A Dual Examination of Learning Through Pedagogical Training and Alzheimer's Disease Pathology

Donielle BreAnna Hutchinson
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ABSTRACT

A Dual Examination of Learning through Pedagogical Training and Alzheimer’s Disease Pathology

Donielle BreAnna Long Hutchinson
Neuroscience Center, BYU
Doctor of Philosophy

Active learning strategies are important for facilitating deep learning that may be carried throughout life, but which is still finding its way into the college setting. Educators are not often trained in effective learning practices, which reduces the cognitive and proficiency gains of their students. By providing such guidance in the formative years of a teacher’s training, we hypothesize that the learning environment will be greatly enriched and enhanced. On the opposite end of the spectrum of life and cognition, the plague of dementia also warrants examination. Alzheimer’s disease (AD), an incurable neurodegenerative disorder progressing from the medial temporal lobe, is the most common form of dementia diagnosed in people over age 65, afflicting 30-40% of those 85 years and older. Despite its prevalence, effective treatments are limited because the principal causes and triggers of AD are not entirely understood. Growing evidence demonstrates that oxidative stress (OS) is an important factor contributing to the initiation and progression of AD. A key player contributing to this OS is iron, an essential trace mineral which is required for proper neuronal function, but which generates reactive oxygen species during redox transitions. Intracellular labile iron pool (LIP) levels are strictly regulated by proteins such as transferrin (import), ferroportin (export), and ferritin (storage). However, when these proteins become dysregulated, excess iron associates with other proteins such as amyloid beta (Aβ) and tau, aggregations of which are hallmarks of AD. In our hypothetical model, under extensive or prolonged OS, as occurs in AD, much larger Aβ plaques form because the stress does not abate. Hyperphosphorylated tau is the last resort to protect the cell against free iron, and aggregates when the LIP is elevated because neither iron storage in ferritin nor iron export through ferroportin can relieve the neurons of the free iron.

We hypothesize that elevations in the LIP in AD are due to altered expression of iron homeostasis proteins. We propose to study these phenomena using transgenic mice models for AD, inducing oxidative stress, and observing the effects on the presence of iron and iron-related proteins via immunohistochemistry, mass spectrometry, and western blot.

Experiment 1: We report the effects of a pedagogical training program on the teaching and learning abilities of novice peer teachers through analysis of self-reporting reflections.

Experiment 2: We report changes in staining presence corresponding to transferrin receptor, ferritin, and ferroportin in ApoE2, ApoE3, and ApoE4 transgenic mice over time.

Keywords: active learning, pedagogy, immunohistochemistry, Alzheimer’s disease, iron, oxidative stress, ferritin, ferroportin, transferrin receptor
ACKNOWLEDGEMENTS

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Sincere thanks to my committee members, Drs. Benjamin Bikman, Michael Brown, JC Price, and Richard Watt, for being excellent exemplars of blending shrewd and honest science, caring and concerned mentorship, and upstanding morality into one cohesive whole. I am obliged to each of them for the generosity of their time and effort to serve on my “board of directors” and strive to emulate the example of excellence they set.

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Special heartfelt thanks to my family for their help, encouragement, patience, and prayers. Deepest appreciation and love to my husband Troy for his unfailing devotion. His selfless dedication, patient understanding, and perpetual reassurance have bolstered me through the greatest difficulties. Finally, I am profoundly grateful to my father for his immense sacrifice of time and effort on behalf of me and my family. Without that, this work would have been impossible.
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Memorization is merely a stepping stone to higher order skills

Education is more involved with improving what a student can think and do, rather than with distributing collections of facts and memorized minutia. While memorization has a place in the realm of learning, it should not be the culminating objective. Rather, it should serve as a foundation upon which higher order skills may be built. Still, students often struggle to attain and perform at these higher levels (Bransford, 2000; Zoller, 1993). Much work has been done to amend this disconnect by moving away from the “sage-on-the-sage” model of traditional lectures in favor of more “scientific teaching,” or teaching based on proven, effective practices (Handelsman et al., 2004; Zoller, 1993). While much has been improved in this work, changing the classroom instruction schema is only one aspect of what is required for true learning to occur.

Fink’s taxonomy serves as a guide for developing higher order skills

Fink and colleagues developed a conceptual framework for significant learning, namely that natural learning occurs in simultaneous cognitive domains of Foundational Knowledge, Application, Integration, Human Dimension, Caring, and Learning How to Learn (Fink, 2003, 2013). Further descriptions of each domain may be found in Table 1.1.

Desired learning goals and course outcomes should address many, if not all the cognitive domains described by Fink to result in one who is truly educated. Effective course design is achieved when course objectives, activities, and assessments work in harmony to produce dynamic learning experiences beneficial for the life-long retention and application of knowledge. Many courses focus heavily on Foundational Knowledge, occasionally mixing in a bit of
Application and Integration, but very rarely are all domains present in implicit or explicit course objectives. Additionally, problems arise when one or more of these elements is incongruous (Crowe, Dirks, & Wenderoth, 2008). For example, if objectives and assessment are aimed at ensuring that students attain a high-order cognitive skill (HOCS) related to integration of subject matter across disciplines, but this expected integration is not modeled, explored, or practiced through course activities, students will find their experience frustrating and will either struggle to succeed on assessments, or else assessments may not adequately reflect a student’s progress and capabilities.

Pedagogy training and the current experience of peer teachers

As the workforce in today’s constantly evolving world shifts from skills-based work toward more knowledge-based work, there is an ever-increasing demand for students to obtain a college degree. By 2020, 65% of jobs in the United States will require some level of
postsecondary education (Carnevale, 2014). This demand puts great pressure on the undergraduate community to provide more and better teaching to adequately prepare students for occupational roles in society. However many, if not most, college and university instructors, while highly trained in their field of study, have little to no formal training where teaching is concerned (Alsop, 2018). Indeed, pedagogical skills and teaching innovations are not the main considerations for hiring new collegiate faculty.

Educational scholarship and scientific teaching are considered a fine pursuit for established faculty members, but many have arrived at their skill through their own trial and error. As such, for universities whose prime focus is undergraduate instruction, there is a gross need to put more value and effort into training the rising generation of faculty in the theoretical

<table>
<thead>
<tr>
<th>Cognitive Domain</th>
<th>Explanation</th>
<th>Objective Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foundational Knowledge</td>
<td>knowledge about phenomena associated with the subject &amp; conceptual ideas associated with those phenomena</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Know the names of each chamber and valve of the heart and the flow of blood through those chambers</td>
<td></td>
</tr>
<tr>
<td>Application</td>
<td>ability to use &amp; think about new knowledge in multiple ways; develop important skills</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Develop skills using a stethoscope and reading an EKG and how those tools reflect heart anatomy</td>
<td></td>
</tr>
<tr>
<td>Integration</td>
<td>connect one body of knowledge with other ideas and bodies of knowledge</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Be able to connect the ideas of the physics of fluid pressure dynamics and blood circulation</td>
<td></td>
</tr>
<tr>
<td>Human Dimension</td>
<td>discovering how to interact more effectively with oneself and with others</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Be able to collaborate in groups to solve problems related to clinical heart problems</td>
<td></td>
</tr>
<tr>
<td>Caring</td>
<td>development of new interests, feelings, and values</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Appreciate the complexities of the circulatory system and of the role of a cardiologist</td>
<td></td>
</tr>
<tr>
<td>Learning How to Learn</td>
<td>develop knowledge, skills, and strategies for continuing one's learning after the course is over</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acquire the knowledge and skills to understand research literature regarding the circulatory system and recognize what can still be learned through research</td>
<td></td>
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</table>
grounding of teaching and learning in their discipline. Because they themselves have had little formal teaching training, most professors are not practiced in providing such support for the teaching assistants (TAs) who work with them. Many TAs are used for general instructional grunt work, but not often trained in the nuances of effective teaching.

Specific Aim 1: Increase understanding and implementation of effective peer teaching strategies in classroom teaching sessions. To determine the effectiveness of formal pedagogical training for peer teachers, self-evaluations of teaching and learning ability improvements were compared between groups of novice peer teachers.

The pathology of learning: prevalence and impact of Alzheimer’s disease

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder with no definitive antemortem diagnosis or cure. AD is the most common form of dementia, seriously affecting intellectual abilities, and interfering with daily life for both patient and caretaker (Jorm, Korten, & Henderson, 1987) In addition to the implications on the lives of individuals, this disease has a huge socioeconomic impact. It is estimated that in 2018 the national cost will add up to $277 billion, rising to an alarming $1.1 trillion by 2050 (Association, 2018). AD is characterized by gradual memory loss, and declining cognitive function that progresses to complete incapacity and death. Patients usually die within 3-9 years of diagnosis, and while there is no definitive cure, some symptoms can be addressed and managed for a time (Querfurth & LaFerla, 2010). The progressive degeneration and development of senility which accompany AD, contrary to popular thought, are not a normal part of aging. However, despite vast efforts in AD research, the exact cause and pathogenesis are not completely understood, and there is no effective long-term treatment for the disease.
The influence of lifestyle choices on disease development

Much effort has gone into examining variables that may have an effect in postponing AD onset. Unlike other losses of function, learning cognition does not recruit other brain regions to compensate for loss (Gould et al., 2006), so it is vital that degeneration be suspended before substantial deficits are developed. It has been shown that remaining physically, socially, and mentally active throughout life, but especially in later years, may protect against normal cognitive decline as well as against dementia and AD (Fratiglioni, Paillard-Borg, & Winblad, 2004; Marx, 2005; Mattson, 2015; Stern, 2012; Vemuri, Lesnick, Przybelski, & et al., 2014).

In the case of AD specifically, it has been suggested that learning may delay disease development as well as slow decline in cognitive loss (Billings, Green, McGaugh, & LaFerla, 2007) The cognitive reserve (CR) hypothesis states that higher education early in life combined with intellectual activity later in adulthood coalesce to give individuals an advantage that allows them to better cope with pathology through the development of more efficient processing networks (Dekhtyar et al., 2015; Fratiglioni et al., 2004; Gold, 2015, 2016; Stern, 2012). Bilingualism is cited as a specific mechanism for increasing cognitive reserve, delaying onset on the scale of years (Bialystok, Craik, & Freedman, 2007; Gold, 2015, 2016). It is thus becoming evident that cognitive interventions both early on and later in life may be important in attenuating AD prevalence.

ApoE variants are the main genetic risk factor for AD

APOE is a gene that codes for a lipoprotein involved in transport of cholesterol throughout the body. This shuttling is particularly important in the brain for functions such as membrane remodeling, dendritic reorganization and myelin maintenance, and ApoE is the
predominant cholesterol transporter expressed in the brain (Riedel, Thompson, & Brinton, 2016). Three isoforms of ApoE are expressed in humans, – E2 (Cys-112, Cys-158), E3 (Cys-112, Arg-158), and E4 (Arg-112, Arg-158) – varying in two positions by one amino acid. The Arg 158 → Cys 158 substitution in E2 causes a structural change which results in hypocholesterolemia and decrease in LDL levels. Conversely, the Cys 112 → Arg 112 substitution in E4 elevates cholesterol and LDL in the blood, increasing the risk of cardiovascular disease and neurodegenerative diseases such as AD (Mahley, Weisgraber, & Huang, 2009). ApoE4 may be attributed to as much as 50% of the genetic risk for late-onset AD (Raber, Huang, & Ashford, 2004), with homozygosity in the E4 allele shifting the AD risk up to ten years earlier as compared to those possessing either of the other variants (Riedel, Thompson, & Brinton, 2016).

Under oxidative stress conditions and other situation of neuronal damage, it has been shown that neurons produce ApoE (Xu et al., 2006), and that increased levels of hydroxyl radicals are present along with the ApoE4 genotype (Ihara et al., 2000). This ApoE, especially ApoE4, may be cleaved to form a fragment which can cause neurodegeneration and is associated with increased tau phosphorylation (Brecht et al., 2004; Harris et al., 2003; Huang et al., 2001). Indeed, ApoE has been found to be associated with plaques and tau tangles, though the exact role of any potential pathogenicity is not fully understood (Brecht et al., 2004; Crowther, 1993; Strittmatter et al., 1993). Overall, it is clear that ApoE4 is linked to an increased risk of AD pathology, particularly when considered in conjunction with gender, age, and other environmental risk factors.

Iron homeostasis and the role of the labile iron pool in neuronal function
Iron is an essential trace mineral found throughout the body, important for various processes related to cellular metabolism. Ferric iron in the (III) form is bound to transferrin, a homodimeric iron-transporting protein, which recognizes and binds to transferrin receptors found on cells, including neurons. The entire complex is endocytosed, and iron is converted to the ferrous (II) form, freed from the endosome, and added to what is known as the labile iron pool (LIP), which is used for normal physiological functions. Any excess free iron imported is stored in ferritin, a spherical iron storage protein consisting of 24 polypeptide subunits, each of which may be either a heavy (H) or light (L) chain. Brain ferritin generally consists primarily of L-chain subunits, which are more conducive to iron storage, while H-chains are active in iron metabolism (Watt, 2013). Iron shuttles between ferritin and the LIP to maintain a constant level.

Figure 1.2: Iron regulation under normal conditions. During normal conditions, 1) transferrin binds to extracellular transferrin receptors. 2) The transferrin/receptor complex is endocytosed. 3) Fe(III) becomes Fe(II) within the endosome and is exported via the divalent metal transporter into the LIP. 4) Excess Fe(II) is stored in ferritin as Fe(III). 5) IRPs prevent transcription of proteins related to iron homeostasis. 6) Low levels of APP and ferroportin expression.
of iron for normal physiological function. Any additional excess free iron may be removed from the cell via ferroportin, a transmembrane channel protein, complexed with APP (McCarthy, Park, & Kosman, 2014). Iron response proteins (IRPs) are peptides that bind to ribosomal binding sites (RBS) of the mRNAs of oxidative stress response genes such as APP, ferritin and ferroportin, thereby preventing their expression until needed. IRPs are closely regulated by the LIP.

Iron associated oxidative stress leads to protein damage and cell death in AD

Definitive etiology is unknown, but several studies have attributed iron-related oxidative stress as a factor promoting neuronal damage contributing to the initiation and progression of AD.

Figure 1.3: Iron regulation under oxidative stress conditions. During oxidative stress conditions, 7) Increased LIP levels cause IRPs to dissociate from their binding sites, allowing transcription of iron homeostasis proteins. 8) Fe(II) is shuttled out via ferroportin and converted to Fe(III). 9) BACE1 cleaves APP to Aβ, which aggregates with the exported Fe(III). 10) HP-tau dissociates from microtubules and creates intracellular tangles which also associate with Fe(III). 11) Hcy causes the release of Fe(III) from ferritin.
Excess free iron in the neurons leads to the formation of reactive oxygen species (ROS) that are neurotoxic. Homocysteine (Hcy) is a molecule known to be elevated in AD which can perform redox chemistry to reduce and release iron from ferritin, leading to elevated cytosolic iron levels (Watt, 2013). This elevated LIP will cause the release of IRPs from RBS, promoting expression of APP, ferritin and ferroportin. The excess iron may then be oxidized and expelled from the cell via ferroportin coordinated with APP. Immunohistochemistry studies have shown co-localization of iron with amyloid plaques and HP-tau (Mondragon-Rodriguez et al., 2013; Stamer, Vogel, Thies, Mandelkow, & Mandelkow, 2002). Such co-localizations are also seen with high-resolution magnetic resonance imaging (MRI) (Meadowcroft, Connor, Smith, & Yang, 2009). Currently, it is thought that if iron were removed from the plaques, it may decrease the severity of the disease. However, no pharmacological approach has yet been effective.

Protein aggregates that define AD pathology

Amyloid-beta (Aβ) is an extracellular peptide fragment resulting from cleavage of amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase, successively. These insoluble peptides accumulate in the extracellular spaces as oligomers and fibrils, the latter of which develop into neurotoxic plaques. Normal aging brains also contain Aβ, but in far smaller amounts than those found in AD brains (Glabe, 2005). Tau is a microtubule-associated protein which undergoes dynamic phosphorylation and dephosphorylation as it aids in maintaining the integrity of neuronal microtubules (Stamer et al., 2002). Another function of tau is to traffic APP to the cell surface (Lei et al., 2012; Stankowski, Dawson, & Dawson, 2012), allowing APP to couple with ferroportin to export iron from the neuron, thereby protecting against the formation
of reactive oxygen species (ROS). Continued oxidative stress induces the increased expression of BACE1, and inhibits phosphatases that dephosphorylate tau, causing tau to become hyperphosphorylated (HP-tau) and aggregate inside the cells to form neurofibrillary tangles (Yamamoto et al., 2002; Zhao & Zhao, 2013). In its aggregated form, tau is unable to assist in APP trafficking, thus leading to an accumulation of intracellular iron (McCarthy et al., 2014). Extensive aggregation of Aβ and HP-tau are the hallmark histopathological changes seen in AD.

Iron Hypothesis for AD

We believe that the hallmark pathological accumulations of Aβ and HP-tau in AD are related to a neuron’s failed attempts to reduce its oxidative stress. Our model suggests that the protein aggregation of Aβ and HP-tau are attempts by the cell to sequester the excessive free iron which we propose is the principal cause of neuronal damage. Our iron hypothesis as a cause for AD is based on the fact that as iron is metabolized, it generates ROS, which promote oxidative damage to tissues (Arosio & Levi, 2002), and that iron is associated with Aβ plaques (Meadowcroft et al., 2009) and HP-tau (Stamer et al., 2002; Yamamoto et al., 2002) in the postmortem brain of AD. However, we do not know the pathophysiological mechanism regarding Aβ and HP-tau iron sequestration. Since there are many proteins involved in iron metabolism which may contribute to pathological accumulation, we want to explore the expression patterns of some of the key proteins involved with the different stages of disease. We propose to study this mechanism using animal models by triggering controlled conditions to elevate iron in the cells and analyzing the resulting change in protein distribution using several imaging and biochemical techniques. Our ultimate goal is to better understand the pathological
A DUAL EXAMINATION OF LEARNING

progression of AD to identify therapeutic targets. We propose the following specific aims to frame our scientific inquiry:

Specific Aim 2: Elucidate the time course of iron homeostasis protein expression. Following induced oxidative stress conditions in AD transgenic murine brains, the localization and extent of ferritin, ferroportin, and transferrin receptor expression will be probed using immunohistochemistry, and relative protein expression levels will be detected by western blot analysis.
CHAPTER 2: Peer Teachers Trained in Specific Active and Self-Directed Learning Strategies Have a Clearer Roadmap for Improving Learning Environments.

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Abstract

Active learning strategies are becoming more important in effective classrooms, successful utilization of which requires understanding of the principles of true learning on the part of the instructor. We developed a pedagogical training program consisting of 12 weekly meetings in which peer teachers learned a variety of techniques related to active and self-directed learning. The goal of these trainings was to increase understanding and implementation of effective peer teaching strategies in classroom teaching sessions. After the training, peer teachers expressed a desire to improve student learning experiences and demonstrated a clear roadmap for accomplishment that utilized specific active learning techniques, which roadmap was lacking in untrained cohorts.

Introduction

Peer teaching provides an environment of learning accountability and is effective at helping learners excel in their courses (Bene & Bergus, 2014). Well-trained peer teachers are essential to the success of any program that uses peer teachers (Horneffer et al., 2016; Shiozawa, Hirt, & Lammerding-Koeppel, 2016). For students to learn anatomy and physiology in an effective manner (Fink, 2003, 2013), peer teachers must be trained to facilitate an active learning environment that promotes the acquisition of life-long learning skills. Although peer teachers are justifiably trained in their respective scientific fields, the effectiveness of pedagogical training is not documented. We have developed a training program over the last three years for peer teachers participating in PDBio 349R at Brigham Young University, with the objective to augment peer teachers’ ability to foster significant learning experiences in classroom teaching sessions. We hypothesize that peer teachers who complete pedagogical skills training will
recognize principles of significant learning (Fink, 2003, 2013) in their learning and teaching activities.

Methods

Peer teacher training program

We developed a pedagogical training program consisting of 12 weekly lessons and two assessments in which lecture, but not lab, peer teachers learned a variety of techniques related to active and self-directed learning. The voluntary program consists of skill building in the following areas: validating students, working with struggling students, leading classroom discussions, developing good discussion questions for all abilities, creating group active learning activities, principles of problem-based learning, principles of team-based learning, writing case studies.

<table>
<thead>
<tr>
<th>Week</th>
<th>Lesson Topic</th>
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<tbody>
<tr>
<td>1</td>
<td>Validating students</td>
</tr>
<tr>
<td>2</td>
<td>Working with struggling students</td>
</tr>
<tr>
<td>3</td>
<td>Leading classroom discussions</td>
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<td>4</td>
<td>Developing discussion questions</td>
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<td>5</td>
<td>Group active learning activities</td>
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<td>6</td>
<td>Problem Based Learning</td>
</tr>
<tr>
<td>7</td>
<td>Writing case studies</td>
</tr>
<tr>
<td>8</td>
<td>Effective PowerPoint slides</td>
</tr>
<tr>
<td>9</td>
<td>Team Based Learning</td>
</tr>
<tr>
<td>10</td>
<td>Analogies and games</td>
</tr>
<tr>
<td>11</td>
<td>Self-directed learning</td>
</tr>
<tr>
<td>12</td>
<td>Writing effective multiple-choice questions</td>
</tr>
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</table>
studies, creating effective PowerPoint slides, the power of using analogies and games, facilitating self-directed learning, and writing effective multiple-choice questions. Each lesson lasted one hour.

Teaching and learning reflections

As part of their training, all first-year peer teachers enrolled in PDBIO 349R (whether they participated in the pedagogy enhancement training or not) completed a semester-long series of 12 reflections on teaching and learning. At the end of the Fall 2016 semester, when the training program was implemented, we asked both our novice lecture (5/5, 100% response) and lab (7/23, 30% response) peer teachers to complete an IRB approved reflection through Qualtrics, comparing and contrasting their skills as a teacher and as a learner between the beginning and end of the semester.

Grounded Theory Approach

Data was downloaded from Qualtrics, then stripped of identifiable information. We analyzed responses using a grounded theory approach (Guzman et al., 2015; Wisco, Thakur, & Stark, 2014; Wisco et al., 2015), beginning with constructing a word cloud to reveal the top three most frequently used words (nouns, verbs, adjectives, and/or adverbs) in responses. Subsequent detailed thematic qualitative analysis was performed, in which multiple raters reviewed the compiled responses containing the top three words and determined themes common amongst the responses. Themes generated were discussed and agreed upon by all raters, and representative quotes were chosen for each theme to reflect the sentiment of the respondents. Themes and
representative quotes were compared between treatment groups to assess similarities and differences which could be explained by the treatment.

Results

Regarding teaching improvements, all peer teachers expressed a desire to improve student learning experiences, but lecture peer teachers described a clear roadmap to improved student learning that utilized specific active learning techniques for the upcoming semester such as working with struggling students, leading thought provoking discussions, creating group activities and games, implementing analogies, facilitating problem and team based learning, writing case studies and multiple choice questions, and promoting self-directed learning. Regarding learning improvements, all peer teachers discovered cognitive bridges that integrated their increasing anatomy knowledge with related concepts in other classes.

Discussion

We showed that a pedagogical training program helps direct peer teachers to learn and implement specific active learning techniques. Such training programs for peer teachers benefit all participants in the learning process. Students profit from interactions with skilled peer teachers who are equipped with the tools, knowledge, and skills to expedite deep learning. Peer teachers become better learning facilitators, which leads to more rewarding experiences with both students and professors. Providing the formal training to start peer teachers on a trajectory to becoming capable educators benefits the peer teachers by giving them a head start in this arena, should they choose to pursue it. Encouraging that pathway on the part of professors providing such training will ultimately generate more competent faculty with which to distribute
the burden of demand for high quality collegiate learning environments. This, in turn will cycle around to benefitting more students, peer teachers, and college and university learning communities.
Acknowledgements

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CHAPTER 3: Histological evidences for changes in iron homeostasis protein expression in a transgenic murine model of Alzheimer’s disease.

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Abstract

Alzheimer’s disease affects an increasing number of individuals and yet little is understood about pathological initiation and progression. Increased oxidative stress due to aberrant iron metabolism is thought to contribute to the observed cellular damage and neurodegeneration underlying the characteristic cognitive decline. To address this hypothesis, transgenic ApoE4 mice were fed a diet high in methionine and cohorts were sacrificed at 3, 6, 9, and 12 months of age. Immunohistochemical assays showed that ApoE4 had a significant reduction in staining for TFNR and Fe, both in the hippocampus and in the subiculum. Methionine treatment significantly increased TFNR in the hippocampus overall. Females overall exhibited an increase in Fe staining. Age trended toward significance for increasing Ftn in both the hippocampus and the subiculum and was a significant factor in decreasing Fe in younger mice. FPN saw a trending decrease in ApoE4 mice in the hippocampus, but no significant changes in the subiculum.

Introduction

Alzheimer’s disease (AD) is the leading cause of dementia in the elderly population, affecting 10% of people age 65 and older, rising to 40% of those age 85 and older (Association, 2018). While there is a genetic risk factor associated the ApoE4 allele and the development of AD, the correlation is not direct; some with the genetic risk factor do not develop disease, and not all who develop the disease possess the deleterious gene. Thus, other risk factors must be examined to more fully understand the condition. Oxidative stress (OS) is a key factor implicated in AD. Homocysteine (Hcy) levels are known to be elevated in AD (Linnebank et al., 2010; McCaddon, Davies, Hudson, Tandy, & Cattell, 1998; Nägga et al., 2003; Regland,
Abrahamsson, Gottfries, & Magnus, 1990; Zhuo, Wang, & Praticò, 2011) and may contribute to OS through its ability to oxidize iron and release it from ferritin (Ftn). Elevated iron contributes to generation of reactive oxygen species (ROS) which trigger cellular damage, leading to cell death. Cell damage and death results in an increase in inflammation, as macrophages and other immune cells are recruited to the area to eliminate debris and address any potential pathogens. Among other effects, increased inflammatory signals will stimulate the expression of hepcidin, a hormone produced by the liver which functions in regulating iron metabolism by promoting ferroportin (FPN) degradation (De Domenico, Lo, Ward, & Kaplan, 2009; Nemeth et al., 2004).

We hypothesize that dyregulation of iron storage due to increased Hcy stimulates oxidative damage to neurons, leading to increased inflammation levels. This inflammation will cause hepcidin-mediated removal of FPN from the membrane. Iron export dysregulation occurs, perpetuating the cycle of damage. We propose that Ftn protein will become more abundant, with a concurrent decrease of FPN. It is also expected that transferrin receptors (TFNR) will either remain the same or decrease over time.

To study these mechanisms, we fed a diet rich in methionine to ApoE transgenic mice from weaning up to 12 months of age. In order to model changes happening over time as disease would progress, cohorts were sacrificed at 3, 6, 9, and 12 months of age and compared to age matched controls fed a normal diet. Immunohistochemical staining was performed for iron, Ftn, FPN, and TFNR and analyzed for staining positivity. Western blot was performed for Ftn and FPN to assess relative expression levels.

Materials and methods
Animals

Breeders transgenic for human genes apoe2, apoe3, and apoE4 were obtained from Jackson Laboratories (Bar Harbor, ME, USA). 40 ApoE2 mice (22M, 18F), 29 ApoE3 mice (14M, 15F), and 42 ApoE4 mice (19M, 23F) were used in the experiment for a grand total of 111. Mice were fed an ad libitum diet of either normal chow or chow enriched with 1.25% methionine obtained from Envigo (Huntingdon, United Kingdom). Diet treatment began at weaning and continued until mice were sacrificed. Cohorts were sacrificed at 3, 6, 9, and 12 months of age using isoflurane followed by decapitation.

Tissue harvesting and preparation

Right brain hemispheres were extracted and fixed in 4% formaldehyde. Fixed hemispheres were processed utilizing a Shandon Excelsior ES tissue processor using a 9-hour xylene-free IPA protocol modified from a Leica protocol (Rolls, 2008). Processed hemispheres were embedded in paraffin wax and sagittal sections were cut at a thickness of 7um. Sections were mounted on charged Unifrost plus slides (Ted Pella, Redding, CA, USA) and stored at room temperature until staining. Left brain hemispheres were extracted and flash frozen on dry ice and stored at -80°C. Homogenates were prepared in the presence of protease inhibitor (Sigma, Saint Louis, MO, USA) and assessed for total protein concentration by BCA assay.

Staining and imaging

Serial sagittal sections were deparaffinized with 100% xylenes and stained for Fe, FPN, Ftn, and TFNR. Fe was stained using a modified Perl’s stain based on the Prussian Blue reaction
between acid ferrocyanide and ionic iron (ab150674, Abcam, Cambridge, United Kingdom), using DAB (3,3’-Diaminobenzidine, SKU: DB801, Biocare Medical, Pacheco, CA, USA) to convert blue staining to brown and substituting a hematoxylin counterstain for the nuclear fast red specified by the kit. In this way, analysis could proceed under the same parameters as protein IHC stains. Primary antibodies for FPN, Ftn, and TFNR were obtained from Sigma (Saint Louis, MO, USA). MACH 2 universal HRP (SKU: M2U522 L, Biocare Medical, Pacheco, CA, USA) was used as the secondary antibody for all proteins and staining was visualized with DAB, followed by hematoxylin counterstaining. Slides were scanned using a Leica Aperio AT2 slide scanner (Leica, Wetzlar, Germany) to produce high resolution images used for analysis.

Western blot

30 µg of total protein for one female sample from each cohort was diluted in 25 mM ammonium bicarbonate (ABC) to a total volume of 15 µL. 15 µL of 2X Laemmli buffer + betamercaptoethanol (BME) (Bio-Rad Laboratories, Hercules, CA, USA) was added for a total loading volume of 30 µL per sample. Samples were heated on a dry heat block at 95°C for 10 minutes and then loaded onto a 4-15% Criterion TGX 18 well gel (Bio-Rad). 1 µg each of purified Ftn and FPN protein were loaded as controls. The gel was run at 150 V for 40 min and transferred to a nitrocellulose membrane at 100 V for 60 min. A Ponceau stain was performed to verify successful transfer, and then the membrane was cut to separate sections for incubation with different antibodies. Membrane sections were blocked in 3% nonfat dairy milk at room temperature for 1 hour on an agitator. Blots were incubated in primary antibodies for Ftn, FPN, and vinculin overnight on an agitator at 4°C. After incubation with secondary antibody at room
temperature for 1 hour, the blots were visualized on a LI-COR Odyssey CLx imager (Lincoln, NE, USA) and signal intensities were analyzed using version 5.2 of Image Studio Lite software.

![Image](image.jpg)

Figure 3.1: Western blot set up. A. Schematic of the gel set up design. Molecular weight ladders were loaded on either side of the gel to aid precise cutting of the membrane for blotting. Purified protein was loaded to inform expected location of sample protein bands. A negative control lane ensured that samples did not spill over, to indicate any nonspecific binding, and to orient the membrane. B. A Ponceau stain was performed after transfer to ensure that transfer was successful and that there were not any irregularities which would interfere with analysis.

Analysis

High resolution slide images were analyzed using Aperio Imagescope v12.3.2.8013 (Leica, Wetzlar, Germany). The hippocampus and the subiculum were sectioned out referencing the Allen Mouse Brain Atlas (Science, 2004). Annotations were analyzed using the Positive Pixel Count v9 algorithm supplied with the standard version of Imagescope. Two to four subjects
per group were used in the statistical analysis. Using SPSS software, a univariate ANOVA was conducted that examined the effect of genotype, gender, diet, and time group on staining positivity between subjects for the hippocampus and subiculum separately, followed by a post hoc multiple comparisons Tukey test to assess mean differences within variables. Western blot signal intensities were measured for each band, subtracting the average surrounding background signal. Vinculin intensities used as normalizing controls. Statistical differences between pairs of variables were examined using univariate ANOVA analysis in SPSS software.

Figure 3.2: Histology Analysis Workflow. A&B. Each section was matched to an atlas image to identify the areas of interest and to preserve analytical consistency across subjects. Image credit: Allen Institute. C. The hippocampus and subiculum were outlined using the free hand pen tool in Aperio Imagescope, each region representing its own annotation layer. D. Annotations were analyzed using the Positive Pixel Count v9 algorithm, generating a visual heat map of staining positivity.
Results

Hippocampal staining

For TFNR, there were main effects of genotype \(F(2,31) = 7.922, p=.002\) and diet \(F(1,31) = 4.419, p=0.044\) on staining positivity, with diet treatment showing an overall increase in mean TFNR positivity, but there were no significant effects of gender \((p=0.875)\) or age \((p=0.722)\). Post hoc testing showed that mean positivity was significantly less for ApoE4 sections as compared to ApoE2 \((p=0.001)\) and ApoE3 \((p=0.003)\).

In the Fe analysis, there was a statistically significant effect of genotype \(F(2,39) = 3.264, p=0.049\) and age \(F(3,39) = 5.776, p=0.002\) on positivity. There was also a significant interaction between genotype, diet, and age \((p=0.026)\) contributing to rejection of the model. Additionally, simple main effects analysis showed that females had significantly more Fe than males \(F(1,39) = 4.221, p=0.047\). Post-hoc multiple comparisons analysis for genotype revealed that the presence of Fe was significantly less for ApoE4 as compared to both ApoE2 \((p=0.018)\) and ApoE3 \((p=0.008)\). Similarly, there was a significant reduction in positivity across the hippocampus overall from ages 3 months to 6 months \((p=0.006)\). However, no difference in diet was observed \(F(1,39) = 0.326, p=0.571\).

For Ftn, a simple main effect of age contributed most to the model \(F(3,29) = 3.109, p=0.042\), with post hoc analysis revealing a decreasing trend from 3 months to 6 months \((p=0.090)\) and an increasing trend between 6 months and 12 months \((p=0.081)\). Ftn positivity was not affected significantly by genotype \((p=0.893)\), gender \((p=0.915)\), or diet \((p=0.250)\).

Finally, analysis of FPN positivity revealed a significant effect of genotype \(F(2,36) = 3.982, p=0.027\), as well as a significant interaction between genotype, gender, diet, and age \(F
A DUAL EXAMINATION OF LEARNING

While there was a main effect of genotype, post hoc multiple comparisons revealed only a trend in difference between ApoE3 and ApoE4 with a p-value of 0.067.

Figure 3.3: Mean staining positivity changes in the hippocampus by genotype. A. Representative pseudo colored heat maps of sections for each protein by genotype. B. Mean staining positivity decreased significantly in ApoE4 mice for TFNR and Fe, while ApoE2 and ApoE3 remained insignificant for all molecules examined. Error bars represent standard errors for each cohort.
Figure 3.4: Mean staining positivity differences in the hippocampus between diet treatments. A. Representative pseudo colored heat maps of sections for each protein by treatment. B. Disregarding genotype, TFNR staining increased significantly with Hcy diet treatment as compared to control diet, while Fe, Ftn, and FPN did not differ significantly with diet. Error bars represent standard errors for each cohort.
Figure 3.5: Mean staining positivity changes in the hippocampus by gender. Females, regardless of genotype or diet group, showed a significant increase in Fe staining overall. All other proteins were not statistically different between genders. Error bars represent standard errors for each cohort.

Figure 3.6: Mean positivity changes in the hippocampus across age cohorts. Genotype not considered, there were no statistically significant differences in positivity for TFNR or FPN as cohorts aged. Ftn positivity showed a trend toward significance between 3 months and 6 months, as well as between 6 months and 12 months. There was a significant decrease in Fe positivity between 3 months and 6 months of age. Error bars represent standard errors for each cohort.
Subiculum staining

In the subiculum, there was a trend toward significance overall for the effect of genotype on TFNR positivity \([F (2,26) = 5.519, p=0.010]\), while neither gender (p=0.913), diet (p=0.107), nor age (p=0.558) had a significant main effect. However, post hoc analysis showed that ApoE4 was significantly lower than both ApoE2 (p=0.006) and ApoE3 (p=0.003).

Genotype was found to exhibit a significant main effect on Fe positivity in the subiculum \([F (2,31) = 3.318, p=0.049]\). Post hoc multiple comparisons revealed a significant difference between ApoE3 and ApoE4 (p=0.050). Additionally, gender \([F (1,31) = 3.771, p=0.061]\) and age \([F (3,31) = 2.682, p=0.064]\) were trending toward significance for Fe positivity.

In the analysis of Ftn positivity, age was trending toward significance \([F (3,17) = 2.955, p=0.062]\) while genotype (p=0.586), gender (p=0.967), and diet (p=0.574) remained insignificant. Similarly, a trending interaction between gender and age \([F (3,17) = 2.819, p=0.070]\) was observed. There were no differences comparisons between gender or between age groups.

The analysis revealed no significant main effects for FPN positivity with genotype (p=0.245), gender (p=0.465), diet (p=0.364), or age (p=0.619). Likewise, there were no differences in the post hoc tests for genotype or age.
Figure 3.7: Mean subiculum staining positivity by genotype. Positivity means were significantly decreased for both TFNR and Fe in ApoE4 mice as compared to either ApoE2 or ApoE3. There was no difference among genotypes for either Fn or FPN positivity levels. Error bars represent standard errors for each cohort.

Figure 3.8: Mean subiculum staining positivity between diet treatments. Treatment exhibited no effects on mean staining positivity for any of the stains performed in the subiculum. Error bars represent standard errors for each cohort.
Figure 3.9: Mean subiculum staining positivity between genders. There were no significant differences in mean staining positivity for any of the iron proteins assessed. There was a trend toward a significant increase in Fe for females. Error bars represent standard errors for each cohort.

Figure 3.10: Mean subiculum staining positivity across age cohorts. Trends for Fe and Ftn were observed across age cohorts, but there were no statistically significant differences between groups. Error bars represent standard errors for each cohort.
Western blot quantification

For Ftn, neither genotype \( F(2,18) = 0.535, p=0.595 \) nor diet \( F(1,18) = 1.257, p=0.277 \) had a significant effect when discounting age. Genotype also did not factor in \( F(2,12) = 1.980, p=0.181 \) when paired with age, discounting diet treatment. However, in this pairing age did have an effect \( F(3,12) = 17.325, p=0.000 \), with multiple comparisons showing a significant increase from 3 to 9 months \( p=0.000 \), from 6 to 9 months \( p=0.001 \), and from 3 to 12 months \( p=0.004 \). There was also a trend toward a significant increase from 6 to 12 months \( p=0.051 \). When discounting genotype, age also had a main effect when paired with diet \( F(3,16) = 14.308, p=0.000 \) and diet trended toward exerting a significant effect \( F(1,16) = 3.841, p=0.068 \).

FPN showed up as three separate bands, which may represent three distinct isoforms, which, in order of decreasing molecular weight, we will refer to as FPN1, FPN2, and FPN3. For FPN1, none of the variables exhibited any significant effects in any combination of comparisons. F-statistics and p-values for these comparisons are reported in Table 3.1. For FPN2, diet had a significant effect when compared with genotype \( F(1,18) = 8.815, p=0.008 \), while genotype did not \( F(2,18) = 0.568, p=0.576 \). Neither

<table>
<thead>
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<th>Variable</th>
<th>df</th>
<th>F</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Genotype &amp; Diet</td>
<td>Genotype</td>
<td>(2,18)</td>
<td>0.483</td>
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<td></td>
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<tr>
<td></td>
<td>Age</td>
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<tr>
<td>Age &amp; Diet</td>
<td>Age</td>
<td>(3,16)</td>
<td>1.668</td>
<td>0.214</td>
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<tr>
<td></td>
<td>Diet</td>
<td>(1,16)</td>
<td>0.607</td>
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genotype \( [F (2,12) = 0.480, \ p=0.630] \) nor age \( [F (3,12) = 0.846, \ p=0.495] \) exerted a significant effect when discounting diet. Statistical measures are reported in Table 3.2.

For FPN3, as with FPN1, none of the variable showed any significant effects in any combination of comparisons. Statistical measures are reported in Table 3.3.

<table>
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<th>F</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
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<td>0.568</td>
<td>0.576</td>
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<tr>
<td></td>
<td>Diet</td>
<td>(1,18)</td>
<td>8.815</td>
<td>0.008*</td>
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<td>0.480</td>
<td>0.630</td>
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<tr>
<td></td>
<td>Age</td>
<td>(3,12)</td>
<td>0.846</td>
<td>0.495</td>
</tr>
<tr>
<td>Age &amp; Diet</td>
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Table 3.3: Statistical measures for FPN3

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<th>p-value</th>
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<td>Diet</td>
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<td>0.837</td>
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Figure 3.11: Western blot for Ftn and FPN across all cohorts. Vinculin was used as an internal normalizing control for each respective lane.
Figure 3.12: Genotype alone does not affect protein quantity. A. Mean signal intensities for Ftn across genotypes. B. Mean signal intensities for three isoforms of FPN. Error bars represent standard errors for each cohort.
Figure 3.13: Diet reduced the amount of FPN2. A. Mean signal intensities for Ftn between diet treatment groups. B. Mean signal intensities for three isoforms of FPN between diet treatment groups. Error bars represent standard errors for each cohort.
Figure 3.14: Ftn, but not FPN quantities increase over time. A. 9 month and 12 month mice show an increase in Ftn levels as compared to 3 and 6 month mice. Inset is a representative portion of the blot. B. There is no significant difference in levels of any FPN isoform as subjects age. Error bars represent standard errors for each cohort.
Discussion

Overall, the histological results given by the positivity analysis are supportive of our model of iron’s role in AD pathology. Iron was significantly higher in females versus males in the hippocampus, and trending toward significance in the subiculum, supporting the facts that women are more likely to develop AD than men (Vina & Lloret, 2010) and that more women are currently living with AD (Association, 2018). A decrease in TFNR in ApoE4 (Figures 2.4 & 2.8) correlates with a decrease in presence of iron, which may be understood as the iron being shuttled less efficiently to storage sites within neurons, leaving it to be taken up by either transferrin or glial cells, which may transport it to recycling locations to be removed from the body. However, this may also represent a dysregulation paradigm in the ApoE4 genotype, as a decrease in intracellular iron has been shown to increase TFNR expression from a transcriptional level (Casey et al., 1988; Rao et al., 1986). It has also been observed that in anemic conditions, TFNR becomes upregulated (Choi & Pai, 2003).

Combined with the trending decrease of FPN and the lack of difference in Ftn, seen in both the histology and western blot data, the decreased iron may truly be the result of less efficient uptake into neurons and therefore left to slowly accumulate extracellularly and extraparanchymally. If this is the case, inflammation and damage could accrue and contribute to triggering formation of Aβ plaques and tipping the scales toward pathology more drastically for ApoE4 than other variants of the gene. It would be advantageous for systems experiencing high levels of oxidative damage to attempt to stop the flow of ROS and those substances that generate them. Considering this, an explanation for why Hcy increases TFNR could be a response to try to transport Fe from outside cells, where it may be more able to cause reactive damage, to inside the cells where there is an abundance of Ftn to keep the redox potential of iron in check.
While not significant, there does appear to be a trend toward increasing both Ftn and FPN with diet treatment, suggesting that the reason Fe does not increase along with TFNR is that the iron taken in is effectively stored and/or exported. Alternatively, the increased TFNR may be due to a greater number of glial cells being recruited to areas of oxidative damage, which cells may also express TFNR to internalize iron for their own cellular processes and to aid in the clearance of excess iron deposits accompanying the damage. This brings to light a weakness in this tissue staining approach in that the positivity detected is not specified to the type of cell expressing the protein of interest. It may however be assumed that the effects are mainly neuronal, given the high concentration of them within the hippocampal and subicular regions. Alternatively, the change may be localized to the microvasculature, which would represent another paradigm to consider. It would be useful and enlightening to include a measure of glial presence and/or activity to shed light on possible inflammatory influences.

When bringing in the factor of time, the mechanisms for dealing with oxidative insult and increased iron load would seem to be effective on the relative short term, as Fe does significantly decrease from 3 to 6 months in the hippocampus, correlating to a trend in a decrease of Ftn during that time as seen in the histology data. However, it does not appear to be effective over a longer time course, as the difference in Fe does not persist past 6 months and seems to trend back upward. This is congruent with our model and population evidence that time is the main risk factor for AD development.

It is possible that the FPN bands we see on the western blot are evidence of a physiological degradation process. As this is an unusual result in our group, we will need to verify with further testing to rule out proteolytic degradation of our samples or other non-specific antibody binding.
Acknowledgements

The authors wish to thank the following funding sources: NIH/NIA 1 R21 AG037843; Brigham Young University, College of Life Sciences, Mentoring Environment Grant; Brigham Young University, School of Family Life, Gerontology Program; Brigham Young University, Magnetic Resonance Imaging Research Facility Seed Grant; Dr. Sarah M. McGinty Neuroscience Graduate Student Research Fellowship; Neurodar, LLC; Limitless Worldwide, LLC.
CHAPTER 4: General Conclusion and Relevance of Research

Our pedagogical findings have served to illustrate that teaching interventions are effective in the realm of peer teaching, which effects should spill over into the classroom and interactions with students. This adds to the body of evidence supporting active learning strategies and the importance of supporting and mentoring teachers and, by extension, all who support students in their efforts to achieve success in their academics and in their personal development. Moving forward, it would be informative to examine more closely the effects such pedagogical interventions have on direct measures of student success.

The histological findings presented here are very encouraging, and very interesting considering our initial model. We found that patterns of Ftn and FPN were consistent with responses that would be typical of cells experiencing oxidative insult for long periods of time. It is possible that the dysregulation of iron may be linked to a dysregulation of TFNR, though from the results presented here it is yet unclear where or why. TFNR has proved to be an interesting protein to study considering the iron hypothesis, and one that has not been examined very commonly, so would be fascinating to continue exploring. The western blot results supported our model, as well as providing a curious new question regarding the possibility of different FPN isoforms, or alternatively the different fragments that result from FPN degradation. Moving forward, it would be interesting to see if the kinetic studies shed light on this question. Together, these findings contribute to further characterizing what has proved to be a puzzling disease and provide a few more avenues of inquiry to explore.

Overall, these findings serve to illustrate the importance of learning to our society and the vital role that environment – both in the educational and lifestyle arenas – play in the cognitive gains and losses that may be experienced.
APPENDIX A: References


McCarthy, R. C., Park, Y. H., & Kosman, D. J. (2014). sAPP modulates iron efflux from brain microvascular endothelial cells by stabilizing the ferrous iron exporter ferroportin. EMBO reports.


APPENDIX B: Curriculum Vitae

Donielle BreAnna Long Hutchinson

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EDUCATION

Doctor of Philosophy in Neuroscience; Neuroscience Center; Family, Home, and Social Sciences. Brigham Young University 2018

(Dissertation: Dysregulation of Iron Homeostasis Proteins in Alzheimer’s Disease)

Bachelor of Science, Physiology and Developmental Biology; Brigham Young University 2014

TEACHING EXPERIENCE

Teaching Assistant: Advanced Neuroscience. Brigham Young University 2014-2017

• Advised individualized study strategies, delivered several lectures on neuronal development and physiology, created learning tools and reference material.

Instructor: Advanced Physiology. Brigham Young University 2015

• Instructed physiology coursework, delivered several lectures on human anatomy and physiology, assisted in lab-work, graded and reviewed final exams.

TEACHING ABSTRACTS


Nguyen SE, Nguyen JL, Hutchinson B, Wisco JJ. The relationship between emotional quotient (EQ) and perception of assessment fairness in pre-professional students. International Association of Medical Science Educators (IAMSE) Abstr 2016 (oral presentation)


RESEARCH EXPERIENCE


- Developed a hypothesis relating oxidative stress of iron on neurons and their direct relation to the development of pathological plaques associated with AD.
- Immunohistochemistry on mice brain.
- Mass spectrometry proteomic analysis of oxidative stress versus genotype in mouse model of AD (Experiment not concluded).

RESEARCH ABSTRACTS


GRANTS AND AWARDS

Dr. Sarah M. McGinty Neuroscience Graduate Student Research Fellowship 2014-Present

Neuroscience Fellowship, College of Life Sciences, BYU 2014-2017

Graduate Student Funding, Department of Physiology and Developmental Biology, BYU 2018
APPENDIX C: A Pathway for the Development of an Integrated Science Educator – My Journey from Learner to Educational Scholar

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Introduction

My academic development has been rather unique in terms of producing a classically trained anatomist versatile in both teaching, scientific research, and educational scholarship. This distinctive skillset is one which bridges the realms of professor and scientist, representing a niche which may serve well the need for science and medical education professionals skilled in cohesively merging content across fields, subjects, and disciplines. Below I describe my journey and submit it as a model for the development of programs and curricula to engender such professionals.

Becoming an educator

I was privileged as an undergraduate student to be selected as one of a team of teaching assistants for PDBio 220: Human Anatomy at Brigham Young University. This in itself was not unusual, as many undergraduates serve as teaching assistants in the course of their experience. What was distinctive about this opportunity was the environment of training and support provided in conjunction with the delineated responsibilities, as well as the latitude to create and improve. More experienced teaching assistants served as models of excellent teaching, and regularly mentored those with less experience, both individually through interviews and observations, as well as in small group discussions and lecture settings.

Small classroom teaching sessions served as an arena where I was able to hone innate skills of linguistic and presentational flexibility in helping students to understand and recall the concepts covered by the course. With each successive semester, I transitioned from merely recreating the main course lectures to presenting the material with my own style and spin. Over the course of the three years involved with this program, I developed a series of 233 PowerPoint
slides aimed to help students to understand, internalize, and recall the information from the course to succeed on their examinations, in addition to another series of 72 slides dedicated to practice questions designed to give students experience with the types of questions they could expect on an exam, as well as to test their knowledge and understanding of the principle concepts. To make these tools available to students who found them useful in their study, I published them on a blog (http://anatomyisos-some.blogspot.com), which site has had a little over 4000 pageviews over its history.

In conjunction with the PowerPoint slides, I also developed a Prezi presentation (http://prezi.com/pge1ytskmy22/anatomy/) framed around the structural hierarchy of anatomy which was geared toward making the information more interactive, as well as mapping out the organization of the information and the connections between them. While this project was never completed, it nonetheless served as an important pedagogical tool in my review sessions and has been viewed a little over 2000 times since its posting. In response to an assignment created for PDBio 220 teaching assistants, I produced two YouTube videos (http://www.youtube.com/channel/UChjIkb7eIG0J84B7EVM8WAg) that demonstrated applications of anatomy concepts, integrating across anatomy, physiology, chemistry, and history. By creating all these resources, I began to see that my passion for improving the learning environment outweighed my enthusiasm for the subject matter, opening the possibility for ambitions beyond merely dispensing information as an instructor. I began to see myself as an educator, one who improves upon systems already in place, rather than merely a good classroom technician.

Pedagogical mentoring in graduate school
With this foundation, I entered graduate school and found myself under the mentorship of Dr. Jonathan Wisco. While I could not focus my entire graduate studies on education, he nonetheless encouraged me to continue to develop my skills in that regard. Throughout my studies I had the opportunity to serve as a teaching assistant for Advanced Neuroscience under the mentorship of Dr. Michael Brown. Over the six semesters thus employed, I developed practice questions and a series of 216 slides, again published to a blog (http://480brainspace.blogspot.com/p/blog-page.html) to aid students as they prepared for discussions centering on a few of the most recent journal articles pertaining to a variety of topics considered in the course. I was also able to participate in development of new literature discussions, as new research came to light and discussions became outdated. There were many conversations about what we could do to improve student participation and understanding during these literature discussions, editing and adjusting the questions and materials to facilitate a smooth, cohesive discussion of the topic.

I had the opportunity to participate in Anatomy Academy, an outreach program created to give elementary school students hands-on experience learning concepts of anatomy, biology, and nutrition, while simultaneously creating an opportunity for college students to improve their skills in directing active learning activities. I was able to participate in a special section of dissection Anatomy Academy, where students had the opportunity to explore anatomy in a very hands-on way, dissecting various animal organs as each body system was discussed.

I continued to expand my understanding of pedagogical theory through workshops with Dr. Wisco and other like-minded graduate students spanning topics such as Fink’s taxonomy and curriculum development, where I was formally introduced to the concepts of learning outcomes, course objectives, active learning activities, team- and problem-based learning strategies, and
formative and cumulative assessments. Not only did I have the opportunity to study these principles from a theoretical perspective, I was also able to put them into practice by designing a curriculum for a hypothetical histology course, as well as to identify them in previous work that I had done, assessing what I had done well and what could be done to improve. In addition to the mentorship I received from Dr. Wisco in this regard, I was also able to take a formal class with Dr. Jamie Jensen, Advanced Topics in Science Education, which provided background on Bloom’s taxonomy, higher-order and lower-order cognitive skills, instructional scaffolding, and the body of literature dedicated to exploring effective strategies in learning transfer. Again, all these theoretical lectures were punctuated with the opportunity to test them actively through development and implementation of a lesson plan, complete with instructor and student guides and an active learning activity.

Armed with a more concrete understanding of the theoretical underpinnings of good teaching, I put these into practice in subsequent teaching opportunities, first as an instructor for an advanced physiology lab, PDBio 363. While the latitude for creation was not as wide as in PDBio 220, PDBio 363 did provide an environment where I could practice skills of just in time teaching – guiding students’ learning by asking them questions, getting them to discuss with each other, and allowing them to struggle and experiment for a little while to discover the answer, rather than jumping in with the answer they needed. I was also able to see how, even though it was a highly hands-on active learning experience, the mismatch in assessment, activity, and outcomes was such to make the overall experience unsavory for students.

Stepping into educational scholarship
This observation of student dissatisfaction in PDBio 363 combined with the exposure to Dr. Jensen’s research illustrated what could be done to experiment within the classroom itself, and I began to find my interest turning toward educational scholarship. I first participated in a study with Sarah Nguyen analyzing data examining the relationship between emotional maturity and students’ perception of exam fairness (Nguyen 2016). This was my first exposure to systematic qualitative analysis, and I was intrigued by the potential spectrum of insights to be gained through this technique.

Lessons learned and skills gained

Each experience along the way has contributed something to my understanding and skillset. In developing and using the Advanced Neuroscience resources, I found that students who participated in reviews in which the paper results were discussed and argued and struggled with before I provided guidance were able to adequately demonstrate mastery of the content through their presentations and comments during the discussion, in comparison to those who merely came to passively copy down the interpretations and “answers” to discussion questions. This latter group of students struggled more consistently with demonstrating deeper understanding of the material and were not often engaged in the deeper discussions of the implications of the research discussed. This helped me to step away from my tendency to come to the rescue with answers and explanations, instead letting the discussions play out and instead asking questions with the intent to guide students to discovery.

I also found the importance of and opportunity to improve my skills in Socratic questioning as I worked to mentor students individually. Learning was much more meaningful if instead of answering students’ questions, I asked them questions in response, or else asked them
to explain what they knew already. Often, they already had the answer, they just needed to be asked the question in a different way or asked a series of simpler questions that all comprised the larger question. When I went about mentoring in this way, students were able to master more content faster, without a lot of repeated explanations of the same concepts.

Through my classroom experiences, I learned that content integration is vital to effective transfer of learning outside of the classroom, which lesson was confirmed to me in my student mentoring interactions. I learned to find ways to apply the content to things the student had learned already, or things that they were naturally interested in or curious about. In that way, new content was connected to things they had already internalized, and it became a part of their understanding more quickly, helping them see the relevance of the new information in their personal paradigm. This ability does not require so much expertise in a given field as it does the breadth of awareness of different fields and subjects as well as the ability to see the connections and interplay between them. Expertise tends to follow as a natural result of subject assimilation.

In my experiences with Anatomy Academy, I really learned the value of active learning design, as students who were previously unresponsive and seemingly uninterested in the lesson begin to light up and engage as soon as they were given this tangible tutorial. Navigating the chaos of such a learning environment was a beneficial growing experience for me, as I was much more accustomed to more traditional lecture-style classrooms, where the instructor is very much in control of the direction of conversation, as well as the volume level. However, it was clear that the excitement of learning in an active way was making a far greater impact than understanding the names and locations of the basic structures of the kidney or lungs.

Future directions for pedagogy and scholarship
In terms of pedagogy, I will soon begin a position as a liaison between Cleveland Clinic and Zygote Media, helping to develop multimedia tools and educational content for their medical school. I hope to be able to work with them to examine how the tool is used and what effect it has on student learning and experience. In this way, I may be able to contribute to educational literature while simultaneously aiding in the dissemination of these and similar tools to other medical schools to improve their curricula by adding an active learning component that will enhance and enrich the learning environment of future medical professionals. I will also continue in collaborations in other areas of educational research, finishing up several studies related to pedagogical training and interventions.
APPENDIX D: Future Research Directions – Kinetic mass spectrometry examination of protein turnover in a murine model of AD.

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Abstract

Alzheimer’s disease (AD) is the leading cause of neurodegenerative dementia, which is increasingly linked to oxidative damage and hippocampal iron dysregulation. It is known that several proteins related to iron metabolism and homeostasis are dysregulated in AD, including ferritin (Ftn) and ferroportin (FPN), but it is unclear whether altered protein levels are due to a synthesis or degradation effect. To address this question, mice were treated with an oxidative stress-inducing diet and labeled with deuterium to probe kinetic rates with mass spectrometric analysis. Experiments are still ongoing, with results yet to be determined.

Introduction

Protein levels in the body are regulated by a delicate balance between synthesis of new proteins and degradation of old and damaged ones. In many diseases, including AD, altered protein levels are observed. Merely measuring the amount of protein present presents a narrow picture of the mechanisms behind the pathologies observed, as it does not provide information about why there is a change. Deuterium exchange mass spectrometry provides an efficient way to examine whether altered expression levels may be due to a change in synthesis regulation or degradation cascades.

We hypothesize that the increased iron load observed in AD may be due to a change in the balance of synthesis and degradation for TFNR, Ftn, FPN, and perhaps other proteins related to iron metabolism or oxidative stress management when faced with a significant cellular stressor. We would expect the mechanism of kinetic regulation to reflect the model and provide insight to what may be explored further to explain the mechanisms of dysregulation in disease.
To study these mechanisms, we subjected wild type and transgenic ApoE4 mice to oxidative stress treatment via a diet rich in methionine for one month, followed by deuterium labeling and analysis of protein turnover via mass spectrometry. Labeling occurred over a time course from 9 hours to 32 days to obtain rates for a wide variety of proteins with varying half-lives.

Materials and methods

Animals

Wild type mice and mice transgenic for human apoE4 (Jackson Labs, Bar Harbor, ME, USA) were given an oxidative stress insult via diet. Diet treatment began at weaning and continued for one month before deuterium labeling began. Diet treatment then continued through the labeling period until sacrifice. Two mice were sacrificed 9 hours, 1 day, 2 days, 4 days, 8 days, 16 days, and 32 days post-injection. An additional mouse in each cohort was sacrificed at 32 days to measure cell proliferation. A grand total of 61 mice were used in the experiment, sacrificed by inhalation of isoflurane followed by decapitation.

Diet

Mice were fed an ad libitum diet of either normal chow or chow enriched with 1.25% methionine obtained from Envigo (Huntingdon, United Kingdom). To allow metabolic effects to reach homeostasis, diet treatment occurred for 30 days before the start of the labeling period. Once deuterium labeling began, mice also had access to a water bottle containing 8% deuterated water.
Deuterium labeling

Thirty days after the start of diet treatment, mice were weighed and given an intraperitoneal injection of 100% deuterated water with 0.9% NaCl at 35µL/g body mass to saturate total body water at ~5% deuterium. Deuterium levels were maintained through ad libitum access to 8% deuterated drinking water. Deuterium enrichment was verified post mortem through spectrometric serum analysis utilizing a liquid water isotope analyzer cavity ring down machine (Los Gatos Research, San Jose, CA, USA).

Tissue collection and sample preparation

Left hemispheres were harvested, flash frozen on dry ice, and stored at -80°C. Hemispheres were homogenized in 1 mL of 25mM ammonium bicarbonate (ABC) in the presence of a protease inhibitor cocktail (Sigma, Saint Louis, MO, USA) using 3mm steel beads and an MP FastPrep-24 Classic tissue homogenizer (Santa Ana, CA, USA). Total protein content of each homogenate was assessed via BCA assay. 50 µg of protein were washed over a 30kD centrifuge spin-filter to remove smaller biomolecules, denatured with 6M guanidine followed by DTT and IAM to maintain linearization. Samples were digested with 1 µL trypsin overnight, washed with 25mM ABC, dried via vacuum centrifugation at room temperature, and resuspended in 80% ACN before transfer to MS vials and re-dried. Dried samples were stored at -20°C until mass spec analysis.
Mass spectrometry analysis

Dried samples were resuspending in MS buffer and run through an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher, Waltham, MA, USA). 9 hour and 1-day time points were analyzed via a tandem mass spectrometry protocol (MS/MS) to generate a list of protein identification for correlating to spectra obtained by MS analysis of all time point samples. MS/MS data was analyzed via Peaks software (Waterloo, ON, Canada) to generate a protein ID list as well as to quantify the amount of each protein. Raw data from the Orbitrap output was extracted through DeuteRater software (JC Price Lab) to correlate the protein IDs with obtains MS spectra and then analyzed in conjunction with deuterium enrichment values to obtain turnover rates for each protein identified.

Results

As yet, no analysis has been completed. Table D.1 represents a collection of potential proteins of interest which we plan to pull from the list of proteins identified by the mass spectrometer.

Discussion

While no definitive conclusions may yet be drawn, preliminary examination of data collected is encouraging. Several hundred proteins are being identified from each sample group, indicating that the preparation methods are sound. Among the proteins identified are several on the list of potential proteins of interest, so it seems that we may be hopeful to be able to find something meaningful in the analysis once it is possible to continue moving forward.
Table D.1: List of potential proteins of interest. A list of proteins which may be of interest in considering the effects of oxidative stress on protein kinetics in AD. Protein ID numbers are UniProt accession numbers, linking the protein name to the UniProt database used in data analysis.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q62120</td>
<td>Tyrosine-protein kinase JAK2</td>
</tr>
<tr>
<td>P09528</td>
<td>Ferritin heavy chain</td>
</tr>
<tr>
<td>P29391</td>
<td>Ferritin light chain 1</td>
</tr>
<tr>
<td>Q62351</td>
<td>Transferrin receptor protein 1</td>
</tr>
<tr>
<td>Q92111</td>
<td>Serotransferrin</td>
</tr>
<tr>
<td>Q9EQ21</td>
<td>Hepcidin</td>
</tr>
<tr>
<td>Q9JIH9</td>
<td>Solute carrier family 40 member 1</td>
</tr>
<tr>
<td>Q811J3</td>
<td>Iron-responsive element-binding protein 2</td>
</tr>
<tr>
<td>P28271</td>
<td>Cytoplasmic aconitate hydratase</td>
</tr>
<tr>
<td>Q9WUB0</td>
<td>RanBP-type and C3HC4-type zinc finger-containing protein 1</td>
</tr>
<tr>
<td>P08226</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>P12023</td>
<td>Amyloid-beta A4 protein</td>
</tr>
<tr>
<td>P56818</td>
<td>Beta-secretase 1</td>
</tr>
<tr>
<td>Q9JL18</td>
<td>Beta-secretase 2</td>
</tr>
<tr>
<td>Q8BVF7</td>
<td>Gamma-secretase subunit APH-1A</td>
</tr>
<tr>
<td>Q3TCV3</td>
<td>Gamma-secretase-activating protein</td>
</tr>
<tr>
<td>O09131</td>
<td>Glutathione S-transferase omega-1</td>
</tr>
<tr>
<td>Q60928</td>
<td>Glutathione hydrolase 1 proenzyme</td>
</tr>
</tbody>
</table>
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