CHARACTERIZATION OF THE MECHANISMS UNDERLYING ALZHEIMER’S DISEASE AND MULTIPLE SCLEROSIS

by

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in Neuroscience

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Abstract

Deposition of the amyloid β protein (Aβ) into neuritic plaques is the neuropathological hallmark of Alzheimer’s Disease (AD). Aβ is generated through the cleavage of the Amyloid Precursor Protein (APP) by β-secretase and γ-secretase. Currently, the evaluation of APP cleavage by β-secretase in experimental settings has largely depended on models that do not replicate the physiological conditions of this process. We have developed a chimeric protein construct, ASGβ, incorporating the β-site cleavage sequence of APP targeted by β-secretase and its intracellular trafficking signal into a Phosphatase-eGFP secreted reporter system. Upon cleavage by β-secretase, ASGβ releases a phosphatase-containing portion that can be measured in the culture medium, and an intracellular fraction that can be detected through western blot. Subsequently, we have generated a cell line stably expressing ASGβ that can be utilized to assay β-secretase in real time. Our findings suggest this system could be a high-throughput tool to screen compounds that aim to modulate β-secretase activity and Aβ production under physiological conditions, as well as evaluating factors that regulate this cleavage.

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system characterized by myelin loss and neuronal dysfunction. Although the majority of patients do not present familial aggregation, Mendelian forms have been described and more recently we have identified novel mutations in 2 genes, NLRP12 and NCOA3 in familial forms of the disease. Our findings show that one of the discovered mutations on NLRP12 affects the maturation of Caspase-1, and that the mutation discovered on NCOA3 leads to a higher basal level of inducible Nitric Oxide Synthase (iNOS). These findings show novel mechanisms causing the disruption of immune and inflammatory responses that could in turn lead to the symptoms observed in MS patients. Our study suggests that further characterization of the pathways affected by the NLRP12 and NCOA3
could lead to a better understanding of the phenomena causing MS, and the development of novel, more effective treatments for the treatment of symptoms.
Lay Summary

Alzheimer's Disease (AD) is the most prevalent form of dementia however the mechanisms regulating one of the main causes of AD, the generation of Aβ, are not fully understood. Methods currently available for evaluating Aβ generation in experimental settings are incapable of replicating the natural generation of Aβ in living beings. Here we detail a novel cell-based system that more closely mimics the endogenous generation of Aβ enabling more detailed studies and the testing of novel treatments.

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system. While MS is not thought to be inherited, mutations have been identified in familial cases and studying these mutations helps us understand the basic mechanisms causing MS. Here we characterize mutations found on two genes, NLRP12 and NCOA3, and how they could affect the symptoms of MS. Understanding the mechanisms involved could lead to the development of better treatments for MS.
Preface

Upon completing my Master of Science degree, I joined Dr. Weihong Song’s team in order to study the molecular mechanisms underlying Alzheimer’s Disease. Among other projects I participated in, I was tasked with developing a novel β-secretase enzymatic assay utilizing a chimeric protein construct that can be used to evaluate β-secretase activity in real-time in living cells. This project and the results obtained from it became the first part of my doctoral dissertation, and are described in detail in Chapter 2. The initial design of the construct was done together with Dr. Zhe Wang, a postdoctoral fellow in Dr. Weihong Song’s lab. I carried out all following design changes, experiments and analyses, under the guidance of Drs. Song and Wang. The results obtained led to the publication of an article in Current Alzheimer Research, of which I am the first author (De Araujo Herculano, Wang et al. 2018).

The second part of this dissertation covers a collaboration project with Dr. Carles Vilariño-Güell, who has with his group carried out extensive analysis of genetic mutations present in familial cases of Multiple Sclerosis. Dr. Vilariño-Güell kindly agreed to collaborate with us, and allowed us to experiment on mutant forms of two of genes identified in these familial cases, NLRP12 and NCOA3. Our experiments and results with these mutations will be the topic of Chapters 3 and 4 of this dissertation. Plasmids and cDNA necessary for this project were kindly ceded by Dr. Beckley Davis (Franklin & Marshall College, USA) and Dr. Vilariño-Güell (University of British Columbia). Experimental design for chapters 3 and 4 was done in collaboration with Dr. Zhe Wang, who also performed preliminary experiments and laid the foundation upon which I could design and carry out further experiments. Dr. Wang also prepared the plasmids and performed part of the experiments on Caspase-1 shown in chapter 3, the binding, intracellular localization and rtPCR experiments shown in chapter 4. I carried out all further
plasmid design, cloning, experimentation and analyses. Part of the data presented in chapters 3 and 4 was included into a journal article that is being prepared for submission in collaboration with Dr. Vilariño-Güell.
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
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<td>AICD</td>
<td>APP intracellular domain</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>ApoE</td>
<td>Apolipoprotein E</td>
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<td>APOE2</td>
<td>Apolipoprotein E allele ε2</td>
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<td>APP</td>
<td>Amyloid-β precursor protein</td>
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<td>APP695</td>
<td>Amyloid-β precursor protein – 695 amino acid isoform</td>
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<td>Aspartic Acid 1 site of Amyloid-β</td>
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<td>Acetyltransferase</td>
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C83 Carboxy-terminal fragment 83
C89 Carboxy-terminal fragment 89
C99 Carboxy-terminal fragment 99
CAPS Cryopyrin-associated periodic syndromes
CARD Caspase-activation and recruitment domain
CATERPILLER Caspase activation and recruitment domain, transcription enhancer, purine binding, pyrin, lots of leucine repeats
cDNA Complementary DNA
CNS Central nervous system
COX2 Cyclooxygenase 2
CTF C-terminal fragment
DAMP Danger-associated molecular pattern
DMSO Dimethyl Sulfoxide
DMEM Dulbecco’s modified Eagle medium
DMT Disease-modifying treatment
DR6 Death receptor 6
DS Down Syndrome
EAE Experimental autoimmune encephalomyelitis
eGFP Enhanced Green fluorescent protein
ELISA Enzyme-linked immunosorbent assay
EpoR Erythropoietin receptor
ER Endoplasmic reticulum
<table>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAD</td>
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</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FRET</td>
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<td>Geneticin</td>
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<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td><strong>MS</strong></td>
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</tr>
<tr>
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<td><strong>NACHT</strong></td>
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<td><strong>PBS</strong></td>
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<td><strong>PFAPA</strong></td>
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<tr>
<td><strong>PHFs</strong></td>
<td>Paired helical filaments</td>
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<tr>
<td><strong>PPAR</strong></td>
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PRR  Pattern-recognition receptor
PS1  Presenilin 1
PS2  Presenilin 2
PYD  Pyrin domain
PYPaf7  PYRIN-containing Apaf1-like protein 7
RE  Restriction enzyme
RIPA-DOC  Radioimmunoprecipitation assay buffer - deoxycholate
RT-PCR  Reverse transcription Polymerase chain reaction
RXR  Retinoid X receptors
sAPPα  Secretory APPα
sAPPβ  Secretory APPβ
sAPPθ  Secretory APPθ
SDS  Sodium dodecyl sulfate
SDS-PAGE  Sodium dodecyl sulfate–Polyacrylamide gel electrophoresis
SEAP  Human Placental Secreted Alkaline Phosphatase
SEM  Standard error of the mean
SEZ6  Seizure protein 6
siRNA  Small interfering RNA
SRC  Steroid receptor coactivator
SRC3  Steroid receptor coactivator 3
tAβ  Truncated Aβ
TACE  Tumor necrosis factor-α-converting enzyme
<table>
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<tr>
<td>TGN</td>
<td>Trans-Golgi Network</td>
</tr>
<tr>
<td>Vb</td>
<td>Verubecstat; MK-8931; BACE1 inhibitor</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin-D receptor</td>
</tr>
<tr>
<td>WES</td>
<td>Whole-exome sequencing</td>
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</table>
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Chapter 1: Introduction

1.1 Alzheimer’s Disease (AD)

1.1.1 General Introduction

Alzheimer’s Disease (AD) was first described in 1907 by Alois Alzheimer, who described the case of Auguste D., a 51-year old female under his care who displayed increasing memory impairment, disorientation, hallucinations and aggressiveness. Post-mortem evaluation of her brain revealed dense bundles of fibrils and numerous focal lesions containing proteinaceous aggregates that were termed senile plaques (Alzheimer, Stelzmann et al. 1995). AD is nowadays the most prevalent cause of dementia and is recognized as one of the major public health problems of our time (Tanzi and Bertram 2005). It is estimated that there were 24.2 million cases of dementia worldwide by 2005, 70% of which were attributed to AD. By 2010, it was estimated that roughly 11% of people over the age of 65 and around one third of people over the age of 85 would develop AD. The current consensus is that the incidence rate for dementia increases exponentially with age, with the most pronounced increase occurring through the 7th and 8th decades of life (Ferri, Prince et al. 2005, Hebert, Weuve et al. 2013, Reitz and Mayeux 2014).

According to the Alzheimer’s Association (www.alz.org; accessed January 20th, 2019), AD affects 5.7 million people in the United States alone, with an estimated worldwide prevalence of 30 million cases.

1.1.2 Clinical features of AD

Currently, the key classification for the diagnosis of AD is the criteria established by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s Disease and Related Disorders association (NINCDS-ADRDA). While initially these criteria encompassed only clinical and neuropathological patterns, they have since been progressively
updated to incorporate more recent findings, aiming to increase diagnosis specificity. One of such changes was the definition of an intermediary state between normal cognition and AD, mild cognitive impairment (MCI), that has aided in identifying patients at risk of developing AD and set guidelines of how to relate it to AD after further research and validation of biomarkers. Additionally, there was the establishment of a preclinical stage, which while not meeting the criteria for diagnosis, sets the stage for the development of hypotheses and the identification of biomarkers and changes in the brain that are consistent with those seen later in MCI and AD (McKhann, Drachman et al. 1984, Perrin, Fagan et al. 2009, Khachaturian 2011, Reitz and Mayeux 2014). In the last stages of the disease, patients are completely dependent on caregivers for all activities of daily living, eventually becoming unable to swallow on their own or retain bowel function (Holtzman, Morris et al. 2011).

1.1.3 Pathological Features

Pathological changes associated with aging and senile dementia were first described in the first years of the 20th century. Plaques in the grey matter were first described by Blocq and Marinesco in 1892, and the term senile dementia was first coined by Simchowicz in 1910. In 1927, Divry demonstrated the presence of amyloid at the core of these senile plaques through Congo red staining. Due to their appearance, Divry termed the main proteinaceous component of these plaques “amyloid”, due to their starch-like appearance (Allsop 2000, Ohry and Buda 2015).

Originally termed “senile plaques” due to their increased presence in the brains of aged individuals, neuritic plaques are extracellular proteinaceous deposits, and are associated with dystrophic neurites and are surrounded by activated microglia and astrocytes (Hyman, Phelps et al. 2012). It was not until much later that the proteinaceous cores of the senile plaques was able to be isolated from the brains of affected patients. Analysis of the composition of these cores revealed
that it was different from other forms of amyloid known at the time (Allsop, Landon et al. 1983, Allsop 2000).

In 1984, the work of two different groups was able to characterize the main component of senile plaques, a 4kDa protein now known as the amyloid-β (Aβ) peptide (Glenner and Wong 1984, Glenner and Wong 1984, Masters, Simms et al. 1985). Neuritic plaques may also present themselves in a “diffuse” form, appearing as granular deposits without a determined condensed core. They usually do not co-localize with dystrophic neurites, and unlike condensed plaques, they mainly consist of Aβ ending at amino acid 42 (Tagliavini, Giaccone et al. 1988, Joachim, Morris et al. 1989, Selkoe 2001). Dystrophic neurites are degenerated neuronal processes that are seen around neuritic plaques, containing dysfunctional mitochondria, dilated lysosomes and paired helical filaments (PHFs) of hyperphosphorylated protein tau (Dickson 1997).

One of the initial challenges in studying the pathophysiology of AD was determining to which extent the symptoms observed are simply an accentuation of the cognitive decline seen during normal aging. The presence of senile plaques was initially suggested as a determinative marker differentiating AD from other types of dementia. However, senile plaques are also present in the brains of healthy elderly individuals, so their mere presence could not be utilized to set AD apart. While their presence alone does not seem to be specific for AD, their distribution seems to be more widespread in AD, with plaques being seen throughout the whole cerebral cortex (Blessed, Tomlinson et al. 1968, Khachaturian 2011).

The definition of AD was soon expanded to include the presence of Neurofibrillary tangles (NFTs), filamentous inclusions in the cell body of neurons consisting of PHFs rich in β-sheets, occupying a large part of the cytoplasm around the nucleus (Selkoe 2001). Immunohistochemical and molecular analyses have revealed NFTs to be composed of hyperphosphorylated protein tau,
a protein integral for the correct assembly of microtubules. Hyperphosphorylation of tau, meaning phosphorylation at residues other than those crucial for its normal functioning, causes it to dissociate from microtubules and renders it insoluble in the cytoplasm (Brion, Couck et al. 1985, Grundke-Iqbal, Iqbal et al. 1986). Currently the presence of both neuritic plaques and NFTs is necessary for the diagnosis of AD, but among neurodegenerative disorders only neuritic plaques are exclusive to AD, with NFTs being found in other neurodegenerative diseases such as frontotemporal dementia (Dubois, Feldman et al. 2007, Hyman, Phelps et al. 2012).

Alongside neuritic plaques and NFTs, AD is also characterized by extensive neuronal loss within the basal forebrain, cholinergic denervation in the cerebral cortex and associated limbic areas, and general brain atrophy. These phenomena are seen as the cause of the cognitive symptoms seen in AD (Whitehouse, Price et al. 1981, Bartus, Dean et al. 1985).

1.1.4 Genetics of AD

AD is classically divided into two subtypes: familial and sporadic. The familial subtype, (FAD) is responsible for less than 1% of all cases, and its onset usually happens between the ages of 30 and 60. The vast majority of AD cases are considered sporadic or “late onset”, with genetic factors not being directly implicated (Holtzman, Morris et al. 2011, Selkoe and Hardy 2016).

Thus far, there are three genes that have been identified as containing pathogenic mutations that lead to the early-onset of FAD: APP, Presenilin 1 (PS1) and Presenilin 2 (PS2) (Tanzi and Bertram 2005, Bekris, Yu et al. 2010, Tanzi 2013, Rosenberg, Lambracht-Washington et al. 2016, Kelleher and Shen 2017, Wang, Zhou et al. 2017). For sporadic AD, Apolipoprotein E (ApoE) is the only genetic factor that has been reported to be a major risk factor for the development of the disease. Carriers of the ε4 allele of ApoE (APOE4) are at higher risk of developing AD, while carriers of the ε2 allele (APOE2) have a lower risk. The specific reasons as
to how these different alleles lead to different outcomes in cognition is as of yet unknown, and the
object of several studies (Corder, Saunders et al. 1993, Rebeck, Reiter et al. 1993, Saunders,

The gene encoding APP was first cloned in 1987, and its first pathogenic mutation was
discovered in hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-Dutch) in
1990. This mutation causes the substitution of the glutamic acid at position 693 of APP for a
 glutamine (APP E693Q) within the Aβ region (Aβ E22Q), affecting the conformation of APP and
triggering apoptosis in endothelial cells, leading to the cerebral hemorrhage phenotype observed
(Goldgaber, Lerman et al. 1987, Kang, Lemaire et al. 1987, Robakis, Ramakrishna et al. 1987,
Levy, Carman et al. 1990, Miravalle, Tokuda et al. 2000). Shortly afterwards the first FAD-
associated mutation was discovered: a substitution of the Valine at position 717 to an isoleucine
(APP V717I), termed the London mutation. This mutation fundamentally alters the cleavage site
preference on APP, leading to a greatly increased generation of the 42 amino acid subspecies of
Aβ (Aβ42), leading to its aggregation and the formation of neuritic plaques (Chartier-Harlin,

Currently the most well-studied APP mutation linked to FAD is the Swedish mutation,
initially described in 1992. This mutation causes the substitution of Lysine at position 670 for an
Asparagine, and the substitution of Methionine at position 671 for a Leucine (K670N/M671L),
greatly increasing the generation of Aβ (Citron, Oltersdorf et al. 1992, Mullan, Crawford et al.

1.1.5 APP Processing

Aβ is generated from the sequential cleavage of amyloid-β precursor protein (APP) by
enzymes termed secretases (Kang, Lemaire et al. 1987, Robakis, Ramakrishna et al. 1987, Tanzi,
Gusella et al. 1987), and its length can vary from 37 to 43 amino acids (Benilova, Karran et al. 2012). Aβ was first purified in 1984 from cerebrovascular amyloid fibrils in both AD and Down Syndrome (DS) patients. It was then already known that DS patients over 40 years of age would inevitably develop AD-like pathology. Given that DS patients carry an extra copy of chromosome 21, it was hypothesized that the gene encoding the protein from which Aβ derives would be located within that chromosome (Olson and Shaw 1969, Glenner and Wong 1984, Glenner and Wong 1984).

APP is a type 1 integral transmembrane protein, and thus far eight isoforms of varying lengths have been identified, being generated by alternative splicing and ranging from 365 to 770 amino acids in length. The three most common isoforms contain 751, 770 and 695 amino acids, with the latter, APP695, being more abundant in nervous tissue. For this reason studies on AD mainly focus on APP695 (Kang, Lemaire et al. 1987, Placido, Pereira et al. 2014). The function of APP is not yet completely understood, but studies have shown that it plays a role in modulating cell survival and growth (Thinakaran and Koo 2008, Brunholz, Sisodia et al. 2012), as well as promoting neurite arborisation, neurite outgrowth and synapse formation in some models (Roch, Masliah et al. 1994, Turner, O'Connor et al. 2003, Lee, Moussa et al. 2010). Additionally, APP may also function as a modulator of cell death, as its ectodomain has been reported to bind to death receptor 6 (DR6) after the cleavage of full length APP by BACE1, being able to induce apoptosis in some models (Nikolaev, McLaughlin et al. 2009).

Currently, it is still unclear in which intracellular organelle the processing of APP takes place. Studies suggest that non-amyloidogenic processing of APP occurs predominantly in the plasma membrane, following its maturation and transport to the cell surface via the secretory pathway (Lammich, Kojro et al. 1999). Another equally likely possibility is that APP located on
the membrane surface is processed after internalization through the endocytic pathway, a process that occurs during the normal life cycle of APP (Haass, Kaether et al. 2012).

During its trafficking from the endoplasmic reticulum (ER) to the plasma membrane, APP undergoes many post-translational modifications. After its synthesis, APP is N-glycosylated in the ER and then transported to the Golgi apparatus where it is then O- and N-glycosylated, phosphorylated and sulphonated at Tyrosine motifs (Lai, Sisodia et al. 1995, Placido, Pereira et al. 2014). Studies have shown that only roughly 10% of total APP is delivered to the plasma membrane, with the remainder staying within the trans-Golgi network (TGN). APP is transported along the axon in post TGN vesicles or elongated tubular structures together with BACE1 and presenilin 1 (PS1), and this transport requires the interaction of APP with kinesin 1 (Kamal, Almenar-Queralt et al. 2001).

Studies suggest APP inserted into the plasma membrane is preferentially cleaved through the non-amyloidogenic pathway, and due to the presence of a YENPTY internalization motif in its C-terminus it could be re-internalized via endocytosis (Perez, Soriano et al. 1999). Following endocytosis, APP fragments can either return to the cell surface, be sorted for degradation in the lysosome or transported back to the TGN in a retromer-dependent manner. Studies have shown that α and β-secretases are present in the ER, indicating that the ER could be a major site of APP processing (Chyung, Greenberg et al. 1997, Shin, Saido et al. 2005).

1.1.6 Amyloidogenic pathway and β-secretase

Amyloidogenic processing of APP is characterized by its cleavage at the β-site by a β-secretase (BACE1) in the first amino acid of the Aβ sequence. This cleavage releases a secreted N-terminal fragment of APP (sAPPβ) and leaves a 99 amino acid-long C-terminal fragment (CTF) anchored to the cellular membrane by the transmembrane domain, termed C99. C99 is then cleaved
by γ-secretase, generating Aβ and the APP intracellular domain fragment (AICD). Beta-amyloid-cleaving enzyme-1 (BACE1) was identified as the major β-secretase in vivo (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999). Data from our group has shown that there is a difference in the cleavage preference site by BACE1 between the wild-type form of APP (APPwt) and the Swedish variant (K670N/M671L; APPSwe). APPwt is cleaved by BACE1 at the glutamic acid at position 682, which is the 11th amino acid in the Aβ peptide (Glu11). This cleavage generates a shorter C-terminal fragment 89 amino acids in length (C89), which is then sequentially cleaved by γ-secretase, generating a truncated form of Aβ (tAβ) together with AICD. The mutations contained in the Swedish form, however, shift this cleavage preference to the aspartic acid at position 672 of APP (Asp1) greatly increasing Aβ generation (Citron, Oltersdorf et al. 1992, Mullan, Crawford et al. 1992, Claeysen, Cochet et al. 2012, Deng, Wang et al. 2013, Kandalepas and Vassar 2014).

BACE1 is a 501-amino-acid type 1 transmembrane aspartyl protease containing 2 catalytic sites that operate in conjunction, located in the lumen of acidic intracellular compartments. It is mostly expressed in neurons, with expression levels in peripheral tissues being reportedly low (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999). Initially it was not thought BACE1 had an important role in the brain besides the generation of Aβ since BACE1 knockout mice are fertile and viable (Luo, Bolon et al. 2001, Roberds, Anderson et al. 2001). Further studies however have shown that BACE1 deficiency results in hypomyelination (Willem, Garratt et al. 2006), decreased neurogenesis with corresponding increased astrogenesis (Hu, He et al. 2013), neurodegeneration and alteration of neuronal activity (Hu, Zhou et al. 2010). Nevertheless, reduction of BACE1 expression has reportedly resulted in reduced Aβ generation, ameliorated cognition and memory and

1.1.7 Non-amyloidogenic pathway and α-secretase

Under physiological conditions, most of APP is cleaved by α-secretase within the Aβ region at leucine 688 (Leu17), precluding the generation of Aβ. This cleavage results in a secreted N-terminal fragment (sAPPα) and an 83 amino acid C-terminal fragment (C83) anchored to the cell membrane (Koike, Tomioka et al. 1999, Lammich, Kojro et al. 1999). After this cleavage, the resulting C83 is then cleaved by γ-secretase, generating a small 3kDa peptide (p3) and AICD (Haass, Hung et al. 1993, Claeyssen, Cochet et al. 2012, Haass, Kaether et al. 2012).

Many α-secretase candidates that have been identified thus far belong to the A Disintegrin and metalloproteinase (ADAM) family. Among these, ADAM9, ADAM10, ADAM17 and ADAM19 are of particular interest (Buxbaum, Liu et al. 1998, Koike, Tomioka et al. 1999, Lammich, Kojro et al. 1999, Asai, Hattori et al. 2003, Tanabe, Hotoda et al. 2007). Overexpression of ADAM10 in human embryonic kidney 293 (HEK293) cells has been reported to result in increased generation of sAPPα. The same study has also shown that expression of a dominant negative form of ADAM10 greatly decreases this generation, confirming its role as an α-secretase (Lammich, Kojro et al. 1999). ADAM17, also known as Tumor necrosis factor-α-converting enzyme (TACE) has been reported to be able to induce the generation of sAPPα in cultured cells (Buxbaum, Liu et al. 1998). Overexpression of ADAM9 (also known as MDC9) has also been reported to increase cleavage of APP695 at the α site (Koike, Tomioka et al. 1999). ADAM19-overexpressing cells have increased levels of sAPPα, and this increase could be countered by ADAM19 small interfering RNA (siRNA) (Tanabe, Hotoda et al. 2007).
Alternatively, APP can also be cleaved within the Aβ region by BACE1, as mentioned in the previous section (Vassar, Bennett et al. 1999). This cleavage generates sAPPβ and C89, the latter being then further cleaved by γ-secretase to generate tAβ and AICD (Vassar, Bennett et al. 1999, Nunan and Small 2000, Zhang, Wang et al. 2017).

Additionally, APP can also be cleaved by β-amyloid cleaving enzyme-2 (BACE2). BACE2 is a homolog of BACE1, sharing 64% of its amino acid sequence. BACE2 expression level is low in neurons, but relatively higher in glial cells. (Bennett, Babu-Khan et al. 2000, Laird, Cai et al. 2005, Kandalepas and Vassar 2014). Given this sequence similarity, BACE2 was initially thought to have the same β-secretase activity as BACE1. Interestingly however, it targets APP at a site within the Aβ region, cleaving it between Phenylalanines 691 and 692 (19 and 20 in the Aβ region; now termed the θ-site) to generate secretory APPθ (sAPPθ) and an 80 amino acid C-terminal fragment (C80) anchored to the membrane. C80 is then further cleaved by γ-secretase, generating a shortened p3 fragment and AICD. Thus, it has been since termed a θ-secretase, with its activity not contributing to the neuropathology of AD (Sun, Wang et al. 2005, Sun, He et al. 2006).

1.1.8 Presenilins and γ-secretase

The importance of Presenilin to the development of AD came to be known with the discovery of missense mutations on two genes, presenilin 1 (PS1) and presenilin 2 (PS2) from 2 families with inherited forms of the disease (Levy-Lahad, Wasco et al. 1995, Rogaev, Sherrington et al. 1995, Sherrington, Rogaev et al. 1995).

Presenilins contain the catalytic site of the γ-secretase complex, which consists of critical aspartate residues at amino acid positions 257 and 385 in the 6th and 7th transmembrane domains, respectively (Wolfe, Xia et al. 1999, Kimberly, Xia et al. 2000, Haapasalo and Kovacs 2011, Zhang,
Presenilins, however, do not appear to be able to function as \(\gamma\)-secretases on their own, requiring the co-expression of Nicastrin, Aph-1 and Pen-2 as cofactors in order to become a functional unit (De Strooper 2003, Edbauer, Winkler et al. 2003, Kimberly and Wolfe 2003, Takasugi, Tomita et al. 2003, Haapasalo and Kovacs 2011).

Deficiency of PS1 in neuronal cultures was then shown to affect normal cleavage of APP, resulting in an accumulation of membrane-bound CTFs and a decrease in the generation of A\(\beta\). This discovery helped cement the status of PS1 as the major component of the \(\gamma\)-secretase complex (De Strooper, Saftig et al. 1998). The role of PS2 in \(\gamma\)-secretase activity was further evidenced by the observation that knockout of both PS1 and PS2 is required to completely abolish the generation of A\(\beta\) in cultured cells (Herreman, Serneels et al. 2000, Zhang, Nadeau et al. 2000).

Presenilins also play a major role in the Notch signalling pathway. PS1-deficient cells display decreased cleavage of a truncated form of Notch that contains only its transmembrane and intracellular domains (Song, Nadeau et al. 1999). The same effect was achieved with a \(\gamma\)-secretase inhibitor, indicating that this effect was due to the \(\gamma\)-secretase activity of PS1 (De Strooper, Annaert et al. 1999). Additionally, endoproteolysis of the Notch Intracellular Domain (NICD) is reported greatly diminished in fibroblasts derived from PS1-knockout mice, an effect that was countered by overexpression of PS1 (Song, Nadeau et al. 1999). A loss-of-function mutation on PS2 reduces A\(\beta\) and Notch cleavage, indicating it has similar functions to PS1 (Steiner, Duff et al. 1999). Taken together, these observations show that presenilins have an indispensable role not only for APP processing, but for other substrates as well. The processing of APP by \(\alpha\), \(\beta\), \(\theta\) and \(\gamma\)-secretases has been summarized in Figure 1.
Figure 1. Processing of APP by secretases. Amyloidogenic processing of APP occurs when it is cleaved by β-secretase (BACE1) at the Asp1 site, generating a secreted N-terminal fragment, termed sAPPβ and a membrane-bound 99-amino acid C-terminal fragment termed C99. C99 is then further cleaved by γ-secretase to generate the APP Intracellular domain (AICD) and Aβ. Under physiological conditions however, β-secretase cleaves APP at the Glu11 site, generating a shorter C-terminal fragment 89 amino acids in length (C89), which is then cleaved by γ-secretase to generate a truncated form of Aβ that does not aggregate. Cleavage of APP by α-secretase at the Leu17 site or by θ-secretase (BACE2) at the Phe20 site also preclude Aβ production. Thickness of arrows indicates relative prevalence of cleavages under normal conditions. Adapted from Zhang and Song, 2017.

1.1.9 Amyloid hypothesis

The amyloid hypothesis dates back to the first discovery of Aβ, and the subsequent tracing of its precursor protein APP, to chromosome 21 (Glenner and Wong 1984, Glenner and Wong 1984). After the successful cloning of APP and the identification of several of its mutations in familial cases of AD, the amyloid hypothesis in its initial form was proposed by Hardy and Higgins in 1992. The hypothesis initially postulated that the generation and consequential accumulation of
Aβ into senile plaques is the core cause underlying the neuropathology seen in AD, leading to the formation of NFTs, neuronal loss and memory deficits (Hardy and Higgins 1992).

It is known that DS patients inevitably develop AD-associated neuropathological features, and this was initially thought to be due the trisomy of chromosome 21 (Olson and Shaw 1969, Mann, Yates et al. 1984, Motte and Williams 1989). While this trisomy invariably means that carriers would possess additional copies of all genes located within that chromosome, it has been reported that a partial trisomy that does not result in an extra copy of the APP gene does not cause the neuropathological changes normally observed in DS and AD, further focusing the spotlight on APP and Aβ as the centerpieces of the AD enigma (Prasher, Farrer et al. 1998).

The APP mutations identified in familial cases of AD were found to mainly affect β and γ cleavages, either generating more total Aβ or changing the ratio of toxic Aβ species, lending credence to the idea that Aβ is the main toxic cause and effect of the pathophysiological changes observed in AD (Citron, Oltersdorf et al. 1992, Hendriks, van Duijn et al. 1992, Mullan, Crawford et al. 1992, Suzuki, Cheung et al. 1994, Scheuner, Eckman et al. 1996, De Jonghe, Esselens et al. 2001, Bentahir, Nyabi et al. 2006, Kumar-Singh, Theuns et al. 2006). Other mutations discovered subsequently also brought to light evidence that corroborated the notion that changes in the sequence of APP that cause its abnormal processing leads to AD. One such mutation in a Japanese family lacking a glutamic acid residue (E693Δ, residue 22 in Aβ) was reported to cause AD by increasing oligomerization, with no fibrillization of Aβ (Tomiyama, Nagata et al. 2008). Another mutation at the same site found in a Swedish family (E693G), termed “Arctic” was observed to cause the same effect (Nilsberth, Westlind-Danielsson et al. 2001).

Another factor to be considered is the role of tau in the neuropathology observed in AD, and how it is affected by Aβ. While hyperphosphorylation of tau and formation of NFTs is a core
component of the neurological changes seen in AD, mutations on the gene encoding tau have been reported to lead to frontotemporal dementia with parkinsonism, not AD (Hutton, Lendon et al. 1998, Poorkaj, Bird et al. 1998, Spillantini, Bird et al. 1998). Studies have shown that injection of fibrillar Aβ into the brains of mice harboring the P301L tau mutation caused a fivefold increase in the formation of NFTs, indicating that Aβ is one of the factors inducing NFT formation in AD (Gotz, Chen et al. 2001). Additionally, it has been reported that double transgenics (APPSwe + tau P301L) have more NFTs than single transgenics for P301L tau, reinforcing the idea that Aβ generation is intrinsically linked to other phenomena observed in AD (Lewis, Dickson et al. 2001).

Apart from the formation of plaques and exacerbation of tau pathology, Aβ has also been reported to exert toxicity on its own, both in vitro and in vivo. In cell cultures, exogenous addition of Aβ has been reported to cause oxidative stress (Behl, Davis et al. 1994), synaptic dysfunction (Lorenzo and Yankner 1994), and overall decreased cell viability (Yankner, Duffy et al. 1990, Pike, Burdick et al. 1993). In animals, injection of either synthetic or cell-generated Aβ induced cholinergic dysfunction (Geula, Mesulam et al. 1998, Vaucher, Aumont et al. 2001), inhibition of Long-term Potentiation (LTP), neuronal death (Geula, Wu et al. 1998) and learning difficulties (Cleary, Walsh et al. 2005).

Immunotherapy using antibodies against Aβ in triple transgenic AD mice (APPSwe, PS1 M146V, Tau P301L) was seen to reduce total Aβ load and tau pathology, reinforcing the idea that the degradation and clearance of Aβ contribute to AD, and that Aβ leads to tau pathology (Oddo, Billings et al. 2004). Taken together with an earlier characterization of neuropathological features in this particular animal model, these results also suggest Aβ accumulation occurs prior to the formation of tangles, and exacerbation of tau pathology could possibly be prevented by preventing the accumulation and aggregation of Aβ (Oddo, Caccamo et al. 2003).
Other conditions that might affect total Aβ levels, such as decreased degradation and clearance seem to have an impact on the progression of the disease in carriers of the ε4 allele of ApoE (APOE4) having a higher risk of developing AD (Rebeck, Reiter et al. 1993, Bales, Verina et al. 1997, Castellano, Kim et al. 2011, Cerf, Gustot et al. 2011). Taken together, these studies suggest that generation and accumulation of Aβ is the main phenomenon causing and exacerbating the range of pathological processes present in AD.

1.1.10 **Limitations of the amyloid hypothesis and the revised hypothesis**

Despite the significance of the amyloid hypothesis and the accuracy with which it describes the progression of the neuropathology of AD, several studies over the years have provided ample evidence to dispute its place as the only, or even the best hypothesis for the etiology and progression of AD.

There is evidence that neuritic plaque load *per se* does not correlate well with cognitive decline and the severity of dementia (Katzman 1986, Terry, Masliah et al. 1991, Nelson, Alafuzoff et al. 2012). Furthermore, transgenic mice expressing human APP display synaptic disruption, behavioral and memory deficits without necessarily having plaque formation (Hsia, Masliah et al. 1999). There is a possibility that the neuritic plaques observed in AD consist of “inert pools” of Aβ, and some studies have gone as far as suggesting they serve a protective role, as their formation would prevent the generation of Aβ oligomers (Yankner, Duffy et al. 1990, Pike, Burdick et al. 1993, Lorenzo and Yankner 1994).

While there is abundant evidence showing that amyloid plaques and NFTs are major contributors to the progression of AD, it is now widely accepted that other factors that lead to neuroinflammation, neuronal, axonal and synaptic dysfunction also play major roles. These
changes appear to occur before there is significant cognitive decline, further complicating the effort in understanding causal relationships between all factors involved (Perrin, Fagan et al. 2009).

Based on these observations, Hardy and Selkoe have then updated the amyloid hypothesis initially proposed in order to incorporate more factors into the overall progression model of AD, summarized in Figure 2 (Hardy and Selkoe 2002, Selkoe and Hardy 2016).
Figure 2. The revised amyloid hypothesis.
Accumulation of Aβ in the brain may occur either due to increased generation in inherited familial forms of Alzheimer’s Disease (AD), or decreased clearance in sporadic forms of the disease. Aβ then triggers other events that compound and exacerbate the effects its toxic effects as it deposits and accumulates into plaques. In its current form, the amyloid hypothesis of AD incorporates several phenomena, directly or indirectly related to Aβ that contribute to cognitive decline. Adapted from Selkoe and Hardy (2016) with permission from the publisher.
1.1.11 The secretase dilemma

As the focus of research moves towards the generation of Aβ as the trigger of cascades of deleterious phenomena that culminate in the complex ensemble of symptoms we see in AD, secretases and their activity came under further scrutiny, as the key to preventing or treating AD could lie in their ability to cleave APP. In this vein, several effective inhibitors of γ-secretase were quickly developed, and their effectiveness, together with their reportedly excellent oral availability and brain penetrance supported the notion that inhibiting secretases was a viable strategy for the treatment of AD (Wolfe 2008, Golde, Petrucelli et al. 2010, Golde, Koo et al. 2013).

The downside to such a treatment strategy however, lies in the fundamental biological functions of γ-secretases due to their ability to cleave a plethora of substrates, affecting a wide range of biological processes (Haass 2004, Ghosh and Osswald 2014). One such process is the Notch signalling pathway. As discussed previously, PS1 and PS2 are important for regulating the Notch signalling pathway responsible for cell proliferation and differentiation during embryonic development, and disrupting this pathway could cause many undesirable side effects (Wolfe, Xia et al. 1999). This importance is underscored by the finding that double knockout of PS1 and PS2 is lethal at the embryonic stage in mice (Shen, Bronson et al. 1997, Herreman, Hartmann et al. 1999, Esler, Kimberly et al. 2000). Single knockout of PS1 causes underdeveloped subventricular areas and cortical dysplasia, as well as severe vascular lesions and hemorrhages, resulting in death soon after birth (Haass and De Strooper 1999, Hartmann, De Strooper et al. 1999). Partial conditional loss of Notch-1 has been reported to lead to vascular tumors, and related lethal hemorrhage in mice (Liu, Turkoz et al. 2011), indicating that loss of this important signalling pathway has potentially lethal consequences not only at the embryonic stage, but also at later stages in life.
These fears were soon confirmed as trials with otherwise promising γ-secretase inhibitors caused a barrage of unwanted side effects. As a result, long-term treatment with γ-secretase is now associated with unacceptable side-effects and a lack of efficacy in clinical settings, leading clinical trials to be halted (Cummings 2010, Samson 2010, Schor 2011, Golde, Koo et al. 2013). Interestingly, outside of the context of AD γ-secretase inhibitors are often thought of as “Notch 1 inhibitors”, being currently tested in that context as a therapeutic strategy in treatment of some types of cancer (Okochi, Steiner et al. 2002, Li, Wen et al. 2007, Rizzo, Osipo et al. 2008, Palomero and Ferrando 2009).

Even if these side effects are ignored and evaluation focuses on the ability of these inhibitors to provide an amelioration of cognitive symptoms, γ-secretase inhibition as a treatment strategy for AD does not seem to be effective. Treatment with Avagacestat, a specific potent γ-secretase inhibitor was seen to cause an increase in total levels of PS1 both in rats and treated cells, suggesting overall inhibition of γ-secretase could have a paradoxical effect that might worsen symptoms (Sogorb-Esteve, Garcia-Ayllon et al. 2018). Another inhibitor, Semagacestat, made it to clinical trials but was unable to provide any measurable improvement in cognitive ability. On the contrary, results show that it actually worsened clinical symptoms in patients (Rosenberg, Lanctot et al. 2016).

Currently, strategies for the treatment of AD focused on γ-secretase are moving away from complete inhibition due to unexpected side-effects and towards modulators that could selectively affect the cleavage of APP while keeping the cleavage of other substrates unaffected (Golde, Koo et al. 2013).

With the failure of γ-secretase inhibitors to treat cognitive decline in AD, research has since distanced itself from γ-secretase, and has instead focused on inhibiting β-secretase activity.
Initially, this was considered a more focused approach, since the cleavage of APP by β-secretase is a rate-limiting step in the generation of Aβ (Ghosh and Osswald 2014, Yan and Vassar 2014, Coimbra, Marques et al. 2018, Zhu, Peters et al. 2018).

General inhibition of the main β-secretase in the brain, BACE1, is not without its drawbacks however. BACE1 is critical for the cleavage of Neuregulin-1 (Willem 2016), and eliminating this cleavage altogether causes a reduction in the myelin sheath thickness of axons of both peripheral sciatic nerves and central optic nerves (Willem, Garratt et al. 2006, Hu, He et al. 2008), and disruption of its cleavage is implicated in schizophrenia (Zhang, Huang et al. 2017). BACE1 is also a major sheddase that cleaves several substrates in mature neurons. Among these are seizure-protein 6 (SEZ6), L1, CHL1 and contactin-2 among others, which are involved in neurite outgrowth, axonal targeting and remyelination (Figure 3). This indicates that cleavage of neuregulin-1 is not the only process affected when there is an inhibition of BACE1 activity (Kuhn, Koroniak et al. 2012). While initially it was reported that a complete knock-out of BACE1 caused no major effects on gross behavioral and neuromuscular function, tissue morphology, histology, blood or urine chemistry in mice (Luo, Bolon et al. 2001, Roberds, Anderson et al. 2001), subsequent studies have revealed complex neurological deficits that point to additional vital functions of BACE1. For example, BACE1 knock-out mice exhibit growth retardation (Dominguez, Tournoy et al. 2005), memory deficits (Ohno, Sametsky et al. 2004, Laird, Cai et al. 2005, Ohno, Cole et al. 2007, Rajapaksha, Eimer et al. 2011), hypomyelination (Willem, Garratt et al. 2006, Hu, He et al. 2008, Hitt, Jaramillo et al. 2010), seizures (Kobayashi, Zeller et al. 2008, Hu, Zhou et al. 2010, Rajapaksha, Eimer et al. 2011), axon guidance defects (Cao, Rickenbacher et al. 2012, Hitt, Riordan et al. 2012, Cheret, Willem et al. 2013), motor coordination deficits (112), schizophrenia-like behavior, reduction in spine density, hyperactivity (Dominguez, Tournoy et al. 2005),

The role of BACE1 in the generation of Aβ however made the idea of utilizing BACE1 inhibitors as a strategy for treating AD especially attractive. As of 2014, there were at least 12 putative inhibitors for BACE1 undergoing clinical trials (Vassar 2014), and initial results showed that their use was able to reduce brain Aβ and rescue cognitive decline in mice (Ghosh and Osswald 2014, Kandalepas and Vassar 2014, Menting and Claassen 2014, Yan and Vassar 2014, Kennedy, Stamford et al. 2016).

BACE1 inhibitors made it as far as clinical trials, but there were several side effects that caused most of them to be halted. Despite being a very specific and potent inhibitor, Verubecestat failed to provide any measurable improvement in cognitive symptoms in clinical trials. That, together with considerable side effects led clinical trials to be halted. Among the side effects were insomnia, anxiety and suicidal ideation (Egan, Kost et al. 2018). It is now known that chronic inhibition of BACE1 causes alterations in synaptic function and loss of dendritic spines, which might be one of the causes of the side effects observed (Blume, Filser et al. 2018, Zhu, Peters et al. 2018). LY2886721, another promising BACE1 inhibitor had its trials stopped due to rising concerns about hepatotoxicity, though it was disputed if that was an effect of BACE1 inhibition itself (Lahiri, Maloney et al. 2014).

Chronic BACE1 inhibition is also linked to retinal toxicity (Fielden, Werner et al. 2015), and some studies went as far as showing BACE1 inhibition negatively affected cognition (Filser,
Ovsepian et al. 2015), which could be derived from impaired function of neuregulin-1 signaling (Hu, Fan et al. 2016).

There are still many unknowns when it comes to BACE1 function and the severity of side effects and lack of improvement in cognitive function in some clinical trials calls into question the validity of the strategy of BACE1 inhibitions as a treatment for AD (Barao, Moechars et al. 2016, Yan 2017, Coimbra, Marques et al. 2018). This means inhibition by itself might not be a good strategy for the treatment of AD, and better models need to be developed and experimented on if we are to understand the factors regulating the cleavage of APP by secretases, and find ways to regulate this cleavage without affecting other substrates.
Figure 3. List of putative substrates of BACE1 in order of affinity.

BACE1 shows high affinity to several substrates involved in neurite outgrowth, axonal targeting and remyelination, and relatively low affinity for APP, causing its inhibition as a means of treating AD to lead to several unwanted side effects. Adapted from Vassar (2014) with permission from the publisher (BioMed Central).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seizure protein 6-like 1</td>
<td>High</td>
</tr>
<tr>
<td>Seizure protein 6</td>
<td>High</td>
</tr>
<tr>
<td>Amyloid precursor-like protein 1</td>
<td>High</td>
</tr>
<tr>
<td>VWFA and cache domain-containing protein 1</td>
<td>High</td>
</tr>
<tr>
<td>Golgi apparatus protein 1</td>
<td>High</td>
</tr>
<tr>
<td>L1</td>
<td>High</td>
</tr>
<tr>
<td>Leucine-rich repeat neuronal protein 1</td>
<td>High</td>
</tr>
<tr>
<td>Plexin domain-containing protein 2</td>
<td>High</td>
</tr>
<tr>
<td>Neurotrimin</td>
<td>High</td>
</tr>
<tr>
<td>Cell adhesion molecule with homology to L1CAM</td>
<td>High</td>
</tr>
<tr>
<td>Peptidyl-glycine α-amidating monooxygenase</td>
<td>High</td>
</tr>
<tr>
<td>Alpha-1,4-N-acetylhexasaminyltransferase EXTL2</td>
<td>High</td>
</tr>
<tr>
<td>Protocadherin γ A11</td>
<td>High</td>
</tr>
<tr>
<td>Amyloid precursor-like protein 2</td>
<td>High</td>
</tr>
<tr>
<td>ST3GAL-1 sialytransferase</td>
<td>High</td>
</tr>
<tr>
<td>Latrophilin-1</td>
<td>High</td>
</tr>
<tr>
<td>Neuroligin-4</td>
<td>High</td>
</tr>
<tr>
<td>Semaphorin-6D</td>
<td>High</td>
</tr>
<tr>
<td>Lysosomal membrane glycoprotein 1</td>
<td>High</td>
</tr>
<tr>
<td>Neurexin I-α</td>
<td>High</td>
</tr>
<tr>
<td>Protocadherin-20</td>
<td>High</td>
</tr>
<tr>
<td>Latrophilin-3</td>
<td>High</td>
</tr>
<tr>
<td>Latrophilin-2</td>
<td>High</td>
</tr>
<tr>
<td>Sodium/potassium-dependent ATPase subunit γ-1</td>
<td>High</td>
</tr>
<tr>
<td>Delta and Notch-like epidermal growth factor-related receptor</td>
<td>High</td>
</tr>
<tr>
<td>Interferon γ/β receptor 2</td>
<td>Low</td>
</tr>
<tr>
<td>Neuroligin-2</td>
<td>Low</td>
</tr>
<tr>
<td>Seizure 6-like protein 2</td>
<td>Low</td>
</tr>
<tr>
<td>Leucine-rich repeat fibronectin type-III domain-containing protein 2</td>
<td>Low</td>
</tr>
<tr>
<td>Cx3C membrane-anchored chemokine</td>
<td>Low</td>
</tr>
<tr>
<td>Contactin-2</td>
<td>Low</td>
</tr>
<tr>
<td>Amyloid precursor protein</td>
<td>Low</td>
</tr>
<tr>
<td>Neuroligin-1</td>
<td>Low</td>
</tr>
<tr>
<td>Transmembrane protein 132A</td>
<td>Low</td>
</tr>
</tbody>
</table>
1.1.12 Importance of BACE1

In order to better understand the intricate relationship of BACE1 with APP and how it exerts its function as a β-secretase, we first need to understand the features of BACE1 and the environment in which it interacts with its substrates. BACE1 is a type I transmembrane aspartyl-protease roughly 70kDa in size related to pepsins and retroviral aspartyl-proteases. While BACE1 is widely expressed throughout the body, its highest expression profile and proteolytic activity is observed in the brain (Seubert, Oltersdorf et al. 1993, Zhao, Paganini et al. 1996, Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999, Lin, Koelsch et al. 2000, Marcinkiewicz and Seidah 2000, Holsinger, Goense et al. 2013). In vitro assays have shown the optimal pH for BACE1 activity to be within a low range (Haass, Hung et al. 1993, Haass, Capell et al. 1995, Knops, Suomensaari et al. 1995, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999) and for this reason most β-secretase activity is reportedly confined primarily to endosomes and the Golgi apparatus (Koo and Squazzo 1994, Haass, Lemere et al. 1995, Thinakaran, Teplow et al. 1996, Morel, Chamoun et al. 2013). Additionally, BACE1 was shown to reside predominantly within acidic intracellular compartments, with its active side facing the luminal side of vesicles (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999, Lin, Koelsch et al. 2000, Kinoshita, Fukumoto et al. 2003). This active side, its luminal domain, contains two aspartyl-protease active sites, both being required for the proteolytic activity of BACE1 (Hussain, Powell et al. 1999, Bennett, Denis et al. 2000).

BACE1 is synthesized as a 501 amino acid pro-enzyme with a short prodomain in the ER (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999, Capell, Steiner et al. 2000, Lin, Koelsch et al. 2000). Within the ER, the
luminal domain of BACE1 is glycosylated and acetylated before being exported to the Golgi apparatus (Hussain, Powell et al. 1999, Bennett, Denis et al. 2000, Capell, Steiner et al. 2000, Benjannet, Elagoz et al. 2001, Creemers, Ines Dominguez et al. 2001). Once in the Golgi, it undergoes maturation, having its prodomain removed and complex carbohydrates attached to its structure (Huse, Pijak et al. 2000, Benjannet, Cromlish et al. 2004). After this maturation, it is transported from the TGN to the cell surface, where it is then internalized into early endosomes. BACE1 is constantly shuttled between the cell surface and endosomes (Walter, Fluhrer et al. 2001, Bonifacino and Traub 2003), being colocalized with APP (Hussain, Powell et al. 1999, Kinoshita, Fukumoto et al. 2003, Araki 2016) under conditions that favor the generation of Aβ (He, Zhu et al. 2003, Tesco, Koh et al. 2007, Prabhu, Burgos et al. 2012, Zhang and Song 2013).

1.2 Multiple Sclerosis

1.2.1 General introduction

Multiple Sclerosis (MS) is a common autoimmune disease of the Central Nervous System (CNS), which affects over two million people in the entire world (Browne, Chandraratna et al. 2014). Recent studies report that the prevalence of MS in Canada could be among the highest in the world, with almost 100,000 people diagnosed (Gilmour, Ramage-Morin et al. 2018). MS seems to disproportionately affect women, although the reasons for this are yet unclear (Orton, Herrera et al. 2006, Ramagopalan, Byrnes et al. 2010). While recognized and described as early as the 14th century, it was only in 1868 that Jean-Martin Charcot made the first correlations between its clinical presentation and pathological features noted post mortem, recognizing it as a distinct condition and recognizing a triad of symptoms, nystagmus, intention tremor and scanning speech, that could be used to diagnose it (Kumar, Aslinia et al. 2011). Currently the definition for MS is considerably broader, with a variety of symptoms, such as vision impairment, numbness, weakness
and mood changes being among those reported in patients (Gilmour, Ramage-Morin et al. 2018). The variety of symptoms, along with the disparity in their severity among patients make the course of MS unpredictable, with some cases presenting reversible neurological deficits, followed by progressive neurological deterioration (Goldenberg 2012). There is no single diagnostic test for MS, and diagnoses are based on the following criteria: presentation of lesions in at least two separate areas of the CNS, recurring symptoms lasting more than 24 hours and at least 1 month apart, and chronic inflammation of the CNS (McDonald, Compston et al. 2001).

While MS was initially thought of as a neurodegenerative disorder, mounting evidence suggests that the immune system plays a large role in the progression of the disease (Yadav, Mindur et al. 2015). Among this evidence is the presence of T and B lymphocytes in demyelinating lesions (Lassmann 2013), the presence of CNS-antigen specific immune response in the peripheral blood of patients (Weissert 2013), and the fact that the symptoms can be treated with immune modulatory therapeutics that eliminate peripheral immune response and promote repopulation of peripheral lymphocytes (Hartung and Kieseier 2014, Menge, Stuve et al. 2014).

Research on MS has advanced considerably in recent years, and the knowledge of the biological mechanisms causing its distinct features has increased accordingly, leading to the development of several disease-modifying treatments (DMTs). These treatments target inflammatory components and have limited impact on regenerative processes. A definitive cure for MS, however, still has not been found (Goldenberg 2012, Wingerchuk and Carter 2014, Torkildsen, Myhr et al. 2016). For this reason, the development of novel treatment strategies that aim at enhancing remyelination is crucial for preventing axonal loss and progressive disability. In order to better develop effective treatments for MS however, it is necessary to better understand
the molecular mechanisms underlying its onset and progression, as well as the potential genetic factors that might affect the presentation and severity of symptoms (Coetzee and Thompson 2018).

### 1.2.2 Genetics of MS

Although the majority of patients suffering from MS do not have a history of the disease in the family, the prevalence of familial aggregation has been estimated at 12.6% globally (Harirchian, Fatehi et al. 2018). Large-scale genome-wide association studies (GWAS) have identified over 200 genes that are reported to moderately affect individual susceptibility to MS (International Multiple Sclerosis Genetics, Wellcome Trust Case Control et al. 2011, International Multiple Sclerosis Genetics, Beecham et al. 2013). Nevertheless, other genetic and environmental factors are expected to play a significant role in the presentation of clinical symptoms, level of disability, disease progression, mutation penetrance and onset age (Wang, Sadovnick et al. 2016, Sadovnick, Traboulsee et al. 2017). A proposed sequence of events in the etiology of MS is shown in Figure 4. The application of whole-exome sequencing (WES) in MS families has already uncovered pathogenic mutations in NR1H3, P2RX4/P2RX7 and NLRP1 (Wang, Sadovnick et al. 2016, Maver, Lavtar et al. 2017, Sadovnick, Gu et al. 2017). While these discoveries have not been found in other families, these mutations have brought a new understanding of the molecular mechanisms involved in MS. Investigating and better understanding how these mechanisms are affected and how they contribute to the systemic inflammation we observe in MS would certainly bring new valuable insight that could lead to better treatments in the future.
An initial event, either exogenous (infection) or endogenous (autoimmune response) causes an inflammatory response. This response, allied to genetic factors and environmental cofactors trigger the induction of MS. The breakdown of myelin sheaths and oligodendrocytes caused by the initial episode of MS triggers an autoimmune response, which generates a self-sustaining cycle of autoimmunity that leads to progressive neurodegeneration. Adapted from Weissert (2013) with permission from the publisher.

1.2.3 Role of the Inflammasome in MS

One of the most widely used models to replicate MS in animals for experimentation is Experimental Autoimmune Encephalomyelitis (EAE), wherein mice are immunized with myelin oligodendrocyte glycoprotein (MOG). This procedure triggers the infiltration of MOG-specific T-cells into the CNS, causing an inflammatory response mediated by the inflammasome (Martinon, Burns et al. 2002, Gris, Ye et al. 2010, Guo, Callaway et al. 2015). The inflammasome is a cytosolic protein complex critical for the maturation and secretion of interleukin-1β (IL-1β) and interleukin-18 (IL-18), which initiate an inflammatory cascade that leads to programmed cell death, termed pyroptosis. Inflammasome assembly occurs when pattern-recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns
(DAMPs) or changes in intracellular cation concentrations (Coutermash-Ott, Eden et al. 2016). The formation of the Inflammasome complex can be activated by PRR sensor molecules such as nucleotide-binding domain (NOD or NACHT)-leucine rich repeat (LRR)-pyrin domain (PYD)-containing proteins (NLRPs) and NACHT-LRR-caspase activation and recruitment domain (CARD)-containing proteins (NLRCs) (Latz, Xiao et al. 2013). Each NOD-like receptor (NLR) is activated by a specific set of stimuli, leading to the formation of specific inflammasomes (Guo, Callaway et al. 2015). The inflammasome complex serves as a scaffold for the recruitment of apoptosis-associated speck-like protein containing a CARD (ASC) adaptor, and oligomerization of the inactive zymogen pro-caspase-1, leading to its autoproteolytic cleavage and subsequent activation (Bauernfeind and Hornung 2013). Once activated, Caspase-1 cleaves cytokine precursor pro-IL-1β and pro-IL-18 into their active forms, which are then secreted, triggering a strong inflammatory response (Broderick, De Nardo et al. 2015, Guo, Callaway et al. 2015). A subgroup of NLRs, including NLRP12, NLRC3 and NOD2 are capable of affecting inflammatory cascades by modulating intracellular signaling pathways such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and extracellular signal-regulated kinase (ERK) pathways (Coutermash-Ott, Eden et al. 2016, Gharagozloo, Gris et al. 2017).

It is known that mutations in the proteins composing the inflammasome can lead to autoinflammatory syndromes. Among these, alterations in NLRP family proteins can lead to disruption in inflammasome assembly, leading to immune disorders. For example, mutations in NLRP3 were reported to cause cryopyrin-associated periodic syndromes (CAPS), characterized by systemic inflammation with fever and blood neutrophilia (Broderick, De Nardo et al. 2015), and other low-penetrance mutations have been discovered in patients suffering from MS,
suggesting that inflammasome assembly could contribute to the onset of autoimmune diseases (Schuh, Lohse et al. 2015).

1.2.4 NLRP12

The nucleotide binding domain leucine-rich repeat containing protein (NLRP) family, also known as CATERPILLER (caspase activation and recruitment domain, transcription enhancer, purine binding, pyrin, lots of leucine repeats), is structurally characterized by a tripartite architecture containing a central nucleotide binding domain (NACHT or NOD), which exhibits ATPase activity and regulates oligomerization, a specific N-terminal effector domain for recruiting downstream effector molecules, and a C-terminal portion consisting of a variable series of leucine-rich repeats implicated in mediating autoregulation, protein–protein interactions, and sensing pathogen-associated molecular patterns (PAMPs) (Ye and Ting 2008, Borghini, Tassi et al. 2011).

NLRP12, also known as Monarch-1 or PYRIN-containing Apaf1-like protein 7 (PYPAF7) is one such protein in the NLRP family, being a regulator of inflammasome formation and function (Ting, Lovering et al. 2008, Vladimer, Weng et al. 2012, Shen, Tang et al. 2017). It is reportedly capable of negatively regulating inflammatory responses by suppressing both canonical and non-canonical NF-κB activation and regulating Caspase-1-dependent processing and secretion of cytokines. (Wang, Manji et al. 2002, Williams, Taxman et al. 2003, Williams, Lich et al. 2005, Lich, Williams et al. 2007, Lukens, Gurung et al. 2015). Mutations on NLRP12 are implicated in common variable immunodeficiency (Borte, Celiksoy et al. 2014) and autoinflammatory diseases (Borghini, Tassi et al. 2011, Shen, Tang et al. 2017, Basaran, Uncu et al. 2018), as well as NLRP12-related periodic fever and periodic fever aphthosis pharyngitis adenitis (PFAPA) syndrome (Ter Haar, Lachmann et al. 2013). Induction of EAE on NLRP12 knock-out mice causes increased expression of cyclooxygenase 2 (COX2) and Interleukin-1β (IL-
LPS stimulation in primary microglia from these mice causes increased expression of Inducible Nitric Oxide Synthase (iNOS), Tumor Necrosis Factor (TNF-α) and Interleukin 6 (IL-6), further evidence of its role as a modulator of immune response (Gharagozloo, Mahvelati et al. 2015).

Recently our group has discovered 2 mutations on NLRP12, one which causes a Leucine to be substituted for a Glutamine at position 475 (L475Q), and another which causes a Leucine to be substituted for a Histidine at position 972 (L972H) in familial cases of MS. While to the present day the exact effect these mutations cause is not known, there is a need to investigate the mechanism through which they might cause or contribute to familial MS in those cases. The structure of NLRP12 with the discovered mutations is detailed in Figure 5, and the pedigree of the affected individuals is detailed in Figure 6.

**Figure 5. Structure of NLRP12 protein with novel mutations highlighted.**
As a member of the NLRP family, NLRP12 presents in its sequence a Pyrin domain in its N-terminus, followed by a nucleotide binding site (NBS) and a Leucine Rich Repeat (LRR) region. The mutations found in familial cases of MS, L475Q and L972H are highlighted, together with their predicted locations in the sequence of NLRP12 as catalogued in the NCBI database under accession number NP_653288.1.
1.2.5 Nuclear Receptor Complexes

Nuclear receptors are ligand-activated transcription factors that play integral roles in many physiological processes, such as metabolism, immunity, homeostasis, cell proliferation and development (Perissi and Rosenfeld 2005). Nuclear receptors bind to promoter-specific DNA sequences and interact with co-repressor complexes to inhibit gene expression. Ligand-induced activation of nuclear receptors triggers the dissociation of inhibitory complexes and the recruitment of nuclear receptor co-activator complex components. These co-activator complexes in turn modify histones or regulate chromatin remodelling to promote gene transcription (Perissi and Rosenfeld 2005).

The Nuclear Receptor family of transcription factors includes the vitamin D receptor (VDR), peroxisome proliferator activated receptors (PPARs), and Liver X Receptors (LXRs), which have been reported to play major roles in the pathological processes involved in MS (Rolf, Damoiseaux et al. 2016). VDR is expressed in immune cells and modulates the innate and adaptive immune responses and vitamin D deficiency is commonly seen in patients suffering from MS, with
this deficiency correlating positively with the severity and progression of the disease (Ascherio, Munger et al. 2014, Jelinek, Marck et al. 2015). Activation of PPARs and LXRαs inhibit the Wnt and NF-κB signalling pathways, leading to disrupted inflammatory response and impaired remyelination in MS (Joseph, Bradley et al. 2004, Makoukji, Shackleford et al. 2011, Vallee, Vallee et al. 2018).

1.2.6 LXRαs and Inflammation

Liver X Receptors (LXRαs) are part of the ligand-activated transcription factor family of nuclear receptors that are activated by oxysterols, oxidized derivatives of cholesterol, acting as transcriptional regulators for the expression of key genes involved in cholesterol homeostasis, cholesterol transport, catabolism and absorption (Repa, Turley et al. 2000, Schultz, Tu et al. 2000, Hu, Li et al. 2003, Zhao and Dahlman-Wright 2010). Both isoforms, LXRα and LXRβ, are expressed in the CNS and immune cells, and part of a subclass that forms heterodimers with retinoid X receptors (RXRs) when activated (Joseph, Bradley et al. 2004, Fan, Kim et al. 2008). Their DNA and amino acid sequences are highly conserved between human and rodent species (Song, Kokontis et al. 1994, Willy, Umesono et al. 1995).

In addition to their functions in cholesterol homeostasis, LXRαs have also been reported to regulate inflammatory gene expression and innate immunity (Zelcer and Tontonoz 2006, Bradley, Hong et al. 2007). Activation of LXRαs by ligands inhibits the upregulation of genes directly implicated in inflammation, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), monocytic chemotactic protein-1 (MCP-1) and Interleukin-6 (IL-6), as well as inhibiting the expression of several genes regulated by NF-κB (Castrillo, Joseph et al. 2003, Castrillo, Joseph et al. 2003, Joseph, Castrillo et al. 2003, Joseph, Bradley et al. 2004, Hong, Walczak et al. 2011). LXRα-mediated signalling was also shown to be active in phagocytes present in MS lesions,
indicating that binding of ligands to LXRs could be at least partially involved in the progression of MS (Mailleux, Vanmierlo et al. 2018). A previous study by our group has shown that a mutation in LXRα (NR1H3) disrupts its binding to ligands, resulting in changes in gene expression that contribute to the pathology observed in a familial case of MS (Wang, Sadovnick et al. 2016).

1.2.7 NCOA3

Nuclear receptor coactivator 3 (NCOA3), also known as steroid receptor coactivator 3 (SRC3), is a transcriptional activator of nuclear receptor complexes, including PPAR and LXR, that is capable of recruiting histone acetyltransferases and methyltransferases to induce chromatin remodeling and activation of gene expression (Berrodin, Shen et al. 2010, Xiao, Xu et al. 2010). NCOA3 belongs to the p160SRC family of proteins and interacts with nuclear receptors and transcriptional factors and possesses intrinsic histone-acetyltransferase activity, being capable of remodeling chromatin to induce active transcription that is involved in the control of several metabolic functions (Chen, Lin et al. 1997, Li, Gomes et al. 1997, Reid, Gallais et al. 2009, Xu, Wu et al. 2009, Stashi, York et al. 2014, Kumar, Das et al. 2015).

NCOA3 has been reported to be involved in inflammatory responses, and plays an important role in innate immunity and maintenance of T-cell function (Li, Niu et al. 2012). NCOA3-deficient mice subjected to experimental autoimmune encephalomyelitis (EAE) display decreased inflammation and CNS infiltration, reduced demyelination, as well as reduced overall severity of symptoms. The cause for the reduction has been reported as being the upregulation of PPAR-β, which induced microglial expression of anti-inflammatory cytokines, opsonins and neurotrophic factors (Xiao, Xu et al. 2010).

Recently our group has discovered a mutation in NCOA3, one which causes an Arginine to be substituted for a Cysteine at position 485 (R485C), in familial cases of MS. While to the
present day the exact effect of these mutations is not known, there is a need to investigate the mechanism through which they might cause or contribute to familial MS in those cases. The structure of NCOA3 with the discovered mutations is detailed in Figure 7, and the pedigree of the affected individuals is detailed in Figure 8.

Figure 7. Structure of NCOA3 protein with novel mutation highlighted.
NCOA3 has in its sequence a basic Helix-Loop-Helix (bHLH) sequence, 2 highly conserved Per-Arnt-Sim (PAS) domains, a Steroid Receptor Coactivator (SRC) domain which it shares with SRC-1, and an Acetyltransferase (AT) domain. The mutations found in familial cases of MS, R485C is highlighted, together with their predicted locations in the sequence of NCOA3 as catalogued in the NCBI database under accession number NP_858045.1.

Figure 8. Pedigrees for families with the NCOA3 R485C mutation.
Males are represented by squares and females by circles, a diagonal line indicates subjects known to be deceased. Black filled symbol, MS; gray filled, unaffected obligate carrier. Heterozygote mutation carriers (M) and wild-type (wt) genotypes are provided. MS patients with inferred genotypes are indicated with an asterisk. Contributed by Prof. Vilarino-Güell (University of British Columbia).
1.3 Overall goal of this research

This thesis addresses two projects. Chapter 2 addresses the first, the development of a novel construct for β-secretase. In the second project, chapter 3 addresses the functional analysis of mutations on the NLRP12 gene in a familial case of MS, and chapter 4 addresses the functional analysis of a mutation on the NCOA3 gene.

1.3.1 A novel β-secretase enzymatic assay in real-time in living cells

Given the number of putative substrates of BACE1, there is a need to identify and investigate the factors that cause it to cleave APP through the amyloidogenic pathway. Techniques currently available for evaluating BACE1 activity rely on in vitro assays, non-mammalian cell systems, western blot, ELISA, Mass Spectrometry, fluorescence resonance energy transfer (FRET) which limit their usefulness either due to the associated cost and required equipment, or overall non-specificity of the model. A great number of strategies employed for the measurement and evaluation of BACE1 activity so far focus more on the ability of the substrate to be cleaved, regardless of whether or not it mimics the cleavage of APP in any way. The main goal of these approaches was to develop a tool for screening the ability of new pharmacological compounds to inhibit BACE1 activity, and as such it was deemed necessary to have a substrate that would be easily cleaved by BACE1. Most of these approaches employed completely artificial sequences, which limit their usefulness as tools for evaluating the cleavage of APP (Tomasselli, Qahwash et al. 2003, Pietrak, Crouthamel et al. 2005). Other approaches that do not use an artificial sequence focus on the Swedish variant of APP, incorporated into a peptide that allows for in-vitro measurement of BACE1 activity (Fernandez-Bachiller, Horatscheck et al. 2013, Lee, Samson et al. 2018, Machalkova, Schejbal et al. 2018). The idea of employing in-vitro enzymatic assays is valid in and of itself when one merely aims to evaluate the dynamics and optimal conditions of
enzymatic activity. However, this general approach optimizes conditions for cleavage, instead of replicating the intracellular conditions under which such an enzyme would be naturally occurring.

Cell-based approaches have been tried as a means of overcoming these limitations, with considerable, if limited success. Folk et al. have published in 2012 (Folk, Torosian et al. 2012) a novel method for assaying BACE1 activity in living cells, though their model had its own limitations, as it relied on the Swedish mutation to ensure cleavage of their semi-artificial substrate, and relied on FRET as a means of measuring results. Older approaches such as the one proposed by Oh et al. in 2003 (Oh, Kim et al. 2003) utilized an approach that was much easier to observe, creating a substrate that relied on the Human Placental Secreted Alkaline Phosphatase (SEAP) as a reporter. This model too was flawed, as it relied on the Swedish mutation and used Drosophila sp. cells, meaning results obtained would not necessarily be the same as one could get from mammalian cells. Volbracht et al. (Volbracht, Penzkofer et al. 2009) have tried to overcome these limitations through the generation of a chimeric protein that incorporated the portion of APP targeted by BACE1 to SEAP and the Erythropoietin receptor (EpoR) transmembrane domain, expressed in mammalian cells. This model aimed to demonstrate BACE1 activity in living mammalian cells by correlating cleavage of this construct by BACE1 to the release of SEAP into the cell culture medium, which could then be objectively measured by an in-vitro phosphatase assay of collected culture media. The usage of this construct achieved limited success, though the model itself was held back by unforeseen non-specific cleavage of the custom construct, which caused a relatively high background during the measurement of phosphatase activity. Furthermore, there were concerns as to its efficacy in mimicking native-state APP, as like previous models theirs relied on the Swedish mutation and required BACE1 overexpression in order to generate a detectable signal.
For this specific project, we have endeavored to develop a novel specific chimeric protein construct that would allow us to easily observe, measure and study the specific cleavage of the β-site of Wild-type APP by BACE1 in living cells, in a manner that allows continuous culturing of cells and real-time evaluation of results. We hypothesize that it is possible to achieve specific cleavage of a reporter protein that incorporates natural sequences of Wild-type APP by BACE1 in a living cells. Generating a good model allows us to better experiment on the factors regulating cleavage, test new strategies for reducing cleavage, and generate a tool that can be used to screen for new compounds that aim at modulating or inhibiting BACE1.

1.3.2 Functional evaluation of mutations in familial cases of MS

Given the complexity of the phenomena that lead to or contribute to the symptoms of MS, there is a growing need to identify and evaluate the molecular mechanisms involved. MS is not normally thought to be inherited, but there is an increasing amount of evidence showing that genetic factors play a large role in determining the risk of developing MS, the specific symptoms presented and their severity (International Multiple Sclerosis Genetics, Wellcome Trust Case Control et al. 2011, International Multiple Sclerosis Genetics, Beecham et al. 2013). Our group has discovered mutations in 2 genes, NLRP12 and NCOA3, which are involved in the regulation of inflammatory and immune responses. For this reason, our goal for this project is to identify and characterize the functions impacted by these mutations. Understanding the molecular mechanisms through which these mutations affect basal inflammatory responses and the mediation of immune responses would further our understanding of the basic processes that when disrupted lead to or contribute to MS. Elucidation of the pathways involved, as well as the extent to which they affect symptoms could lead to the development of better experimental models for studying MS, as well as better DMTs and would aid in identifying targets for clinical intervention in the future.
1.3.2.1 NLRP12

As introduced in previous sections, NLRP12 is involved in the formation and proper function of the inflammasome (Ting, Lovering et al. 2008, Vladimer, Weng et al. 2012, Shen, Tang et al. 2017) and regulation of inflammatory responses (Wang, Manji et al. 2002, Williams, Taxman et al. 2003, Williams, Lich et al. 2005, Lich, Williams et al. 2007). Previously described mutations on NLRP12 have been implicated in immune and autoinflammatory diseases (Borghini, Tassi et al. 2011, Ter Haar, Lachmann et al. 2013, Shen, Tang et al. 2017, Basaran, Uncu et al. 2018). In this vein, there is a possibility that the mutations more recently identified, L475Q and L972Q, could affect basic inflammatory processes that could cause or contribute to the development of MS. In order to achieve this, we have decided to focus on whether these mutations can affect the ability of NLRP12 to suppress NF-kB-mediated gene transcription, and its ability to activate Caspase-1 activation.

1.3.2.2 NCOA3

NCOA3 is a transcriptional activator of nuclear receptor complexes such as PPAR and LXR that is also capable of modulating gene expression either by its intrinsic acetyltransferase activity or by recruiting histone acetyltransferases (Berrodin, Shen et al. 2010, Xiao, Xu et al. 2010, Stashi, York et al. 2014, Kumar, Das et al. 2015). While the extent of the functions of NCOA3 is not completely known, there is a possibility that a point mutation such as the one we have discovered (R485C) could affect the protein’s normal functioning leading to a disruption in the modulation of processes in which it is involved. For this reason, in this project we aim to evaluate which of the functions of NCOA3 are affected by the R485C mutation.

The disruption of ligand binding to LXR caused by a mutation alters the expression of genes implicated in MS (Wang, Sadovnick et al. 2016). Mutations such as R485C could affect the
binding of NCOA3 to LXR leading to similar effects. As such, we propose to evaluate whether the R485C mutation of NCOA3 affects its binding to LXR, as well as its translocation to the nucleus.

Another function of NCOA3 that could be affected by the R485C mutation is its ability to affect gene transcription by promoting chromatin remodeling, either through its acetyltransferase domain, or through the recruitment of histone acetyltransferases. We aim to evaluate whether or not the R485C mutation of NCOA3 affects the acetylation of the H3 histone, thus leading to altered gene regulation.

Another possibility we will investigate is whether this mutation is capable of affecting the expression of proteins associated with an inflammatory response, such as COX-2 and iNOS in cultured microglial cells. We also aim to investigate the mechanisms through which NCOA3 affects the expression of these proteins.
Chapter 2: A novel β-Secretase enzymatic assay in real-time in living cells

2.1 Introduction

For this project our goal was to develop a sensitive method for assaying BACE1 activity in living cells that mimics the cleavage of endogenous APP by BACE1 as closely as possible while still generating an easily detectable reporter signal. Our method of choice was generating a chimeric protein construct that incorporates key features of APP necessary for its maturation, secretion, internalization and cleavage by BACE1, while removing those that would lead to cleavage by other secretases or cause undesired or unintended interactions with other proteins.

APP is a structurally complex protein, being capable of interacting with several other proteins (Russo, Venezia et al. 2005, Bai, Markham et al. 2008, Perreau, Orchard et al. 2010). To ensure the construct would only be cleaved specifically by BACE1, we have chosen to restrict the proximal luminal domain of APP to a 48-amino acid sequence (PVDARPAADRGLTTRPGSGLTNKTEIESEVKM-DAEFRHDSGYEVHHQ; from here on referred to as β-region for brevity) that theoretically preserves the natural helical structure of APP while precluding cleavage at any site other than the β-site. To further preserve the natural processing of APP, we have decided to also incorporate its 47 amino acid C-terminal tail (KKQYTSIHGHVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN) containing the YENPTY motif responsible for proper internalization (Perez, Soriano et al. 1999, Thinakaran and Koo 2008). Additionally, the generation of this soluble fragment after the cleavage of the construct by BACE1 allows the correlation of phosphatase readouts to intracellular BACE1 activity by measuring the intracellular concentration of the fragment through western blot.

Despite several reports detailing stronger cleavage of APP by BACE1 at the β-site when there are mutations present (Citron, Oltersdorf et al. 1992, Mullan, Crawford et al. 1992, Zhang,
Wang et al. 2017), these mutations were also seen to impact the normal regulation and processing of APP (Koo and Squazzo 1994, Thinakaran, Teplow et al. 1996, Ben Halima, Mishra et al. 2016, Zhang, Wang et al. 2017), introducing several unknown factors that cannot be accounted for. For this reason, we have chosen to utilize sequences derived from the APP in its Wild-type configuration. The complexity of APP, however, extends to its transmembrane domain as well. The APP transmembrane domain has been reported to be capable of interacting with other proteins (Gibson Wood, Eckert et al. 2003, Perreau, Orchard et al. 2010), and as such could affect observable results due to unknown factors. For this reason, we have decided to replace APP’s native transmembrane domain with that of the Human Major Histocompatibility Complex, Class I, C (HLA-C; VGVAGLAVLAVLAVLGAVMAVVMC). We hypothesize that this sequence, β-site, HLA-C, C-terminus of APP would limit unwanted protein interactions while keeping the desired features intact.

2.2 Version 1 (APP propeptide + Luciferase)

2.2.1 Introduction

As discussed in the introduction, APP and BACE1 follow similar internalization and cycling pathways (Hussain, Powell et al. 1999, Kinoshita, Fukumoto et al. 2003, Araki 2016), and as such, the construct would need to have similar features to APP to ensure the cycling pathway is conserved. For this reason, in this initial iteration of our construct we have chosen to keep the N-terminus of APP intact, as it contains the propeptide that guides APP through its normal modification and maturation pathway. Luciferase was chosen as a reporter due to the ease of manipulation and readout that is conferred by the existing protocols in our laboratory. Structurally, this construct follows the following sequence: APP propeptide, Luciferase, β-site, HLA-C transmembrane domain, APP C-terminus. The structural sequence is illustrated in Figure 9.
**Figure 9. Schematic of APP Luciferase Reporter Construct.**
The construct contains the 47 amino acid region of Wild-type APP with the β cleavage site targeted by BACE1. The propeptide of APP is at its N-terminus to ensure the construct is exported from the ER and follows the same trafficking pathway as endogenous APP. The propeptide is followed by Luciferase as a reporter, the aforementioned β cleavage region of APP, and is anchored to the membrane by the HLA-C transmembrane domain. The transmembrane domain is followed by the 48 amino acid C-terminus of APP containing its internalization signal. Upon cleavage by BACE1 at the β-site (C99), the N-terminal fragment containing Luciferase is released into the cell culture medium, where it can be measured as a means of estimating BACE1 activity. The remaining C-terminal half is retained in the cell, and can be observed through western blot as a means of validating observed results.

2.2.2 Materials and methods

2.2.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. Dulbecco-Modified Eagle Medium (DMEM), and pcDNA4 vectors were purchased from Invitrogen.

2.2.2.2 Plasmids

Plasmids containing the coding region for APP 695 and BACE in pcDNA4 vectors (pzAPP Wt and pzBACE1 respectively) were previously generated in our lab as previously described (Qing, Zhou et al. 2004).
2.2.2.3 Generation of custom protein construct

The custom protein construct was generated by sequentially fusing its different parts through sequential Polymerase Chain Reactions (PCR).

Step 1: The 19 amino acid propeptide of APP was generated using the sequence 5' taatacgactcactatatgagacc as forward primer and the sequence 5' ctttatgttttggcgctttcatctcagcgccagccgcttccaggcc as a reverse primer, using a pcDNA4 plasmid containing the full-length coding sequence of APP previously generated in our lab as a template. The reverse primer utilized partially complements the coding sequence for Luciferase to allow a fusion of the sequences. The end product of this reaction was a sequence encoding the propeptide of APP and the first 24 base pairs of the sequence encoding Luciferase. The forward primer complements the T7 promoter region of the pcDNA4 vector, and introduces Restriction Enzyme (RE) sites in that region of the vector, facilitating cloning.

Step 2: The sequence for Luciferase was cloned from a PGL3 reporter plasmid (Promega), utilizing 5' gcgcgcctggacggctcgctgggctggagatggaagacgccaaaaacataaag as a forward primer, and the sequence 5' ggtcagtctgcctgcagccagccggtttcgcagccc as a reverse primer. For this instance, the forward primer partially complemented the sequence for the APP propeptide, and the reverse primer partially complemented the coding region for the β-site of APP to allow for sequential fusion. The end product of this step was a sequence containing the full coding sequence for Luciferase, flanked partially by the sequence for the APP propeptide on its 5’ end, and the sequence for the 48-amino acid β-site-containing region of APP on its 3’ end.

Step 3: PCR products from Steps 1 and 2 were fused by using the Forward primer for the former, and the reverse primer for the latter, yielding a combined sequence.
Step 4: The 48-amino acid region of APP containing its β cleavage sites was generated using the same template as Step 1, utilizing 5' ggcgcgaagatgcggctgctggtgtgatgcggcgccctgctgccgaccgaggactgace as the forward primer and 5' gccaggccagcaacgatgcccaactgtgatgacaactcatatcc as the reverse primer. The end product consisted of a 186-base pair sequence encoding the 48-amino acid region of APP containing its β cleavage sites, flanked partially by the sequence for the C-terminus of Luciferase on its 5’ end, and the partial sequence for the HLA-C transmembrane domain on its 3’ end.

Step 5: The HLA-C transmembrane domain was generated by utilizing complementing primers to reconstitute its coding sequence artificially (5' gtgggcatcgttgctggcctggctgtcctggctgtcctagctgtcctaggagctgtgatggctgttgtgatgtgt). The sequence 5' ggtatatgaagttcatcatcaagtgggcatcgttgctggcctggctgtcctggctgtcc was used as the forward primer, and the sequence 5' ccgggatccacacatcacaacagccatcacagctcctagg was used as its reverse. The primers utilized added portion of the coding region for the β-region of APP to the 5’ end of the sequence, and a BamHI RE site to its 3’ end. The RE site was added to facilitate fusion with the C-terminus of APP through PCR.

Step 6: PCR products from Steps 4 and 5 were fused utilizing the forward primer for the former, and the reverse primer of the latter.

Step 7: PCR products from Steps 3 and 6 were fused through PCR using the forward primer of Step 1 and the reverse primer of Step 5.

Step 8: The coding region for the 47-amino acid C-terminus of APP was amplified using the same template as Step 1, with 5' cccgggatcaagaagaacagcagcatcattcatgtag as the forward primer and 5' ctagaagggcagctggtgctctcttgtc as a reverse primer. The reverse primer complements the BGH terminator sequence in the pcDNA4, introducing RE sites useful for cloning.
Step 9: PCR products of Steps 7 and 8 were subjected to digestion with the BamHI RE (New England Biolabs) as per the manufacturer’s instructions. After digestion, fragments were combined and ligated utilizing Quick-Stick Ligase (Bioline), following the protocol supplied by the manufacturer.

Step 10: The resulting DNA sequence from Step 9 was digested with the REs XbaI and HindIII and cloned into a pcDNA4 Vector.

2.2.2.4 Cell culture

HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. For transfection, cells were grown to 70% confluence in 35mm plates and transfected with a total of 2.5μg plasmid DNA (2μg Construct; 0.5μg BACE1) and Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio.

2.2.2.5 Luciferase assay

Cell cultured medium was collected 24 hours after transfection, and measurement of Luciferase activity was carried out utilizing the Dual-Glo Luciferase Assay System (Promega) following the protocol supplied by the manufacturer.

2.2.2.6 Statistical analysis

Two-tailed Student’s t-test was used to compare group differences. Analyses were performed using the GraphPad Prism software.
2.2.3 Results

Transfection of HEK cells with the Luciferase-APP construct as well as BACE1 generated a significantly detectable amount of Luciferase activity in the medium when compared to mock transfected cells (0.00125 ± 0.001010 SEM for mock transfected cells vs. 0.01058 ± 0.002587 for cells transfected with the construct and BACE1; p=0.0283). Cells transfected with the Luciferase-APP construct alone displayed a detectable signal, but were not statistically different from either the mock transfected cells (0.00125 ± 0.001010 SEM for mock transfected cells vs. 0.007167 ± 0.003703 SEM for cells transfected with the construct; p=0.1981) or the cells transfected with both the construct and BACE1 (0.007167 ± 0.003703 SEM for cells transfected with the construct vs. 0.01058 ± 0.002587 SEM for cells transfected with the construct and BACE1; p=0.4916) (Figure 10).
Figure 10. Luciferase Assay of Version 1 of the construct.
HEK cells were transfected with a plasmid encoding the APP-Luciferase construct either alone or with a plasmid encoding BACE1. Empty Vector (pcDNA4) was used as a control. 24 hours after transfection culture media from transfected cells was collected and Luciferase activity in the media was measured using a luminometer. *: p=0.0283 when compared to pcDNA4. N=3 experiments done in triplicate; Bars represent SEM.

2.2.4 Considerations

The main goal of the generation of a custom protein construct such as this one is to allow for easy observation of BACE1 activity in living cells through the use of a reporter that could be detected in the cell culture medium with minimal disturbance to cultured cells. One of the goals of the initial design of the construct was ensuring that it would follow the same secretory pathway as
APP, and in doing so, allow it to encounter BACE1 and be selectively cleaved by it at the Asp1 β-site included in its sequence. In order to achieve that, we decided to include the propeptide of APP on its N-terminus, which theoretically would cause it to undergo the same processing as APP. We have also removed all other parts of APP that to our knowledge could cause unexpected interactions, in the hope of generating a simple, effective and reliable reporter for BACE1 activity in cells.

The significant increase in Luciferase activity in the medium when BACE1 is overexpressed indicates that the construct is being cleaved, and the N-terminal fragment containing Luciferase is being released outside the cells. However, the fact that the construct being overexpressed on its own generates a signal that is high enough to be detected and comparable to that of cells also overexpressing BACE1, yet not high enough to be distinguishable from mock transfected cells is concerning, though it could be due to one or several different factors. First, there is the possibility that contrary to our expectations, the considerably artificial nature of the construct causes it to undergo a processing pathway that is fundamentally different from that of APP. This would mean that the results observed here are artifacts, and not consequences of the cleavage of the construct. Second, there is the possibility that the construct is cleaved not by BACE1 but other proteases, and this non-specific cleavage could generate a detectable signal regardless of actual BACE1 activity. Third, there is a possibility that transfection and cell manipulation cause enough cell death and membrane rupture, allowing the full-length form of the construct to leak into the medium. This, together with a relatively low sensitivity of Luciferase as a reporter could be enough to make it difficult to distinguish the signal generated by the cleavage of the construct from the noise generated by other non-specific processes. Fourth and finally, there is a possibility that the construct does undergo the same maturation and secretion pathway as APP
and BACE1, but Luciferase as a reporter does not retain its activity. Interestingly the stability and activity of Luciferase are negatively affected by low pH (Lundin 2014, England, Ehlerding et al. 2016) and the endosomal pathway that APP, and therefore the construct undergoes is a relatively acidic environment. This evidence suggests that if we are to rework the construct in order to generate a reliable reporter for BACE1 activity, it is necessary to replace Luciferase with a reporter that would better survive the conditions to which the protein construct would be subjected to prior to secretion.

2.3 Version 2 (APP propeptide + eGFP)

2.3.1 Introduction

Based on the results of the initial version of the construct described in the previous section, we decided to replace the initial reporter, Luciferase, with one that would be more resilient to the intracellular conditions the custom protein construct would be subjected to before its secretion. Enhanced Green Fluorescent Protein (F64L variant; eGFP) is widely used for biological applications, can be detected through many different methods, and is resistant to low pH environments such as the one encountered in endosomes (Kneen, Farinas et al. 1998, Elsliger, Wachter et al. 1999). The remainder of the construct remained unchanged, and its structure is detailed in Figure 11.
Figure 11. Schematic of APP eGFP Reporter Construct.
The construct contains the 47 amino acid region of Wild-type APP with the β cleavage site targeted by BACE1. The propeptide of APP is at its N-terminus to ensure the construct is exported from the ER and follows the same trafficking pathway as endogenous APP. The propeptide is followed by eGFP as a reporter, the aforementioned β cleavage region of APP, and is anchored to the membrane by the HLA-C transmembrane domain. The transmembrane domain is followed by the 48 amino acid C-terminus of APP containing its internalization signal. Upon cleavage by BACE1 at the β-site, the N-terminal fragment containing eGFP is released into the cell culture medium, where it can be measured as a means of estimating BACE1 activity. The remaining C-terminal half is retained in the cell, and can be observed through western blot as a means of validating observed results.

2.3.2 Materials and methods

2.3.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA4 vectors were purchased from Invitrogen. The plasmid vector encoding eGFP used as a template (pEGFP-N3) was purchased from Clontech.

2.3.2.2 Generation of custom protein construct

The custom protein construct was generated by sequentially fusing its different parts through sequential PCR described as follows:

Step 1: The propeptide of APP was amplified utilizing the T7 primer described in the previous section as the forward primer, and 5’ gtcctgcctggttcctcgacgcagcgccggcggcct as the
reverse primer. As a result, a small sequence complementing the coding sequence of eGFP was attached to the 3’ end of the propeptide.

Step 2: The coding sequence for eGFP was amplified from pEGFP-N3 using 5’ ggacggctgggcgctggagatggtgagcaagggcgagg as the forward primer, and 5’ ggcatggacgagctacaagccttgatgcggccgctgc as the reverse primer. These primers introduced overlapping regions that complement the APP propeptide on the 5’ end and complement the sequence for the 48-amino acid β-site-containing region of APP on its 3’ end.

Step 3: PCR products from the previous steps were fused using the forward primer from Step 1 and the reverse primer from Step 2, generating a sequence encoding the propeptide of APP followed by the full coding sequence of eGFP, with a sequence overlapping the sequence for the 48-amino acid β-site-containing region of APP on its 3’ end.

Step 4: Using the APP-Luciferase construct from the previous section as a template, the final 21 nucleotides of the coding sequence of eGFP were fused to the β-site-containing region of APP using 5’ ggcatggacgagctacaagccttgatgcggccgctgc as the forward primer, and the BGH primer from the previous section as the reverse primer.

Step 5: PCR products from Steps 3 and 4 were fused utilizing T7 and BGH primers.

Step 6: The resulting DNA sequence from Step 5 was digested with the REs XbaI and HindIII and cloned into a pcDNA4 Vector.

2.3.2.3 Cell culture

HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50µg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. For transfection, cells were grown to 70% confluence in 35mm plates and transfected with a total of
2.5μg plasmid DNA (2μg Construct; 0.5μg BACE1) and Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio.

2.3.2.4 Drug treatment

4 hours after transfection, culture medium was changed and cells were treated with either BACE1 inhibitor IV (C3; EMD Millipore) diluted in DMSO to a final concentration of 5μM, or DMSO at a 1:1000 dilution.

2.3.2.5 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics) 24, 48 or 72 hours post-transfection. Protein concentration was determined by Bradford assay (Bio-rad) and each sample was diluted with 4x SDS loading buffer (200mM Tris-HCl pH 6.8, 400mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol). Cell culture media samples were collected 24, 48 and 72 hours post-transfection and diluted with 4x SDS loading buffer to a final volume of 50μl. Samples were resolved by SDS-PAGE on 10% Tris-glycine or 16% Tris-tricine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. Primary antibodies diluted in the blocking medium were incubated overnight at 4℃. The primary antibodies are detailed as follows: C20, a rabbit antibody against the last 20 amino acids of APP’s C-terminus developed in our lab (Ly, Wu et al. 2013), was used to detect APP and its C-Terminal fragments (CTFs) at a 1:1000 dilution. Mouse anti-β-actin monoclonal antibody AC-15 (Sigma, 1:5000). Mouse anti-GFP sc-9996 (Santa Cruz Biotechnology, 1:1000 dilution). 9E10, monoclonal mouse antibody against the myc tag was used to detect BACE1 (1:1000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye
800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

2.3.2.6 Statistical analysis

Two-tailed Student’s t-test was used to compare group differences. Analyses were performed using the GraphPad Prism software.

2.3.3 Results

Transfection of HEK cells with the variant eGFP-APP construct together with BACE1 generated a C-terminal fragment (CTF) that is readily observable through western blot as early as 48 hours post-transfection. Inhibition of BACE1 with C3 eliminated CTF generation, indicating that the construct is being cleaved by BACE1. Presence of the N-terminal fragment (NTF) containing GFP was low in the cell culture medium, and high in cell lysates, indicating low efficiency of secretion of the reporter fraction of the construct after cleavage (Figure 12)
Figure 12. Western blot detection of the Version 2 construct after transfection.
HEK cells were transfected with the APP eGFP construct either alone or with BACE1, cells were harvested and culture media collected 24, 48 or 72 hours post-transfection. Cells were treated with C3, a BACE1 inhibitor, at a concentration of 5μM to confirm the cleavage of the construct by BACE1. Cleavage of the construct by BACE1 generated a C-terminal fragment (CTF) detectable with C20, and an N-terminal fragment (NTF) detectable with an antibody against GFP. The relatively low amount of GFP-containing fragment in the medium could be a consequence of the low efficiency of secretion, or retention of the GFP-positive NTF in the cells (N=3).

2.3.4 Considerations

Given the limitations of Luciferase as a reporter, we have redesigned our protein construct to use utilize eGFP, as it is resilient to the intracellular conditions under which cleavage occurs, and is more easily detectable. While GFP was detected in the culture medium of cells transfected with the construct and BACE1, the presence of a GFP-positive C-terminally truncated form of the construct in cell lysates indicates much of the resulting N-terminal fragment is not secreted outside cells after cleavage, severely limiting the usefulness of this version of the construct as a reporter for BACE1 activity.
These results do however help us understand the limitations of the design of the construct thus far. In the previous section we detailed how the initial version of the construct, which utilized Luciferase as a reporter, didn’t generate a strong enough signal to be detectable by a luminometer. The results shown here with the use of a modified version utilizing GFP make it unmistakably evident that cleavage is taking place due to the generation of a CTF detectable with the C20 antibody and a GFP-positive NTF, whose generation are abolished when BACE1 is inhibited. These results indicate that the main issue we face with the design of this construct as a reporter is not cleavage itself, but the secretion of the reporter-containing NTF after cleavage. This means that there are two aspects that need to be taken into consideration if the construct is to be used to assay BACE1 in cultured cells. First, we need to ensure the construct and its resulting cleavage products can be properly secreted from cells. Second, we need to choose a reporter that can be more easily detected in solution without the need to concentrate the medium or subject samples to additional steps for detection.

2.4 Version 3 (SEAP + eGFP)

2.4.1 Introduction

Building upon the results obtained from the previous iterations of the construct, we decided to change the reporter and the secretion signal, as it has become evident that the initial design had considerable issues with secretion efficiency. The reporter we have chosen in an attempt to rectify these shortcomings was the Secreted form of Human Placental Alkaline Phosphatase (SEAP). SEAP is widely used in biological applications as a reporter due to its sensitivity, strong secretion profile, heat resistance and resistance to low pH (Birkett, Done et al. 1966, Berger, Hauber et al. 1988, Tannous and Teng 2011), features which if maintained when SEAP is expressed as part of a longer chimeric protein would greatly enhance the usability of the
construct as a tool for measuring BACE1 activity. We have chosen to keep Enhanced Green Fluorescent Protein (eGFP) as a secondary reporter, as it could be used to confirm results. As the goal of this version of the construct was optimizing secretion, we decided to forego the usage of the APP propeptide, and rely on the propeptide and secretion signal of SEAP alone to guide the full length construct into the secretory pathway. The general structure of this version of the construct is described in Figure 13.

Figure 13. Schematic of APP SEAP eGFP Reporter Construct.
The construct contains the 47 amino acid region of Wild-type APP with the β cleavage site targeted by BACE1. Secreted Alkaline Phosphatase (SEAP) is utilized as both a reporter and a means of promoting secretion of the full-length protein after expression. SEAP is followed by eGFP as a secondary reporter, the aforementioned β cleavage region of APP, and is anchored to the membrane by the HLA-C transmembrane domain. The transmembrane domain is followed by the 48 amino acid C-terminus of APP containing its internalization signal. Upon cleavage by BACE1 at the β-site, the N-terminal fragment containing SEAP and eGFP is released into the cell culture medium, where they can be measured as a means of estimating BACE1 activity. The remaining C-terminal half is retained in the cell, and can be observed through western blot as a means of validating observed results.
2.4.2 Materials and Methods

2.4.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA4 vectors were purchased from Invitrogen.

2.4.2.2 Plasmids

The plasmid generated for the previous version of the construct (APP+eGFP) was used as a template. The sequence for SEAP was cloned from the pSEAP2-Basic plasmid (Clontech).

2.4.2.3 Generation of custom protein construct

The coding sequence for SEAP was amplified from pSEAP2-Basic plasmid utilizing 5’tcggaagcttgccaccaatgctgctgctgctgctgctgggcctgaggctac as the forward primer, introducing a HindIII RE site and a Kozak sequence (gccacc) to drive expression in mammalian cells, and 5’gcccttgctcaccatggtggtgcc as a reverse primer to add a short sequence complementing the coding sequence for eGFP on its 3’ end. The remainder of the construct, consisting of eGFP, the β-site of APP, the transmembrane domain and the C-terminus of APP was amplified using 5’ggcaccaccatatgtggtgagcaagggc as the forward primer, adding a short sequence complementing that of SEAP on its 5’ end, and BGH as the reverse primer. The resulting PCR products of the 2 reactions were fused utilizing the forward primer used to amplify SEAP as the forward primer and BGH as the reverse primer. The resulting full sequence was then cloned into a pcDNA4 vector between the HindIII and XbaI sites.

2.4.2.4 Cell culture

HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. For
transfection, cells were grown to 70% confluence in 35mm plates and transfected with a total of 2.5μg plasmid DNA (2μg Construct; 0.5μg BACE1) and Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio.

2.4.2.5 Drug treatment

4 hours after transfection, culture medium was changed and cells were treated with either BACE1 inhibitor IV (C3; EMD Millipore) diluted in DMSO to a final concentration of 5μM, or DMSO at a 1:1000 dilution.

2.4.2.6 Phosphatase assay

Phosphatase activity in the culture media collected from transfected cells was measured with the Tropix Phospha-Light Kit from Thermo-Fisher. Assay was carried out as per the manufacturer’s instructions.

2.4.2.7 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics) 24, 48 or 72 hours post-transfection. Protein concentration was determined by Bradford assay (Bio-Rad) and each sample was diluted with 4x SDS loading buffer (200mM Tris-HCl pH 6.8, 400mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol). Cell culture media samples were collected 24 hours post-transfection and diluted with 4x SDS loading buffer to a final volume of 50μl. The samples were resolved by SDS-PAGE on 10% Tris-glycine or 16% Tris-tricine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. Primary antibodies diluted in the blocking medium were incubated overnight at 4°C. The primary antibodies are detailed as follows: C20, a rabbit antibody against
the last 20 amino acids of APP’s C-terminus developed in our lab (Ly, Wu et al. 2013), was used to detect APP and its C-Terminal fragments (CTFs) at a 1:1000 dilution. Mouse anti-β-actin monoclonal antibody AC-15 (Sigma, 1:5000). Mouse anti-GFP sc-9996 (Santa Cruz Biotechnology, 1:1000 dilution). 9E10, monoclonal mouse antibody against the myc tag was used to detect BACE1 (1:1000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

2.4.2.8 Statistical analysis

Two-tailed Student’s t-test was used to compare group differences. Analyses were performed using the GraphPad Prism software.

2.4.3 Results

The construct generated a clear, easily detectable C-terminal fragment (CTF) when transfected together with BACE1. Generation of this CTF was abolished when cells were treated with C3, a BACE1 inhibitor (Figure 14). After cleavage by BACE1, the N-terminal part of the construct containing GFP and SEAP was secreted into the cell culture medium, where it could be detected through western blot by using an antibody against GFP. Inhibition of BACE1 activity with C3 abolished the generation and secretion of this fragment (Figure 15).

Cleavage of the construct by endogenous BACE1 generated a secreted N-terminus containing SEAP, whose activity could be easily detected through a phosphatase assay (0.02715 ± 0.003263 SEM for mock transfected cells vs. 100.0 ± 6.875 SEM for cells transfected with the construct; p<0.0001). Co-transfection with BACE1 caused a roughly 50% increase in the readout, indicating that phosphatase activity in the medium increases with the amount of active BACE1 in cells (100.0 ± 6.875 SEM for cells transfected with the construct alone vs. 156.1 ± 11.57 SEM for
cells transfected with the construct and BACE1; \( p=0.0019 \). Treating cells with C3, a BACE1 inhibitor reduced the observed activity to levels comparable to cells transfected with the construct alone (156.1 ± 11.57 SEM vs. 87.71 ± 7.751 SEM; \( p=0.0006 \)) (Figure 16).

**Figure 14. Western blot detection of the Version 3 construct post-transfection.**
HEK cells were transfected with the APP eGFP construct either alone or with BACE1, cells were harvested 24, 48 or 72 hours post-transfection. Cells were treated with C3, a BACE1 inhibitor, at a concentration of 5\( \mu \)M to confirm the cleavage of the construct by BACE1. Samples were resolved by SDS-PAGE in a 10% Tris-glycine gel for the detection of the Full-length construct and BACE1. The C-terminal fragment (CTF) generated from the cleavage of the construct by BACE1 was resolved in a 16% Tris-tricine gel (\( N=6 \)).
Figure 15. Western blot detection of secreted Version 3 construct post-transfection.
HEK cells were transfected with the APP SEAP eGFP construct either alone or with BACE1. Cells and culture media were collected 24 hours post-transfection. Cells were treated with C3, a BACE1 inhibitor, at a concentration of 5μM to confirm the cleavage of the construct by BACE1. Samples were resolved by SDS-PAGE in a 10% Tris-glycine gel (N=2).
Cell culture media was collected from cells transfected with an empty vector (Control), the SEAP eGFP APP construct either alone or with BACE1 24 hours post-transfection. Cells were treated with 5μM C3, a BACE1 inhibitor to check if the observed effect was due to BACE1 activity. N=6 experiments done in triplicate; **: p<0.01; ***: p<0.0001, Two-tailed Student’s t-test.

2.4.4 Considerations

One of the biggest limitations of the previous versions of the construct was that the NTF containing reporters generated after cleavage by BACE1 was not released into the cell culture medium, making observation and quantification of results difficult. Our goal with the use of SEAP in this version of the construct was to incorporate a reporter with a better secretion profile, and by
doing so create a sensitive, easily observable and measurable tool to evaluate BACE1 activity in living cells. Replacing the secretion signal of APP with that of SEAP did not seem to provide any measurable improvement in secretion of the construct as seen through western blot in Figure 15, though the results obtained through a measurement of phosphatase activity in the medium tell a different story.

While secretion as measured through western blot did not show any improvement, measuring phosphatase activity in the medium caused by the presence of SEAP-containing NTFs shows us that perhaps the issue we are facing is not necessarily one of secretion efficiency, but one of sensitivity of measurements and the protocol chosen to evaluate results. Measuring release of reporters in the medium through a phosphatase assay is visibly more sensitive than methods previously attempted in this study, with cells expressing the construct either alone or with BACE1 generating a high readout and mock transfected cells generating no detectable signal.

One issue does stand out with this methodology, the relatively high signal generated from cells transfected with the construct alone when compared to cells transfected with both the construct and BACE1. There is a possibility that cells are lysed during manipulation and leak full-length construct, generating a basal background phosphatase activity that is not derived from cleavage. Another possibility is that endogenous BACE1 activity is strong enough to generate a detectable signal, though the fact that BACE1 inhibition does not completely negate this potential endogenous cleavage suggests otherwise. It is possible however that the BACE1 inhibitor employed is not specific or potent enough to eliminate all BACE1 activity, meaning we would have to experiment with better and more specific inhibitors to rule out this possibility. Another possibility is that the construct is being cleaved by proteases other than BACE1, and this needs to be investigated if the construct is to be used as an indicator of BACE1 activity.
The results obtained are promising nonetheless, though there is room for improvement. SEAP is resistant to the conditions to which APP, and therefore the construct is subject to, but its secretion profile is reportedly somewhat limited (Tannous and Teng 2011), meaning secretion could be optimized by substituting the secretion signal of SEAP with one that is more efficient, or an additional secretion signal could be attached to the N-terminus of the construct.

2.5 Version 4 (Albumin + SEAP + eGFP)

2.5.1 Introduction

As discussed in the previous section, while the basic concept of the construct worked as theorized, its usefulness and sensitivity was hindered by an apparent low efficiency of secretion, which created a concerning high background that could affect future experiments. Here we have attempted to rectify this shortcoming by employing an additional secretion signal. The sequence we have chosen for this purpose is the 18 amino acid propeptide/secretion signal of human albumin (MKWVTFISLLFLFSSAYS), which was chosen due to its strong secretion-promoting profile, and because it is not entirely artificial to mammalian cells unlike other potentially stronger signals (Kober, Zehe et al. 2013, Attallah, Etcheverrigaray et al. 2017). Due to the sequence of the construct being Albumin-SEAP-eGFP-β-site at its N-terminus, we have named the construct ASGβ, as a means of simplifying terminology and setting this version of the construct apart from previous iterations. We have decided to change the expression vector from pcDNA4 to pcDNA3.1, as a means of ensuring better co-transfection with BACE1, which utilizes pcDNA4, and minimizing possible interference.

Another significant change we have decided to make in our methodology is the usage of a new, highly specific BACE1 inhibitor, Verubecestat (MK-8931). Verubecestat possesses a stronger BACE1 inhibition profile, being effective at much lower concentrations (Menting and
Claassen 2014, Vassar 2014, Scott, Li et al. 2016), and was being actively investigated in clinical trials before being discontinued due to adverse effects (Hawkes 2017, Egan, Kost et al. 2018). Nevertheless, its potency and specificity makes it a good candidate for experimentation in cells, especially if we are focusing on BACE1 activity specifically. The sequence and structure of ASGβ is detailed in Figure 17, and its theoretical mechanism of cleavage and secretion is illustrated in Figure 18.

![Figure 17. Schematic of ASGβ.](image)

This construct contains the 47 amino acid region of Wild-type APP with the β cleavage site targeted by BACE1. Secreted Alkaline Phosphatase (SEAP) is utilized as a reporter, followed by eGFP as a secondary reporter, the aforementioned β cleavage region of APP, and is anchored to the membrane by the HLA-C transmembrane domain. The transmembrane domain is followed by the 48 amino acid C-terminus of APP containing its internalization signal. The secretion signal of human Albumin (MKWVTISLLFLFSSAYS) was added to the N-terminus of SEAP to optimize secretion. Upon cleavage by BACE1 at the β-site, the N-terminal fragment containing SEAP and eGFP is released into the cell culture medium, where they can be measured as a means of estimating BACE1 activity. The remaining C-terminal half of ASGβ is retained in the cell, and can be observed through western blot as a means of validating observed results.
Upon transfection into cells alongside BACE1, both it and ASGβ are expressed and anchored to the plasma membrane. BACE1 recognizes the β-cleavage site of APP on ASGβ and cleaves it, releasing its N-terminus containing SEAP and eGFP into the extracellular cell culture medium where it can be collected for further analyses. The remaining C-terminal part of ASGβ remains anchored to the membrane by its transmembrane domain. The YENPTY internalization signal present in the C-terminus of APP replicated on ASGβ causes the C-terminal fragment (CTF) to be internalized. The internalized CTF can then be measured and quantified through western blot as a means of confirming BACE1 activity by correlating its levels with SEAP activity in the extracellular media.

2.5.2 Materials and Methods

2.5.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA3.1 A (-) vector were purchased from Invitrogen. Verubecestat was purchased from MedChemExpress.

2.5.2.2 Generation of custom protein construct

Plasmid ASGβ (Albumin-SEAP-eGFP-β site) was generated by sequentially fusing its different parts through sequential Polymerase Chain Reactions (PCR). The 54 amino acid Albumin
signal peptide was generated through PCR using 5’ccgctagcgcaccatgaagtggttaacctttatttcccttttttc as the forward primer and 5’cccagcagcagcagcagcagcagcatctagaggaataagccgagctaaagagaaaaagaagggaaa as the reverse primer. This PCR product was then fused to the full length sequence utilizing the forward primer from the reaction above and 5’cggaagctttcactagtctgtcgctcttgtctaa as the reverse primer. The resulting PCR product was then cloned into a pcDNA3.1 A (-) vector between NheI and HindIII.

2.5.2.3 Cell culture

HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. For transfection, cells were grown to 70% confluence in 35mm plates and transfected with a total of 2.5μg plasmid DNA (2μg ASGβ; 0.5μg BACE1) and Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio.

2.5.2.4 Drug treatment

24 hours after transfection, culture medium was changed and cells were treated with either Verubecestat diluted in DMSO to a final concentration of 2.5nM, or DMSO at a 1:1000 dilution.

2.5.2.5 Phosphatase assay

Phosphatase activity in the culture media collected from transfected cells was measured with the Tropix Phospha-Light Kit from Thermo-Fisher. Assay was carried out as per the manufacturer’s instructions.

2.5.2.6 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease
inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine or 16% Tris-tricine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. APP was detected with C20, a rabbit antibody against the last 20 amino acids of APP’s C-terminus (Ly, Wu et al. 2013), to detect APP and its C-Terminal fragments (CTFs). SEAP was detected with a Rabbit anti-Alkaline Phosphatase antibody (NBP1-57907; NOVUS Biologicals, 1:200 dilution). GFP was detected with a Mouse anti-GFP sc-9996 (Santa Cruz Biotechnology, 1:1000 dilution). β-actin was detected with a mouse anti-β-actin monoclonal antibody (AC-15; Sigma; 1:5000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

2.5.2.7 Statistical analysis

Two-tailed Student’s t-test was used to compare group differences. Analyses were performed using the GraphPad Prism software.

2.5.3 Results

Cloning of the coding sequence for ASGβ was successful, as indicated by its high level of expression in HEK cells detected with antibodies against the C-terminus of APP (C20), GFP or SEAP (Figure 19). Transfection of cells with ASGβ generated easily detectable phosphatase activity in the medium 24 hours post-transfection (0.04792 ± 0.01269 SEM in mock transfected cells vs. 100 ± 0 SEM in cells transfected with ASGβ; p<0.0001) (Figure 20A). Inhibiting BACE1 with Verubecstat caused a roughly 80% reduction of phosphatase activity in the medium (100 ± 0 SEM in cells transfected with ASGβ vs. 19.57 ± 1.791 SEM in cells transfected with ASGβ treated with Verubecstat; p<0.0001) (Figure 20A). Overexpression of BACE1 together with
ASGβ caused a roughly 3-fold increase in phosphatase activity in the medium (100 ± 0 SEM in cells transfected with ASGβ vs. 304.2 ± 27.96 SEM in cells transfected with ASGβ and BACE1; p<0.0001) (Figure 20B). Inhibition of BACE1 with Verubecestat was able to counter this increase, bringing the phosphatase readout to levels comparable to when BACE1 is inhibited without overexpression (100 ± 0 vs. 16.95 ± 2.649 SEM; p<0.0001) (Figure 20C).
Figure 19. Characterization of ASGβ construct.
(A) Plasmid schematic. ASGβ was cloned into a pcDNA3.1 A (-) vector for transfection into mammalian cells, generating a plasmid roughly 8kb in size that can be used in mammalian cells. (B) Agarose gel electrophoresis of the pcDNA3.1 A (-) vector containing the ASGβ sequence. Digestion of the plasmid using the NheI and HindIII restriction enzymes yields a sharp insert band of roughly 2.5kb. (C) Western blot of HEK cells after transfection with ASGβ. ASGβ is highly expressed in HEK cells after transfection, and its full-length form can be detected with antibodies against the C-terminus of APP (C20), GFP, or Human Placental Alkaline Phosphatase (SEAP).
2.5.4 Considerations

The addition of the Albumin secretion signal to the N-terminus of ASGβ resulted in stronger secretion, making the cleaved N-terminal part containing SEAP and GFP more easily detectable in the medium. This enhanced secretion caused the phosphatase signal in the medium resulting from co-expression with BACE1 to become significantly higher, making it more evident that phosphatase activity can be correlated to the amount of active BACE1 in cells.

Inhibition of BACE1 with Verubecestat also yielded very interesting results. Despite its failings in clinical trials, Verubecestat is a very specific and potent BACE1 inhibitor, and that shows in the experiments we have done so far. Application of Verubecestat to cells transfected with ASGβ and BACE1 caused a dramatic reduction in phosphatase activity in the medium, confirming that the increase in phosphatase activity in the medium does indeed occur as a function of intracellular BACE1 activity. Interestingly, Verubecestat was also capable of markedly reducing the amount of phosphatase activity in the medium generated when ASGβ is transfected alone by
roughly 80%, which suggests that the signal generated in that case comes not from leakage of full-length ASGβ, but largely from the activity of endogenous BACE1 present in HEK cells.

The remainder of activity when BACE1 is inhibited, however, remains unaccounted for, and we need to run further experiments to investigate whether or not this is unavoidable background noise caused by overexpression and manipulation of cells, or due to unspecific cleavage of ASGβ by other proteases. We will address these possibilities in the following section.

2.6 Specificity of ASGβ to BACE1

2.6.1 Introduction

From the very beginning of this project, we have aimed to incorporate into ASGβ only the parts of APP that would be relevant for its cleavage by BACE1, while removing any others that might cause non-specific results, such as interactions with other proteins, or non-specific cleavage by other proteases. Nevertheless, unless there is extensive experimentation to rule all other possibilities, we cannot rule out the possibility of it being targeted, cleaved or degraded by other intracellular proteases. BACE1 is part of the family of aspartyl proteases (Vassar, Bennett et al. 1999, Lin, Koelsch et al. 2000, Holsinger, Goense et al. 2013), of which its close homolog, BACE2, is also a member (Sun, He et al. 2006, Kandalepas and Vassar 2014). This means that there is a possibility that aspartyl proteases other than BACE1 could target and cleave ASGβ, generating background noise and possible false positives, and for that reason it needs to be investigated.

Another possibility is that ASGβ could be cleaved by Cathepsins. Cathepsins are divided into Serine proteases (Cathepsins A and G), Aspartic proteases (D and E) and Cysteine proteases (B, C, F, H, K, L, O, S, V, X and W) (Turk, Stoka et al. 2012), some of which have been reported to be capable of cleaving APP and generating Aβ in some models and were thought of as secretases
until relatively recently (Higaki, Catalano et al. 1996, Klein, Felsenstein et al. 2009, Schechter and Ziv 2011). This ability could cause some Cathepsins to recognize ASGβ and cleave it, a possibility that needs to be investigated if ASGβ is to be used as a tool for specifically measuring BACE1 activity.

There is also the possibility that the inhibitor we have chosen to use, Verubecestat, has off-target effects, and could inhibit proteases other than BACE1. We shall address this possibility by employing an alternative BACE1 inhibitor, LY-2886721 and comparing it to results obtained with Verubecestat. LY-2886721 is another potent and specific BACE1 inhibitor, which was used in clinical trials before being pulled due to concerns with the side effects experienced by patients (Lahiri, Maloney et al. 2014, May, Willis et al. 2015).

Finally, but not less importantly, there is the possibility that even if ASGβ is cleaved specifically and selectively by BACE1, this cleavage does not take place at the Asp1 site (β-site; C99). In this section we will also address this possibility.

2.6.2 Materials and methods

2.6.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM, pcDNA3.1 and pcDNA4 vectors were purchased from Invitrogen. Verubecestat and LY-2886721 were purchased from MedChemExpress.

2.6.2.2 Cell culture

HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). For transfection, cells were grown to 70% confluence in 35mm plates and
transfected with a total of 2.5μg plasmid DNA (2μg ASGβ; 0.5μg BACE1) and Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio.

2.6.2.3 Drug treatment

24 hours after transfection, culture medium was changed and drugs were added to the cell culture media. Drugs used and their concentrations are as follows: Verubecestat (2.5nM); LY-2886721 (30nM); E64 (2.5μM); E64D (5μM); Pepstatin-A (2μM). DMSO diluted at a 1:1000 ratio was used as a control treatment.

2.6.2.4 Phosphatase assay

Phosphatase activity in the culture media collected from transfected cells was measured with the Tropix Phospha-Light Kit from Thermo-Fisher. The assay was carried out as per the manufacturer’s instructions.

2.6.2.5 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine or 16% Tris-tricine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. Membranes were incubated with C20, a rabbit antibody against the last 20 amino acids of APP’s C-terminus (Ly, Wu et al. 2013), to detect APP and its C-Terminal fragments (CTFs). SEAP was detected with a Rabbit anti-Alkaline Phosphatase antibody (NBP1-57907; NOVUS Biologicals, 1:200 dilution). GFP was detected with a Mouse anti-GFP sc-9996 (Santa Cruz Biotechnology, 1:1000 dilution). 9E10, a monoclonal mouse antibody against the myc tag was used to detect BACE1 (1:1000 dilution). Detection of CTFs derived from cleavage of ASGβ at the
β site was carried out using a streptavidin-conjugated 82E1 antibody (IBL America; 1:1000 dilution). β-actin was detected with a mouse anti-β-actin monoclonal antibody (AC-15; Sigma; 1:5000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

2.6.2.6 Statistical analysis

Two-tailed Student’s t-test was used to compare group differences. Analyses were performed using the GraphPad Prism software.

2.6.3 Results

As shown in the previous section, overexpression of BACE1 alongside ASGβ caused a roughly three-fold increase in active phosphatase in the culture medium of transfected HEK cells. Application of LY2886721 yielded similar results as Verubecestat in reduction of the enzymatic activity (277.3±16.02% SEM vs. 42.74±3.10% SEM for LY2886721; and 277.3±16.02% SEM vs. 41.35±1.647% SEM for Verubecestat). E64, a Cysteine protease inhibitor, and E64D, an inhibitor of Cathepsins B, H and L, were incapable of suppressing the increase in phosphatase activity caused by the ectopic expression of BACE1. Pepstatin A, an Aspartic protease was capable of partially preventing this increase (277.3±16.02% vs. 160.1±52.25%, p<0.01) (Figure 21).

Upon transient transfection, ASGβ has a high expression level, being easily detectable in cells using antibodies against the C-terminus of APP (C20), SEAP or GFP (Figure 22A). Co-transfection of ASGβ with BACE1 generates an intracellular C-terminal fragment (CTF), which could be detected with C20. Inhibition of BACE1 with Verubecestat eliminated the generation of this fragment. The CTF generated by cleavage of ASGβ is also detected by 82E1, an antibody raised against the N-terminus of APP cleaved specifically at the Asp1 site (C99) (Figure 22B).
Figure 21. Specificity of cleavage of ASGβ by BACE1.
HEK cells were transfected with ASGβ either alone or together with BACE1 (B1), and inhibitors against different proteases were added to the cell culture medium 24 hours post-transfection. Medium samples were collected 24 hours after the addition of inhibitors, and tested for phosphatase activity. Specific inhibition of BACE1 with LY-2886721 (LY) and Verubecestat (Vb) significantly decreased phosphatase activity in the medium. Cathepsin inhibitors E64 and E64D had no observable effect on phosphatase activity caused by overexpression of ASGβ and BACE1. Aspartic-protease inhibitor Pepstatin A (Pep) caused a partial, non-significant reduction in phosphatase activity in the medium. Values are expressed as percentages, and variations are shown as SEM of repeat experiments carried out in triplicate. N≥2; *p<0.01 when compared to ASGβ alone.
Figure 22. Cleavage of ASGβ at the β-site by BACE1.
(A) ASGβ is highly expressed upon transfection, and can be detected using antibodies against the C-terminus of APP (C20), or antibodies against GFP and SEAP. (B) Western blot of HEK cells after transfection with ASGβ. Expression of ASGβ generates a C-terminal-fragment band that can be recognized by both C20 and a specific antibody against the C-terminal fragment of APP cleaved at the β-site (82E1). Specific inhibition of BACE1 with Verubecestat (Vb) abolished the generation of this band (N=3).

2.6.4 Considerations

As shown in previous sections, co-expression of ASGβ generates an easily measurable phosphatase activity in the medium. While it was still unknown to which extent this activity was related to intracellular BACE1 activity, preliminary data had shown that the release of active phosphatase into the medium could be greatly reduced by the application of BACE1 inhibitors. Our results here show that the results observed were not due to off-target effects of our chosen inhibitor, Verubecestat, as we were able to obtain similar results by utilizing a competitor’s inhibitor, LY-2886721.

As Cathepsins are reportedly capable of cleaving APP in some models, this created a possibility that they could in some manner cleave ASGβ, generating false positives and...
compromising its usefulness as a tool for measuring BACE1 activity. Our results show however, that inhibiting Cathepsins with E64 or E64D did not reduce phosphatase readout, indicating that if such non-specific cleavage of ASGβ occurs, it is not to the extent that it causes measurable changes to the observed results. Interestingly, treatment of cells with Pepstatin-A caused a partial, albeit not significant reduction in the phosphatase signal generated from cleavage of ASGβ. While certainly this could indicate other Aspartic proteases cleave ASGβ, it is likely that this result is derived from the partial inhibition of BACE1 caused by Pepstatin-A, as BACE1 is one such Aspartic protease.

There remains the possibility that while ASGβ is cleaved specifically by BACE1, the site of cleavage is not specific, which would limit the potential of ASGβ as a tool and experimental model. We aimed to address this concern by verifying whether or not the CTF generated after its cleavage could be identified by 82E1, an antibody specific against the N-terminus of APP cleaved specifically at the Asp1 site (Horikoshi, Mori et al. 2004). As our results show, the CTF generated can be identified by both 82E1 and C20, an antibody against the C-terminus of APP, indicating that the cleavage by BACE1 is indeed specific at the Asp1 site. This result shows that not only ASGβ can be used to screen BACE1 activity, it can be used to evaluate cleavage at the Asp1 site specifically. This is especially intriguing due to the fact ASGβ contains both the Asp1 (C99; KM-DA) and Glu11 (C89; GY-EV) sites and there are no mutations that would either be conducive or counter to cleavage at the Asp1 site.

These findings lead us to two conclusions that are worth investigating in the future. First, that it is indeed possible to observe preferential cleavage at the Asp1 site utilizing an APP sequence devoid of any mutations or alterations that enhance cleavage. This indicates ASGβ could be a great model to investigate and measure the cleavage of Wild-type, non-mutated APP by BACE1. Second,
these results indicate that there are possible features in the structure of APP that dictate cleavage site selection by APP, and those would be located in regions we have chosen to omit from the whole sequence of ASGβ. This generates exciting possibilities for ASGβ as a tool for studying the regulatory factors surround the cleavage of Wild-type APP by BACE1 in the future.

For such possibilities to become reality however, we need to better establish ASGβ as a model, and make it into a tool that can be readily used for future experimentation and measurement of BACE1 in living cells.

2.7 Development of cell line stably expressing ASGβ

2.7.1 Introduction

As shown in previous sections, ASGβ is usable as a tool to easily measure BACE1 cleavage at the Asp1 site of APP. The fact that its experimental use requires transient transfection either alone or alongside BACE1 however creates room for considerable variability in results, either due do the inherent harm caused to cultured cells during transfection, or the efficiency of transfection efficiency used. Lower transfection efficiency, low expression due to unsuitable culture conditions, or variations in the rate of effectively transfected ASGβ or BACE1 are sources of variation and error that could confound future results and limit the usefulness of the model. Aiming to overcome these limitations, we have generated a cell line that stably expresses both ASGβ and BACE1, which could then be used directly as a means of reliably measuring BACE1 activity without the need to transfec
t cells.

2.7.2 Materials and methods

2.7.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM, pcDNA3.1 A (-) and pcDNA4 vectors were purchased from Invitrogen.
2.7.2.2 Cell culture

HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). For transfection, cells were grown to 70% confluence in 35mm plates and transfected with a total of 2.5μg plasmid DNA (2μg ASGβ; 0.5μg BACE1) and Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio. Selection of stable cell lines after transient transfection was carried out using Zeocin (100μg/ml) and Geneticin (200μg/ml). Cells were subjected to selection conditions until few cells remained. After colonies started to grow from the few remaining cells, colonies were picked and allowed to grow in a maintenance medium containing Zeocin (50μg/ml) and Geneticin (G418; 100μg/ml).

2.7.2.3 Phosphatase assay

Phosphatase activity in the culture media collected from transfected cells was measured with the Tropix Phospha-Light Kit from Thermo-Fisher. Assay was carried out as per the manufacturer’s instructions.

2.7.2.4 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine gels, then transferred to Nitrocellulose membranes. Membranes were incubated with C20, a rabbit antibody against the last 20 amino acids of APP’s C-terminus (Ly, Wu et al. 2013), to detect ASGβ. 9E10, monoclonal mouse antibody against the myc tag was used to detect BACE1 (1:1000 dilution). β-actin was detected with a mouse anti-β-actin monoclonal antibody (AC-15; Sigma; 1:5000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-
labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

2.7.3 Results

Culture of transfected cells with the use of selection agents Zeocin and Geneticin resulted in complete elimination of cells that did not incorporate the transfected plasmids into their genome. Of these colonies, 12 were picked and allowed to grow in isolation. Phosphatase activity in the culture medium of selected colonies was measured as a means of evaluating whether or not cells are expressing transfected sequences. Of the 12 colonies picked, only colony 11 showed detectable phosphatase activity in the medium (Figure 23A). Western blot of cell lysates from picked colonies showed that while most had become capable of expressing BACE1 at high levels, only colony 11 was expressing ASGβ at a detectable level (Figure 23B).

Figure 23. Selection of Colonies transfected with ASGβ and BACE1.
HEK cells were transfected with a pcDNA3.1 plasmid encoding ASGβ and a pcDNA4 plasmid encoding BACE1. 24 hours after transfection culture medium was changed and selection agents Zeocin and G418 were added. Concentration of selection agents in culture media was maintained until survival of cells was limited to a few slowly growing colonies of cells still attached to the culture dish. (A) Phosphatase assay of colonies of cells subjected to selection. 12 colonies were labeled, picked and allowed to grow in isolation from one another. Cell culture medium was harvested from these cells and phosphatase activity measured. Of 12 colonies picked, only colony 11 had detectable phosphatase activity in the medium. Culture medium from non-transfected cells was used as a negative control (Ctrl). N=2 repeats; numbers on the graph represent readout in arbitrary units. (B) Western blot of picked colonies after selection. After selection colonies were passaged and allowed to grow, whereupon cells were lysed and expression of ASGβ and BACE1 was checked through western blot. While all colonies had considerable expression of BACE1, only colony 11 had a strong expression and cleavage of ASGβ (N=2).
2.7.4 Considerations

During the generation of the version of ASGβ detailed in the previous section, we had foreseen that selection of cells expressing both ASGβ and BACE1 would be difficult unless we changed the expression vector of either of them to allow for selection utilizing different selection agents. As results have shown, incorporation of both sequences into the genome of cells was a difficult process, with most of the colonies failing to properly incorporate ASGβ, and the entire selection process lasting upwards of 10 months. This could be due to the nature and size of the sequences being transfected, as well as discrepancies between the efficiency of selection agents Zeocin and G418 when used concomitantly. Nevertheless, the colony expressing both ASGβ and BACE1 at high levels became usable in normal experimental settings, as we will demonstrate in the following section.

2.8 Evaluation of intracellular BACE1 activity

2.8.1 Introduction

Following the generation of a cell line stably expressing ASGβ and BACE1, it has become necessary to evaluate its usefulness as a method to measure intracellular BACE1 activity. We have evaluated here how our stable cell line would respond to different concentrations of BACE1 inhibitors in regards to secretion of phosphatase in the medium. For this goal, we have decided to use Verubecestat and LY-2886721 as BACE1 inhibitors due to their potency and efficacy at low concentrations (Menting and Claassen 2014, Vassar 2014, Yan and Vassar 2014, May, Willis et al. 2015, Scott, Li et al. 2016). As a control we have elected to utilize Pepstatin-A, as it does not affect BACE1 activity, as we demonstrated in previous sections.
2.8.2 Materials and methods

2.8.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM was purchased from Invitrogen. Verubecestat and LY-2886721 were purchased from MedChemExpress.

2.8.2.2 Cell culture

ASGβ/BACE1 stable cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, 50µg/ml Streptomycin sulfate, 50µg/ml Zeocin and 100µg/ml Geneticin (G418) (Invitrogen).

2.8.2.3 Drug treatment

ASGβ/BACE1 stable cells were lightly trypsinized and seeded on 96-well plates. 24-hours after seeding, Verubecestat (2.5nM), LY-2886721 (30nM) or Pepstatin-A (2µM) were added to the culture media.

2.8.2.4 Phosphatase assay

Phosphatase activity in the culture media collected from transfected cells was measured with the Tropix Phospha-Light Kit from Thermo-Fisher. Assay was carried out as per the manufacturer’s instructions.

2.8.2.5 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour.
Membranes were incubated with C20, a rabbit antibody against the last 20 amino acids of APP’s C-terminus (Ly, Wu et al. 2013), to detect ASGβ. 9E10, a monoclonal mouse antibody against the myc tag was used to detect BACE1 (1:1000 dilution). β-actin was detected with a mouse anti-β-actin monoclonal antibody (AC-15; Sigma; 1:5000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

2.8.2.6 Statistical analysis

Two-tailed Student’s t-test was used to compare group differences. Analyses were performed using the GraphPad Prism software.

2.8.3 Results

As previously demonstrated, ASGβ/BACE1 stable cells express both ASGβ and BACE1 at high levels, being easily detectable through western blot (Figure 24A). BACE1 inhibitors Verubecestat and LY2886721 caused a significant drop in measurable phosphatase activity even at their lowest concentration (0.1nM). Phosphatase activity further decreased as the concentration of either Verubecestat or LY2886721 increased, indicating phosphatase activity in the medium is correlated to intracellular BACE1 activity. Increasing the concentration of Pepstatin-A, however, did not produce any significant dose-dependent effect on phosphatase activity (Figure 24B).
Figure 24. Effects of BACE1 inhibition on measurable phosphatase activity.

(A) Western Blot of HEK cells stably expressing ASGβ and BACE1 (B1). Stable cells strongly express both BACE1 and ASGβ, the latter being easily detected by an antibody against the C-terminus of APP, and presenting itself as a sharp band roughly 120kDa in size when resolved in a 10% acrylamide gel. (B) Stable cells were treated with different concentrations of the specific BACE1 inhibitors LY2886721 (LY) and Verubecestat (Vb), and the Aspartyl-protease inhibitor Pepstatin-A (Pep). 24 hours after addition of the inhibitors, medium samples were collected and assayed for phosphatase activity. Phosphatase activity in the medium decreased in a predictable manner with increased concentrations of either LY2886721 or Verubecestat, indicating Phosphatase activity can be correlated with BACE1 inhibition. Increasing the concentration of Pepstatin used did not produce any significant dose-dependent effect on phosphatase activity. N=3. *p<0.05; **p<0.01; ***p<0.001.

2.8.4 Considerations

Our goal with the present experiment was to evaluate the usefulness of ASGβ/BACE1 stable cells as tools to screen compounds that could inhibit BACE1 activity. Our results show that Phosphatase activity in the medium decreases as the concentration of BACE1 inhibitor increases, indicating that the phosphatase readout can be reliably correlated with intracellular BACE1 activity. For this particular set of experiments we decided to downscale the experiment, utilizing 96-well plates instead of the usual 35mm dishes. There are two main reasons for this decision. First, we wanted to evaluate whether or not the signal generated by the cleavage from ASGβ would still be easily detectable with a much reduced number of cells per well. As we could see, the
phosphatase reporter system is sensitive enough to allow easy observation of results at smaller scales. Second, we wanted to evaluate whether or not the system we designed could still be utilized in a manner that would allow for high-throughput assay that allows for continuous culture of cells with chronic treatments. As demonstrated here, results can be obtained without the need to lyse cells or interrupt treatments, which potentially allows for easier, real-time trials with several different compounds at the same time.

2.9 Swedish variant of ASGβ

2.9.1 Introduction

As demonstrated in previous sections, it is possible to achieve specific cleavage of a substrate by BACE1 utilizing the sequence of Wild-type APP, without the need to introduce mutations or other factors that might enhance cleavage. Given the specificity of cleavage at the Asp1 (C99) site instead of the non-amyloidogenic Glu11 (C89) despite both being present in the sequence, it raises the question of how mutations might affect cleavage under the more controlled and limited conditions imposed by the sequence of ASGβ. Familial mutations such as the Swedish one (K670N/M671L) reportedly and unmistakably increase cleavage preference at the Asp1 site (Citron, Oltersdorf et al. 1992, Mullan, Crawford et al. 1992), though the factors other than the amino acid substitution that cause this shift in cleavage preference are not known. The sequence of ASGβ limits its interaction with BACE1 to the 48 amino acids that match the β-region of APP, and therefore could possibly allow us to study this interaction in isolation, without any other confounding factors that might affect the cleavage of endogenous APP by BACE1. For this reason, here we propose to introduce the Swedish mutation into the sequence of ASGβ, and evaluate how that mutation changes cleavage as observed through the previously shown methods of phosphatase
activity and western blot. The comparative sequences of Wild-type and Swedish variants of ASGβ are detailed in Figure 25.

![Figure 25. Introduction of Swedish mutation into ASGβ.](image)

In order to evaluate the effects of the Swedish mutation (K670N/M671L) on BACE1 cleavage of ASGβ, the Swedish mutation was introduced to the β-cleavage site of ASGβ by point-directed mutagenesis. No further alterations were made to the sequence of the ASGβ construct.

### 2.9.2 Materials and Methods

#### 2.9.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA3.1 vector were purchased from Invitrogen.

#### 2.9.2.2 Plasmids

Plasmids containing the coding regions for BACE1 and ASGβ were generated as described in previous sections.
2.9.2.3 Cloning

The Swedish mutation of APP (K670N/M671L) was introduced into the sequence of ASGβ through site-directed mutagenesis. The mutation was introduced by amplifying the N-terminal part of ASGβ using the T7 forward primer and the sequence 5’ cggaattgtcaggttcacttcagatctctcc as the reverse primer. The resulting PCR product was then digested with EcoRI and NheI and inserted into the pz-ASGβ plasmid between these cloning sites, replacing the Wild-type N-terminus of ASGβ with the one containing the mutation.

2.9.2.4 Cell culture

HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). For transfection, cells were grown to 70% confluence in 35mm dishes and transfected with a total of 2.5μg plasmid DNA (2μg ASGβ; 0.5μg BACE1) and Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio.

2.9.2.5 Phosphatase assay

Phosphatase activity in the culture media collected from transfected cells was measured with the Tropix Phospha-Light Kit from Thermo-Fisher. Assay was carried out as per the manufacturer’s instructions.

2.9.2.6 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine or 16% Tris-tricine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature
for 1 hour. Membranes were incubated with C20, a rabbit antibody against the last 20 amino acids of APP’s C-terminus (Ly, Wu et al. 2013), to detect APP and its C-Terminal fragments (CTFs). GFP was detected with a Mouse anti-GFP sc-9996 (Santa Cruz Biotechnology, 1:1000 dilution). β-actin was detected with a mouse anti-β-actin monoclonal antibody (AC-15; Sigma; 1:5000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

2.9.2.7 Statistical analysis

Two-tailed Student’s t-test was used to compare group differences. Analyses were performed using the GraphPad Prism software.

2.9.3 Results

Cloning of the Swedish mutation into ASGβ yielded a sequence that is similar to the Wild-type form of the protein and that exhibits a detectable phosphatase activity in the cell culture medium when transfected into HEK cells. Unlike the Wild-type form however, co-expression of BACE1 does not cause a significant increase in phosphatase activity in the cell culture medium (Figure 26). Western blot of lysates of cells transfected with the Swedish variant of ASGβ together with BACE1 shows strong cleavage of Swedish ASGβ as seen by the large increase in CTF generation together with a decrease in the amount of full-length protein. Cleavage does however also generate a C-terminally truncated form of the protein that can be detected by an antibody against GFP, but not one against the CTF of APP (C20) (Figure 27). This indicates that the N-terminus of Swedish ASGβ is retained in cells after cleavage.
Figure 26. Phosphatase assay of the Swedish variant of ASGβ.
Cell culture media was collected from cells transfected with an empty vector (Control), ASGβ Wild-type (Wt) or Swedish (Swe) either alone or with BACE1 (B1) 24 hours post-transfection, and phosphatase activity in the medium was measured. Co-transfection with BACE1 caused a significant increase in phosphatase activity from Wild-type ASGβ. No such effect was observed with the Swedish variant. N=8 experiments done in triplicate; ***: p<0.001, Two-tailed Student’s t-test.
2.9.4 Considerations

Mutations such as Swedish causing a stronger cleavage of APP at the Asp1 site is a well-established fact in the field of AD. The particular causes for this stronger cleavage are not completely elucidated however, and here we described our attempt to investigate the role of that mutation in isolation by using a reporter system of our design. As expected, the Swedish mutation did cause a considerably stronger cleavage of ASGβ, though surprisingly that stronger cleavage was not accompanied by a corresponding increase in phosphatase activity in the medium. The marked increase in CTF generation, along with a decrease in total levels of the full-length modified
ASGβ unmistakably shows that cleavage by BACE1 is taking place. Cleavage by BACE1 generated a considerable amount of an N-terminal fragment containing SEAP and GFP, though western blot shows that this fragment is contained within cells, not being secreted as you would expect given the results obtained from Wild-type ASGβ. Given that the only confirmed difference in sequence between the Wild-type and Swedish variants of ASGβ is the KM>NL substitution, this creates the unique possibility that substitution of these 2 amino acids alone dramatically affects the trafficking and processing of the full-length protein, therefore impeding its secretion. This is a possibility that we will explore further in the following section.

2.10 Secreted ASGβ and the Swedish Mutation

2.10.1 Introduction

As shown in the previous section, the introduction of the Swedish mutation to ASGβ increases its cleavage by BACE1 without generating a corresponding increase in phosphatase signal in the medium after cleavage. One possible explanation for this result is that the last 2 amino acids of the resulting N-terminal fragment of the cleaved protein affect its secretion. Here we propose to investigate this possibility by generating truncated variants of ASGβ that mimic the resulting N-terminal fragment post-cleavage. These forms of ASGβ therefore end at the Asp1 (C99) site, with the last 2 amino acids being KM for the Wild-type, and NL for the Swedish variant, lacking the transmembrane domain and C-terminus of APP. A scheme for the proposed sequence of these truncated forms is given in Figure 28. We will evaluate whether or not these variants differ in terms of retention rate in cells.
Figure 28. Secreted truncated forms of Wild-type and Swedish ASGβ.
In order to evaluate the effects of the Swedish mutation on the secretion of ASGβ, altered forms of the protein that lack the portion C-terminal to the Asp1 site were designed and cloned. The resulting proteins lack a C89 site, the transmembrane domain and the internalization motif present in the full-length versions of ASGβ.

2.10.2 Materials and methods

2.10.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA3.1 vector were purchased from Invitrogen.

2.10.2.2 Plasmids

Plasmids containing the coding regions for BACE1 and ASGβ were generated as described in previous sections.
2.10.2.3 Cloning

Truncated Wild-type ASGβ was generated using the plasmid encoding Wild-type ASGβ using the T7 sequence as a forward primer, and 5’ cgggaagctttcatcttctcagagatctcctc as the reverse primer. Truncated Swedish ASGβ was generated using the plasmid encoding Swedish ASGβ using the T7 sequence as a forward primer, and 5’ cgggaagctttcacaagttcacttcagagatctcctc as the reverse primer. Resulting sequences were then cloned into pcDNA3.1 vectors between NheI and HindIII.

2.10.2.4 Cell culture

HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). For transfection, cells were grown to 70% confluence in 35mm dishes and transfected with a total of 2.5μg plasmid DNA (2μg ASGβ; 0.5μg BACE1) and Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio. 24 hours after transfection, cell culture medium was collected, and cells lysed in RIPA-DOC lysis buffer (1%Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics).

2.10.2.5 Phosphatase assay

Phosphatase activity in the culture media collected from transfected cells and total cell lysates was measured with the Tropix Phospha-Light Kit from Thermo-Fisher. Assay was carried out as per the manufacturer’s instructions.

2.10.2.6 Statistical analysis

Two-tailed Student’s t-test was used to compare group differences. Analyses were performed using the GraphPad Prism software.
2.10.3 Results

Truncated forms of ASGβ are readily secreted, and generate an easily detectable phosphatase activity in the cell culture medium, with no significant differences between the Wild-type and Swedish variants when they are either transfected alone or with BACE1 (Figure 29A). Transfected cells were lysed 24 hours after transfection, and phosphatase activity in cell lysates was measured as a means of estimating the amount of truncated ASGβ retained in cells. No significant difference between groups was observed in cell lysates (Figure 29B). In order to estimate whether or not the Swedish mutation caused a higher rate of retention of truncated ASGβ, the ratio in phosphatase activity between the medium and cell lysates was calculated (Figure 29C). No significant differences were observed between the groups, indicating that the mutation does not affect the retention of the truncated fragment in cells.

Figure 29. Phosphatase activity of Secreted truncated forms of Wild-type and Swedish ASGβ.

In order to evaluate whether or not the Swedish form of secreted ASGβ gets retained in cells, cells were transfected with Wild-type (Wt) or Swedish (Swe) ASGβ, either alone or with BACE1 (B1). Phosphatase activity was measured in (A) cell culture medium from transfected cells, and (B) whole cell lysates from these same cells. While it appears that the Swedish mutation causes some retention of truncated ASGβ, there is no significant difference in the ratio of phosphatase activity between the medium and cell lysates (C). N=3.
2.10.4 Considerations

In the previous section, we demonstrated that the Swedish mutation increases cleavage of ASGβ without a corresponding increase in the amount of phosphatase activity in the medium. Our initial hypothesis was that the amino acid substitution caused by the mutation would cause the resulting N-terminal fragment to be retained. We aimed to test this hypothesis by creating truncated forms of both Wild-type and Swedish ASGβ that theoretically mimic the N-terminal fragments generated by cleavage of their full-length counterparts at the Asp1 site. If our assumptions were correct, the change in the final amino acids of this N-terminal fragment from the normal KM to the mutant NL would cause the truncated form of the protein to be retained, indicating that these two amino acids are crucial for secretion of the protein after cleavage. To our surprise, this substitution did not cause any observable differences, indicating these two amino acids by themselves do not affect secretion.

While an apparent contradiction, these results hint at a possibility that it is not the secretion itself that is affected, but perhaps the intracellular location where cleavage occurs. The Swedish mutation could be causing ASGβ to be cleaved at an earlier stage in its maturation or cycling, generating an N-terminal fragment in an environment that would not be conducive to its secretion outside the cell. While the data presented here is too limited to allow us to make any assertion with confidence, it could indicate that the Swedish mutation of APP causes increased Aβ generation not only due to a more efficient cleavage by BACE1, but also by altering the stage in the cycling and maturation of both APP and BACE1 where they can encounter each other and cleavage can occur. As it stands however, further experimentation is necessary to confirm or deny this possibility.
Chapter 3: Effects of NLRP12 mutations L475Q and L972H on inflammation

3.1 Introduction

As discussed in the Introduction, nucleotide-binding oligomerization domain-like receptor (NLR) Family Pyrin Domain Containing protein 12 (NLRP12), is a regulator of immune inflammatory responses, and mutations that affect its normal functioning being reported as causing either immunodeficiency (Borte, Celiksoy et al. 2014) or autoinflammatory disorders (Borghini, Tassi et al. 2011, Ter Haar, Lachmann et al. 2013, Shen, Tang et al. 2017, Basaran, Uncu et al. 2018). One possible mechanism through which NLRP12 could be affecting overall inflammatory response is through its ability to regulate NF-κB-mediated gene transcription (Wang, Manji et al. 2002, Williams, Lich et al. 2005, Lich, Williams et al. 2007). NF-κB activation is a crucial step in the activation of proinflammatory signalling pathways, with both its canonical and alternative pathways being heavily involved in the expression of other proinflammatory genes such as cytokines, chemokines and adhesive molecules (Lawrence 2009). There is a possibility that the mutations we have discovered are capable of disrupting this basal regulation of inflammatory response. For this reason, here we propose to evaluate the effects the L475Q and L972H mutations have on NF-κB activation by means of a Luciferase reporter-based assay.

NLRP12 is also, like other proteins in the NLRP family, involved in the assembly and functioning of the inflammasome, a function that is crucial for proper immune response to infection (Martinon, Burns et al. 2002, Guo, Callaway et al. 2015). Inflammasomes are crucial parts of cellular response to proinflammatory molecules, regulating inflammation and immune responses by participating in the production of pro-inflammatory cytokines such as IL-1β and interleukin-18 (IL-18) (Martinon, Mayor et al. 2009). The majority of inflammasome assemblies consist of a NOD-like receptor (NLRP3, NLRP6, NLRP7, NLRC4 or NLRP12), an apoptosis-
associated-speck-like protein with a carboxyterminal CARD (ASC) and pro-Caspase-1. Assembly of the inflammasome causes cleavage of pro-Caspase-1 into its active form, which then proceeds to cleave IL-1β and IL-18 into their active forms. Catalytic activity of Caspase-1 is tightly regulated by inflammasomes (Martinon, Burns et al. 2002, Schroder and Tschopp 2010) and this regulation is a critical function that could be affected by mutations such as L475Q and L972H. Here we propose to evaluate the effects the aforementioned mutations have on activation of Caspase-1 by NLRP12.

3.2 Materials and methods

3.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA4 vectors were purchased from Invitrogen.

3.2.2 Plasmids

Plasmids containing FLAG-tagged full length Wild-type, L475Q and L972H human NLRP12 we kindly donated by Dr. Beckley Davis (Franklin & Marshall College, USA) and Dr. Carles Vilariño-Güell (University of British Columbia). NF-κB p65 expression plasmid and pNF-κB-Luciferase Reporter were generated in our lab as previously described (Chen, Zhou et al. 2012).

3.2.3 Cell culture

HEK293 cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. Cells were transfected with 200ng of a pcDNA4 vector containing the coding region for either Wild-type NLRP12, or its L475Q or L972H mutant forms, together with 200ng of a reporter PGL3 plasmid with a response element for NF-κB. Cells were co-transfected with a vector to express p65 in order to induce NF-
κB activation. 15ng of a vector containing Renilla Luciferase was transfected into cells as an internal control. Empty PGL3 vector plasmid was used instead of NLRP12 and p65 as a negative control. For the evaluation of Caspase-1 activation, murine microglial BV2 cells were transfected with 200ng of a pcDNA4 vector containing the coding region for either Wild-type NLRP12, or its L475Q or L972H mutant forms.

3.2.4 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. The primary antibodies used are as follows: Caspase-1/p10 (Santa Cruz; sc-56036, 1:1000 dilution), FLAG (for NLRP12) (Sigma; F7425, 1:1000 dilution) and β-actin (Sigma; A5316, 1:1000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences). Quantification of relative protein amounts after western blot was carried out using Quantity One software.

3.2.5 Luciferase assay

Cell cultured medium was collected 24 hours after transfection, and measurement of Luciferase activity was carried out utilizing the Dual-Glo Luciferase Assay System (Promega) following the protocol supplied by the manufacturer.
3.2.6 Statistical analysis

Two-tailed Student’s t-test and analysis of variance (ANOVA) followed by Tukey’s posthoc test were used to compare group differences. Analyses were performed using the GraphPad Prism software.

3.3 Results

Transfection of HEK cells with Wild-type NLRP12 together with an expression vector for NF-κB activator p65 and a Luciferase reporter with an NF-κB response element severely reduced expression of the reporter, a result that corresponds to the reported function of NLRP12 as a repressor of NF-κB-activated gene transcription. Transfection of NLRP12 L475Q or L972H did not significantly affect this reduction, indicating this function of NLRP12 is not affected by these mutations (Figure 30).

Overexpression of Wild-type NLRP12 in BV2 caused a significant increase in levels of Pro-Caspase-1 and activated Caspase-1 (p10). Similar results were obtained with overexpression of the L475Q mutant. Overexpression of the L972H mutant elicited an increased expression of Pro-Caspase-1 without a corresponding increase in p10 levels, indicating impaired Caspase-1 activation when this variant is expressed (Figure 31).
Figure 30. Inhibition of NF-κB activation by NLRP12 L475Q and L972H mutants.
HEK cells were transfected with vectors containing either the Wild-type (Wt), L475Q or L972H mutant forms of NLRP12, together with a Luciferase-expressing vector driven by an NF-κB response element and a vector containing the coding sequence for p65 in order to induce NF-κB activation. Empty vectors were used as negative controls. Neither the L475Q nor the L972H mutations were capable of reducing the effects of NLRP12 on NF-κB activation. Values are represented as percentages of control. N=3 repeats; bars represent SEM.
Figure 31. Effects of NLRP12 mutants on Caspase-1 activation.
(A) Western blot showing expression of pro-caspase-1, caspase-1, NLRP12 and β-actin in microglial (BV2) cells transfected with an empty vector (vector), wild-type NLRP12 (WT) or mutant constructs (L475Q or L972H).
(B) Quantification of caspase-1 expression in BV2 cells transfected with either vector, wild-type NLRP12 or its mutant forms. Mean caspase-1 expression ± standard error is provided. *Tukey’s HSD post hoc p-value < 0.001. r.u. relative units (N≥3).

3.4 Considerations

Experimentation with the L475Q and L972H variants of NLRP12 has shown us that these specific point mutations do not affect the ability of NLRP12 to inhibit NF-κB signalling. While the exact mechanism through which NLRP12 is capable of affecting NF-κB is not yet completely understood, studies suggest that it does so through its nucleotide binding site (NBS) (Lich, Williams et al. 2007, Lukens, Gurung et al. 2015). While it is not possible to make a confident claim without thorough structural evaluation and experimentation, it is possible that the aforementioned mutations do not affect NF-κB suppression since they are not located in the NBS domain proper.

Interestingly however, the L972H mutation negatively impacted the maturation of Caspase-1, which points towards impaired inflammasome assembly as a possible mechanism. Inflammasome assembly requires oligomerization of NLRs through their NBS (NOD/NACHT) domains after being activated by a ligand, which then recruits ASC as an adaptor protein. ASC is
thought to function as a molecular platform upon which Caspase-1 can finally be activated (de Alba 2009, Vanaja, Rathinam et al. 2015). While the initial oligomerization of NLRs such as NLRP12 is thought to occur through its NBS domain, there is a possibility that a mutation within its LRR domain such as L972H could affect pairing and oligomerization, therefore disrupting inflammasome assembly. This creates the distinct possibility that any changes that might affect structural conformation of NLRs and their pairing and oligomerization negatively affect the assembly and proper functioning of the inflammasome. As it stands however, further experimentation and comparison with other mutations and evaluation of protein binding would be necessary to confirm or deny this possibility.

In summary, the results herein show that the novel NLRP12 mutation L972H affects immune response by causing decreased activation of Caspase-1. This discovery shows that impaired inflammasome assembly of function could be one of the factors that affect the progression and severity of MS.
Chapter 4: Effects of NCOA3 mutation R485C on inflammation

4.1 Introduction

As previously discussed in the Introduction, as a member of the p160SRC family of proteins NCOA3 plays a role in a wide range of biological processes and metabolic pathways. (Stashi, York et al. 2014, Pecenova and Farkas, 2016). Among its functions, of particular interest is its ability to regulate inflammatory responses to infection, with the lack of NCOA3 reportedly leading to impaired immune response (Chen, Chen et al. 2010, Li, Niu et al. 2012). There is a possibility that the newly discovered R485C mutation in familial cases of MS affects its ability to properly regulate immunity, and if that is the case, it is first necessary to determine which function of NCOA3 is impaired, and how it would contribute to the development of the symptoms in MS.

NCOA3 is known to exert its effects on gene transcription by activating nuclear receptor complexes such as LXR (Berrodin, Shen et al. 2010, Xiao, Xu et al. 2010), and a previous study by our group has shown that a mutation found in a familial case of MS disrupted proper LXR-mediated signaling (Wang, Sadovnick et al. 2016). Based on these results, we hypothesize that the R485C mutation of NCOA3 affects its ability to bind to LXR, thus leading to disrupted LXR-mediated gene regulation, a possibility that we explored and will explain in this chapter.

Another mechanism through which NCOA3 affects gene regulation is the induction of chromatin remodeling, either by recruiting histone acetyltransferases and methyltransferases or through its intrinsic histone-acetyltransferase activity (Berrodin, Shen et al. 2010, Xiao, Xu et al. 2010). One of the targets of NCOA3 is histone H3 (Wagner, Koslowski et al. 2013, Drazic, Myklebust et al. 2016), and if the R485C mutation affects the ability of NCOA3 to acetylate histones, it is possible that the pattern of acetylation on histone H3 would be significantly altered, a possibility that we also investigated.
It is also possible that the R485C mutation could affect the activation of enzymes involved in inflammatory response, such as inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2), through mechanisms other than the ones previously mentioned. Enzymes iNOS and COX-2 have been shown to play pivotal roles in the development of inflammatory diseases (Cedergren, Forslund et al. 2002, Cross and Wilson 2003, Murakami and Ohigashi 2007), and studies have shown higher levels of iNOS and COX-2 in MS lesions from patients as well as in experimental models of MS (Rose, Hill et al. 2004, Carlson, Rojas et al. 2010). This creates the possibility indicating that the R485C mutation of NCOA3 could be contributing to the symptoms of MS by upregulating these enzymes, a possibility which we also addressed.

Finally, we have attempted to characterize the mechanisms through which R485C NCOA3 causes an increased expression level of iNOS. We evaluated the transcriptional regulation of iNOS by NCOA3, and analyzed the ability of R485C NCOA3 to activate the promoter region of iNOS, approaches which we will detail in this chapter.

4.2 NCOA3 and LXR binding

4.2.1 Introduction

Among other functions, NCOA3 is a transcriptional activator of nuclear receptor complexes such as the Liver X Receptors (LXRs), through which it is capable of regulating gene expression (Berrodin, Shen et al. 2010, Xiao, Xu et al. 2010). LXRs have been implicated in the regulation of inflammation and immune response due to their ability to control the expression of genes such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), monocytic chemotactic protein-1 (MCP-1) and Interleukin-6 (IL-6) (Castrillo, Joseph et al. 2003, Joseph, Castrillo et al. 2003, Joseph, Bradley et al. 2004, Zelcer and Tontonoz 2006, Bradley, Hong et al. 2007). LXRs have strong interactions with co-repressors such as NCoR, and modulating this
binding plays an important role in regulating gene expression (Hu, Li et al. 2003). Activation of LXR with specific agonists such as the sulfonamide T0901317 causes it to dissociate from co-repressors and recruit coactivators, with whom it forms complexes (Schultz, Tu et al. 2000, Albers, Blume et al. 2006). A previous study has shown that disruption of the binding of LXR to ligands causes changes in gene expression that contribute to the pathology observed in a familial case of MS (Wang, Sadovnick et al. 2016). Given that NCOA3 is a known ligand of LXR, and this binding is required for the regulation of gene expression (Albers, Blume et al. 2006) there is a possibility that the R485C mutation could disrupt this binding. NCOA3 is a mostly nuclear protein, shuttling between the nucleus and the cytoplasm by binding to nuclear receptors (Amazit, Pasini et al. 2007), and disruption of binding could also affect its translocation to the nucleus. For this reason here we propose to evaluate whether or not the R485C mutation in NCOA3 affects its binding to LXR, and in doing so provide some insight as to the mechanism through which it could affect the progression of MS.

4.2.2 Materials and methods

4.2.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA4 vectors were purchased from Invitrogen.

4.2.2.2 Cloning

The coding sequence for wild type NCOA3 was PCR amplified from total human brain cDNA (generously contributed by Dr. Carles Vilariño-Güell at the University of British Columbia), and inserted into pcDNA4 between KpnI and XbaI sites. The mutation causing R485C mutation was introduced into wild type NCOA3 cDNA by fusion PCR. Sequence coding for FLAG tag was
fused to the 5’ end of the forward primer for PCR amplification in order to allow identification of transfected NCOA3 protein.

4.2.2.3 Cell culture

HEK293 cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. Cells were transfected with 200ng of a pcDNA4 vector containing the coding region for either Wild-type or R485C NCOA3, and a vector containing the coding region for LXR tagged with GFP (kindly provided by Dr. Makoto Mikishima and Dr. Carles Vilarriño-Güell) using Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio.

4.2.2.4 Binding assays

FLAG-tagged full length human Wild-type and mutant NCOA3 and GFP-tagged LXRα were used for binding assays. For immunoprecipitation studies, pz-LXRα-GFP was co-transfected with Wild-type or R485C NCOA3 plasmid into HEK293 cells and treated overnight with Dimethyl Sulfoxide (DMSO), or 10μM of a synthetic LXR agonist (T0901317). Cells were lysed in lysis buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 1mM MgCl2, 1% Triton X-100) supplemented with protease inhibitor (Roche), and lysates were incubated with anti-FLAG (M2) beads (Sigma Cat # F1804) for three hours at 4°C with rotation. Beads were washed and incubated with SDS sample buffer without reducing agent for 10 min, and the supernatants were withdrawn, supplemented with 2% β-mercaptoethanol and boiled.

4.2.2.5 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1%Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease
inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. The primary antibodies used are as follows: Mouse anti-GFP sc-9996 (Santa Cruz Biotechnology, 1:1000 dilution), FLAG (for NCOA3) (Sigma; F7425, 1:1000 dilution) and HDAC1 (Santa Cruz Biotechnology, 1:1000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

4.2.2.6 Sub-cellular fractioning

HEK293 cells over-expressing vector, wild type or mutant NCOA3 were homogenized (20mM Tris pH 7.4, 10mM NaCl, and 1mM MgCl2), and the homogenates were centrifuged at 1,000g for 10min. The pellet nuclear fraction and the supernatant cytosol fraction were western blotted for FLAG-NCOA3s and HDAC1 as a nuclear marker.

4.2.2.7 Immunostaining

HEK293 cells expressing wild type and mutant FLAG-NCOA3 were fixed with 4% Paraformaldehyde (PFA) for 15 minutes at room temperature. After fixation, cells were briefly washed with PBS, permeabilized with 0.2% Triton-X100, and blocked with 3% BSA for 30min. Cells were co-stained with anti-FLAG antibody (Sigma; F7425, 1:1000 dilution) and NCOA3 specific antibody (Cell Signaling Technology; 2126S, 1:1000 dilution). Alexa 594 Goat anti-rabbit and Alexa 488 goat anti-mouse secondary antibodies were used for visualization.

4.2.3 Results

Upon activation by a ligand, LXR dissociates from repressors and recruits coactivators such as NCOA3 before translocating to the nucleus, where it then regulates gene expression. Here
we attempted to demonstrate the interaction of LXR and NCOA3 through immunoprecipitation targeting the FLAG tag attached to the transfected NCOA3. We could not observe any differences between the binding of Wild-type and R485C NCOA3 to LXR when they are transfected into HEK cells. Activation of LXR with the agonist TO901317 increased binding of NCOA3 to LXR, but no significant difference was observed between the Wild-type and R485C mutant forms of NCOA3 (Figure 32).

We also attempted to evaluate whether or not the R485C mutation would affect the localization of NCOA3 in the cells. We did not observe any significant difference in the levels of NCOA3 in the nuclear fraction of the cells between the Wild-type and R485C NCOA3, either when they were transfected alone or together with LXR (Figure 33A). Immunostaining of NCOA3 in transfected cells has revealed the transfected FLAG-tagged R485C localizes to the cell nucleus, though we were not able to detect NCOA3 in the cytosol of stained cells (Figure 33B).
Figure 32. Binding of R485C NCOA3 to LXR.
HEK cells were transfected with LXR-GFP and either empty vector, wild-type NCOA3 (NCOA3 Wt) or R485C NCOA3 (NCOA3 R485C) tagged with a Flag tag. An LXR agonist, TO901317 was used to induce activation of LXR and its binding to coactivators. Cells were lysed 24 hours after transfection, and LXR-NCOA3 conjugates were immunoprecipitated (IP) using agarose beads coated with antibodies against the FLAG tag. The R485C mutation in NCOA3 did not affect its binding to LXR (N=3).
Figure 33. Nuclear translocation of R485C NCOA3.
(A) Western blot of cellular fractions. HEK293 cells were transfected with vector, Wild-type NCOA3 (Wt) or R485C NCOA3 (R485C) either alone or with LXR, and then homogenized. After homogenization, the nuclear fraction was separated through centrifugation. The R485C mutation did not negatively affect the binding of NCOA3 to LXR and its translocation to the nucleus. H: whole homogenate; C: cytosolic fraction; N: nuclear fraction. Histone deacetylase 1 (HDAC1) is used as a positive control for nuclear separation. (B) Immunostaining of R485C NCOA3 in cultured cells. HEK293 cells were transfected with R485C NCOA3 (NCOA3), stained with antibodies against total NCOA3 and the FLAG tag on the transfected NCOA3 and visualized under a fluorescence microscope. Green: Transfected R485C NCOA3 stained with an antibody against the FLAG tag and visualized with Alexa 488 secondary antibody. Red: Total NCOA3 stained with an antibody against total NCOA3 and visualized with Alexa 594 secondary antibody. Merge: Superposition of the Green and Red images showing the distribution of both transfected and endogenous NCOA3. Blue: DAPI-stained nuclear DNA. The R485C mutation did not affect the localization of NCOA3 to the nucleus (N=2 experiments).

4.2.4 Considerations

As previously discussed, NCOA3 is capable of regulating gene expression through its ability to bind to nuclear receptors such as LXR. As disruption of LXR-mediated gene transcription caused by impaired binding to ligands had been previously reported by our group (Wang, Sadovnick et al. 2016), we considered the possibility of the NCOA3 R485C mutation exerting its...
effects by affecting its ability to act as an LXR coactivator. As our results clearly show, there is no apparent difference in the binding of NCOA3 to LXR between Wild-type and R485C, indicating that this specific point mutation does not affect this particular function of NCOA3.

Another possibility we considered was the R485C mutation affecting the ability NCOA3 to translocate to the nucleus upon an activating stimulus. We did not find any differences in nuclear localization between Wild-type and R485C NCOA3 either when they are transfected alone or with LXR. Immunofluorescence staining of cells transfected with tagged R485C NCOA3 confirmed that this mutation does not affect the ability of NCOA3 to be translocated to the nucleus. It was surprising however, that we were not able to observe either endogenous or tagged NCOA3 in the cytosol of transfected cells. While NCOA3 locates primarily to the cell nucleus, it does shuttle between the nucleus and cytosol (Amazit, Pasini et al. 2007), and therefore we should be able to observe some of it in the cytosol. It’s possible that the PFA fixation method utilized to prepare the cells irreversibly damage the epitopes targeted by the antibodies in the cytosol, therefore impeding their detection.

In summary, the R485C mutation of NCOA3 does not affect its binding to LXR and its translocation to the nucleus, indicating that if this mutation affects the function of NCOA3, it does so through other mechanisms.

4.3 NCOA3 and Histone acetylation

4.3.1 Introduction

As discussed briefly in the Introduction (chapter 1), NCOA3 possesses intrinsic acetyltransferase activity (Chen, Lin et al. 1997), and is also capable of recruiting other acetyltransferases and methyltransferases, allowing it to regulate gene transcription (Berrodin, Shen et al. 2010, Xiao, Xu et al. 2010). Acetylation of histones modifies chromatin structure and
provides a more accessible promoter environment for recruitment of components of the general transcriptional machinery (McKenna and O'Malley 2002), and that opens a distinct possibility that the R485C mutation of NCOA3 could affect this specific function of NCOA3. As acetylation of histones makes certain regions of the chromatin more likely to be targeted by the transcription machinery, a mutation that affects this important regulatory function could affect the expression of several genes, some of which could severely affect the development of symptoms and the progression of MS.

One of the targets of NCOA3 and its recruited acetyltransferases is histone H3 (Wagner, Koslowski et al. 2013, Drazic, Myklebust et al. 2016), and if the acetyltransferase function of NCOA3 is impacted by the R485C mutation, it is possible that the pattern of acetylation on histone H3 would be significantly affected. For this reason, here we propose to evaluate the differences caused by the R485C NCOA3 mutation on the acetylation of Lysine residues of histone H3.

4.3.2 Materials and methods

4.3.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA4 vectors were purchased from Invitrogen.

4.3.2.2 Plasmids

Plasmids encoding FLAG-tagged Wild-type and R485C NCOA3 were obtained and generated as detailed in previous sections.

4.3.2.3 Cell culture

HEK293 cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. Cells were transfected with
200ng of a pcDNA4 vector containing the coding region for either Wild-type or R485C NCOA3, using Lipofectamine 2000 Reagent (Invitrogen) at a 1:3 ratio.

### 4.3.2.4 Western blot

Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine gels then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. The primary antibodies used are as follows: Acetyl-Histone H3 Antibody Sampler Kit (Cell Signaling Technology; #9927, 1:200 dilution). IRDye 680RD-labelled goat anti-rabbit antibody was applied as a secondary antibody. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

### 4.3.3 Results

As NCOA3 is capable of acetylating histones as a means of regulating gene expression we have attempted to evaluate whether or not the R485C mutation of NCOA3 would affect the acetylation pattern of Lysine residues on histone H3. Cells were transfected with either an empty vector, Wild-type NCOA3 or R485C NCOA3. After transfection cells were lysed, and acetylation of Lysine residues 9, 14, 18, 27 and 56 was evaluated through western blot with the use of a Histone Acetylation Antibody kit containing antibodies specific to these acetylated residues. Our results didn’t show any differences in the acetylation patterns between cells transfected with an empty vector, Wild-type or R485C NCO3, indicating this function of NCOA3 is not affected by the mutation (Figure 34).
Figure 34. Effects of NCOA3 R485C on Histone acetylation.
HEK293 cells were transfected with either an empty pcDNA4 vector (Vector), NCOA3 wild-type (Wt) or NCOA3 R485C. Acetylation of lysine (Lys) residues 9, 14, 18, 27 and 56 of histone 3 (H3) was evaluated through western blot utilizing specific antibodies. No differences were observed between the groups (N=3).

4.3.4 Considerations

One of the mechanisms through which NCOA3 affects gene expression is its ability to acetylate histones either through its intrinsic acetyltransferase domain or by recruitment of other acetyltransferases, affecting the accessibility of chromatin to transcription factors. Due to the reported role of NCOA3 in regulating this important mechanism, we have investigated whether or
not the R485C mutation in NCOA3 affected the methylation of several Lysine residues in histone H3. As a result we could see that the R485C mutation by itself does not affect the acetylation pattern of H3, indicating that if R485C negatively affects the functioning of NCOA3, it does so through a mechanism that is not the acetylation of histones.

Surprisingly however, overexpression of NCOA3 by itself did not affect acetylation patterns, which as a result is counter-intuitive to the idea that NCOA3 itself is capable of acetylating histones and its overexpression should cause an observable change in acetylation patterns. This could be due to NCOA3 expression being strictly regulated, with transfection being incapable of causing a measurable increase in total levels of intracellular NCOA3, as we will demonstrate and elaborate on in the following section.

4.4 NCOA3 and inflammation: Response to LPS

4.4.1 Introduction

In addition to its function as a coactivator of nuclear receptors and its ability to acetylate histones, NCOA3 is reportedly involved in the regulation of inflammatory responses, playing an important role in innate immunity and maintenance of T-cell function (Li, Niu et al. 2012). Experimentation in some models has shown that NCOA3 plays a role in regulating immune response to infection (Chen, Chen et al. 2010).

Chronic and dysregulated activation of inducible enzymes such as inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) have been shown to play pivotal roles in the development of inflammatory diseases (Cedergren, Forslund et al. 2002, Cross and Wilson 2003, Murakami and Ohigashi 2007). Studies have shown higher levels of iNOS and COX-2 in MS lesions from patients as well as in experimental models, indicating that the upregulation of these
enzymes could be intimately linked to the proinflammatory phenomena of MS (Rose, Hill et al. 2004, Carlson, Rojas et al. 2010).

There is a possibility that the R485C mutation of NCOA3 affects its role in regulating the immune response, leading to a heightened level of basal inflammation that could contribute to the overall autoinflammatory condition of MS. Here we propose to evaluate the effects of the R485C mutant of NCOA3 on the expression of proinflammatory enzymes iNOS and COX-2.

4.4.2 Materials and methods

4.4.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA4 vectors were purchased from Invitrogen.

4.4.2.2 Plasmids

Plasmids encoding FLAG-tagged Wild-type and R485C NCOA3 were obtained and generated as detailed in previous sections.

4.4.2.3 Cell culture

Murine Microglial BV2 cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. Cells were transfected with 200ng of a pcDNA4 vector containing the coding region for either Wild-type or R485C NCOA3, using Polyethylenimine at a 1:3 ratio. 4 hours post transfection the cell culture medium was changed and cells were treated with either E. coli derived Lipopolysaccharide (LPS; EMD Millipore) to trigger an inflammatory response or 10μM of a synthetic LXR agonist (T0901317) to block such response.
4.4.2.4 Western blot

24 hours after transfection cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. The primary antibodies used are as follows: NCOA3 specific antibody (Cell Signaling Technology; 2126S, 1:1000 dilution), iNOS (Cell Signalling; D6B6S, 1:1000 dilution), COX-2 (Cell Signaling; 4842S, 1:1000 dilution) and β-actin (Sigma; A5316, 1:1000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences). Quantification of relative protein amounts after western blot was carried out using Quantity One software.

4.4.2.5 Statistical analysis

Two-tailed Student’s t-test and analysis of variance (ANOVA) followed by Tukey’s posthoc test were used to compare group differences. Analyses were performed using the GraphPad Prism software.

4.4.3 Results

In order to evaluate the effects of both Wild-type and R485C NCOA3 on the expression of iNOS and COX-2, we have transfected murine microglial cells, BV2, with either an empty vector, or the aforementioned forms of NCOA3. As a control for positive induction of activation, we treated cells with bacterial LPS. As a complete negative control, we have treated cells with both LPS and a synthetic LXR agonist, TO901317, which is capable of negating the upregulation of
both iNOS and COX-2 triggered by LPS. Overexpression of Wild-type NCOA3 did not trigger upregulation of either iNOS or COX-2 above the levels induced by a mock transfection. Overexpression of R485C NCOA3 however caused a strong upregulation of the expression of iNOS, but not COX-2. This increase in iNOS levels could not be countered by treatment with TO901317, indicating that this increase is not caused by the same mechanism as LPS (Figure 35).

Further analysis through quantification of the iNOS bands obtained by western blot has shown that NCOA3 R485C causes a roughly 3.5-fold increase in the expression levels of iNOS when compared to Wild-type NCOA3 and Vector (Figure 36).

**Figure 35. Effect of NCOA3 R485C on iNOS and COX-2 expression.**
BV2 cells were transfected with either an empty vector, wild-type NCOA3 (NCOA3 Wt) or R485C NCOA3 (NCOA3 R485C). Lipopolysaccharide (LPS) was added to cells after transfection to induce activation of inflammation through iNOS and COX-2. The LXR agonist TO901317 is capable of negating the increase in iNOS and COX-2 expression induced by LPS. Overexpression of NCOA3 R485C caused increased expression of iNOS in the absence of an activator like LPS, and this increase was not able to be countered by TO901317, indicating this effect occurs through a different pathway than the one activated by LPS (N=7 for iNOS, N=2 for COX-2).
Figure 36. Quantification of the effect of NCOA3 on iNOS levels.
(A) Western blot showing expression of iNOS, NCOA3 and actin in microglial (BV2) cells transfected with an empty pcDNA4 vector (Vector), Wild-type NCOA3 (WT) or NCOA3 R485C (R485C). (B) Software-based quantification of iNOS expression observed through western blot. Mean iNOS expression ± standard error is provided. N=3. *Tukey’s HSD post hoc p-value < 0.001. r.u. relative units (folds).

4.4.4 Considerations

Despite our previous results showing that the NCOA3 R485C mutation did not affect its ability to bind to LXR and translocate to the nucleus, or its ability to directly affect chromatin organization by acetylating histones, the results obtained here show that this mutation is capable of affecting inflammatory response, as seen by its ability to induce iNOS expression. This ability to trigger an inflammatory response seems to be, however, specifically tied to iNOS expression, as R485C did not cause any measurable increase in COX-2 levels. This indicates that NCOA3 is affecting iNOS, though the mechanism through which it accomplishes that is not clear and will be addressed in the following sections. More interestingly, the use of an LXR agonist, TO901317, did not counter this increase in iNOS levels, despite it clearly being capable of abolishing the increase in both iNOS and COX-2 caused by LPS. This suggests that the mechanisms through which NCOA3 and LPS affect iNOS expression are different and independent, warranting further investigation.
Another interesting observation is that this effect was achieved despite total NCOA3 levels in cells not increasing significantly after transfection, indicating that the effects observed are due to an intrinsic functional difference between the Wild-type (or endogenous) NCOA3 and the R485C mutant. NCOA3 is a relatively large protein (1424 amino acids, roughly 157kDa), and its transient transfection is not without its difficulties. After extensive experimentation, we have found that increasing the amount of plasmid that is transfected does not necessarily increase the amount of NCOA3 actually expressed in cells. Furthermore, the amount of transfected NCOA3 (FLAG-tagged) seems to be barely detectable above basal levels of total NCOA3 (data not shown), indicating that the total amount of NCOA3 is well-regulated in cells. This suggests that the differences observed are caused by a minute amount of transfected NCOA3 replacing the endogenous protein, and this amount is apparently sufficient to cause such results.

In summary, the NCOA3 R485C mutant is capable of causing increased expression of iNOS, which could then be causing a higher basal level of inflammation in microglia that would contribute to the overall autoinflammatory syndrome seen in MS. In the following sections we will explore the mechanisms through which the regulation of iNOS by NCOA3 occurs.

4.5 Regulation of iNOS expression by NCOA3

4.5.1 Introduction

As demonstrated in the previous section, the NCOA3 R485C mutant is capable of upregulating the expression of iNOS in mouse microglial cells, though the exact mechanisms through which this effect is achieved are not yet known. Here we propose to evaluate whether or not the NCOA3 R485C mutant is capable of regulating the expression of iNOS at a transcriptional level. First, we investigated whether or not it is capable of increasing the levels of iNOS RNA in mouse microglial BV2 cells. Following that, we investigated whether or not it is capable of
regulating iNOS expression by employing a reporter whose expression is driven by a fragment of the promoter of endogenous mouse iNOS.

4.5.2 Materials and methods

4.5.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA4 vectors were purchased from Invitrogen. PGL3 vector was purchased from Clontech.

4.5.2.2 Cloning

A roughly 1.7kb region containing the promoter region of murine iNOS (GenBank accession number L23806.1) (Lyons, Orloff et al. 1992) was cloned from total DNA extracted from BV2 cells utilizing 5’ gcggctaccggtgcattgtatgcttgaaatccataagctg as the forward primer and 5’ gcgaagcttgactaggctactccgtggagtgaacaagacc as the reverse primer. The resulting sequence was then inserted into the multiple cloning region of a PGL3 Luciferase reporter vector between sites KpnI and HindIII.

4.5.2.3 Plasmids

Plasmids encoding FLAG-tagged Wild-type and R485C NCOA3 were obtained and generated as detailed in previous sections.

4.5.2.4 Cell culture

Murine Microglial BV2 cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. Cells were transfected with 275ng of a pcDNA4 vector containing the coding region for either Wild-type or R485C NCOA3, 25ng of a PGL3 reporter plasmid containing a 1.7kb fragment of the mouse iNOS promoter, and 5ng of a reporter plasmid encoding Renilla Luciferase, using Polyethylenimine at a
1:3 ratio. 4 hours post transfection the cell culture medium was changed and cells were treated with *E. coli* derived Lipopolysaccharide (LPS; EMD Millipore) to trigger an inflammatory response.

4.5.2.5 **Luciferase assay**

Cell cultured medium was collected 24 hours after transfection, and measurement of Luciferase activity was carried out utilizing the Dual-Glo Luciferase Assay System (Promega) following the protocol supplied by the manufacturer.

4.5.2.6 **Western blot**

24 hours after transfection cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 10% Tris-glycine gels, then transferred to Nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk at room temperature for 1 hour. The primary antibodies used are as follows: NCOA3 specific antibody (Cell Signaling Technology; 2126S, 1:1000 dilution), iNOS (Cell Signalling; D6B6S, 1:1000 dilution), COX-2 (Cell Signaling; 4842S, 1:1000 dilution) and β-actin (Sigma; A5316, 1:1000 dilution). IRDye 680RD-labelled goat anti-rabbit antibodies and IRDye 800CW-labelled goat anti-mouse antibodies were applied as secondary antibodies. Membranes were scanned in the Odyssey system (LI-COR Biosciences).

4.5.2.7 **RT-PCR**

Total RNA was extracted from BV2 cells by TRI reagent (Sigma), and reverse transcription was carried out using ThermoScript RT-PCR system (Invitrogen) according to the manufacturer’s instructions. The primers used are detailed in Table 1.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miNOS Forward</td>
<td>5’ cccttccgaagtttctggcagcgc</td>
</tr>
<tr>
<td>miNOS Reverse</td>
<td>5’ ggctgtcagacgcctgtgcttttg</td>
</tr>
<tr>
<td>mActin Forward</td>
<td>5’ atctgaaagacctctatgc</td>
</tr>
<tr>
<td>mActin Reverse</td>
<td>5’ aacgcagctcagtaacagtc</td>
</tr>
<tr>
<td>mNCOA3 Forward</td>
<td>5’ tccgaagatgtccagaggttc</td>
</tr>
<tr>
<td>mNCOA3 Reverse</td>
<td>5’ aagaaagtgggctgagatgaag</td>
</tr>
<tr>
<td>hNCOA3 Forward</td>
<td>5’ aaagccatacatttaattgcgc</td>
</tr>
<tr>
<td>hNCOA3 Reverse</td>
<td>5’ acctttgttttccaaaggac</td>
</tr>
</tbody>
</table>

Table 1. Primers for RT-PCR of NCOA3 and iNOS.

4.5.3 Results

RT-PCR analysis of mRNA expression levels shows that total mouse iNOS (miNOS) mRNA levels in BV2 cells is increased when NCOA3 R485C is transfected, indicating that NCOA3 R485C expression induces increased transcription of iNOS (Figure 37). In order to better evaluate the effects of the R485C mutant on transcriptional regulation of iNOS, we have cloned a 1.7kb fragment of the mouse iNOS promoter in front of a Luciferase reporter, and proceeded to evaluate whether or not this promoter could be differentially activated by the NCOA3 R485C mutant. Our results show that the 1.7kb fragment clone displays promoter activity when expressed in BV2 cells, and addition of LPS strongly activates this promoter, indicating that this sequence does behave like the endogenous promoter of iNOS. Co-expression of this promoter with Wild-type NCOA3 did not cause any increase in activation over cells transfected with the reporter and an empty vector, whereas co-transfection of the reporter with the NCOA3 R485C mutant caused a roughly 2.5-fold increase in activation (Figure 38A), indicating that the mutant activates the
iNOS promoter. Such an effect was not observed when the reporter and NCOA3 were transfected into HEK cells (Figure 38B), indicating that this effect requires cell-specific factors to occur.

**Figure 37. Effect of NCOA3 R485C on iNOS RNA and protein levels.**
(A) Western blot of BV2 cells transfected with either an empty vector (Vector), Wild-type NCOA3 (Wt) or NCOA3 R485C. Overexpression of the R485C mutant of NCOA3 causes an increase in the total protein level of iNOS in cells.
(B) RT-PCR evaluation of iNOS expression. Overexpression of R485C NCOA3 causes an increase in mRNA levels of iNOS, indicating that it affects iNOS at the transcriptional level. miNOS: mouse iNOS; mActin: mouse Actin; hNCOA3: human NCOA3; mNCOA3: mouse NCOA3. (N=3).
Figure 38. Activation of the iNOS Promoter by NCOA3 R485C.
A 1.7kb fragment of mouse iNOS upstream from its transcription start site (TSS) was cloned in front of a Luciferase reporter, and transfected into a mouse microglial cell line, BV2 (A) and HEK cells (B). E. coli Lipopolysaccharide (LPS) was used as a means of inducing activation of iNOS transcription. The NCOA3 R485C mutant was able to induce activation of the iNOS promoter in BV2 cells, but not in HEK, indicating it requires cell line-specific co-activators to induce expression. Vector: pcDNA4; Wt: NCOA3 Wt; R485C: NCOA3 R485C; Basic: PGL3 reporter plasmid with a basic promoter; Promoter: PGL3 reporter plasmid containing a 1.7kb mouse iNOS promoter. N=6 for all groups; **: p<0.001 ***: p<0.0001.

4.5.4 Considerations

Building upon the results shown in the previous section, here we aimed to determine how NCOA3 R485C is capable of regulating the expression of iNOS. While we had already demonstrated that NCOA3 is capable of affecting total iNOS levels in transfected cells, here we show evidence that it achieves this effect by regulating iNOS at a transcriptional level. Evaluation of RNA levels through RT-PCR here shows that unmistakably increases the level of mouse iNOS RNA, which corresponds to an increased level of expressed iNOS in cells.

In order to confirm that NCOA3 R485C is actively increasing the transcription of iNOS we generated a reporter that expresses Luciferase driven by a 1.7kb long promoter of mouse iNOS.
Experimentation with this reporter has shown that NCOA3 R485C causes specific activation of the promoter when compared to Wild-type NCOA3, confirming that it targets the iNOS promoter specifically. This effect was observed only when the reporter and NCOA3 were expressed in mouse microglial cells, indicating that NCOA3 requires cofactors or transcription factors that are cell-type specific, which warrant further investigation if the pathway exploited by NCOA3 to achieve this effect is to be fully characterized.

The results here indicate that NCOA3 R485C causes a higher basal expression level of iNOS that could result in a perpetually activated immune response in the microglia of patients with this specific mutation. This by itself already creates conditions for dysregulated response to inflammation that could be the cause, or at the very least contribute to the increased immune response seen in MS. Further evaluation of the mechanisms through which NCOA3 achieves this effect could possibly generate new insights into the pathways that when dysregulated contribute to MS. We will explore the specific regulatory pathways and transcription factors that affect the upregulation of iNOS expression by NCOA3 R485C in the following section.

4.6 Analysis of the iNOS promoter activity

4.6.1 Introduction

As demonstrated in the previous sections, NCOA3 R485C is capable of increasing the total amount of iNOS in cells by regulating at a transcriptional level. Further experimentation has shown that NCOA3 R485C achieves this effect by activating the iNOS promoter. Gene regulation in itself is a complex phenomenon, and NCOA3 could be regulating expression through one or several indirect factors. Before we are able to pinpoint the factors and pathways affected, we need to figure out which region of the promoter is being targeted by the NCOA3 R485C mutant.
Here we will attempt to identify the sequences in the iNOS promoter that are being affected by NCOA3 by doing sequential deletions of the promoter sequence, as well as fusing fragments and altering the promoter sequence and evaluating how those changes affect the expression of a Luciferase reporter driver by these shortened and altered promoters. Additionally, we will utilize software transcription factor binding prediction to isolate possible transcription factors that could be directly driving expression of iNOS. Finally, we will attempt to isolate and block the action of these transcription factors in order to confirm whether or not they affect the expression of reporters driven by the iNOS promoter and its variations.

Succeeding in isolating the transcription factors that cause iNOS to be expressed when NCOA3 R485C is expressed will allow us to discern the intracellular pathways that are affected by NCOA3 and expand our knowledge on how inflammation can be regulated at a cellular level, possibly enabling the development of novel strategies for the treatment and management of symptoms of MS.

4.6.2 Materials and methods

4.6.2.1 Reagents

All reagents were purchased from Thermo-Fisher unless otherwise specified. DMEM and pcDNA4 vectors were purchased from Invitrogen. PGL3 vector was purchased from Clontech.

4.6.2.2 Cloning

A PGL3 vector containing a 1.7kb-long promoter region of murine iNOS (GenBank accession number L23806.1) was cloned as described in the previous section and utilized as a template for the cloning of plasmids with shorter promoter fragments. Primers utilized for cloning are described in Table 2. The pairing of primers used to generate the sequences is detailed in Table
3. All resulting sequences were inserted into the multiple cloning region of a PGL3 Luciferase reporter vector between sites KpnI and HindIII.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>1.7kb Forward</td>
<td>5’ gccggtacctgcgttgactttgatagatgcttagaatccataagctg</td>
</tr>
<tr>
<td>1.65kb Forward</td>
<td>5’ gccggtacctgcgtgcgtgcagtcgtgcttggtgcacatgagtg</td>
</tr>
<tr>
<td>1.6kb Forward</td>
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</tr>
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<tr>
<td>900bp Forward</td>
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</tr>
<tr>
<td>700bp Forward</td>
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</tr>
<tr>
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</tr>
<tr>
<td>1.5-900bp Fuse Forward</td>
<td>5’ gggcagaaacccttagctcccttccactcttgtagtttgcaggggcc</td>
</tr>
<tr>
<td>1.1-900 Del Forward</td>
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<tr>
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<tr>
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Table 2. Primers for cloning of iNOS Promoter fragments.
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<th>Reaction B (3’ end)</th>
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</tr>
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<td>-</td>
</tr>
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<td>1.65kb Forward</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>miNOS Promoter Reverse</td>
<td>-</td>
<td>-</td>
</tr>
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<td>1.6kb Forward</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>miNOS Promoter Reverse</td>
<td>-</td>
<td>-</td>
</tr>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>miNOS Promoter Reverse</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>1.1kb Forward</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>miNOS Promoter Reverse</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>900bp</td>
<td>900bp Forward</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>miNOS Promoter Reverse</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>700bp</td>
<td>700bp Forward</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>miNOS Promoter Reverse</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>1.7kb Forward</td>
<td>1.5-1.1kb Fuse Forward</td>
<td>1.7kb Forward</td>
</tr>
<tr>
<td></td>
<td>1.5-1.1kb Fuse Reverse</td>
<td>miNOS Promoter Reverse</td>
<td>miNOS Promoter Reverse</td>
</tr>
<tr>
<td>1.7-1.5kb + 900bp</td>
<td>1.7kb Forward</td>
<td>1.5-900bp Fuse Forward</td>
<td>1.7kb Forward</td>
</tr>
<tr>
<td></td>
<td>1.5-900bp Fuse Reverse</td>
<td>miNOS Promoter Reverse</td>
<td>miNOS Promoter Reverse</td>
</tr>
<tr>
<td>1.1-900 Del</td>
<td>1.7kb Forward</td>
<td>1.1-900 Del Forward</td>
<td>1.7kb Forward</td>
</tr>
<tr>
<td></td>
<td>1.1-900 Del Reverse</td>
<td>miNOS Promoter Reverse</td>
<td>miNOS Promoter Reverse</td>
</tr>
</tbody>
</table>

Table 3. Primer pairing for iNOS Promoter Fragments.
4.6.2.3 Plasmids

Plasmids encoding FLAG-tagged Wild-type and R485C NCOA3 were obtained and generated as detailed in previous sections.

4.6.2.4 Cell culture

Murine Microglial BV2 cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 50 U/ml penicillin G sodium, and 50μg/ml Streptomycin sulfate (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO2. Cells were transfected with 275ng of a pcDNA4 vector containing the coding region for either Wild-type or R485C NCOA3, 25ng of a PGL3 reporter plasmid containing fragments of the mouse iNOS promoter, and 5ng of a reporter plasmid encoding Renilla Luciferase, using Polyethylenimine at a 1:3 ratio. 4 hours post transfection the cell culture medium was changed and cells were treated with either 10μM of a synthetic LXR agonist (T0901317) to block exogenous activation of iNOS and NF-κB-mediated activation of transcription or 8nM Cyclosporin-A to block Calcineurin and NF-AT-mediated activation of transcription.

4.6.2.5 Luciferase assay

Cell culture medium was collected 24 hours after transfection, and measurement of Luciferase activity was carried out utilizing the Dual-Glo Luciferase Assay System (Promega) following the protocol supplied by the manufacturer.

4.6.2.6 Transcription factor binding prediction

Prediction of putative binding sites for transcription factors on the sequence for the mouse iNOS promoter was carried out utilizing the ALGGEN PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3; accessed April 7th, 2018) prediction software.
Parameters used were *Mus musculus* transcription factors, *Mus musculus* binding sites, 5% maximum dissimilarity rate.

### 4.6.3 Results

Progressive deletion of 200bp fragments from the 5’ end of the iNOS promoter caused a gradual reduction in promoter activity, with marked drops from the 1.7kb to 1.5kb deletion, followed by gradual decrease with further deletions. Deletion of the first 200bp eliminated the differences between the activation by the Wild-type NCOA3 and the R485C mutant, indicating that these first 200bp are crucial for the differential activation by the R485C mutant (Figure 39).

We proceeded to make smaller deletions of the first 200bp of our promoter region, generating 1.65kb, 1.6kb and 1.55kb fragments. Surprisingly, deletion of the first 50bp caused a drastic reduction in promoter activity, which then appeared to recover when the following 50bp were deleted as well (1.6kb). No differences were observed between the 1.6kb, 1.55kb and 1.5kb fragments, indicating that the 100bp removed do not affect transcription. Interestingly, fusing the first 200bp of the promoter region to the 900bp shortened promoter (1.7-1.5kb + 900bp) further decreased its activity, indicating that the initial 200bp could be targeted by transcription repressors (Figure 40).

We have proceeded to use the ALGGGEN PROMO software to analyze the 800bp sequence of the iNOS promoter (between 1.7kb and 900bp) to identify possible unique transcription factor binding sites. The software identified a unique NF-AT4 binding site in the first 50bp of the sequence, and a unique NF-AT1 binding site in the last 200bp of the queried sequence (Figure 41).

Based on the above prediction, we experimented with further deletions of these last 200bp (1.1-900bp Del), as well as fusing the first 200bp of the promoter to the shortened promoter that contained these 200bp (1.7-15kb Fuse 1.1kb). At the same time, we have attempted to evaluate
whether or not NF-AT was capable of activating the iNOS promoter fragments by treating cells with Cyclosporin-A, which is capable of inhibiting the activation of NF-AT. Our data shows that deletion of the 200bp between 1.1kb and 900bp (1.1-900bp Del) dramatically decreased promoter activity, whereas fusing the first 200bp of the promoter to the 1.1kb fragment did not significantly affect its activity. More interestingly, treatment with NF-AT blocker Cyclosporin-A did not cause any measurable effect on the promoter activity of any of the fragments tested, suggesting NF-AT does not interact with any of the promoter fragments tested (Figure 42).

Figure 39. Activation of iNOS Promoter fragments by NCOA3 R485C. BV2 cells were transfected with either Wild-type (NCOA3 Wt) or R485C NCOA3 (NCOA3 R485C), together with reporter plasmids whose expression was driven by a basic promoter (Basic), the Full-length 1.7kb promoter of mouse iNOS (Full), or fragments thereof. Cells were treated with 10μM of the LXR agonist TO901317 to prevent non-specific activation of the iNOS promoter. The NCOA3 R485C mutant caused an increased activation of the Full-length iNOS promoter when compared to Wild-type NCOA3. No significant effects were seen with shorter fragments of the iNOS promoter. N=6 for each group; ***: p<0.001.
Figure 40. Effects of deletion of fragments on the activity of the iNOS Promoter.
BV2 cells were transfected with R485C NCOA3 (NCOA3 R485C), together with reporter plasmids whose expression was driven by a basic promoter (Basic), the Full-length 1.7kb promoter of mouse iNOS (Full), or fragments thereof. Cells were treated with 10μM of the LXR agonist TO901317 to prevent non-specific activation of the iNOS promoter. Deletion of the first 50bp of the mouse iNOS promoter severely diminishes activation by R485C NCOA3, but further shortening of the promoter on its 5’ end reverts this apparent change. Appending the first 200bp for the promoter sequence to a 900bp-long promoter severely reduced activation, indicating this fragment possesses repressor activity. N=3 for all groups. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 when compared to Full length. #: p<0.05; ## p<0.01 when compared to the 1.6kb promoter. %: p<0.05 when compared to the 900bp promoter. Two-tailed student’s t-test.

Figure 41. Transcription Factor binding site prediction for the promoter region of mouse iNOS.
ALGGGEN-Promo Transcription Factor binding site software prediction with a dissimilarity matrix at 5% dissimilarity for mouse transcription factors identified 2 unique NF-AT1 and NF-AT4 (dissimilarity <4%) putative binding sites on the 1.7kb mouse iNOS promoter utilized in this study.
Figure 42. Effects of NFAT blocking on the activation of the iNOS Promoter by NCOA3. BV2 cells were transfected with either Wild-type (NCOA3 Wt) or R485C NCOA3 (NCOA3 R485C), together with reporter plasmids whose expression was driven by a basic promoter (Basic), the Full-length 1.7kb promoter of mouse iNOS (Full), or fragments thereof. Cells were treated with 10μM of the LXR agonist TO901317 to prevent NF-κB-mediated activation, or 8nM of Cyclosporin A to prevent activation mediated by Calcineurin, PP2B or NF-AT. No differences between the 2 treatments were observed.

4.6.4 Considerations

As discussed previously, transient transfection of NCOA3 is not without its difficulties, and there appears to be a limit to the amount of plasmid that can be transfected for optimal expression before the total amount of NCOA3 plasmid starts affecting the transfection efficiency of the reporter plasmids. For this reason we have limited the amount of NCOA3 plasmid transfected into cells to 275ng, as any lower amount results in insufficient NCOA3 expression as total levels of intracellular NCOA3 seem to be tightly regulated and the transfected NCOA3 struggles to compete with the endogenous protein, and higher amounts affect the transfection efficiency and usefulness of the Luciferase reporters.

Regardless, expression of NCOA3 R485C in BV2 cells causes an upregulation of iNOS expression, and the promoter analysis here confirms that observation. As seen in previous sections,
R485C causes a higher basal expression of iNOS, which cannot be countered by treatment with an LXR agonist. This indicates that whatever the mechanism affected by NCOA3 that results in iNOS expression, it does not seem to be the canonical NF-κB pathway through which iNOS is reported to be activated (Li, Yan et al. 2000, Uffort, Grimm et al. 2009, Arias-Salvatierra, Silbergeld et al. 2011, Oussaief, Ramirez et al. 2011, Jia, Liu et al. 2013). Interestingly, LXR activation also did not seem to always counter the apparent random increases in promoter activity with shorter fragments of the iNOS promoter, which appeared to occur more frequently with the 1.5kb and 1.1kb fragments despite all care being taken so conditions for transfection would be the same. Our initial assumption was that low residual concentrations of external contaminants, physical cell-cell interaction or other factors were the source of these variations. As an attempt to mitigate these interfering factors, we used the LXR agonist TO901317 in our experiments, due to prior observations that it was capable of limiting exogenous activation of iNOS. Unfortunately however, use of the agonist was not completely effective in eradicating these sources of error, indicating there are other factors at play that are yet unknown.

Predictive analysis of the promoter sequence revealed that there were unique NF-AT1 and NF-AT4 binding sites, indicating a possibility that either of these transcription factors could be the ones causing the increase in iNOS expression when NCOA3 R485C is expressed. Calcineurin has been reported to trigger iNOS expression through NF-AT in some models (Kim, Moon et al. 2004), and the Calcineurin and NF-AT blocker Cyclosporin-A was reported to be able to inhibit iNOS expression in an NF-κB-independent manner in some models (Hamalainen, Lahti et al. 2002, Diaz-Ruiz, Vergara et al. 2004, Eckstein, Van Quill et al. 2005, Lessio, de Assuncao Silva et al. 2005, Nomura, Vilalta et al. 2017). Based on these reports, we attempted to utilize Cyclosporin-A as a means of blocking NF-AT activation in our model, and compare it to the results obtained with the
ones achieved by blocking NF-κB-mediated activation using the LXR agonist TO901317. Surprisingly, yet somewhat disappointingly, treatment with these compounds did not seem to significantly alter the activation of the iNOS promoter by NCOA3 R485C. This creates the possibility that NCOA3 R485C triggers the activation of iNOS through an as of yet unknown non-canonical pathway. While the results herein presented do not allow us to make such a claim with certainty, this possibility needs to be further explored and investigated. Other mechanisms that could lead to the activation of iNOS expression could be explored, though further caution must be exercised in order to eliminate pathways that have been evaluated previously. For example, ligands for peroxisome proliferator-activated receptor γ (PPARγ), such as rosiglitazone, PPARδ (GW1516) and PPARα (GW7647) were reported to have minimal effects on iNOS expression in some models (Joseph, Castrillo et al. 2003), but experimentation might lead us to better results when NCOA3 is involved.

In summary, regulation of iNOS expression appears to be significantly more complex than initially thought, with different sequences of the promoter region being able to differentially drive expression under certain conditions and unknown factors influencing expression when fragments of the promoter sequence are deleted or altered. More interestingly however, NCOA3 R485C seems to induce activation of the iNOS promoter through a pathway that is not the canonical NF-κB pathway, nor the alternative Calcineurin/NF-AT pathway. These findings suggest that NCOA3 is capable of modulating iNOS expression through a novel pathway that remains to be identified and characterized.
Chapter 5: Conclusions

5.1 A novel β-Secretase enzymatic assay in real-time in living cells

5.1.1 Conclusions

In Chapter 2 of this dissertation we have detailed the generation of a novel reporter system for BACE1 activity in living cells, a chimeric protein construct that we have named ASGβ. While several assay systems have been designed to screen BACE1 activity, most of them rely on artificial in vitro conditions that do not replicate the natural conditions that lead APP to be cleaved by BACE1, have concerns as to their sensitivity, or are over-reliant on mutations that exacerbate BACE1 cleavage of a specific substrate. Here we have demonstrated the possibility of a sensitive, cell-based, real-time reporter system that is able to be cleaved by BACE1 utilizing sequences from the Wild-type form of APP, without any mutations to induce stronger cleavage or prevent it altogether.

While mutations such as Swedish cause stronger cleavage of APP by BACE1 (Citron, Oltersdorf et al. 1992, Mullan, Crawford et al. 1992, Zhang, Wang et al. 2017), these mutations were also seen to impact the normal regulation and processing of APP (Koo and Squazzo 1994, Thinakaran, Teplow et al. 1996, Ben Halima, Mishra et al. 2016, Zhang, Wang et al. 2017), which could mean any data obtained with their addition might not be applicable to the Wild-type APP most patients with AD carry.

Interestingly, despite ASGβ having no mutations and possessing both Asp1 (C99) and Glu11 (C89) sites, its cleavage by BACE1 appears to occur predominantly at the Asp1 site. This could indicate that the regions of APP flanking the β-site that we have removed from the construct are the ones affecting cleavage preference of BACE1. Cleavage site selection is important for the generation of Aβ with regions of APP not directly targeted by BACE1 dictating cleavage dynamics...
(Zhang, Wang et al. 2017), and that could very well be the case with ASGβ as well. Volbracht et al arrived at similar conclusions during the development of their custom BACE1 substrate, indicating the need to evaluate the whole sequence of APP more carefully and investigating which alterations can cause meaningful changes in cleavage patterns (Volbracht, Penzkofer et al. 2009). Regardless, our results could be utilized to further investigate what factors influence the targeting of BACE1 cleavage, and perhaps incorporated into similar constructs to study these differences.

5.1.2 Limitations and Future directions

ASGβ is usable in its current form, and is a valuable tool for real-time evaluation of BACE1 activity in cultured cells. It does however, have room for modifications or improvements if one aims to further investigate the intricate mechanisms regulating cleavage. Volbracht et al. (2009) experimented with a shorter version of the β-site, and achieved promising results (Volbracht, Penzkofer et al. 2009). We have tried replicating their sequence by generating a shorter version of ASGβ that does not include the C89 site, but results were not significantly different than the ones obtained with the slightly longer form we have chosen to use (data not shown). Furthermore, the lack of the endogenous region of APP flanking the β-site terminally did not negatively affect cleavage, indicating that if it has any effect it is not conducive to cleavage, but rather counter to it. Other experiments carried out in parallel in our lab have hinted at the possibility that the region of APP closer to the membrane on the luminal side is critical for selection. We have generated modified versions of ASGβ including the Swedish mutation, and surprisingly it has affected the observed results, albeit in unexpected ways. The Swedish mutant promoted CTF generation with no corresponding phosphatase release, indicating that either the Swedish mutation fundamentally alters the cycling of the protein, or that it promotes cleavage at a stage of the trafficking or maturation pathway of the protein that is not conducive to its proper secretion. We
have investigated the possibility of the two C-terminal amino acids of the N-terminus of ASGβ altering secretion by transfecting cells with truncated versions of the protein ending at KM or NL on its C-terminus. Surprisingly, the Swedish variant (NL) had a similar secretion profile to its wild-type counterpart, indicating that if the N-terminus of ASGβ is retained after cleavage it is not due to the substitution in those amino acids. This suggests an interesting scenario wherein these substitutions in the full forms of either ASGβ or APP cause them to encounter BACE1 at different stages in its trafficking pathway. As a result, this creates a possibility that the maturation, trafficking and transport of both APP and BACE1 are major factors in determining the total amount of Aβ that could be secreted. Granted, our observations in this regard have been somewhat limited given the scope of this study, but they hint at the possibility that much more could be achieved and investigated through modification and adaptation of ASGβ. If studies utilizing our model are to be continued, further mutations could be introduced, and further alterations could be done to evaluate what exactly in the sequence or structure of ASGβ causes it to be preferentially cleaved at the Asp1 site by BACE1. This could lead to the discovery of specific features that affect this cleavage alone, but not of other potential substrates, allowing for more specific, better targeted approaches to modulating BACE1.

5.1.3 Significance of this study

Despite decades of research on AD and the phenomena that lead to the changes causing its complex combination of symptoms, it is still not clear what causes BACE1 to targets the Asp1 (C99) site over other possible sites. The generation of Aβ being the main phenomenon leading to the neuropathological changes that we see in AD, understanding the factors regulating the cleavage of APP by BACE1 would greatly aid in understanding the circumstances under which this cleavage takes place. While it is currently known that several mutations can increase cleavage at that site
(Thinakaran, Teplow et al. 1996, Citron, Westaway et al. 1997, Ben Halima, Mishra et al. 2016, Zhang, Wang et al. 2017), the results herein suggest that such mutations are not necessary for this differential cleavage to occur, and that other factors could affect substrate selection. We also cannot discount the possibility that our choice of intracellular domain causes the construct to undergo a processing pathway that makes it more likely to be cleaved by BACE1 at that site. As such, we also believe that knowledge obtained from the use of our construct when compared and used together with other models will allow us to better understand the mechanisms responsible for the amyloidogenic processing of APP.

The artificial nature of ASGβ could be utilized to argue against its ability to accurately replicate the cleavage of APP by BACE1, but the results shown here indicate that it is nonetheless at the very least a tool with which we can easily observe this cleavage taking place in real time. Furthermore, the fact that it only incorporates a limited sequence of APP helps us better understand the sequences and structures of APP that aid or guide the cleavage site selection by secretases. In that vein, the results herein reported indicate that the regions flanking the Asp1 (C99) site play a major role in determining its cleavage, since despite the presence of the Glu11 (C89) site in the sequence of ASGβ, BACE1 seemed to overwhelmingly favor the former as a cleavage target. There are studies showing that altering the fundamental structure of APP can alter cleavage efficiency, and consequently the amount of total Aβ generated (Qahwash, He et al. 2004, Tang, Hu et al. 2014, Zhang, Wang et al. 2017), and as such we cannot completely discount the possibility that the sequence we have chosen to incorporate into ASGβ inadvertently affects cleavage site selection.

Other than the cleavage site itself, one potentially major factor affecting the generation of Aβ is the intracellular compartment where cleavage takes place. Amyloidogenic processing of
APP reportedly takes place in the ER and endosomes (Vetrivel and Thinakaran 2010, Schon and Area-Gomez 2013), though that might not be the case when mutations are introduced into the sequence of APP. Experimentation with introduced mutations to ASGβ have shown that while introducing the Swedish mutation into the sequence caused stronger cleavage by BACE1 at the C99 site there was very little increase, if any, of SEAP activity in the medium following cleavage. This potentially indicates that the presence of the mutation causes ASGβ, and perhaps APP, to encounter BACE1 at a point in its trafficking route that makes secretion more difficult, or makes it more likely for cleavage products to be retained in the cell. Further experimentation with the Swedish mutation has shown that the mutation by itself has no negative impact on secretion, if anything it apparently enhances it when introduced to a C-terminally truncated form of ASGβ. This piece of data suggests that while cleavage site selection is important, there are other factors at play regulating when, where, and how effectively ASGβ and to some extent APP is cleaved by BACE1. Unfortunately, as of the present day this is mostly speculation, and we do not have conclusive data to make any assertion confidently.

In summary, ASGβ shows that the theoretical idea of mimicking APP cleavage attaching parts of APP to reporters is viable, and allows us to better visualize an intracellular process that up until now required more costly and less sensitive procedures. While thus far we have focused on the feasibility of utilizing the Wild-type sequence of APP as a tool to measure BACE1 activity, the successes achieved indicate that much could be learned by experimenting with variations of ASGβ. By changing the sequence and optimizing certain conditions we could fine-tune the extent to which cleavage occurs, which indicates that it is not the sequence per se that guides total cleavage, but parallel mechanisms influence it as well. Experimenting with factors that might influence transport, trafficking or secretion could enable a better understanding of the factors
underlying the generation of Aβ, and help in finding better treatments for the prevention or treatment of AD.

5.2 Functional evaluation of mutations in familial cases of Multiple Sclerosis

5.2.1 Conclusions

In chapters 3 and 4 we have explored the effects of mutations on the NLRP12 and NCOA3 genes, both found in familial cases of MS. The current scientific consensus is that given the complexity of MS and the variability of presentation and severity of symptoms, no single mutation on its own is capable of causing MS. Nevertheless, these mutations demonstrably are capable of affecting several aspects of the disease (Wang, Sadovnick et al. 2016, Wang, Sadovnick et al. 2016, Sadovnick, Gu et al. 2017, Sadovnick, Traboulsee et al. 2017), and can teach us much about the minute changes in molecular pathways that can lead or contribute to the etiology of the disease.

Studies so far have demonstrated that inflammation, while not necessarily the cause, is a main effector in the progression of the pathophysiological changes we see in MS. Given that inflammation is not necessarily contained to one specific tissue or area of the brain it could explain why the presentation of MS varies so much from one patient to another (Lassmann 2013, Weissert 2013). Inflammation is admittedly a rather broad term for a series of phenomena that might be happening in different places at different times, under different circumstances, creating a pressing need to define, identify and characterize the minute pieces that play a role in the development of an inflammatory condition. Understanding what pieces of the molecular puzzle lead to the onset of inflammation in some cell lines might help us understand what processes are at risk and under which conditions, allowing us to focus our studies on those processes and better tailor treatments for specific cases in human patients.
5.2.2 Limitations and Future directions

5.2.2.1 NLRP12

Our results show that while neither the L475Q nor the L972H mutations of NLRP12 affect its ability to repress NF-κB-mediated gene activation, the L972H mutation negatively affected the maturation and activation of Caspase-1. This finding suggests this mutation is capable of affecting the ability of NLRP12 to properly assemble the inflammasome complex, though the exact mechanism through which this happens is not yet known. Inflammasome assembly requires oligomerization of NLRs through their NBS (NOD/NACHT) domains after activation by a ligand, and recruiting of ASC as an adaptor protein upon which Caspase-1 can finally be activated (de Alba 2009, Vanaja, Rathinam et al. 2015). The initial oligomerization of NLRP12 is thought to occur through its NBS domain, a region that is not affected by either mutation. The L972H mutation however is predicted to occur within the LRR region of NLRP12, which could affect its pairing and oligomerization, and in doing so disrupt inflammasome assembly.

It could be argued that impaired maturation and activation of Caspase-1 would have an anti-inflammatory rather than pro-inflammatory effect, thought that cannot be stated with certainty without a more meaningful amount of research and data on how the disruption of basic inflammasome function affects overall inflammation. One possible way of investigating the ramifications of this disruption would be to compare the effects of the L972H mutation to the effects of other mutations that have been reported to lead to autoinflammatory and immune disorders (Borghini, Tassi et al. 2011, Ter Haar, Lachmann et al. 2013, Borte, Celiksoy et al. 2014, Shen, Tang et al. 2017, Basaran, Uncu et al. 2018). This would have to be done in an experimental rather than clinical setting however, with a more detailed and minute characterization of these
other mutations, which would help to understand which functions are altered and how they affect broader inflammatory and immune responses.

5.2.2.2 NCOA3

As NCOA3 is a ligand of nuclear receptors, our first hypothesis was that the R485C mutation discovered would affect its binding to receptors such as LXR. As our results have clearly shown, the R485C mutation does not affect this binding. That is not to say, however, that binding to other possible ligands such as estrogen and androgen receptors (Gnanapragasam, Leung et al. 2001, Wong, Komm et al. 2001, Zhou, Suino-Powell et al. 2010) is not disrupted, a possibility that could be explored in further studies.

Another possibility we considered was the R485C mutation affecting the ability of NCOA3 to translocate to the nucleus upon an activating stimulus (Amazit, Pasini et al. 2007). Our results did not show any differences in nuclear localization between Wild-type and R485C NCOA3 either when they are transfected alone or together with LXR as a ligand. While this indicates that the R485C mutation does not disrupt this binding as seen by the observation of the cellular localization of NCOA3 through immunostaining, there is a possibility that the preparation method we utilized made it difficult to observe the transfected NCOA3 in the cytosol.

One of the mechanisms by which NCOA3 is capable of affecting gene expression is through its ability to acetylate histones either through its intrinsic acetyltransferase domain or by recruitment of other acetyltransferases, affecting the accessibility of portions of the chromatin to transcription factors. As such, we have investigated whether or not the R485C mutation in NCOA3 affected the acetylation of several Lysine residues in histone H3. We concluded that the R485C mutation by itself does not affect the acetylation pattern of H3, indicating that if R485C negatively affects the functioning of NCOA3, it does so through a mechanism that is not the acetylation of
histones. There is however the possibility that R485C could affect the acetylation of histone residues other than the ones we elected to evaluate, which could be further explored. Surprisingly however, overexpression of NCOA3 in our experiments by itself did not significantly alter acetylation patterns, which runs counter to the notion that NCOA3 by itself is capable of acetylating histones. One possibility is that NCOA3 expression is strictly regulated, and transfection does not cause a measurable increase in total levels of intracellular NCOA3.

Our next step was to evaluate the effects of the R485C mutation on the expression level of proteins associated with inflammatory response, namely iNOS and COX-2. We were able to demonstrate that expression of R485C NCOA3 was capable of inducing an increase in expression levels of iNOS, but not COX-2. This indicates that NCOA3 is affecting iNOS, though the mechanism through which it accomplishes that is not yet clear. More interestingly, the use of an LXR agonist, TO901317, was not capable of countering this increase in iNOS levels, despite it clearly being capable of abolishing the increase in both iNOS and COX-2 caused by treatment with LPS. This suggests that the mechanisms through which NCOA3 and LPS affect iNOS expression are different and independent, raising the possibility of NCOA3 inducing an increased level of basal inflammation. Another interesting observation is that this effect was achieved despite total NCOA3 levels in cells not increasing significantly after transfection, indicating that these effects are due to functional differences between Wild-type NCOA3 and the R485C mutant, and even a minute amount of mutant is capable of triggering a response as seen through the increase in iNOS expression. At present, it is not known whether this is an effect that can only be observed in experimental settings, and further analyses utilizing data and samples from human patients and animal models would certainly help confirm or discredit the notion that altered NCOA3 function
leads to a higher basal level of iNOS, and therefore a higher basal level of inflammation that could cause or exacerbate other inflammatory processes.

Subsequent evaluation of RNA levels through RT-PCR shows that NCOA3 R485C is capable of causing an increase in total mouse iNOS RNA, which points towards NCOA3 having a role in the transcriptional regulation of iNOS. In order to confirm that, we generated a Luciferase reporter driven by a 1.7kb-long mouse iNOS promoter. Experimentation with this reporter has shown that NCOA3 R485C causes specific activation of the promoter when compared to Wild-type NCOA3, confirming that it targets the iNOS promoter specifically. This effect was observed only when the reporter and NCOA3 were expressed in mouse microglial cells, indicating that NCOA3 requires cofactors or transcription factors that are cell-type specific. As demonstrated previously the higher basal expression of iNOS caused by NCOA3 R485C cannot be countered by treatment with an LXR agonist, indicating that the mechanism through which this effect occurs is not the canonical NF-κB pathway (Li, Yan et al. 2000, Uffort, Grimm et al. 2009, Arias-Salvaterra, Silbergeld et al. 2011, Oussaief, Ramirez et al. 2011, Jia, Liu et al. 2013). This means other pathways must be explored if we are to elucidate the exact mechanism through which NCOA3 affects iNOS.

We have attempted to identify the pathway responsible through predictive analysis of the promoter sequence for iNOS. This analysis revealed that there were unique NF-AT1 and NF-AT4 binding sites, indicating a possibility that either of these transcription factors could be the ones causing the increase in iNOS expression when NCOA3 R485C is expressed. Calcineurin has been reported to trigger iNOS expression through NF-AT in some models (Kim, Moon et al. 2004) and likewise the Calcineurin and NF-AT blocker Cyclosporin-A was reported to inhibit iNOS expression in an NF-κB-independent manner in some models (Hamalainen, Lahti et al. 2002, Diaz-
Ruiz, Vergara et al. 2004, Eckstein, Van Quill et al. 2005, Lessio, de Assuncao Silva et al. 2005, Nomura, Vilalta et al. 2017). Based on these studies, we have attempted to utilize Cyclosporin-A as a means of blocking NF-AT activation in our model, comparing it to the results obtained with the ones achieved by blocking NF-κB-mediated activation using the LXR agonist TO901317. Surprisingly, treatment with these compounds did not significantly alter the activation of the iNOS promoter by NCOA3 R485C. This creates the possibility that NCOA3 R485C triggers the activation of iNOS through an as of yet unknown non-canonical pathway that remains to be described and characterized.

In summary, regulation of iNOS expression appears to be significantly more complex than initially thought, with different sequences of the promoter region being able to differentially drive expression under certain conditions and unknown factors influencing expression when fragments of the promoter sequence are deleted or altered. More interestingly however, NCOA3 R485C seems to induce activation of the iNOS promoter through a pathway that is not the canonical NF-κB pathway, nor the alternative Calcineurin/NF-AT pathway. These findings suggest that NCOA3 is capable of modulating iNOS expression through a novel pathway that remains to be identified and characterized. While not conclusive, the results herein presented hint at an intermediary regulatory mechanism that is not directly activated by NCOA3, which should be sought out. Identifying and understanding what this pathway is would certainly help to understand which pathways are vulnerable to over-activation, and when put together with other pieces of data give us a better insight into the molecular effectors of inflammation. From here onwards we could try exploring what other mutations of pathways affect iNOS expression, and from there we could work our way back up to NCOA3, and perhaps other regulatory mechanisms that would be better targets for pharmacological intervention. That means the key might not be in
the NCOA3 pathway, but rather in one that is shared with other effectors and produces the same effects. Regardless, discovering what causes this higher basal level of iNOS expression and finding ways to counter it is a step in the right direction for the prevention of MS, or at least for the treatment of symptoms.

5.2.3 Significance of this study

While mutations causing familial forms of MS are rare, these mutations nevertheless point us in the right direction, showing us which mechanisms when disrupted could lead to the development of autoinflammatory and autoimmune conditions. Discovering which molecular mechanisms are altered by these mutations leads to new avenues for scientific inquiry and exploration, as we can then start investigating other pathways and effectors that further our understanding of the phenomena involved in complex diseases such as MS.

Our findings on the disrupted function of NLRP12 caused by the L972Q mutation could, when put together with findings from other studies characterizing other mutations help us better understand how NLRPs regulate immune response, and how inflammasome assembly and function contribute to larger inflammatory phenomena implicated in complex diseases such as MS.

The discovery of increased iNOS expression caused by the R485C mutation of NCOA3 is novel, and shows us that changes in minute regulatory mechanisms could have wide-ranging effects such as leading to a higher basal level of inflammation which could contribute to development of the symptoms of MS. Building upon this finding and discovering which exact mechanisms are responsible for this change could create new targets for future pharmacological treatments.

Overall, the discoveries presented herein are considerable advances in understanding the complex molecular puzzle that leads to the multitude of symptoms seen in MS. Elaborating and
investigating further upon the results presented here could lead to the development of better, more specific strategies for the prevention or treatment of MS.
Bibliography


amyloid-beta precursor protein-cleaving enzyme 1 (BACE1), and shifting the cleavage site to Abeta Asp1 contributes to Alzheimer pathogenesis." Eur J Neurosci 37(12): 1962-1969.


Fielden, M. R., J. Werner, J. A. Jamison, A. Coppi, D. Hickman, R. T. Dunn, 2nd, E. Trueblood, 

Filser, S., S. V. Ovsepyan, M. Masana, L. Blazquez-Llorca, A. Brandt Elvang, C. Volbracht, M. B. 
synaptic plasticity and cognitive functions." Biol Psychiatry 77(8): 729-739.

secretase activity in living cells with a membrane-anchored FRET probe." Angew Chem Int Ed Engl 
51(43): 10795-10799.

tangles, and loss of cortical cholinergic fibers in Alzheimer disease." J Neuropathol Exp Neurol 
57(1): 63-75.


Gharagozloo, M., K. V. Gris, T. Mahvelati, A. Amrani, J. R. Lukens and D. Gris (2017). "NLR-
Dependent Regulation of Inflammation in Multiple Sclerosis: Dependent Regulation of 
Inflammation in Multiple Sclerosis." Front Immunol 8: 2012.


interactions with membranes and cholesterol: causes or casualties of Alzheimer's disease." 
Biochim Biophys Acta 1610(2): 281-290.


unique cerebrovascular amyloid fibril protein." Biochem Biophys Res Commun 122(3): 
1131-1135.

characterization of a novel cerebrovascular amyloid protein." Biochem Biophys Res Commun 
120(3): 885-890.

"Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer." Br J 

Goate, A., M. C. Chartier-Harlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. 
amyloid precursor protein gene with familial Alzheimer's disease." Nature 349(6311): 704-
706.

inhibitors and modulators." Biochim Biophys Acta 1828(12): 2898-2907.


"Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation." Nat Neurosci 4(3): 231-232.


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precursor protein in transgenic mice is efficient in neurons but inefficient in astrocytes. " J Biol Chem 271(49): 31407-31411.