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Lipid Regulation as a Critical Factor in the Development of Alzheimer's Disease

Michael A. Castello

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Loma Linda University
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Lipid Regulation as a Critical Factor in the Development of Alzheimer’s Disease

by

Michael A. Castello

A Dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy in Physiology

June 2015
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy in Physiology.

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DEDICATION

To my wife, my family, and those who believed. I carry you in my heart.
CONTENTS

Approval Page.......................... iii
Acknowledgements............................... iv
Dedication..................................... v
Table of Contents................................ vi
List of Figures ................................ x
List of Tables ................................ xii
List of Abbreviations.............................. xiii
Abstract........................................ xv

Chapter

1. Introduction........................................... 1
   Alzheimer’s Disease in the United States............... 1
   AD Characteristics................................. 1
   The Amyloid Cascade Hypothesis........................... 2
   Shortcomings of the Amyloid Cascade Hypothesis.......... 3

      Targeting Aβ Has Failed to Produce Effective AD Therapies ........................................ 3
      Defining AD on the Basis of Aβ is Fundamentally Flawed ........................................... 5
      Understanding Aβ Function is Critical for Understanding AD ......................................... 9

   A New Direction: Moving Beyond the Amyloid Cascade ................. 10

      APP Functions as Part of the Brain’s Response to Stress................................. 10
      Lipid Dysregulation as a Stress and Pathogenic Trigger of Late-Onset AD .......................................................... 13

      Lipid Metabolism in the Brain................................. 13
      Relationship Between Lipid Regulation and AD........................... 14
      Lipid Dysregulation Insights from Niemann-Pick Type C Disease........................................ 16

   Cholesterol-Related Stress Drives APP Dysregulation in the Pathogenesis of Late-Onset AD ......................... 18
Adaptive Response: A Unifying Hypothesis of Neurodegeneration ..........19

Statement of Hypothesis ........................................................................19
The Adaptive Response Hypothesis of Neurodegeneration ..........20

Summary and Research Design ...............................................................25
Specific Aim One ..................................................................................25
Specific Aim Two ..................................................................................26
Specific Aim Three ...............................................................................27

2. Identification of Genes Involved in Regulation of Brain Cholesterol by Amyloid Precursor Protein .........................................................28

Specific Aim One ..................................................................................28
Introduction ............................................................................................28
Results .....................................................................................................30

Initial Microarray Analysis Confirms Loss of APP Affects Expression of Genes in Cholesterol-Related Pathways .............................................30

Cholesterol-Associated Genes Differentially Expressed in the Absence of APP: Cerebellum .................................................................30
Cholesterol-Associated Genes Differentially Expressed in the Absence of APP: Cortex ..............................................................................36

IPA Analysis Connects Cholesterol Synthesis and Endocytosis Pathways .................................................................................................39
Proteomic Analysis ..................................................................................47
Discussion ...............................................................................................54

3. Identification of Specific Mechanisms Through Which APP Influences Cholesterol Homeostasis .............................................................58

Specific Aim Two ..................................................................................58
Introduction ............................................................................................58
Results .....................................................................................................65

Filipin Levels in Primary Fibroblasts .......................................................65
Filipin Levels and Cellular Damage in Differentiated SH-SY5Y Cells ...70
TTM Biosimulation ...............................................................................74
Discussion ...............................................................................................78

Filipin Fluorescence Correlates with Disease Severity .........................79
27-OHC Treatment Produces AD-Like Filipin Fluorescence in Differentiated SH-SY5Y Cells .................................................. 79
Biosimulation Validates Impact of APP Loss on Cholesterol Metabolism ............................................................................. 80

4. Confirmation of Clinical Relevance and Practical Application of APP-Cholesterol Relationships in the Brain ...................................... 85

Specific Aim Three ...................................................................... 85
Introduction .................................................................................. 85
Results .......................................................................................... 87

Filipin Levels in Human Brains ...................................................... 87
Cholesterol Dysregulation in Peripheral Blood Mononuclear Cells .... 88
Myo-Parkinson’s Project ................................................................. 93

Discussion .................................................................................... 96

5. Discussion .................................................................................. 98

APP is a Regulator of Brain Cholesterol Homeostasis ..................... 100
Direct Clinical Application of Findings ........................................ 103
Supporting the Adaptive Response Hypothesis .............................. 104
Concluding Remarks ..................................................................... 107

6. Materials and Methods ............................................................... 108

Introduction .................................................................................. 108
Cell Culture ................................................................................... 108

Human Fibroblasts and B-Lymphocytes ........................................ 108
Peripheral Blood Mononuclear Cells ............................................. 110
SH-SY5Y Cells ............................................................................. 110

Immunofluorescence ..................................................................... 111

Preparation of Filipin ..................................................................... 111
Filipin Staining for FACS Analysis ............................................... 111
Filipin Staining of Human Tissue ..................................................... 111
Filipin staining and Immunofluorescence for Microscopy ............... 112

Flow Cytometry ........................................................................... 112
Microarray Analysis ...................................................................... 113
Transcriptome-to-Metabolome Biosimulation ................................ 113

Initial Simulations ......................................................................... 113
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simulation Refinement</td>
<td>114</td>
</tr>
<tr>
<td>Preparation of Protein Lysates</td>
<td>115</td>
</tr>
<tr>
<td>Tissue Lysis</td>
<td>115</td>
</tr>
<tr>
<td>Cell Lysis</td>
<td>115</td>
</tr>
<tr>
<td>Proteomic Analysis</td>
<td>116</td>
</tr>
<tr>
<td>Trypsin Digestion and Isotope Labeling</td>
<td>116</td>
</tr>
<tr>
<td>Strong Cation Exchange Fractionation</td>
<td>116</td>
</tr>
<tr>
<td>Desalting</td>
<td>117</td>
</tr>
<tr>
<td>Result Analysis</td>
<td>117</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>118</td>
</tr>
<tr>
<td>U18666A and Cholesterol Treatment</td>
<td>118</td>
</tr>
<tr>
<td>Chemical Preparation</td>
<td>118</td>
</tr>
<tr>
<td>Cytotoxicity Assay</td>
<td>119</td>
</tr>
<tr>
<td>Western Blotting</td>
<td>119</td>
</tr>
<tr>
<td>References</td>
<td>120</td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
</tr>
<tr>
<td>A. Patent Filings</td>
<td>143</td>
</tr>
<tr>
<td>Methods and Kits for Determining Risk for Developing</td>
<td></td>
</tr>
<tr>
<td>Alzheimer’s Disease and Prevention or Treatment Thereof</td>
<td>143</td>
</tr>
<tr>
<td>Tremor Detection and Correction System</td>
<td>153</td>
</tr>
<tr>
<td>B. Publications</td>
<td>136</td>
</tr>
<tr>
<td>Rational Heterodoxy: Cholesterol Reformation of the Amyloid Doctrine</td>
<td>172</td>
</tr>
<tr>
<td>On the Origin of Alzheimer’s Disease. Trials and Tribulations of</td>
<td></td>
</tr>
<tr>
<td>the Amyloid Hypothesis</td>
<td>180</td>
</tr>
<tr>
<td>Filipin Levels as Potential Predictors of Alzheimer’s Disease Risk</td>
<td>184</td>
</tr>
</tbody>
</table>
# FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Comparison of Amyloid and Adaptive Response Hypotheses</td>
<td>7</td>
</tr>
<tr>
<td>2. Visual Representation of the Adaptive Response Hypothesis</td>
<td>24</td>
</tr>
<tr>
<td>3. Reelin-Related Interactions in Three Mouse Genotypes</td>
<td>40</td>
</tr>
<tr>
<td>4. Consequences of APP Loss on Lipid Regulation Pathways</td>
<td>42</td>
</tr>
<tr>
<td>5. Network Map of CD59 Relationships</td>
<td>44</td>
</tr>
<tr>
<td>6. Network Map of ERMN-Related Genes</td>
<td>46</td>
</tr>
<tr>
<td>7. Relative GAPDH Expression</td>
<td>53</td>
</tr>
<tr>
<td>8. <em>In Vitro</em> Testing of APP Mechanisms</td>
<td>60</td>
</tr>
<tr>
<td>9. Fibroblast Western Blots</td>
<td>66</td>
</tr>
<tr>
<td>10. Filipin Staining of NPC Fibroblasts</td>
<td>68</td>
</tr>
<tr>
<td>11. Filipin Staining of AD Fibroblasts</td>
<td>69</td>
</tr>
<tr>
<td>12. Effect of Oxysterols on Cell Survival and Filipin Staining</td>
<td>72</td>
</tr>
<tr>
<td>13. Cholesterol Biosynthesis in ( App^{ko} ) Mice</td>
<td>75</td>
</tr>
<tr>
<td>14. Cholesterol Biosynthesis in ( Npc1^{-/-} ) Mice</td>
<td>76</td>
</tr>
<tr>
<td>15. Cholesterol Biosynthesis in ( App^{ko}/Npc1^{-/-} ) Mice</td>
<td>77</td>
</tr>
<tr>
<td>16. Simplified Cholesterol Biosynthesis in ( App^{ko} ) mice</td>
<td>82</td>
</tr>
<tr>
<td>17. TTM Biosimulation Uncovers Possible Experimental Approaches</td>
<td>84</td>
</tr>
<tr>
<td>18. Cholesterol Dysregulation in Human PBMCs</td>
<td>89</td>
</tr>
</tbody>
</table>
19. Filipin Staining of B-Lymphocytes .................................................................91

20. Concept for Detection of Movement Abnormalities Using Myo Device ............95

21. Results from Testing the Adaptive Response Hypothesis ................................106
TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Genes of Interest Differentially Expressed in Mouse Cerebellum Samples</td>
<td>32</td>
</tr>
<tr>
<td>2. Genes of Interest Differentially Expressed in Mouse Cortex Samples</td>
<td>38</td>
</tr>
<tr>
<td>3. Representative Sample of Proteomics Data</td>
<td>50-52</td>
</tr>
<tr>
<td>4. List of Cells Purchased from Coriell Cell Repository</td>
<td>109</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta peptide</td>
</tr>
<tr>
<td>SP</td>
<td>Senile plaques</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<tr>
<td>NPC1</td>
<td>Neimann-Pick type C disease gene 1</td>
</tr>
<tr>
<td>NPC</td>
<td>Neimann-Pick type C disease</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>T-TBS</td>
<td>1% Triton X-100 in tris-buffered saline</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>TEAB</td>
<td>Triethylammonium buffer</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>AcN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>TTM</td>
<td>Transcriptome-to-Metabolome biosimulation algorithm</td>
</tr>
<tr>
<td>COPASI</td>
<td>COMplex PATHway SIMulator</td>
</tr>
<tr>
<td>NodeXL</td>
<td>Network Overview, Discovery, and Exploration for Excel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
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<td>24-OHC</td>
<td>24-hydroxycholesterol</td>
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<tr>
<td>27-OHC</td>
<td>27-hydroxycholesterol</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>U18</td>
<td>U18666A compound</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium-chloride-potassium lysing buffer</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>BNDF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver x receptor</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
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</tbody>
</table>
ABSTRACT OF THE DISSERTATION

Lipid Regulation as a Critical Factor in the Development of Alzheimer’s Disease

by

Michael. A. Castello

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University, June 2015
Dr. Salvador Soriano-Castell, Chairperson

Alzheimer’s disease (AD) is the most common form of dementia in the United States, representing around eighty percent of all cases. For more than two decades, researchers have been led by the amyloid cascade hypothesis, which assumes that accumulation of the amyloid peptide Aβ, derived by proteolytic processing from the amyloid precursor protein (APP), is the key pathogenic trigger in AD. To date, therapies have largely focused on removing Aβ from the brain, an approach that has produced disappointing clinical outcomes.

I present an alternative hypothesis in which Aβ production and aggregation is a symptom of a larger, systemic disease affecting the regulation of lipids, including cholesterol. In addition to assigning a physiological function for APP and Aβ generation, my hypothesis suggests that lipid dysregulation would likely occur early in the disease process, making it an ideal target for identification of disease risk or even intervention.

Using a mouse model, I show that expression of APP is involved in the regulation of cholesterol synthesis, endocytosis, and myelination pathways. Using human cell culture models, I demonstrate that fibroblasts and peripheral blood mononuclear cells taken from AD patients show signs of lipid dysregulation, and that neuron-like cells...
develop this dysregulation when exposed to oxysterols. Finally, I developed and characterized a method of quantifying these detrimental changes using a fluorescence compound, filipin, which could form the basis of a commercial test to evaluate the potential risk of conversion from mild cognitive impairment to AD.
CHAPTER ONE

INTRODUCTION

Alzheimer’s Disease in the United States

Alzheimer’s disease (AD) is the most common form of dementia in the United States, representing around eighty percent of all cases. As the country’s sixth leading cause of death, AD currently affects 5.4 million Americans, a figure that translates to one in eight people age 65 or older and nearly half of Americans over 85 (Alzheimer’s Association 2013). While deaths from well-known diseases such as HIV, stroke, and heart disease have decreased over the past decade, AD deaths continue to increase, incurring an estimated annual cost of $183 billion. As the US population ages, the number of individuals with AD is projected to increase to 7.7 million over the next twenty years (Alzheimer’s Association 2013). Despite years of research, AD continues to have no cure or effective treatments.

AD Characteristics

Clinically, AD begins with difficulty remembering names and events. Later stages feature symptoms such as confusion and difficulty walking and swallowing, eventually ending in death. Affirmative diagnosis of the disease is only possible at autopsy, when specific pathological hallmarks can be visualized in the brain: extracellular deposits termed senile plaques (SPs), comprised of the amyloid-beta peptide (Aβ), and neurofibrillary tangles (NFTs), made from abnormally phosphorylated tau protein.
Aβ is 38 to 43 amino acids in length, formed through sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases (J. Hardy and Selkoe 2002). The 40 (Aβ40) and 42 (Aβ42) amino acid forms are the most abundant, the latter having the greater tendency to aggregate (Jarrett, Berger, and Lansbury 1993).

NFTs are formed when the tau protein is abnormally phosphorylated and aggregates into paired helical fragments (Grundke-Iqbal et al. 1986). Tau is a cytoskeletal protein with a key role in axonal transport; formation of NFTs disrupts this transport and structurally disables neurons, leading to cell loss, cognitive decline, and patient death (Roy et al. 2005).

The Amyloid Cascade Hypothesis

Characterization of the process by which Aβ is produced from APP led Hardy, Higgins, and Selkoe to propose linking SPs and NTFs in the initial form of the Amyloid Cascade Hypothesis (J. A. Hardy and Higgins 1992). The hypothesis states that the pathogenic process of AD initiates upon accumulation, oligomerization, and aggregation of Aβ in SPs. Aβ accumulation subsequently promotes the hyperphosphorylation of tau protein, leading to formation of NFTs and neurodegeneration, causing the pathological presentation and dementia symptoms (J. A. Hardy and Higgins 1992; D J Selkoe 1991). The overwhelming majority of research in the field has been driven by this premise, which has held a firm grip on the field since AD research began gaining momentum in the 90s (Karran, Mercken, and Strooper 2011; Karran and Hardy 2014).

When the amyloid hypothesis was formed, strong evidence appeared to support its assumptions. All forms of the rare (representing approximately 1% of AD cases) familial early-onset AD are linked to specific mutations in APP or its cleavage enzymes that
promote increased Aβ production (Alzheimer’s Association 2013; Hendriks et al. 1992; Scheunert et al. 1996; Goate et al. 1991; Mullan et al. 1992). Trisomy 21 (Down syndrome, DS) usually results in three copies of the APP gene and formation of Aβ plaques identical to those found in AD patients (Masters et al. 1985). Since both FAD and DS exhibit pathology clearly linked to Aβ production, it seems reasonable to conclude that late onset AD – which also has abnormal Aβ – must also begin with Aβ. Thus, the amyloid cascade hypothesis implicates overproduction and aggregation of Aβ as the most likely cause of observed neurodegeneration in late onset AD, FAD, and DS, a pathological process that is accelerated in FAD and DS due to genetically abnormal APP. Subsequently, the amyloid cascade hypothesis has been supported by innumerable animal and cell culture studies in which pathology is induced by Aβ and rescued by its removal (Jin et al. 2011; Li, Ebrahimi, and Schluesener 2013; Bloom 2014; Bignante et al. 2013). Focusing primarily on these findings, the thinking currently guiding clinical trials concludes that removing Aβ from the brain is the expedient strategy for the treatment of AD.

**Shortcomings of the Amyloid Cascade Hypothesis**

**Targeting Aβ Has Failed to Produce Effective AD Therapies**

Despite initial optimism, attempts to cure or slow progression of AD by targeting Aβ have resulted in an ever-growing list of miscarriages. Guided by the amyloid cascade hypothesis, some of the first AD drugs to reach clinical trial targeted Aβ production by inhibiting the enzymes involved in its generation (β- or γ-secretases), or by driving γ-secretase activity toward the production of shorter, less aggregation-prone forms of Aβ in lieu of Aβ42. More recently, strategies have included the inhibition of Aβ aggregation or
enhancing mechanisms of Aβ removal, the most promising of these being Aβ removal via
the passive induction of an anti-Aβ immune response. Discouragingly, all of these
therapeutic approaches have had a negligible effect on cognitive functioning – even when
successfully reducing “Aβ load” in the brain – and in some cases have even accelerated
disease progression (Karran, Mercken, and Strooper 2011; Benilova, Karran, and De
Strooper 2012; R. A. Sperling, Jack, and Aisen 2011; Mullard 2012; Aisen, Vellas, and
Hampel 2013; Golde, Petrucelli, and Lewis 2010; Vellas 2010; S. Salloway et al. 2011;
Doody et al. 2014; Stephen Salloway et al. 2014; Karran and Hardy 2014). Throughout
its lifespan, the immune approaches have been limited by the unintended consequences of
increased vascular Aβ deposition and edema, both features of the common AD
comorbidity cerebral amyloid angiopathy (CAA) in which vascular Aβ deposition leads
to edema, hemorrhage, and increased cell loss (C. Holmes et al. 2008; Delrieu et al. 2011;
Karran and Hardy 2014; Stephen Salloway et al. 2014; R. Sperling et al. 2012; M. Zabel
et al. 2013; Cupino and Zabel 2014).

As trial after trial has failed, culling the once-promising roster of therapeutic
approaches, several of the highest profile remainders are representatives of the passive
immunotherapy approach: anti-Aβ antibodies bapineuzumab and solanezumab. However,
according to reports published in the New England Journal of Medicine, even these
heavyweights appear destined to disappoint. Phase 3 clinical trials of both drugs have
failed to improve clinical outcomes in patients with late onset AD, with bapineuzumab
treatment continuing to be limited by edema formation (Doody et al. 2014; Stephen
Salloway et al. 2014; Karran and Hardy 2014). Rather than prompting a critical re-
examination of the amyloid hypothesis, clinical trial frustrations have been attributed not
to shortcomings in the hypothesis, but to weaknesses in the experimental design: patients are treated too late in the disease process for drugs to be effective, damage is caused by undetectable Aβ oligomers prior to plaque formation, or the wrong patients have been selected for treatment (Karran and Hardy 2014; Benilova, Karran, and De Strooper 2012; Castellani and Smith 2011; Castello and Soriano 2014). Unfortunately, this line of reasoning overlooks numerous other observations collected since the amyloid hypothesis was first proposed that contradict its assumptions for treatment and the definition of AD itself.

Defining AD on the Basis of Aβ is Fundamentally Flawed

A major contribution to the continued failure of anti-Aβ clinical trials is that affirmative diagnosis of AD can only occur when the presence of Aβ accumulation in the brain is confirmed, a definition contradicted by an increasing amount of data (Castello and Soriano 2013). Imaging studies confirm previous post-mortem observations of Aβ accumulation in a significant proportion of non-demented individuals (Jack et al. 2013; Hulette et al. 1998; Aizenstein H et al. 2008; Price et al. 2009; Crystal et al. 1988). Conversely, a sizable proportion of patients clinically diagnosed with AD do not display Aβ accumulation—even though neurodegeneration is in progress (Jack et al. 2013; Hyman et al. 2012). Based on this information, many in the medical community have concluded that Aβ status is not a reliable marker for the early stages of clinical AD, instead relying more heavily on neurological function in diagnosis (Jack et al. 2013; Hyman et al. 2012; Dubois et al. 2010). Remarkably, AD researchers have instead reached a consensus in which clinically diagnosed AD patients without Aβ accumulation are classified as not suffering from AD. Other than Aβ, there is no evidentiary basis for the assumption that
clinical AD cases with and without Aβ accumulation are etiologically different. Such reasoning effectively perpetuates a tautological argument in which an *a priori* assumption that Aβ is the cause of AD is used to reject any clinical case in which no Aβ increase is apparent.

This defective logic, employed by many involved in AD research and clinical trials, is further illustrated by figure 1. Cognitive status assessment and Aβ imaging data lead, according to the amyloid hypothesis, to a division of the population into four distinct groups (Fig 1A), which are: patients who are cognitively healthy (normal cognition, NC; Fig 1A, 4), patients who are cognitively healthy but accumulate Aβ (normal cognition with Aβ, NC-Aβ; Fig 1A, 2), patients with neurodegeneration who have clinical AD symptoms but no Aβ accumulation (neurodegeneration-first AD, NDF-AD; Fig 1A, 3), and finally, patients who have neurodegeneration, clinical AD symptoms, and Aβ accumulation (Aβ-first AD, AF-AD; Fig 1A, 5) (Karran and Hardy 2014; Jack et al. 2013; Hyman et al. 2012). According to the amyloid hypothesis, of all four observed populations, only those with Aβ-first AD can be considered by definition as suffering from AD, and only the group with normal cognition and no Aβ should be considered as appropriate cognitively normal controls in clinical trials. This is the reasoning used in the EXPEDITION 3 phase of the ongoing solanezumab trial to justify the exclusion of approximately 25% of patients in the study – all of whom were clinically diagnosed with mild AD, but whose imaging data showed no Aβ accumulation (Doody et al. 2014; Karran and Hardy 2014).
Figure 1. Comparison of Amyloid and Adaptive Response Hypotheses. A, Amyloid Hypothesis. Cognitive tests and amyloid imaging separate the total population into four distinct groups (1). These groups are: Normal Cognition (NC; 4), NC with Aβ accumulation (NC-Aβ; 2), Neurodegeneration-First AD (NDF-AD; 3), and Amyloid-First AD (AF-AD; 5). Under this hypothesis, only the AF-AD and NC groups (4,5) are going to be studied moving forward in EXPEDITION 3 as disease state and control, whereas the NC-Aβ and NDF-AD groups (2,3) are ignored, as they cannot be explained and do not fit the paradigm. B, Adaptive Response Hypothesis. The total population (1) is differentiated by a set of stress variables (2) which may include, but are not limited to, oxidative stress, metabolism dysregulation (cholesterol homeostasis, insulin resistance, etc.), genetic factors, and inflammatory response. These variables elicit an adaptive response in the brain and, depending on the nature and intensity of such response, the population falls into two groups, either Normal Cognition (NC) (3) or AD (4), both of which contain Aβ positive and negative subpopulations.
Logically, a decision to exclude patients in this manner is inconsistent with the available data. It has been known for some time that Aβ plaques do not correlate with areas of neurodegeneration, which is why the amyloid hypothesis implicates soluble Aβ oligomers as the cause of AD (Terry et al. 1991; Dickson et al. 1995; Walsh et al. 2002; Dennis J Selkoe 2008; Knopman et al. 2003; Heiko Braak and Del Tredici 2011). Solanezumab is thought to remove these soluble oligomers, yet plaques are the only form of Aβ detectable by imaging methods (Holland et al. 2014; Esparza et al. 2012). There is no obvious reason to continue to follow and treat patients in whom Aβ plaques are already detectable, since the presence of those plaques occurs too late in the disease for anti-Aβ treatment to be effective (S. Salloway et al. 2011; Golde, Schneider, and Koo 2011; Karran and Hardy 2014). By eliminating patients diagnosed with clinical AD who lack Aβ plaques, all patients in early stages of neurodegeneration that would potentially benefit from the solanezumab trial have effectively been removed.

Some scientists attempting to abandon the Aβ-centric view of AD by focusing on the tau protein in NFTs nonetheless fall into identical thought patterns. “Amyloid hypothesis” reasoning has been offered for a tau hypothesis in which hyperphosphorylated tau protein replaces Aβ as the pathogenic trigger of AD, perhaps by acting as a prion protein to spread its aggregation-prone structure from neuron to neuron (Heiko Braak and Del Tredici 2011). There has been a rush to develop antibodies to aggressively remove “toxic” tau, in the hope that by doing so AD progression will be slowed or halted (Yanamandra et al. 2013). As with the Aβ trials, these studies are defeated before they begin. At this point it seems increasingly unlikely that targeting
proteins for removal from the brain is a viable method of attacking AD. Instead, AD treatments will need to address the disease process as part of the wider brain physiology.

Understanding Aβ Function is Critical for Understanding AD

Following the reasoning of the amyloid hypothesis, every *in vitro* and *in vivo* experimental model of AD begins with overexpression of Aβ itself (Jin et al. 2011; Li, Ebrahimi, and Schluesener 2013; Bloom 2014; Bignante et al. 2013). Ostensibly, these models validate the hypothesis by demonstrating harm when Aβ is overproduced and subsequent improvement when it is removed; however, while all cases of FAD can be linked to a relatively small number of mutations directly affecting APP processing, this is not the case with late-onset AD (J. A. Hardy and Higgins 1992; Blennow, de Leon, and Zetterberg 2006). Because FAD only represents approximately 1% of all AD cases, it is more likely that its unique Aβ-producing mutations represent a subset of possible pathways for developing AD, rather than a mechanistic model of the late onset disease affecting the vast majority of AD patients (Alzheimer’s Association 2013; Hendriks et al. 1992; Scheuner et al. 1996). In the largest analyses of late-onset AD to date, nearly all of the genetic polymorphisms most frequently observed occur in genes associated with cholesterol metabolism, endocytosis (an essential part of cholesterol processing), and inflammation (Naj et al. 2011; Bali et al. 2012; Hollingworth et al. 2011). Along with studies showing that Aβ accumulation has little relationship to AD initiation or symptoms, this evidence strongly argues against Aβ as a suitable therapeutic target or even as a reliable marker for defining the disease (Hulette et al. 1998; Aizenstein H et al. 2008; Hyman et al. 2012; Jack et al. 2013; Knopman et al. 2003; H Braak and Braak 1991). Unfortunately, by considering Aβ a toxic byproduct of APP metabolism, the
amyloid cascade hypothesis offers little insight into the physiological generation of Aβ. Neither the physiological role of Aβ nor the mechanisms of its accumulation are well understood and without this information, we will not be able to explain why Aβ is generated in the healthy brain, let alone place it within the pathological process of AD.

A New Direction: Moving Beyond the Amyloid Cascade

New, evidence-based thinking is needed to replace the Aβ-focused model provided by the amyloid cascade hypothesis, a perspective that begins by considering Aβ as a protein with a physiological function whose production from APP is, like other cellular processes, susceptible to dysregulation. As is the case with inflammation or cell division, Aβ production can be both necessary and harmful, and the logical way for potential harm to be assuaged is to strive to understand its purpose – beginning with its parent, APP. Lamenting the slow progress of AD research, John Hardy, one of the initial proponents of the amyloid hypothesis, stated that “If I was a young researcher now, I would try to work out what the function of APP is (J. Hardy and Mayer 2011).” Despite the wealth of data on the cellular and molecular mechanisms of APP trafficking and processing into Aβ, the primary function of APP in the brain remains unknown.

APP Functions as Part of the Brain’s Response to Stress

Several hypotheses that are not Aβ-centric have been proposed, although few have gained significant traction (Obrenovich et al. 2002; Castellani et al. 2009; Castellani and Smith 2011; H. Lee et al. 2007; Benilova, Karran, and De Strooper 2012; Marchesi 2011). Unlike in the past, however, numerous independent researchers have now gathered sufficient information to strongly support a reworked conceptualization of late onset AD.
Synthesizing this information, I propose that Aβ accumulates as part of a response to chronic brain stress stimuli (Castello and Soriano 2013; Castello and Soriano 2014). These stress stimuli constitute the *bona fide* pathogenic triggers of late onset AD and, therefore, would be suitable candidates for therapeutic intervention (Herrup 2010b; Castello and Soriano 2013; Castello and Soriano 2014; Castellani and Smith 2011; Stranahan and Mattson 2012). That vision can be understood in the context of hormesis, the idea that the brain benefits greatly from low levels of cognitive or physical stress that elicit a response from biological repair systems (Stranahan and Mattson 2012). “Common sense” interventions, such as exercise, aid successful brain aging by stimulating the brain’s adaptive response mechanisms, promoting restorative activity that otherwise would not occur (Stranahan and Mattson 2012). In this scenario, the presence of excessive Aβ is evidence of an ongoing stress process, rather than a marker of disease initiation as the amyloid hypothesis assumes.

Applying this model to AD, as illustrated in figure 1B, the total population (Fig 1B, 1) can be affected by chronic stress stimuli (Fig 1B, 2) which may include, but are not limited to, oxidative stress, metabolic dysregulation (cholesterol homeostasis, insulin resistance, etc.), genetic factors, and inflammatory response (Castello and Soriano 2014; Stranahan and Mattson 2012). Each of these stimuli is capable of eliciting a response in which Aβ is produced, and the nature of that response (not the total amount of Aβ that may accumulate in parallel) determines progression into clinical AD (Castello and Soriano 2013; Castello and Soriano 2014). Ultimately this leads to the observed division, shown in figure 1B, into individuals with normal cognition (NC; Fig 1B, 3) and those clinically diagnosed with AD (AD; Fig 1B, 4), both of which may be further divided into
Aβ positive and Aβ negative subpopulations (Castello and Soriano 2013; Castellani and Smith 2011). According to this view, therapeutic approaches must address the biology of the chronic stressors that initiate the disease, not the Aβ accumulation that, unlike in FAD, may or may not occur. This offers numerous potential avenues to explore in the battle against AD. In fact, research into aging, cholesterol regulation, and metabolic disorders such as diabetes all can potentially be applied to AD. Conceiving of the disease in this open-ended, systemic fashion will allow clinicians and scientists to identify new patterns and possibilities for therapy.

In further support of this view, acute stress such as traumatic brain injury causes an increase in Aβ production that is inversely correlated with hyperphosphorylated tau (the basis of NFTs) and signs of neuronal injury (Magnoni et al. 2012; Tsitsopoulos and Marklund 2013). A similar effect is observed after ischemia, possibly in response to high oxidative stress (Pluta et al. 2013). Even low levels of stress can promote a response from APP, with synaptic activity producing Aβ, and APP itself aiding in plasticity and axon pruning (Bero et al. 2011; Cirrito et al. 2005; Hoe et al. 2009; Nikolaev et al. 2009). Other stressors likely include metabolism and cholesterol regulation, which would account for the significantly increased risk of AD in individuals with the less-efficient ε4 allele of the cholesterol transporter apolipoprotein E (ApoE) (Corder et al. 1993; Akram et al. 2010). Diabetes symptomatology also significantly accelerates both Aβ and tau pathology in a mouse model of aging, while systematic cholesterol dysregulation directly alters tau in the absence of APP (Currais et al., 2012; Nunes et al., 2011). If Aβ is produced from APP as part of its involvement in the brain’s adaptive response to stress, it is easy to see how Aβ accumulation may (but not necessarily) occur over time both in
successful aging and in parallel with disease progression, in accordance with what has been clinically observed in elderly patients.

Lipid Dysregulation as a Stress and Pathogenic Trigger of Late-Onset AD

**Lipid Metabolism in the Brain**

Although the human brain accounts for 2% of body mass, it contains about 25% of total body cholesterol (Chobanian and Hollander 1962). In cells, membrane cholesterol is concentrated in the lipid rafts, with lower levels present in the intracellular membranes of organelles (Simons and Ikonen 1997; Schroeder et al. 2001; Reitz 2013). Lipids, such as cholesterol and sphingolipids, appear to be tightly regulated in the brain, with APP and its cleavage products, including Aβ, acting through multiple functional pathways to maintain balance (Grimm, Zimmer, et al. 2013). It is well documented that cholesterol, for example, is a key regulator of cellular processes in the brain, including the formation of myelin sheaths, synaptic function, membrane fluidity and neurosteroid biosynthesis; cholesterol homeostasis has also been linked to learning and memory function in both humans and animals (Chris M. Valdez et al. 2010; Schreurs 2010).

Cholesterol turnover in the brain is slow, with a half-life of five years in humans and four to six months in rodents (I. Björkhem et al. 1998; Sérougne-Gautheron and Chevallier 1973; Bogdanovic et al. 2001; Ingemar Björkhem and Meaney 2004). Due to blood-brain barrier separation, most brain cholesterol is synthesized endogenously in glial cells and astrocytes, with a smaller portion occurring in neurons (M. Saito et al. 1987; Chris M. Valdez et al. 2010). Cholesterol synthesis begins with the conversion of coenzyme A (CoA) into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in the cytoplasm (Tymoczko and Stryer 2002). In the ER, HMG-CoA is converted into mevalonate by
HMG-CoA reductase (HMGCR), from mevalonate into 5-phospho-mevalonate by mevalonate kinase, and from 5-phospho-mevalonate to cholesterol by 7-dehydrocholesterol reductase (7-DHCR) (Tymoczko and Stryer 2002). Because HMGCR catalyzes the rate-limiting step, it is highly regulated by feedback inhibition: high cholesterol levels trigger HMGCR ubiquitination and degradation by the proteosome, while low cholesterol levels stimulate sterol regulated element binding protein (SREBP) to promote HMGCR transcription (Canevari and Clark 2007). Cholesterol synthesized in astrocytes is attached to apolipoprotein E (ApoE) and secreted as high-density lipoprotein particles, which are taken up by neurons via the endosomal-lysosomal system and recycled to the plasma membrane or delivered to other organelles (Wahrle et al. 2004; Brown and Goldstein 1986; Benarroch 2008). Cholesterol is removed from the brain by first being converted into oxysterols, which are capable of crossing the blood-brain barrier; once in the periphery oxysterols are metabolized by the liver (I. Björkhem et al. 1998).

Relationship between Lipid Regulation and AD

Lipids appear to be highly dysregulated in AD, suggesting the kind of chronic stress environment that could cause APP to continuously produce Aβ (Wells et al. 1995; Lars Svennerholm, Boström, and Jungbjer 1997; Prasad et al. 1998). While physiological cholesterol synthesis rates and levels in the healthy brain decline more than 40% with age, reflecting the different needs and roles for brain cholesterol at different stages of life, this phenotype is reversed in late-onset AD (Thelen et al. 2006). At the same time, membrane lipids involved in myelination are decreased (Lars Svennerholm, Boström, and Jungbjer 1997; L. Svennerholm and Gottfries 1994). Furthermore, there is a strong
correlation between levels of brain cholesterol and disease severity in the AD brain (Phelix et al. 2011).

Studies of APP physiology also support cholesterol-related stress as a key causative factor in the pathogenesis of the disease. Processing of APP into Aβ occurs in the cholesterol-rich lipid rafts, and cholesterol accumulation can enlarge lipid raft size, thereby increasing Aβ generation (Cordy et al. 2003; M. Burns et al. 2003; O. Holmes et al. 2012). Because of this, it is possible for cholesterol dysregulation to occur prior to, and increase, Aβ production, a concept supported by animal studies in which rabbits and APP transgenic mice fed a cholesterol-enriched diet showed increases in Aβ (Sparks et al. 2000; Shie et al. 2002; Ghribi et al. 2006). Conversely, α-cleavage of APP, which prevents APP from producing Aβ, is increased in the presence of polyunsaturated fatty acids; in mice, dietary stigmasterol reduces the cholesterol composition of lipid rafts and decreases β-cleavage (Grimm, Haupenthal, et al. 2013; Burg et al. 2013). Anomalies in cholesterol levels and distribution are able to influence the phosphorylation state of tau and create NFTs independently of Aβ (Q. Liu et al. 2010; Koudinov and Koudinova 2001; Nunes et al. 2011). Taken together, this means that cholesterol is capable of inducing the production of Aβ (and its aggregation into SPs) and the formation of NFTs independently of the Aβ-tau cascade mechanism proposed by the amyloid hypothesis. As previously mentioned, there is also a well-established link between AD and ApoE, a protein that, in the brain, is involved in transporting cholesterol and other lipids (Bu 2009). Individuals homozygous for the ε4 allele of the APOE gene, which produces a protein product that is a less efficient transporter than its counterparts, are as much as 18-fold more likely to develop AD (Corder et al. 1993). More recent genetic evidence in
support of cholesterol dysregulation as causative of late-onset AD has been provided by genome-wide association studies (GWAS). Remarkably, in addition to APOE, four other genes are associated with cholesterol metabolism and/or transport: CLU, ABCA7, LDLR, SORL1 (Hollingworth et al. 2011; Naj et al. 2011; Jones et al. 2010). A second associated pathway is that of endocytosis, also linked to cholesterol biology (Hollingworth et al. 2011; Jones et al. 2010; Naj et al. 2011). In that respect, the gene PICALM is of particular interest, as it has been shown to confer risk predominantly in the presence of APOE ε4 positive subjects (Jun et al. 2010). TP53INP1, a stress-inducible protein that mediates apoptotic cell death, and SUCLG2, involved in metabolism via the citric acid cycle, are also risk factors for AD (Escott-Price et al. 2014; Ramirez et al. 2014).

*Lipid Dysregulation Insights from Niemann-Pick Type C Disease*

Other neurodegenerative conditions can also be informative with regards to a potential causative role for cholesterol in brain pathogenesis. Of particular interest is the lysosomal storage disorder Niemann-Pick type C disease (NPC). In NPC, mutations in either of two genes, NPC1 and NPC2, results in intracellular accumulation of unesterified cholesterol and other lipids in late endosomes and lysosomes across all cell types (Lloyd-Evans and Platt 2010; Vance 2006). In the brain, NPC causes dendritic and axonal abnormalities and neuronal death (particularly Purkinje cells in the cerebellum); patients present with splenomegaly, abnormal movement, seizures, and dementia (Ory 2004; Vance 2006).
In common with AD, APP is disproportionately metabolized to Aβ in NPC, even forming AD-like plaques in patients carrying the ApoE ε4 allele; tau has been observed both in early stages of hyper-phosphorylation and as NFTs that are indistinguishable from those appearing in AD brains (Y. Saito et al. 2002; Auer et al. 1995; Treiber-Held et al. 2003). NFTs are also more readily detectable in cell populations most affected by cholesterol accumulation, often forming without any prior deposition of Aβ into plaques, indicative of a direct causative link between cholesterol dysregulation and NFT formation (Love, Bridges, and Case 1995; Treiber-Held et al. 2003). Increased expression of NPC1 has been reported in cortex and hippocampus from AD patients, and some NPC1 polymorphisms have been linked to increased risk of developing AD (Kågedal et al. 2010; Rodríguez-Rodríguez et al. 2010). Loss of one copy of the NPC1 gene in AD mice harboring PS1 and APP mutations leads to accelerated Aβ42 accumulation, compared to control mice with two copies of NPC1 (Borbon and Erickson 2011).

Healthy functioning of the brain appears to depend heavily on lipid regulation – a process that is markedly dysregulated in AD. Sustained dysregulation of brain lipids, such as cholesterol, could play a key role in beginning the pathological processes that lead to AD. Not only does cholesterol have the ability to directly impact Aβ production and tau aggregation, a significant number of genes involved in cholesterol homeostasis are associated with AD. Other neurodegenerative conditions such as NPC provide formal proof that cholesterol dysregulation can precede, and lead to, Aβ and tau abnormalities similar to those commonly observed in AD. If these relationships between APP and lipid regulation are functional, rather than merely coincidental, they would support a model in which AD is a consequences of sustained activation of the brain’s adaptive response to
stress. If this is the case, identifying and treating these stressors would be a far more effective avenue for AD therapy than targeting its symptoms, such as Aβ production.

**Cholesterol-Related Stress Drives APP Dysregulation in the Pathogenesis of Late-Onset AD**

As previously mentioned, the biological function of APP in the brain is not well understood, in particular with regards to the physiological generation of Aβ and the mechanisms leading to its accumulation. An alternative to the amyloid cascade hypothesis should provide new areas for research and potential therapy by attempting to explain the role of APP and Aβ in both health and disease. I have suggested that the evidence accumulated to date indicates that APP functions to regulate lipid homeostasis in the brain in response to stress, through mechanisms including, but not limited to, Aβ generation.

The evidence in support of APP as a lipid regulator in the brain is multifold. In rat neurons, APP has been shown to regulate endogenous cholesterol synthesis via the rate-limiting enzyme, HMG CoA reductase (HMGCR), with decreased cholesterol turnover leading to inhibition of neuronal activity (Pierrot et al. 2013). In a mouse model of NPC that faithfully recreates the disease phenotype and cholesterol accumulation observed in human patients, loss of APP leads to an exacerbation of the cholesterol abnormalities seen in NPC brains, which in turn leads to accelerated progression of the disease (Treiber-Held et al. 2003; Nunes et al. 2011).

APP contains a cholesterol-sensing domain shown to be functional *in vitro*, thereby providing a potential molecular mechanism by which APP could be involved in regulation of brain cholesterol through HMGR, LRP1, and other cholesterol-associated
pathways (Barrett et al. 2012; Beel et al. 2010; Beel et al. 2008). The cleavage products of APP have been shown to directly affect lipid regulation pathways. Cholesterol synthesis is increased by α-cleaved APP, a product of APP cleavage that does not produce Aβ (Grimm, Grimm, and Hartmann 2007). This APP product may even be involved in extracellular signaling by selectively binding to phosphoinositides (PIPs) in the lipid rafts of neurons, which would make it highly sensitive to PIP levels (Dawkins et al. 2014). Aβ acts to reduce the size of lipid rafts by decreasing sphingomyelin and inhibiting cholesterol synthesis by HMGR (Grimm et al. 2005; Grimm, Grimm, and Hartmann 2007; Wang et al. 2014). Interestingly, neprilysin, a major protein responsible for degrading Aβ, is decreased in both level and activity in AD patients (Grimm, Mett, et al. 2013). The remaining APP intracellular domain (AICD) acts as a transcription factor to suppress production of the cholesterol transporter LRP1 (Q. Liu et al. 2007).

Furthermore, Aβ production is increased in response to 27-hydroxycholesterol (27-OHC), an oxidized, neurotoxic form of cholesterol that is increased in AD brains (Shafaati et al. 2011; Jaya R. P. Prasanthi et al. 2011).

**Adaptive Response: A Unifying Hypothesis of Neurodegeneration**

**Statement of Hypothesis**

Collectively, the evidence described above leads me to propose the hypothesis that

Lipid dysregulation is a key stressor in the early pathogenesis of AD, and APP metabolism – including Aβ production – is part of an adaptive response to stress in the brain.
A corollary to this hypothesis is that heritable mutations cause early-onset AD by dysregulating the adaptive response.

The Adaptive Response Hypothesis of Neurodegeneration

In this hypothesis, illustrated in figure 2, AD occurs as a result of long-term stress that is capable of overwhelming the defense mechanisms in the brain. Because APP functions as part of the defense mechanisms, stress factors, including lipid dysregulation, are the more likely *bona fide* pathogenic triggers in AD – rather than arbitrary Aβ accumulation. Stresses to the brain can originate from numerous sources: physical trauma, illness, metabolic stress, vascular pathology, genetic polymorphisms, even regular neuronal activity (Herrup 2010b; J.-P. Liu et al. 2010; Sato et al. 2012; Jaya R. P. Prasanthi et al. 2011; Kirsch et al. 2003; Mattson 2009; Stranahan et al. 2011; Xu et al. 2011; Stranahan and Mattson 2012; Suzuki et al. 2010; Bero et al. 2011; Castellani et al. 2009; Castellani and Smith 2011; Castello and Soriano 2013; Castello and Soriano 2014; Brody et al. 2008). Whether benign or severe, these stressors provoke a response from APP as part of the brain’s defense mechanisms (Herrup 2010b; Stranahan and Mattson 2012). APP responds to stress by producing α-cleavage or β-cleavage products, which act in signaling pathways, as transcription factors, or to regulate other proteins (Magnoni et al. 2012; Tsitsopouulos and Marklund 2013; Pluta et al. 2013; Bero et al. 2011; Cirrito et al. 2005; Hoe et al. 2009; Akram et al. 2010; Hölscher 2011). Lipid regulation is particularly sensitive to stress, exhibiting changes in levels, localization, or oxidation that both cause inflammation and provoke a response from APP (J.-P. Liu et al. 2010; Sato et al. 2012; Jaya R. P. Prasanthi et al. 2011; Kirsch et al. 2003; Mattson 2009; Stranahan et
al. 2011; Xu et al. 2011; van den Kommer et al. 2009). Importantly, the presence of Aβ and inflammation in this scenario is evidence of an ongoing stress process, rather than a marker of disease initiation, explaining why Aβ plaques are found in many cognitively normal patients. As long as the response remains functional, the stress is alleviated and homeostasis in the brain is restored. This has been observed in traumatic brain injury, where Aβ is inversely correlated with increased tau pathology as neurological status improves – perhaps due to increased synaptic activity (Brody et al. 2008; Magnoni et al. 2012).

Sustained stress can overwhelm the adaptive response, causing it to remain active despite being insufficient to counteract the imbalances. It is likely that numerous genetic and environmental factors affect the brain’s ability to resist stress before the adaptive response transitions from functional to dysfunctional, which would explain why brain injury, diabetes, the ε4 allele of ApoE, polymorphisms in lipid regulation genes, and microglial clearance of Aβ can all increase risk for later development of AD (Magnoni and Brody 2010; Bennett et al. 2013; Conejero-Goldberg et al. 2011; Hölscher 2011; Cheng et al. 2011; Naj et al. 2011; Hollingworth et al. 2011; M. Zabel et al. 2013). In the dysfunctional response, Aβ continues to be produced and can aggregate into oligomers and plaques, where it affects already-vulnerable neurons by perpetuating inflammation, extending oxidative damage, and contributing to tau hyperphosphorylation and aggregation (Morgan 2009).

A dysfunctional response contributes to a disease state in which the brain experiences major cellular damage as a consequence of destructive inflammation, axonal transport disruption, tau phosphorylation and aggregation, reactive oxygen species, and
neuronal commitment to aberrant entry into the cell cycle – all of which have been shown to occur as a consequence of unchecked lipid dysregulation (Thirumangalakudi et al. 2008; Pappolla et al. 2002; Stranahan et al. 2011; M. Zhang et al. 2010; Nunes et al. 2011; Koudinov and Koudinova 2001; Conejero-Goldberg et al. 2011; Currais, Hortobágyi, and Soriano 2009; Herrup 2010a). Once in the disease state, inflammation can have additional destructive effects on lipid regulation (Block and Hong 2005).

Vascular Aβ deposition would be prominent at this stage, contributing to cerebral amyloid angiopathy (CAA) and microbleeds. A plausible mechanism for cholesterol-induced cell cycle reentry in AD involves miR-33, a microRNA encoded within SREBP-2 that is involved in the regulation of cholesterol synthesis and uptake, as well as the expression of cell cycle genes – including cyclin D1 (Mandas et al. 2012; Cirera-Salinas et al. 2012). Specifically, downregulation of miR-33 leads to increased cyclin D1 levels, and could conceivably result in neuronal reentry into the cell cycle (Cirera-Salinas et al. 2012; Currais, Hortobágyi, and Soriano 2009). SREBP-2, which interacts with APP cleavage products, is downregulated in AD; since miR-33 levels mirror those of SREBP-2, the resulting increase in cyclin D1 would contribute to an aberrant entry into the cell cycle (Mandas et al. 2012; Bommer and MacDougald 2011; Wang et al. 2014).

Ultimately, the disease state results in synaptic collapse, loss of dendritic mass, and neuronal destruction that causes dementia and death (Arendt 2009; Uylings and de Brabander 2002).

Figure 2 also emphasizes the fundamental differences between early-onset AD, Down syndrome, and late-onset AD. In the former two cases, Aβ is constitutively overproduced throughout life (or the Aβ42/Aβ40 ratio is significantly higher), in the
absence of stress to cholesterol regulation. In such instances, the protective role of APP metabolites in the brain is not available, and the sustained overexpression of Aβ accelerates the rate at which cell dysfunction occurs, through a combination of a weaker adaptive response and a lower threshold for transition from functional to dysfunctional response. Neurodegeneration is further exacerbated by the impact of a higher Aβ load on the pathogenic cascade.
Figure 2. Visual Representation of the Adaptive Response Hypothesis. Through various factors including metabolism, trauma, and synaptic activity, the environment produces stress on the brain. Stress promotes a response from APP and inflammatory processes that can be both functional and dysfunctional. A functional response corrects the stress, while a dysfunctional response contributes to a disease state in which continuous activation of the response system leads to tau tangles, cell cycle reentry, and Aβ deposition, ultimately resulting in cell death. Any stage of this process can be affected by genetics.
Summary and Research Design

Several decades of research have failed to produce effective treatments for AD. Based on the amyloid cascade hypothesis, which implicates Aβ production and aggregation as causative to AD, numerous potential therapies have advanced to clinical trial only to demonstrate negligible benefit to AD sufferers (J. A. Hardy and Higgins 1992; Mullard 2012; Karran and Hardy 2014; Karran, Mercken, and Strooper 2011). Such failures highlight a major shortcoming of the amyloid cascade hypothesis: it ignores any possible physiological function for Aβ. Aβ is cleaved from the larger precursor protein, APP, a molecule relevant to cholesterol regulation (Nunes et al. 2011; Barrett et al. 2012; Kosicek et al. 2010) Synthesizing this information, I proposed the adaptive response hypothesis, which suggests that APP is centrally involved in brain lipid regulation, especially in response to stress (Castello and Soriano 2013). In fulfillment of my PhD requirements, I proposed to experimentally test my hypothesis through three specific aims, the results of which are discussed in the following three chapters.

Specific Aim One

To identify genes involved in brain cholesterol homeostasis that are regulated by the amyloid precursor protein, APP, I performed genetic microarray analysis of cerebellum and cortex collected from pre-symptomatic mice of the following genotypes: non-functional NPC1 (Npc1−/−); lack of APP (Applox); double mutant carrying non-functional NPC1 and lacking APP (Applox/Npc1−/−), and wildtype controls. Microarray changes were validated by proteomic analysis using a shotgun approach.

In my original proposal, I planned to validate microarray changes at the RNA and protein level using real-time quantitative PCR and Western blotting, respectively.
However, these approaches were replaced by proteomics as this technique could potentially measure protein-level changes across thousands of proteins simultaneously. Specific Aim One is discussed in Chapter Two.

Specific Aim Two

To identify specific mechanisms through which APP influences cholesterol homeostasis, I developed an in silico model in which to measure the systemic impact of manipulating APP on the cholesterol homeostasis pathways identified in SA1. Because entire molecular pathways can be observed in silico, this technique will reduce dependency on both in vitro and in vivo models for observation of system-wide consequences of genetic changes.

In my original proposal, I also planned to use primary fibroblasts from NPC and AD patients and age-matched controls as an in vitro approach to study the role of APP on cholesterol homeostasis. However, in the process of establishing the model, I discovered that the intensity of filipin staining in NPC cells appeared to correlate with the severity of the NPC case, and that AD fibroblasts displayed increased baseline filipin staining relative to their age-matched controls.

These findings suggested to me that filipin could be a marker of both cellular damage and disease status. Furthermore, if cellular damage, as detected by filipin, were to begin at presymptomatic stages of AD, and its severity were to increase with disease progression, it might be possible to differentiate mild cognitive impairment (MCI) sufferers according to their risk of conversion into AD by monitoring filipin staining. Consequently, I moved away from the original idea of using primary fibroblasts to test the in vitro role of APP in cholesterol homeostasis and explored instead the link between
filipin fluorescence and cellular damage, to obtain proof of concept that filipin is not only associated with an increase in cholesterol levels, but also can be a measure of cellular damage and, by extension, of disease status.

In summary, my second specific aim consists of the in vitro characterization of the link between filipin staining and cellular damage, as well as the generation of a biosimulation model to identify pathway-wide mechanisms through which APP influences cholesterol homeostasis. Specific Aim Two is discussed in Chapter Three.

Specific Aim Three

To determine if filipin levels can be used as potential predictors of Alzheimer’s disease risk, I tested whether filipin intensity correlates with dementia status in peripheral blood mononuclear cells and B-lymphocytes obtained from AD patients. In my initial proposal, I planned to investigate the relevance of the results obtained in SA1 and SA2 to human AD by measuring levels and tissue distribution of proteins, identified in the previous two aims as associated with APP function, in human autopsy brain samples. However, the in vitro findings in SA2 lead to a more sophisticated implementation of this aim, in which I now lay the foundation for a larger-scale clinical study of filipin-detectable cellular cholesterol abnormalities as a predictor of AD risk. This work confirms that my hypothesis and work in model systems is relevant to clinical AD while also having potential diagnostic utility. The practical and clinical work involved in these experiments was instrumental in the creation and patenting of two diagnostic tests for AD, both of which have detailed roadmaps for clinical validation in the near future. Specific Aim Three is discussed in Chapter Four.
CHAPTER TWO

IDENTIFICATION OF GENES INVOLVED IN REGULATION OF BRAIN CHOLESTEROL BY AMYLOID PRECURSOR PROTEIN

Specific Aim One

To identify genes involved in brain cholesterol homeostasis that are regulated by the amyloid precursor protein, APP, I performed genetic microarray analysis of cerebellum and cortex collected from pre-symptomatic mice of the following genotypes: non-functional NPC1 (\textit{Npc1}^{-/-}); lack of APP (\textit{App}^{ko}); double mutant carrying non-functional NPC1 and lacking APP (\textit{App}^{ko}/\textit{Npc1}^{-/-}), and wildtype controls. Microarray changes were validated by proteomic analysis using a shotgun approach.

Introduction

Initial work by Nunes \textit{et al} has demonstrated the importance of APP in a mouse model of severe cholesterol dysregulation (Nunes et al. 2011). While the impact of removing APP from healthy mice includes modest age-dependent cognitive and locomotor deficits, reactive gliosis, and synaptic degeneration, APP loss in NPC1 mice results in an exacerbation of their aberrant cholesterol phenotype (Zelcer et al. 2009; Kang and Fölsch 2011). These double mutant mice show decreased myelination and impaired cholesterol trafficking (Nunes et al. 2011). These findings suggest that APP has a specific role in the metabolism, transport, and/or usage of cholesterol in the brain – collectively defined as cholesterol regulation. I propose that this role is likely to be part of
an adaptive response to stress, such that if cholesterol function is *not* impaired, APP is not necessary and its loss would have a negligible impact. Thus, the NPC mouse model provides a unique opportunity to perform a targeted, systematic search through cholesterol regulatory pathways to find molecular targets for APP function in the absence and presence of stress to cholesterol regulation.

In Specific Aim One, I use this mouse model to identify genes involved in brain cholesterol homeostasis regulated by APP. Depending on their genetic background, NPC mice begin to show symptoms six weeks after birth, with rapid decline until death around ten weeks (Nunes et al. 2011). In order to limit observed genetic changes to early factors, I proposed to perform genetic microarray analysis of cerebellum and cortex collected from pre-symptomatic mice (younger than six weeks) of the following genotypes: non-functional NPC1 (Npc1<sup>-/-</sup>); lack of APP (App<sup>ko</sup>); double mutant carrying non-functional NPC1 and lacking APP (App<sup>ko</sup>/Npc1<sup>-/-</sup>), and wildtype controls. In addition to manual analysis, Ingenuity Pathway Analysis (IPA) software will be used to process the full dataset of gene expression changes and link them to common regulatory or functional pathways.

I initially proposed to validate gene expression changes at the RNA and protein level using real-time quantitative PCR and Western blotting, respectively. Although ongoing work will utilize these approaches, they are replaced here by proteomic analysis using a shotgun approach, as this technique could potentially measure protein-level changes across thousands of proteins simultaneously. The approaches in this specific aim will test my hypothesis that APP regulates cholesterol pathways by gathering gene
expression information, with the goal of using any identified genes and pathways in the design of mechanistic experiments in Specific Aim Two.

Results

Initial Microarray Analysis Confirms Loss of APP Affects Expression of Genes in Cholesterol-Related Pathways

Western blots for APP and NPC1 proteins were used by Nunes et al. to confirm the genotypes of the App\textsuperscript{ko}, Npc1\textsuperscript{-/-}, and App\textsuperscript{ko}/Npc1\textsuperscript{-/-} mice used for the microarray analysis (Nunes et al. 2011). I confirmed genotype identity as well in the samples that I used in order to rule out handling and/or labeling errors (not shown). I subsequently prepared cerebellum and cortex tissue for microarray processing (as described in Materials and Methods under Microarray Analysis) and received from GenUs Biosystems (Northbrook, IL) the list of gene expression levels across App\textsuperscript{ko} and Npc1\textsuperscript{-/-} genotypes.

While the anatomical structure and known functions of cortex and cerebellum are dramatically different, it is reasonable to propose the study of a potential link of APP to cholesterol homeostasis in both regions. Their biological significance is evident: Cerebellum is the earliest and most affected tissue in the NPC brain; cortex is initially and severely affected in AD. In addition, Nunes et al. described widespread cholesterol and myelin dysregulation in both regions, suggesting that, despite their wide differences, there might be shared mechanisms of cholesterol homeostasis (Nunes et al. 2011).

Cholesterol-Associated Genes Differentially Expressed in the Absence of APP: Cerebellum

The list of genes that are involved in cholesterol biosynthesis and transport and are differentially expressed in cerebellum across App\textsuperscript{ko} and Npc1\textsuperscript{-/-} genotypes is presented
Amongst them, the gene for 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR, http://www.ncbi.nlm.nih.gov/gene/3156), which is reduced in the App<sup>ko</sup> brain, is perhaps the most important, as it is the rate-limiting enzyme for cholesterol biosynthesis, it has been linked to AD, and it is regulated in part by Aβ (Grimm et al. 2005; Grösgen et al. 2010). HMGCR is not affected in the Npc1<sup>−/−</sup> brain, consistent with the idea that cholesterol impairment in Npc1<sup>−/−</sup> mice is primarily linked to transport defects. However, it is downregulated in App<sup>ko</sup>/Npc1<sup>−/−</sup> brains, confirming the impact of APP loss on the gene and suggesting that the exacerbated cholesterol phenotype seen in the double mutant brain may reflect in part the additive impact of HMGCR downregulation from APP loss and the trafficking defects seen in Npc1<sup>−/−</sup> mice.

Other genes involved in cholesterol biosynthesis that are differentially expressed in the absence of APP are hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7, http://www.ncbi.nlm.nih.gov/gene/51478) and lanosterol synthase (LSS, http://www.ncbi.nlm.nih.gov/gene/4047). Hsd17b7 is a 3-ketosteroid reductase in the post-squalene biosynthesis of cholesterol and is downregulated in all non-control genotypes (Marijanovic et al. 2003). Double mutation of App and Npc1 has a greater effect on Hsd17b7 levels than loss of either gene alone. Lanosterol synthase is part of the biosynthetic pathway of lanosterol, which itself is an intermediate in cholesterol biosynthesis. It is not affected by the loss of Npc1, but shows a 2-fold decrease in its expression in App<sup>ko</sup> tissue that does not reach statistical significance (p=0.057). Nevertheless, this gene is of interest because loss of both App and Npc1 results in a significant reduction in its levels; therefore, it could contribute to the changes in cholesterol levels and distribution seen in the double mutant mice. Because lanosterol
Table 1. Genes of Interest Differentially Expressed in Mouse Cerebellum Samples. Statistically significant values (p < 0.05) are highlighted in dark green.

| Gene Name    | Pathway                  | Cerebellum
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Npc1&lt;sup&gt;-/-&lt;/sup&gt;</strong></td>
<td></td>
<td><strong>App&lt;sup&gt;ko&lt;/sup&gt;</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Fold change</strong></td>
<td><strong>p-value</strong></td>
<td><strong>Fold change</strong></td>
<td><strong>p-value</strong></td>
</tr>
<tr>
<td>HMGCR</td>
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<td>LSS</td>
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<td>0.59047</td>
<td>-2.25352</td>
<td>0.13120</td>
</tr>
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<td>APOE</td>
<td>Membrane Trafficking</td>
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<td>0.14494</td>
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<td>0.48902</td>
</tr>
<tr>
<td>IDOL (Mylip)</td>
<td>Membrane Trafficking</td>
<td>1.50719</td>
<td>0.06621</td>
<td>1.34162</td>
<td>0.01059</td>
</tr>
<tr>
<td>LDLRAP1</td>
<td>Membrane Trafficking</td>
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<td>0.30474</td>
<td>-1.40333</td>
<td>0.00418</td>
</tr>
<tr>
<td>RAB15</td>
<td>Membrane Trafficking</td>
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<td>0.49950</td>
<td>-1.26104</td>
<td>0.04301</td>
</tr>
<tr>
<td>RAB28</td>
<td>Membrane Trafficking</td>
<td>1.17341</td>
<td>0.00686</td>
<td>1.22143</td>
<td>0.03559</td>
</tr>
<tr>
<td>RAB3GAP1</td>
<td>Membrane Trafficking</td>
<td>-1.02824</td>
<td>0.60069</td>
<td>1.62916</td>
<td>0.00145</td>
</tr>
<tr>
<td>REP15</td>
<td>Membrane Trafficking</td>
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<td>0.04464</td>
<td>-1.94922</td>
<td>0.00121</td>
</tr>
<tr>
<td>VLDLR</td>
<td>Membrane Trafficking</td>
<td>-3.08933</td>
<td>0.00579</td>
<td>-2.69968</td>
<td>0.01007</td>
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<td>VPS16</td>
<td>Membrane Trafficking</td>
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<td>0.42265</td>
<td>227.03531</td>
<td>0.00230</td>
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<td>VPS52</td>
<td>Membrane Trafficking</td>
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<td>0.84696</td>
<td>4.90361</td>
<td>0.00892</td>
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<td>ERMN</td>
<td>Myelination</td>
<td>-1.84502</td>
<td>0.05713</td>
<td>-1.94087</td>
<td>0.03265</td>
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<td>OMG</td>
<td>Myelination</td>
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<td>0.29316</td>
<td>1.59659</td>
<td>0.01396</td>
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<tr>
<td>QK, v1</td>
<td>Myelination</td>
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<td>0.03758</td>
<td>-1.34727</td>
<td>0.00640</td>
</tr>
<tr>
<td>QK, v3</td>
<td>Myelination</td>
<td>-1.24712</td>
<td>0.04122</td>
<td>-1.34609</td>
<td>0.01372</td>
</tr>
<tr>
<td>TSPAN2</td>
<td>Myelination</td>
<td>-1.97175</td>
<td>0.01914</td>
<td>-1.81330</td>
<td>0.02248</td>
</tr>
</tbody>
</table>
synthase has been shown to redistribute from the ER to mitochondria in a mouse model of Parkinson’s disease, further analysis to test the relevance of this gene will involve the study of intracellular redistribution changes in addition to measurement of its protein levels (Lim et al. 2012).

Another pathway affected in the cerebellum in the absence of App involves membrane traffic throughout the endosomal/lysosomal membrane system, which is the key initial subcellular location affected by Npc1 mutations (Karten, Peake, and Vance 2009). This pathway contains the gene most affected by the loss of App, vacuolar protein sorting homolog 16 (VPS16, http://www.ncbi.nlm.nih.gov/gene/64601), which is increased a remarkable 227-fold. While loss of Npc1 has no impact on Vps16, double loss of App and Npc1 leads to further increase of Vps16 to a final 355-fold. Vps16 is a component of the multivesicular body (multivesicular endosomes; MVB) and an essential regulatory element for endosome-lysosome fusion (Nickerson, Brett, and Merz 2009). Because MVB formation and dynamics depend on appropriate cholesterol homeostasis, changes in Vps16 levels may also contribute to the NPC phenotype exacerbated in double mutants – perhaps through a mechanism linked to aberrant endosomal formation and lysosomal cargo delivery (Brankatschk et al. 2011; Kobuna et al. 2010). Consistent with this concept, Vps52 (http://www.ncbi.nlm.nih.gov/gene/6293), which is required for traffic from endosomes to the trans-Golgi network, is significantly upregulated both in App<sup>ko</sup> and App<sup>ko</sup>/Npc1<sup>−/−</sup> brains.

Furthermore, Rab proteins involved in endocytosis are also affected by the loss of App. Rab28 (http://www.ncbi.nlm.nih.gov/gene/9364), necessary for efficient turnover of endocytosed proteins and lysosomal delivery of cargo, increases in response to both Npc1
and App losses, an increase that appears to have an additive effect in double mutant mice (Stenmark and Olkkonen 2001). Rab3gap1 (http://www.ncbi.nlm.nih.gov/gene/22930), a Rab3 GTPase activating protein, is upregulated in App\(^{ko}\) and App\(^{ko}/Npc1^{-/-}\) brains. This is a key finding because Rab3gap1 is expressed exclusively in the brain; mutations are linked to Warburg micro syndrome, a disease characterized by microcephaly and mental retardation (Morris-Rosendahl et al. 2010). Rab3gap1 is essential for neurotransmitter release through vesicular exocytosis, a part of the synaptic vesicle cycle that involves endocytosis and further recycling. In contrast, Rab15 (http://www.ncbi.nlm.nih.gov/gene/376267), also exclusive to the brain and involved in early and recycling endosomal traffic, is downregulated in App\(^{ko}\) brain; its effector, Rep15 (http://www.ncbi.nlm.nih.gov/gene/387849), decreases in response to single loss of App and Npc1, as well as in double mutants.

In addition to genes involved in the basic mechanism of molecular transport through the endosomal/lysosomal system, I have identified genes that are specifically linked to cholesterol transport through this pathway. Of these, perhaps the most interesting is Mylip, also called IDOL, for Inducible Degrader Of the LDL receptor (IDOL, http://www.ncbi.nlm.nih.gov/gene/29116), which decreases in the absence of APP and is further downregulated in the double mutant brain. IDOL acts as a sterol-dependent inhibitor of cellular cholesterol uptake by mediating ubiquitination and subsequent degradation of LDLR through a mechanism involving liver x receptors (LXRs) (Zelcer et al. 2009). Also of interest, low density lipoprotein receptor adapter protein 1 (LDLRAP1 or ARH, http://www.ncbi.nlm.nih.gov/gene/26119) decreases in App\(^{ko}\) and App\(^{ko}/Npc1^{-/-}\) mice. Ldlrap1 is well known for its function in clathrin-mediated
endocytosis of low density lipoprotein receptors (LDLRs), so its downregulation, together with an increase in IDOL, could potentially affect cholesterol traffic through endosomes – a pathway that is already compromised in the NPC brain (Kang and Fölsch 2011).

Another key player in cholesterol transport in the brain is the previously-mentioned apolipoprotein E (APOE, http://www.ncbi.nlm.nih.gov/gene/348), a significant risk factor for developing AD (Bu 2009). I found that, while it is not affected by the absence of either App or Npc1 separately, it is robustly upregulated in double mutant mice (2.25-fold; p=0.0016). Furthermore, very low density lipoprotein receptor (VLDLR, http://www.ncbi.nlm.nih.gov/gene/7436), an ApoE receptor, is significantly downregulated across genotypes, indicating that both Npc1 and App roles are linked to this gene (Sakai et al. 2009). Interestingly, there is no additive effect in App$^{ko}$/Npc1$^{-/-}$ mice, suggesting that they may function in the same pathway (Go and Mani 2012).

Overall, it is evident that App loss correlates with widespread impairment of endosomal/lysosomal dynamics and cargo delivery, including cholesterol. Because these subcellular compartments, as well as the biological functions associated with them, are inherently affected in the NPC brain, loss of this novel APP role is likely to contribute to the exacerbation of the NPC phenotype seen in double mutant mice (Ganley and Pfeffer 2006).

I also explored the possibility that genes involved in myelination could be affected by APP function. Myelin contains large amounts of cholesterol and other lipids, while being essential to neuronal function, making pathways related to its formation and maintenance highly likely to be sensitive to cholesterol dysregulation. Identifying genes
affected by knocking out APP may help to explain the decreases in myelination and motor function in the App^ko/Npc1^−/− mice. Oligodendrocyte myelin glycoprotein (OMG, http://www.ncbi.nlm.nih.gov/gene/4974) is increased in samples from App^ko^ mice, but not Npc1^−/− mice. This may fit with OMG being related to autoimmune reduction of myelination and the potential role of APP products, specifically Aβ, as anti-inflammatory in certain cases (D.-H. Lee and Linker 2012; Grant et al. 2012).

Other myelination-associated genes that warrant further investigation include quaking (QK, http://www.ncbi.nlm.nih.gov/gene/19317), tetraspanin 2 (TSPAN2, http://www.ncbi.nlm.nih.gov/gene/10100), and ermin (ERMN, http://www.ncbi.nlm.nih.gov/gene/57471), all of which are downregulated in all three non-control genotypes. Quaking is part of a pathway that controls CNS myelination. TSPAN2 is still largely uncharacterized, but its similarity to other proteins suggests that it may be involved in oligodendrocyte differentiation into myelin-producing cells; its downregulation in App^ko^ mice could affect myelin production. ERMN is involved in the late stages of myelinogenesis, as well as in myelin stability in the adult brain (Brockschnieder et al. 2006).

**Cholesterol-Associated Genes Differentially Expressed in the Absence of APP: Cortex**

Table 2 shows the genes that are differentially expressed in cortex across all three mouse genotypes. For the purpose of comparison, the genes differentially expressed in the cerebellum (table 1) are also included. The most striking observation is that the expression patterns of Vps16, Vps52 and Rab3gap1 show identical behavior in both cortex and cerebellum, in the absence of APP and regardless of Npc1 status. That these key players in the regulation of membrane traffic into endosomes, and through the
endosomal/lysosomal system, are equally affected in both tissues by the loss of APP suggests a novel function for APP in this pathway.

Other genes involved in membrane or cholesterol traffic are not equally affected in both tissues. Indeed, of all the genes in these pathways identified in cerebellum that are responsive to APP, only VLDLR appears to be significantly reduced in double mutant mice in both brain regions; it is not reduced in the single mutants. Conversely, I found that only one additional gene involved in cholesterol biosynthesis appears to be affected by APP loss in the cortex: NSDHL (http://www.ncbi.nlm.nih.gov/gene/50814), an enzyme involved in post-lanosterol cholesterol biosynthesis (“NSDHL-Related Disorders - GeneReviews™ - NCBI Bookshelf” 2012). The relatively few genes affected in the cortex is consistent with the observation that, in NPC, cholesterol dysregulation in cortex is less dramatic than in cerebellum (Treiber-Held et al. 2003). Nevertheless, further analysis will be necessary to determine whether these results reflect differences in cholesterol homeostasis between both tissues, or represent experimental artifacts.

Another pathway of interest that shows similarities between both tissues is myelination. Table 2 shows that, similar to what occurs in cerebellum, OMG increases and TSPAN2 decreases in response to APP loss in both tissues, whereas QK and ERMN, both affected by APP loss in cerebellum, only increase in the cortex in double mutants. Further analysis of these genes at RNA and protein levels will help to determine whether they are physiologically linked to APP and/or NPC1 or if they represent experimental artifacts.
Table 2. Genes of Interest Differentially Expressed in Mouse Cortex Samples. Statistically significant values (p < 0.05) are highlighted in dark green.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Pathway</th>
<th>Cortex</th>
<th>Npc1&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>p-value</th>
<th>App&lt;sup&gt;ko&lt;/sup&gt;</th>
<th>p-value</th>
<th>App&lt;sup&gt;ko&lt;/sup&gt;/Npc1&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCR</td>
<td>Cholesterol Biosynthesis</td>
<td>1.2819</td>
<td>0.26695</td>
<td>-1.0017</td>
<td>0.99046</td>
<td>-1.1907</td>
<td>0.15375</td>
<td></td>
</tr>
<tr>
<td>HSD17B7</td>
<td>Cholesterol Biosynthesis</td>
<td>1.1502</td>
<td>0.54615</td>
<td>-1.2444</td>
<td>0.38189</td>
<td>-1.0676</td>
<td>0.75585</td>
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<tr>
<td>LSS</td>
<td>Cholesterol Biosynthesis</td>
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<td>-1.4002</td>
<td>0.02441</td>
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<tr>
<td>NSDHL</td>
<td>Cholesterol Biosynthesis</td>
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<td>0.00360</td>
<td>-2.4208</td>
<td>0.02924</td>
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<tr>
<td>APOE</td>
<td>Membrane Trafficking</td>
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<td>0.20150</td>
<td>1.0701</td>
<td>0.65921</td>
<td>1.3729</td>
<td>0.05622</td>
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</tr>
<tr>
<td>IDOL (Mylip)</td>
<td>Membrane Trafficking</td>
<td>1.3067</td>
<td>0.00238</td>
<td>-1.1963</td>
<td>0.07663</td>
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<td>LDLRAP1</td>
<td>Membrane Trafficking</td>
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<td>0.39859</td>
<td>-1.5071</td>
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<tr>
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<td>Membrane Trafficking</td>
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<td>0.40199</td>
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<td>0.20115</td>
<td>1.1869</td>
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<td>0.54004</td>
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<td>4.8750</td>
<td>0.01831</td>
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<td>ERMN</td>
<td>Myelination</td>
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<td>0.00196</td>
<td>-1.9777</td>
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<td>-28.3279</td>
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<td>Myelination</td>
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<td>1.9029</td>
<td>0.03231</td>
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<td>Myelination</td>
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<td>-1.5165</td>
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<tr>
<td>QK, v3</td>
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<td>0.12463</td>
<td>1.0195</td>
<td>0.83774</td>
<td>-1.1466</td>
<td>0.21538</td>
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<tr>
<td>TSPAN2</td>
<td>Myelination</td>
<td>-8.70801</td>
<td>0.01797</td>
<td>-1.2350</td>
<td>0.58881</td>
<td>-11.25114</td>
<td>0.00346</td>
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IPA Analysis Connects Cholesterol Synthesis and Endocytosis Pathways

More in-depth examination and discussion of pathways affected by removal of APP is possible using Ingenuity Pathway Analysis (IPA) software. This package draws from the body of published literature across diseases and model systems to suggest relationships between molecules. Gene expression changes observed via microarray can be overlaid on these relationships, allowing the researcher to infer potential systemic consequences as a result of the changes. Recalling that VLDLR is one of the membrane trafficking genes affected in the three mouse genotypes (table 1), I used IPA to find that a relationship between VLDLR and APP exists via reelin signaling. Expression changes in each genotype can be compared to infer how the combination of the App<sup>ko</sup> and Npc1<sup>−/−</sup> genotypes interact (figure 3). Although reelin is not directly affected by any of the genotypes, it appears from this network map that when the interaction of APP and reelin via ApoE is removed from the Npc1<sup>−/−</sup> mouse, downstream targets of VLDLR are also downregulated; these targets are not downregulated in either genotype alone.
Figure 3. Reelin-Related Interactions in Three Mouse Genotypes. Comparison of gene expression of genes related to reelin signaling among three mouse genotypes. In the App\textsuperscript{ko}/Npc1\textsuperscript{−/−} mouse (bottom), downstream targets of VLDLR are downregulated (red) that are unaffected (grey) by either genotype alone.
It is also interesting to see how changes in VLDLR, which I initially identified as belonging to membrane trafficking pathways (table 1), is related to other molecules and pathways via the broader pathway of lipid regulation. In this network, VLDLR (membrane trafficking) overlaps with cholesterol biosynthesis via HMGCR, both of which decrease expression as a consequence of APP loss (figure 4). IPA network maps highlight transcriptional changes in numerous other molecules that might otherwise be overlooked, including crossover with inflammatory molecules such as proinflammatory cytokines and CD59. CD59 relates to inflammation due to its function as a key inhibitor of cell lysis by complement, part of the innate immune system (Huang et al. 2006). CD59 has been found to be decreased in expression in AD brains, and has been described as a potential target for therapeutic upregulation to inhibit pathological inflammation in the brain (Yang et al. 2000; Kolev et al. 2009). As can be seen in figure 4, although a number of the molecules that are downregulated in the App^ko mice return to control expression levels in the App^ko/Npc1^-/- mice, CD59 expression remains down. At the same time, C1q, an initiator of the compliment activation cascade, increases expression, suggesting that some of the inflammatory pathology in these mice may be due to compliment activation (M. K. Zabel and Kirsch 2013)
Figure 4. Consequences of APP Loss on Lipid Regulation Pathways. Expression changes in the App\(^{ko}\) and App\(^{ko}/Npc1^{-/-}\) mice relative to control mice are shown in green (increased), red (decreased), and gray (no change). Increased size highlights the notable molecules VLDLR, HMGCR, and CD59.
Using IPA to map a network in the App<sup>ko/Npc1</sup>−/− mice beginning with CD59, rather than VLDLR and lipid regulation, reveals a separate set of connections. In this map, shown in figure 5, CD59 is related to cholesterol biosynthesis via HSD17B7, previously highlighted in table 1. It is also related once again to lipid regulation through phosphatidylethanolamine N-methyltransferase (PEMT). PEMT, which is increased in these mice, is a protein involved in phospholipid metabolism; in cancer studies its increase is associated with pathological lipid metabolism and predicts shorter patient survival (Zinrajh et al. 2014).
Figure 5. Network Map of CD59 Relationships. Expression changes in the App<sup>ko</sup>/Npc1<sup>−/−</sup> mice relative to control mice are shown in green (increased expression) and red (decreased expression). Gray indicates no change relative to control. Increased size highlights CD59 and a gene involved in cholesterol biosynthesis, HSD17B7.
Finally, I used IPA to examine genetic relationships involving ERMN, a member of the myelination pathway of genes in table 1 (figure 6). Here, the network map shows an unexpected close connection between ERMN and APP expression, suggesting that there is a mechanistic, rather than simply consequential, relationship between APP expression and neuronal myelination.
Figure 6. Network Map of ERMN-Related Genes. Expression changes in the App<sup>ko</sup>/Npc1<sup>−/−</sup> mice relative to control mice are shown in green (increased expression) and red (decreased expression). Gray indicates no change relative to control. Increased size highlights ERMN and APP, which appear to share a mechanistic relationship.
These findings provide a solid basis for further mechanistic experiments in cultured cells (Specific Aim 2), which will help to separate *bona fide* changes from experimental artifacts and contribute to the understanding of what specific role APP has in endosome and cholesterol biology and how its loss may contribute to pathology.

**Proteomic Analysis**

For proteomic analysis, brain tissue samples were selected from mice of the same genotypes as those used for the microarray study: control, App<sup>ko</sup>, Npc1<sup>-/-</sup>, and App<sup>ko</sup>/Npc1<sup>-/-</sup>. Choosing different animals of the same genotype eliminates the possibility that overlap between the microarray and proteomic studies is limited to specific animals, strengthening the argument that any overlap between the microarray and proteomic datasets is a result of the genetic differences.

Due to limitations in tissue availability, proteomic analysis could only be performed on tissue from the cerebellum, rather than both cortex and cerebellum as in the microarray analysis. Despite this limitation, it was likely that any observable changes at the pre-symptomatic stage would be the most apparent in this region: while at later stages the NPC phenotype affects large parts of the brain, the region most significantly affected at early stages is the cerebellum (Treiber-Held et al. 2003).

To process samples for protein analysis, tissues were lysed according to standard protocol and a BCA assay used to determine the protein concentrations of each sample, diluting samples as necessary to equalize the concentrations. These samples were allowed to digest in trypsin, a serine protease that cleaves proteins between the amino acids lysine and arginine (Darbre 1986). Because of this well-documented activity, if a protein of
known sequence is digested by trypsin, the sequence can be re-assembled from individual peptide fragments based on where trypsin cleavage sites are located.

After digesting the proteins into peptide fragments, the fragments from each animal sample were labeled with a six-plex series of tandem mass tags (TMT), a system of peptide labeling based on isobaric variation of an otherwise-identical structure (Dayon et al. 2008). Labeled peptides were combined into three sample sets, each containing proteins from four animals. These combined samples were subsequently divided into six fractions, based on pH, and the fractions analyzed by mass spectrometry. All copies of a single peptide are detected simultaneously, yielding a unique spectral signature that corresponds to the exact amino acid sequence of that peptide. This reading can be subdivided based on the TMT labeling, allowing for the calculation of a ratio between a given peptide sequence with one label (and thus from one sample) and the identical peptide sequence with one of the other five labels (and thus from a separate sample).

Known peptide sequences and their mass spectrometry signatures are stored in a database that can be accessed using Thermo Proteome Discoverer (Thermo Fisher Scientific, Inc.), a software package that takes raw mass spectrometry data files as input and, using the SEQUEST algorithm, identifies possible peptide sequence signatures and searches for information on those signatures (and the possible proteins to which they belong) in the database (Eng, McCormack, and Yates 1994). These search results are presented as a list of identified peptide sequences and possible proteins. Proteins are assigned a confidence score based on the signal quality of the individual peptides and how many peptides of the whole protein were identified (“coverage”).
This data was inconclusive, potentially due to sub-optimal protein concentration in the samples analyzed; however, shown here is a representative sample of the data I was able to obtain (table 3). The table includes potential proteins that have a 20% or greater confidence of being accurate based on the number of times the protein was identified and the number of individual peptides found that belonged to that protein.

My analysis did reveal apparent decreasing trends in glyceraldehyde-3-phosphate dehydrogenase expression (GAPDH) in the two APP knockout mouse genotypes (figure 7, one-way ANOVA, 0.06 > p > 0.05). GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate into glycerate 1,3-bisphosphate as part of glycolysis.
Table 3. Representative Sample of Proteomics Data. Values presented here are taken from the second set of tissue samples analyzed.

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<th>Accession Number</th>
<th>Description</th>
<th>Score</th>
<th>Coverage</th>
<th># Unique Peptides</th>
<th># Peptides (NPC 130/wt 131)</th>
<th>Var. [%]</th>
<th># Peptides (APP 128/wt 131)</th>
<th>Var. [%]</th>
<th># Peptides (dKO 129/wt 131)</th>
<th>Var. [%]</th>
<th># AAs</th>
<th>MW [kDa]</th>
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<td>9.4</td>
<td>1053</td>
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<td>4</td>
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<td>95% CI High</td>
<td>95% CI Low</td>
<td>95% CI High</td>
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<td>19.8</td>
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<td>19.2</td>
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**Legend:**
- **OS:** Organism
- **PE:** Protein Entrez Gene ID
- **SV:** Statistical variation
- **Value:** Log2 fold change
- **Fold Change:** Log2 fold change
- **p-value:** Statistical significance
- **Odds Ratio:** Odds ratio
- **95% CI Low:** Lower confidence interval
- **95% CI High:** Higher confidence interval
| P56480 | ATP synthase subunit beta, mitochondrial OS=Mus musculus GN=Atp5b PE=1 SV=2 - [ATPB_MOUSE] | 22.1 | 34.40% | 4 | 12 | 0.923 | 20.1 | 0.91840796 | 94.8 | 0.718094 | 66.2 | 8.7 | 529 | 56.3 |
| Q504P4 | Heat shock cognate 71 kDa protein OS=Mus musculus GN=Hspa8 PE=2 SV=1 - [Q504P4_MOUSE] | 22 | 26.00% | 4 | 14 | 1.06 | 22.9 | 1.20317821 | 20.9 | 0.91054 | 20.4 | 29 | 627 | 68.7 |
| P51881 | ADP/ATP translocase 2 OS=Mus musculus GN=Slc25a5 PE=1 SV=3 - [ADT2_MOUSE] | 21 | 13.09% | 1 | 6 | 1.083 | 1.13166144 | 1.185885 | 298 | 32.9 |
| Q9D6F9 | Tubulin beta-4A chain OS=Mus musculus GN=Tubb4a PE=1 SV=3 - [TBB4A_MOUSE] | 20.1 | 22.97% | 1 | 7 | 1.68 | 1.32701422 | 1.95552 | 444 | 49.6 |
Figure 7. Relative GAPDH Expression. Graph of relative expression of GAPDH protein in three knockout mouse genotypes compared to expression of control mice. Both $\text{App}^{\text{ko}}$ and $\text{App}^{\text{ko}}/\text{Npc1}^{-/-}$ mice have decreased GAPDH expression, while GADPH expression levels in $\text{Npc1}^{-/-}$ mice are comparable to that of controls.
Discussion

My adaptive response hypothesis posits that APP functions as a critical regulatory molecule in the brain, responding to stresses that threaten the homeostasis of systems such as lipid metabolism. This hypothesized role for APP is supported by published work by Nunes et al using a mouse model: although loss of the App gene alone (App\(^{ko}\)) appeared to have no significant effect on neurodegeneration, when it was combined with the loss-of-function Npc1 mutation (Npc1\(^{-/-}\)), the pathological phenotype appeared both at an earlier time point and with more severe consequences than in the Npc1\(^{-/-}\) mice. In addition to significant neurological pathology, measured by behavioral testing, the App\(^{ko}/\)Npc1\(^{-/-}\) mice showed increased cholesterol accumulation, inflammation, and tau phosphorylation compared to either mutation alone (Nunes et al. 2011). I expanded these findings, and strengthened the support of my hypothesis, by examining the molecular consequences of APP removal via transcriptomic and proteomic analysis.

Microarray analysis allowed me to obtain a transcriptome for each mouse genotype, a dataset that formed the foundation of a search for mechanistic clues to why apparently small changes in cholesterol regulatory processes can have such a large impact. Notably, the pathways affected in these severely degenerative double knockout mice fall into similar groups as those identified by human genome-wide association studies (GWAS) of AD patients: cholesterol synthesis, intracellular transport, endocytosis, myelination, and immune response (Waring and Rosenberg 2008; Naj et al. 2011; Hollingworth et al. 2011; Jun et al. 2010). Transcriptomic analysis has also revealed interesting information supporting the observational data that APP function includes the regulation of cholesterol or cholesterol-related processes. APP loss alone is sufficient to cause gene expression changes in a number of pathways either directly
associated with cholesterol, such as synthesis and transport (HMGCR, LDLRAP1; tables 1 and 2), and indirectly, such as those affecting myelination (ERMIN, TSPAN2; tables 1 and 2). Using the IPA software reveals that altered cholesterol synthesis and transport genes VLDLR and HMGCR connect through the broader pathway of lipid regulation, which also connects to inflammation via CD59 (figures 5 and 5). APP may also have a mechanistic relationship to myelination through ERMN (figure 6).

Unfortunately for the proteomic analysis, I made the post hoc discovery that my protein samples were not concentrated enough to give conclusive data. Although I worked closely with the LLU Mass Spectrometry core facility to prepare the samples according to their established protocol, it is possible that overall experience with preparing tissue samples for analysis was limited, as much of the previous work had been conducted using cell lysates (Xiong et al. 2011). My samples were also limited to small portions of mouse cerebellum, limiting my maximum possible protein concentration from the outset. Had I known in advance that protein concentration was an issue, I would have pooled several samples from identical mouse genotypes and performed fewer runs. In reviewing the literature, I also found that many techniques involve additional fractionation of the mixed protein samples using either a wider acidic gradient or isoelectric focusing (Xiong et al. 2011; Dayon et al. 2008; Licker et al. 2014). With increased fractionation, the likelihood that two different proteins are detected simultaneously is decreased, reducing background signal and making any changes in amount between animals more apparent. As a result of this experience, any future proteomic studies performed by the lab will have increased chance of success, and the
technique remains a valuable way to collect information on potential systemic changes between experimental groups.

Despite the complications with the proteomics experiments, at least one protein of interest appeared to be decreased in both the APP knockout and the double knockout mouse genotypes: GAPDH. GAPDH is well known for its role in catalyzing one of the steps in glycolysis; however, what is most interesting about a change in GAPDH expression is the more recent association of the protein with oxidative stress. The molecule glutathione is critical for the elimination of reactive oxygen species (ROS). After this reaction takes place, glutathione is regenerated by a reaction with nicotinamide adenine dinucleotide phosphate (NADPH) (Rush et al. 1985). These reactions are important because under conditions of high oxidative stress, more NADPH is needed to continuously regenerate glutathione. Because this additional NADPH must be generated by metabolic pathways other than glycolysis, GADPH is involved in vesicular and axonal transport, the latter of which is likely crucial for neuronal function (Zala et al. 2013). GAPDH production is curtailed in order to force a shift in reactions in response to oxidative stress, and could also be occurring in the genetic knockout mice as part of the response to lipid dysregulation (Ralser et al. 2007; Agarwal et al. 2012).

Data collected by microarray here was critical for establishing the biosimulation model described in the subsequent chapter. Additionally, the work I performed here provided validation that cholesterol systems were disrupted by the loss of APP even in the absence of an obvious phenotype, fully consistent with the APP role postulated by the adaptive response hypothesis. This finding will inform any future mechanistic study of
APP, as members of the cholesterol pathways can be used as reporters or specifically manipulated themselves.
CHAPTER THREE
IDENTIFICATION OF SPECIFIC MECHANISMS THROUGH WHICH APP INFLUENCES CHOLESTEROL HOMEOSTASIS

Specific Aim Two
To identify specific mechanisms through which APP influences cholesterol homeostasis, I developed an \textit{in silico} model in which to measure the systemic impact of manipulating APP on the cholesterol homeostasis pathways identified in SA1. Because entire molecular pathways can be observed \textit{in silico}, this technique will reduce dependency on both \textit{in vitro} and \textit{in vivo} models for observation of system-wide consequences of genetic changes.

Introduction
In SA1 (discussed in the previous chapter), analysis of microarray and proteomic data in the APP and NPC1 mouse models highlighted functional pathways and interacting networks of genes and proteins that supported my hypothesis that APP regulates lipid homeostasis in the brain, especially in situations of cellular stress. In my original Specific Aim Two, I proposed to identify specific mechanisms through which APP influences cholesterol homeostasis in response to that stress. To achieve that goal, I had proposed to develop and validate an \textit{in vitro} model in which lipid dysregulation could be induced, APP and its products manipulated, and the resulting consequences observed. In such a model, lipid dysregulation could take the form of loss of NPC1 function, inhibition of
HMGCR, or exposure to 27-hydroxycholesterol, an oxysterol that accumulates early in the AD brain (as discussed in chapter one). Manipulation of APP could be achieved through RNA interference or the use of secretase inhibitors.

Figure 8A shows a schematic of such an experimental design, in which HMGCR inhibition by U18666A, or treatment with oxysterols in a variety of cell lines, with and without manipulation of APP function, is followed by measuring parameters related to cell stress and apoptosis (i.e. LDH release or filipin fluorescence), as well as the impact on signaling pathways identified in SA1 as being APP-dependent.

However, while characterizing primary fibroblasts from NPC and AD patients, I obtained results that led to the modification of SA2. Specifically, I found that the intensity of filipin staining in NPC cells appeared to correlate with the severity of the NPC case, and that AD fibroblasts displayed increased baseline filipin staining relative to their age-matched controls.

These findings suggested to me that filipin staining could reflect cellular damage linked to disease status. Furthermore, if that cellular damage were detectable at presymptomatic stages of AD, and its severity were to increase with disease progression, it might be possible to differentiate mild cognitive impairment (MCI) sufferers according to their risk of conversion into AD by monitoring filipin staining. Consequently, I shifted my focus from the original idea of using primary fibroblasts to test the in vitro role of APP in cholesterol homeostasis and explored instead the link between filipin fluorescence and cellular damage, to obtain proof of concept that filipin is not only associated with an increase in cholesterol levels, but also can be used to measure cellular damage and by extension, disease status.
In vitro Testing of APP Mechanisms

A. Original Experimental Design

APPLIED STRESS

secretase inhibition  siRNA knockdown

APP AND NPC1 MANIPULATION

Fibroblasts
SH-SY5Y cells
PBMCs

LDH assays
protein levels
filipin fluorescence

CELLULAR DAMAGE

U18666A oxysterols

B. Modified Experimental Design

APPLIED STRESS

U18666A oxysterols

CELLULAR DAMAGE

filipin fluorescence

TTM SIMULATION

unlimited genetic manipulation

SYSTEMIC CHANGES
Figure 8. *In vitro* Testing of APP Mechanisms. A: Original experimental design for SA2. Stress to lipid regulation (U18666A, oxysterols) would be applied to cell culture models (fibroblasts, SH-SY5Y cells, peripheral blood mononuclear cells [PBMCs]) in which APP and NPC1 expression had been manipulated (using techniques such as siRNA knockdown and secretase inhibition). The resulting consequences to the cells would be observed by measuring cell death (LDH assays), the expression of proteins identified in SA1, and cholesterol accumulation (using filipin). B: Revised experimental design for SA2. In one set of experiments, cells are stressed and the impact of that stress measured using filipin. This design mechanistically links the stressor to an AD-like phenotype in cells. In a second set of experiments, individually assessing the impact of APP regulation on proteins of interest is replaced by *in silico* simulation, in which changes to APP and resulting impact can be simultaneously observed across every protein in a metabolic pathway.
Thus, my overall specific aim 2 was modified to include both the *in vitro* characterization of the link between filipin staining and cellular damage, as well as the generation of a biosimulation model to identify pathway-wide mechanisms through which APP influences cholesterol homeostasis. The modified experimental design is described in figure 8B. In order to find whether filipin intensity may be a marker of cellular damage, I measured the correlation between LDH release and levels of filipin fluorescence in the neuron-differentiated neuroblastoma SH-SY5Y cells following several cholesterol-associated stress stimuli.

I specifically chose to assess the impact of side-chain oxidized cholesterols 24-OHC and 27-OHC as forms of stress stimuli that are relevant to AD. Unlike cholesterol, 27-OHC can cross the blood-brain barrier, and has been shown in SH-SY5Y cells to influence APP and Aβ production (Ghribi et al. 2006; Jaya Rantham Prabhakara Prasanthi et al. 2008; Jaya R. P. Prasanthi et al. 2009; Burg et al. 2013). Outside of the brain, oxysterols like 27-OHC activate liver x receptors (LXRs), which are also capable of regulating Aβ production (M. P. Burns et al. 2006; Jiang et al. 2008). Because it is physiologically capable of directly stressing neurons, and is known to be dysregulated in AD, treatment with physiologically relevant concentrations of 27-OHC is an ideal experimental stress stimulus (Shafaati et al. 2011). In order to control for any binding of filipin to oxysterols, I also treated cells with another oxysterol, 24-OHC. 24-OHC is the most prominent cholesterol metabolite in the brain and has been shown to have no effect on cells at equivalent doses to 27-OHC (Jaya Rantham Prabhakara Prasanthi et al. 2008; Jaya R. P. Prasanthi et al. 2009; Matsuda et al. 2013).
The second part of this aim is the setup of an *in silico* metabolic model using Transcriptome-to-Metabolome (TTM) biosimulation (figure 8B), a process developed by Phelix *et al* and initially tested on glycolysis in AD brains (Phelix *et al.* 2011). Leveraging the accumulated knowledge of biochemistry, TTM can generate a metabolic model based on gene transcription data. With the use of the model, entire metabolic pathways can be observed simultaneously, rather than the individual gene or protein changes identified by classical techniques. Recalling my initial goal in this aim of mechanistically characterizing the changes observed in SA1, TTM provides another method of re-creating the effects of APP and NPC1 manipulation, and offers a pathway-wide perspective to generate predictions that can form the basis of a hypothesis for subsequent experiments. As highlighted in figure 8B, the serial work of stressing cells, manipulating APP expression, and measuring consequential protein changes could be performed in parallel. Rather than examining proteins identified in SA1 individually, TTM could test the effect of APP manipulation on *all* proteins in a pathway and show changes not only in proteins I had identified as significant, but also proteins that would have otherwise been overlooked.

TTM simulations account for as many relevant reactions as can be practically included: the model of cholesterol metabolism that TTM uses to study the AD hippocampus has been published and includes fifty-one synthesis reactions and two degradation reactions – a total of fifty-three reactions (Christopher M Valdez *et al.* 2011). Each reaction is catalyzed by one or more enzymes that are themselves encoded by genes; the transcription values from the control microarray are used to apply a modification constant to each reaction. From this baseline model, percent changes in
transcription as a result of disease or genotype are used to modify the constants. Thus, the metabolic consequences of the transcriptional changes can be visualized.

Models generated by TTM should additionally be validated, by comparing predictions to metabolic information available in literature. In the specific case of the mouse model, it has been experimentally documented that free cholesterol accumulates in vesicles in brain tissue from the \( Npc^{-/-} \) and \( App^{ko}/Npc^{-/-} \) mice (Nunes et al. 2011). If the \textit{in silico} model does not also show this occurring, the simulation must be corrected accordingly. Refinement of the model is an ongoing process, especially given that many of the metabolic consequences in NPC involve abnormal localization rather than simply abnormal levels or synthesis (Vanier 1999). As well as adding additional reactions to the model, I, along with others in the Soriano and Phelix laboratories, are working to add compartmentalization and transport into the model. Subcellular compartments, such as the lysosome, would take the form of reaction groups that have limited input and output, while transport rate of metabolites between subcellular locations can be modeled as an additional type of reaction. Thus far, simulation results have shown unexpected metabolic alterations in the \( App^{ko} \) mouse genotype. It is possible that these kinds of changes create a weakness to metabolic stress, such that the further addition of the \( Npc{1}{\text{-}}^{-/-} \) mutation causes such significant cellular distress (Nunes et al. 2011).

In summary, the focus of my work has shifted from my original proposal of using an \textit{in vitro} model to study the role of APP in modulating the cellular responses to cholesterol stress. Instead, the modified experimental setup presented here widens the scope of my aim, by exploring the possibility that filipin could be of potential value as a marker of cholesterol-induced cellular damage and disease status. In addition, TTM
allows testing the impact of APP manipulation on all the proteins within a full pathway, not only on proteins identified by microarray analysis as significant.

**Results**

Filipin Levels in Primary Fibroblasts

Primary fibroblasts from AD, NPC, and DS patients obtained from Coriell Cell Repositories (table 4, chapter 6) were selected for their unique aspects as a means of testing my adaptive response hypothesis: AD cells as examples of the disease, NPC as a model of cholesterol dysregulation, and DS as a model of APP overexpression. Via Western blot, I confirmed the DS fibroblasts as a model of APP overexpression (figure 9, A), and confirmed that the increased Aβ production found in NPC brains is also active in fibroblasts, as evidenced by the presence of β-cleaved C-terminal fragment (CTF) of APP (figure 9, B) (Nixon 2004).
Figure 9. Fibroblast Western Blots. A, Western blot confirmed overexpression of full-length APP in DS fibroblasts compared to age-matched controls. B, Western blot confirmed increased β-cleaved APP in NPC fibroblasts compared to age-matched controls.
I began the characterization of different cell lines by measuring intracellular levels of filipin in response to different cellular stressors. Using NPC fibroblasts as a model of cells that are constitutively under stress secondary to impaired cholesterol transport, I found that filipin staining in cells from a patient experiencing severe NPC symptoms at one year of age was more intense than staining in cells from a milder case of a twenty three year-old patient (figure 10), a finding that is consistent with reports of a spectrum of NPC disease phenotypes (Tängemo et al. 2011). This led to the intriguing possibility that filipin staining may correlate with cellular damage status and disease severity, rather than simply serving as a marker of cholesterol accumulation. I also found that AD fibroblasts had increased baseline filipin staining relative to their age-matched controls, a result that confirms observations that lipid abnormalities are a critical part of AD pathology and that these abnormalities are detectable even in peripheral cells (Pani, Mandas, et al. 2009; Pani, Dessì, et al. 2009; Mandas et al. 2012). Following up on this result, I repeated the experiment with additional AD patient and control fibroblasts from Coriell Repositories, and carried out a more objective quantification of results using fluorescence-activated cell sorting (FACS) analysis, in addition to the manual quantification of filipin fluorescence at the microscope. Unlike microscopy methods, FACS can quantitatively measure fluorescence in thousands of cells across numerous patients in a relatively short amount of time. As is clearly visible via fluorescence microscopy, and quantifiable by FACS, filipin fluorescence in fibroblasts from AD patients is higher than in age-matched control individuals (figure 11).
Figure 10. Filipin Staining of NPC Fibroblasts. Fibroblasts from patients with NPC symptoms at a young age (1 year) show more intense filipin staining than fibroblasts from patients with later onset of symptoms (23 years). All NPC fibroblasts have more filipin staining than their age-matched controls.
Figure 11. Filipin Staining of AD Fibroblasts. A, Control (top left) and AD (top right) fibroblasts were stained with filipin to visualize cholesterol abnormalities. Despite similar cellular densities, shown by transmitted light overlay, Alzheimer’s disease (AD) fibroblasts (bottom right) have a significant increase in filipin-positive cells compared to control (bottom left). B, Flow cytometry quantitation of fibroblasts demonstrates a significant increase over baseline autofluorescence in each AD patient as compared to age-matched control (one-way ANOVA, p = 0.01).
Filipin Levels and Cellular Damage in Differentiated SH-SY5Y Cells

The finding that AD fibroblasts show filipin staining that is similar to that of NPC fibroblasts, without additional applied stress, suggested that filipin changes could be used as an indicator of AD-like cellular pathology. In accordance with my adaptive response hypothesis, I predicted that cholesterol stressors would be capable of inducing cellular damage and that this damage was being detected by filipin. Such a mechanistic demonstration would be in contrast to the thinking of the amyloid cascade hypothesis, which would suggest that the filipin staining in AD cells was a consequence related to Aβ pathology in the brain.

For these experiments, healthy cells were needed so that filipin staining could be used to measure cellular damage as a consequence of stress. Rather than continuing to use control fibroblasts, I switched to SH-SY5Y neuroblastoma cells, which, when differentiated, have the cellular architecture of neurons (Encinas et al. 2000; Xie, Hu, and Li 2010). I used a more specific model stressor of lipid regulation in the brain, the side-chain oxidized form of cholesterol, 27-hydroxycholesterol (27-OHC). Unlike cholesterol, 27-OHC can cross the blood brain barrier, and has been shown in SH-SY5Y cells to influence APP and Aβ production (Ghribi et al. 2006; Jaya Rantham Prabhakara Prasanthi et al. 2008; Jaya R. P. Prasanthi et al. 2009; Burg et al. 2013). Using 27-OHC allowed me to test a mechanistic link between cholesterol-related stress and the filipin staining I had observed in AD fibroblasts, using a molecule with the physiological capability of causing damage, known abnormalities in actual AD, and in a cell culture model that was close to human neurons. As a positive control, I used the cholesterol synthesis inhibitor U18666A to generate filipin-detectable pathology analogous to what is observed in NPC cells. To control for the addition of oxysterols to the cells, I used 24-
OHC, another oxidized form of cholesterol that has been shown to not be damaging to cells at equivalent concentrations (Jaya Rantham Prabhakara Prasanthi et al. 2008; Jaya R. P. Prasanthi et al. 2009).

Using an assay for lactate dehydrogenase (LDH), I confirmed the detrimental effects of U18666A and 27-OHC on cell survival, compared to control and 24-OHC (figure 12, A). Quantifying filipin fluorescence with FACS, I found that 27-OHC, but not 24-OHC, led to a statistically significant increase in filipin staining, comparable to the impact of U18666A (figure 12, B). Representative graphs of the FACS data show this increase in filipin fluorescence compared to control (shaded) cells (figure 12, C). Because the LDH assay showed that treatment also induced cell death, this confirmed that increased filipin staining was indeed a direct consequence of cellular damage. Additionally, the differential results with 27-OHC and 24-OHC treatment indicate that, in this neuronal model, filipin fluorescence does not correlate with overall levels of oxidized cholesterol, because the control oxysterol 24-OHC does not lead to significant changes in filipin signal. In support of my hypothesis, filipin fluorescence correlates with the cellular damage caused by 27-OHC and the positive control U18666A, which presents as changes in the subcellular distribution of free cholesterol and ganglioside GM1 that filipin detects (Arthur, Heinecke, and Seyfried 2011).
A  
LDH release (A.U.)

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<tr>
<td>24OHC</td>
<td>2</td>
</tr>
<tr>
<td>27OHC</td>
<td>2.5</td>
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B  
Average Filipin Positive Cells (%)

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<tr>
<th>Treatment</th>
<th>Filipin Positive Cells (%)</th>
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</thead>
<tbody>
<tr>
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<tr>
<td>U18666A</td>
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</tr>
<tr>
<td>24OHC</td>
<td>30</td>
</tr>
<tr>
<td>27OHC</td>
<td>50</td>
</tr>
</tbody>
</table>

C  
Filipin Fluorescence of Differentiated SH-SY5Y Cells (# cells)

- **U18666A**
  - Filipin - 52.5%
  - Filipin + 47.5%

- **24OHC**
  - Filipin - 68.7%
  - Filipin + 31.3%

- **27OHC**
  - Filipin - 48.1%
  - Filipin + 51.9%
Figure 12. Effect of Oxysterols on Cell Survival and Filipin Staining. A, LDH assay shows that 27-OHC causes cell death comparable to that of the positive control, U18666A, while 24-OHC does not. B, Filipin increases in differentiated SH-SY5Y cells treated with 27-OHC but not with 24-OHC (one-way ANOVA, $p = 0.01$). 27-OHC, but not 24-OHC, appears to have a similar effect on cell survival and filipin staining as U18666A, a compound known to cause cholesterol and GM1 dysregulation. C, Representative flow cytometry histograms of differentiated SH-SY5Y cells treated with U18666A, 24-OHC, and 27-OHC. During analysis, a gate is set to include all cells to the immediate right of the peak fluorescence from control (shaded) cells; both control and experimental cells contained by this gate are considered filipin-positive.
TTM Biosimulation

Using the datasets obtained from the microarray analysis described in SA1, gene expression values from control mice were used to assign constants to each reaction in the metabolic pathway for cholesterol synthesis. These constants were subsequently modified based on the percent change in gene expression, relative to control, found in the $App^{ko}$, $Npc1^{-/-}$, and $App^{ko}/Npc1^{-/-}$ mice. Basic simulation results are visualized in figures 13, 14, and 15, where the cholesterol metabolic pathways are mapped with spheres indicating metabolites and arrows indicating reactions. Color and size indicate percent change in metabolite compared to control, with red indicating percent increases and green representing percent decreases.
Figure 13. Cholesterol Biosynthesis in \textit{App}^\textit{ko} Mice. Metabolic network map of biosimulation results showing metabolic changes in \textit{App}^\textit{ko} mice compared to control mice. Spheres represent metabolites, while arrows indicate reactions. Color and size of spheres represent percent difference over control; green represents the lowest (negative) percent differences while red represents the highest (positive) percent differences.
Figure 14. Cholesterol Biosynthesis in Npc1^{-} Mice. Metabolic network map of biosimulation results showing metabolic changes in Npc1^{-} mice compared to control mice. Spheres represent metabolites, while arrows indicate reactions. Color and size of spheres represent percent difference over control; green represents the lowest (negative) percent differences while red represents the highest (positive) percent differences.
Figure 15. Cholesterol Biosynthesis in $App^{ko}/Npc1^{-/}$ Mice. Metabolic network map of biosimulation results showing metabolic changes in $App^{ko}/Npc1^{-/}$ mice compared to control mice. Spheres represent metabolites, while arrows indicate reactions. Color and size of spheres represent percent difference over control; green represents the lowest (negative) percent differences while red represents the highest (positive) percent differences.
The simulation model used to generate these figures is continually being refined to add additional detail that may more accurately represent the metabolic dynamics observed in AD. A large piece of this detail will be the addition of compartmentalized reactions, representing how many cellular reactions take place in specialized organelles, with metabolites transported between them. Within the simulation, compartments are modeled as groups of reactions with limited input and output, while transport steps are added as additional reactions. With these changes, I am specifically hoping to be able to model some of the reactions that take place in the lysosome, the organelle responsible for much of the intracellular cholesterol degradation reactions, and the stage at which most cholesterol accumulates in NPC (Vance 2006; Nixon 2004).

**Discussion**

In this chapter I have focused on identifying potential mechanisms through which APP might influence cholesterol homeostasis. Beginning with the information and experimental results I collected in SA1, I have tried to link the pieces together in a way that allows for the formation and testing of hypotheses. As there continues to be a dearth of mechanistic information on AD, any insight I am able to find represents a potential avenue for earlier identification of disease risk, or even future intervention strategies. Importantly, during my work on this aim I found that filipin staining could be used as an indicator of the cellular damage that occurs in AD. On a basic science level, this repurposing of filipin staining provided a means to demonstrate that cellular stress stimuli, such as oxysterols, are capable of inducing AD-like cellular damage, which is of direct relevance to my adaptive response hypothesis. At the same time, I was able to
demonstrate the strong potential for filipin staining to be used clinically to diagnose AD risk.

Filipin Fluorescence Correlates with Disease Severity

Using NPC fibroblasts, I found that filipin staining in cells from a severely affected one year-old NPC patient was more intense than staining in cells from a less severely affected twenty three year-old NPC patient (figure 10). This finding is consistent with reports of a spectrum of NPC disease phenotypes (Tängemo et al. 2011). I also found, and quantified using FACS, that AD fibroblasts had increased baseline filipin staining relative to their age-matched controls (figure 11), a result that confirms observations that peripheral lipid abnormalities are a critical part of AD pathology (Pani, Mandas, et al. 2009; Pani, Dessì, et al. 2009; Mandas et al. 2012).

27-OHC Treatment Produces AD-Like Filipin Fluorescence in Differentiated SH-SY5Y Cells

Using differentiated SH-SY5Y cells as a neuronal model, I showed that the oxysterol 27-OHC, but not the oxysterol 24-OHC, is capable of inducing similar changes in cholesterol homeostasis to those observed in AD cells as well as changes artificially induced by U18666A (figure 12). This experimental design allows me to conclude that filipin is detecting changes in cholesterol distribution caused by 27-OHC, a view supported by the fact that such changes also occur and are detected by filipin in NPC (Distl et al. 2003). This begins to build a picture of oxysterols having an impact on the brain, a picture supported by the fact that 27-OHC is one form of cholesterol that is capable of crossing the blood-brain-barrier and is elevated in AD brains (Ingemar
Björkhem 2012; Jaya R. P. Prasanthi et al. 2009; Shafaati et al. 2011; Leoni and Caccia 2011). Thus, I have not only provided a potential mechanism by which systemic cholesterol abnormalities can cause stress in the brain, I have demonstrated for the first time that the damage caused by this stress can be detected and quantified using filipin and FACS. Ongoing work will continue to use this model to manipulate the expression of APP as originally proposed, and identify gene and protein-level changes.

Biosimulation Validates Impact of APP Loss on Cholesterol Metabolism

A large portion of my work in this aim involved using TTM biosimulation to leverage the gene transcription data collected in specific aim one, as well as the accumulated knowledge of biochemistry, to generate a metabolic model. Using biosimulation, complete metabolic pathways can be observed simultaneously prior to performing hands-on experiments with in vitro or in vivo models, rather than the individual gene or protein changes identified by more classical molecular approaches. Additionally, biosimulation provided another method of re-creating the effects of APP and NPC1 manipulation, supporting my aim of mechanistically characterizing the changes observed in SA1.

Simulation of the interrelated endogenous cholesterol synthesis pathways showed, unsurprisingly, that mutation of Npc1 changes how this pathway is regulated (figure 14). I was surprised, however, to discover the extent of changes in this pathway due solely to the removal of APP (figure 13), especially since these mice appear functionally normal compared to their counterparts with the Npc1/− mutation (Nunes et al. 2011). I previously noted, as part of my microarray analysis, that a number of genes were changed that fit
into a cholesterol metabolism pathway grouping (tables 1 and 2). On top of this, the simulation suggests that actual changes in the metabolic process are taking place that could lead to excess metabolites – metabolites that themselves could have detrimental effects. This finding is easier to visualize in a simplified presentation of the simulation data (figure 16). It is interesting to see here that the simulation predicts an increase in desmosterol, an immediate precursor to cholesterol that may have its own functions in myelination and brain development (Porter and Herman 2011; Herman 2003).

This information begins to explain the surprising knock-on effect of combining APP knockout with NPC mutation in the App$^{ko}$/Npc1$^{-/-}$ mice (Nunes et al. 2011). If APP is responsible, even in part, for the regulation of these pathways, it appears both from the mice and from the simulation that its importance is magnified when the pathway is under stress (figure 15). Without APP, an important protective mechanism is eliminated, causing stress damage from the NPC mutation to more rapidly cause negative consequences.

Applied to human AD, it makes sense that a similar process might be underway as people age. As suggested by my hypothesis, metabolism, genetics, and environment all are able to cause varying levels of stress that can affect the brain. Action from APP is required to maintain a stable environment. Chronic stress over many years would prevent the APP mechanism from completely responding, and allowing damage to slowly build up until more severe consequences are noticeable. This would explain the appearance of APP products in high amounts in the brains of AD patients: evidence of a decades-long struggle to protect the brain from stressors.
Figure 16. Simplified Cholesterol Biosynthesis in App\textsuperscript{ko} Mice. In this simplified representation of biosimulation results, metabolic changes in the cholesterol synthesis pathway are compared between APP knockout and control mice. Decreased expression of enzymes converting isopentenyl pyrophosphate (IPP) to cholesterol leads to an increase in the metabolite desmosterol.
In addition to the results already generated, the simulation model is continually being refined to add details, such as compartmentalized reactions, that will more represent metabolic dynamics with greater granularity. As the model becomes increasingly complete, more pathways are likely to emerge in which APP is central to cholesterol metabolism and transport. This will create more opportunities to test my hypothesis and may even suggest avenues for pharmacological intervention – interventions that may already exist as therapies for rare metabolic diseases. One way of visualizing the powerful aspect of simulation is shown in figure 17. Several post-squalene reactions in cholesterol synthesis are catalyzed by CYP51A1, an enzyme targeted by novel approaches to systemic cholesterol reduction that expand on what can be accomplished using statins (L. Zhang et al. 2012). This information would have been overlooked without the simulation, and, hypothetically, a validated technique for altering cholesterol (like targeting CYP51A1) could become a useful manipulation technique for experimentally characterizing APP mechanisms, and could even be a possible means of treating AD abnormalities.
Figure 17. TTM Biosimulation Uncovers Possible Experimental Approaches. CYP51A1 has been targeted as part of a unique approach to reducing systemic cholesterol levels. Such an approach could be repurposed in the laboratory as part of experiments designed to better characterize APP function, or even become new AD therapies.
CHAPTER FOUR
CONFIRMATION OF CLINICAL RELEVANCE AND
PRACTICAL APPLICATION OF APP-CHOLESTEROL
RELATIONSHIPS IN THE BRAIN

Specific Aim Three

To determine if filipin levels can be used as potential predictors of Alzheimer’s disease risk, I tested whether filipin intensity correlates with dementia status in peripheral blood mononuclear cells and B-lymphocytes obtained from AD patients.

Introduction

Results gathered in Specific Aims One and Two are significant on a basic science level, especially as they contribute to testing a hypothesis of AD that, unlike the amyloid cascade hypothesis, has the potential to guide researchers toward novel therapeutic approaches. However, the genetic and mechanistic work of the previous two aims relies heavily on model systems. Demonstrating that these findings are reflective of human AD validates the significance of the work as a contribution to the scientific understanding of the disease. I have utilized the results from my hypothesis testing in the design of two potential tests for AD risk, which has yielded an exciting opportunity to promote the clinical application of basic science findings.

Initially, this third specific aim was centered on the validation of my molecular findings in human brain tissue. However, the work on SA1 (presented in chapter two)
provided a strong confirmation of the hypothesis that APP is part of an adaptive response to stress in the brain, identifying networks of genes involved in processes such as lipid regulation and transport that are disrupted when APP is removed. A prediction of this hypothesis is that lipid dysregulation will occur, and become apparent, in the AD process well before dementia symptoms.

My work in cultured cells for SA2, presented in chapter three, demonstrated that peripheral cells of AD patients show severe lipid abnormalities that can be detected using filipin, a compound used to detect similar abnormalities in the diagnosis of other diseases such as NPC (Arthur, Heinecke, and Seyfried 2011; Argoff et al. 1990; Karten, Peake, and Vance 2009; Tängemo et al. 2011). My mechanistic work in neuron-like differentiated SH-SY5Y cells showed that 27-OHC, an oxidized form of cholesterol that has been shown to stress cells and is upregulated in AD, is capable of inducing similar abnormalities that can also be detected using filipin (Jaya R. Prasanthi et al. 2009; Jaya Rantham Prabhakara Prasanthi et al. 2008; Shafaati et al. 2011).

In this chapter, I synthesized this information in the design of a potential test for AD risk, using filipin to detect lipid abnormalities in peripheral cells. My rationale for this approach was that both my adaptive response hypothesis, as well as the results from the first two specific aims, support the prediction that lipid abnormalities can be identified prior to dementia symptoms. If this were the case, testing for these abnormalities would allow patients to seek further advice from a neurologist or be placed into trials for experimental MCI and AD interventions. A clinical study to test this prediction is ideal; however, collection of fibroblasts requires an invasive punch biopsy, while differentiating neurons from individual patients is time and cost prohibitive. Before
a clinical study could be proposed and approved, I needed to demonstrate that the idea could be tested on patients in a non-invasive manner. In order to work toward clinical validation of a filipin-based test for AD risk, I showed that PBMCs are capable of showing similar filipin fluorescence to what was observed in fibroblasts, and that there are distinct differences in filipin fluorescence between B-lymphocytes (a subset of PBMCs) from control and AD patients. At the same time, I worked on a separate non-invasive test for AD risk based on observations that movement abnormalities may occur early in the disease. This test utilizes the Myo, a commercial computer input device that is capable of detecting both physical movement and electrical signals from muscles (Digman 2014; Etherington 2014). Both tests have been granted provisional patents, and the clinical study of the filipin test is underway.

Results

Filipin Levels in Human Brain

I initially used filipin to stain human brain tissue samples kindly provided by Dr. Kirsch here at LLU, with the goal of measuring a potential correlation between the patterns of distribution and intensity of filipin staining and dementia status. Unfortunately, while some filipin fluorescence was visible, the areas of staining did not conform to cellular boundaries. Instead, it appeared that filipin was labeling lipids associated with Aβ plaques in affected tissue, and did not label intracellular lipids in either control or AD patients. Given the inconclusive results with brain samples, I began to focus on cellular models that could provide information regarding the potential correlation of filipin intensity and dementia status.
Cholesterol Dysregulation in Peripheral Blood Mononuclear Cells

In order to more readily test the clinical applications of my findings with filipin, described in the previous chapter, I needed to determine first if peripheral blood cells (PBMCs) displayed the range of cholesterol levels that is subject to dysregulation, as I showed with fibroblasts and SH-SY5Y cells. The rationale for this initial test is that, if such a range were not present, there would be no obvious basis for the study of cholesterol dysregulation in these cells. Furthermore, such testing is also important because PBMCs consist of dozens of cellular subtypes. That complexity is potentially advantageous, both in providing mechanistic insight into the system-wide pathology of AD that disproportionately affects cognitive function (as each subtype is functionally defined) and to fine-tune potential clinical tests based on these cells.

To test the susceptibility of PBMCs to cholesterol dysregulation, I treated human PBMCs with U18666A. Figure 18 shows a dose-dependent effect of this HMGCR inhibitor on filipin staining, confirming that cholesterol dysregulation can be induced in PBMCs (figure 18, A). Representative FACS histograms (figure 18, B) reflect this increase in filipin fluorescence over control (shaded) cells.
Figure 18. Cholesterol Dysregulation in Human PBMCs. A, Human peripheral blood mononuclear cells (PBMCs) treated with increasing concentrations of U18666A to induce cholesterol and GM1 dysregulation also show a dose-dependent increase in the percentage of filipin-positive cells, indicating that PBMCs are also sensitive to dysregulation visible via filipin staining (one-way ANOVA, p = 0.03). B, Representative flow cytometry histograms of PBMCs treated with U18666A. During analysis, a gate is set to include all cells to the immediate right of the peak fluorescence from control (shaded) cells; both control and experimental cells contained by this gate are considered filipin-positive.
Next, I asked whether cholesterol dysfunction is present in PBMCs from AD patients, as such occurrence would be consistent with cholesterol-associated cell damage correlating with cognitive status, while providing strong justification for the initiation of a longitudinal clinical study to test the predictive value of filipin. Using FACS, I measured filipin fluorescence in B-lymphocytes from AD patients and appropriate controls purchased from Coriell cell repository (table 4, chapter 6). A significantly larger proportion of filipin-positive B-lymphocytes occurs in AD patients as compared to controls, again consistent with the concept that there is a continuum of cholesterol-associated damage in these cells as the disease progresses that is detectable by filipin (figure 19, A). Additionally, I determined the average mean fluorescence intensity in individual cells, as a measure of the severity of intracellular abnormalities (figure 19, B). Figure 19B shows a dramatic, significant increase in that average, indicating that B-lymphocytes populations from AD patients not only show larger numbers of filipin-positive cells, but also display greater severity of intracellular, filipin-measurable abnormalities. A representative flow cytometry histogram (figure 19, C) shows the increased filipin fluorescence in AD B-lymphocytes compared to control (shaded) cells.
A

Filipin-Positive B-Lymphocytes (%)

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B

Average Mean Intensity of Filipin Fluorescence (A.U.)

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<td></td>
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<tr>
<td>16000</td>
<td></td>
<td></td>
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</tbody>
</table>

C

Filipin Fluorescence of Human B-Lymphocytes (# cells)

[Graph showing Filipin fluorescence intensity distribution by Alzheimer's disease status]
Figure 19. Filipin Staining of B-Lymphocytes. A, Flow cytometry analysis of B-lymphocytes from control and Alzheimer’s disease (AD) patients shows that AD cells show a significantly higher percentage of filipin-positive cells (two-tailed t-test, p = 0.01). B, Mean filipin fluorescence intensity of B-lymphocytes is significantly increased in AD patients compared to control (two-tailed t-test, p = 0.02). C, Representative flow cytometry histogram of B-lymphocytes. During analysis, a gate is set to include all cells to the immediate right of the peak fluorescence from control (shaded) cells; both control and AD cells contained by this gate are considered filipin-positive.
Myo-Parkinson’s Project

This project applies my theoretical breakthroughs in Alzheimer’s disease to a new area of research. With the previous patent project, I was looking for an early signal of AD that could be objectively defined and used to develop a risk threshold; filipin staining filled this role. When I first encountered the Myo device, I saw an opportunity to collect more detailed movement data than ever before, which suggested that a similar approach could be used to objectively define thresholds based on tremor, an idea that has immediate application to Parkinson’s disease (PD). Of greater relevance to my work on AD, it has been documented for some time that AD develops motor abnormalities in later stages (Parakh et al. 2004; Nishikata et al. 2013). More recently, imaging studies have presented evidence that areas of the brain involved in coordinating motor actions are affected at early stages (Schroeter et al. 2009; Jacobs et al. 2011). These findings suggested to me that small movement abnormalities could be beneficial as an early sign of AD. Several studies using specific motor testing or simple accelerometer-based actigraphy have already shown that a motor-based test is capable of differentiating MCI patients from healthy controls of similar age (Hawkins and Sergio 2014; Kirste et al. 2014).

Given the high sensitivity of the Myo for movement and muscular activity, it seems plausible that patients could be asked to wear the device for a specific length of time, or perform a series of actions while visiting their physician, and subsequently analyze the collected data for patterns associated with cognitive dysfunction. A system such as this (concept shown in figure 20) has the potential to detect these signs earlier than other available testing methods, and is similar to my work on the filipin test in that it attempts to find ways to generate a reliable signal that can be analyzed as objectively as
possible to separate low-risk and high-risk MCI patients. Ideally, with early detection, high-risk patients can be entered into clinical trials for pharmacological approaches, or make lifestyle changes before additional irreversible damage occurs.
Figure 20. Concept for Detection of Movement Abnormalities Using Myo Device. 1) Movements from individual control and Parkinson’s patients are recorded using the Myo device produced by Thalmic Labs, Inc. 2) Raw data on motion and muscular activity is collected from the device. 3) Raw data is processed using a signal-noise isolation algorithm to identify distinct patterns produced by individuals with tremor. 4) Identified tremor patterns are recorded. 5) Data collected from many patients using the Myo device is processed and compared against previously identified tremor patterns. 6) The process correctly identifies individuals with and without tremor.
I immediately took the lead on this project and began making the necessary contacts at Thalmic, the company developing the Myo device. I was able to file a provisional patent on the concept which has since been approved. I filed a grant application to use the Myo in a study of essential tremor, the most common form of tremor. In discussions with LLU neurologists, the possible applications for Myo data were expanded beyond tremor alone to include even earlier markers such as asymmetrical movement. Currently this project is in the process of securing working legal agreements between LLU and Thalmic, after which special-access editions of the devices will be made available for our research.

**Discussion**

In this chapter I took the molecular mechanistic insight from the preceding two chapters and tested their clinical potential in human AD. This work has yielded the encouraging finding that the changes in cholesterol regulation highlighted by filipin staining persist even in the peripheral blood cells of AD patients. Using the HMGCR inhibitor U18666A to artificially create cholesterol abnormalities, I was able to demonstrate that cholesterol regulatory changes are detectable by filipin in peripheral blood cells isolated directly from human patients. Using lymphocytes from Coriell, I was able to show that the lymphocytes of AD patients have increased filipin staining, echoing my prior data in fibroblasts (presented in Specific Aim 1) and suggesting significant dysregulation of cholesterol-related processes in AD. This finding strengthens the argument that AD is, in fact, a systemic disease, and suggests – as my hypothesis predicts – that cholesterol regulation is a large part of the early disease process. The application of filipin as a potential early diagnostic for AD has been patented by the lab and limited
clinical trials are underway here at LLU. In these trials, I will be collecting peripheral blood cells from patients with MCI and staining with filipin. In this ongoing study, I will be able to determine if high filipin staining in MCI correlates with eventual conversion to AD, as well as verify my current findings that AD patients have significantly more filipin staining than normal controls of the same age.

Navigating the process of filing a patent and designing a clinical test for the filipin study also allowed me to pursue a related idea using a new type of wearable sensor, the Myo. Using an “unlocked” developer version of the device, I will be working with several collaborators to analyze data captured by this device for signs of movement abnormalities, such as tremor, that could be early warning signs of Parkinson’s or other neurological diseases. This has also been patented and pending legal agreements between LLU and the manufacturer of the device will soon be tested on human patients.

Participating in translational work is extremely rewarding for a student on an MD/PhD track such as myself: my doctoral work has given me the opportunity to not only add to the scientific understanding of AD, but also to develop applications with the potential to help those at risk of developing the disease.
CHAPTER FIVE

DISCUSSION

Despite several decades of research, effective treatments for AD remain elusive. Numerous major clinical trials of potential AD therapies had failed as I began my doctoral work, with several more failing at phase three within the past year (Mullard 2012; Karran and Hardy 2014; Karran, Mercken, and Strooper 2011). A common thread connecting all of these treatments is the amyloid cascade hypothesis, which implicates Aβ production and aggregation as causative to AD (J. A. Hardy and Higgins 1992). In addition to failing to produce workable clinical treatments for the disease, this hypothesis conspicuously omits any consideration of a physiological function for Aβ. Aβ is cleaved from the larger precursor protein, APP, the physiological importance of which was emphasized by the work of Nunes et al. In their work, App<sup>ko</sup> mice showed no obvious abnormalities, yet when this seemingly benign mutation was combined with the Npc1<sup>−/−</sup> mouse, the results were severe (Nunes et al. 2011). Npc1<sup>−/−</sup> mice model the human disease NPC, characterized by significant lipid accumulation in endosomes (Vanier 1999). At later stages, the disease also exhibits increased Aβ production and tau phosphorylation, both considered hallmarks of AD (Nixon 2004). In the combined App<sup>ko</sup>/Npc1<sup>−/−</sup> mice, the NPC phenotype appeared both more severely and at an earlier time point, with widespread inflammation in the brain, behavioral deficits, and increased tau phosphorylation (Nunes et al. 2011).
These observations, coupled with the failures of Aβ-centric therapeutic approaches, led me to incorporate the work of numerous other researchers into the adaptive response hypothesis, which suggests that APP is centrally involved in brain lipid regulation, especially in response to stress (Castello and Soriano 2013). In order to experimentally test this hypothesis, I first attempted to identify genes involved in cholesterol regulatory pathways that might be regulated by APP using microarray and proteomic analysis in Appko mice. Both of these techniques allow for the simultaneous consideration of thousands of genes and proteins, which allows for the observation of common pathways rather than a more limited focus on isolated changes. In performing analysis of the microarray dataset, I found that in the absence of APP, expression of genes involved in cholesterol regulation, endocytosis, and myelination pathways was altered (tables 1 and 2, figures 3, 4, 5, and 6), while analysis of proteomic data (table 3) suggested a change in expression of the metabolic enzyme GAPDH (figure 7). Taken together, these pieces of data begin to explain why the removal of APP in the mouse model of NPC was so severe: if APP is involved in the regulation of these pathways, their regulation is missing in the Appko/Npc1−/− mice. As a direct consequence, the brain is prevented from correcting ongoing imbalances and neurodegeneration progresses more rapidly (Nunes et al. 2011).

In my second specific aim, I also demonstrated that filipin is a marker of cholesterol-associated cellular damage (figures 10, 11, and 12). This is a key novel finding because, although cholesterol-related abnormalities in peripheral cells of AD patients have been described (Pani et al. 2009; Mandas et al. 2012), it was previously unknown whether such abnormalities are reflective of pathogenesis or are instead a
marker for pleiotropic effects – an unanswered question with obvious implications for therapeutic intervention. Addressing that question, my finding that filipin fluorescence increased in response to 27-OHC (figure 12) cannot be accounted for by an overall increase in oxidized cholesterol levels per se, because an excess of 24-OHC, which is not cytotoxic, does not result in an increase in the filipin signal. In addition, I used the mouse microarray data and TTM biosimulation to construct a metabolic model of cholesterol metabolism. Using this model, I showed that loss of APP function causes significant abnormalities in cholesterol metabolism, including the accumulation of cholesterol intermediates (figures 13, 14, and 15). This information provides strong evidence in support of the argument that APP is a bona fide regulator of lipid metabolism.

In my third specific aim, I began the foundational work of translating my laboratory findings into clinical applications. With the finding that filipin is a marker for cholesterol-associated cellular damage (figures 10 and 12), and the identification of filipin-sensitive lipid abnormalities in AD peripheral cells (figure 11), I designed a blood test for potential AD risk based on the premise that the abnormalities being measured begin at presymptomatic stages and therefore their clinical value is superior to that of the amyloid peptide Aβ (figures 18 and 19).

**APP is a Regulator of Brain Cholesterol Homeostasis**

While a relationship between cholesterol and AD has been repeatedly suggested, few researchers have focused on it as a useful source of information for the understanding and eventual treatment of AD. Initial insight into the cholesterol-APP relationship came from the mouse work by Nunes et al., which demonstrated that simply removing APP
from cholesterol-abnormal NPC mice was sufficient to increase both the severity and rate of decline of the pathology (Nunes et al. 2011).

In SA1, microarray analysis revealed numerous transcriptional changes in networks of genes related to cholesterol and lipid synthesis, endocytosis, and even myelin formation (tables 1 and 2, figures 3, 4, 5, and 6). Many of these changes take place with APP knockout alone, and are compounded in an environment of severe stress such as that created by the loss of NPC1 function in the NPC mice. Proteomic analysis also revealed that APP knockout alone is sufficient to cause a decrease in GAPDH (table 3 and figure 7), suggesting that a stress response pathway involving this metabolic enzyme is also affected by APP. These findings provide evidence for APP as a regulatory molecule capable of influencing metabolism, transport, and the use of lipids in tissues such as myelin.

My work with biosimulation studies further confirmed a key role for APP in brain cholesterol metabolism by demonstrating significant widespread alterations in cholesterol synthesis pathways as a result of loss of APP function (figures 13, 14, and 15). Such an impact by APP on cholesterol metabolism suggests that while symptoms of dysfunction are not readily apparent in APP knockout mice (i.e. they do not have an overt phenotype), they are “primed” for more severe pathologies when subjected to biological stressors. If I had access to a colony of these mice, I would be interested in challenging them with a series of biological stressors. One such stressor might be the kind of high-fat diet used by Ghribi et al. in rabbit studies, while another might involve injection of a compound to destroy pancreatic islet cells, a technique used by Currais et al as a model of diabetes (Currais et al. 2012; Ghribi et al. 2006). These treatments attempt to model the kinds of
stressors that would be encountered by humans over the course of a lifetime, and which epidemiologically appear to confer some amount of AD risk. I would predict that in both of these cases, loss of APP would cause the brains of the mice to be more sensitive to the consequences of the stress. I would expect to observe increases in inflammation, as shown by GFAP, perhaps a decrease in lifespan compared to treated mice with a functional APP gene, and possibly even non-Aβ pathological hallmarks of AD, such as changes in brain cholesterol metabolism, loss of myelination, phosphorylation of tau, NFT formation, and ultimately neuronal loss.

If these kinds of experiments continue to be performed, they represent a new avenue of pursuit for AD researchers. Cholesterol-related pathways have been independently studied for decades, and many compounds exist to manipulate various aspects of these pathways, either for the treatment of rare diseases or for more routine management of cholesterol levels. This suggests that pharmaceuticals already in existence could possibly be capable of treating AD at very early stages, prior to any measurable Aβ pathology (figures 16 and 17). Given the diversity of genes affected in the microarray (tables 1 and 2) as well as large-scale GWAS studies, it is also possible that individual patients could be profiled by microarray and the information used as the basis for TTM biosimulation. Biosimulation data would then identify the cholesterol pathways most at risk for future dysregulation, and treatment or lifestyle recommendations could be made immediately that may prevent or delay the onset of AD.

Overall, the accumulated evidence shown here strongly supports my hypothesis that AD is in actuality a systemic disease that is detectable throughout the body. While the brain is the organ most sensitive to AD, signs of the pathology are apparent in the
periphery, suggesting that identification of these signs, as well as intervention, could also be performed systemically. There is even the possibility that sustained changes in peripheral cholesterol regulation can positively affect AD risk, something strongly suggested by the observation that individuals who aggressively regulate high cholesterol in middle age are significantly less likely to develop AD decades later (Xu et al. 2011).

**Direct Clinical Application of Findings**

In SA2, I was able to successfully differentiate between AD and control fibroblasts on the basis of filipin staining (figure 11), I carried this research further to see if it had the potential to be used diagnostically. If cholesterol abnormalities are indeed as early in the AD process as I predict, such a test would be extremely useful for identifying at-risk patients and starting any potential interventions. While the fibroblasts study and FACS characterization described in SA2 (figure 11) was informative, the test would be far more clinically useful if it could be performed using blood cells. To that end, I first showed that filipin can be measured in PBMCs and that it displays a range of levels that corresponds to cholesterol dysregulation (figure 18). This offered proof of concept that PBMCs are of potential clinical use in filipin-based tests. Next, I used lymphocytes from AD and control patients (table 4), and successfully identified both populations based on their filipin levels (figure 19). It is also noteworthy that the choice of B-lymphocytes was determined by their commercial availability. Further work in larger clinical studies, which are already underway, will determine differences in filipin sensitivity between these cells and different subpopulations of PBMCs, which may show comparable or superior clinical value than B-lymphocytes to diagnose cognitive status and/or identify at-risk MCI populations.
The second clinical application I have been exploring involves using the Myo armband as a novel way of detecting and characterizing neuromuscular tremor, both in Parkinson’s disease patients and potentially as an early sign of AD (figure 20). This also represents a novel idea and I have been able to take the steps to translate it from concept to workable study over the past several years.

In the future, I would like to see these two projects continue to move forward – the filipin study should also look at characterizing the types of cholesterol abnormalities with additional fluorophores, working toward the creation of a custom fluorophore to identify the most significant abnormalities as a replacement for the promiscuous staining patterns and volatility of filipin. With the Myo, I would like to see an expansion of the project to not only identify tremor signals, but isolate them from user input to a computer. In this way the device can be not only diagnostic, but also interventional, improving quality of life for patients who are already suffering from severe movement impairment.

**Supporting the Adaptive Response Hypothesis**

The results produced by the work presented here support the adaptive response hypothesis of AD I initially proposed (figure 2 and figure 21). Environmental factors ranging from metabolism, trauma, and neuronal activity combine to cause stress in the brain – stress that I replicated experimentally using Npc1-/ mice, which have a nonfunctional NPC1 protein (tables 1 and 2), and by treating cells with 27-OHC, a form of cholesterol that could physiologically link peripheral cholesterol dysregulation to the brain (figure 12). This stress prompts a response from APP that can be both functional and dysfunctional as it causes transcriptional changes in genes with lipid regulation, transport, and myelination function (here observed via transcriptomic analysis of APP
function in the mouse brain, tables 1 and 2) as well as altered cholesterol metabolism (predicted via biosimulation, as shown in SA2). My microarray data, as well as GWAS studies of AD patients, suggest that inflammation has a significant role in both functional and dysfunctional responses, perhaps due to connections between inflammation and APP (figures 3, 4, 5, and 6). In the event of a dysfunctional response, or when the stress is great enough to overwhelm the adaptive response driven by APP, disease occurs – which I observed in cells using filipin fluorescence (figure 11). In this disease state, other pathologies such as NFT formation and vascular Aβ deposition (CAA) are likely to occur, ultimately resulting in the death of neurons and loss of cognitive function.

A major next step for testing my hypothesis will be to manipulate APP function under similar stress conditions to the ones I describe here. If APP levels are downregulated (i.e. via RNA interference), or its binding to cholesterol is inhibited, what are the transcriptional consequences? Are there measurable changes in filipin fluorescence? Conversely, if APP is overexpressed, or cholesterol metabolism prevented from changing, are cells more resistant to stress stimuli? These kinds of questions provide enormous opportunity to make use of both my in vitro and in silico work to gain greater mechanistic insight into AD. Ideally, this insight will lead to higher quality experimental design and therapeutic application in the AD field, which, unlike the current Aβ approaches, have a possibility of successful clinical outcomes.
Figure 21. Results from Testing the Adaptive Response Hypothesis. In this representation of the Adaptive Response Hypothesis, experimentally tested elements have been included. Non-functional NPC1 protein and 27-OHC were used as stressors (A), which tested a response from APP by observing transcriptional changes and altered cholesterol metabolism (B). Evidence of the disease state was found using filipin fluorescence (C), which greatly increased in response to stress.
Concluding Remarks

In somewhat of a less-traditional path, I started my doctoral work with a large amount of theoretical research, attempting to assemble the collected pieces of information on APP and its relationship to AD. As a direct result, I was able to contribute my own hypothesis to the field beginning early in my career in the form of a published paper, followed by two more articles in which I drew attention to the shortcomings of the amyloid hypothesis and proposed viable alternatives to study the basic mechanisms of AD pathogenesis. Establishing a theoretical model also gave me a hypothesis-driven project, asking questions that could directly apply to the clinical understanding of AD. I was also able to publish results showing the potential clinical value of filipin in blood tests (Castello et al. 2014).

Over the course of my PhD studies I have had to learn how to adapt my experimental goals to be more attainable or to work around setbacks, and in doing so have contributed at least a small part to my field of study by strengthening the argument for APP as a protein with cholesterol regulatory function. I am proud that my work has set up future opportunities for the lab to pursue, as the work of Dr. Ana Nunes did before me. Finally, I have tried to start the process of converting my discoveries into practical clinical applications, a project I hope to continue over the coming years.
CHAPTER SIX
MATERIALS AND METHODS

Introduction

What follows is the complete documentation of the methods used to generate the presented data. Original sources are cited where possible, along with any modifications.

Cell Culture

Except where otherwise noted, cells were maintained in DMEM (fibroblasts, SH-SY5Y) or RPMI (PBMCs, B-lymphocytes) with 15% FBS and 1% pen/strep and grown for experiments on 6-well or 24-well plates.

Human Fibroblasts and B-Lymphocytes

Human cell samples (fibroblasts and B-lymphocytes) were obtained from Coriell Institute for Medical Research (www.coriell.org). Fibroblasts were taken from patients with AD, Down syndrome (DS), NPC, and age-matched healthy controls, while B-lymphocytes were taken from patients with AD and age-matched controls (table 4).
Table 4. List of Cells Purchased from Coriell Cell Repository.

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<th>Group</th>
<th>Age</th>
<th>Cell Type</th>
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Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) from adult donors were obtained from Leuko-pak leukocyte filters (Fenwal Laboratories, Lake Zurich, IL) and donated by the Blood Processing and Quality Control Lifestream (San Bernardino, CA). Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) and red blood cell lysis density gradient centrifugation were used to isolate PBMCs from blood collected from filters, as described (Tamul et al. 1995).

For collection of PBMCs from AD patients, whole blood was collected by venipuncture in EDTA-treated collection tubes. 4 mL of whole blood was added to 20 mL ammonium-chloride-potassium (ACK) lysing buffer and incubated 5 min at RT. Samples were centrifuged at 300 g for 5 min at RT, supernatant aspirated, and the pellet suspended in cold phosphate buffered saline (PBS). Peripheral blood cells were collected by centrifugation at 300 g for 5 min at 2-8 °C.

For experimentation, PBMCs were cultured in a 96-well plate at a density of 400k cells per well.

SH-SY5Y Cells

SH-SY5Y human neuroblastoma cells (CRL-2266, American Type Culture Collection) were maintained in DMEM with 15% FBS and 1% pen/strep. Differentiation of SH-SY5Y cells was based on an established protocol involving sequential treatment of the culture with retinoic acid (RA) and brain-derived neurotrophic factor (BDNF), characterized by Encinas et al (Encinas et al. 2000). Cells were plated in plastic 6-well plates at 400,000 cells/well (day 1). After 24 hrs (day 2), media was changed to DMEM with 3.75% FBS, 1% pen/strep, and 10 μM retinoic acid (Sigma-Aldrich, St. Louis, MO).
On day 5, media was discarded and replaced with fresh DMEM, again with 3.75% FBS, 1% pen/strep, and 10 µM RA. On day 6, media was changed to DMEM without FBS, and containing 10 µM RA with 50 ng/mL of BDNF (Sigma-Aldrich, St. Louis, MO). Differentiated cells were experimentally treated on day 8.

**Immunofluorescence**

**Preparation of Filipin**

Filipin was prepared as a 50 mg/mL solution in DMSO protected from light. Aliquots were stored in a light-protected container at -80 °C. Unused portions of thawed aliquots were discarded.

**Filipin Staining for FACS Analysis**

For FACS analysis, cells were fixed in paraformaldehyde (PFA) at room temperature (RT; fibroblasts and peripheral blood cells, 1% PFA 30 min at RT; B-lymphocytes, 4% PFA 1 hr at RT) and treated with filipin for 30 min at RT (fibroblasts and peripheral blood cells, 50 µg/ml filipin; B-lymphocytes, 100 µg/ml filipin).

**Filipin Staining of Human Tissue**

Fixed post-mortem human brain tissue sections from patients with AD, vascular dementia, and an age-matched control were provided by the Kirsch lab. Prior to staining, sections were hydrated in increasingly dilute aqueous ethanol solutions, beginning with 100% ethanol and ending with ethanol-free PBS. Sections were stained in 0.05 mg/mL filipin in PBS for one hour at RT, protected from light.
 Filipin Staining and Immunofluorescence for Microscopy

For fluorescence microscopy, filipin visualization, and measurements, cells were grown on glass coverslips and fixed in 4% PFA 15 min at RT. Filipin staining was carried out on fixed cells using 5 μg/ml of filipin (Sigma-Aldrich, St. Louis, MO) for 1 hr at RT in PBS + 1% BSA.

Cells that had not been stained with filipin were blocked in 1% BSA 30 min at RT before treatment with primary antibody. Filipin-stained cells skipped additional blocking. Primary antibody at desired concentration was dispensed as a 50 μl droplet of antibody in PBS + 1% BSA solution on parafilm. Coverslips were placed facedown onto individual droplets and incubated in a humidified chamber 1 hr at RT. After incubation, coverslips were lifted off of the parafilm by adding additional PBS to droplets. Coverslips were washed in PBS three times for 5 min at RT. Addition of fluorescent secondary antibodies (Alexa fluorophores, Life Technologies) followed the same droplet procedure as for the primary antibody, while ensuring that the coverslips were protected from light during incubation. Cells were imaged on an EVOSfl fluorescence microscope (Advanced Microscopy Group) with excitation at 350 nm (filipin), 594 nm (red), or 488 nm (green).

Flow Cytometry

Flow cytometry was performed using a MACSQuant Analyzer (Miltenyi Biotec) with excitation at 350 nm. Average percentage of filipin-positive to total number of cells was calculated with FlowJo software (TreeStar, Inc.) using cut-off parameters based on cells from control patients (fibroblasts, B-lymphocytes) or treated with treatment vehicle (SH-SY5Y and peripheral blood cells). In all cases, control and experimental cell populations are first normalized to mode. A gate is set to include all cells to the
immediate right of the peak fluorescence from control cells; both control and experimental cells contained by this gate are considered filipin-positive.

**Microarray Analysis**

Brain tissue was collected from genotyped male and female mice between the ages of 6 and 13 weeks and flash-frozen in liquid nitrogen. Frozen samples were sent to GenUs (GenUs Biosystems, Northbrook, IL) to perform RNA extraction and microarray using the Agilent mouse v2 platform. Raw data received from GenUs was analyzed using Excel (Microsoft) and Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA).

**Transcriptome-to-Metabolome Biosimulation**

**Initial Simulations**

Transcriptomic data acquired by microarray was processed for biosimulation in Excel by removing nonessential metadata, leaving only gene identifiers and raw expression values (as opposed to the relative expression values used to compare each mouse genotype to control). This data was converted into simulation values by the Transcriptome-to-Metabolome (TTM) algorithm maintained by Clyde Phelix and used by Phelix and others to analyze metabolic changes in AD brains (Phelix et al. 2011; Chris M. Valdez et al. 2010; Christopher M Valdez et al. 2011).

Because the enzymes involved in catalyzing the reaction are known, the gene expression value for that enzyme can be used to modify the rate of reaction. A value, based on the gene expression of an enzyme, is added to the chemical reaction catalyzed by that enzyme as a constant. These values are initially configured using the gene
expression data from a control population, in this case the control mice. This set of reactions and constants forms a dataset that can be processed using the Complex Pathway Simulator software (COPASI), an open source biochemical network simulator (Hoops et al. 2006). Once any additional parameters such as length of simulation are set, COPASI simulates metabolic flux through the reactions, adjusting each reaction based on the constants, returning a new dataset of metabolites and their predicted amounts.

Once the constants have been established to create a baseline model, the reaction constants are modified based on the percent change in gene expression that occurs between the control and experimental groups, allowing new simulations to be run and the resulting metabolite values compared between groups. Using Excel, the final dataset from COPASI can be imported into the Network Overview, Discovery, and Exploration for Excel (NodeXL) software, which is used to convert the raw numbers into a visual network map (Smith et al. 2010).

Simulation Refinement

After performing initial simulations using the above methods, the TTM model is refined by comparing the output with known metabolic outcomes. In the initial work by Phelix et al., simulations were compared with metabolic data for glycolysis products acquired from human brains (Phelix et al. 2011). TTM output was compared with what has been experimentally shown in the mice, such as the demonstration with filipin that free cholesterol accumulates in endosomes in both the Npc1⁻/⁻ and App⁻/⁻/Npc1⁻/⁻ mouse brains (Nunes et al. 2011). At this point, the gene expression-derived constants can be modified to better reflect the impact of transcriptional changes on metabolic output.
Additional reactions can be added to the overall simulation, or compartments can be simulated by limiting input and output of metabolites to groups of reactions.

**Preparation of Protein Lysates**

**Tissue Lysis**

Fresh-frozen mouse tissue samples from cortex and cerebellum were microdissected and collected into Triton X-100 lysis buffer (1% Triton X-100, 150 mM sodium chloride, 50 mM tris pH 7.5 in dH₂O) supplemented with protease inhibitor cocktail containing EDTA (Amresco, 1:100 from stock) and phosphatase inhibitors (10 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate). Samples were sonicated until homogenous, centrifuged (15,000 rpm, 15 min, 4 °C), and the supernatant collected. Nuclear material remaining in the pellet was stored at -80 °C and lysed separately in RIPA lysis buffer (1% Triton X-100, 150 mM sodium chloride, 50 mM tris pH 7.5, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS] in dH₂O) with protease inhibitor cocktail and phosphatase inhibitors, sonicated, centrifuged (15,000 rpm, 15 min, 4 °C), and the supernatant collected.

**Cell Lysis**

To generate lysates from cells, culture plates were removed from incubator and immediately placed on ice. Media was removed and immediately frozen at -80 °C or discarded. Remaining cells were washed in PBS containing Ca²⁺ and Mg²⁺ and lysed for 10 min in Triton X-100 lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor compounds. Attached cells were scraped from plates and lysate was
collected and centrifuged. Supernatent was alliquoted and prepared for Western blotting, while the remaining pellet was frozen at -80 °C.

**Proteomic Analysis**

**Trypsin Digestion and Isotope Labeling**

Protein was prepared for mass spectrometry according to a protocol established by Sowers *et al* in conjunction with the LLU Mass Spectrometry Core Facility (Xiong *et al*. 2011). Lysed mouse tissue samples were reduced with 1 M dithiothreitol (DTT) 1 hr at 50 °C. 0.5 M iododecamide was added and the samples incubated 2 hr at RT in the dark. 5 volumes of 100% acetone were added and the samples incubated overnight at -20 °C. Samples were centrifuged (14,000 rpm for 10 min at 4 °C) and the acetone decanted. Remaining acetone was allowed to evaporate before adding 20 mM triethylamonium buffer (TEAB) and vortexing. Trypsin was added to the samples and allowed to digest overnight at 37 °C. Samples were centrifuged (14,000 rpm for 15 min at 4 °C) and the supernatant collected. A solution of acetonitrile (AcN) and one of six isobaric Tandem Mass Tags (Thermo) was added to each sample and incubated for 2 hr at RT. Reaction was quenched by incubating 15 min in 5% hydroxyamine at RT. Four of twelve total samples with different TMT tags were combined for fractionation to create three sample sets.

**Strong Cation Exchange Fractionation**

Strong cation exchange fractionation was performed by adding 1% formic acid (FA) to the combined labeled samples until pH reached 3-4 range. Strong cation exchange tips (TT1000 PSC TopTips, Poly LC) were prepared by passing 250 μl binding
solution (1% FA and 20% AcN) through the tip three times. Solutions were passed through the prepared tip and collected to generate individual fractions, beginning with the sample (flowthrough fraction) and followed by 0.05 M potassium chloride (KCl) in 20% AcN (fraction 1), 0.15 M KCl in 20% AcN (fraction 2), 0.3 M KCl in 20% AcN (fraction 3), 0.5 M ammonium hydroxide in 20% AcN (fraction 4), and 50% AcN (fraction 5). At this point the tip was regenerated for repeated use by washing 3 times with 0.5 mL 5% ammonium hydroxide and three times with 1% FA. Samples were vacuum-dried 3 hr at RT and resuspended in 1% FA for desalting.

Desalting

Desalting tips (TopTip T1000 MC18 C18 gel-filtration, Poly LC) were prepared by washing three times with 1% FA. Each resuspended fraction was checked for a pH of 3-4, passed through the prepared tip, and washed with 1% FA. Proteins were eluted by passing 60% AcN through the tip and collecting the flowthrough. Samples were vacuum-dried, reconstituted in 0.1% FA, centrifuged (1 min 10,000 rpm) and transferred to tubes for mass spectrometry. Prepared fractions were run individually on a mass spectrometer by the LLU Mass Spectrometry core facility.

Result Analysis

Raw mass spectrometry data received from the LLU Mass Spectrometry core facility was imported into Thermo Proteome Discoverer™ (Thermo Fisher Scientific, Inc.) and searched against a database of known protein fragments using the published SEQUEST algorithm (Eng, McCormack, and Yates 1994). The resulting lists of
identified proteins and peptide sequences were exported to Excel (Microsoft) for further analysis.

**Statistical Analysis**

All statistical analysis was performed in SPSS Statistics (version 22, IBM Corp.) and Excel (2010, Microsoft). For single comparisons, homoscedasticity of variance was confirmed using an f-test and a two-tailed student’s t-test was performed. To compare more than one group to control and for comparison between groups, a one-way ANOVA was used with the Bonferroni post-hoc analysis.

**U18666A and Cholesterol Treatment**

Chemical Preparation

U18666A was stored in aliquots at -80 °C as an aqueous solution at a concentration of 4.7163 M (2 mg/mL). Aliquots were diluted further in the appropriate culture media prior to treatment.

24-hyrdoxycholesterol (24-OHC) and 27-hydroxycholesterol (27-OHC) were prepared as 10 mM solutions in 100% ethanol and stored in aliquots at -80 °C. Aliquots were diluted further in the appropriate culture media prior to treatment.

Except where otherwise noticed, cells were treated with media containing approximately 5 mM U18666A, 10 μM 24-OHC, or 10 μM 27-OHC and incubated between 16-18 hours at 37 °C. This U18666A concentration is taken from its wide use in studies of NPC, while the cholesterol treatment concentrations are taken from work by Ghribi *et al* characterizing their effect in SH-SY5Y cells (Neufeld et al. 1999; Jaya Rantham Prabhakara Prasanthi et al. 2008; Jaya R. P. Prasanthi et al. 2009).
Cytotoxicity Assay

Lactate Dehydrogenase (LDH) assays were purchased from Thermo Scientific (cat # 88953) and performed on plated cells according to the manufacturer’s instructions after treatment with 24-OHC (15 μM), 27-OHC (15 μM), or U18666A (300 μM) for 12 hours. LDH levels were quantified using a BioTek ELx800 plate reader.

Western Blotting

Lysate samples were prepared for running on tris-glycine gels (4-12% gradient, 4-20% gradient, or 10-20% gradient, depending on the size of the proteins of interest) by combining with tris-glycine SDS sample buffer (2X, Life Technologies) and reducing agent (10X, Life Technologies). For tricine gels, sample was combined with tricine SDS sample buffer (2X, Life Technologies) and reducing agent.

Equivalent volumes of sample were heated (tris-glycine: 10 min, 85 °C; tricine: 2 min, 85 °C) and loaded onto the gels for separation (tris-glycine: 2.5 hr, 125 V; tricine: 90 min, 125V) and transfer (tris-glycine: 30 min, 100V; tricine: 75 min, 75V) of proteins onto nitrocellulose membranes. Membranes were incubated in blocking solution (tris-glycine: 5% milk in T-TBS; tricine: 5% BSA in T-TBS) 30 min at RT and incubated under parafilm overnight at 4 °C in the appropriate dilution of primary antibody (prepared in T-TBS). Membranes were washed in T-TBS three times for 10 min and incubated in the appropriate secondary antibody (1:15,000, Li-Cor) 1 hr at RT protected from light. After incubation, membranes were again washed in T-TBS three times for 10 min, transferred to TBS, and imaged on an Odyssey scanner (Li-Cor). Intensity of bands was analyzed using ImageStudio (Li-Cor).
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APPENDIX A

PATENT FILINGS

Methods and Kits for Determining Risk for Developing Alzheimer’s Disease and Prevention or Treatment Thereof

METHODS AND KITS FOR DETERMINING RISK FOR DEVELOPING ALZHEIMER’S DISEASE AND PREVENTION OR TREATMENT THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present disclosure relates to methods and kits for assessing a patient’s risk for developing Alzheimer’s disease, for preventing or delaying the onset of Alzheimer’s disease, or for reducing the symptoms of Alzheimer’s disease.

Description of the Related Art

[0002] Alzheimer’s disease (“AD”) is an irreversible, progressive brain disease and is a form of dementia, the loss of cognitive functioning and behavioral abilities that affects daily life and activities. Alzheimer’s disease affects 5.4 million Americans. Because of no known cure or effective treatment, the disease incurs an estimated annual cost of $183 billion. Affirmative diagnosis of the disease is only possible at autopsy. Previous studies suggested that the accumulation of the amyloid peptide was responsible for the onset of the disease, and consequently, research in the last 20 years has primarily focused on the prevention of amyloid peptide accumulation in the brain to ameliorate or halt the disease. These approaches have not been successful.

SUMMARY OF THE INVENTION

[0003] Methods and kits for determining a patient’s risk of developing Alzheimer’s disease (“AD”), prevention or delay of onset of AD, and reducing the symptoms of AD in a patient in need thereof are disclosed.

[0004] In some embodiments, a method for determining a patient’s risk of developing Alzheimer’s disease is provided. The method comprises obtaining a test sample from the patient, determining a level of intracellular cholesterol in the test sample, and comparing the level of intracellular cholesterol in the test sample with a control level of intracellular cholesterol, wherein a greater level of intracellular cholesterol in the test sample compared to the control indicates an increased risk of developing Alzheimer’s disease.
[0005] In some embodiments, the method comprises obtaining a test sample from
the patient, determining a level of intracellular cholesterol distress and/or defect in the test
sample, and comparing the level of intracellular cholesterol distress and/or defect in the test
sample with a control level of intracellular cholesterol distress and/or defect, wherein a greater
level of intracellular cholesterol distress and/or defect in the test sample compared to the
control indicates an increased risk of developing Alzheimer’s disease.

[0006] In other variations, a method for preventing or delaying the onset of
Alzheimer’s disease, or reducing the symptoms of Alzheimer’s disease in a patient in need
thereof is provided. The method comprises obtaining a test sample from the patient,
determining a level of intracellular cholesterol in the test sample, comparing the level of
intracellular cholesterol in the test sample with a control level of intracellular cholesterol,
identifying at least one cholesterol defect or distress of the patient, and administering at least
one therapeutic agent which targets the at least one cholesterol defect or distress.

[0007] In some embodiments, the method comprises obtaining a test sample from
the patient, determining a level of intracellular cholesterol distress and/or defects in the test
sample, comparing the level of intracellular cholesterol distress and/or defects in the test
sample with a control level of intracellular cholesterol distress and/or defects, identifying at
least one cholesterol defect or distress of the patient, and administering at least one
therapeutic agent which targets at least one cholesterol distress and/or defect.

[0008] Further provided is a kit for assessing a patient’s risk of developing
Alzheimer’s disease comprising: a tissue collecting assembly; an intracellular cholesterol
detection reagent; and a composite control sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] Figure 1 shows how cholesterol distress, rather than Aβ accumulation, may
be the more likely candidate for a bona fide pathogenic trigger in most cases of late-onset AD.
Cholesterol distress may be defined as some combination of abnormal amounts of cholesterol
or its precursors and metabolites, aberrant subcellular localization, or oxidation state (green
arrow 1a), and is primarily caused by multiple factors, including age, metabolic stress, and
gene polymorphisms. In turn, cholesterol distress prompts activation of an APP-driven
adaptive response, through a mechanism involving, in part, the generation of Aβ (green arrow 1b). If the adaptive response from APP is insufficient, cholesterol distress can result in cell dysfunction (red arrow 2a). Cell dysfunction can be defined by the presence of major cellular damage as a consequence of axonal transport disruption, tau phosphorylation and aggregation, inflammatory response, reactive oxygen species, and neuronal commitment to aberrant entry into the cell cycle. Cell dysfunction can in turn affect cholesterol regulation via a mechanism mostly driven by inflammation (red arrow 2b). Note that the pathogenic cascade only initiates after cell dysfunction, leading to synaptic collapse, loss of dendritic mass, and neuronal destruction that results in dementia and death (pathway defined by dark red arrow 4). Aβ oligomers and plaques form when there is an excessive defensive response from APP to chronic cholesterol distress. In a brain where cell dysfunction is already ongoing (i.e. within the pathogenic cascade leading to dementia; dark red arrow 4), excess Aβ affects already-vulnerable neurons, further inducing inflammatory responses, extending oxidative damage, and contributing to tau hyperphosphorylation and aggregation. Vascular Aβ deposition would be prominent at this stage, contributing to cerebral amyloid angiopathy and microbleeds, exacerbating the pathogenic cascade (dark red loop 5). Note that, where plaques are present in cognitively functional individuals, an excessive adaptive response from APP has generated a large amount of Aβ, while successfully defending against cholesterol distress. In this case, cell dysfunction is absent and the formation of Aβ oligomers and plaques does not have an obvious impact on neuronal function (not shown in figure). In early-onset AD and Down syndrome, Aβ is constitutively overproduced throughout life (or the Aβ40/Aβ42 ratio is significantly higher), to levels that are sufficient to cause dysfunction.

Figure 2 depicts representative images of a filipin test for a patient with (A) severe Niemann-Pick type C (“NPC”) disease (“NPC 1 year” or “NPC01”) and (B) less severe Niemann-Pick type C disease (“NPC 21 years” or “NPC21”). For comparison, Figure 2 also depicts representative images of a filipin test for a healthy patient (C) age-and-sex matched to patient NPC1 year (“Control 1 year” or “CTL01”) and (D) age-and-sex matched to patient NPC 21 years (“Control 23 years” or “CTL23”). Figure 2E is a graph showing the filipin-positive percentages for patients NPC01, CTL01, NPC21, and CTL23. Because NPC is a
disease involving cholesterol distress, filipin staining in NPC cases can be used as a positive control in the measurement of filipin staining in AD cases.

0011 Figure 3 depicts representative figures of a filipin test of (A) a 73-year-old patient with Alzheimer’s disease (“AD 73 years” or “AD73”) and (B) a healthy patient age- and-sex matched to patient AD73 (“Control 73 years” or “CTL73”). Figure 3C is a graph showing the filipin-positive percentages for patients CTL73 and AD73. The severity of the disease presentation as measured by random chromosomal loss and/or gain may correlate with the degree of filipin staining.

0012 Figure 4 shows the filipin score of fibroblasts taken from three patients affected with Alzheimer's disease (“08245(AD),” “08243(AD),” “07375(AD)”) and one age-matched cognitively healthy patient (“0919(CTL)”). Filipin score was determined by hand-counting filipin-positive cells. The patient with less DNA damage (07375(AD)) also has a lower filipin score (further description of patients in Table 1). As shown in Figure 4, 07375(AD) has more filipin staining than control but less than the other two AD patients; this patient also has only 2% random chromosomal loss.

0013 Figure 5 depicts a fluorescence-activated cell sorting (“FACS”) analysis of fibroblasts taken from three patients affected with Alzheimer's disease (“AG08245,” “AG08243,” “AG07375”) and one age-matched cognitively healthy patient (“AG0919”). The leftmost graph in each panel indicates baseline signal, while the rightmost graph shows filipin intensity. The two peaks are clearly separated in the three clinically affected patients, while in the healthy patient, the two peaks largely overlap. This differential response to filipin may allow for separation of Alzheimer's and healthy patients.

0014 Figure 6 depicts an exemplary graph of the prophetic data displayed in Table 2 below. Alzheimer’s Disease patients may have the highest filipin score, while healthy patients may have the lowest filipin score. Patients presenting with Mild Cognitive Impairment can be sorted into low and high risk of developing Alzheimer’s Disease based on their filipin score.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Disease and Anatomy
Figure 3
Figure 5
Tremor Detection and Correction System

TREMOR DETECTION AND CORRECTION SYSTEM

SUMMARY

[0001] This disclosure relates generally to detection and measurement of tremors in a human subject. In some embodiments, a system senses tremor signals using sensors attached to a patient suffering from muscular tremors and corrects for the tremors by distinguishing between voluntary motion and involuntary tremors. Tremors can be a symptom of neurological disease, including neurodegenerative Parkinson’s disease. The technology disclosed herein can substantially improve the quality of life of subjects suffering from tremors.

[0002] In some embodiments, signal processing can be applied to data generated by a motion sensing device to identify tremors. The motion sensing device can sense actual motion and/or electrical signals of the subject using the device. In some embodiments, tremor signals identified by the system can be used to eliminate tremor signals from motion-based computer input. Detection hardware and signal processing software can identify individuals with early signs of tremor.

BRIEF DESCRIPTION OF THE DRAWINGS

[0003] Figure 1 shows a first flow chart of an example embodiment of a method for identifying the presence of tremor in a person.

[0004] Figure 2 shows a second flow chart of an example embodiment of a method for identifying the presence of tremor in a person.

[0005] Figure 3 shows a schematic block diagram example embodiment of a tremor detection system.

[0006] Figure 4 shows a schematic block diagram example embodiment where an instruction signal from a motion input device can pass through a tremor signal filter to remove interfering tremor signal before being interpreted by a device driver.

[0007] Figure 5 shows a flow chart of an example embodiment for calibrating methods of detecting tremor.
DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[0008] Identifying a tremor in patients and differentiating between types of tremor sometimes requires neurological evaluation by a trained physician. Unfortunately, many people do not visit a neurologist until significant neurodegeneration has occurred and therapies are less effective. Thus, automated approaches that can be performed with commercially available devices are beneficial as they can identify those people who are at risk and direct them to seek out medical care. Automated approaches of sufficient sensitivity may also be used to assist in tremor diagnosis by a neurologist. Such automated approaches for detection of tremor (or Parkinson’s disease, the most common form of tremor in the elderly) can include vocal analysis and the use of accelerometers, such as those found on modern smartphones. Movement can be detected by using an accelerometer, possibly with the additional use of a gyroscope. Use of accelerometer data for detecting tremor (such as via a smartphone) relies on detecting movement abnormalities, which may already be hindering a person’s ability to function normally, and can be confounded by fatigue or the surrounding environment. Using both movement data and muscular electrical impulse data for tremor detection improves the accuracy and sensitivity of tremor detection.

[0009] Electrical impulses can be measured to detect muscle movements indicating tremor. Muscle activity can create electrical impulses, which may be measured to detect muscle movement even if no movement is detected visually or by an accelerometer. Electrical impulses can also be detected to identify physical actions such as arm rotation or forming a fist. By measuring electrical impulse data in addition to movement data, sensitivity and accuracy of tremor detection can be greatly increased and can take place at an earlier time in the disease process when the individual may be less impaired. With sufficiently sensitive detection hardware, tremor may be detected even when visual diagnosis of tremor may not be possible. Tremors can be detected by comparing a detected electrical impulse signal and movement signal to identify discrepancies or inconsistencies. Tremors can also be detected by evaluating a motion signal built from both movement data (such as from an accelerometer) and an electrical impulse signal.

[0010] Hardware for detecting movement and electrical impulses can be worn on the arm or another body portion of a user. The detection hardware can in some embodiments
be worn as a bracelet. Detection hardware can be capable of sensing three-dimensional movement as well as detecting small electrical impulses generated by muscle movement. A gyroscope and accelerometer can be used to track three-dimensional movement. Electrode leads attached to a user can detect small electrical impulses generated by muscle movement. The detection hardware can in some embodiments include an accelerometer, electrode leads, and a means of communicating data with a computer. Other sensors for detecting movement or detecting electrical impulses generated by muscle movement can also be used. Data can be communicated from the detection hardware with a computer in many ways including through a wired connection, through a wireless local area network, through a Bluetooth connection, or through a cellular telephone link. In some embodiments the detection hardware can comprise of the MYO device developed by Thalmic Labs, Inc. of Kitchener, Ontario, Canada.

[0011] Based on movement and electrical impulse data, early signs of tremor can be detected. Signal isolation algorithms can be used to identify some patterns specific to Parkinsonian tremor or other varieties of tremor. Software features of some embodiments can include any combination of means of acquiring movement and/or electrical impulse data from hardware, processing that data to identify tremors, and filtering tremor signal from the final data stream before sending it to a computer.

[0012] Figure 1 shows a flow chart example of a method for detecting tremors 100 utilized in some embodiments of the present disclosure. First, both electrical impulse data is detected 102, and movement data is detected 104. Detection 102 and 104 can include identifying the presence of movement or electrical impulses, and in some embodiments can include measuring any of the direction, magnitude, frequency or duration of movement or electrical impulses. In some embodiments the detection of the movement data 104 and the electrical data 102 can be accomplished by a single article of detection hardware capable of detecting both movement and electrical impulses. In some embodiments the detection of the movement data 104 can be accomplished by a separate article of detection hardware from the detection of electrical impulses 102.

[0013] Movement data and electrical impulse data for tremor detection 104 and 102 can be detected in a variety of settings. Detection hardware worn by a user can passively detect movement and electrical impulse data during the course of normal daily user activity.
In some embodiments, movement data and electrical impulse data can be detected during supervised user activity specifically for the purpose of evaluating potential tremors. Movement instructions can be given to a user to facilitate tremor detection. A neurologist or other medical professional can observe or direct the movements of a user wearing detection hardware. A user can wear detection hardware in accordance with a medical professional’s instruction between visits to the medical professional in order to provide data to the medical professional for the purpose of diagnosing tremor. In some embodiments, a user can provide input regarding motion to be performed while wearing the detection hardware.

[0014] Second, detected electrical impulse data and detected movement data from the same timeframe can be compared. Detected electrical impulses from muscle movements can be correlated to specific detected movement. Correlated data can be compared. Electrical impulses from muscles can be detected shortly before any movement. Some muscle movements can produce known or predictable electrical impulses. For comparison with detected data 106, in some embodiments, specific muscle activity and associated electrical impulses can be predicted based on a detected movement. Predictions of electrical impulse data can in some embodiments be made based on previously collected movement and electrical impulse data from the same user or other users. Predictions of electrical impulse data can also be based on any known properties of the detected movement, such as what movement (if any) was intended by the user at the time of the data detection.

[0015] Third, irregularities or inconsistencies in the detected data can be identified 108. In some embodiments, instances can be identified where the detected electrical signal may not be as expected based on the detected movement. Deviations in the detected electrical impulses from the expected or predicted electrical impulse for a detected movement can be measured. Aberrations in the detected electrical impulse data preceding movement can also be detected. What qualifies as an irregularity or inconsistency for identification 108 can in some embodiments be determined differently for an individual user. Previously collected data from users both exhibiting tremor and those not exhibiting tremor can be considered to determine if detected data reflects an irregularity or inconsistency. The direction, magnitude, frequency, or duration of any irregularity in the detected data can also be identified 108. In
some embodiments signal isolation algorithms can be applied to the detected data in order to identify potential tremors or Parkinsonian tremor.

[0016] Fourth, based on any identified irregularities or inconsistencies, a tremor or potential tremor can be identified 110. In some embodiments the identification of tremor or tremor pattern 110 can be based on variations in detected electrical impulse data from the electrical impulses expected with a detected movement. Where detected electrical impulses indicate unusual muscle activity for a detected movement, a tremor could potentially be present. If detected electrical impulses would normally be associated with a movement that was not detected, the impulses could indicate tremor or a potential tremor not yet detectable by accelerometer or by visual inspection. Identification of tremor 110 can include identification of a potential tremor, the probability of tremor, or the need to follow up with a neurologist. Where tremor is not indicated by any irregularities or inconsistencies, identifying tremor 110 can include identifying an absence of tremor.

[0017] Differences between tremors can be identified based on the detected electrical impulse and movement data. A tremor can be characterized by identifying the magnitude, duration, or frequency of movements detected correlating to an irregularity in the electrical impulse data. A tremor can also be characterized by identifying the magnitude, duration, or frequency of an identified irregularity in the electrical impulse data. In some embodiments tremors can be differentiated based on the context that the data indicating a tremor was detected. If a tremor is indicated when a user wearing the detecting hardware is at rest, then the tremor may potentially be characterized as a resting tremor. If a tremor is indicated when a user is making some volitional movement, then the tremor may potentially be characterized as an intention tremor. Other differentiations may be made between identified tremors based on known context for an identified tremor and any determinable features of an identified tremor.

[0018] Figure 2 shows a different flow chart example of the method for detecting tremors 100 utilized in some embodiments of the present disclosure. First, both electrical impulse data is detected 102, and movement data is detected 104. The detection of data 102 and 104 for the method of Figure 2 can be accomplished in any manner described above for Figure 1. Detection of data 102 and 104 can include measuring any of the direction,
magnitude, frequency, or duration for movement or electrical impulses. As described above in regards to the method of Figure 1, the detection of data 102 and 104 can be accomplished in some embodiments by a single article of detection hardware, or multiple articles of detection hardware. Similarly, movement data and electrical impulse data for tremor detection can be detected in a variety of settings, including the settings and embodiments described above for Figure 1.

[0019] Second, a motion signal can be developed based on the detected data 112. A motion signal can reflect the resulting motion associated with both the detected movement and the detected electrical impulses from muscle activity. In some embodiments motion signal development 112 can consist of identifying electrical impulse data and movement data. In some embodiments the motion signal can be developed 112 by adjusting detected movement by applying motion corrections for detected electrical impulses indicating muscle activity. For example if a small muscle action is detected via electrical impulses, a small motion may be inferred, and, if not already reflected in the detected movement, the inferred motion could be used to adjust and develop the motion signal 112. The amount of motion to infer from a detected electrical impulse can be determined in some embodiments based on previously collected data from the same user, similar users, or other users. By including indications of muscle movement from detected electrical impulses, the motion signal development 112 can in some embodiments include both motion large enough to be detected visually and motion not large enough to be detected visually.

[0020] Third, the developed motion signal can be evaluated 114. Evaluation of a motion signal 114 can include isolating or locating patterns within a motion signal. Small or large irregularities in a motion signal can be identified. Delays or interruptions in the progression of a motion can also be identified in the signal. In some embodiments the direction, magnitude, frequency, and duration of patterns in a motion signal can be evaluated. The evaluation of the motion signal 114 can include comparisons to previously collected data or other developed motion signals from different sets of data. In some embodiments a motion signal can be compared to a previously developed motion signal known to indicate the presence of tremor. Motion signal evaluation 112 can also include consideration of any known context for the detected data such as the known intended motions of a user while
wearing the detection hardware. In some embodiments signal isolation algorithms can be applied to the motion signal to locate potential tremors or potential Parkinsonian tremor.

[0021] Fourth, based on the evaluation of the developed motion signal, the presence or absence of tremor can be identified 110. In some embodiments tremor identification 110 can include comparing patterns found in the motion signal to patterns found in previously developed motion signals known to be associated with tremor. In some embodiments tremor identification 110 can include detecting irregularities, delays, or interruptions in motion. Features such as magnitude, frequency, and duration of patterns in a motion signal can be used to identify tremor 110. The criteria applied to an evaluated motion signal to identify tremor can 110 be specific to an individual user or based on previously known features of motion signals associated with tremor. In some embodiments a neurologist or physician can use a developed motion signal to identify a tremor, with or without using other diagnostic tools.

[0022] As described with regards to the identification of tremor in the method of Figure 1, the identification of tremor 110 can include differentiating between types of tremor. Features of the motion signal including the frequency, magnitude, or duration of any patterns in the signal can indicate characteristics of an identified tremor. Any known context for user motion associated with a motion signal can be used to characterize an identified tremor. Identification of tremor 110 can also include identification of the probability that tremor is present, or the need to follow up with a neurologist on potential tremor. As described above, the identification of tremor 110 can include the identification of the absence of tremor.

[0023] While described in separate flow charts, in some embodiments multiple methods for detecting tremor 100 can be utilized. A single set of detected electrical impulse and movement data can be both for comparing data 106 in the method of Figure 1, and used for developing a motion signal 112 in the method described in Figure 2. Tremor detection can be accomplished through a combination of the elements described in relation to the methods of Figure 1 and Figure 2. In some embodiments a tremor can be identified and characterized both by deviations of the electrical impulse data from the expected electrical impulse data for a detected movement, and by evaluating features of a developed motion signal. The same

-7-
detection hardware and processing means can be used to perform both the method of Figure 1 and of Figure 2.

[0024] Comparisons of data 106, development of a motion signal 112, identification of irregularities or inconsistencies 108, evaluation of a motion signal 114, identification of tremor 110, and any other manipulation of the detected data can in some embodiments be performed on one or more processors. In some embodiments a hardware processor can be used to perform one or more manipulation of the detected data. Data from the detection hardware can be communicated to a personal or hand-held computer including at least one processor. In some embodiments some manipulations of the detected data can occur in one or more processors within the detection hardware. While described in separate steps of the flow charts, each step can be performed on the same processor, or in some embodiments multiple processors can be used.

[0025] Figure 3 shows a schematic example of an embodiment of a tremor detections system 200. The tremor detection system 200 can apply tremor detection module 210 to data detected by movement and electrical impulse detector 202. Any tremors detected or identified by the tremor detection module can in some embodiments be presented to a user through the front end 204. The data store 206 can maintain a record of user data, data regarding identified tremor patterns, tremor detection data from tremor detection module 210, detected movement data or electrical impulse data from movement and electrical impulse detector 202, measurements of detected data or tremor, relationships between sets of data, other medical data, or a combination of different types of data.

[0026] The movement and electric impulse detector 202 can be any detection hardware capable of both detecting electrical impulses generated by muscle activity, and detecting movement. Movement and electrical impulse detector 202 can comprise one article of detection hardware or multiple articles of detection hardware. The movement and electrical impulse detector 202 can comprise of a single article of detection hardware. In some embodiments movement and electrical impulse detector 202 can comprise of multiple articles of detection hardware. Although not pictured, in some embodiments vocal analysis for tremor can also be incorporated in tremor detection system 200 to provide additional tremor detection capabilities.

-8-
[0027] A tremor detection module 210 can apply a variety of methods to identify a tremor based on the detected data. Electrical impulse data and movement data detected by the movement and electrical impulse detector 202 can be evaluated or compared by tremor detection module 210 to detect tremor. The tremor detection module can apply the method for detecting tremor described in Figure 1. In some embodiments the tremor detection module can apply the method for identifying tremor described in Figure 2. The tremor detection module 210 can also apply multiple methods for identifying tremor, including any combination of the elements of the methods described in Figure 1 and Figure 2. The tremor detection module 210 can include one or more processors. In some embodiments data recorded in data store 206 such as previously identified tremor patterns can also be used by tremor detection module 210 to identify tremor.

[0028] Once tremor, or the absence of tremor, is identified by the tremor detection module 210, a user can in some embodiments access information about any detected tremor through the front end 204. The front end 204 can provide information including (among other possibilities): the probability a tremor was detected, the correlated movement data and electrical signal data associated with an identified tremor, any possible characterizations of an identified tremor, or an indicated absence of tremor. The tremor detection module 210 can in some embodiments provide the front end 204 with depictions of the data relied on for identification of tremor. As described above, data relied on for identification of tremor can include (among other possibilities) detected movement and electrical impulse data, expected electrical impulse data associated with a detected movement, previously collected electrical impulse data for movement data known to be associated with tremor, or a previously evaluated motion signal.

[0029] The front end 204 can in some embodiments include a display module. The display module can display information related to both the detected data and any identified tremors or potential tremors. A display module can include one or more screens for displaying data. A display module can in some embodiments be part of a computer display, a tablet display, or a smartphone display. In some embodiments front end 204 can also include audio features to provide information to a user or neurologist. Audio features can include alerts for the detection of tremor or detection of tremor meeting user specified criteria.
[0030] In some embodiments a user can interact with the front end 204 to select what results or correlating data is displayed. The level of detail displayed regarding the tremor identification process can be adjusted for different users. If, as in some embodiments, the tremor detection system 200 is being used for personal evaluation of the likelihood of tremor, then the front end 204 can provide a calculation of the percent chance of tremor detection along with a recommendation of whether to follow up with a neurologist. If the tremor detection system 200 is being used to assist a neurologist or other medical professional in diagnosing a tremor, more detail can be displayed. A user can in some embodiments interact with the front end 204 to alter the focus, signal processing, or time portion displayed for any data or results being displayed.

[0031] The movement and electrical impulse detector 202, the front end 204, the data store 206, and the tremor detection module can connect to one another through a network 208. The network 208 can include a local area network, a wide area network, a wired network, a wireless network, a local bus, a Bluetooth connection, connection to a cellular phone network, or any combination thereof. In some embodiments, one or more components of the system 200 connect another component of the system 200 over the Internet. While movement and electrical impulse detector 202, front end 204, data store 206 and tremor detection module 210 are pictured as separate modules, in some embodiments features of each module can overlap. In some embodiments one or more processors may be utilized by one or more of movement and electrical impulse detector 202, front end 204, data store 206 and tremor detection module 210.

[0032] When used by a neurologist or other medical professional, some embodiments of the disclosure may allow for a more accurate and detailed understanding of a patient’s tremor. As described above, the methods for detecting tremor using both electrical impulse and movement detection can provide a greater degree of sensitivity in identifying signs of tremor because small motions of muscles indicated only by electrical impulses may not be detectable by visual inspection. In some embodiments where a patient wears detection hardware for passive electrical impulse and movement detection between visits to a neurologist, the collected data may allow for a more accurate track of the progression of
tremor symptoms. Features of a patient’s identified tremors or potential tremors can also be tracked by a neurologist to help determine how tremor symptoms are progressing.

[0033] In some embodiments the features of an identified tremor or potential tremor can be evaluated to determine the risk of mild cognitive impairment (MCI) progressing to Parkinson’s disease or to Alzheimer’s disease. While tremor and MCI can both be indicative of a risk to progress to Parkinson’s disease or Alzheimer’s disease, the tremors exhibited may be used to distinguish whether Parkinson’s or Alzheimer’s is likely to develop. By tracking the features of a patient’s tremors with some embodiments of the present disclosure, a neurologist can assess the risk that MCI will progress to Alzheimer’s. Enabling early differentiation between patients with MCI and tremor can increase treatment options or efficacy and has the potential to improve a patient’s prognosis.

[0034] In some embodiments the tremor correction system can compensate for specific tremor patterns that impact a user’s ability to use a computer. Figure 4 shows a schematic example embodiment of an input filtering system 300 where motion based input to a computer is filtered to remove the distorting effect of tremor. Tremor can negatively impact a person’s ability to provide motion based input to a computer by distorting the motion signal. By correcting for a specific tremor pattern an impaired person can accurately and conveniently operate a computer.

[0035] User input in the form of motion can be detected by a motion input sensor 302. A motion input sensor 302 can be part of a motion input device. A motion input device can include a tradition computer mouse or any device that converts physical motion by a user into instructions for a computer. In some embodiments the detection hardware can also be configured to provide motion based input to a computer. When operated, a motion input sensor 302 can generate a distorted instruction signal that includes both motion intended by a user as instructions and motion created involuntarily by tremor.

[0036] A tremor signal filter 306 can receive a distorted instruction signal 304 from a motion input sensor 302 and produce a corrected instruction signal 308 to a device driver. The tremor signal filter 306 can be software. In some embodiments signal isolation algorithms can be applied by tremor signal filter 306 to detect tremor patterns in a distorted instruction signal 304. The tremor signal filter 306 can utilize identified patterns associated
with tremor to identify whether a detected signal should be associated with tremor. Identified tremor patterns can be from a user’s specific tremor pattern history, or from a generic identified tremor pattern. A generic identified tremor pattern can be specific to users with certain types or degrees of tremor. Once a tremor pattern is detected in the distorted instruction signal 304, that pattern can be removed from the signal by the tremor signal filter 306. The tremor signal filter 306 can in some embodiments apply Bayesian logic to determine the probability that a detected pattern is associated with tremor. In some embodiments the tremor signal filter 306 can apply other methods of heuristic problem solving to identify tremor patterns in a computer instruction signal.

[0037] The corrected instruction signal 308 can be sent to a device driver 310 in the same manner that instructions from the motion input sensor 302 would be. A motion input device including motion input sensor 302 can be associated with a device driver 310. A device driver 310 can be a computer program that operates or controls a particular type of associated device that can be attached to a computer. A device driver 310 can communicate with an associated device through the computer bus or communications subsystem. When a calling program invokes a routine in the device driver 310, the device driver 310 can issue commands to the associated device. Once the associated device sends back data to the device driver 310, the device driver 310 may invoke routines in the original calling program. Data sent back to the device driver 310 from motion input sensor 302 and the associated motion input device can be filtered by tremor signal filter 306.

[0038] The apparatus operated by motion based instructions can be a personal computer for individual use. In some embodiments the operated device could be part of a larger system being operated at least in part by a person with tremor such as an interface in an industrial setting or an office computer that is part of a larger network. The tremor signal filter 306 can be housed on or utilize one or more processors. In some embodiments the tremor signal filter can involve one or more processors that are part of the motion input device or part of the computer receiving the instructions. Motion input sensor 302 and device driver 310 can be housed on or utilize one or more processors which can include processors housing or utilized by tremor signal filter 306.
[0039] A signal filter can be utilized in some embodiments even where a user is not suffering from a clinical tremor. In some contexts such as laparoscopic surgery a filter identifying and correcting for minimal involuntary muscle movement noise in instruction signals could be useful. The signal corrections in non-tremor settings can in some embodiments be based on previously recorded motion signal input, which can be associated as either a noise input or an intentional input. In some embodiments the same features which could be used to identify a potential tremor if on a larger magnitude can be used to identify instruction signal noise in non-tremor users.

[0040] Figure 5 shows a flow chart of an example embodiment for calibrating methods of detecting tremor. Initially, movements from individual control and tremor patients can be recorded using detection hardware 402. Detection of data can be accomplished according to a variety of embodiments including any embodiment described above in connection with Figures 1, 2, or 3. Next movement data and electrical impulse data from muscle activity can be collected 404. Data collection can in some embodiments involve transferring data detected by one or more piece of detection hardware to a data store.

[0041] Data can be processed 406 to identify distinct patterns produced by individuals with tremor, in some embodiments by using a signal-noise isolation algorithm. Tremor patterns can then be identified 408. In some embodiments any of the above methods described in connection with Figures 1, 2, or 3 for identifying a tremor pattern can be used to identify tremor pattern. Data collected from many patients can next be processed and compared 410 against data collected from previously identified tremor patterns. Finally, the system can distinguish 412 between individuals with and without tremor.

[0042] The systems and methods disclosed herein can be implemented in hardware, software, firmware, or a combination thereof. Software can include computer-readable instructions stored in memory (e.g., non-transitory, tangible memory, such as solid state memory (e.g., ROM, EEPROM, FLASH, RAM), optical memory (e.g., a CD, DVD, Blu-ray disc, etc.), magnetic memory (e.g., a hard disc drive), etc.), configured to implement the algorithms on a general purpose computer, special purpose processors, or combinations thereof. For example, one or more computing devices, such as a processor, may execute program instructions stored in computer readable memory to carry out processes disclosed.
herein. Hardware may include state machines, one or more general purpose computers, and/or one or more special purpose processors. In some embodiment, multiple processors can be used, and in some implementations the processors can be at different locations (e.g., coupled via a network). While certain types of user interfaces and controls are described herein for illustrative purposes, other types of user interfaces and controls may be used.

[0043] The embodiments discussed herein are provided by way of example, and various modifications can be made to the embodiments described herein. Certain features that are described in this disclosure in the context of separate embodiments can also be implemented in combination in a single embodiment. Conversely, various features that are described in the context of a single embodiment can be implemented in multiple embodiments separately or in various suitable subcombinations. Also, features described in connection with one combination can be excised from that combination and can be combined with other features in various combinations and subcombinations. Various features can be added to the example embodiments disclosed herein. Also, various features can be omitted from the example embodiments disclosed herein.

[0044] Similarly, while operations are depicted in the drawings or described in a particular order, the operations can be performed in a different order than shown or described. Other operations not depicted can be incorporated before, after, or simultaneously with the operations shown or described. In certain circumstances, parallel processing or multitasking can be used. Also, in some cases, the operations shown or discussed can be omitted or recombined to form various combinations and subcombinations.
Fig. 1

1. Detect Electrical Impulse
2. Detect Movement
3. Compare Movement and Electrical Impulse Data
4. Identify Irregularities
5. Identify Tremor

Tremor Detection
APPENDIX B

PUBLICATIONS

Rational Heterodoxy: Cholesterol Reformation of the Amyloid Doctrine

Review
Rational heterodoxy: Cholesterol reformation of the amyloid doctrine
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ABSTRACT
According to the amyloid cascade hypothesis, accumulation of the amyloid peptide Aβ, derived by proteolytic processing from the amyloid precursor protein (APP), is the key pathogenic trigger in Alzheimer’s disease (AD). This view has led researchers for more than two decades and continues to be the most influential model of neurodegeneration. Nevertheless, close scrutiny of the current evidence does not support a central pathogenic role for Aβ in late-onset AD. Furthermore, the amyloid cascade hypothesis lacks a theoretical foundation from which the physiological generation of Aβ can be understood, and therapeutic approaches based on its premises have failed.

We present an alternative model of neurodegeneration, in which sustained cholesterol-associated neuronal stress is the most likely pathogenic trigger in late-onset AD, directly causing oxidative stress, inflammation and tau hyperphosphorylation. In this scenario, Aβ generation is part of an APP-driven adaptive response to the initial cholesterol distress, and its accumulation is neither central to, nor a requirement for, the initiation of the disease. Our model provides a theoretical framework that places APP as a regulator of cholesterol homeostasis, accounts for the generation of Aβ in both healthy and demented brains, and provides suitable targets for therapeutic intervention.

1. Introduction
Alzheimer’s disease (AD) is the most common form of dementia in the United States, representing around eighty percent of all cases. As the country’s sixth leading cause of death, AD currently affects 5.4 million Americans, or one in eight people 65 or older (Alzheimer’s Association, 2011). With no cure or effective treatment, AD incurs an estimated annual cost of $183 billion (Alzheimer’s Association, 2011). As the US population continues to age, the number of individuals with AD is projected to increase to 7.7 million over the next twenty years (Alzheimer’s Association, 2011).

A definitive diagnosis of the disease is possible at autopsy, where specific pathological hallmarks can be visualized in the brain: extracellular senile plaques (SPs), comprised of the amyloid peptide Aβ, and neurofibrillary tangles (NFTs), made from abnormally phosphorylated tau protein. Aβ is a 38–43 amino acid peptide formed through the sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases (Hardy and Selkoe, 2002). The 40 (Aβ40) and 42 (Aβ42) amino acid forms are the most abundant, the latter having the greater tendency to aggregate (Jarrett et al., 1993).

NFTs are formed when the tau protein is abnormally phosphorylated and aggregates into paired helical fragments (Grundke-Iqbal et al., 1986). Tau is a cytoskeletal protein with a key role in axonal transport; formation of NFTs disrupts this transport and structurally disables neurons, leading to cell loss, cognitive decline, and patient death (Roy et al., 2005).

2. The amyloid cascade hypothesis
The identification of SPs and NFTs in AD led Hardy, Higgins, and Selkoe to propose the Amyloid Cascade Hypothesis. This hypothesis states that AD initiates when Aβ accumulates and aggregates to form SPs, which in turn induce the formation of NFTs and cell death, thereby causing the pathological presentation and dementia (Selkoe, 1991; Hardy and Higgins, 1992). For the past several decades, researchers have been guided by the primary prediction of this hypothesis: preventing Aβ aggregation will ameliorate or halt progression of the disease.

Supporting this hypothesis, trisomy 21 (Down syndrome) usually results in three copies of the APP gene, and consistently coincides with AD-like pathology (Masters et al., 1985). Mechanistic studies have also shown that Aβ accumulation can precede, and lead to, tau phosphorylation and aggregation into NFTs (Hardy and Selkoe, 2007; Jin et al., 2011). Additionally, rare forms of familial early-onset AD are linked to specific mutations in APP or to enzymes that promote Aβ aggregation (Hendriksen et al., 1992; Schmeier et al., 1996). Proponents of the amyloid cascade hypothesis have assumed...
that late-onset AD is mechanically identical to early-onset, consistently placing Aβ as the origin of pathogenesis in all cases.

2.1. Shortcomings of the amyloid cascade hypothesis

Conversely, there is an increasing amount of data contradicting the notion that Aβ is the key pathogenic trigger in late-onset AD, which represents the vast majority of cases (Bennouna et al., 2006). Notably, there is no significant correlation between Aβ accumulation and cognitive deterioration in either humans or in mouse models, and the appearance of SPs has been reported in the brains of approximately thirty percent of individuals with no dementia (Cryطالب et al., 1988; Price et al., 2000). Such findings have prompted some to reasonably advocate restructuring AD diagnosis to focus on symptomatic changes rather than the neuropathological identification of SPs and NFTs (Dubois et al., 2010).

Discouragingly, the clinical trials of these drugs have produced lackluster outcomes. Recent compounds targeting β-secretase, such as tarenflurbil, even when successfully reducing Aβ levels, have shown no benefit to cognitive function (Vellas, 2010). While antibodies targeted against aggregating Aβ have managed to decrease Aβ plaques, there has been no significant change in survival or cognition. NFTs have remained in plaque-free areas, and vascular deposition of amyloid, known as cerebral amyloid angiopathy (CAA), has actually increased (HOLMES et al., 2008).

Other immunotherapy treatments have caused vasogenic edema while showing no evidence of therapeutic benefit (Detlieu et al., 2001). Drugs like scyllo-inositol and homotaurine, aimed at preventing Aβ aggregates from forming into SPs, have failed to mitigate clinical AD progression (Goldie et al., 2010; Salowoy et al., 2011).

The amyloid hypothesis is also lacking at the conceptual level, due to the absence of a theoretical framework through which the physiological generation of Aβ and the mechanisms leading to its accumulation are understood. This shortcoming stems from our poor understanding of APP biology, despite the wealth of data on the cellular and molecular mechanisms of APP trafficking and processing, we do not know what the function of APP is in the brain. Until we do, we will not be able to understand why Aβ is generated in the healthy brain, let alone place it within the pathological cascade of AD. Nevertheless, current evidence argues against a central pathogenic role for Aβ in late-onset AD; this has been best summarized by Karl Herup, who recently proposed a model in which neurodegeneration begins with an initiating injury, subsequently leading to microglial dysregulation and inflammatory responses, and, eventually, resulting in a so-called "charge-of-state" in cells that primes them for neurodegeneration (Herup, 2010a). While this model does not provide specifics on the nature of an initiating injury that would be relevant to the majority of AD cases, it represents a comprehensive interpretation of available data; it identifies Aβ accumulation as a potential part of the process of microglial dysregulation and inflammation that characterizes the AD brain, while rejecting it as neither central nor a requirement for, the initiation and progression of the disease (Herup, 2010a).

3. Moving beyond the amyloid cascade hypothesis

The amyloid cascade hypothesis defines AD as a series of pathological hallmarks that are causally linked, both in familial (early-onset) and sporadic (late-onset) cases. It assumes that erasing these hallmarks will restore cognitive function. Current evidence does not support this concept for sporadic cases; rather, the data suggest that Aβ is either too peripheral or too close to the final stages of the disease to be considered the primary initiator of pathogenesis, making it of little therapeutic use. If this view is correct, then likely candidates for bona fide pathogenic triggers in late-onset AD have yet to be identified.

3.1. Cholesterol dysregulation: a common pathogenic trigger in late-onset Alzheimer's disease

It is well documented that cholesterol is a key regulator of cellular processes in the brain, including the formation of myelin sheaths, synaptic function, membrane fluidity and neurosteroid biosynthesis; cholesteryl esters is also been linked to learning and memory function in both humans and animals (Schmeur, 2010; Valdez et al., 2010).

It is noteworthy that, while physiological cholesterol synthesis rates and levels in the healthy brain decline more than 40% with age, reflecting the different needs and roles for brain cholesterol at different stages of life, this phenotype is reversed in late-onset AD (Thelen et al., 2006). Furthermore, there is a strong correlation between levels of brain cholesterol and disease severity in the AD brain (Phelix et al., 2011).

That brain cholesterol levels may evolve differently in healthy, elderly individuals and late-onset AD patients is suggestive of a causative role for cholesterol in the pathogenesis of the disease. This causative role is more clearly illustrated by several other lines of evidence. At the cellular level, cholesterol dysregulation can both precede and affect SPs and NFTs, and upregulate hallmarks of AD: The amyloidogenic processing of APP occurs in cholesterol-rich lipid rafts, and cholesterol accumulation can enlarge lipid raft size, thereby increasing Aβ generation (Burns et al., 2003; Carty et al., 2003). Supporting this concept, rabbits and APP transgenic mice fed a cholesterol-enriched diet showed increases in Aβ (Sparks et al., 2000; Shie et al., 2002; Ghebi et al., 2006). Furthermore, anomalies in cholesterol levels and distribution are able to influence the phosphorylation state of tau and create NFTs independently of Aβ (Kourkova and Kourkova, 2001; Iuel et al., 2001; Nunes et al., 2011).

There is also evidence suggesting a link between AD and apolipoprotein E (APOE), a protein that, in the brain, is involved in transporting cholesterol and other lipids (Lu, 2009). Individuals homozygous for the e4 allele of the APOE gene, which produces a protein product that is a less efficient transporter than its counterparts, are as much as 18-fold more likely to develop AD (Corder et al., 1993). More recent genetic evidence in support of cholesterol dysregulation as causative of late-onset AD has been provided by genome-wide association studies. Remarkably, in addition to APOE, four other loci are associated with cholesterol metabolism and/or transport: CIU, AIRA7, DIDK, and SIM1 (Jones et al., 2010; Hollingworth et al., 2011; Naj et al., 2011). A second associated pathway is that of endocytosis, also linked to cholesterol biology (Jones et al., 2010; Hollingworth et al., 2011; Naj et al., 2011). In that respect, PICALM is of particular interest, as it has been shown to confer risk predominantly in the presence of APOE e4 positive subjects (van et al., 2010).

Other neurodegenerative conditions can also be informative with regards to a potential causative role for cholesterol in brain pathogenesis. Of particular interest is the case of Niemann–Pick type C disease (NPC). In NPC, mutations in one of two genes,
Fig. 1. Cholesterol distress, rather than AP accumulation, is the more likely candidate for a long-fide pathogenic trigger in most cases of late-onset AD. Cholesterol distress is defined as some combination of abnormal amounts of cholesterol or its precursors and metabolites, abnormal subcellular localization, or oxidation state (green arrow 1a), and is primarily caused by multiple factors, including age, metabolic stress, and gene polymorphisms. In turn, cholesterol distress prompts activation of an APP-driven adaptive response (1b). If the adaptive response from APP is insufficient, cholesterol distress can result in cell dysfunction (red arrow 2a). Cell dysfunction can be defined by the presence of major cellular damage as a consequence of altered transport regulation, tau phosphorylation and aggregation, inflammatory response, reactive oxygen species, and neuronal commitment to aberrant entry into the cell cycle. Cell dysfunction can in turn affect cholesterol regulation via a mechanism mostly driven by inflammation (red arrow 2b). Note that the pathogenic cascade only initiates after cell dysfunction, leading to lysosomal collapse, loss of dendritic mass, and neuronal destruction that results in dementia and death (pathway defined by dark red arrow 4). AP oligomers and plaques form when there is an excessive defensive response from APP to chronic cholesterol distress. In a brain where cell dysfunction is already ongoing (i.e., within the pathogenic cascade leading to dementia), dark red arrow 4), excess AP affects already vulnerable neurons, further inducing inflammatory responses, extending oxidative damage, and contributing to tau/hyperphosphorylation and aggregation. Vascular AP deposition would be
NPC1 and NPC2, results in cholesterol and other lipids accumulating in late endosomes and lysosomes (Vance, 2006; Lloyd-Evans and Hatzi, 2010). In common with AD, APP is disproportionately metabolized to Aβ in NPC, even forming AD-like plaques in patients carrying the ApoE4 allele; tau has been observed both in early stages of hyperphosphorylation and as NFTs that are indistinguishable from those appearing in AD brains (Auer et al., 1995; Sato et al., 2002). NFTs are also more readily detectable in cell populations most affected by cholesterol accumulation, often forming without any prior deposition of Aβ into plaques, indicative of a direct causative link between cholesterol dysregulation and NFT formation (Lowe et al., 1995).

In addition, increased expression of NPC1 has been reported in cortex and hippocampus from AD patients, and some NPC1 polymorphisms have been linked to increased risk of developing AD (Kajedal et al., 2010; Rodríguez-Krížan et al., 2010). Finally, loss of one copy of the NPC1 gene in AD mice harboring PS1 and APP mutations leads to accelerated Aβ accumulation, compared to control mice with two copies of NPC1 (Borbon and Erickson, 2011).

In summary, cholesterol is causally linked to neurodegeneration in late-onset AD at several levels. Besides its direct impact on amyloidogenesis and tau aggregation, a significant number of genes involved in cholesterol homeostasis are associated with AD, and other neurodegenerative conditions such as NPC provide formal proof that cholesterol dysregulation precedes, and leads to, Aβ and tau abnormalities.

3.2. Dysregulation of APP as a key contributor to cholesterol-driven pathogenesis in late-onset AD

The biological function of APP in the brain, in particular with regards to the physiological generation of Aβ and the mechanisms leading to its accumulation, are not well understood; this shortcoming weakens the theoretical persuasiveness of the amyloid cascade hypothesis. At the same time, it hinders the progress of alternative views that better explain the role of APP and Aβ in health and disease.

We suggest a close look at the evidence available indicates a potential role for APP as a regulator of cholesterol homeostasis in the brain through mechanisms including, but not limited to, Aβ generation. The evidence in support of APP as a cholesterol regulator in the brain is multifaceted. In a mouse model of NPC that faithfully recreates the cholesterol accumulation and disease phenotype observed in human patients, loss of APP leads to an exacerbation of the cholesterol abnormalities seen in NPC brains, which in turn leads to accelerated progression of the disease (Trotter-Held et al., 2003; Nunes et al., 2011). Furthermore, a comparison of gene expression profiles in pre-symptomatic cerebella of control mice to NPC mice with and without APP reveals that the latter is necessary for appropriate cholesterol synthesis in the post-mevalonate pathway; cholesterol transport; myelination, and cholesterol regulation through modification of hindbrain deacetylation (Castello and Soriano, unpublished).

The cleavage products of APP have also been shown to affect cholesterol synthesis and transport directly: Aβ acts to reduce the size of lipid rafts and decreases cholesterol synthesis by inhibiting the rate-limiting enzyme, HMGCra reductase (HMGR; Grimm et al., 2005, 2007). The remaining intracellular domain (AICD) acts as a transcription factor to suppress production of the cholesterol transporter LRPI (Liu et al., 2007). Furthermore, Aβ production is increased in response to 27-hydroxycholesterol, an oxidized, neurototic form of cholesterol that is increased in AD brains (Prasanthi et al., 2011; Shafqat et al., 2011). Finally, it is noteworthy that APP contains a cholesterol-sensing domain shown to be functional in vitro, thereby providing a potential molecular mechanism by which APP could be involved in regulation of brain cholesterol through HMGR, LRPI, and other cholesterol-associated pathways (Castello and Soriano, unpublished; Ble et al., 2008, 2010; Barrett et al., 2012).

4. A new focus: the cholesterol hypothesis of neurodegeneration

The fact that cholesterol dysregulation is causally linked to pathogenesis in the AD brain and that APP may function as a regulator of cholesterol homeostasis lead us to propose a novel model of neurodegeneration. In this model, outlined in Fig. 1, sustained cholesterol distress, rather than Aβ accumulation, is the more likely candidate for beta-amyloid pathogenic trigger in most cases of late-onset AD.

We define cholesterol distress as some combination of abnormal amounts of cholesterol or its precursors and metabolites, aberrant subcellular localization, or oxidation state, all of which are involved in the pathogenesis of neurodegeneration (Fig. 1, green arrow 1a) (Kirsch et al., 2003; Mattson, 2009; van den Kommer et al., 2009; Liu et al., 2012a; Prasanthi et al., 2011; Stranahan et al., 2011; Xu et al., 2011; Sato et al., 2012). This cholesterol distress is primarily caused by multiple factors, including aging, metabolic stress, and gene polymorphisms (top of Fig. 1) (Kirsch et al., 2003; Mattson, 2009; Liu et al., 2012a; Suzuki et al., 2010; Prasanthi et al., 2011; Stranahan et al., 2011; Xu et al., 2011; Sato et al., 2012; Stranahan and Mattson, 2012). In turn, cholesterol distress prompts activation of an APP-driven adaptive response, through a mechanism involving, in part, the generation of Aβ (Fig. 1, green arrow 1b). The view of Aβ as a protective molecule in the brain has been proposed before, perhaps most vocally and systematically by Smith et al., who have argued that Aβ accumulation is a byproduct of upstream pathogenic events and instigates as a protective response to neuronal insult (Castello et al., 2008). Such a notion is also consistent with data showing that Aβ is neuroprotective at physiological concentrations and that Aβ can be generated in response to acute damage caused by ischemia and head trauma (Whitson et al., 1989; Fössl et al., 2010). Nevertheless, the nature of the stress stimuli that would initiate an Aβ response in late-onset AD remains unclear. Smith et al. have proposed that the underlying stress is of an energetic nature through mechanisms involving oxidative stress (Lee et al., 2007). In the model we present here, APP is a key part of the protective response against cholesterol distress via HMGR regulation of HMGR and LRPI, and potentially other mechanisms involving cholesterol synthesis, transport or hoxone deacetylation regulation (Castello and Soriano, unpublished; Grimm et al., 2005, 2007; Grisken et al., 2010).

If the adaptive response from APP is insufficient, cholesterol distress can result in cell dysfunction. Cell dysfunction can be defined by the presence of major cellular damage as a consequence of axonal transport disruption, tau phosphorylation and aggregation, inflammatory response, reactive oxygen species, and neuronal commitment to aberrant entry into the cell cycle – all of which have been shown to occur as a consequence of cholesterol distress (Fig. 1, red arrow 2a). (Koulakov and Koulakov, 2001;
Of particular interest is the link between cholesterol and the neuronal cell cycle, an early event in AD known to be a cause, rather than a consequence, of neurodegeneration (Carras et al., 2009; Herrup, 2010b). Disruption of cell cycle reentry genes has been reported in young adults homozygous for the ε4 allele of the APOE gene (Conjeero-Goldberg et al., 2011; Nunes et al., 2011; Stanahan et al., 2011). A plausible mechanism for cholesterol-induced cell cycle reentry in AD involves miR-33, a microRNA encoded within SREBP-2, which is involved in the regulation of cholesterol synthesis and uptake, as well as the expression of cell cycle genes -- including cyclin D1 (Cirera-Salinas et al., 2012; Mandas et al., 2012). Specifically, downregulation of miR-33 leads to increased cyclin D1 levels, and could conceivably result in new reentry into the cell cycle (Cirera-Salinas et al., 2012; Cirera-Salinas et al., 2012). SREBP-2 is downregulated in AD, and since miR-33 levels mimic those of SREBP-2, the resulting increase in cyclin D1 would contribute to an aberrant entry into the cell cycle (Sommer and MacDougall, 2011; Mandas et al., 2012).

Cell dysfunction can in turn affect cholesterol regulation via mechanisms mostly driven by inflammation (Fig. 1, red arrow 2b) (Block and Hong, 2005). Note also that the pathogenic cascade only initiates after cell dysfunction, leading to synaptic collapse, loss of dendritic mass, and neuronal destruction that results in dementia and death (Fig. 1, pathway defined by dark red arrows) (Ylijalkas and de Braeherder, 2002; Avendt, 2009).

Our model accounts for Aβ oligomers and plaques forming when there is an excessive defensive response from APP to chronic cholesterol distress (Fig. 1, red arrow 3). In a brain where cell dysfunction is already ongoing (i.e. within the pathogenic cascade leading to dementia: dark red arrow 4 in Fig. 1), excess Aβ affects already vulnerable neurons, further inducing inflammatory responses, extending oxidative damage, and contributing to tau hyperphosphorylation and aggregation (Morgan, 2009). Vascular Aβ deposition would be prominent at this stage, contributing to cerebral amyloid angiopathy and microbleeds, exacerbating the pathogenic cascade (Fig. 1, pathway defined by dark red arrows).

Where plaques are present in cognitively unaffected individuals, an excessive defensive response from APP has generated a large amount of Aβ, while successfully defending against cholesterol distress. In this case, cell dysfunction is absent and the formation of Aβ oligomers and plaques do not have an obvious impact on neuronal function (not shown in Fig. 1).

In early-onset AD and Down syndrome are fundamentally different from late-onset AD. In the former two cases, Aβ is constitutively overproduced throughout life (or the AβAPP/β ratio is significantly higher), in the absence of cholesterol distress. In such instances, the protective role of APP may be overwhelmed in the absence of Aβ, and the sustained overexpression of Aβ accelerates the rate at which cell dysfunction occurs, through a combination of a weaker adaptive response (represented by green arrow 1b in Fig. 1) and an accelerated rate at which cell dysfunction occurs (represented by red arrow 2a in Fig. 1). Neurodegeneration is further exacerbated by the impact of a higher Aβ load (red arrow 3 in Fig. 1) on the pathogenic cascade (represented by dark red arrow 4 in Fig. 1).

The opposite situation occurs in the case of the recently reported rare mutation within the APP gene, A677T (Jonsson et al., 2012). This mutation is less common in AD patients than in non-demented individuals, and elderly carriers perform better in cognitive tests than do non-carriers. Although the impact of this mutation on the APP and cholesterol metabolism profile in the brain is unknown, amyloidogenesis from A677T APP is significantly reduced in cultured cells, whereas α-secretase cleavage is modestly decreased. These findings are also best accommodated within our model. In mutant carriers, Aβ is underproduced throughout life, while preserving the overall function of APP within the adaptive response to cholesterol distress. As a consequence, there is no Aβ excess secondary to a sustained adaptive response (illustrated in red arrow 3, Fig. 1), and the potential contribution of Aβ to the pathogenic cascade (dark red arrow 4 in Fig. 1) and CAA (loop 5 in Fig. 1) is drastically reduced, accounting for the slower rate of neurodegeneration and the delay in age of onset, as reported. Note also that the A677T mutation creating a stronger adaptive response is also consistent with the superior performance in cognitive tests of patients with the mutation, who retain cognitive ability for a longer period of time even when they develop AD.

4.1. Broader applications of the cholesterol hypothesis

The status of cholesterol as a critical functional element in the brain predicts that it should also emerge as a risk or causative factor for neurodegenerative conditions other than AD, and the current evidence supports that prediction. In Parkinson’s disease (PD) and the related Lewy body dementia, for example, there is a progressive degeneration of dopaminergic neurons, accompanied by intraneuronal deposition of α-synuclein (α-syn) (Galpern and Lang, 2006). Increases in cholesterol metabolites have been found in these cells, which, in vitro, promote α-syn aggregation (Bonco et al., 2006; Barceiz-Cohijn et al., 2007). As is the case with APP, α-syn is able to associate with the cholesterol-containing lipid rafts, and is upregulated in the brain in response to 27-hydroxysterol cholesterol (suggesting that it may be part of an adaptive response to cholesterol distress analogous to that occurring in AD (Fortin et al., 2004; Bar-On et al., 2008).

Other diseases that have been proposed to be causally linked to cholesterol distress are Huntington’s disease, schizophrenia, and autism spectrum disorder (ASD) (del Toro et al., 2010; Ota et al., 2011; Seneff et al., 2011). Interestingly, ASD has a high coincidence with Smith-Lemli-Opitz, a direct consequence of a defect in cholesterol synthesis (Slikora et al., 2006).

Finally, in support of cholesterol involvement in a variety of neurodegenerative conditions, ApoE, one of the critical genetic risk factors for AD, also appears as a risk or exacerbating factor for Parkinson’s disease, cerebral amyloid angiopathy, tauopathies and other dementias, and even multiple sclerosis (Greenberg et al., 1995; Josephs et al., 2004; Masters and Hilbert, 2004; Martinez et al., 2005).

In short, there appear to be many instances of neurodegeneration that initiate or interface with a failure of cholesterol regulation. Applying our model, molecules such as α-syn, histin, and others yet to be discovered would fill the role of APP and Aβ as part of an adaptive response analogous to that proposed for AD. An excessive response to correct cholesterol imbalances would result in protein aggregation, with phenotypic differences determined by the type and nature of cholesterol stress and the adaptive response available in susceptible neuron populations.

5. Conclusions: a new direction for neurodegenerative research

In 2011, President Barack Obama signed the National Alzheimer’s Prevention Act, legislation that aims to establish a coordinated plan to overcome the AD crisis in the United States (Baeh, 2011). Completion of such a plan is timely manner requires us to widen the scope of inquiry. Even though current evidence does not support a role for Aβ as an initiators of pathogenesis in late-onset AD, debate continues unabated on the finer details of Aβ pathology, diverting resources from broad, comprehensive hypotheses that fit more strongly with the available data (Karran et al., 2011; Lemkova et al., 2012).
We have provided evidence that cholesterol disturbances is likely to be a common early pathogenic factor in late-onset AD and a better target for intervention than AB. From a therapeutic standpoint, the presence of atherosclerosis in patients with cholesterol disturbances can occur in many individuals with high cholesterol levels, these drugs will not work for patients for whom cholesterol disturbances is not specifically linked to increased cholesterol levels – a conclusion supported by current studies of the NHANES-CFN study. As with cancer treatments, the research and pharmaceutical companies will need to develop cost-effective methods to test for a spectrum of cholesterol abnormalities, including levels, trafficking, oxidation, and synthesis that will form the basis of patient-specific medical care.

References


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On the origin of Alzheimer’s disease. Trials and tribulations of the amyloid hypothesis

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The amyloid cascade hypothesis, which implicates the amyloid Aβ peptide as the pathological initiator of both familial and sporadic, late onset Alzheimer’s disease (AD), continues to guide the majority of research. We believe that current evidence does not support the amyloid cascade hypothesis for late onset AD. Instead, we propose that Aβ is a key regulator of brain homeostasis. During AD, while Aβ accumulation may occur in the long term in parallel with disease progression, it does not contribute to primary pathogenesis. This view predicts that amyloid-centric therapies will continue to fail, and that progress in developing successful alternative therapies for AD will slow until closer attention is paid to understanding the physiological function of Aβ and its precursor protein, Aβ.

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Recently the Alzheimer’s Association held its 25th annual conference in Boston, Massachusetts. Despite new knowledge about the mechanisms affecting aging and cognitive decline in general, it was striking to note how little progress has been made over the past quarter century regarding our understanding of Alzheimer’s disease (AD). The overwhelming majority of research in the field has been driven by the premises of the amyloid hypothesis, which has held a firm grip on the field since AD research began gaining momentum in the 90s. The amyloid hypothesis states that the pathogenic cascade of AD initiates upon accumulation, oligomerization, and aggregation of the amyloid-beta peptide (Aβ) in extracellular deposits termed senile plaques. This accumulation subsequently promotes the hyperphosphorylation of tau protein, leading to neurofibrillary tangles and neurodegeneration. The tenets of the amyloid hypothesis are largely based on the existence of rare autonomic dominant, early onset forms of the disease (FAD), all of which involve mutations that affect APP processing and result in increased production and aggregation of Aβ. Based on these observations, the hypothesis assumes that preventing, halting, or reversing this process will cure the disease (Karran et al., 2011). As we have previously discussed in detail, current evidence does not support this view for the sporadic, late onset form of AD currently affecting five million Americans over age 65 (Alzheimer’s Association, 2013; Castello and Soriano, 2013). Importantly, therapeutic approaches based on an amyloid-centric perspective have had negligible effect on cognitive functioning and in some cases have even accelerated disease progression (Alsen et al., 2013; Benilova et al., 2012; Karran et al., 2011; Mullard, 2012; Sperling et al., 2011).

Similar arguments have been offered for a tau hypothesis of AD, whose main tenet is that, regardless of Aβ status, hyperphosphorylated tau protein is the bona fide pathogenic trigger of the disease, perhaps by acting as a prion protein to spread its aggregation-prone structure from neuron to neuron (Brink and Del Tredici, 2011). There has been a rush to develop antibodies to aggressively remove “toxic” tau, in the hope that by doing so AD progression will be slowed or halted (Yamananda et al., 2013). Although the impact of tau dysregulation on neuronal function is beyond doubt, the more pertinent observation is that tau aggregation occurs very late in the pathological process of AD, where intervention will be largely ineffective. For a tau hypothesis to be valid, one would need to assume, against all the evidence, that tau dysregulation is both the main disease-causing process and that it occurs sufficiently early in the disease to be of therapeutic value.

The key flaw of the amyloid hypothesis is its assumption that FAD represents an accelerated model of AD pathology, when in fact it is more likely that FAD represents a subset of AD presentations. To begin to understand this distinction we must consider first that Aβ plays a physiological role that is susceptible to dysregulation. As is the case with inflammation or cell division, Aβ production can be both necessary and harmful, and the logical way for potential harm to be amissed is to strive to understand its purpose. This idea is highly intuitive and, indeed, John Hardy, one of the initial proponents of the amyloid hypothesis, has recently stated, in the context of the slow progress in the field, that “If I were a young researcher
now, I would try to work out what the function of APP is” (Hardy and Mayer, 2011). Following that line of reasoning, understanding the biology of APP and Aβ in the brain leads to a broad vision of their role in neurodegeneration that is relevant to AD. That vision can be understood in the context of hormesis, as recently described by Stranahan and Mattson (2012). They have examined the potential mechanisms by which “common sense” interventions, such as exercise, aid successful brain aging, and propose that the brain benefits greatly from hormesis—low levels of cognitive or physical stress that elicit a response from biological repair systems. Stimulating the brain’s adaptive response mechanisms promotes restorative activity that otherwise would not occur.

Expanding on the sound reasoning of this adaptive response hypothesis, APP and its metabolites, including Aβ, can be considered as potential components of the brain’s adaptive response to stress. A solid body of evidence supports such an adaptive response hypothesis, including the work of Perry, Smith, and Castellani (Castellani and Smith, 2011; Castellani et al., 2009; Castello and Serrano, 2013). The presence of excessive Aβ in this scenario is evidence of an ongoing stress process, rather than a marker of disease initiation. In further support of this view, acute stress such as traumatic brain injury causes an increase in Aβ production that is inversely correlated with hyperphosphorylated tau and signs of neuronal injury (Magoun et al., 2012; Tsitsopoulou and Marklund, 2013). A similar effect is observed after ischemia, possibly in response to high oxidative stress (Plata et al., 2013). Even low levels of stress can promote a response from APP, with synaptic activity producing Aβ, and APP itself aiding in plasticity (Bero et al., 2011; Cirrito et al., 2005; Hoe et al., 2009). Other stressors likely include metabolism and cholesterol regulation, which would account for the increased risk of AD in individuals with diabetes or the ε4 allele of the cholesterol transporter APOE (Akkar et al., 2010; Hölscher, 2011). Diabetes symptomatology significantly accelerates both Aβ and tau pathology in a mouse model of aging, while systematic cholesterol dysregulation directly alters tau in the absence of APP (Corrao et al., 2012; Nunes et al., 2013).

We argue that, in the brain, Aβ is part of an adaptive response to both acute and chronic stress stimuli that may eventually lead to the development of AD. This view stands in fundamental contrast with the disease-causing role for Aβ proposed by the amyloid hypothesis, illustrated by Fig. 1. According to the amyloid hypothesis, Aβ is a toxic, misfolded peptide that needs to be maintained at low levels to avoid impaired brain homeostasis and neurodegeneration that are causative of disease. According to our vision of the adaptive response hypothesis, Aβ is part of a defense mechanism (represented by a shield in Fig. 1) that supports appropriate brain homeostasis, and its accumulation may occur over the long term in parallel with disease progression as part of its involvement in the adaptive response; however, such accumulation does not contribute to primary pathogenesis. Note also that our view of the role of Aβ in health and disease does not require a correlation between Aβ accumulation and cognitive status. This is consistent with evidence showing the presence of excess Aβ in cognitively functional individuals and, conversely, the absence of Aβ accumulation in cases that would otherwise be classified as dementia of the AD type (Decas et al., 2012; Petrovitch et al., 2001; Price et al., 2009).

It is discouraging that, despite the experimental and conceptual shortcomings of the amyloid hypothesis, as well as the multiple clinical failures associated with it, the field appears reluctant to re-evaluate its premises. Instead, the goals of research have been shifted to include the concept of “practical” Alzheimer’s disease, an assumption of Aβ accumulation and aggregation without corresponding evidence for neurodegeneration or even detectable cognitive symptoms (Gold et al., 2011). This notion is the primary intellectual justification for treatment with anti-Aβ therapy in the absence of pathological evidence. In our view, the obvious fact that brain pathology can advance as people age is not logically followed by the notion that Aβ is the causative agent of such pathology. The scientific method requires the critical evaluation and testing of hypotheses, and subsequent rejection of those that do not fit the evidence. However, it seems as if the reverse argument prevails in the field of AD, where it appears that, if the evidence does not fit the hypothesis, it follows that the evidence must be wrong. This abandonment of the scientific method led George Perry and the late Mark Smith to, at one point, describe the field as the “Church of the Holy Amyloid” in an indictment that remains relevant over a decade later (Joseph et al., 2001).

Several clinical trials are underway that use current Aβ-modifying strategies on pre-symptomatic individuals at high risk
of developing AD later in life, and their outcomes may shed light on the validity of our views on the role of Aβ in neurodegeneration. The Alzheimer’s Prevention Initiative APOE4 Trial, recently funded by NIH, will test anti-Aβ drugs in cognitively normal vol-
teers carrying two e4 alleles of the cholesterol transporter ApoE, 
a genotype that confers an 18-fold increase in AD risk (Corder et al. 1993). If the adaptive response hypothesis is accurate and Aβ supports brain homeostasis, the combination of Aβ depletion and the presence of the stress stimuli associated with APoE e4 (likely derived from impaired cholesterol homeostasis) could lead to accelerated neurodegeneration. In the landmark California clin-
tical trial, a collaborative effort involving Cenestech, the Banner Alzheimer’s Institute and NIH, participants with a FAD genotype (in this case, mutations in the presenilin-1 gene) will be treated with anti-Aβ drugs beginning in their 30s. Unregulated, constitutive aberrant expression of Aβ leaves these individuals with a deficient adaptive response to stress stimuli throughout their lives. Rather than preventing the development of AD, drug treatments will result in depletion of brain Aβ, leading to a similar outcome in which the brain is left with a poor adaptive response to chronic stress. Given the current data from genome-wide association studies, which show that cholesterol, endocytosis and immune response pathways may be most commonly associated to the etiology of late onset AD, it is likely that deregulation of these pathways may be accelerated in these individuals (Hollingsworth et al. 2011; Raj et al. 2011).

In summary, after twenty-five years of exhaustive research, it is increasingly obvious that accumulation of Aβ is neither necessary or sufficient to initiate pathogenesis in sporadic Alzheimer’s disease. We must heed that evidence and accept that the amyloid hypothesis has run its course and does not work at the bench nor at the clinic, it is intellectually flawed, and it has become a sink for valuable resources that could be better spent elsewhere.

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Filipin Levels as Potential Predictors of Alzheimer’s Disease Risk

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Abstract

To date, therapies to prevent or treat Alzheimer’s disease (AD) have largely focused on removing excess aggregation-prone amyloid peptide Aβ from the brain, an approach that has produced disappointing clinical outcomes. An alternative hypothesis proposes that Aβ production and aggregation is a symptom of a larger, systemic disease affecting the regulation of lipids, including cholesterol. In this scenario, lipid dysregulation would likely occur early in the disease process, making it an ideal target for predicting risk of mild cognitive impairment (MCI) to AD conversion. Here, we report that levels of filipin, a fluorescent polyelectrolyte macrolide widely used as a diagnostic tool for diseases of lipid dysregulation, correlate with cellular damage caused by 27-hydroxycholesterol and with dementia status in human peripheral blood cells. These results provide strong preliminary data suggesting that filipin could be of use in the development of a quick and inexpensive method to measure the risk of AD conversion in patients with MCI, complementing existing testing strategies that focus on the consequences of Aβ accumulation.

Keywords

Alzheimer’s Disease, Lipid Dysregulation, Cholesterol, Mild Cognitive Impairment, Filipin Levels

1. Introduction

Alzheimer’s disease (AD) is the most common form of dementia in the United States, representing around eighty percent of all cases. As the country’s sixth leading cause of death, AD currently affects 5.4 million Americans and is projected to increase to 7.7 million over the next several decades [1]. Conclusive diagnosis of AD is made post mortem, when specific pathological hallmarks are visualized in the brain: extracellular senile plaques (SPs), comprised of the amyloid-beta peptide (Aβ), and neurofibrillary tangles (NFTs), made from abnormally phos-
phosphorylated tau protein. To date, AD research and therapy has largely focused on removing excess aggregation-prone Aβ from the brain. However, while numerous drugs have successfully reduced the burden of Aβ in the brain by removing it or preventing its aggregation, they have failed to produce significant clinical improvements [2]. These lackluster outcomes have added weight to criticism from a growing number of scientists casting doubt on the validity of the amyloid hypothesis [3] [4].

One alternative that has been proposed by ourselves and others suggests that Aβ production and aggregation is a symptom of a larger, systemic disease that affects the exquisitely-regulated balance of lipids in the brain, including cholesterol [5]. Genome-wide association studies (GWAS), as well as epidemiological and biochemical evidence, place cholesterol regulation as a likely early pathogenic trigger of late onset Alzheimer’s disease, while biochemical and histochemical studies from human autopsy brains clearly show evidence for widespread brain cholesterol dysregulation in late onset AD [6]-[9]. Of particular interest is the presence of high levels of the side-chain oxidized form of cholesterol, 27-hydroxycholesterol (27-OHC), in the AD brain. Unlike cholesterol, 27-OHC can cross the blood brain barrier, providing a mechanism to account for the link between hypercholesterolemia and the risk of AD [10].

Studies using fibroblasts and peripheral blood cells from AD patients have added weight to this argument by supplying histological and biochemical evidence of lipid dysregulation taking place in those cells [11] [12]. At the same time, APP and Aβ production has been shown to be influenced by sterols including 27-OHC [13] [16]. If dysregulation begins at presymptomatic stages of AD, and its severity increases with disease progression, it might be possible to differentiate mild cognitive impairment (MCI) sufferers according to their risk of conversion into AD by monitoring dysregulation of cholesterol or other lipids in peripheral cells. Ideally, one would want an inexpensive test that can provide quick quantification of cholesterol dysregulation using a system amenable to high throughput approaches.

Here, we report that filipin, a polycyclic macrolide widely used as a diagnostic tool for diseases of lipid dysregulation, such as Niemann-Pick type C disease (NPC), can be of potential use to develop such a test [17] [18]. We show that filipin fluorescence, as quantified by flow cytometry, can be measured in human peripheral blood mononuclear cells (PBMCs) and that its levels correlate with dementia status. These results provide strong support for larger clinical studies to test whether our filipin-based method can in fact predict the risk of AD conversion in MCI patients, an approach that would supplement existing strategies that focus on the consequences of Aβ accumulation.

2. Materials and Methods

2.1. Reagents

U18666A (Sigma-Aldrich, St. Louis, MO) was stored in aliquots at -80°C as an aqueous solution at a concentration of 4.7163 M (2 mg/ml). Working concentrations were prepared in culture medium immediately prior to experiments.

24-hydroxycholesterol (24-OHC) and 27-hydroxycholesterol (27-OHC) (Cayman Chemical, Ann Arbor, MI) were prepared as 10 mM stock solutions in 100% ethanol (Sigma-Aldrich, St. Louis, MO) and stored in aliquots at –80°C. Working concentrations were prepared in culture medium immediately prior to experiments.

Except where otherwise noted, cells were treated with media containing 5 mM U18666A, 10 μM 24-OHC, or 10 μM 27-OHC and incubated for 16 hours at 37°C.

2.2. Cell Culture and Fluorescence Microscopy

B-lymphocytes were obtained from Coriell cell repository (Camden, NJ, Table 1) and cultured in RPMI (Life Technologies, Carlsbad, CA) with 15% FBS (Atlanta Biologicals, Flowery Branch, GA) and 1% pen/strep (Life Technologies, Carlsbad, CA). According to information available from Coriell, AD patients were not known to be homozygous for ApoE4.

SH-SY5Y human neuroblastoma cells (CRL-2266, American Type Culture Collection) were cultured in DME (Life Technologies, Carlsbad, CA) with 15% FBS and 1% pen/strep. For neuronal differentiation, cells were plated in 6-well plates at 400,000 cells/well (day 1). After 24 hrs (day 2), media was changed to DMEM with 3.75% FBS, 1% pen/strep, and 10 μM retinoic acid (RA, Sigma-Aldrich, St. Louis, MO). On day 5, media was discarded and replaced with fresh DMEM, again with 3.75% FBS, 1% pen/strep, and 10 μM RA. On day 6,
media was changed to DMEM without FBS, and containing 10 μM RA with 50 ng/ml of brain-derived neurotrophic factor (BDNF, Sigma-Aldrich, St. Louis, MO). Differentiated cells were experimentally treated on day 8.

Peripheral blood mononuclear cells (PBMCs) from adult donors were obtained from Leuko pak leukocyte filters (Fenwal Laboratories, Lake Zurich, IL) and donated by the Blood Processing and Quality Control Life- stream (San Bernardino, CA). Ficol Hysapc (GE Healthcare, Pittsburgh, PA) and red blood cell lysis density gradient centrifugation were used for PBMC isolation exactly as described [19].

2.3. Flow Cytometry

For FACS analysis, cells were fixed in paraformaldehyde (PFA) at room temperature (RT; peripheral blood cells, 1% PFA 30 min at RT; B-lymphocytes, 4% PFA 1 hr at RT) and treated with filipin for 30 min at RT (peripheral blood cells, 50 μg/ml filipin; B-lymphocytes, 100 μg/ml filipin). Flow cytometry was performed using a MACS Quant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) with excitation at 350 nm. Mean fluorescence intensity was calculated with FlowJo software (TreeStar, Inc., Ashland, OR). Average percentage of filipin-positive to total number of cells was determined in FlowJo using filipin fluorescence in cells from control patients (B-lymphocytes) or vehicle-treated cells (SH-SY5Y and PBMCs) to determine cut-offs (as described in Figure S1). Statistical analysis of the percentages was performed in SPSS Statistics (version 22, IBM Corp.) using one-way ANOVA and Bonferroni post-hoc analysis.

3. Results

To assess the impact of cholesterol dysregulation on cytotoxicity, we treated neuron-differentiated SH-SY5Y cells with side-chain oxidized cholesterol forms 24-OHC or 27-OHC, and measured extracellular lactate dehydrogenase (LDH) as an indicator of cell damage. In addition, we treated cells with U18666A, a compound that inhibits cholesterol synthesis and intracellular trafficking and leads to apoptosis in a variety of cell lines, including neurons, thus serving as positive control for cholesterol-associated cytotoxicity [20] [21].

As shown in Figure 1(a), treatment of cells with 27-OHC, but not 24-OHC, led to significantly higher levels of LDH release than in untreated control cells, in agreement with previous work from the Ghribi laboratory [15]. Note also that the cytotoxic impact of 27-OHC was comparable to that seen in cells treated with U18666A.

Next, we assessed the correlation between the cellular damage, as detected by LDH release, and levels of filipin fluorescence. Neuron-differentiated SH-SY5Y cells were treated as above with 24-OHC, 27-OHC, or U18666A, and fluorescence from filipin was measured by fluorescence-activated cell sorting (FACS). As seen in Figure 1(b), 27-OHC, but not 24-OHC, led to a statistically significant increase in filipin staining that was comparable to the impact of U18666A. This differential result indicates that, in our SH-SY5Y neuronal model, filipin fluorescence does not correlate with overall levels of oxidized cholesterol, because 24-OHC does not lead to significant changes in filipin signal. Rather, filipin fluorescence correlates with the cytotoxicity status that accompanies the changes in subcellular distribution of the lipids that it detects [22]. This correlation is consistent with the idea of
cholesterol dysregulation contributing to early pathogenesis in late-onset AD, as we have proposed [5]. Further supporting this correlation, Pani et al. have shown that skin fibroblasts from AD patients display higher levels of filipin fluorescence [11], a result that we have confirmed in our laboratory (not shown).

Overall, these results suggest that there is a continuum of filipin-sensitive cell damage that expands incrementally between healthy cognition and severe dementia. If that were the case, it might be possible to design a method to differentiate MCI sufferers according to their risk of conversion into AD by monitoring intracellular filipin levels. To be of clinical value, such a method would have to be quick and inexpensive and involve minimal risk to the patient. Thus, the use of fibroblasts is not optimal, as it involves invasive punch biopsies that are not a standard part of clinical care. In contrast, if the use of filipin could be applied to blood cells, the clinical application of our findings could be explored. To test that idea, we first determined whether filipin fluorescence can be detected in peripheral blood mononuclear cells (PBMCs) and whether it displays a range of levels that correspond to cholesterol dysregulation, as we found with SH-SY5Y cells (Figure 1). To that end, we treated them with U18666A, widely used in vitro to generate an NPC-like aberrant cholesterol phenotype [20] [21]. Figure 2 shows a dose-dependent effect of U18666A on filipin staining in PBMCs, indicating that dysfunction of cholesterol metabolism can occur in PBMCs and can be measured by filipin fluorescence.

Having determined that filipin-sensitive cholesterol dysregulation occurs in blood cells, we asked whether differences could exist between AD patients and cognitively healthy controls. In this case, we chose to study B-lymphocytes because they are commercially available (Coriell Cell Repositories, Camden, NJ), and allow for quick confirmation of the feasibility of a filipin-based test in blood cells to determine cognitive status as well as the potential risk of MCI to AD conversion. As shown in Figure 3, when compared to controls, B-lymphocytes from AD patients show a significantly larger proportion of filipin-positive cells (3A). In addition to measuring population differences between groups, we also determined the average mean fluorescence intensity in individual cells, as a measure of the severity of intracellular abnormalities. As shown in Figure 3(b), the average is significantly higher in AD cells, indicating that B-lymphocytes from AD patients not only show higher numbers of filipin-positive cells, but cells also display greater severity of intracellular, filipin-measurable abnormalities.

4. Discussion

We have recently proposed an Adaptive Response Hypothesis of neurodegeneration to account for the etiology of late onset Alzheimer’s disease [5]. Our hypothesis states that familial and late onset forms of the disease represent ecologically different subsets of dementia and, furthermore, that dysregulation of lipids, including aberrant cholesterol oxidation patterns, is a key early pathogenic trigger of the late onset form of AD. Such dysregulation would begin at presymptomatic stages of AD, and its severity would increase with disease progression such that increasing levels of dysregulation would correlate with a worsening of cognitive status. Here, we rea-
Cholesterol Dysregulation in Human PBMCs

![Graph showing cholesterol dysregulation in human PBMCs.]

Figure 2. Human peripheral blood mononuclear cells (PBMCs) treated with increasing concentrations of U18666A to induce lipid dysregulation also show a dose-dependent increase in the percentage of filipin-positive cells, indicating that PBMCs are also sensitive to filipin-sensitive dysregulation (one-way ANOVA, p = 0.03).

![Graph showing filipin-positive B-lymphocytes.]

Figure 3. (a) Flow cytometry analysis of B-lymphocytes from control and Alzheimer’s disease (AD) patients shows higher filipin fluorescence in AD cells (two-tailed t-test, p = 0.01). (b) Mean filipin fluorescence intensity of B-lymphocytes is also significantly increased in AD patients compared to control (two-tailed t-test, p = 0.02). Details on the control and AD cells used for these experiments are contained in Table 1.

It is noted that filipin, which is already in use as a diagnostic tool in fibroblasts for other lipid dysregulation diseases, such as NPC [18], could be used to test whether such correlation exists in AD using blood cells, with the ultimate goal of designing a method of predictive value in assessing the risk of MCI to AD conversion. Our results presented here are consistent with that view, showing that the presence of intracellular abnormalities, as detected by filipin fluorescence, correlates with cellular damage (Figure 1) and is a part of AD pathology that can be readily detected in B-lymphocytes (Figure 3).

The fact that cholesterol-associated cellular damage can be detected by filipin is a key novel finding. Although cholesterol-related abnormalities in peripheral cells of AD patients have been described [1] [12] [23], whether such abnormalities are reflective of pathogenesis or are instead a marker for pleiotropic effects is an important but still unanswered question with obvious implications for therapeutic intervention. In that regard, this is, to our knowledge, the first report showing that cellular damage induced by oxidized cholesterol can be measured with filipin fluorescence. Increased filipin fluorescence in response to 27-OHC cannot be accounted for by an overall increase in oxidized cholesterol levels per se, because an excess of 24-OHC, which is not cytotoxic (Figure 1a) does not result in an increase in the filipin signal (Figure 1b) [14] [15]. This suggests that filipin is detecting changes in cholesterol distribution secondary to an excess of 27-OHC, a view supported by the fact that such changes also occur and are detected by filipin in NPC [18]. The specific mechanisms by which filipin may detect cholesterol-related abnormalities beyond its established affinity for unesterified cholesterol remain...
unknown and their study is outside of the scope of this report [22]. Of more direct relevance is the fact that filipin fluorescence correlates with changes elicited by U18666A and by exposure to 27-OHC, known to be upregulated in AD, both of which result in measurable cytotoxicity.

We have also shown that human PBMCs are susceptible to cholesterol dysregulation as induced by U18666A and that the range of dysregulation is measurable by filipin using flow cytometry (Figure 1). These findings suggest that filipin could be of use to develop a test to compare cholesterol dysregulation levels between AD patients and cognitively normal controls. The results shown in Figure 3 provide proof of principle that this is indeed possible, showing a higher number of filipin-positive B-lymphocytes, as well as higher average mean intensity of fluorescence, in the AD population (Figure 3). Note also that cells from the control population showed greater heterogeneity compared to AD patients by both measures. Variance in the controls is predicted from a population whose cognitive function levels are heterogeneous below the threshold of cognitive impairment. In contrast, the AD population has an extremely low variance, also as expected, because their cognitive function is compromised and they have crossed the “filipin threshold”. These differences in variance are consistent with the notion that MCI patients might be separated out in terms of their risk of conversion to AD, strongly supporting a potential clinical application for filipin testing.

It is also noteworthy that the choice of B-lymphocytes was determined by their commercial availability. Further work in larger clinical studies will determine differences in filipin sensitivity between these cells and different subpopulations of PBMCs, which may show comparable or superior clinical value than B-lymphocytes to diagnose cognitive status and/or identify at-risk MCI populations.

In summary, we conclude that quantifying cellular filipin levels can accurately differentiate between control and AD patients in blood cells. Our findings may help define AD in the blood by focusing on markers of cellular pathology, and they provide strong preliminary data in support of larger clinical studies to test whether filipin-based method can indeed predict the risk of AD conversion in MCI patients.

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References


Abbreviations

AD, Alzheimer’s disease; SPs, senile plaques; Aβ, amyloid-β; NFTs, neurofibrillary tangles; GWAS, genome-wide association studies; 27-OHC, 27-hydroxycholesterol; MCI, mild cognitive impairment; NPC, Niemann-Pick type C disease; PEmCs, peripheral blood mononuclear cells; 24-OHC, 24-hydroxycholesterol; RA, retinoic acid; BDNF, brain-derived neurotrophic factor; FFA, paraformaldehyde; RT, room temperature; LDH, lactate dehydrogenase; FACS, fluorescence-activated cell sorting.
Filipin Fluorescence of neuron-differentiated SH-SY5Y Cells

(a)

Filipin Fluorescence of Human PBMCs

(b)

Filipin Fluorescence of Human B-Lymphocytes

(c)

Figure S1. Representative flow cytometry histograms of filipin fluorescence in neuron-differentiated SH-SY5Y (a), human PBMCs (b) and human B lymphocytes (c). In all cases, the number of cells counted is graphed by log intensity of filipin fluorescence. Control (shaded) and experimental cell populations are normalized to mode before comparison. A gate is set to include all cells to the immediate right of the peak fluorescence from control (shaded) cells. Both control and experimental cells contained by this gate are considered filipin-positive. Flow cytometry data was analyzed by FlowJo software.