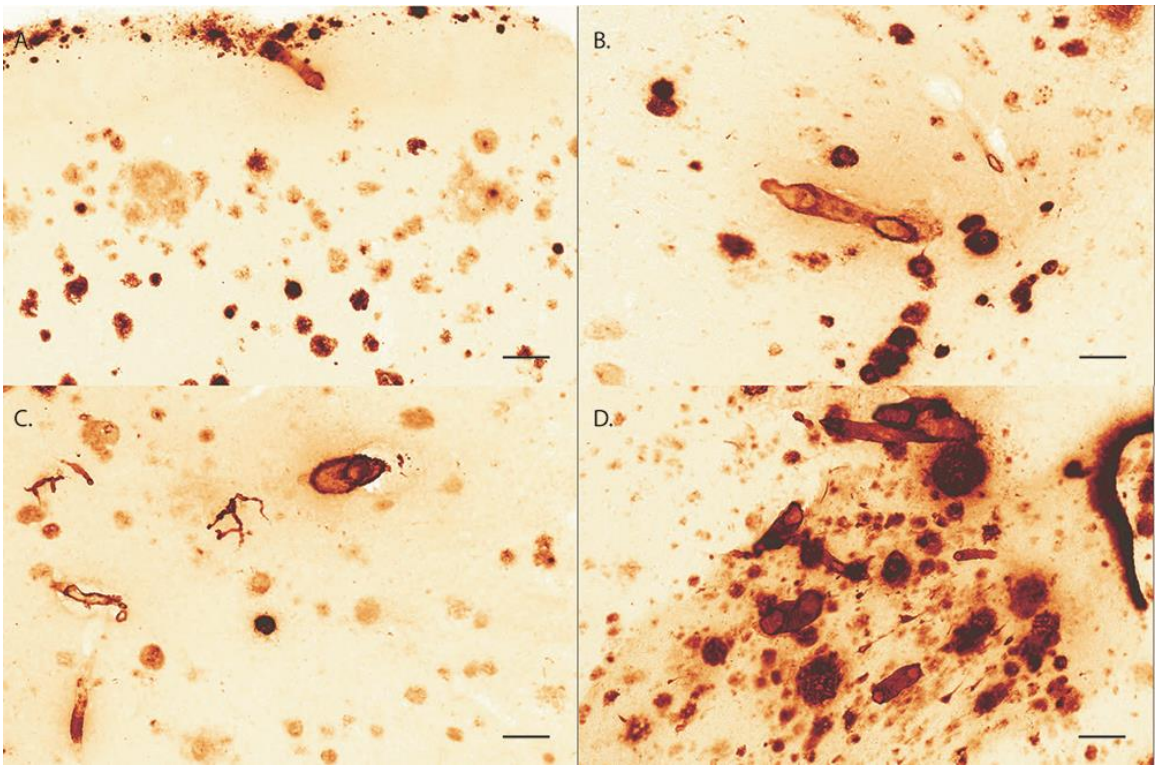

Figure 3.4: Scoring of CAA.
CAA was scored on a scale of 0-4 in the FC and OCC, where 0=no deposition (not pictured), 1=scattered segmental deposition of amyloid **(A)**, 2=circumferential deposition in up to 10 vessels **(B)**, 3=widespread, strong, circumferential deposition in up to 75% of vessels **(C)** 4= deposition in over 75% of region (includes dyshoric changes) **(D)**.

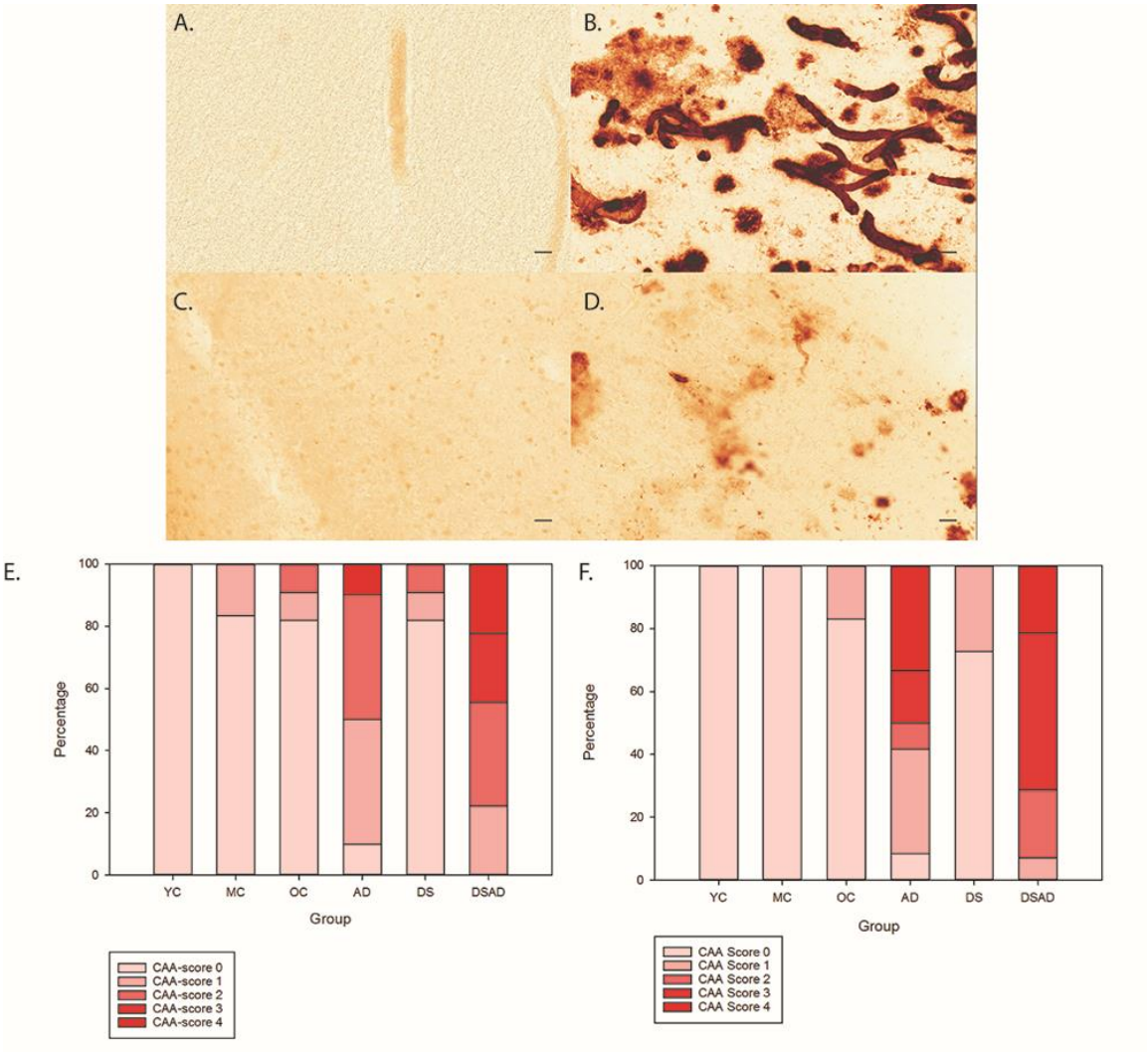

Figure 3.5: DSAD and AD individuals have more severe CAA than age-matched controls.

(A-D) $A\beta_{1\text{-}40}$ (1:500) immunohistochemistry stain; all images taken at 20x magnification and in the OCC **(A)** MC (age=51), **(B)** DSAD (age=51), **(C)** OC (age=78), **(D)** AD (age=78). In the FC **(E)**, The DSAD group did not have significantly higher CAA scores than the AD group (p=0.097), all individuals with DS (DS+DSAD) had higher CAA scores than age matched controls. In the OCC **(F)**, individuals in the DSAD and AD groups had high counts of CAA, although these groups were not significantly different from each other (OR = 9.0, 95% CI 2.3-35.4). Overall, we found that based on an unadjusted ordinal

regression model, people with DS across all ages have more severe CAA scores than their age-matched controls in the OCC (OR=57.5, 95% CI 6.5-509.7).

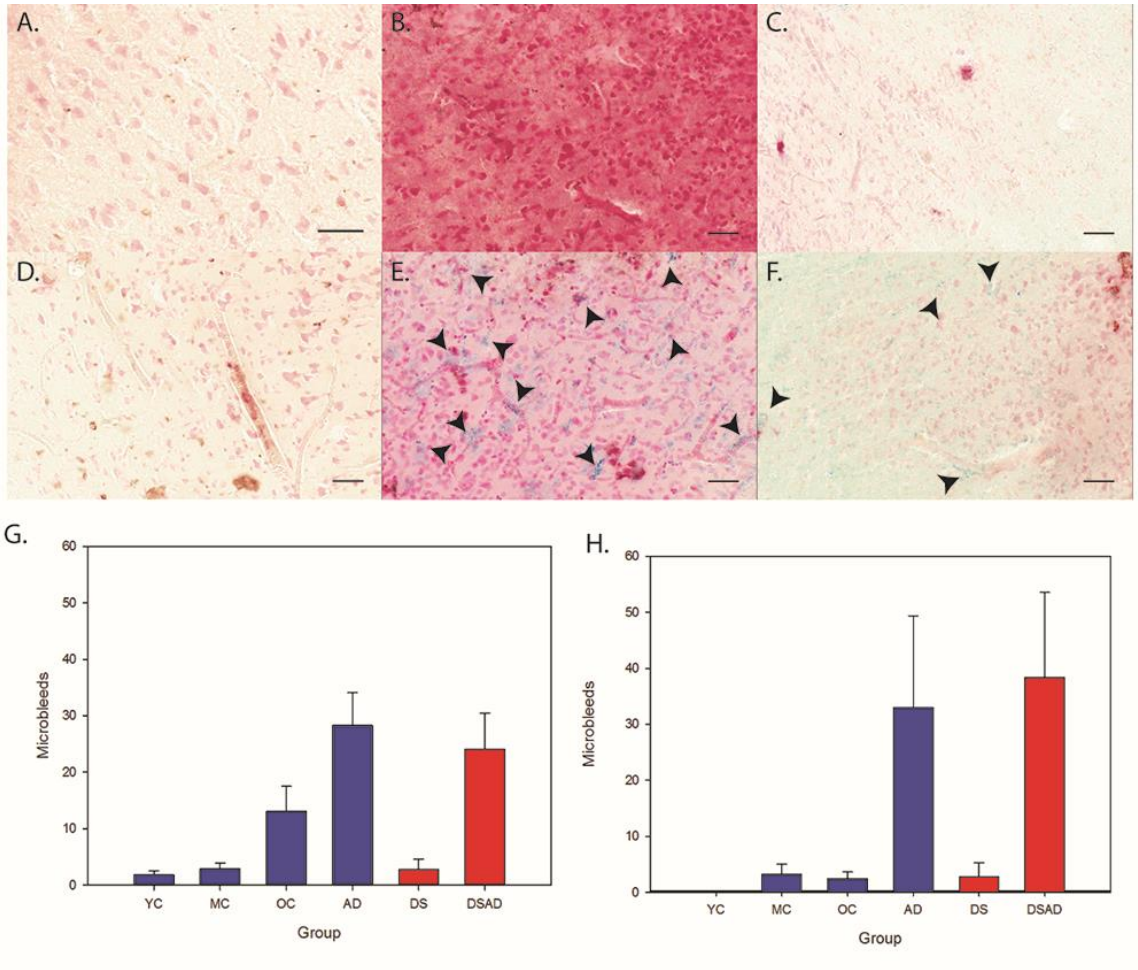

Figure 3.6: Microbleeds increase with age in the FC and OCC.
Prussian blue staining in our control **(A-C)** and non-control **(D-F)** groups, with positive MB labeling marked with arrowheads; all images taken at a 20x magnification. **(A)** YC (Age=39), **(B)** MC (age=56), **(C)** OC (age=76), **(D)** DS (age=2), **(E)** DSAD (age=57), **(F)** AD (age=76). MB counts were highest in the AD and DSAD group for both the FC **(G)** and OCC **(H)**.

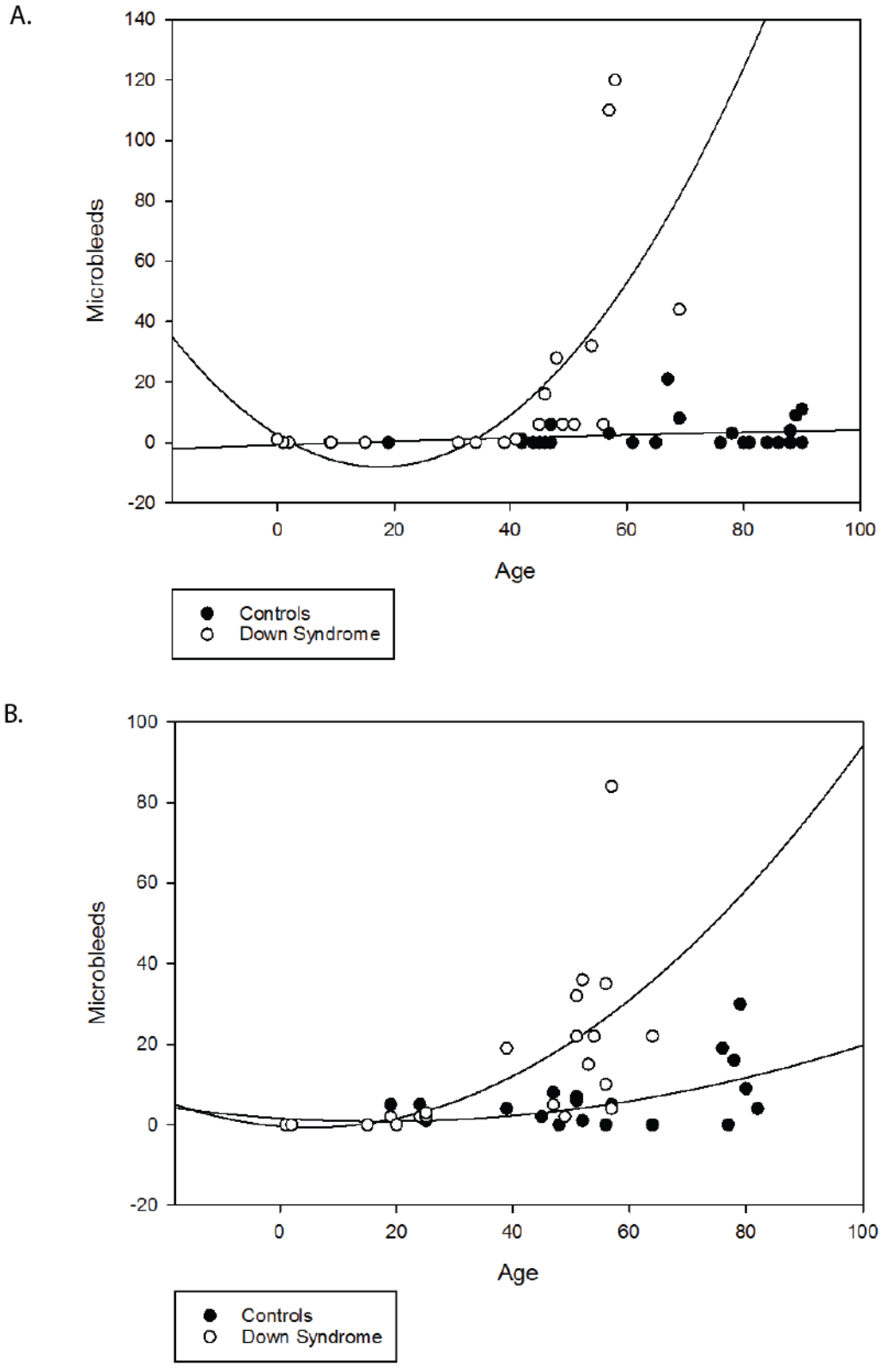

Figure 3.7: Microbleeds increase with age in individuals with Down syndrome.

In the FC **(A)**, the number of MB increases with age, starting around 40 years old, whereas the number of MB in control cases stays fairly consistent. In the OCC **(B)**, MB start increasing in individuals with DS in their 30s, which is about a decade earlier than in the FC. In addition, control cases also appear to have a slight increase in number of MB with age, although not as severely and at a much later age.

Chapter 4: Microglial Phenotypes in the Occipital and Posterior Cingulate Cortex in the Brains of People with Down syndrome with Alzheimer's Disease

4.1 - Introduction

There is growing recognition that microglia, as the primary immune cells in the brain, play a key role in maintaining normal brain function, and concurrently, play a role in disease pathogenesis. Differences in microglial morphology, which are thought to reflect their function, were first described in 1928 (Rio-Hortega and Penfield 1932), when it was noted that ramified microglia were abundant throughout the brain and that their morphology changed in response to injury to take on an amoeboid morphology (Kettenmann, Hanisch et al. 2011). Since this early description, it has become well established that microglial morphology changes in response to disease or injury, and this has been extensively studied in AD (Aguzzi, Barres et al. 2013, Prokop, Miller et al. 2013, Biber, Owens et al. 2014, Derecki, Katzmarski et al. 2014, Mosher and Wyss-Coray 2014).

However, there are few studies describing microglial morphology in DS, particularly in people with DSAD. Griffen et al. were the first to describe differences in microglial morphology in DS, finding significantly more IL-1 activated microglia in brain tissue from young people with DS relative to age-matched controls (Griffin, Stanley et al. 1989). More recent work has confirmed and extended this initial report, showing activated microglia along with other neuroinflammatory markers in the brains of people with DS (Mann, Iwatsubo et al. 1995, Xue and Streit 2011). While activated microglial may suggest an inflammatory cascade similar to that seen in AD (Mann, Iwatsubo et al. 1995, Stoltzner, Grenfell et al. 2000), we have reported a neuroinflammatory phenotype in DSAD that is distinct from sporadic AD (Wilcock, Hurban et al. 2015). Thus, the goal of the current

study was to further characterize microglial morphology as a function of age and AD neuropathology in DS.

4.2 - Methods

4.2.1 - Human Tissue Samples

Fixed tissue samples were acquired from the OCC and the posterior cingulate cortex (PCC) from the University of California, Irvine, Alzheimer's disease Research Center, the NIH NeuroBioBank, and the University of Kentucky Alzheimer's Disease Center. Human tissue collection and handling adhered to the University of Kentucky and/or University of California, Irvine Institutional Review Board guidelines.

Six autopsy groups were included in this study (Tables 4.1 and 4.2): young controls (YC; age-matched to young DS group; PCC:n=10; OCC: n=10), middle-aged controls (MC; age-matched to DSAD group; PCC: n=10; OCC: n=10), old controls (OC; age-matched to AD group; PCC: n=6, OCC=6), DS (PCC: n=10; OCC: n=10), DSAD (PCC: n=17, OCC: n=14), and sporadic AD (PCC: n=6, OCC: n=11). Since people with DSAD come to autopsy at younger ages than those with sporadic AD, we were not able to match for age between these two groups. All control cases were selected to match for PMI to the DS, DSAD and AD cases. Our groups contained both males and females, but due to the limited availability of cases, we were not able to match for sex across groups.

4.2.2 - Immunohistochemistry

Tissue was sectioned on a vibratome (Leica Biosystems, Buffalo Grove, IL) at 50 μm. Sequential

sections were collected and stored in PBS with 0.02% sodium azide until used. Following standard immunohistochemistry protocols and using Iba-1 antibody (Abcam,

Cambridge, MA, 1:800) the sections were incubated in the primary antibody overnight and followed by incubation in an anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA). This was followed by amplification of the signal using an avidin-biotin complex peroxidase kit (Vector Laboratories, Burlingame, CA), and 3,3' diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). Following immunohistochemistry, each tissue section was mounted on a glass slide and coverslipped with Depex mounting media.

4.2.3 - Image Analysis

The Aperio ScanScope XT digital slidescanner was used to scan the entire slide at 20x magnification. The Aperio ImageScope (v11.1.2.752) software was used to draw five random 250 x 250 micron boxes in the white matter (WM) and five random 250 x 250 micron boxes in the gray matter (GM) for each tissue section. Five microglia morphological types were assessed, as defined in previous work (Boche, Perry et al. 2013, Bachstetter, Van Eldik et al. 2015): 1) ramified microglia, which have thin, branched processes to actively "survey" changes in their environments (Kettenmann, Hanisch et al. 2011, Boche, Perry et al. 2013, Bachstetter, Van Eldik et al. 2015); 2) hypertrophic microglia (also known as activated microglia), which may have enlarged, short processes and thicker bodies (Streit, Xue et al. 2014, Bachstetter, Van Eldik et al. 2015); 3) dystrophic microglia that have fragmented or "beaded" processes possibly due to microglial dysfunction due to ageing (Streit, Sammons et al. 2004, Streit 2006, Boche, Perry et al. 2013, Bachstetter, Van Eldik et al. 2015); 4) rod-shaped microglia that have elongated nuclei, few processes, and are most notable in chronic disorders (Boche, Perry et al. 2013, Bachstetter, Van Eldik et al. 2015); and 5) amoeboid microglia which have a round body,

lack processes, and appear in response to acute destruction of central nervous system tissue (Boche, Perry et al. 2013, Bachstetter, Van Eldik et al. 2015).

The number of ramified, hypertrophic, dystrophic, rod-shaped, and amoeboid microglia in each box was counted by hand and averaged for each case, separating WM and GM (Figure 4.1). The categorical analysis used to quantify the microglia expanded upon previously established guidelines and figures (Bachstetter, Norris et al. 2012, Bachstetter, Rowe et al. 2013, Bachstetter, Van Eldik et al. 2015). Counts were made while blind to all samples groups and case histories.

4.2.4 - Statistics

IBM SPSS Statistics Software (Version 24) was used for statistical analysis. Overall group differences were assessed for the GM and WM separately using a 2-way ANOVA on these groups to examine interactions between age group (young vs middle aged) and genotype (DS vs non-DS). We directly compared OC and AD groups by t-tests made a-priori. Individual microglial phenotype counts are provided in Tables 4.1 and 4.2 (Means ± SD).

4.3 - Results

4.3.1 - DS vs Controls

4.3.1.1 - Ramified Microglia

A 2-way ANOVA comparing ramified microglial counts in the GM of the PCC suggests a main effect of age independent of genotype (DS vs CTL) ($F(1,46)=5.827$, $p=0.020$). Figure 4.2A shows that the number of ramified microglial cells is lower overall with age (MC, DSAD) relative to younger cases (YC, DS). Figure 4.2A also shows that

the number of ramified microglial cells is lower overall in DS (DS and DSAD) relative to age-matched controls (YC, MC). Additionally, the DSAD group had significantly fewer ramified microglia in the PCC GM than MC (p=0.037) and significantly less than the DS group (p=0.014). In contrast, a 2-way ANOVA suggests an overall genotype effect (F(1,44)=9.095, p=0.004) but not a significant age effect for ramified microglial cell counts in the OCC GM. In the OCC GM, DS cases had fewer ramified microglial cells than YC (p=0.011), but we did not find any significant differences in ramified microglia between DSAD cases and MC, nor DS and DSAD. In the WM, DSAD cases had the fewest ramified microglial cells relative to all other groups. No differences were observed for the PCC WM counts. Note that the number of ramified microglial cells in the WM in both the PCC and OCC was lower than that observed in the GM for both regions

4.3.1.2 - Hypertrophic Microglia

We did not see any significant differences in the average number of hypertrophic microglia in the GM of either the PCC or OCC with age or genotype (Figure 4.2C).A 2-way ANOVA in the WM of both the PCC (F(1,46)=6.239, p=0.016) and OCC (F(1,44)=4.907, p=0.032) suggests a main effect overall of age. We also see an overall effect of genotype in the OCC (F(1.44)=10.574, p=0.002), although no significant interaction between age and genotype (Figure 4.2D). The DSAD group had significantly higher numbers of hypertrophic microglial cells in the OCC WM relative to MC (p=0.005) and DS (P<.0005). The number of hypertrophic microglial cells appeared similar in the WM of the PCC across groups although the DS and DSAD groups were higher overall than their age-matched controls.

4.3.1.3 - Dystrophic Microglia

A 2-way ANOVA of the dystrophic microglial cell counts in the GM of the PCC suggests an overall effect of age ($F(1,46)=4.488$, $p=0.04$), but not a significant genotype effect. As shown in Figure 4.3A, these differences are largely driven by an increase in the number of dystrophic microglia in the DSAD cases relative to MC ($p=0.019$) and to DS cases ($p=0.007$). In contrast, there were no significant differences in the number of dystrophic microglial cells in the GM of the OCC nor in the WM of either brain region.

4.3.1.4 - Rod-shaped Microglia

As consistent with the literature, few rod-shaped microglia were found in either the PCC (Table 4.1) or OCC (Table 4.2), thus the average counts for rod-shaped microglia are relatively low (Bachstetter, Van Eldik et al. 2015, Bachstetter, Ighodaro et al. 2017). However, we decided to run an analysis on this data because there are few descriptions of this cell type. We did not see any statistically significant group differences in the number of rod-shaped microglia in the GM or WM of either region.

4.3.1.5 - Amoeboid Microglia

Our counts for amoeboid microglia were consistently very low across all of our groups. However, we did find several amoeboid microglia in a subset of our DSAD cases. A 2-way ANOVA in the GM of the PCC suggests a main effect of genotype overall ($F(1,46)=5.645$, $p=0.022$) and an effect of age that is trending towards significance ($F(1,46)=3.868$, $p=0.056$). We also saw a significant interaction between age and genotype ($F(1,46)=6.449$, $p=0.015$). This was driven primarily by the higher average number of amoeboid microglia in the PCC GM of DSAD cases compared to the other groups. No significant differences in the number of amoeboid microglial cells were observed in the

GM of the OCC. Although no significant differences in the number of amoeboid microglial cells were observed in the WM of PCC, the WM of the OCC showed a significant genotype effect ($F(1,44)=5.906$, $p=0.02$) with a 2-way ANOVA, indicating that the number of amoeboid microglial cells is increased overall in DS (DS and DSAD) relative to YC and MC.

4.3.2 - AD vs Controls

Additionally, we see fewer microglia in our cases with AD pathology. However, we did not see many differences in microglia morphology between sporadic AD cases and age-matched controls. In the GM of the OCC, sporadic AD cases had significantly less amoeboid microglia than age-matched controls ($p=0.024$). Conversely, the WM of the same region, sporadic AD cases had significantly more amoeboid microglia than controls ($p=0.024$). We did not see any significant differences in ramified or hypertrophic microglia, as seen in the DSAD cases.

4.3 - Discussion

The aim of this study was to characterize the distribution of several microglial phenotypes in the brains of people with DS. We used autopsy tissue from the PCC, as these are regions where we have seen neurodegenerative changes in DSAD cases (Lin, Powell et al. 2016). Using IBA1 (ionized calcium binding adaptor molecule 1) that labels both resting and activated microglia we were able to identify and quantify different microglia morphologies in our tissue samples. DS and DSAD is associated with a microglial phenotype that distinguishes them from non-DS control cases in the PCC and OCC. Microglial phenotypes vary as a function of brain region, differ between GM and WM of these regions, as well as age and genotype. Consequently, differences in the

phenotype of microglial cells in DS may be due to gene overexpression (lower number of ramified microglial cells in the GM of PCC, higher numbers of hypertrophied microglia in the WM of OCC, higher numbers of amoeboid microglia in the GM of PCC and WM of OCC) or the presence of AD neuropathology, or synergy between both.

The number of ramified microglial cells was significantly lower in the GM of both the PCC and OCC in older people with DS who have AD neuropathology. Further, in the OCC, we see individuals with DS (including both DS and DSAD groups) have fewer ramified microglia than controls in the GM, which supports a previous small study on microglia in DS that showed fewer ramified microglia in individuals with DS than age-matched controls (Xue and Streit 2011). This study independently supports that of a recent paper, which found fewer ramified microglia in individuals with dementia (including AD, hippocampal sclerosis of aging, hippocampal sclerosis of aging with AD, and dementia with lewy bodies) than age matched controls without dementia (Bachstetter, Van Eldik et al. 2015). Interestingly, we did not observe statistically significant lower numbers of ramified microglia in the WM, this may be due to lower numbers overall, leading to a floor effect. Ramified microglia, which have thin, branched processes are thought to be actively "surveying" changes in their environments (Kettenmann, Hanisch et al. 2011, Boche, Perry et al. 2013, Bachstetter, Van Eldik et al. 2015).

We observed significant differences in the number of hypertrophic microglia, in the WM of our DS cases. In the WM of both the PCC and OCC, individuals with DS had significantly more hypertrophic microglia than age matched controls.. We hypothesize that increased numbers of hypertrophied microglial cells in the OCC may be associated with vascular pathology with age in individuals with DS, and the microglia in this region may

be activated in a response to this pathology (Jellinger 2002, Nelson, Pious et al. 2013). This increase in hypertrophic microglia contrasts with other published findings on microglia in DS, which show individuals with DS having fewer hypertrophic microglia than controls (Streit, Sammons et al. 2004, Xue and Streit 2011). However, these studies looked only at the GM in the temporal cortex, a trend, which interestingly, we also observed in the OCC, albeit non-significant. Our findings highlight the importance of examining both the WM and the GM. Hypertrophic microglia (also known as activated microglia), which have enlarged, short processes and thicker bodies are thought to be actively responding to injury (Streit, Xue et al. 2014, Bachstetter, Van Eldik et al. 2015);

It has previously been reported that there is an increase in the number of dystrophic microglia in the temporal cortex of individuals with DS compared to controls (Xue and Streit 2011). Therefore, we hypothesized that we would see more dystrophic microglia in our DS subjects compared to controls. We found higher numbers of dystrophic microglia in our DSAD cases than age-matched controls or young individuals with DS in the GM of the PCC, but not in the GM of OCC or in the WM of either region. Observing the highest numbers of dystrophic microglial cells in the GM of the PCC in DSAD is consistent with a previous report showing an increase in the number of dystrophic microglia in the hippocampus of three different type of dementia: AD, hippocampal sclerosis of aging, and dementia with lewey bodies (Bachstetter, Van Eldik et al. 2015). We found this to be true in the GM of the PCC, but not in the OCC. We also did not find any significant difference in the number of dystrophic microglia in the WM of either region. Dystrophic microglia that have fragmented or "beaded" processes may be due to microglial dysfunction or

senescence due to ageing or exposure to AD neuropathology (Streit, Sammons et al. 2004, Streit 2006, Boche, Perry et al. 2013, Bachstetter, Van Eldik et al. 2015).

Descriptions of rod-shaped microglia are sparse in the literature. While the relationship between AD pathology and rod-shaped microglia is still unknown, the presence of rod-shaped microglia following traumatic brain injury has been suggested to serve a role in neuronal survival (Ziebell, Taylor et al. 2012). Further, rod-shaped microglia have shown strong immunoreacitivty to tau in a small subset of AD patients (Odawara, Iseki et al. 1995). Therefore, it is possible that aggregates of hyperphosphorylated tau, known as neurofibrillary tangles, initiate the formation of neuroprotective rod-shaped microglia in AD. Based on previous studies showing an increase in rod-shaped microglia with age (Bachstetter, Van Eldik et al. 2015, Bachstetter, Ighodaro et al. 2017), we hypothesized that we would see an increase in rod-shaped microglia with age in our controls and DS autopsy cases. However, we did not see any significant age-related increases in rod-shaped microglia. Additionally, we hypothesized that we would see a significant increase in the number of rod-shaped microglia with the presence of AD pathology, based on previous literature (Wierzba-Bobrowicz, Gwiazda et al. 2002, Bachstetter, Van Eldik et al. 2015). In DS and DSAD, we did not find any significant differences in rod-shaped microglia with the presence of AD pathology. Rod-shaped microglia that have elongated nuclei, few processes, and are most notable in chronic disorders (Boche, Perry et al. 2013, Bachstetter, Van Eldik et al. 2015). Thus, although rod-shaped microglia are present in individuals with DS, their role in aging and AD in DS remains unclear.

Very little is known about the function of amoeboid microglia, as they are present in the brain at birth, before transforming into ramified microglia (Kettenmann, Hanisch et al. 2011). They are phenotypically very similar to macrophages found in the rest of the body and it is still unknown whether these microglia are senescing microglia found in the brain, or if they originate from monocytes entering the brain in response to injury (Boche, Perry et al. 2013). We found that our DSAD cases had significantly more amoeboid microglia than age-matched controls and individuals with young DS. We found increased numbers of amoeboid microglia to be a feature of DS, as we saw an overall significant genotype effect in both the GM of the PCC and the WM of the OCC. While further work needs to be done to explore the clinical significance of amoeboid microglia, this is the first description, to or knowledge, of amoeboid microglia as a feature of DS. It is possible that monocytes from the blood are entering the brains of people with DS as vascular neuropathology (Scott, Collins et al. 1994) may lead to small microhemorrhages in the brain leading to the presence of more amoeboid microglial cells.

Overall, individuals with DS, particularly those with DSAD, have different microglial phenotypes than age-matched controls (MC) (Figure 4.4). In DSAD, there appears to be a shift towards the presence of higher numbers of dystrophic microglial cells and fewer ramified microglial cells suggesting fewer resting state microglia and more damage to microglia. Further, individuals with DS appear to have microglial changes that are independent of AD pathology, including increases in both hypertrophic and dystrophic microglia. These data reinforce the differences in neuroinflammation seen in individuals with DS. Previous work from our group showed that individuals with DSAD have a different neuroinflammatory phenotype than sporadic AD or age-matched controls, which

is shifted towards an M2b profile (Wilcock, Hurban et al. 2015). Further, given that there are a variety of inflammatory genes located on chromosome 21, these different gene expression profiles may be affecting the morphology of the microglia.

The microglial changes also appear to be region dependent, with different microglial morphologies in at least the two cortical regions described here: the OCC and PCC. It is also notable that we see more changes in microglia morphology in the GM rather than the WM overall. This regional variability has been shown previously in the protein expression profile of microglia, which differed greatly by WM index, indicating that the myelin environment requires a microglial environment that is different than that of the GM (de Haas, Boddeke et al. 2008). These findings are notable for future studies, as they suggest that we should be focusing more on comparing microglia across brain regions and that we need to examine the GM and WM separately.

It is interesting to note that magnetic resonance spectroscopy (MRS) studies in the PCC of DS indicate an age-dependent increase in myoinositol, thought to partially reflect neuroinflammation (Lin, Powell et al. 2016). Also in MRS studies, levels of N-aceytlaspartate decline with age in DS and indicate increasing levels of neuronal dysfunction. Thus, the functional consequences of fewer ramified microglia and higher numbers of dystrophic microglia at autopsy may be observable *in vivo.* Interventions that may reduce neuroinflammation in DS and may lead to fewer hypertrophied microglia, may be a valuable target to prevent AD pathogenesis in DS.

Table 4.1: Case characteristics for PCC tissue obtained from brain banks (n=60).

Results presented are mean (SD). YC= young control (control for DS), MC=middle-age control (control for DSAD), OC=old control (control for AD), DS=Down syndrome, DSAD=Down syndrome with neuropathological Alzheimer's disease, AD=sporadic Alzheimer's disease; GM=grey matter; WM=white matter. F and p values are reported for a one-way ANOVA followed by a Fisher's LSD test to compare differences between four groups: YC, DS, MC, and DSAD.

PCC	Microglial Phenotype	YC (n=11)	DS (n=10)	MC (n=10)	DSAD (n=17)	F	p	OC (n=6)	AD (n=6)
	Age at death, y	17.89 (11.83)	19.5 (15.54)	53.5 (6.65)	57.00 (8.28)	n/a		78.67 (2.16)	78.83 (1.83)
	Male/Female (n/n)	9/2	7/3	6/4	8/9			3/3	4/2
	Post Mortem Interval (PMI), h	20.45 (5.05)	19.3 (6.15)	16.2 (6.78)	5.98 (6.41)			9.00 (6.54)	12.86 (10.13)
GM	Ramified	8.84 (5.80)	7.50 (6.76)	6.68 (5.08)	2.32 (3.19)	4.33	0.01	5.30 (2.98)	1.13 (1.34)
	Hypertrophic	3.84 (2.60)	5.62 (3.63)	4.64 (3.76)	4.69 (4.11)	0.40	0.76	5.27 (3.09)	5.53 (3.42)
	Dystrophic	6.50 (5.50)	6.52 (2.97)	7.42 (3.29)	13.41 (8.15)	4.20	0.01	6.33 (2.25)	9.63 (3.86)
	Rod-Shaped	0.06 (0.19)	0.06 (0.10)	0.12 (0.38)	0.18 (0.32)	0.53	0.66	0.10 (0.17)	0.00 (0.00)
	Amoeboid	0.48 (0.66)	0.44 (0.44)	0.34 (0.42)	1.54 (1.15)	6.88	0.00	0.43 (0.53)	0.33 (0.33)
WM	Ramified	0.10 (0.25)	0.12 (0.32)	0.62 (1.41)	0.21 (0.49)	1.11	0.36	0.41 (0.88)	0.47 (1.05)
	Hypertrophic	0.36 (0.59)	3.04 (5.87)	1.14 (0.86)	2.94 (2.02)	2.23	0.10	2.62 (1.35)	2.93 (1.89)

	Dystrophic	17.44 (6.29)	15.9 (8.85)	17.00 (5.26)	14.59 (3.29)	0.63	0.60	13.62 (2.82)	14.73 (4.26)
	Rod-Shaped	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.21 (0.59)	1.27	0.30	0.53 (1.31)	0.20 (0.31)
	Amoeboid	0.50 (0.49)	0.66 (0.78)	0.70 (0.67)	1.02 (0.61)	1.60	0.20	0.73 (0.74)	0.83 (0.63)

Table 4.2: Case characteristics for OCC tissue obtained from brain banks (n=63).

Results presented are mean (SD). YC= young control (control for DS), MC=middle-age control (control for DSAD), OC=old control (control for AD), DS=Down syndrome, DSAD=Down syndrome with neuropathological Alzheimer's disease, AD=sporadic Alzheimer's disease; GM=grey matter; WM=white matter. F and p values are reported for a one-way ANOVA followed by a Fisher's LSD test to compare differences between four groups: YC, DS, MC, and DSAD.

OCC	Microglial Phenotype	YC (n=10)	DS (n=11)	MC (n=10)	DSAD (n=14)	F	p	OC (n=6)	AD (n=12)
	Age at death, y	17.20 (12.40)	19.91 (14.80)	53.50 (6.65)	53.29 (4.48)	n/a		78.67 (2.16)	79.67 (1.56)
	Male/Female (n/n)	8/2	8/3	6/4	4/10			3/3	6/6
	Post Mortem Interval (PMI), h	21.50 (3.87)	19.73 (6.0)	16.20 (6.78)	9.82 (8.56)			9.00 (6.54)	7.92 (8.58)
GM	Ramified	7.40 (9.35)	1.20 (2.10)	3.42 (5.46)	0.04 (0.12)	4.27	0.01	1.93 (2.44)	0.56 (1.74)
	Hypertrophic	3.32 (6.70)	5.08 (9.17)	3.14 (3.10)	2.00 (2.22)	0.56	0.64	2.13 (2.25)	1.44 (1.46)
	Dystrophic	9.70 (10.28)	14.26 (8.01)	13.98 (8.07)	16.79 (3.66)	1.71	0.18	13.17 (7.28)	15.47 (6.43)
	Rod-Shaped	0.02 (0.06)	0.02 (0.06)	0.18 (0.45)	0.11 (0.28)	0.86	0.47	0.07 (0.10)	1.25 (3.78)
	Amoeboid	0.82 (2.59)	0.40 (1.06)	0.24 (0.58)	0.59 (0.66)	0.32	0.81	1.63 (3.53)	0.67 (0.74)
WM	Ramified	4.14 (7.73)	3.40 (4.22)	4.22 (5.24)	1.09 (1.30)	1.11	0.36	3.57 (1.78)	1.78 (2.91)

	Hypertrophic	1.02 (1.37)	2.82 (2.07)	2.06 (1.50)	5.03 (3.46)	6.09	0.00	3.50 (2.47)	3.04 (2.07)
	Dystrophic	7.88 (7.16)	9.26 (7.38)	10.58 (5.08)	8.60 (4.35)	0.38	0.77	4.40 (3.76)	7.69 (4.68)
	Rod-Shaped	0.02 (0.06)	0.12 (0.27)	0.02 (0.06)	0.13 (0.23)	1.14	0.35	0.03 (0.08)	0.15 (0.20)
	Amoeboid	0.00 (0.00)	0.20 (0.57)	0.08 (0.14)	0.76 (0.92)	4.19	0.01	0.13 (0.16)	0.56 (0.73)

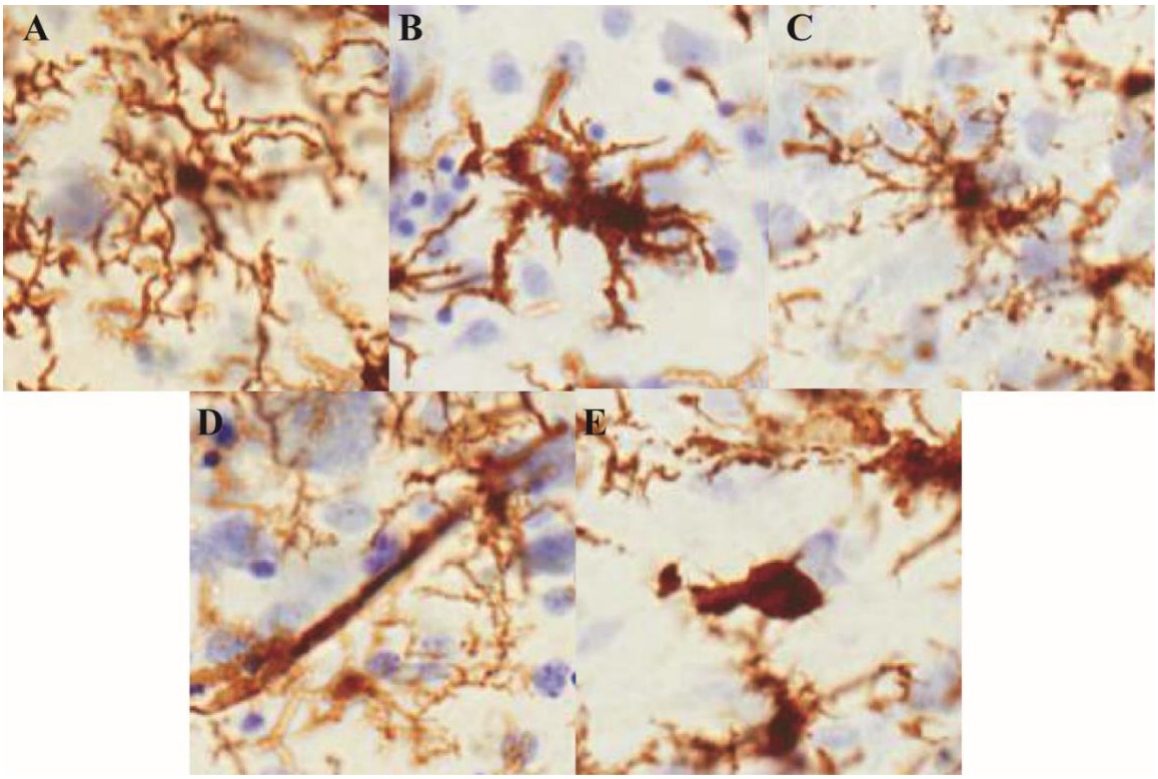

Figure 4.1: Different microglia morphologies were evaluated in the PCC and OCC. In order to further understand the role of microglia in DS, we examined 5 microglia morphologies: A) ramified microglia, which have thin, branched processes to actively "survey" changes in their environment; B) hypertrophic microglia (also known as activated microglia), which may have enlarged, short processes and thicker bodies; C) dystrophic microglia that have fragmented or "beaded" processes possibly due to microglial dysfunction due to ageing; D) rod-shaped microglia that have elongated nuclei, few processes, and are most notable in chronic disorders; and E) amoeboid microglia which have a round body, lack processes, and appear in response to acute destruction of central nervous system tissue.

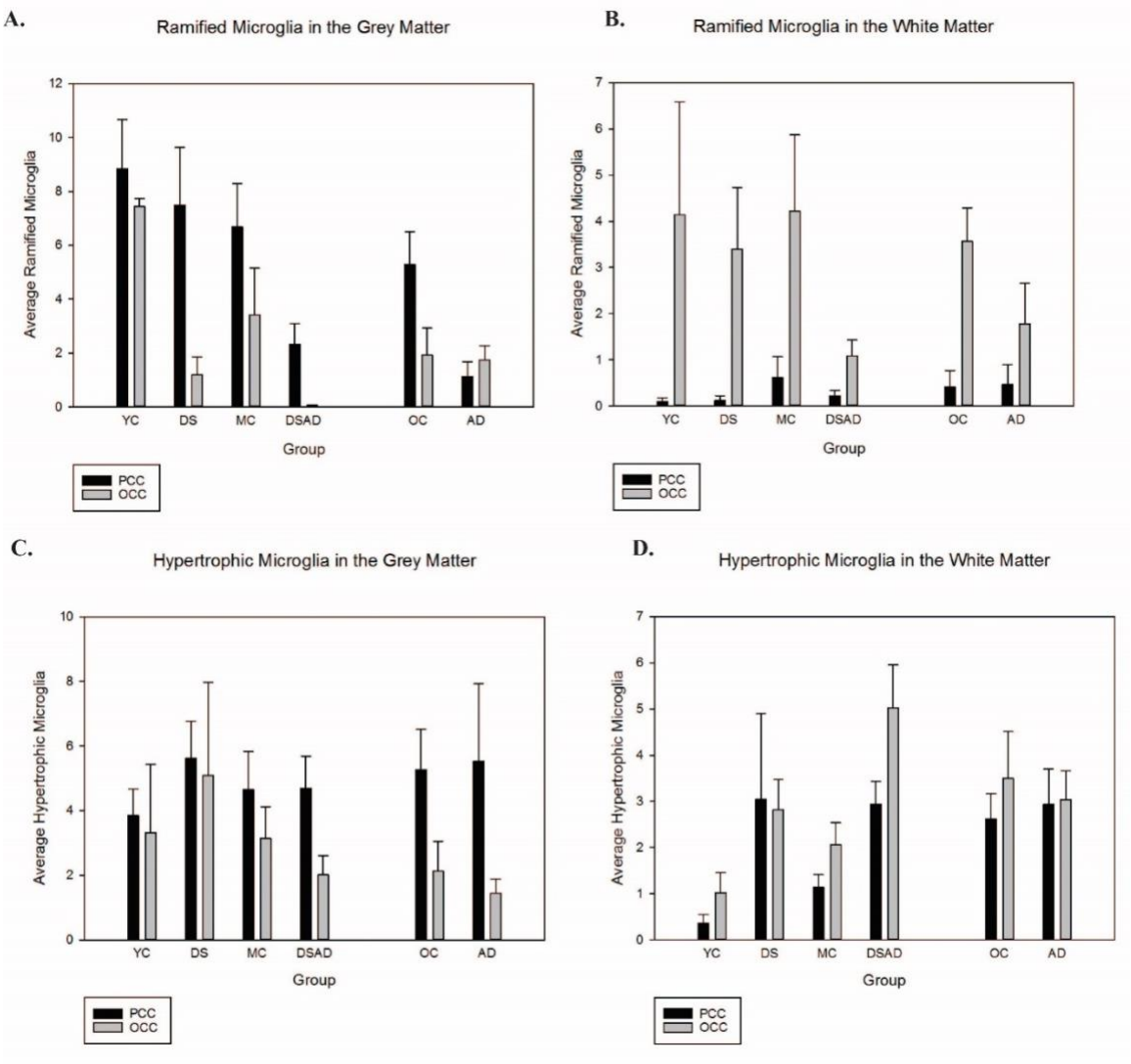

Figure 4.2: Fewer ramified microglia are present in the GM and more hypertrophic microglia are in the white matter of DSAD cases.
(A) There are significant group differences in the number of ramified microglia in the GM of the PCC ($F(3,46)=4.331$, $p=0.009$) and OCC ($F(3,43)=4.27$, $p=0.01$). Specifically, the DSAD group had significantly less ramified microglia than age-matched controls ($p=0.037$) and significantly less than the young DS group ($p=0.014$). **(B)** We did not see any significant group differences in the number of ramified microglia in the WM of either region. **(C)** We did not see any significant group differences in the number of hypertrophic microglia in the GM of the OCC or PCC. **(D)** In the WM of the OCC, we saw significant

group differences in the number of hypertrophic microglia ($F(3,43)=6.09$, $p=0.00$). In this region, we found individuals with DSAD had significantly more ramified microglia than age-matched controls ($p=0.005$) and young individuals with DS ($p=0.000$). While not statistically significant, we saw similar trends in the PCC.

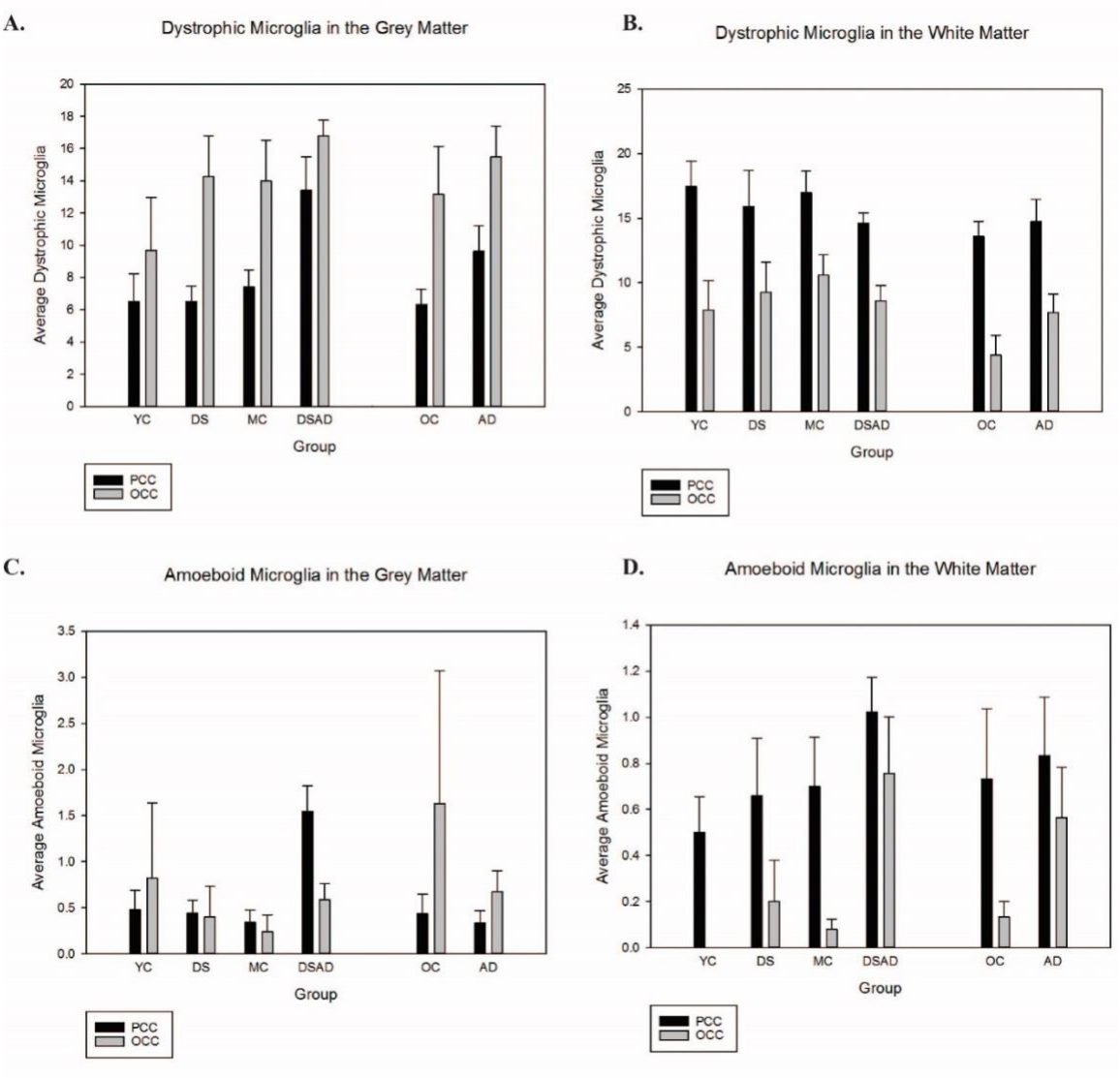

Figure 4.3: DSAD cases have significantly more dystrophic and amoeboid microglia than age-matched controls.
(A) There were significant group differences in the GM of the PCC ($F(3,46)=4.20$, $p=0.01$) and found that there were significantly more dystrophic microglia in our DSAD cases than age-matched controls ($p=0.019$) and young DS cases ($p=0.007$). **(B)** We did not see any significant group differences in the WM of the PCC or OCC. **(C)** We saw statistically significant group differences in the GM of the PCC ($F(3,46)=6.88$, $p=0.00$) and the **(D)** WM of the OCC ($F(3,43)=4.19$, $p=0.01$). In both of these regions, we saw significantly

more amoeboid microglia in our DSAD cases compared to age-matched controls (PCC: p=0.001; OCC: p=0.009) and young individuals with DS (PCC: p=0.002; OCC: p=0.029).

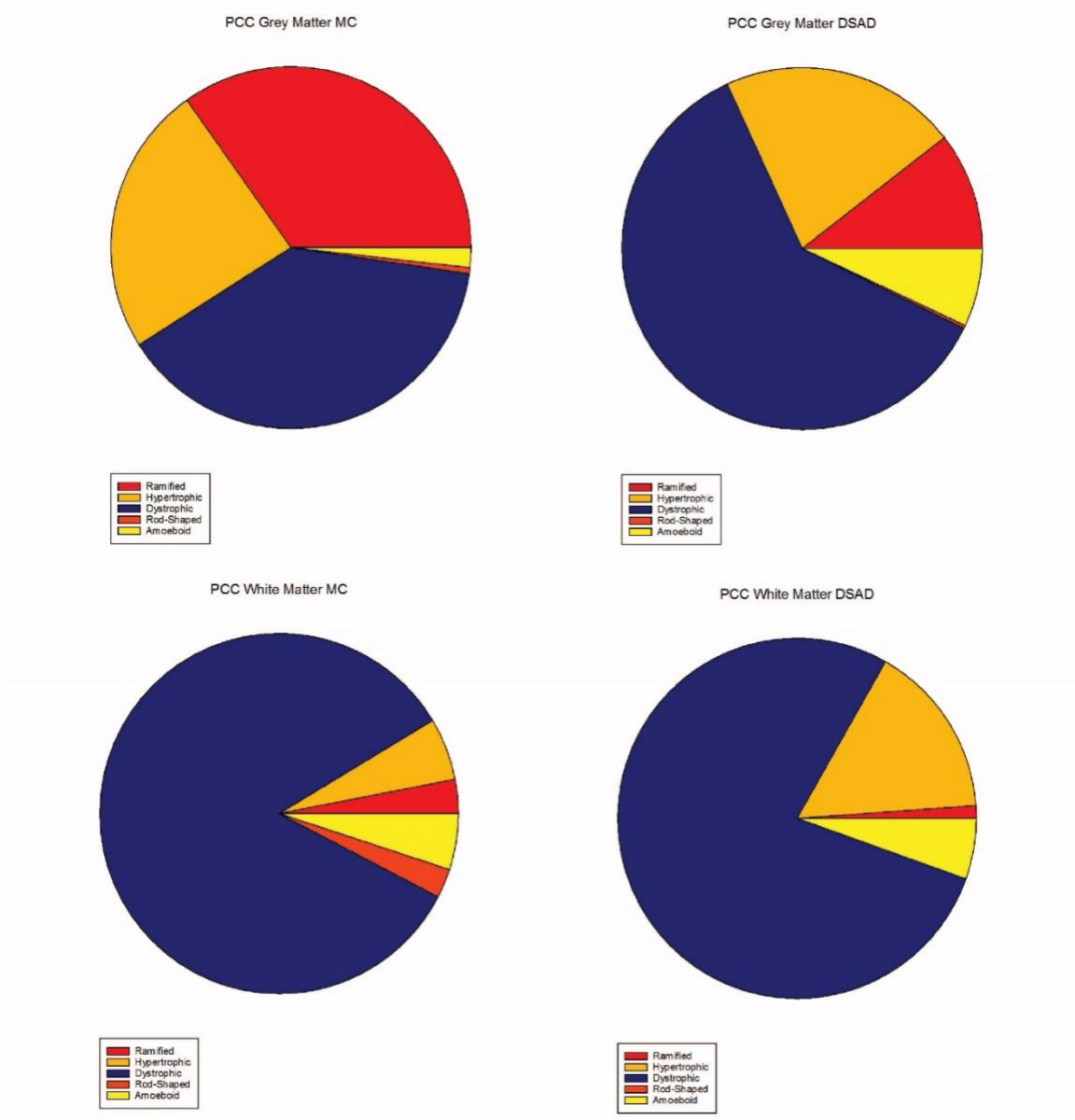

Figure 4.4: DSAD cases have different microglia phenotypes than age-matched controls.

The graphs above represent microglia phenotypes in the PCC of the GM and WM of MC and DSAD groups. In both the GM and WM we see an increase in dystrophic microglia in DSAD cases. In the GM of DSAD cases, we see fewer ramified microglia and more amoeboid microglia than age-matched controls. In the WM of DSAD cases, we see more

hypertrophic microglia than age-matched controls. We see similar changes occurring in the OCC.

Chapter 5: Conclusions and Future Directions

5.1 – NHE1 Discussion

5.1.1 - Summary

In chapter two, we evaluated the possible role of NHE1 in VCID. We started looking at NHE1 because gene that encodes it, *SLC9a1*, was highly upregulated in a microarray and follow-up RT-PCR study performed on our db/AD mice (Figure 2.1F). When we followed this up with protein studies, we consistently saw moderate, but significant group effects with NHE1 levels increasing, particularly in our db/AD mice.

Despite the extensive data we have collected on NHE1 and its role in VCID, we have yet to elucidate a mechanism for the role of NHE1 in cerebrovascular disease or cognitive impairment. While we do consistently see a modest, but significant increase in NHE1 in the db/AD mice, it has proven difficult to establish what this means for the development of pathology.

When we attempted to expand our NHE1 studies into human cases, we were not able to find a connection between NHE1 and VCID pathology. This could be because we were not entirely sure what kind of pathology we were looking for in connection to NHE1. We did see NHE1 levels decrease with age in individuals with DS, but it is possible that the observed decrease is due to neurodegeneration and that the older DS subjects have less NHE1 because they have less brain matter.

Given our data showing that NHE1 and pTau are correlated in the db/AD animals and that these proteins increase together following injury (Figure 2.4C-E), it is possible that NHE1 links to aspects of AD-related pathology through tau. However, we did not see

any increases in NHE1 levels our human AD or DS cases, which would both have high levels of pTau (Table 2.1). It is likely we will not understand this connection until further studies are done.

5.1.2 - Future Directions

To understand how NHE1 and tau pathology may be related, we could measure tau pathology and cognitive dysfunction in NHE1 knockdown mice. This would involve crossing commercially available $NHE1^{+/-}$ and $NHE1^{+/+}$ mice (Hwang, Yoo et al. 2008) to generate knock-down and WT mice ($NHE1^{-/-}$ mice are not viable) and giving pups an intracerebroventricular injection of adeno-associated virus- Tau^{P301L} (AAV-Tau^{P301L}), following previously published methods (Chakrabarty, Rosario et al. 2013). If NHE1 function or activity is at least partly responsible for the development of tau pathology, we should see less tau pathology and cognitive dysfunction in the $NHE1^{+/-}$ mice.

To better understand whether NHE1 is at least partly responsible for cerebrovascular pathology in a mouse model of VCID, we could use an NHE1 inhibitor, such as Cariporide, which is well tolerated for chronic dosing in mice (Kilic, Velic et al. 2005, Luo, Chen et al. 2005, Ferrazzano, Shi et al. 2011, Shi, Chanana et al. 2011, Leng, Shi et al. 2014), to pharmacologically suppress NHE1. We could treat db/AD mice, which are known to develop stroke pathology around 10 months of age (Niedowicz, Reeves et al. 2014), with Cariporide. We could then measure cognition in these mice using Morris Water Maze and evaluate cerebrovascular pathology in post-mortem tissue. If NHE1 is involved in VCID, then vascular pathology will be less pronounced in the Cariporide treated animals than control animals.

5.1.3 - Conclusions

NHE1 is a highly abundant protein throughout the entire body, including the brain. It is nearly ubiquitous in the plasma membrane of virtually all mammalian cell types (Fliegel 2001) and serves a wide-variety of physiologically important functions, including maintaining intracellular pH (Pouyssegur, Sardet et al. 1984, Grinstein, Rotin et al. 1989), cell volume after osmotic shrinkage (Grinstein, Rotin et al. 1989, Shrode 1996), cell growth (Grinstein, Rotin et al. 1989, Hoffmann and Simonsen 1989), cell differentiation (Rao, de Roux et al. 1991), and acts as a structural anchor that helps determine cell shape and membrane integrity (Denker and Barber 2002). Given that NHE1 serves an array of functions that are critical for survival (Bell, Schreiner et al. 1999), it is difficult to mechanistically explain why these levels increase in our db/AD animals. However, it is possible that NHE1 levels vary widely in the brain, which is why we see NHE1 both increase and decrease with age and why we only see a marginally significant increase in the db/AD animals. With all of the data we have gathered and the failed clinical trials targeting NHE1 (Avkiran, Cook et al. 2008), I do not see reason to pursue NHE1 as a therapeutic target in VCID. While it is possible that there is a connection with tau or with other cerebrovascular pathologies, we have little significant data to stand on to ask non-speculative, hypothesis-driven questions that could determine NHE1's role in VCID.

We did a microarray in a mouse model of VCID and hoped to take this to human cases, which ultimately was not successful. Mouse models are one of the most important tools for studying diseases, but it is difficult to make the leap from animal models to humans. This challenge is apparent given the difficulties of developing therapeutics in the AD field (Hall and Roberson 2012). As discussed in chapter one, the heterogeneity of

VCID makes it a difficult disease state to model. Therefore, we decided to move from mice to studying as cerebrovascular pathology in humans.

One of the only significant pathology related effects we saw in the human cases from the NHE1 project was a decrease in NHE1 in the DS cases with age (Figure 2.5F). Since individuals with DS develop a marked amount of cerebrovascular pathology with age, we wondered if we could further investigate which forms of vascular pathology are better predictors of VCID.

5.2. – Cerebrovascular Pathology in Individuals with Down Syndrome

5.2.1 – Summary

In chapter 3, we studied the extent of MBs in DS as a function of age and AD in the FC and OCC. Using a Prussian blue stain to identify MBs, we observed MBs in our DSAD cases with similar frequency with AD cases in both the FC and OCC (Figure 3.6). Additionally, individuals with DS (DS and DSAD) had significantly more frequent MBs than similarly aged controls in both regions examined. When we plotted MBs in individuals with DS against age, we found that the number of MBs increased significantly with age in both the FC and OCC (Figure 3.7). However, MBs appear earlier in the OCC of individuals with DS (in their 30s), than in the FC (during their 40s), indicating that the OCC is perhaps more vulnerable to early cerebrovascular changes than the FC.

We also scored CAA in these cases on a scale of 0-4 (Figure 3.4) with the hypothesis that the presence of CAA and MBs are linked. We found CAA to be more severe in individuals with DS (combined DS and DSAD) relative to their aged matched controls in both regions (Figure 3.5). Further, we found that all individuals with DS, regardless of

age, have more severe CAA scores than controls, which confirms previous data published in our lab, showing more severe CAA in individuals with DS (Head, Phelan et al. 2017). The data in chapter 3 indicates that individuals with DSAD have CAA scores that are equally as severe as people with sporadic AD, despite a 27 year age difference. Additionally, We found CAA to be associated with MBs, indicating that MBs and CAA appear to be significant contributors to the development of dementia.

5.2.2 – Future Directions

Autopsy cases that we were working with were not cognitively characterized prior to death. Therefore, we were not able to evaluate how the presence of MBs and CAA contributes to cognitive impairment in individuals with DS. This would be an interesting next step for this work and one that we have already begun working on with the University of Kentucky's Down Syndrome and Aging Study. The goal of this study is to follow people with DS over the age of 25 as they get older. Every year, participants come in for cognitive testing, where we measure learning and memory, blood draws, and MRI. Participants currently undergo a series of MRI scans, including T2* imaging, which show low signals due to disruptions to the main magnetic field. This includes iron from de-oxygenated hemoglobin, which allows us to detect hemorrhages *in vivo* (Greenberg, Vernooij et al. 2009, Charidimou, Jäger et al. 2012, Van der Flier and Cordonnier 2012). We hypothesize that given the number of MBs we see in our autopsy cases, that we would see more MBs present in our older participants and that this would be correlated with cognitive impairment.

5.2.2.1 – Methods

5.2.2.1.1 – Subjects

T2* images were collected from visits for an ongoing longitudinal study evaluating cognitive decline in adults with DS (Powell, Caban-Holt et al. 2014, Lin, Powell et al. 2016). Participants over the age of 25 were recruited through local DS support groups and residential facilities in Kentucky and southern Ohio since 2010. All participants completed informed written consent or assent with guardian consent. The study and research procedures were approved by the University of Kentucky Institutional Review Board. Only participants with usable T2* scans (images acquired without too much movement) were included in this analysis. This included 47 participants, 25 without dementia and 22 with a dementia diagnosis at consensus.

5.2.2.1.2 – Imaging Acquisition

MRI was performed on a 3T TIM Siemens scanner at the Magnetic Resonance Imaging Spectroscopy Center at the University of Kentucky. 2D GRE images were used to acquire T2* weighed images rather than SWI images to minimize time and motion artifacts, using a 20 channel head coil. The T2* imaging parameters were: TR 620ms, TE 20ms, TA 2:06, BW 200Hz, 25 slices, 4mm thick/1.2mm gap, FOV 220x220 mm2, matrix 205 x 256, Flip 20 degrees, axial. MPRAGE T1 weighted images were acquired for high resolution anatomical comparison with parameters: TR 2530ms, TI 1260ms, TE 2.9ms, TA 4:39, BW 235Hz, 1mm isotropic resolution, FOV, 241 x 281 mm2 , sagittal.

5.2.2.1.3 – Image Analysis

All MRI scans were evaluated by myself, and any that were questionable were discussed with Dr. Powell and a consensus decision was made. MBs were defined as round

lesions, of less than 10 mm in diameter. Using the Microbleed Anatomical Rating Scale (MARS), we characterized the presence, number, and anatomical distribution of MB across MRI sequences for 47 participants (Gregoire, Chaudhary et al. 2009). We decided to use the MARS system because it has good reliability and gave information on number of MB as well as location. MBs were counted in the lobar region (frontal, temporal, parietal, occipital, and insular cortices) and deep region (brainstem, cerebellum, basal ganglia, thalamus, internal capsule, external capsule, corpus callosum, and deep periventricular white matter).

Due to the severity of hemorrhaging in select DS cases, we followed up the MARS system with our own preliminary rating system of cerebrovascular pathology. In this rating system, we evaluated all hemorrhaging, including macrohemorrhages, superficial siderosis (hemosiderin deposition on the pial surface of the brain (Yamawaki and Sakurai 2013)), and MBs. First, we determined whether or not there was any hemosiderin present in the scan. If there was pathology, we evaluated it as mild, moderate, or severe. A classification of mild was made if there was bleeding present in 1-3 slices per brain (Figure 5.1 A), a moderate classification was made if bleeding was present in 3-6 slices per brain (Figure 5.1 B), and a severe classification was made if bleeding was extensive (i.e. it was not possible to count the number of bleeds) (Figure 5.1 C). This methodology has not been published, nor has it been verified by independent evaluation. However, it was meant to serve as a preliminary analysis of the T2* images.

5.2.2.2 – Results

Initially, we evaluated all subjects on the MARS rating system. However, due to the severity of hemorrhaging in several of our cases (Figure 5.1 C), we found it difficult to

count and determine the distribution of bleeds. Therefore, the analysis from the MARS system was inconclusive (data not shown).

The preliminary data analysis from our own rating system, which evaluated images based on a severity of mild, moderate, or severe cerebrovascular pathology, showed that the majority (64%) of participants without dementia had no vascular pathology present in their images (Figure 5.1 D). However, 24% of participants without dementia had mild vascular changes, and 12% had moderate cerebrovascular pathology. This indicates that hemorrhaging on a T2* scan is not a direct indicator of dementia. Further, of those participants with dementia, 36% had no cerebrovascular pathology, indicating that at least some individuals with DS develop dementia without any hemorrhaging, or at least hemorrhaging that is detectable by T2*. All of the participants with a score of severe cerebrovascular pathology had dementia.

Unrelated to the bleeding, we found a significant amount of flow voids present in our people with DS. Often, it was difficult to determine if these flow voids were MBs or flow voids because they appear so large. This was an unexpected finding, but one that we found unusual.

5.2.2.3 - Discussion

The MARS system was unable to be applied in a subset of cases. However, we feel that it is an important finding that MARS is not an appropriate scoring mechanism for individuals with DS. We have several cases where we see multiple bleeds in a single slice, often overlapping each other. In these cases, it is difficult to determine whether this is a single bleed that continues to hemorrhage, leading to a distorted signal loss in the MRI, or

whether these are multiple bleeds from separate blood vessels. Additionally, we found that many of our participants have more severe hemorrhaging than just MBs, a finding that thus far has only been published in case reports (Donahue, Khurana et al. 1998, McCarron, Nicoll et al. 1998, Naito, Sekijima et al. 2008, Jastrzebski, Kacperska et al. 2015). These data give a more extensive examination of hemorrhaging in this population and provides important clinical information for treating individuals with DS as they age.

We debated doing a load analysis on these subjects, which would give us a percent of signal loss in the brain. However, this process would be labor intensive, as there are several mimics of bleeds in T2*-weighted images. These include calcifications, iron deposits, flow voids in pial vessels, and others (Greenberg, Vernooij et al. 2009). These mimics are easily detectable by the human eye, but would be difficult to differentiate from a bleed by a computer. For example, a flow void often has the appearance of a MB, until you track the vessel and flow through multiple slices (Figure 5.1E). Occasionally this involves referring back to the structural three dimensional magnetization prepared rapid acquisition GRE (MPRAGE) sequence to determine whether a vessel of that size could structurally fit in that exact location. Additionally, a load analysis would tell us the percent area with signal loss, but would still not give us a count on the amount of hemorrhages that are present. In this case, we would need to evaluate which is more important: the number of hemorrhages or the volume of the brain that is hemorrhaging. At this time, we do not know which is more important, but would make an interesting and important follow-up study.

An additional follow-up to this study would be to further characterize the large volume of flow voids present in our DS cases (Figure 5.1E). These flow voids are

occasionally so large that we suspect there may be some structural vascular issues occurring in our DS participants. Time permitting, it would be interesting to start collecting magnetic resonance venography's (MRV) to image the blood vessels in the brain. This would allow us to determine whether there are abnormalities in the vasculature in individuals with DS.

5.2.3 – Conclusions

Based on the autopsy and MRI data we have collected on MBs and CAA in DS, it is clear that MBs are a consistent feature in older individuals with DS. This is an important finding in that it highlights the need to consider cerebrovascular pathologies when studying adults with DS. Taking into consideration cerebrovascular neuropathology is especially important as we think about designing clinical trials in this population, particularly trials that involve immunotherapies (van Dyck 2017). Further, we need to continue the preliminary work described above to elucidate the role that cerebrovascular pathology plays in the onset of dementia in this population.

5.3 – Microglia Morphology in DS Discussion

5.3.1 – Summary

Using autopsy cases from the PCC and OCC of individuals with DS, we characterized the distribution of several microglial phenotypes in these regions. We used IBA1 to label both resting and activated microglia and counted ramified, hypertrophic, dystrophic, rod-shaped, and amoeboid microglia in our cases. We separately examined the GM and WM in young individuals with DS, DSAD, AD, and age-matched controls for each of these groups.

Overall, we found that individuals with DS, particularly those with DSAD, have a different microglial phenotype than age-matched controls. In individuals with DSAD, we saw higher numbers of dystrophic microglia and fewer ramified microglia. This suggests that the surveying microglial cells are likely senescing and becoming dystrophic, which may contribute to some of the neuroinflammatory changes we see in this population (Abraham 2001).

We also found the makeup of microglia morphology to be region dependent, with significant differences in the morphology of microglia in the PCC and OCC. Additionally, we overall saw more changes in microglial morphologies in the GM rather than the WM.

5.3.2 – Future Directions

Describing microglial changes in individuals with DS as they age is important for understanding neuroinflammatory changes in this population. We were only able to examine two regions of interest, the OCC and PCC, in this study. However, it would be interesting to be able to understand the regional heterogeneity across the brain. The complexity of a study of this size is noted, given the regional variability across both brain regions and GM and WM. Additionally, microglial changes in humans over the course of the lifespan has yet to be fully defined, so doing so in DS would certainly be a challenge. However, this information would help us to understand the microglial response to various types of neuropathology.

In chapter 4, we found that our DSAD cases had significantly more amoeboid microglia than age matched controls and higher numbers than younger individuals with DS. However, very little is known about amoeboid microglia and their clinical significance.

Further studies to examine the role of amoeboid microglia as a function of aging and cognitive impairment would be important in understanding neuroinflammatory changes in DS.

It would also be interesting to be able to create a pathological cascade model of dementia in AD, similar to the one made by Jack et. al in 2010 for AD (Jack, Knopman et al. 2010). In order to do so, we would need to complete similar studies with other markers of neurodegeneration, such as Aβ and tau, and including *in vivo* techniques, such as MRI. This would allow us to better understand changes in pathology over the lifespan for individuals with DS, which is important as we think about designing interventions for this population. Understanding when pathology appears in this population will allow us to design better and more effective clinical trials.

5.3.3 – Conclusions

In chapter 4, we found that individuals with DS, particularly those with DSAD, have different microglia morphologies than age-matched control in the OCC and PCC. This work supports previous data from our lab, showing that individuals with DSAD have a different inflammatory profile than controls or individuals with sporadic AD (Wilcock, Hurban et al. 2015). Overall, this work highlights the importance of examining regional differences and including data on both the GM and WM when examining microglia.

5.4 - Conclusions

VCID is a complex, heterogeneous form dementia, involving aspects of several different neurodegenerative diseases to cause cerebrovascular-driven cognitive impairment. This heterogeneity makes VCID difficult to model, given that it encompasses

a number of combinations of pathology. While there are a variety of animal and cell culture models than can aid in our understanding of VCID, there is currently no model that accurately reflects this condition in humans.

This dissertation has examined several cerebrovascular contributions to aging, initially, in an animal model of VCID. However, the data gathered from these animals did not necessarily reflect what we observed in the human cases. Therefore, we moved into examining autopsy cases from individuals with DS. Given that individuals with DS develop cerebrovascular pathology with age, independent of certain risk factors for vascular pathology (Vis, Duffels et al. 2009), this population provides a unique way to study VCID.

As the field of VCID becomes more widely recognized, we must start to specify different neuropathological and cognitive aspects of this condition. This will allow us to better model, and therefore develop better treatments, for individuals with VCID. Further, we must examine how different aspects of VCID drive cognitive impairment in aging populations. Understanding how different pathologies contribute to cognitive decline will be critical in order to develop early interventions to dementia. By building on these findings, we may be able provide critical information about aging in DS and help tease apart some of the complexities of VCID.

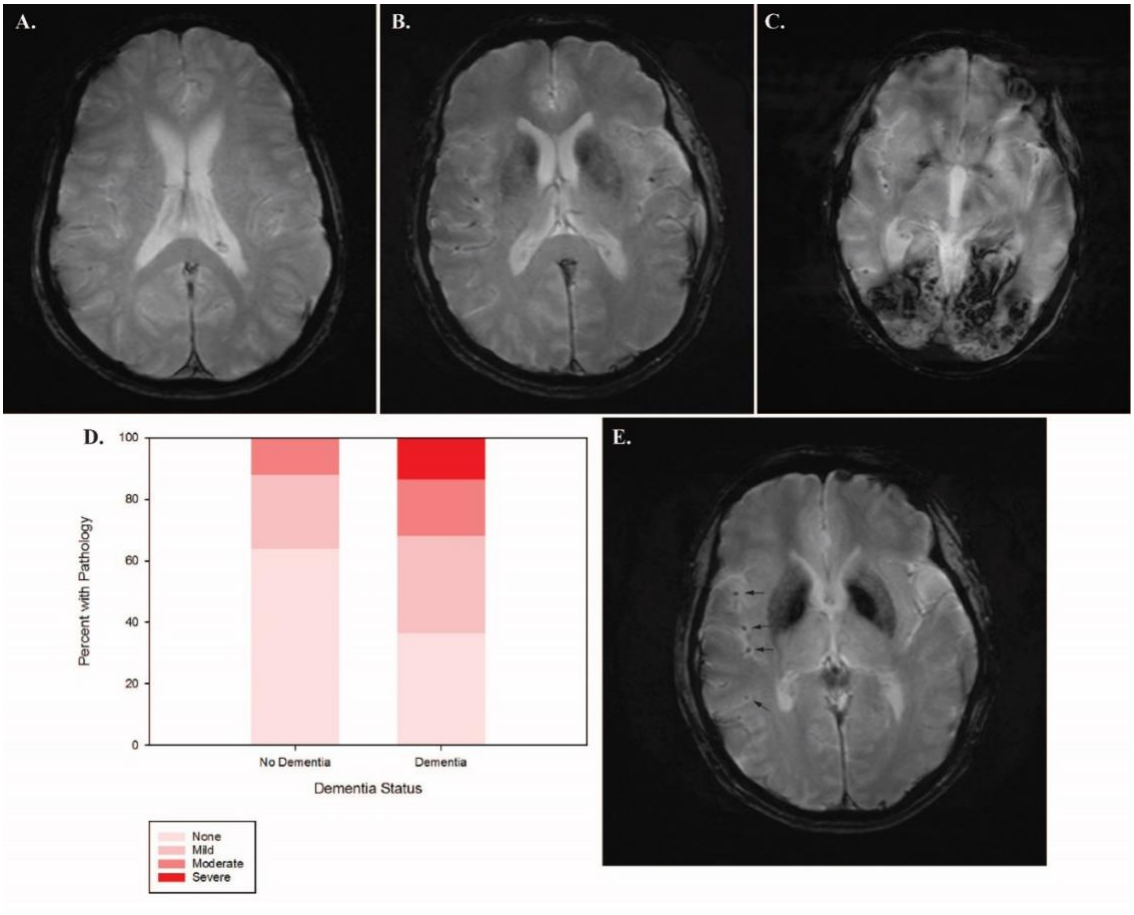

Figure 5.1: T2*-weighted images detect hemorrhages in individuals with DS. We evaluated our images based on the rating scale of mild, moderate, and severe. A mild diagnosis **(A)** was made if there was bleeding present in 1-3 slices per brain, a moderate diagnosis **(B)** was made if bleeding was present in 3-6 slices per brain, and a severe diagnosis **(C)** was made if bleeding was so extensive, that it was not possible to count the number of bleeds. Dementia status was used to categorize the ratings of the scans (**D)**, and as hypothesized, we saw more severe pathology in our participants with dementia. Rating of cases was done by hand to ensure mimics, such as flow voids (arrows) or iron deposition seen in the ventricles, were not counted as bleeds **(E)**.

Appendix 1: List of Abbreviations

4VO	Four vessel occlusion
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AAV	Adeno-associated virus
Aβ	Amyloid-beta
AD	Alzheimer's disease
AICD	Amyloid precursor protein intracellular domain
APP	Amyloid precursor protein
BACE	β-Site APP-cleaving enzyme
BBB	Blood brain barrier
BCAS	Bilateral common carotid artery stenosis
CAA	Cerebral amyloid angiopathy
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
Caln	Calcineurin
CCA	Common carotid artery
CCH	Chronic cerebral hypoperfusion
CHI	Closed head injury
CHO	Chinese hamster ovary
CSF	Cerebrospinal fluid
DS	Down syndrome
DSAD	Down syndrome with AD
DSCR	Down syndrome critical region
DTI	Diffusion tensor imaging
Dyrk1a	Dual-specificity tyrosine-(y)-regulated kinase 1a
FC	Frontal cortex
FTD	Frontotemporal dementia

GM	Gray matter
GOM	Granular osmiophilic material
Hcy	Homocysteine
HUVECs	Human umbilical vein endothelial cells
IACUC	Institutional Animal Care and Use Committee
IHC	Immunohistochemistry
IPSCs	Induced pluripotent stem cells
LC	Locus coeruleus
MARS	Microbleed Anatomical Rating Scale
MC	Middle-aged controls
MCI	Mild cognitive impairment
MMSE	Mini mental score exam
MPRAGE	Magnetization prepared rapid acquisition GRE
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MRV	Magnetic resonance venography
NbM	nucleus basalis of Meynert
NHE1	Sodium-hydrogen exchanger 1
NHP	Non-human primates
NFAT	Nuclear factor of activated T-cells
NFTs	Neurofibrillary tangles
NOTCH3	Notch homolog 3
OC	Old controls
OLAC	Office of Laboratory Animal Welfare
PCAD	Preclinical AD
PET	Positron emission tomography
PiB	Pittsburgh Compound B
PMI	Post mortem interval

RCAN1	Regulator of calcineurin 1
SHRSP	Spontaneously hypertensive rat
SMTG	Superior middle temporal gyri
STZ	Streptozotocin
SVD	Small vessel disease
T2DM	Type 2 diabetes mellitus
VCID	Vascular contributions to cognitive impairment and dementia
WM	White matter
YC	Young controls

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1. **Helman AM**, Siever M, McCarty KM, Lott IT, Doran E, Abner E, Schmitt FA, Head E, Cerebral Amyloid Angiopathy is Associated with Microbleeds in Down Syndrome with Alzheimer's Disease, Journal of Alzheimer's Disease (2018) (In review)

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