Co-Localization Patterns of Aquaporin-4 with Amyloid Beta and CD68 in Alzheimer's Disease and Cerebrovascular Disease

Anthony Bryan Crum
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Co-Localization Patterns of Aquaporin-4 with Amyloid Beta and CD68 in Alzheimer’s Disease and Cerebrovascular Disease

Anthony Bryan Crum

A thesis submitted to the faculty of Brigham Young University
in partial fulfillment of the requirements for the degree of Master of Science

Jonathan Jayme Wisco, Chair
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Neuroscience Center
Brigham Young University

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ABSTRACT

Co-Localization Patterns of Aquaporin-4 with Amyloid Beta and CD68 in Alzheimer’s Disease and Cerebrovascular Disease

Anthony Bryan Crum
Neuroscience Center, BYU
Master of Science

Aquaporin-4 (AQP4) is a key component in maintaining proper glymphatic flow. In recent years, the glymphatic system has been discovered and approached as a major factor in amyloid plaque clearance in Alzheimer’s disease. This study examines the depolarization of AQP4 from the astrocytic endfeet and subsequent co-localization with amyloid plaques in the hippocampus and subiculum. Results show a significant pattern of co-localization in advanced Alzheimer’s disease, as well as increases in AQP4 in cerebrovascular disease. This pattern shows AQP4 should be approached as a promising therapeutic area in future research.

Keywords: aqp4, alzheimer’s, co-localization, cerebrovascular, dementia
ACKNOWLEDGMENTS

First and foremost, I want to thank my wife, Laura. Without her constant support and understanding, this project would never have come to fruition. I would also like to thank my parents, Wayne and Cindy Crum, and my in-laws, Mark and DeAnne Vickers, for their continued prayers and support.

I would like to thank my committee for their guidance and willingness to mold my fledgling ideas into a legitimate research question. I would also like to specifically thank Dr. Jonathan Wisco for his mentoring and advice. He always believed I could complete this project in a condensed time frame and never doubted I could push through at a strenuous pace. And finally, I would like to thank my grandmother, Kathryn E. W. Gambrel. Were it not for the experience of watching her struggle through the depths of dementia, I may never have started on this lifelong path.
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Introduction

History of the Amyloid Hypothesis

Over a century ago, Alois Alzheimer presented his ground-breaking work in which he identified neurofibrillary tangles in the cerebral cortex of a woman in her mid-fifties showing signs of disorientation in her home, paranoia, and rapid memory loss (Stelzmann, Schnitzlein, & Murtagh, 1995). It would be only a few short years later that this illness would be classified as its own dementia and given the name Alzheimer’s disease (AD). Alzheimer was not alone in the field and his work was rapidly corroborated by research from the likes of Oskar Fischer with papers in 1907, 1910, and 1912 describing neurofibrillary tangles in hundreds of cases of dementia, as well as neuritic plaques (Goedert, 2009). However, it was not until the invention of the electron microscope and studies by Michael Kidd and Robert Terry that researchers began to better understand the pathology of AD. For the first time, researchers were able to see the senile plaques were actually two distinct types: intracellular neurofibrillary tangles (NFT) and extracellular neuritic plaques (Dickson, 1997; Dikranian, 2012; Selkoe, 2001). While NFTs and plaques had differing locations, further research indicated a common progenitor of amyloid-β (Aβ) protein and suggested Aβ first accumulates intracellularly before spilling over to the extracellular space (Masters et al., 1985). This research has given rise to the amyloid clearance hypothesis of AD.

In 1992, John Hardy and Gerald Higgins offered the first amyloid cascade explanation showing when amyloid precursor protein (APP) was cleaved by lysosomes, instead of by the endogenous APP secretase, a completely intact Aβ remained and was able to be deposited in the
brain. Researchers were able to show the deposition of Aβ disrupted calcium levels in the brain and lead to cytotoxicity resulting in cell death and, in the presence of tau protein, lead to an over-accumulation of hyper-phosphorylated tau proteins in neurofibrillary tangles (Hardy, J. A., & Higgins, 1992). As research into the production of Aβ continued, mutations in the APP gene were identified and it was suggested these mutations were responsible for the rapid accumulation of Aβ in AD patients (Goate et al., 1991; Haass et al., 1992; Hardy, J., & Mullan, 1992; Hendriks et al., 1992; Mullan et al., 1992). Perhaps one of the greatest supports for the amyloid clearance hypothesis came less than a decade later when mutations in the genes responsible for the coding of tau protein were shown to precede frontotemporal dementia with Parkinson’s disease-like symptoms but were not sufficient on their own to induce AD (Grover et al., 1999; Hutton et al., 1998). When all of the previous research is considered together, the root cause associated with the development of AD appears to be an imbalance between the production and clearance of Aβ protein. As the brain does not have a traditional lymphatic drainage system to remove toxins and solutes, researchers were left with the daunting task of discovering a clearance system for the brain prior to determining the role Aβ would potentially play in disrupting proper clearance.

**The Glymphatic System**

The circulation and removal of cerebrospinal fluid (CSF) has been well established from production in the choroid plexus to flowing through the subarachnoid spaces to draining into the blood through arachnoid villi in the walls of the venous sinuses (Milhorat, 1975; Weller, 1998). However, the understanding of how solutes are cleared from the interstitial space of the brain has remained an area needing greater elucidation as interstitial fluid (ISF) pathways are not very well mapped out. Recent studies have shown CSF enters the brain rapidly via cortical pial arteries and an influx into the Virchow-Robin spaces along penetrating arterioles follows (Iliff et al., 2012).
CSF has also been shown to enter the parenchyma through a periarterial pathway encircling the vascular smooth muscle, which is then encased by perivascular astrocytic endfeet. It is at this point, CSF is able to undergo constant exchange with ISF and facilitate the clearance of solutes through the blood-brain barrier and out of the brain by means of basement membranes. It has also been shown this fluid circulatory pathway is larger and more active during sleep (Iliff et al., 2012; Mestre, Kostrikov, Mehta, & Nedergaard, 2017; Xie et al., 2013). One of the most crucial aspects of the CSF-ISF exchange exists in the endfeet region of the astrocytes: the water channel aquaporin-4 (AQP4).

AQP4, the most abundant water channel found in the mammalian brain, expresses as a heterotetramer of the M1 and M23 variations and has been shown to cluster into orthogonal arrays of particles (OAPs) (Neely, Christensen, Nielsen, & Agre, 1999; Wolburg, Wolburg-Buchholz, Fallier-Becker, Noell, & Mack, 2011; Yang, Brown, & Verkman, 1996). OAPs were first determined to exist in skeletal muscle and astrocytes over 40 years ago after using the freeze-fracture electron microscopy method (Rash, Staehelin, & Ellisman, 1974). These rectangular arrangements of particles were later found to actually be the different forms of AQP4 arranged in rectangular patterns throughout the plasma membrane (Frigeri, Gropper, Turck, & Verkman, 1995). In fact, recent research has determined the M23 variation is the most commonly found isoform in the densely packed OAPs (Nicchia et al., 2010). Mouse models using AQP4 deletions have shown the importance of AQP4 in the formation of cytotoxic edema and in the elimination of vasogenic edema. As such, current research has implicated the proper location and polarization of AQP4 in various models of cerebrovascular disease and neuroinflammation (Chu et al., 2016; Fukuda & Badaut, 2012). Furthermore, as the water channel responsible for maintaining ISF-CSF exchange homeostasis, there has been a great deal
of interest in the role of AQP4 in maintain proper lymphatic flow, as well as the role of lymphatic flow dysfunction in the pathogenesis of AD. Recent studies have shown the importance of properly functioning AQP4 in the astrocytic endfeet regions by comparing the bulk flow rate and solute clearance levels in AQP4 knockout mice and found the solute clearance rate of Aβ was reduced by over half (Iliff et al., 2012). In addition AQP4 deficiency in a transgenic Aβ precursor protein/presenilin 1 (APP/PS1) model exacerbates the deposition of Aβ in the brain parenchyma as well as displaying as cerebral amyloid angiopathy (CAA) (Xu et al., 2015). Moreover, as the deletion of AQP4 correlates to an increase in the amyloid burden, other studies have approached the relationship between Aβ and AQP4 by examining the effects of Aβ plaques on AQP4 location. In both mouse and human models, the presence of Aβ can lead to the depolarization of AQP4 from their typical location anchored in the astrocytic endfeet (Duncombe et al., 2017; Yang et al., 2011).

In 2010, Parham Moftakhar and colleagues examined the expression of AQP4 in subjects with the diagnosis of CAA and found statistically significant clustering of AQP4 around vessels containing high levels of amyloid deposits when compared to healthy controls, as well as clustering around Aβ plaques in those subjects with AD (Moftakhar, Lynch, Pomakian, & Vinters, 2010). By using tissues from the frontal, occipital, and temporal lobes for the immunohistochemistry, Moftakhar and colleagues were able to demonstrate the presence of AQP4 throughout the brain and show the localization of it around Aβ plaques. However, this broad approach failed to focus on sections affected in the early stages of AD, such as the hippocampus (Convit et al., 1993). Thus, it follows an investigation into the co-localization pattern of AQP4 and Aβ specifically in the hippocampus should be carried out. Furthermore, as research has implicated AQP4 expression as a major contributor to neuroinflammation, an
examination must be conducted of the co-localization pattern of AQP4 and CD68 (a common immunohistochemistry marker for inflammation) as well. To the author’s knowledge, prior researchers have carried out no such studies specifically targeting the hippocampus.

The Role of the Hippocampus in Disease Pathology

Alzheimer’s Disease

The hippocampus has long been implicated as one of the first areas in the brain affected by NFTs and neuritic plaques. More than three decades ago, researchers determined three specific characteristics exhibited by the hippocampus: 1) at least 20 tangle-bearing nucleolated neurons per cubic millimeter, 2) at least 55 nucleolated neurons per millimeter showing granulovaculolar degeneration of Simchowicz, and 3) a population of less than 5600 nucleolated nerve cells per cubic millimeter (Ball et al., 1985). These characteristics clearly show the presence of neuronal death in the hippocampus in subjects diagnosed with AD. Furthermore, investigators had also shown the presence of significant amounts of NFTs in the subiculum and CA1 subfield while the majority of the CA3 and CA4 subfields were void of tangles. As the subiculum is the primary output formation from the hippocampus, this lead to the conclusion of NFTs in the subiculum contributing to the memory impairments found in AD (Hyman, Van Hoesen, Damasio, & Barnes, 1984). In addition to these findings, researchers compiled a large neuropathological study using autopsied brain samples in an effort to determine how different subfields of the brain are affected by AD by dividing the medial temporal lobe into hippocampal subfields and the subiculum. NFTs existed in significantly different amounts and allowed for the characterizing of AD into six specific categories (Braak & Braak, 1991). The pyramidal cells in the CA1 subfield of the hippocampus showed the earliest evidence of NFTs at stage II. This result differed greatly from other subfields with pyramidal cells in the CA4/CA3 subfields failing
to show trace amounts of NFTs until stage V. The subiculum was found to be more similar to the CA4/CA3 pyramidal cells by showing isolated NFTs during stage IV (Braak & Braak, 1991).

The deposition of Aβ plaques were found to remain relatively isolated throughout the hippocampus; however, there was found a densely packed area of amyloid in the subiculum which was greatly diminished as it transitioned into the CA1 region (Braak & Braak, 1991).

Neuronal loss appears to also follow this same pattern of increased levels in CA1 and the subiculum when compared to the other hippocampal subfields (West, Coleman, Flood, & Troncoso, 1994). The level of neuronal loss directly correlates with the decrease in hippocampal volume which has been validated by several magnetic resonance imaging (MRI) studies to be significantly greater in subjects diagnosed with AD (Bobinski et al., 1999; Carlesimo et al., 2015; Convit et al., 1993). As the hippocampus and subiculum are clearly affected early in the progression of AD by Aβ aggregation and the presence of NFTs, research into underlying mechanisms must also target these areas.

Cerebrovascular Disease

As adults continue to age, the comorbidity of AD and CVD increases dramatically (Jellinger, 2002, 2013; Jellinger & Attems, 2010). Categories of cerebrovascular disease include cerebral artery atherosclerosis (AS), cerebral small vessel disease (SVD), and CAA. AS primarily affects the large and medium arteries by accumulating cholesterol in the vessel walls and may lead to large brain infarcts. SVD, as the name implies, affects primarily small vessels but is similar to the vessel wall changes in larger vessels. These changes can lead to micro-hemorrhaging, lacunar infarcts, and cerebral microbleeds. Due to SVD contributing to memory impairment, researchers have investigated the effects of SVD on neuronal loss in the hippocampus. They found very similar results to those in AD with areas showing statistically
significant neuronal loss again located in the subiculum and CA1, while CA2, CA3, and CA4 were similar to healthy controls (Kril, Patel, Harding, & Halliday, 2002). In instances of CAA, investigators have demonstrated through MRI and transcranial Doppler ultrasound a decrease in blood flow due to a loss of vascular integrity which is exacerbated by Aβ deposition (Peca et al., 2013). This loss of vascular integrity may be directly correlated with the depolarization of AQP4 surrounding cerebral vessels (Chu et al., 2016; Marchant et al., 2012). These findings suggest a common underlying mechanism between SVD, CAA, and AD and should be investigated further.

**AQP4 in the Hippocampus and Subiculum**

AQP4 has long been understood to assist in regulating fluid homeostasis following CNS insults such as ischemic stroke (Manley et al., 2000; Zador, Bloch, Yao, & Manley, 2007) and expression levels are increased in hippocampal sclerosis – a significant loss of neurons and increased gliosis in the CA1 subfield of the hippocampus and the subiculum – associated with temporal lobe epilepsy (Lee et al., 2004). As has been previously shown, hippocampal sclerosis is also a common finding in AD; therefore, the same changes in AQP4 expression and function should be exhibited. In addition, as the CA1 subfield and subiculum are especially prone to neuronal degeneration in AD, it stands to reason the properties of AQP4 in these areas should be particularly investigated.

**Study Design**

In order to determine whether AQP4 undergoes a depolarization as previously described, we have compared three serial slices of post mortem brain tissue from 23 different human subjects. Each subject had immunohistochemistry (IHC) staining completed for the presence of Aβ plaques, AQP4, and CD68. Depolarization of AQP4 will be determined by breakdowns in
OAPs and clustering behavior determined by staining intensity. Breakdowns in OAPs will be demonstrated by images showing a loss of even distribution of AQP4 staining. Co-localization patterns will be determined by comparing areas in subjects where Aβ plaques are exhibited with the identical area in slices stained for AQP4 and CD68.

**Hypothesis**

AQP4 has been shown previously to demonstrate clustering behavior around Aβ plaques throughout the cerebral cortex in subjects with CAA (Moftakhar et al., 2010). As the hippocampus and the subiculum have been shown to exhibit AD pathology differently, the clustering behavior of AQP4 should also exhibit differently. We believe the hippocampus will show greater levels of AQP4 clustering (demonstrated by decreased intensity) than the subiculum in areas with Aβ plaques. Furthermore, as Aβ deposition increases in later stages of AD progression, an increased breakdown of OAPs causing more sporadic distributions of AQP4 will be exhibited within the higher diagnosis categories.
Methods

Sample Collection

Post-mortem hippocampal and subicular sections of 23 human subjects were examined: 4 age- and sex-matched controls, 3 AD Braak stage I-III, 4 AD Braak stage IV-V, 7 AD Braak IV-V + CVD, 2 AD Braak stage VI, and 3 AD Braak stage VI + CVD. For demographic data, see Table 1. Each sample was formalin-fixed paraffin-embedded and sliced to 5 µm thickness prior to mounting. All specimens were obtained from the Alzheimer’s Disease Research Center (ADRC) Neuropathology and Molecular Genetics Core (Dr. Harry Vinters, Core Chief and Chief of the Division of Neuropathology) at UCLA.

Table 1: Demographic Data for All Subjects. Subject data showing subject number, the subject age in years at time of death, sex (male or female), official diagnosis upon autopsy, and the post-mortem interval in hours if known.

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<th>Diagnosis</th>
<th>PMI, hours</th>
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<td>80</td>
<td>M</td>
<td>AD Braak I-III</td>
<td>35</td>
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<tr>
<td>2</td>
<td>73</td>
<td>F</td>
<td>AD Braak I-III</td>
<td>108</td>
</tr>
<tr>
<td>3</td>
<td>82</td>
<td>M</td>
<td>AD Braak I-III</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>M</td>
<td>AD Braak IV-V</td>
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<td>M</td>
<td>AD Braak IV-V</td>
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</tr>
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<td>6</td>
<td>111</td>
<td>F</td>
<td>AD Braak IV-V</td>
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<td>82</td>
<td>M</td>
<td>AD Braak IV-V + CVD</td>
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<td>9</td>
<td>115</td>
<td>F</td>
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<td>23</td>
<td>53</td>
<td>M</td>
<td>Control</td>
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Immunohistochemistry

Tissues were incubated for 1 hour at 37°C before undergoing deparaffinization and rehydration. To remove any endogenous activity, 3% hydrogen peroxide/peroxidase was applied to each tissue. Antigen retrieval was completed next by immersing the tissues in Diva Decloaker 10X (1:10, BioCare Medical, Pacheco, CA) at 95°C for 20 minutes. Following antigen retrieval, the tissue underwent blocking of nonspecific antibody sites with two washes of 10 minutes each with tris-buffered saline/Polysorbate 20 (TBST) followed by goat serum for 15 minutes. Each tissue was then stained with an unconjugated polyclonal antibody to AQP4 (1:2000, no. HPA014784; Sigma-Aldrich, St. Louis, MO) and incubated for 1 hour at room temperature. Sections were treated with MACH 2 Universal HRP-Polymer Detection (BioCare Medical, Pacheco, CA) and incubated for 30 minutes at room temperature. Diaminobenzidine (DAB) was used as the chromagen and each section was then counterstained with hematoxylin. Following counterstaining, all slides were dehydrated and mounted. All staining procedures for Aβ and CD68 were performed previously following similar protocols.

Image and Data Analysis

All slides were imaged using a Leica Biosystems Aperio AT2 scanner (20x objective lens, Leica Biosystems, Inc., Buffalo Grove, IL). Scanned slides were then uploaded into Aperio ImageScope (v12.3.2.8013, Leica Biosystems, Inc., Buffalo Grove, IL) where the hippocampus was digitally sectioned from the brain tissue and stain intensity was analyzed using the Positive Pixel Count v9 (PPCv9) algorithm with modified input settings (see Table 2).
Table 2: Positive Pixel Count v9 Algorithm Input Values

Values for Intensity Threshold WEAK (Upper Limit), Intensity Threshold WEAK (Lower Limit), Intensity Threshold MEDIUM (Upper Limit), Intensity Threshold MEDIUM (Lower Limit), and Intensity Threshold STRONG (Upper Limit) were all adjusted from default to establish stricter threshold values for positive staining intensity.

<table>
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<tr>
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<td>View Height</td>
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<td>Overlap Size</td>
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</tr>
<tr>
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<td>Classifier</td>
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</tr>
<tr>
<td>Class List</td>
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<tr>
<td>Classifier Neighborhood</td>
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</tr>
<tr>
<td>Pixel Area (millimeter-squared)</td>
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</tr>
<tr>
<td>Hue Value (Center)</td>
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<tr>
<td>Hue Width</td>
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<tr>
<td>Color Saturation Threshold</td>
<td>4.e-002</td>
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<td>Intensity Threshold WEAK (Upper Limit)</td>
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<tr>
<td>Intensity Threshold WEAK (Lower Limit)</td>
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<tr>
<td>Intensity Threshold MEDIUM (Upper Limit)</td>
<td>110</td>
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<tr>
<td>Intensity Threshold MEDIUM (Lower Limit)</td>
<td>30</td>
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<tr>
<td>Intensity Threshold STRONG (Upper Limit)</td>
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</tr>
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<td>Intensity Threshold Negative Pixels</td>
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Intensity is a measure of the brightness of the pixel with 0 = black and 255 = white, meaning the higher the intensity value, the greater the brightness. Image analysis outputs were shown in a color-coded overlay image with pixel staining showing as blue, yellow, orange, and red for negative, weak positive, positive, and strong positive pixels respectively.

Outputs from PPCv9 were recorded for each slide and categorized into AQP4, Aβ, and CD68 stains. Negative and weak positive intensities were excluded after adjusting the lower limit threshold for weak and medium intensities to 110 and 30 respectively. This adjustment in values maintained a much more stringent qualifier than the default settings and allowed for greater certainty when obtaining counts for positive and strong positive pixels. Average intensities were obtained by combining positive and strong positive total intensities and dividing by the number of positive and strong positive pixels. Average positive intensities were then normalized for area of the hippocampus. A one-way ANOVA was then run to compare the average intensities for each of the diagnostic categories and a post-hoc Tukey-Kramer HSD test was run to determine
differences between each category. Significance was determined by p-values < 0.05. All statistical analyses were completed using JMP Pro 14.0.0 (SAS Institute, Inc., Cary, NC).
Results

Intensity

**Hippocampus**

We performed a one-way ANOVA and applied a Tukey-Kramer HSD post hoc analysis. After controlling for differences in hippocampal area, there was a statistically significant main effect of average intensity (Iavg) for AQP4 [F (5,25) = 6.6421, P = 0.0009] (See Table 3) and a trend toward significance for intensity in Aβ [F (5,18) = 2.3088, P = 0.0871] (See Table 4) and CD68 [F (5,12) = 2.6901, P = 0.0744] (See Table 5).

Table 3: One-way ANOVA Results for Hippocampal AQP4 Iavg. Statistical output for comparing the average intensity values for AQP4 staining for each of the diagnosis categories from hippocampal sections. (*) denotes a statistically significant relationship between the average intensity of the AQP4 staining and which diagnosis category the stain is a part of. DF = Degrees of Freedom.

<table>
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<th>Source</th>
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<td>C. Total</td>
<td>25</td>
<td>98.099317</td>
<td></td>
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</tbody>
</table>

Table 4: One-way ANOVA Results for Hippocampal Aβ Iavg. Statistical output for comparing the average intensity values for Aβ staining for each of the diagnosis categories from hippocampal sections. (*) denotes a statistically significant relationship between the average intensity of the Aβ staining and which diagnosis category the stain is a part of. DF = Degrees of Freedom.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis Category</td>
<td>5</td>
<td>11.254561</td>
<td>2.25091</td>
<td>2.3088</td>
<td>0.0871</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>17.548623</td>
<td>0.97492</td>
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<tr>
<td>C. Total</td>
<td>23</td>
<td>28.803183</td>
<td></td>
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</tbody>
</table>

Table 5: One-way ANOVA Results for Hippocampal CD68 Iavg. Statistical output for comparing the average intensity values for CD68 staining for each of the diagnosis categories from hippocampal sections. (*) denotes a statistically significant relationship between the average intensity of the CD68 staining and which diagnosis category the stain is a part of. DF = Degrees of Freedom.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis Category</td>
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<td>5.22994</td>
<td>2.6901</td>
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<tr>
<td>C. Total</td>
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<td>49.479431</td>
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</table>
Post hoc analysis showed significant differences in intensity between AD Braak VI + CVD and AD Braak IV-V (P = 0.0013), AD Braak VI + CVD and AD Braak IV-V + CVD (P = 0.0015), AD Braak VI + CVD and AD Braak VI (P = 0.0068), and AD Braak VI + CVD and Control (P = 0.0212) (See Table 6). Further evaluation with the Tukey-Kramer HSD post hoc failed to return any statistically significant relationships between groups for intensity in Aβ and CD68 stains.

Subiculum

We performed a one way ANOVA and applied a Tukey-Kramer HSD post hoc analysis. After controlling for differences in subicular area, there was a statistically significant main effect of Iavg for AQP4 [F (5,25) = 3.8394, P = 0.0134] (See Table 7). Post hoc analysis showed a significant difference between AD Braak VI + CVD and Control (P = 0.0219) and AD Braak VI + CVD and AD Braak I-III (P = 0.0222) (See Table 8). Aβ and CD68 analyses failed to return any statistically significant difference.
Co-Localization Patterns

Among the control subjects, the distribution pattern of AQP4 followed the expected OAP distribution with organized filaments throughout the tissue (See Figure 1). As the control subjects did not exhibit Aβ plaques, there was no observed co-localization of AQP4 and Aβ. There was an absence of CD68 as well due to a lack of neuroinflammation in these subjects.
Figure 1: Immunohistochemistry Slides for the Control Diagnostic Category. (Top) Slides show typical patterns from control subjects stained for AQP4 (Left), Aβ (Middle), and CD68 (Right) at 20X magnification. (Bottom) Output overlay from Aperio ImageScope Positive Pixel Count. Blue = Negative, Yellow = Weak Positive, Orange = Positive, and Red = Strong Positive. As very little Aβ is present, co-localization patterns are not observed and OAPs remain intact for AQP4.

In subjects with a diagnosis of AD in Braak stages I-III, a breakdown of OAP structure can be recognized as AQP4 begins to have a more sporadic distribution with some areas beginning to show areas of higher intensity (See Figure 2). For these subjects, the presence of Aβ
CO-LOCALIZATION PATTERNS OF AQP-4

plaques are still uncommon and, as such, instances of co-localization between AQP4, Aβ, and CD68 are rare.

Figure 2: Immunohistochemistry Slides for the AD Braak I-III Diagnostic Category. (Top) Slides show showing typical patterns from subjects in the AD Braak I-III diagnostic category stained for AQP4 (Left), Aβ (Middle), and CD68 (Right) at 20X magnification. (Bottom) Output overlay from Aperio ImageScope Positive Pixel Count. Blue = Negative, Yellow = Weak Positive, Orange = Positive, and Red = Strong Positive. Breakdown of OAPs are beginning to be noticed with small areas of clustering beginning in AQP4.
Subjects in the diagnosis category of AD Braak stages IV-V show significant instances of co-
localization between AQP4, Aβ, and CD68 with AQP4 exhibiting a strong clustering behavior
around Aβ plaques (See Figure 3).

![Image](image1.png)

**Figure 3:** Immunohistochemistry Slides for the AD Braak IV-V Diagnostic Category. (Top) Slides show typical patterns from subjects in
the AD Braak IV-V diagnostic category stained for AQP4 (Left), Aβ (Middle), and CD68 (Right) at 20X magnification. (Bottom) Output
overlay from Aperio ImageScope Positive Pixel Count. Blue = Negative, Yellow = Weak Positive, Orange = Positive, and Red = Strong
Positive. Strong clustering behavior is observed in presence of Aβ plaque with co-localization of CD68 as well.

![Image](image2.png)
Samples from subjects with the diagnosis of AD Braak stages IV-V + CVD show decreased clustering behaviors of AQP4 when compared to those in the same Braak stages without CVD; however, CD68 continues to show co-localization with AQP4 and Aβ plaque (See Figure 4).

Figure 4: Immunohistochemistry Slides for the AD Braak IV-V + CVD Diagnostic Category. (Top) Slides show typical patterns from subjects in the AD Braak IV-V + CVD diagnostic category stained for AQP4 (Left), Aβ (Middle), and CD68 (Right) at 20X magnification. (Bottom) Output overlay from Aperio ImageScope Positive Pixel Count. Blue = Negative, Yellow = Weak Positive, Orange = Positive, and Red = Strong Positive. While Aβ plaques are present, clustering of AQP4 does not appear to be occurring. However, breakdown of OAPs is present.
Subjects in the diagnosis category of AD Braak stage VI continue to show co-localization patterns of AQP4, Aβ, and CD68; however, in the sample subjects there does not appear to be a similar strong clustering pattern which was observed in Braak stages IV-V (See Figure 5).
Figure 5: Immunohistochemistry Slides for the AD Braak VI Diagnostic Category. (Top) Slides show typical patterns from subjects in the AD Braak VI diagnostic category stained for AQP4 (Left), Aβ (Middle), and CD68 (Right) at 20X magnification. (Bottom) Output overlay from Aperio ImageScope Positive Pixel Count. Blue = Negative, Yellow = Weak Positive, Orange = Positive, and Red = Strong Positive. Clustering behavior of AQP4 is not as apparent; however, co-localization patterns are still observed between AQP4, Aβ, and CD68.

As with the other samples with CVD, samples from the diagnosis category AD Braak stage VI + CVD again show strong co-localization between Aβ and CD68 with weak clustering of AQP4 around Aβ plaques (See Figure 6).
When looking at the subiculum, a similar pattern of AQP4 distribution was discovered. The control subjects demonstrated a well-organized OAP distribution which can be seen to
breakdown as AD Braak stage progresses. This process of OAP breakdown is even more pronounced when CVD is present (See Figure 7).

Figure 7: AQP4 Immunohistochemistry Slides from the Subiculum. Slides show the Aperio ImageScope overlay for AQP4 staining in the subiculum. 20X magnification. Blue = Negative, Yellow = Weak Positive, Orange = Positive, and Red = Strong Positive A) Control Diagnostic Category. Staining shows areas of OAPs consistent with AQP4 distribution in healthy individuals. B) AD Braak I-III Diagnostic Category. Breakdown of OAPs are becoming apparent by more disorganized distribution of AQP4 staining. C) AD Braak IV-V Diagnostic Category. Small areas of clustered AQP4 are beginning to form. D) AD Braak IV-V + CVD Diagnostic Category. Areas of clustered AQP4 are rare with decreased organization of AQP4 distribution. E) AD Braak VI Diagnostic Category. Areas of tight AQP4 clustering are apparent. F) AD Braak VI + CVD Diagnostic Category. Clustering behavior of AQP4 is no longer apparent. Distribution appears increasingly random.
Discussion

Implications

Aquaporins are key features in maintaining proper glymphatic flow and the integrity of the blood-brain barrier (BBB) (Chu et al., 2016; Moftakhar et al., 2010; Nakada, Kwee, Igarashi, & Suzuki, 2017) This study has shown AQP4 co-localizes with Aβ and CD68 with increased frequency as AD stage progresses. The pattern of co-localization is evidenced by the change in intensity values for AQP4 stains. When comparing the AQP4 stain intensity from the control subjects (See Figure 1) to those in the AD Braak IV-V diagnosis category (See Figure 3), the presence of orange pixels in the PPCv9 overlay become apparent. The location of these positive pixels is clearly influenced by the location of Aβ plaques in the hippocampus and offers additional support to prior research which suggests AQP4 undergoes depolarization from the astrocytic endfoot region (Cai, Wan, & Liu, 2017; Zeppenfield et al., 2017). This depolarization may be a critical factor in the decreased water and solute clearance characteristic of advanced AD. As result of this decreased glymphatic flow and solute clearance, recent research has suggested the use of ventriculo-peritoneal shunts to maintain appropriate flow levels in subjects with AD (Silverberg, Mayo, Saul, Caravalho, & McGuire, 2004; Yasar et al., 2017). This approach to maintaining proper glymphatic flow is extremely invasive and remains in preliminary studies; however, this increased understanding of AQP4 co-localization patterns may provide a more effective approach in developing therapeutics for AD in the future. By specifically targeting the depolarization of AQP4 in AD, future therapy may be able to maintain appropriate levels of glymphatic flow thereby increasing solute clearance to a level comparable to pre-AD symptoms. This approach could either mitigate or remove the negative effects of Aβ
aggregation in the brain such as decreased cognition, neuronal death, and chronic neuroinflammation.

This research has also shown when CVD is combined with advanced stages of AD that AQP4 fails to exhibit the same level of clustering behavior observed in similar stages of AD without CVD. The decreases in clustering behavior suggest the addition of CVD causes an increase in AQP4 expression as suggested in previous research (Badaut et al., 2010; Badaut, Ashwal, & Obenaus, 2011). One potential explanation of this phenomenon is due to the breakdown of vessel integrity with CVD. As vessels become compromised, more fluid can easily leak into the VRS and an increase in AQP4 could be the brains way of mitigating the damage of too much fluid entering the VRS. This increase in AQP4 expression levels could be used as a differential diagnostic measure when determining if CVD is, in fact, co-morbid with AD. It is interesting to note this potential increase in AQP4 expression appears to disregard the location of Aβ plaques suggesting the edema often associated with CVD is a more powerful regulator of AQP4 than the amount of amyloid in the brain. By using TGN-020, a radioactive 11C positron emission tomography (PET) tracer, Suzuki and colleagues were able to successfully image AQP4 in the astrocytic endfeet of subjects (Suzuki et al., 2013). When used in conjunction with 18F amyloid PET tracers, such as florbetaben or florbetapir, physicians would be able to accurately identify the level of co-localization between AQP4 and Aβ to supplement the current diagnostics using hyper-phosphorylated tau proteins.

Future Directions

One key area needing further research is the mechanism by which AQP4 co-localizes with Aβ plaques. As previously mentioned, AQP4 is a plasma membrane protein typically anchored in the astrocytic endfoot region in healthy individuals. This study has shown AQP4
CO-LOCALIZATION PATTERNS OF AQP-4

does exhibit clustering behavior in the presence of Aβ plaques; however, the underlying mechanism behind this clustering is not understood. One potential mechanism is tied to the increased expression of glial fibrillary acidic protein (GFAP) in the presence of Aβ plaques in CAA (Moftakhar et al., 2010). Co-localization analysis between AQP4 and GFAP must be carried out. It is suggested the samples again be taken from the hippocampus and subiculum to aid in early AD detection. Each sample would then undergo immunofluorescence staining to demonstrate co-localization of AQP4 and GFAP without the need for serial slices.

As our results also showed a decrease in clustering behavior in subjects with AD + CVD, research also needs to be undertaken to determine potential explanations for this change in behavior. Due to CVD decreasing the integrity of the BBB, this may be a portion of the explanation. It follows, therefore, an examination of BBB integrity must be conducted in subjects with AD + CVD. The most common method of this would be using Evans blue to perform IHC staining. This staining would allow investigators to determine whether or not the vessels in AD + CVD subjects are, in fact, compromised. Following this process, serial slices should also be stained for AQP4 to determine if the water channels have increased presence around vessels with greater integrity deficiencies.

As the frequency of AD continues to rise, and with current estimates suggesting the number of elderly adults over the age of 65 will increase by 1.25 billion by 2050 (Brookmeyer, Johnson, Ziegler-Graham, & Arrighi, 2007), further studies into all aspects of AD must be undertaken. By illuminating the nature of co-localization patterns between AQP4, Aβ, and CD68, this study has provided valuable information for the future diagnosis and treatment of AD and CVD. Future studies must continue to investigate the nature of AQP4 in an effort to unlock
the mechanisms of depolarization and co-localization. In doing so, researchers may achieve a
dramatic breakthrough which has been sought after for more than a century.
References


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RESEARCH EXPERIENCE

2017 – Present  Graduate Research Assistant, Dr. Jonathan J. Wisco, Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT
Research topics: Identification of tau proteins and beta amyloid plaques in mice, meningeal vasculature, and cerebrospinal fluid flow measurement.

2016 – 2017  Medical Research Associate, PRA Health Sciences, Salt Lake City, UT
Research topics: Phase I Alzheimer’s disease clinical trials and Phase I Major Depressive Disorder clinical trials.

2015 – 2016  Staff Research Assistant, Dr. Man Hung, Department of Orthopaedics, University of Utah School of Medicine, Salt Lake City, UT
Research Topics: Patient Reported Outcomes, mental health, PROMIS scale validation, and geriatric health care.

EDUCATION

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