The Humanization of Rodent Beta-Amyloid: The Aggregative and Toxic Properties of Amino-Terminal Beta-Amyloid Mutations

by

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ABSTRACT

Human β-amyloid$_{1-42}$ (hAβ_{1-42}) peptides are known to self-aggregate into oligomers that contribute to the degeneration of neurons and development of Alzheimer's disease (AD) pathology. Unlike humans, rodents do not develop AD, possibly due to differences in three amino acids (R5G, Y10F and H13R) within the hydrophilic N-terminal domain of Aβ_{1-42}. This is partly supported by evidence that hAβ_{1-42} is more prone to fibrillization and has a higher cellular toxicity than rodent Aβ_{1-42} (rAβ_{1-42}). Mutagenesis studies, however, have shown that correlation between fibrillization potential and toxicity is not always direct. Thus, to understand better how N-terminal mutations can affect hAβ_{1-42} toxicity through oligomerization, we evaluated fibrillization kinetics, oligomer sizes and toxicity profiles of double mutant (human towards rodent) Aβ_{1-42}. Additionally, we tested the mutant peptides in combination with hAβ_{1-42}, to assess effects on hAβ_{1-42} aggregation/toxicity. Our results clearly show that double mutations to humanize rAβ_{1-42} result in a significantly reduced efficiency of fibril formation, as determined by Thioflavin-T aggregation assays and confirmed with electron micrographic studies. Interestingly, the mutants are still able to aggregate into oligomers, which are predominantly larger than those comprised of hAβ_{1-42}. Our cell viability experiments further showed a rank order of oligomer toxicity of hAβ_{1-42}>rAβ_{1-42}>>mutant Aβ_{1-42}, suggesting that toxicity can be influenced by N-terminal Aβ_{1-42} mutations *via* reduction of fibril formation and/or alteration of oligomer size. These results, taken together, confirm that N-terminal mutations can affect Aβ fibril and oligomer formation with reduced toxicity despite lying outside the core amyloid region of Aβ peptide.

PREFACE

Chapter 2 of this thesis has been published as Foroutanpay, B. V., Kumar, J., Kang, S. G., Danaei, N., Westaway, D., Sim, V. L., & Kar, S. (2018). The Effects of N-terminal Mutations on β-amyloid Peptide Aggregation and Toxicity. *Neuroscience*, 379, 177–188. B.V.F. and J.K. have equal contributions. B.V.F. did the experiments of Aβ aggregation kinetics and cell toxicity, whereas J.K. performed the experiments related to light scattering and E.M. S.G.K. and D.W. performed and analyzed LDH-based cell toxicity data with the help of N.D. Both B.V.F. and J.K. analyzed the data and prepared the manuscript draft. V.L.S. and S.K., as supervisors, designed the study, helped in analyzing the data and wrote the manuscript.

This thesis is dedicated to my late mother

"The most beautiful word on the lips of mankind is the word "Mother," and the most beautiful call is the call of "My Mother." It is a word full of hope and love, a sweet and kind word coming from the depths of the heart. The mother is everything - she is our consolation in sorrow, our hope in misery, and our strength in weakness. She is the source of love, mercy, sympathy, and forgiveness."

-by Kahlil Gibran

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AD	Alzheimer's disease
ADAM	A Disintegrin and Metalloproteinase
AICD	APP intercellular domain
APH1	Anterior pharynx defective phenotype-1
APL-1	Amyloid precursor-like
APLP1	Amyloid precursor protein-like protein 1
APLP2	Amyloid precursor protein-like protein 2
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APPL	Amyloid precursor protein-like
Aβ	Amyloid-beta
BACE	β-site APP cleaving enzyme
CTF	Carboxyl-terminal fragment
DLS	Dynamic light scattering
EGCG	Epigallocatechin gallate
EM	Electron microscopy
ER	Endoplasmic Reticulum
IDE	Insulin Degrading Enzyme
LDH	Lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCT	Nicastrin
NEP	Neprilysin
NFT	Neurofibrillary tangles
PEN-2	Presenilin enhancer-2
PS1/PS2	Presenilin 1/Presenilin 2
ROS	Reactive oxygen species
sAPP	Soluble APP
ThT	Thioflavin T
TMD	Transmembrane domain

LIST OF PUBLICATIONS BY THE AUTHOR

1. Foroutanpay, B. V., Kumar, J., Kang, S. G., Danaei, N., Westaway, D., Sim, V. L., & Kar, S. (2018). The Effects of N-terminal Mutations on β-amyloid Peptide Aggregation and Toxicity. *Neuroscience*, 379, 177–188. https://doi.org/10.1016/j.neuroscience.2018.03.014

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CHAPTER – 1

General Introduction

1.1 Introduction

Alzheimer's disease (AD), the most common cause of senile dementia, is a multifactorial progressive neurodegenerative disorder depicted by a gradual loss of memory followed by deterioration of higher cognitive functions (Selkoe and Hardy, 2016; Lane et al., 2018). AD was initially described in 1906 by German psychiatrist Alois Alzheimer who identified both intracellular neurofibrillary tangles (NFT) and the extracellular β-amyloid (Aβ) peptide-containing neuritic plaques as the two pathological hallmarks of the disease which result in neuronal neurodegeneration (Goedert and Spillantini, 2006). It is estimated that as of 2011 there are approximately 750,000 Canadians living with AD or other related dementias (Alzheimer's Society of Canada, 2012). When considered globally, this number reaches approximately 50 million affected individuals, and is expected to double in 20 years (Brookmeyer et al., 2007; Bondi et al., 2017). The prevalence of AD rises exponentially with age; epidemiological studies present a 15-fold increase of dementia related cases between the ages of 60 and 85. The worldwide increased prevalence of AD is attributed to the advancing age of society combined with the length of the illness (Brookmeyer et al., 2007; Mayeux, 2012; Bondi et al., 2017; Wolteres and Ikram, 2018). The economic burdens posed by AD affect not only the healthcare system but also drastically impact the families and caregivers of AD patients, where social and caregiving costs amount to billions (Takizawa et al., 2015; Wolteres and Ikram, 2018). At present, there is no remission in the progression of the disease nor are there any effective treatments. Therefore, a better understanding of AD pathogenesis to search for reliable biomarkers and treatments are highly sought after in order to combat AD prior to the onset of symptoms.

Etiologically, AD is heterogeneous - a minority (<10%) of all AD cases segregate with genetic abnormalities, while the majority of cases are believed to be sporadic (Bertram and Tanzi, 2012; Rosenberg et al., 2016). Mutations in three genes, the amyloid precursor protein (*APP*) gene on chromosome 21, the presenilin 1 (*PSEN1*) gene on chromosome 14, and the presenilin 2

(*PSEN2*) gene on chromosome 1, have been identified as the cause of a large proportion of early-onset/familial AD cases (Bertram and Tanzi, 2012; Karch and Goate, 2015). Additionally, inheritance of the ε4 allele of the Apolipoprotein E (APOE) gene on chromosome 19 increases the risk of late-onset/sporadic AD (Corder et al., 1993; Poirier et al., 1993). Other contributors that play an important role in AD include age, and possibly some environmental factors such as diet, exercise, head injury, or stress (St George-Hyslop and Petit, 2005; Leduc et al., 2014; Karch and Goate, 2015). The neuropathological features associated with AD include the presence of extracellular Aβ-containing neuritic plaques, intracellular tau-positive NFT, and the loss of synapses and neurons in defined brain regions.

1.2 Amyloid cascade hypothesis

The principle theoretical concept behind AD pathogenesis known as the "*Amyloid cascade hypothesis"* suggests that increased level/deposition of Aβ peptides can directly trigger the development of AD pathology through a cascade of events leading to synaptic dysfunction, inflammation, tau pathology, neuronal loss and ultimately dementia (Hardy and Higgins, 1992; Hardy and Allsop, 1991). The amyloid cascade hypothesis has since been adapted to include the accumulation of intracellular and soluble oligomeric structures of the Aβ. An important facet to the amyloid cascade hypothesis is that all other features of AD including NFT, inflammation, synaptic dysfunction, and cognitive impairment, are consequent to the increased level and deposition of Aβ-related peptides (Selkoe and Hardy, 2016).

1.3 APP gene, structure, and function

The discovery of genes and proteins involved in AD began with studies conducted on patients affected with Down Syndrome or Trisomy 21, where it was observed that these patients invariably suffered from AD-like neuropathology and dementia (Olson and Shaw, 1969). The similarities between AD and Down Syndrome allowed research groups to target and isolate a ~4.2-kDa amyloid protein known as Aβ from neuritic plaques present in both AD and Down Syndrome patients. This unveiling provided researchers with substantial evidence that the genetic information that is responsible for AD and AD-like symptoms in Down Syndrome and that encodes for Aβ, may reside on chromosome 21 (Glenner and Wong, 1984a, 1984b; Masters et al., 1985). Shortly afterward, the human gene encoding the precursor protein of Aβ peptide

i.e., *APP* was identified and sequenced through the generation of a cDNA library from human AD brain samples (Kang et al., 1987). Located on chromosome 21 (21q21.3), the *APP* gene contains 18 exons and encompasses approximately 240 kilobases (Kang et al., 1987; Yoshikai, Sasaki et al., 1990). Although APP is encoded by a single gene, alternative splicing produces various isoforms of which the principal isoforms expressed in the brain are APP770, APP751 and APP695 (O'Brien and Wong, 2011; Dawkins and Small, 2014). APP695 is the predominate isoform expressed within the neuronal cells (Kang et al., 1987; Neve et al., 1988), while APP751 and APP770 are expressed primarily in non-neuronal cells such as glia and endothelial cells (De Silva et al., 1997).

APP is a member of a gene family that is evolutionarily conserved across various species. The emergence of the earliest APP orthologs found in species with primitive nervous systems containing functional synapses such as Amyloid Precursor Protein-Like (*APPL*) in the fly *D. melanogaster* (Rosen et al., 1989) and Amyloid Precursor-Like (*APL-1*) in the worm *C. elegans* (Daigle and Li, 1993). The evolutionary continuance of the APP family gives rise to its mammalian counterparts that include, APP and Amyloid Precursor Protein-Like Protein 1 and 2 (*APLP1* and *APLP2*) (Shariati and De Strooper, 2013). While APP and APLPs are subjected to the same proteolytic processing, it is important to distinguish that only APP, and not any other APP-related genes, encode for the Aβ sequence (Thinakaran and Koo, 2008; O'Brien and Wong, 2011; Dawkins and Small, 2014; van der Kant and Goldstein, 2015; Müller et al., 2017), therefore research into APP is paramount to decipher its role in AD pathogenesis.

Constitutively expressed, APP is a type-I transmembrane glycoprotein with a single transmembrane domain (TMD) that separates a large amino-terminal ectodomain from a small carboxyl-terminal cytoplasmic tail (Reinhard et al., 2005; Dawkins and Small, 2014). The N-terminus of APP contains an extracellular region composed of various subdomains, in particular the E1 and E2 domains. The E1 is a cysteine-rich globular region that encompasses a heparin-binding domain along with a metal binding domain specific for copper and zinc. While similar to the E1 domain, the E2 domain, known as the central APP domain, presents with an unique α-helix-rich composition presumed to function as an interaction site for binding partners or APP dimerization/self-association (Reinhard et al., 2005; Müller and Zheng, 2013; Dawkins and

Small, 2014; van der Kant and Goldstein, 2015). The E1 and E2 domains have been shown to mediate the dimerization (homo and heterodimers) of APP, significant in APP's ability to form cell-cell adhesion and its role in synaptogenesis (Rossjohn et al., 1999; Müller and Zheng, 2013; Dawkins and Small, 2014; Hoefgen et al., 2014).

A relatively unstructured flexible acidic region composed of a high content of glutamic and aspartic acid residues separates the E1 and E2 domain. While the relevance and function of the acidic region is not well understood, E1 domain plus the acidic region are sufficient to stimulate neurite outgrowth (Reinhard et al., 2005). Furthermore, the acidic region of longer APP isoforms (APP751) contain a Kunitz protease inhibitor (KPI), believed to protect APP protein from protease digestion and promote cell growth, while full length APP770 contains an supplementary OX2 binding domain, which is believed to mediate cell to surface receptor interactions (Dawkins and Small, 2014). The Aβ sequence, present in all APP isoforms, resides within the extracellular juxtamembrane domain and partially continues into the TMD (Müller and Zheng, 2013; Dawkins and Small, 2014).

The final structural domain at the carboxyl-terminal region of APP, known as the APP intercellular domain (AICD), constitutes a relatively short cytosolic component that encodes a YENPTY sorting motif (O'Brien and Wong, 2011; Müller and Zheng, 2013; Dawkins and Small, 2014). This YENPTY motif facilitates clathrin-mediated endocytosis, vital for the recycling of APP at the plasma membrane and is believed to promote synaptogenesis as it serves as a binding site for the majority of APP interacting proteins such as Fe65, JIP, and X11/Mint. Additionally, the YENPTY motif is responsible for the trafficking of APP to the synaptic buttons, as mutations to the conserved motif such as Y682G residue of the "Y682G"ENPTY, result in synaptic defects (Barbagallo et al., 2011). AICD has been suspected to initiate various signalling pathways such as calcium signalling, apoptosis, and gene transcription (Chen et al., 1990; Barbagallo et al., 2011; Müller and Zheng, 2013; Dawkins and Small, 2014; van der Kant and Goldstein, 2015).

1.4 APP trafficking and metabolism

As an integral type-I membrane protein, APP is incorporated within the Endoplasmic Reticulum (ER) after synthesis and then follows the secretory pathway through the Golgi apparatus to the

plasma membrane (Koo et al., 1996). During transit, APP undergoes various post-translational modifications, primarily within the Golgi apparatus, which include glycosylation, sulfation, phosphorylation and palmitoylation (Selkoe, 2001; Haass et al., 2012; Bhattacharyya et al., 2013). At the plasma membrane, the majority of APP is rapidly internalized *via* clathrin-mediated endocytosis and incorporated into the endosomal pathway (Weidemann et al., 1989; Koo and Squazzo, 1994; Yamazaki et al., 1996). APP can then be differentially regulated through recycling endosomes or targeted for degradation through endosomal-lysosomal compartments (Haass et al., 1992; Yamazaki et al., 1996; Andersen et al., 2005; Rogaeva et al., 2007). Only mature or post-translationally modified (N- and O-glycosylation) APP molecules are expressed at the plasma membrane where APP can be metabolized by various enzymes i.e., α-, β- and γ-secretases (Weidemann et al., 1989). The two primary alternate pathways mediated by specific secretases include; (1) α-secretase pathway mediated by α- and γ-secretases that lead to the generation of peptides which do not contain full-length Aβ peptide (i.e., non-amyloidogenic pathway) and (2) β-secretase pathway mediated by β- and γ-secretases that leads to the generation of intact Aβ peptide (i.e., amyloidogenic pathway) (Glenner and Wong, 1984a; Masters et al., 1985; Selkoe et al., 1986; Haass et al., 2012). This intricate proteolytic procedure generates various proteolytic fragments along multiple alternative pathways; exacerbating the complexities of examining APP and the many related proteolytic polypeptides produced. Fig. 1.1 provides a detailed depiction of APP metabolism, secretases and proteolytic fragments.

1.4.1 *α-secretase*

The non-amyloidogenic pathway constitutes a group of proteases known as A Disintegrin and Metalloproteinases (ADAMs) involved in the cell adhesion and cellular signalling through the proteolysis of the ectodomains of a diverse number of cell surface receptors and ligands (Dawkins and Small, 2014). Many ADAMs partake in proteolytic processing of APP (ADAM-8/9/10/17/19), whereas ADAM10 is considered to be the primary metalloproteinase involved in the proteolysis of APP between the lysine 16 and leucine 17 of the Aβ domain giving rise to a membrane bound α-carboxyl-terminal fragment (α-CTF) (C83) and a soluble APPα (sAPPα) (Esch et al., 1990; Thinakaran and Koo, 2008). The majority of non-amyloidogenic processing occurs at the cell surface due to the plasma membrane bound/associated ADAM10 (Sisodia, 1992; Haass et al., 2012).

1.4.2 *β-secretase*

The amyloidogenic pathway, which leads to the formation of intact Aβ peptides, is mediated through β-secretase, a transmembrane aspartyl protease known as β-site APP cleaving enzyme-1 (BACE1). BACE1 performs juxtamembrane cleavage of APP at the amino-terminus of Aβ peptide domain generating a membrane bound Aβ-containing β-carboxyl-terminal fragment (β-CTF) (C99) and a soluble APPβ (sAPPβ) (Hussain et al., 1999; Lin et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Thinakaran and Koo, 2008). Although BACE1 is present at the plasma membrane, it is known to be localized predominately within endosomal-lysosomal compartments where it conducts the majority of its APP endoproteolytic activity, possibly due to a higher concentration and proximity of APP and BACE1 or an optimal acidic environment (Vassar et al., 1999; Kinoshita et al., 2003; Thinakaran and Koo, 2008; Sannerud et al., 2011; Haass et al., 2012; van der Kant and Goldstein, 2015). Contrary to BACE1, homologous aspartyl protease BACE2 primarily processes APP through non-amyloidogenic pathways, where it cleaves APP between phenylalanine-19 and phenylalanine-20 of the Aβ peptide domain thus precluding the formation of an intact Aβ peptide species (Farzan et al., 2000).

1.4.3 *γ-secretase complex*

The γ-secretase is a tetrameric protein complex comprised of i) presenilin 1 (PS1) or 2 (PS2), an aspartyl protease functioning as the catalytic subunit of the complex, ii) nicastrin (NCT), believed to have roles in substrate recognition, iii) presenilin enhancer-2 (PEN-2), thought to act as a stabilizing subunit for the catalytic functions of PS1/2, and iv) anterior pharynx defective phenotype-1 (APH1), suggestive scaffolding roles for the γ-secretase complex (Edbauer et al., 2003; Kimberly et al., 2003; Haass et al., 2012). All components of the γ-secretase complex are essential for secretase complex function (Edbauer et al., 2003; Kimberly et al., 2003). The γ-secretase complex has a unique role in APP metabolism in that a series of sequential transmembrane cleavage events of the membrane anchored αCTF/C83 or βCTF/C99 lead to P3 and AICD or Aβ peptide and AICD respectively. Interestingly, γ-secretase is a unique enzyme in that it mediates the regulated intramembrane proteolysis of substrates that have already undergone ectodomain shedding (Lichtenthaler et al., 2011). In addition to APP, γ-secretase

complex mediates proteolysis of over 80 type-I membrane proteins, most famously the Notch receptors involved in neurogenesis (Haapasalo and Kovacs, 2011).

1.4.4 *η-secretase*

In addition to the aforesaid canonical APP processing *via* α- and β-secretases, recent reports have indicated APP is processed by a membrane-type 5-matrix metalloproteinase (MT5-MMP), called η-secretase, to generate η-carboxyl-terminal fragment (η-CTF). The η-CTF is subsequently cleaved by ADAM10 or BACE1 to generate lower molecular mass soluble peptides Aη-α or Aη-β peptides, respectively (Wang et al., 2015; Willem et al., 2015). Although Aη-α can inhibit long-term potentiation and the η-CTF fragment is found to be enriched in dystrophic neurites in animal models as well as human AD brains (Wang et al., 2015; Willem et al., 2015; Baranger et al., 2016), the potential role of CTFs generated by η-secretase in AD pathogenesis remains unclear (Willem et al., 2015; Andrew et al., 2016; Cheng et al., 2018; Maulik et al., 2018).

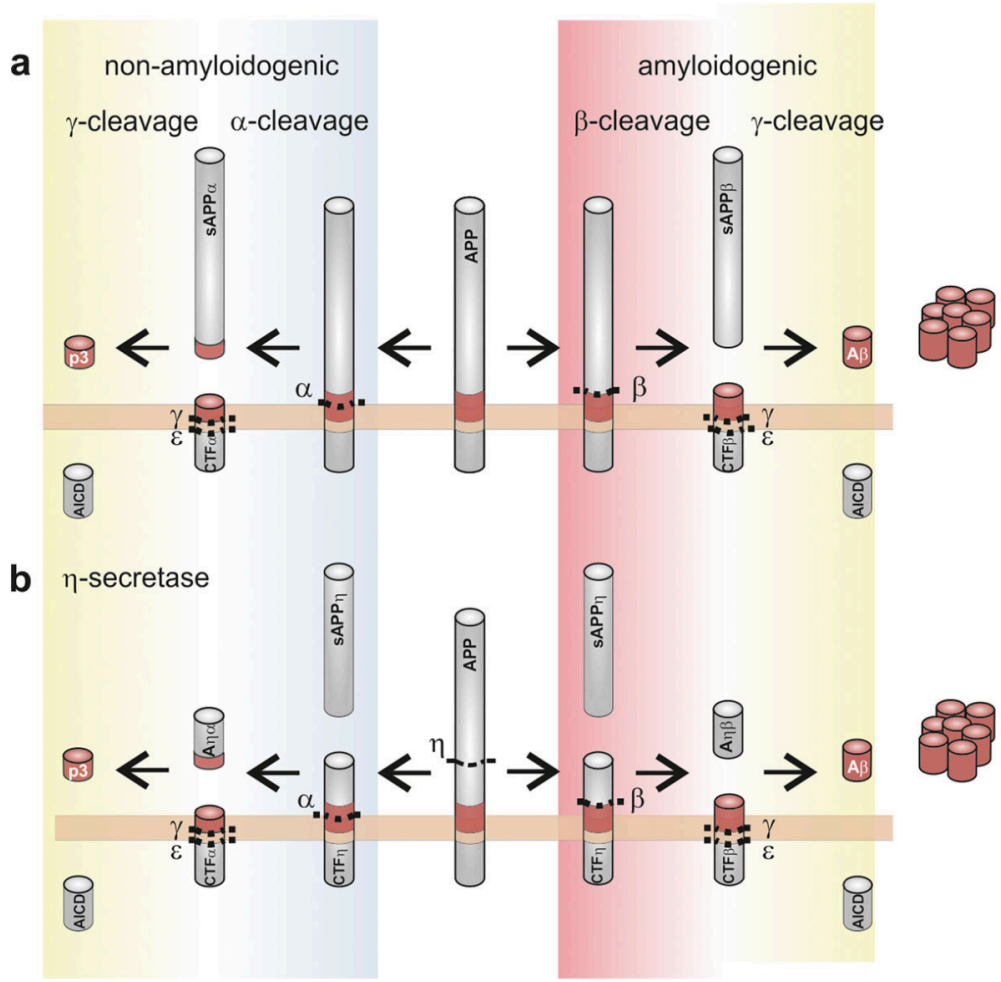

Figure. 1.1. Representation of different APP metabolism pathways. (A) The non-amyloidogenic and amyloidogenic metabolism of APP. (B) *η-secretase* proteolytic actions (adapted from Eggert et al. (2018) *Mol. Neurobiol.* 55:5809-5829).

1.5 Aβ peptides

Aβ production is a natural physiological process in APP expressing cells (Haass et al., 1992; Shoji et al., 1992; Walsh et al., 2000; Haass et al., 2012). While Aβ peptides can be produced to some extent at the plasma membrane, the majority of Aβ is generated in the endosomal-lysosomal compartments, which are involved in the internalization, recycling, and degradation of cellular proteins (Golde et al., 1992). Aβ peptides produced in the endosomal-lysosomal system can either be released extracellularly by secretory pathway/exosomes *via* multivesicular bodies or can be targeted to lysosomes for degradation. Like synthesis, degradation of Aβ peptide is primarily conducted by two zinc metallopeptidases known as neprilysin (NEP) and Insulin Degrading Enzyme (IDE). Other key enzymes/protein complexes that participate in the degradation of Aβ are cathepsins B and D, and proteasomes (Chesneau et al., 2000; Leissring et al., 2003; Huang et al., 2006; Poirier et al., 2006; Rajendran et al., 2006; Rajendran and Annaert, 2012).

The variability in enzymatic cleavage by γ-secretase from the β-CTF produce varying lengths of Aβ peptides containing approximately 39 to 43 amino acids residues; the most predominate and physiologically relevant species are $Aβ_{1-40}$ (90%) and $Aβ_{1-42}$ (10%) (Glenner and Wong, 1984a; Masters et al., 1985; Selkoe et al., 1986; Kang et al., 1987; Thinakaran and Koo, 2008; Haass et al., 2012; van der Kant and Goldstein, 2015). These two Aβ isoforms share an identical sequence other than two additional hydrophobic amino acids, isoleucine (Ile/I) at position 41 and alanine (Ala/A) at position 42 of the carboxyl-terminus of the $Aβ_{1-42}$ peptide (Teplow et al., 2006). $Aβ_{1-42}$ is hypothesized to be the main culprit in AD pathogenesis as it is the principal constituent of amyloid plaques and is prone to aberrant folding and aggregation leading to greater neurotoxicity (Irie et al., 2005; Masters and Selkoe, 2012; Zheng et al., 2015). Dyshomeostasis in the production and clearance of Aβ results in the abnormal accumulation of these proteinaceous species therein eliciting benign reactions that become more persistent. This results in pathological cellular reactions that induce irreparable impairment to the brain ultimately resulting in the clinical phase of the disease (De Strooper and Karran, 2016). Furthermore, clinical evidence demonstrates that deviation from the normal 1:10 ratio between Aβ42:Aβ40 is relevant to AD pathogenesis and correlates with the severity of AD progression (Irie et al., 2005) because

the accretion of the pathogenically associated $A\beta_{1-42}$ peptide is believed to initiate and contribute to neuronal degeneration and development of AD pathology.

1.6 Structure and function of Aβ peptide

Structurally, Aβ peptides contain a hydrophilic N-terminus (1-16), the hydrophobic central domain (17-21), the hydrophilic linker region (22-30) and hydrophobic C-terminus (31-42) (Kang et al., 1987). Despite being relatively unstructured in monomeric form, structural studies have shown that the Aβ peptide adopt two β-hairpin motif in its core and C-terminus (between residue 22 and 23 and residue 33 and 34 of Aβ42) along with two β-strands (between residues 15-21 and 24-32 of Aβ42 – forming two parallel β-sheets key for nucleation, oligomerization, and toxicity), whereas the N-terminus of Aβ remains unstructured and flexible due to its hydrophilic nature (Irie et al., 2005; Lührs et al., 2005; Ahmed et al., 2010). The β-hairpin motif of the Aβ is able to self-aggregate into a range of stable structures - a quality attributable to its amphiphilic nature and hydrophobic properties (Masters and Selkoe, 2012; Thal et al., 2015). While the amino acid backbone is responsible for the β-pleated sheet stacking structure characteristic of amyloid fibrils, the amino acid side chains indicative of the primary peptide sequence, play a vital role in the intermediate structures leading up to fibril formation (Irie et al., 2005).

The constitutive production of Aβ in the normal brain indicates its possible involvement in normal physiological functions such as neuronal growth, neurogenesis, regulating synaptic scaling, synaptogenesis and neurotransmitter release at low picomolar concentrations (Koo et al., 1993; Kamenetz et al., 2003; Kar et al., 1996, 2004; Abramov et al., 2009; Puzzo et al., 2008, 2011), while the overproduction or lack of clearance of Aβ peptides disrupts physiological homeostasis and increases the levels of Aβ-related peptides that ultimately leads to AD pathogenesis (Selkoe, 1991; Hardy and Higgins, 1992; De Strooper and Karran, 2016). Supporting this notion, a number of *in vitro* studies have shown that prolonged exposure to Aβ peptides at μM concentrations can cause neuronal toxicity. At present, however, mechanisms associated with Aβ-mediated toxicity are not clearly defined but appear to involve alterations in intracellular $[Ca^{2+}]$, production of toxic free radicals and activation of a caspase-cascade

culminating in cell death (Smith et al., 2006; Song et al., 2008; Cavallucci et al., 2012; Kayed and Lasagna-Reeves, 2013).

1.7 Aβ aggregation and its implication in AD

AD is principally characterized by fibrillar Aβ deposits in the brain parenchyma and cortical blood vessels. The term amyloid is in reference to tissue deposits composed of aggregated proteins that are distinguished by a shared β-pleated sheet fibril composition ranging from 8-10nm in diameter: the β-pleated sheets are oriented perpendicular to the long axis of the fibril (Irie et al., 2005). Specifically, in the case of AD, amyloid fibrils are extracellular molecular structures with diameters of approximately 10nm that are composed of Aβ peptides stacked upon one another as a cross-β-sheet structure with "characteristic dye-binding properties" (Goedert and Spillantini, 2006). These amyloid fibrils have the distinct attribute of being extremely stable proteinaceous structures; fractions of Aβ peptides isolated from post-mortem AD patients have proven to be insoluble (Roberts et al., 2017). The pathogenesis of Aβ in AD parallels other similar human neurodegenerative disorders such as Huntington's disease (Huntingtin), Parkinson's disease (α-synuclein) and prionopathies (prion protein), which link disordered protein folding and aggregation to toxic mechanisms (Haass and Selkoe, 2007; Teplow, 2013).

Although the pathogenic properties of Aβ peptides were generally attributed to their ability to form insoluble aggregates, it is now accepted that the most detrimental structural isoforms of Aβ peptides are soluble oligomers (Lambert et al., 1998; Dominic M et al., 2007; Benilova et al., 2012; De Strooper and Karran, 2016). Monomeric Aβ does not pose any cellular threat, however, self-associating into oligomers can result in neurodegeneration (Pike et al., 1991). Aβ oligomers are not only present up to two decades prior to AD onset but there is an increased correlation between disease and extracted oligomers from human AD brain tissue compared to amyloid plaque load (Hayden and Teplow, 2013). There is also a strong correlation between the quantities of soluble Aβ and the extent of neurodegeneration, synaptic loss and cognitive decline (Lue et al., 1999; McLean et al., 1999). These oligomeric assemblies of Aβ are responsible for the induction of neurotoxicity that drive neurodegeneration rather than the insoluble amorphous aggregates and fibrils, which represent a less harmful inactive form of the peptide (Irie et al.,

2005; Hayden and Teplow, 2013; Tipping et al., 2015). Amyloid plaques are thought to be inert and act as a dormant source of oligomeric Aβ, where a dynamic equilibrium exists between oligomeric Aβ and fibril Aβ (Hepler et al., 2006; Benilova et al., 2012). Furthermore, Aβ load, whether it be fibrillar or oligomeric, does not correlate to AD progression, but rather the length/isoform of Aβ peptides (pathogenic isoforms Aβ42 and Aβ43) could provide seeds for Aβ nucleation and amyloidosis (Bart De Strooper and Karran, 2016).

Although ubiquitously expressed, Aβ peptides are classified as unfolded proteins with various factors contributing to possible non-fibril assemblies or fibril precursors, pivotal to the formation of Aβ aggregates. Generation of Aβ aggregate is based on nucleation-dependent polymerization reactions, which consist of a protracted nucleation stage, monomers-oligomers responsible for the "lag phase", the expeditious elongation stage and fibril formation. The latency phase is described as the initial step where a small group of Aβ monomers form a nuclei/oligomers that subsequently aggregate to form amyloid fibrils. This process is driven by increased concentrations or mutations of Aβ that enhance its aggregation propensities based on the peptide charge, hydrophobicity and β-pleated sheet propensity (Chiti et al., 2003). Low micromolar concentrations of Aβ are sufficient to form stable fibrils *in vitro*, which can be examined through fibrillization kinetic assays such as thioflavin fluorescence binding assays that bind to the β-pleated sheet assemblies of the amyloid fibrils.

1.8 Mutations of AD-related genes and their association with Aβ peptides

While there is no specific cause for AD, etiologically it can be classified, as mentioned earlier, into either early-onset inherited or late-onset sporadic forms (Bhadbhade and Cheng, 2012). Of the many gene variants/mutations associated with early-onset AD, specific amino acid modifications within the Aβ domain of *APP* are essential for investigation in order to establish the relationship among various intrinsic factors, such as structural conformation, aggregation and toxic properties of Aβ peptide (Irie et al., 2005). The majority of APP mutations occur near α-, β-, or, γ-secretase processing sites. Mutations near the β-secretase, such as the APP Swedish double mutations (K670N, M671L) or (E682K), increase the affinity for β-secretase leading to enhance the production of Aβ peptide (Citron et al., 1992; Mullan et al., 1992; Zhou et al., 2011).

Whereas mutations near the α-secretase cleavage location (K687N – K16N of the Aβ domain), make APP a poor α-secretase substrate, thus encouraging its processing *via* β-secretase pathway (Kaden et al., 2012). The majority of mutations exist between the γ-secretase cleavage region and the carboxyl-terminal of the TMD (L723 and K724 – also known as the epsilon site). These increase the predisposition of γ-secretase complex to the generation of longer Aβ peptides that are considered to be more prone to aggregation and cell toxicity, which play a critical role in the development of AD pathology (Hardy, 1997).

A large number of disease-associated mutations are also located adjacent to the central hydrophobic domain of Aβ peptide. These mutations include Flemish (A692G – A21G of the Aβ domain) (Hendriks et al., 1992), Osaka (E693Δ – E22Δ of the Aβ domain) (Tomiyama et al., 2008), Arctic (E693G – E22G of the Aβ domain) (Nilsbeth et al., 2001), Italian (E693K - E22K of the Aβ domain) (Tagliavini et al., 1999), Dutch (E693Q – E22Q of the Aβ domain) (Levy et al., 1990), and Iowa (D694N – D23N of the Aβ domain) (Grabowski et al., 2001) mutations – all of which have been shown to alter the aggregative and/or neurotoxic properties of both Aβ40 and Aβ42 peptides (Dahlgren et al., 2002; Irie et al., 2005; Benilova et al., 2012). Since the majority of Aβ mutations concentrate at a β-turn at position 22-23 of the peptide, it is proposed that this region is a vital secondary structure highly associated to the aggregative and neurotoxic potentials of Aβ (Irie et al., 2005).

While many of these mutations are located in and around the hydrophobic central domain, directly relevant to aggregative events, the N-terminal mutations of the Aβ peptides are also relevant in the context of AD pathology. These mutations are critical as they are located adjacent to α- and β-secretase cleavage sites, thereby potentially influencing the generation of Aβ peptide. The N-terminal region is hydrophilic and observed as an unstructured flanking region outside of the amyloid core. Mutations identified within the N-terminal region include the (A673V – A2V of the Aβ domain) (Di Fede et al., 2009), English (H677R – H6R of the Aβ domain) (Janssen et al., 2003), Taiwanese (D678H – D7H of the Aβ domain) (Chen et al., 2012), Tottori (D678N – D7N of the Aβ domain) (Wakutani et al., 2004), and Leuven (E682K – E11K of the Aβ domain) (Zhou et al., 2011). The most interesting among N-terminal mutations are at position 2 of the Aβ

domain, which contain the A2V and A2T mutations (Jonsson et al., 2012). While A2T mutation has both pathological and cognitive protective effects, sequence variant modification to valine at the same position (A2V) results in early disease onset (Benilova et al., 2014; Maloney et al., 2014; Murray et al., 2016). Thus, distinctive mutations can result in unique features that are derived from properties gained by the amino-acid substitution; this may provide an insight not only about the neuropathology associated with AD but also the absence of disease pathology in a variety of other mammals including rodents. A general summary of disease-causing mutations spanning the Aβ domain of APP is demonstrated in Fig. 1.2.

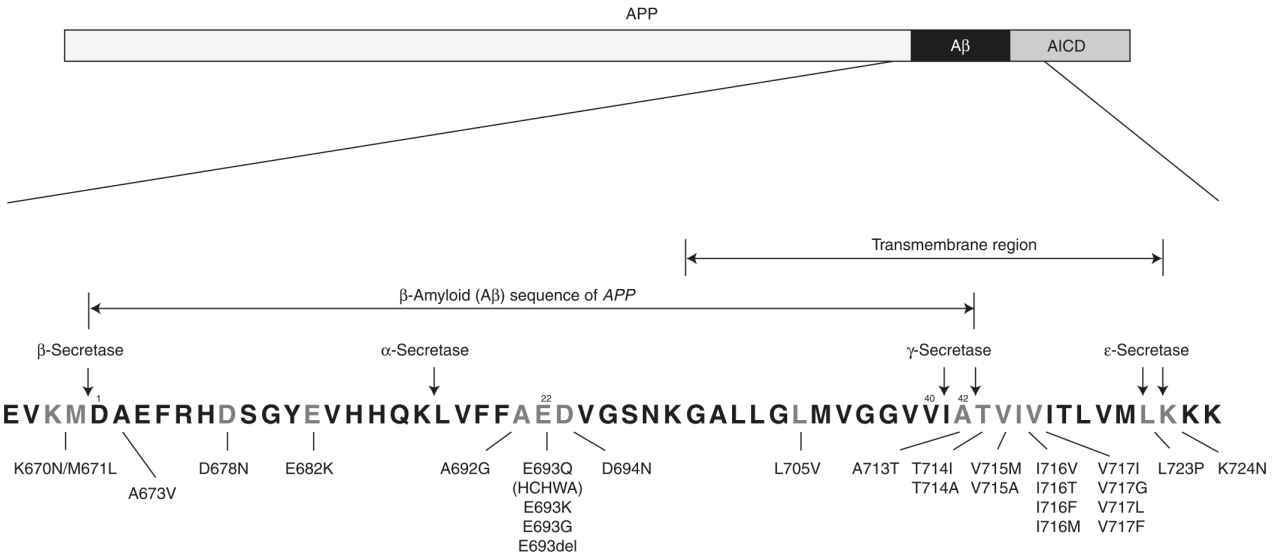

Figure. 1.2. Location of pathogenic mutations with the *APP* sequence spanning the Aβ domain (adapted from TCW and Goate (2017) *Cold Spring Harb. Persp. Med.* 7)

1.9 Aβ peptides in human *vs.* rodent

Although mice (*Mus musculus*) and rats (*Rattus norvegicus*) (i.e., rodent) $A\beta_{1\text{-}42}$ hold a high degree of sequence similarity to human $A\beta_{1\text{-}42}$ (i.e., 89% nucleotide sequence of the protein coding region and 97% amino acid homology), human Aβ compared to its rodent counterpart varies in only three amino acid residues [Gly(G)5Arg(R), Phe(F)10Tyr(Y), Arg(R)13His(H)] located at the amino-terminus of the peptide. None of these amino acid substitutions are believed to affect the secondary or tertiary structures of the Aβ, suggesting that the gene is highly conserved in mammalian evolution and functionally relevant. The only stipulation is that rodents do not spontaneously develop Aβ protein deposition or neuritic plaques, therefore the rodent APP is either metabolized differently or the rodent Aβ variant (three amino acid substitutions) could be responsible for altered Aβ aggregation/peptide stability – both of which may contribute to lack of AD pathogenesis in rodent (Yamada et al., 1987; Shivers et al., 1988). These subtle interspecies amino acid differences, however, provide further supports for a role for the amino-terminus of Aβ in AD pathogenesis.

While rodents may not develop neuritic plaques in their brains, rodent Aβ amyloidogenesis has been demonstrated *in vitro* where the peptides were able to form stable filaments (Hilbich et al., 1991). Thus, the lack of Aβ-containing neuritic plaques in the brains of aged rodents cannot be attributed to an inability to aggregate. It may partly relate to the fact that human Aβ is capable of forming aggregates at low concentrations, whereas rodent Aβ homolog requires considerably higher peptide concentrations (Otvos et al., 1993). Studies demonstrate that the rodent Aβ is less aggregative because of the tyrosine and histidine residue substitution, at positions 10 and 13 respectively, in the amino-terminal region of Aβ which is believed to have a vital role in amyloidogenesis (Dyrks et al., 1993). Additionally, one specific site-directed mutant (G5R – towards human) resulted in a three-fold increase of mutant Aβ production in comparison to its rodent counterpart (De Strooper et al., 1995). Previous studies have also shown that human Aβ can induce higher cellular toxicity than rat Aβ, reiterating the notion that amino acid variation at positions 5, 10 and 13 may have a key role in structural, functional, and cytotoxic responses of the Aβ peptide (Edrey et al., 2013; Lv et al., 2013). At present, however, very little is known

about the aggregation kinetics of human *vs*. rodent Aβ peptides and how humanization of rodent Aβ peptide can influence its aggregation properties and/or cell toxicity.

1.10 Hypothesis and aim

On the basis of the aforesaid information, we hypothesize that subtle amino acid differences in the N-terminal region of Aβ peptide between human and rodent may play a critical role in the development of AD pathology. To address this issue, we generated double mutants (human toward rodent – Fig. 2.1) of $A\beta_{1-42}$ peptide and assessed their ability to aggregates and induce cell toxicity in comparison to the human peptide.

CHAPTER – 2

The Effects of N-terminal Mutations on β-Amyloid Peptide Aggregation and Toxicity

A version of this chapter has been published in Neuroscience as follows:

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2.1 Introduction

Alzheimer's disease (AD), the most common cause of dementia affecting the elderly, is a progressive neurodegenerative disorder characterized pathologically by the accumulation of intracellular neurofibrillary tangles and extracellular neuritic plaques (Masters and Selkoe, 2012; De Strooper and Karran, 2016). The primary components of the plaques are 39-43 amino acid long amyloid-β (Aβ) peptides, derived from the amyloid precursor protein (APP) by sequential cleavage *via* β- and γ-secretases (Haass et al., 2012; Maulik et al., 2013; Andrew et al., 2016). Multiple lines of experimental evidence indicate that the accumulation of Aβ in the brain contributes to the loss of neurons and subsequent development of AD pathology (Poduslo et al., 2010; Revett et al., 2013; De Strooper and Karran, 2016). Physiologically, Aβ peptide predominantly exists in two isoforms, $A\beta_{1-40}$ and $A\beta_{1-42}$. $A\beta_{1-42}$ is hypothesized to be the main culprit involved in AD pathology as it exhibits greater neuronal toxicity and is believed to be the principle constituent of diffuse/neuritic plaques (Irie et al., 2005; Masters and Selkoe, 2012; Zheng et al., 2015). Due to its amphiphilic nature and high hydrophobic properties, $A\beta_{1-42}$ is able to self-aggregate into a variety of stable structures ranging from oligomers to amyloid fibrils (Irie et al., 2005; Masters and Selkoe, 2012; Thal et al., 2015). It is generally accepted that oligomeric forms of $A\beta_{1-42}$ are the predominant isoform involved in cell toxicity (Irie et al., 2005; Hayden and Teplow, 2013; Tipping et al., 2015), but given the many different types of oligomeric Aβ (Benilova et al., 2012) it is not clear if or how their specific aggregate structure relates to cell toxicity and AD pathogenicity.

The nature of an Aβ aggregate can be influenced by its primary sequence. The normal peptide contains a hydrophilic N-terminus (residues 1-16), a hydrophobic central domain (residues 17-21), a hydrophilic linker region (residues 22-30) and a hydrophobic C-terminus (residues 31-42) (Fig. 2.1) (Kang et al., 1987). While monomeric Aβ is largely flexible, its amyloid form contains a C-terminal core β-hairpin motif with a flexible hydrophilic N-terminus (Lührs et al., 2005; Ahmed et al., 2010). Mutations within the Aβ sequence can be associated with disease; many of these mutations are located adjacent to the central hydrophobic domain [Flemish (A21G), Dutch (E22Q), Arctic (E22G), Italian (E22K), Iowa (D23N)] where they can predictably interfere with amyloid core formation (Dahlgren et al., 2002; Irie et al., 2005). However, there are a number of

N-terminal mutations that can also affect disease susceptibility and may do so by affecting oligomer formation. For example, the English (H6R) and Tottori (D7N) mutant Aβ peptides produce larger oligomers (Hayden and Teplow, 2013), and the Taiwanese (D7H) isoform has been shown to produce more stable oligomers (Chen et al., 2012).

More evidence for a role of the N-terminus in Aβ pathogenesis comes from studies of mice and rats. These rodents produce Aβ but do not develop extracellular plaques or AD, possibly due to their differences in three amino acids (R5G, Y10F, and H13R) situated within the hydrophilic N-terminal domain of the peptide (De Strooper et al., 1995). Previous studies have demonstrated differences in Aβ aggregation rates and cytotoxicity between the human ($hA\beta_{1-42}$) and rat ($rA\beta_{1-42}$), where the human peptide was more prone to fibrillization and induced higher cellular toxicity (Edrey et al., 2013; Lv et al., 2013). Interestingly, single mutation studies of H13R (human towards rodent), rather than showing an intermediate aggregation profile, showed increased fibrillization of the mutant but with a reduced toxicity (Poduslo et al., 2010). Single mutation studies of Y10F (human towards rodent) also demonstrated increased aggregation in the mutant despite a lower toxicity (Dai et al., 2012). Thus, the correlation between fibrillization potential and toxicity is not always direct. To better understand whether N-terminal mutations can affect $hA\beta_{1-42}$ toxicity through influences on fibril formation or oligomerization, we generated double mutants (human towards rodent - Fig. 2.1) to contrast with the existing literature, which has largely examined single mutants (human towards rodent). Apart from determining fibrillization kinetics, oligomer sizes and toxicity profiles of these peptides, we also tested the peptides in combination with $hA\beta_{1-42}$, to assess for dominant negative or positive effects upon $hA\beta_{1-42}$ aggregation and toxicity.

2.2 Experimental Procedures

2.2.1 Materials

Time-pregnant Sprague-Dawley rats were obtained from the Biological Sciences Centre, University of Alberta (Edmonton, Alberta, Canada). Dulbecco's modified Eagle's medium (DMEM), neurobasal medium, fetal bovine serum (FBS), B27 and N2 supplement were purchased from Gibco (Waltham, Massachusetts, USA), whereas penicillin-streptomycin (P/S) was from Hyclone of GE Healthcare Life Sciences (Logan, Utah, USA). All isoforms of Aβ peptides including human $A\beta_{1-42}$ (i.e. $hA\beta_{1-42}$ - product number: 62-0-80), rat $A\beta_{1-42}$ (product number: 62-0-84) and various mutant rat $A\beta_{1-42}$ (i.e. Gly5Arg - product number: 316885 - referred to as Rfr; Phe10Tyr - product number: 365508 - referred to as gYr; Arg13His - product number: 365509 - referred to as gfH) were purchased from American Peptide (Sunnyvale, California, USA). Hexafluoro-2-Propanol (HFIP), Thioflavin T (ThT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis Missouri, USA), and lactate dehydrogenase (LDH)-based cytotoxicity assay kit was purchased from Promega (Wisconsin, USA). Electron microscopy grids (Formvar/Carbon 300 mesh, Copper with grid hole size 63μm) and uranyl acetate stain were purchased from TedPella (Redding, California, USA). All other chemicals were from Thermo Fisher Scientific (Montreal, QC, Canada) or Sigma-Aldrich.

2.2.2 Preparation of $A\beta_{1-42}$

All lyophilized $A\beta_{1-42}$ peptides stored at -80°C were first equilibrated at room temperature for 30min prior to dissolving in HFIP to obtain a 1mM solution. Once dissolved, peptide aliquots were quickly dried down using a SpeedVac to remove HFIP and moisture and then restored at -80°C for subsequent use. For experimental purpose, all isoforms of $A\beta_{1-42}$ peptides were thawed at 4ºC, diluted first with dimethyl sulfoxide (DMSO) to 5mM concentration and then to 100µM or 200µM concentrations with sterile dH_2O. For the preparation of Aβ fibrils, diluted peptides were incubated at 37°C overnight in phosphate-buffered saline (PBS, pH 7.4), whereas for the oligomer formation, peptides were incubated at 4°C in PBS overnight.

2.2.3 $A\beta_{1-42}$ kinetic reaction

For kinetic experiments, different isoforms of $A\beta_{1-42}$ at 5μM, 10μM or 20μM concentration were prepared in 100μL reaction buffer (10mM Na_2HPO_4 with 100mM NaCl) containing 0.001% ThT. The samples were then loaded into 96-well black walled plates with clear bottoms. All kinetic reactions were performed in triplicate and were carried out at 37°C. The fluorescence was continuously measured every 15min (with a 30sec shaking prior to measurement) over 48-72hrs using a Spectramax M5 spectrophotometer with excitation at 444nm and emission at 482nm with a cutoff filter at 475nm. Raw data for each set of experiments were normalized to $hA\beta_{1-42}$. The highest ThT fluorescence values for $hA\beta_{1-42}$ were set as 100% while lowest values were set to zero. The kinetics traces of other isoforms of $A\beta_{1-42}$ were normalized accordingly and represented as percentage fluorescence.

Raw data was fitted using the following equation (Nielsen et al., 2001) to determine the lag phase based on ThT fluorescence, where y_0 and y_f represent initial and final ThT fluorescence values respectively:

$$y = (y_0 + m_0\, x) + \left[(y_f + m_f\, x\,)/(1 + e^{-(t-t_0)/\tau}\,)\right]$$

Lag phase (L) was calculated using t_0-2τ.

The change in ThT fluorescence for co-incubated $A\beta_{1-42}$ and mutant peptide was calculated by averaging the normalized data for their maximum ThT fluorescence.

2.2.4 Electron microscopy (EM)

Aliquots of 5μL $A\beta_{1-42}$ reaction solutions were placed on 300 mesh carbon-coated copper grids for 2min followed by two washes with water. After removal of excess liquid, samples were negatively stained using 2% uranyl acetate. The dried samples were examined in a Hitachi H-7650 transmission electron microscope at 80 kV.

2.2.5 Light scattering

DLS experiments were performed with a Malvern Zetasizer-Nano S. A 633nm wavelength HeNe laser was used to detect backscattered light at a fixed angle of 173°. The software (DTS v6.20)

provided both the mean size and polydispersity by cumulants analysis. We assumed the solution viscosity and refractive index (1.33) to be that of water for calculation purposes. The cell holder was maintained at 4°C for the measurement of 4°C generated oligomers. Data were collected using a 3mm x 3mm quartz cuvette filled with 45μL of sample (100μM) and 45μL of mineral oil on top to avoid evaporation. The data were collected without attenuation and a minimum number of 10 consecutive runs of 10seconds each were averaged to obtain the autocorrelation function. Particle size was calculated by the manufacturer's software through the Stokes-Einstein equation assuming spherical shapes of the particles.

2.2.6 Cultures of rat cortical neurons

Rat primary cortical neurons were cultured from embryonic 18-day fetuses in accordance with the University of Alberta and the Canadian Council for Animal Care guidelines. In brief, the pregnant rats were anesthetised with halothane and then the frontal cortical region of the brain was dissected out and digested with TrypLE Express. Subsequently, cells were centrifuged and re-suspended in growth medium containing neurobasal medium supplemented with 2% B27, HEPES 10mM, P/S 25U, and L-Glutamine 0.5mM. The cell suspension was filtered through a cell strainer and plated (5×10^3 cells/well) on 96-well plates. The medium was replaced every three days and all experiments were performed on day 6 after initial plating, when neurobasal medium supplemented with B27 was replaced with 1% N2 as described earlier (Song et al., 2008).

2.2.7 MTT and LDH assays

Viability of rat cortical neurons following treatment with various isoforms of $A\beta_{1\text{-}42}$ was assessed using MTT and LDH assays as described earlier (Song et al., 2008; Wang et al., 2015). For MTT assays, rat cortical neuronal cultures were treated with or without various isoforms of $A\beta_{1\text{-}42}$ at different concentrations (5μM, 10μM or 20μM) for 24hr. In some cases, cultured neurons were co-treated with equimolar ratios of 5μM $hA\beta_{1\text{-}42}$ plus 5μM Rfr, gYr, or gfH mutant $A\beta_{1\text{-}42}$ for 24hr. Subsequently, media from control and various Aβ-treated cultured neurons was replaced with new media containing 0.5mg/mL MTT and then cultures were incubated for 4hr at 37°C with 5% CO_2/95% air. The formazan was then dissolved in DMSO and absorbance was measured at 570nm with a Spectramax M5 spectrophotometer. To substantiate MTT data, rat

cortical cultured neurons were treated with 10μM hAβ_{1-42}, rAβ_{1-42}, Rfr, gYr, or gfH mutant Aβ_{1-42} as mentioned above and then cytotoxicity was determined based on the measurement of LDH activity released into the conditioned medium from the cytosol of damaged cells. The absorbance was measured at 490nm with a Spectramax M5 spectrophotometer. Both MTT and LDH experiments were repeated three to five times with three technical replicates per sample.

Statistical analysis

All data were collected from a minimum of 3 biological repeats and expressed as means ± SEM. Kinetics of peptide aggregation as well as cell viability data from cultured neurons were analyzed by one-way ANOVA followed by Bonferroni's *post-hoc* analysis for multiple comparisons with a significance threshold set at $p < 0.05$. All statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., CA, USA).

2.3 Results

2.3.1 Fibril aggregation kinetics of $hA\beta_{1-42}$, $rA\beta_{1-42}$ and mutant $A\beta_{1-42}$

To assess aggregation kinetics of mutant $A\beta_{1-42}$, 10μM of all isoforms of Aβ peptides [$hA\beta_{1-42}$, $rA\beta_{1-42}$, Rfr (Gly5Arg), gYr Phe10Tyr) and gfH (Arg13His)] were subjected to thioflavin T (ThT) fluorescence assays as previously described (Kumar et al., 2015). Despite some variability, $hA\beta_{1-42}$ exhibited the shortest lag phases (7.24 ± 3.80hrs) and highest ThT levels (948.8909 +/- 215.8747 absorbance units) at all time points studied. Therefore, for each experiment, final ThT fluorescence values for $hA\beta_{1-42}$ were averaged and normalized to 100% and readings of other isoforms of $A\beta_{1-42}$ were normalized accordingly and represented as percentage fluorescence (Fig. 2.2A, B). The kinetics of $rA\beta_{1-42}$, in keeping with earlier results (Edrey et al., 2013; Lv et al., 2013) depicted a longer lag phase (17.59 ± 9.66hrs) and lower ThT values (77.58 ± 14.75%) than those observed with $hA\beta_{1-42}$. Interestingly, our mutant Rfr, gYr and gfH peptides, rather than presenting with intermediate kinetic profiles, showed no appreciable exponential growth curves (Fig. 2.2A, B), therefore lag phase calculations were not applicable. Additionally, the maximum changes observed in ThT fluorescence over a period of 48hrs for Rfr, gYr and gfH peptides were significantly less than those observed for either $hA\beta_{1-42}$ or $rA\beta_{1-42}$ (Rfr: 14.49 ± 3.54%; gYr: 17.78 ± 2.95%; and gfH: 12.38 ± 6.45%) (Fig. 2.2C).

2.3.2 Morphological analysis of Aβ fibrils

To determine whether the Rfr, gYr and gfH peptides formed fibrils or aggregates despite low final ThT fluorescence values, we examined the end-products of 48hr kinetic reactions with electron microscopy (EM). Surprisingly, electron micrographs revealed the presence of fibrils in each group, although the number of fibrils in the mutant peptide preparations was markedly less (Fig. 2.3A-E), consistent with the low ThT fluorescence values. As expected, $hA\beta_{1-42}$ formed long fibrils while $rA\beta_{1-42}$ displayed fewer fibrils overall, with a combination of long fibrils and shorter fragments, consistent with previous findings (Lv et al., 2013). In contrast, the mutant peptide preparations contained sparse populations of fibrils (Fig. 2.3C-E). Of note, there were no amorphous aggregates in any of the preparations.

2.3.3 Morphological analysis of Aβ oligomers

To assess characteristics of the mutant peptides, 10μM of the mutant Aβ peptides (Rfr, gYr and gfH) were put under oligomer-forming conditions as previously described (Messa et al., 2014; Kumar et al., 2015). After 24hrs at 4°C, oligomers could be detected by dynamic light scattering (DLS) for all the mutants, each producing a distinct size distribution (Fig. 2.4; area under the curve equals 100% of the distribution of the particles for each peptide). $hA\beta_{1-42}$ was used as a control and, consistent with prior studies indicating that $hA\beta_{1-42}$ generates oligomers with hydrodynamic radii between 5 and 20nm (Ahmed et al., 2010; Cizas et al., 2010; Sakono and Zako, 2010), 53.5% of our $hA\beta_{1-42}$ oligomeric peptide preparation formed aggregates less than 10nm in radius (maxima 5.848nm) with 33.8% of the remaining oligomers existing between 10 and 40nm. In contrast, the majority of mutant $A\beta_{1-42}$ peptides generated oligomers larger than 10nm. Rfr and gfH were similar to each other with respect to their oligomer size distributions, with 45.1% and 53.0% of the total oligomer population, respectively, existing between 10 and 20nm with maxima of 14.11nm. Interestingly, gYr displayed two distinct polydisperse populations, one with a maximum at 39.41nm and the other at 741.9nm.

2.3.4 Mutant $A\beta_{1-42}$ oligomers and viability of cortical cultured neurons

To determine the neurotoxic potency of mutant $A\beta_{1-42}$ oligomers, rat primary cortical cultured neurons were treated with various concentrations (5, 10 and 20μM) of human, rat and mutant oligomeric isoforms of $A\beta_{1-42}$ for 24hrs and then cell viability was assessed using an MTT assay. As expected, both $hA\beta_{1-42}$ and $rA\beta_{1-42}$ induced toxicity in a dose-dependent manner in cortical cultured neurons, with the potency of effect being significantly higher for $hA\beta_{1-42}$ than $rA\beta_{1-42}$ (Fig. 2.5A, B). The mutant isoforms Rfr, gYr and gfH also produced dose-dependent toxicity, but to a lesser extent than $hA\beta_{1-42}$ or $rA\beta_{1-42}$, with only concentrations of 10 and 20μM significantly affecting cell viability in all cases. Treatment with 5μM of Rfr and gYr, but not gfH, also caused some toxicity (Fig. 2.5A). The cell viability results obtained with MTT assay were also validated with LDH assay, which confirmed the same hierarchy of neurotoxicity; $hA\beta_{1-42}$ was more toxic than $rA\beta_{1-42}$, and the mutant forms affected cell viability to a much lesser extent (Fig. 2.5C).

2.3.5 Effect of mixing hAβ_{1-42} with mutant peptide oligomers on neuronal viability

Given that 5μM Rfr, gYr and gfH oligomer preparations were least toxic to neurons, we wanted to determine whether the use of these preparations could protect cultured neurons from hAβ_{1-42}-induced toxicity. To address this issue, rat primary cortical cultures were treated for 24hrs with 5 or 10μM hAβ_{1-42} oligomers, 5μM of mutant Aβ_{1-42} oligomer, or a combination of 5μM hAβ_{1-42} + 5μM mutant Aβ_{1-42} oligomer preparations. Cell viability was assessed using MTT assay (Fig. 2.6). As expected, exposure to 5 and 10μM hAβ_{1-42} oligomers caused a significant reduction in cell viability (64.89%±4.58% and 58.33%±1.05, respectively), whereas the effect of 5μM mutant Aβ_{1-42} was much less pronounced. Interestingly, adding 5μM mutant Aβ_{1-42} to 5μM hAβ_{1-42} generated a similar cell viability as observed with 5μM hAβ_{1-42} alone (Fig. 2.6), suggesting that none of the mutant Aβ_{1-42} oligomer preparations were able to protect cortical neurons from hAβ_{1-42} oligomer-induced toxicity.

2.3.6 Effect of mixing hAβ_{1-42} with mutant peptides on hAβ_{1-42} aggregation

To test whether the mutant peptides were able to interact with and affect aggregation properties of hAβ_{1-42}, we measured fibril aggregation kinetics and DLS oligomer profiles from co-incubated reactions. For fibril reactions, we combined increasing molar ratios of Rfr, gYr and gfH with 5 or 10μM hAβ_{1-42}. There was marked variability between experiments with no consistent or significant effects on the aggregation kinetics of hAβ_{1-42}, suggesting that the aggregation is dominated by hAβ_{1-42} (data not shown).

For DLS measurements of oligomer formation, we combined equimolar amounts (50μM each) of hAβ_{1-42} plus mutant and incubated at 4ºC (Fig. 2.7) overnight. As expected, the majority (56.4%) of oligomers generated by hAβ_{1-42} alone had hydrodynamic radii of less than 10nm with a prominent maximum at 5.484nm (14.2%). There were also minor peaks at 14.11nm (3.8%) and 25.37nm (4.7%). The area under these three peaks equalled 87.3% of the total population; the remainder of the population was less than 500nm, with two small peaks with maxima at 147.7nm (1.3%) and 356.2nm (0.8%). For all co-incubated reactions, the overall aggregate sizes were larger than for hAβ_{1-42} alone, with the proportion of aggregates below 10nm in radius falling from 56.4% to 7.7%, 16% and 12.5% when co-incubated with Rfr, gYr, and gfH respectively (Fig. 2.7A). In addition, the dominant 5.484nm hAβ_{1-42} peak disappeared in each case and was

either replaced with a dominant peak at 14.11nm (for Rfr), 12.18nm (for gYr), or three roughly equal peaks at 10.52nm, 16.34nm and 29.39nm (for gfH). Co-incubation with Rfr produced two polydisperse populations and closely resembled the profile of Rfr alone. The first population spanned 7.843 - 34.03nm with maxima at 14.11nm (11%). The second covered a larger range, spanning 477.7 - 2780nm with maxima at 1335nm (3.1%). Co-incubation with gYr produced four populations. The first spanned 9.803 - 16.34nm with maxima at 12.18nm (14.8%). The second had a maximum at 34.03nm (3.3%) and the third had a maximum at 171.0nm (1.6%). The fourth had a wide range from 477.7 - 2073nm and a maximum at 995.1nm (8.1%). Co-incubation with gfH produced three populations. The first spanned 6.772 - 70.89nm with three similarly populated maxima at 10.52nm (6.3%), 16.34nm (6.4%) and 29.39nm (5.4%). The second had a maximum at 147.7nm (2%), and the third had a maximum at 741.9nm (4.5%).

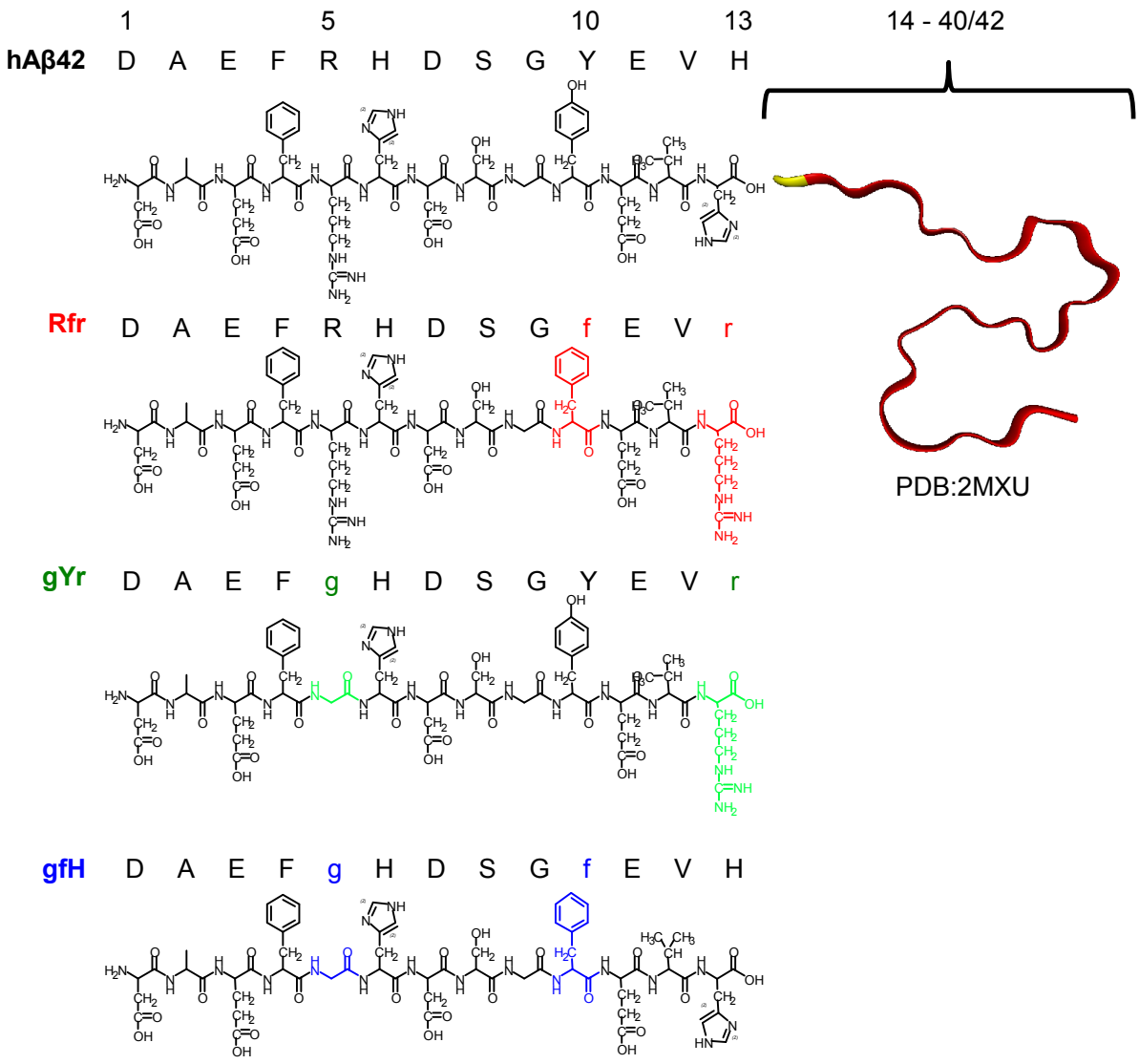


Figure 2.1. Structure of human $A\beta_{1-42}$ (hAβ42) peptides with and without N-terminal rodent sequence substitutions. N-terminal sequences (1-13) of Aβ42 are shown highlighting the mutant residue structures beside a ribbon structure of residues 14-42 (taken from PDB:2MXU). Upper case letters represent human sequence and lower case represent rodent sequence.

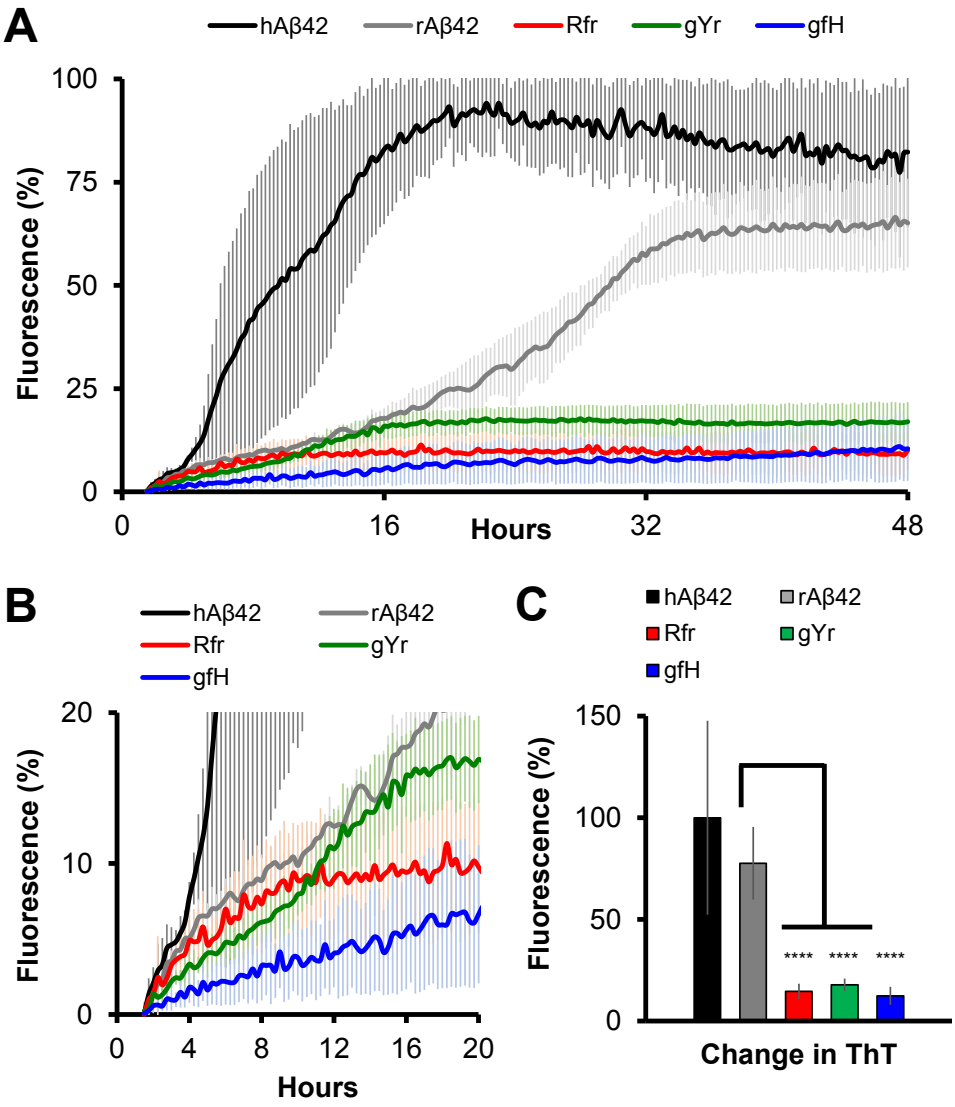


Figure 2.2. Aggregation kinetics of 10μM peptides over 48hrs at 37°C. (A) Averaged kinetic traces for rodent Aβ, hAβ42, Rfr, gYr, and gfH. (B) Average percent changes in ThT fluorescence relative to hAβ42 alone. (C) Inset of panel A showing details of early kinetics. Rank order of rate of aggregation and increase in ThT fluorescence is hAβ42> rodent Aβ >> mutant peptides. The data shown is from three independent experiments (total n = 10 for hAβ42). Error bars = SD. ****$p < 0.0001$

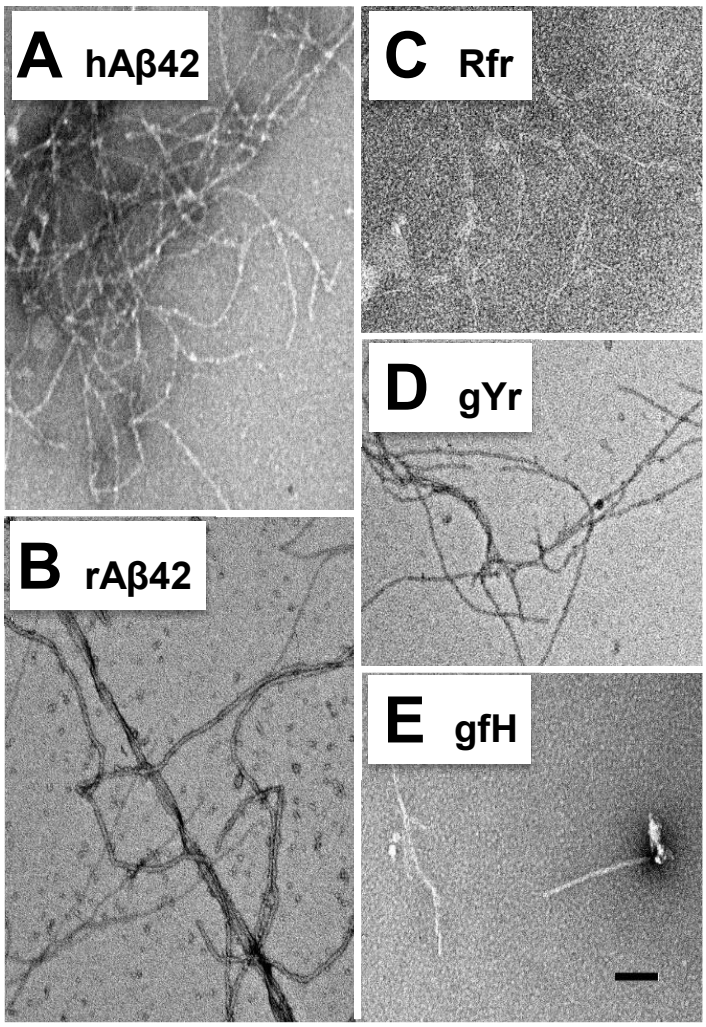


Figure 2.3. **Transmission electron micrographs of end-products of 48hr $A\beta_{1-42}$ fibril (37°C) reactions.** (A) human Aβ42 fibrils (B) rodent $A\beta_{1-42}$ fibrils (C) Rfr fibrils (D) gYr fibrils and (E) gfH fibrils. The fibril abundance was very low in Rfr, gYr and gfH fibrils preparations. Scale bar = 100nm.

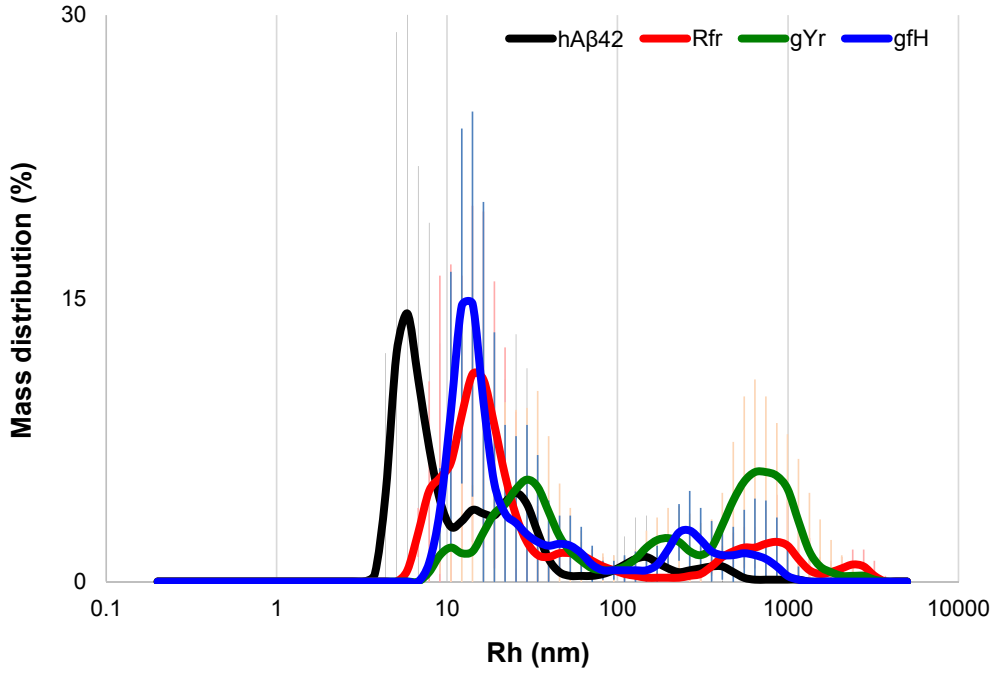
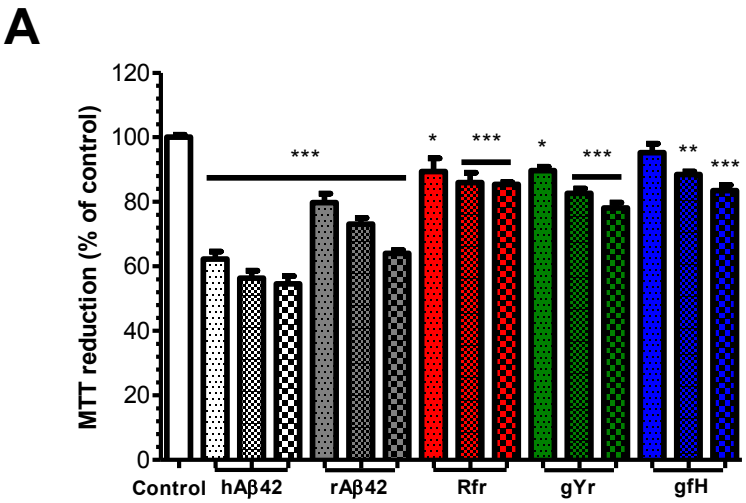
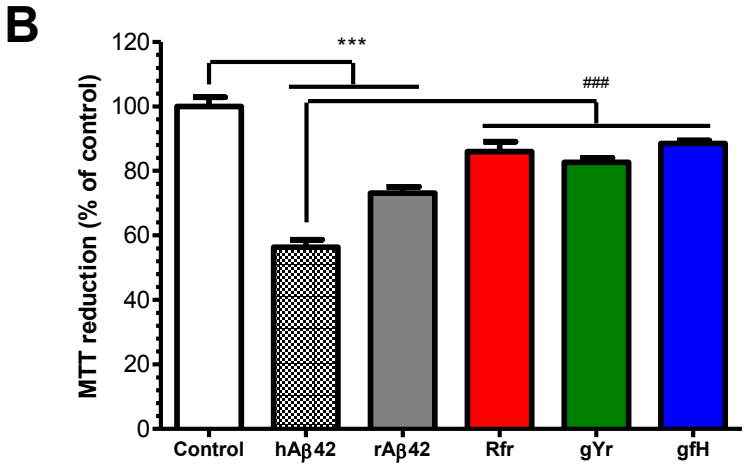


Figure 2.4. Dynamic light scattering analysis of end-products of 24hr oligomer (4°C) preparations. Dynamic light scattering traces showing that Rfr, gYr and Rfr oligomers are larger than hAβ42 oligomers. The size (hydrodynamic radius (Rh)) distribution by mass has been plotted. Averages of ten readings are shown with error bars representing standard deviation.


A
MTT reduction (% of control)
120
100
80
60
40
20
0
***
*
***
*
***
**
***
Control
hAβ42
rAβ42
Rfr
gYr
gfH


B
MTT reduction (% of control)
120
100
80
60
40
20
0
***
###
Control
hAβ42
rAβ42
Rfr
gYr
gfH

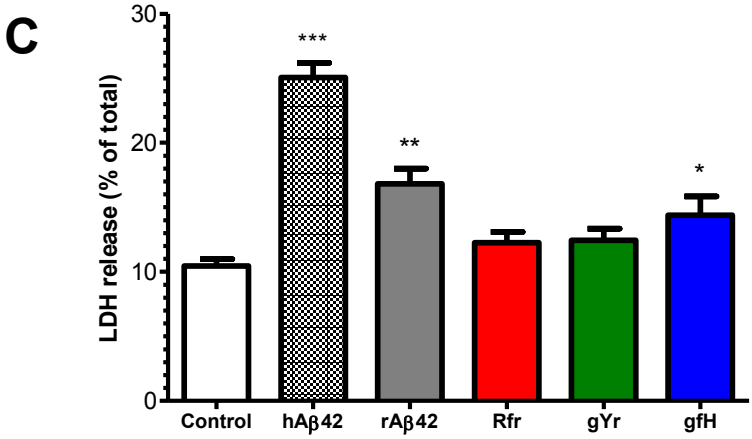

C
LDH release (% of total)
30
20
10
0
***
**
*
Control
hAβ42
rAβ42
Rfr
gYr
gfH

Figure 2.5. Neuronal viabilities with various $A\beta_{1-42}$ peptides. (A) Histograms showing the dose-dependent (5, 10 and 20μM) effects of 24hr exposure to various mutants $A\beta_{1-42}$ compared to $hA\beta_{1-42}$ as well as $rA\beta_{1-42}$ on viability of cortical cultured neurons as revealed by MTT assay. (B) Histograms depicting that 24hr exposure to various mutant $A\beta_{1-42}$ peptides at 10μM concentration are significantly less toxic to cultured neurons than equimolar concentration of rodent or human $A\beta_{1-42}$. (C) Histograms depicting that exposure to various mutant $A\beta_{1-42}$ peptides at 10μM concentration is significantly less toxic to cultured neurons than equimolar concentration of rodent or human $A\beta_{1-42}$ as revealed by LDH assay. Data presented as % of control (means ± S.E.M.) were obtained from three to five separate experiments, each performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{\#\#\#}p < 0.001$.

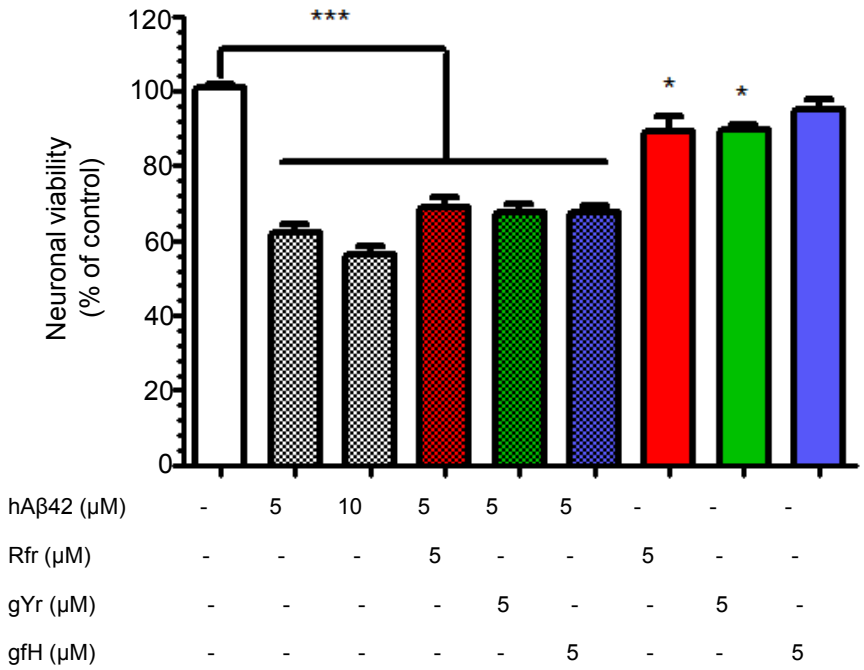


Figure 2.6. Neuronal viabilities with mixture of mutant and hA$\beta_{1\text{-}42}$ peptides. Histograms showing the effects of hA$\beta_{1\text{-}42}$ with or without various mutant A$\beta_{1\text{-}42}$ peptides on viability of cortical cultured neurons as revealed by MTT assay. As evident from the histogram, 24hr exposure to 5μM mutant A$\beta_{1\text{-}42}$ along with 5μM hA$\beta_{1\text{-}42}$ was unable to enhance cell viability compared to those observed with 5μM hA$\beta_{1\text{-}42}$ alone. Data presented as % of control (means ± S.E.M.) were obtained from three to five separate experiments, each performed in triplicate. *$p <$ 0.05, ***$p < 0.001$.

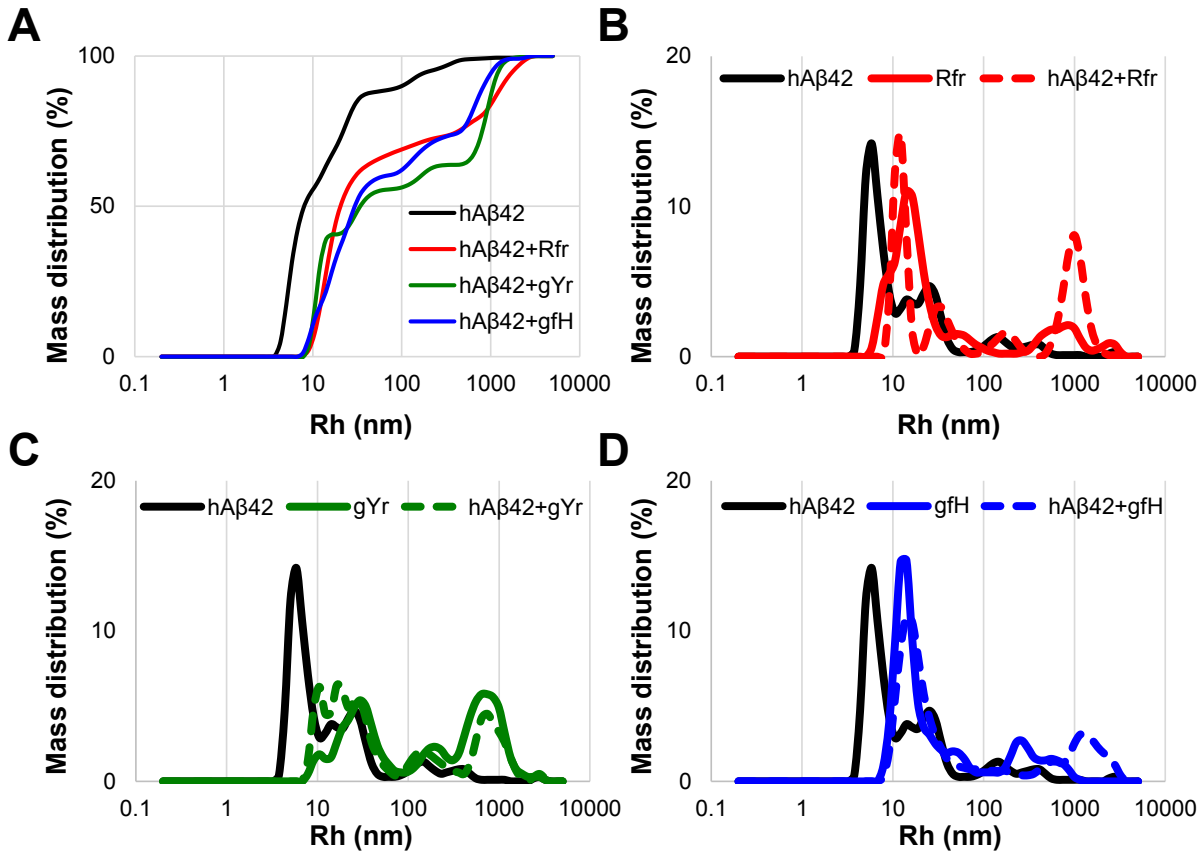


Figure 2.7. Dynamic light scattering analysis of end-products of 24hr oligomer (4°C) preparations in the presence of mutant peptides. (A) Undersize graph showing a shift to larger oligomer populations when hAβ42 is co-incubated with Rfr, gYr and Rfr. (B-D) The shift in oligomer size distribution when hAβ42 is co-incubated with (B) gfH, (C) Rfr or (D) gYr. The size (hydrodynamic radius (Rh)) distribution by mass has been plotted. Averages of ten readings are shown with error bars representing standard deviation.

2.4 Discussion

Our biophysical and cellular analyses of mutant $A\beta_{1-42}$ peptides support a role of the N-terminus of Aβ in peptide aggregation and toxicity. We demonstrate that double mutations constructed to humanize the rodent $A\beta_{1-42}$ sequence result in a significantly reduced efficiency of fibril formation, as determined by kinetic ThT aggregation assays and further confirmed with our electron micrographic studies, which revealed sparse fibrils that were morphologically shorter and thinner than both $hA\beta_{1-42}$ and $rA\beta_{1-42}$. Interestingly, the mutants were readily able to aggregate into oligomers, but each mutant formed oligomers that were predominantly larger than those comprised of $hA\beta_{1-42}$. Cell viability deduced from MTT and LDH assays, showed a rank order of oligomer toxicity of $hA\beta_{1-42}$>$rA\beta_{1-42}$>>mutant $A\beta_{1-42}$, demonstrating that toxicity can be influenced by N-terminal $A\beta_{1-42}$ mutations *via* reduction of fibril formation and/or alteration of oligomer size.

There is evidence that the efficiency of Aβ fibril formation does not predict toxicity, and the effect of N-terminal Aβ mutation can further depend on whether the construct being used is $A\beta_{1-40}$ or $A\beta_{1-42}$. Peptides containing the single mutation H13R (created within human $A\beta_{1-40}$) (Poduslo et al., 2010) or Y10F (also created within human $A\beta_{1-40}$) (Dai et al., 2012) were more likely than $hA\beta_{1-40}$ to aggregate and yet had lower toxicity. By contrast, studies of naked-mole rat Aβ, which is the equivalent to an H13R mutation within $hA\beta_{1-42}$, revealed a disconnect between aggregation propensity and toxicity, with the peptide aggregating more slowly yet having the same toxicity as $hA\beta_{1-42}$ at 10μM (Edrey et al., 2013).

Rather than propensity for fibril formation, oligomer characteristics such as size may be more relevant to the endpoint measure of cell viability. It is well known that levels of oligomers correlate more with the symptoms of AD (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999; Näslund et al., 2000) and are more toxic *in vitro* (Benilova et al., 2012). Aβ lacking residue 22 causes AD and, while it is unable to form fibrils *in vitro*, it can produce oligomers that inhibit long-term potentiation (Tomiyama et al., 2008). The N-terminal mutation A2V causes AD, possibly *via* an altered pathway of oligomerization (Messa et al., 2014), and in a study of D-phenylalanine substitution in Aβ, toxicity was correlated with oligomer size, with the presence of very large aggregates associated with reduced toxicity (Kumar et al., 2015). A study of the H13R

mutation in $hA\beta_{1-42}$ also demonstrated an inverse relationship between oligomer size and toxicity, with H13R peptides forming larger oligomers with less effect on cell viability, as measured by MTT (Roychaudhuri et al., 2015).

Many different types of oligomers can be generated *in vitro* or isolated from AD-affected brains (Benilova et al., 2012). It is now well established that Aβ peptide exists in multiple forms including monomers, dimers, trimers and oligomers to protofibrils and fibrils that range in size from 4kD to more than 100kD which vary in morphology and conformation (Rushworth and Hooper, 2010; Jarosz-Griffiths et al., 2016). Soluble Aβ oligomers appear to be the most neurotoxic species, triggering various processes that underlie AD including synaptic dysfunction, impairment of long-term potentiation, Ca^{+2} dysregulation, mitochondrial dysfunction, ER stress, lysosomal breakdown and activation of pro-apoptotic pathways leading to cell death (Walsh and Selkoe, 2007; Ferreira and Klein, 2011; Benilova et al., 2012; Thal et al., 2015). Although several experiments using primary neurons or neuronal cell lines have shown that cytotoxicity induced by Aβ peptide correlates with its β-sheet structure and fibrillar state, the underlying mechanism by which extracellular Aβ damages neurons remains unclear (Iversen et al., 1995; Xia et al., 2016). There is evidence that Aβ peptide can bind to the cell membrane and form ion channels or pores that induce membrane disruption followed by neuronal damage. In fact, some studies have reported pore-like structures of Aβ under *in vitro* conditions as well as in cell membranes of AD brains and mice (Bhatia et al., 2000; Inoue, 2008; Kawahara et al., 2009). Additionally, soluble Aβ oligomers, but not monomers or fibrils, have been shown to increase membrane permeability and thus dysregulate Ca^{+2} signals associated with neurotoxicity (Demuro et al., 2005). Other lines of experimental evidence indicate that Aβ binding to neurons may involve single or multi-protein cell surface receptor complex which can subsequently trigger a variety of downstream signalling pathway leading to cell toxicity. The cell surface protein/receptors that can regulate Aβ-mediated toxicity include cellular prion protein, receptor for advanced glycation end-products, the α_7 nicotinic acetylcholine receptor, the p75 neurotrophin receptor, the β2 adrenergic receptor, the low-density lipoprotein receptors, the amylin 3 receptors, Fcγ receptor II-b (FcγRIIb), scavenger receptors, the Eph receptors and the paired immunoglobulin-like receptor B (Yan et al., 1996; Wang et al., 2000; Husemann et al., 2001; Hashimoto et al., 2004; De Felice et al., 2007; Lauren et al., 2009; Wang et al., 2010;

Cisse et al., 2011; Basak et al., 2012; Fu et al., 2012; Kim et al., 2013; Kam et al., 2014; Jarosz-Griffiths et al., 2016; Xia et al., 2016). However, the role of several of these receptors in mediating Aβ toxicity is somewhat controversial or yet to be reproduced and considering the heterogeneity and dynamic nature of Aβ peptide, it is possible that different receptors may interact with different species of Aβ to trigger a specific signalling cascade. Many of the signalling pathways initiated by these ligand-receptor interactions then converge into a common downstream target that is ultimately responsible for neurotoxicity and cell death (Kam et al., 2014; Jarosz-Griffiths et al., 2016).

While there is structural information on the core of the amyloid fibril, less is known about oligomer structure. There is evidence that the C-terminal of Aβ may form β-barrels in some oligomer species (Tay et al., 2013; Do et al., 2016), or may exist as loosely aggregated strands in other oligomers (Ahmed et al., 2010). The first 11 N-terminal residues appear to remain disordered (Breydo et al., 2016) but molecular dynamic studies of the N-terminal mutation A2T has revealed that the N-terminus may interact with hydrophobic residues in the central and C-terminal domains (Das et al., 2017). Thus, while N-terminal residues may not be incorporated into the growing amyloid core, they can affect oligomer formation and stability. Whether they can be involved in pore formation described in some toxicity studies is unknown.

There is also evidence that the N-terminus of Aβ may directly influence toxicity. The N-terminal region of $A\beta_{1-42}$ interacts with the FcγRIIb receptor and through this interaction it can regulate toxicity of cultured neurons as well as memory impairment in an animal model of AD (Kam et al., 2013). Additionally, neurotoxicity induced by oligomeric Aβ isolated from AD patients were reduced by Aβ N-terminal antibodies but not Aβ C-terminal antibodies, thus highlighting the significance of N-terminal region in regulating the toxicity of Aβ peptide (Shankar et al., 2008).

Others and we demonstrate that $rA\beta_{1-42}$ is much less potent than $hA\beta_{1-42}$ in reducing cell viability (Boyd-Kimball et al., 2004). Of the three residues that differ between mouse and human, the 5th position residue has been proposed to be most important in conveying toxicity because "humanizing" this residue alone (the equivalent of our Rfr) was sufficient to restore its toxic effect in one study (De Strooper et al., 1995). A problem with this conclusion is that it was based

on a study in primary neurons infected with mutated APP. While these cells suffered reduced viability, this was associated with increased production of Aβ and higher Aβ 42/40 ratios so we cannot conclude whether the structure of Aβ oligomer produced or its ability to aggregate into fibrils influenced toxicity, or whether this was simply due to the presence of more $A\beta_{1-42}$. Given the characteristics of the amino acids in question, a charged amine (R) at position 5 in human, a phenolic residue (Y) at position 10 in human, and a charged amine (H) at position 13 in human, the mutation with the most significant electrostatic or steric effect should be replacement of position 5 R with G, a smaller uncharged residue. Replacing Y with F (both phenolic) and H with R (both charged amines) would not be predicted to affect structure as much. All the mutants, however, formed oligomers that were larger than $hA\beta_{1-42}$ oligomers and were the dominant species when co-incubated with $hA\beta_{1-42}$. This suggests that the mutant oligomers may have incorporated or absorbed the $hA\beta_{1-42}$ aggregates into their larger structures, as has been proposed previously (Kumar et al., 2015). This interaction between mutant and $hA\beta_{1-42}$ oligomers must have been relatively loose because combining mutant and $hA\beta_{1-42}$ oligomers did not rescue cells from the toxic effects of $hA\beta_{1-42}$ oligomers.

Given the range of above mentioned possible mechanisms by which Aβ aggregates may affect cell viability, it remains unclear exactly how aggregate size influences pathogenesis, but larger mutant oligomers may be unable to incorporate into pore-forming structures in the membrane or interact with binding partners on the cell surface because of steric hindrance. Alternately, the N-terminal exposed residues, containing the mutations studied here, may electrostatically prevent binding to receptors or even facilitate mutant Aβ clearance. Because there was neither a rescue effect nor a competitive inhibitory effect on cell viability when mutant oligomers were added to preformed human Aβ oligomers, we can conclude that the larger mutant oligomers did not induce a protective response and did not compete for the same binding partners as $hA\beta_{1-42}$ oligomers.

2.4.1 Conclusion

Our study confirms that N-terminal mutations can affect Aβ fibril and oligomer formation, despite lying outside the core amyloid region of Aβ. Of the three factors that may influence Aβ-mediated toxicity (primary structure of Aβ, assembly structure and cellular responses), our

results suggest that it is more the assembly structure that correlates with effects on cell viability. N-terminal mutations produced three peptides, all with reduced propensity to form fibrils, increased oligomer size distributions, and reduced toxicities. Because the mutant oligomers formed were larger and less toxic but unable to rescue the damaging effects of $hA\beta_{1-42}$ oligomers, we conclude that larger assembly size and/or alteration of N-terminal binding sites prevented interaction with the cell membrane and binding receptors required to induce toxicity. Whether distinct cell types (e.g., neurons, glia or non-neuronal cells) would respond differently to these Aβ assemblies was beyond the scope of this study, but if the N-terminus of Aβ can be targeted such that oligomer size is increased, that may be sufficient to block downstream effects on cell viability.

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CHAPTER – 3

General Discussion

3.1 Summary of results

In order to establish a structure-activity relationship between Aβ assemblies and toxicity, the significance of the unstructured hydrophilic N-terminal domain of Aβ on peptide aggregation and toxicity was investigated by humanizing rodent $A\beta_{1-42}$ at positions 5, 10, and 13 *via* mutations. Our results indicate a shift in aggregation dynamics towards oligomeric assemblies while discouraging fibril formation as depicted by ThT binding assay, morphological analysis of Aβ fibrils by EM and measuring the hydrodynamic radius of Aβ oligomers by DLS. This structural alteration in Aβ peptides by mutagenesis leads to a significant increase in cellular viability compared to human or rodent $A\beta_{1-42}$ as assessed by LDH and MTT cell viability/death assays. Collectively, these results demonstrate that the N-terminus contributes to overall peptide assembly and cellular viability, indicating that amino acid sequences influencing the quaternary structure of the peptide may underlie the toxic properties of the peptide and subsequent development of AD pathology.

3.2 Species variations of Aβ sequence: comparisons between human and rodent Aβ

APP and its proteolytic fragments are highly conserved in vertebrates and Aβ aggregates have been identified not only in AD patients, but also in aged brains of a variety of animals (Braidy et al., 2015). Since oligomers are the focal point of AD research, the lack of amyloid plaques in rodent brains provides a unique opportunity to establish the significance of oligomeric species in neuronal homeostasis. Rodent Aβ differs from human Aβ by three amino acids mutations (R5G, Y10F, H13R) in the N-terminus of the peptide. While early research into the causes of sequence variation between rodent and human Aβ suggested that a specific site-directed mutation towards human Aβ (G5R) resulted in favouring the amyloidogenic pathway (De Strooper et al., 1995), not much else has been ascertained about the other residues in question within the N-terminus of Aβ until several years later. This flexible linker region comprises of a critical role in metal binding; histidines at position 6, 13, and 14 of the human Aβ sequence bind Cu/Zn/Fe metal

ions, an act that promotes Aβ aggregation and conformational change (Curtain et al., 2001). Furthermore, a tyrosine residue at position 10 mediates the redox reaction of metal ions to generate reactive oxygen species (ROS) with the help of the neighbouring histidine residues in the amino-terminal region of Aβ (Barnham et al., 2004). Thus, residues 10 and 13 are critical to examine the variance between human and rodent $A\beta_{1-42}$.

Rodent $A\beta_{1-42}$, as depicted by our ThT studies, displayed lower aggregation kinetics compared to human $A\beta_{1-42}$, corresponding to existing literature (Lv et al., 2013). Our mutant Aβ peptides also demonstrated negligible amounts of ThT fluorescence, indicating that these peptides lack the ability to generate stable β-sheet fibril structures. Thus, it would be noteworthy to investigate the aggregation behaviours of these mutant peptides in the presence of metal ions, specifically Cu^{2+} or Zn^{2+}, that have roles in promoting peptide aggregation. Furthermore, neuronal cell viability studies should be conducted using mutant Aβ peptides in the presence of metal ions to define the roles of these amino acid substitutions on ROS production. Previous studies evaluating the effects of Y10F substitution in $A\beta_{1-40}$ depicted an increase in fibril formation with a reduction on neurotoxicity suggesting that tyrosine substitution is most likely responsible for reduced ROS production in rodent $A\beta_{1-42}$ compared to its human counterpart (Lv et al., 2013).

Most of the commonly used animal models of AD are transgenic mice that express human genes with mutations link to familial AD. However, some recent studies have been carried out on the South American rodent, *Octodon degu*, known as the naked-mole rat, which spontaneously develops AD with age as observed in human population. In fact, there is a high degree of homology (97.5%) between naked-mole rat and human Aβ amino acid sequence, varying by only a single amino acid at position 13 (His13Arg → similar to rodent sequence). The brains of naked-mole rats, as observed in human, exhibit the presence of neurofibrillary tangles as well as Aβ-containing neuritic plaques as a function of normal aging process (Inestrosa et al., 2005). Considered as a natural animal model for AD, naked-mole rats demonstrate an age-related increase in the levels of soluble Aβ oligomers that induce postsynaptic dysfunction, precipitating the synaptic and memory impairment as observed in AD (Ardiles et al., 2012). Roychaudhuri et al. (2015) examined the biophysical and biological properties of human, mouse and naked-mole

rat Aβ, revealing that while the primary structure of the protein is correlated to its aggregation propensity and toxicity, protein quaternary structure may also have an important role in determining the toxic properties of the protein.

3.3 Effect of Aβ mutations on its nucleation and oligomerization

The amyloid cascade hypothesis, which depicts the functional importance of Aβ in AD aetiology, has been revised to emphasize toxic properties of the soluble oligomeric forms of Aβ and their ability to instigate the impairment of synaptic communication and subsequent cognitive deterioration in AD patients (Hardy and Allsop, 1991; Hardy and Higgins, 1992; Cline et al., 2018). As Aβ peptides are known to exhibit a myriad of structures ranging from monomeric state to various structural assemblies i.e., small soluble oligomers with the capabilities to diffuse throughout the brain to large insoluble fibrils that assemble into amyloid plaques, new insights are being gained into the dynamics these assemblies to AD pathogenesis. Protein molecules, such as amyloids, are structurally flexible entities that adopt a diversity of conformational states between synthesis and degradation based on their thermodynamic and kinetic equilibriums determined primarily by their amino acid sequence and the environment (Lu et al., 2009). Furthermore, the range of Aβ structures that exist in a dynamic equilibrium make it difficult to elucidate precisely the structure and activity relationships in pathological and physiological conditions (Bemporad and Chiti, 2012; Benilova et al., 2012). Additionally, the diversity of Aβ proteoform provides hurdles in assessing individual oligomers as well as structural conformations of Aβ that are toxic to neurons (Wildburger et al., 2017). Thus, further insight into the biochemical properties of Aβ will enhance our understanding into the molecular intricacies of AD.

Amyloidogenesis or Aβ aggregation is thought to be governed through a nucleation-dependent polymerization model classically initiated through a two-step self-assembly process dependent on the hydrophobic nature of the peptide (Jarrett and Lansbury, 1992). To create the complex β-sheet structure, the hydrophobic domains must interact with one another (folds upon itself) followed by the interpeptide hydrogen bonding interactions that further stabilize the protein assembly. This defines the primary nucleation process, which states that monomeric forms of

peptides bind to form a nucleus that continues to grow faster with the recruitment of additional monomeric species in a concentration-dependent manner. Elongation is characterized as the process that follows nucleation and instigates the extension of fibrillar aggregates through monomer incorporation. However recent discoveries have reported the existence of a secondary nucleation, a process by which fibrils themselves breakdown and contribute to the oligomeric population or act as seeds for further nucleation by facilitating monomers to form a nucleus on the surface of pre-existing aggregate, therefore creating a detrimental positive feedback loop. Secondary nucleation combined with fibril fragmentation produce a successive sequence of aggregation that considerably contributes to the proliferation of toxic oligomer and fibrillar Aβ species, thereby unifying the products of the Aβ peptide (Cohen et al., 2013). Contrary to the disease related aggregates; off-pathway oligomers are regarded as stable unstructured amorphous assemblies that are considered to be non-toxic (Hartl, 2017). Therefore Aβ peptide oligomerization/aggregation follows two pathways; i) on-pathway oligomerization, which consists of protein oligomer complexes or polymorphs formed during the nucleation phase (comprising both primary and secondary nucleation) and the elongation phase (encompassing small oligomers to fibrils), and ii) off-pathway oligomerization, which comprise stabilized protein oligomers that refrain from fibril formation. Fig 3.1. Illustrates the production, nucleation, elongation, fragmentation, and off pathway assembly of Aβ peptides.

While a role for Aβ oligomers in AD pathogenesis is widely accepted, a relationship between the structural properties and toxicity of Aβ oligomers has yet to be defined clearly. Insights into the Aβ oligomers' assembly, structural characteristics, and toxicity have been impeded due to the heterogeneous nature of the polymorphic intermediates present in the complex entity. Aβ peptides lack stabilized secondary and tertiary structures and are considered natively disordered, leading them to completely or partially folded states contingent to internal (mutations/amino acid substitutions) or external (pH, temperature, concentration, solubility) factors. Partially folded or misfolded peptides have unstructured regions exposing hydrophobic amino acid residues that encourage the concentration-dependent aggregation of β-sheet structures (Teplow, 2013).

The nucleation potentials required for Aβ to form aggregates from monomeric species are based on sequence length as well as mutations. A minor two additional amino acids on the carboxyl-terminus of human $A\beta_{1-42}$ compared to $A\beta_{1-40}$ is sufficient to produce distinct aggregation and toxicity profiles, depicted through a stronger β-hairpin structure responsible for an intensified propensity to aggregate (Bitan et al., 2003; Irie et al., 2005). This is believed to be liable for the crucial transformation from α-helical to β-sheet structure within the hydrophobic domain associated with Aβ. Additionally, mutations within Aβ domain such as Osaka mutation (E22Δ), can influence elongation, fragmentation, or secondary nucleation, thereby potentially altering the production of toxic intermediates.

A single amino acid substitution at the 2nd position of Aβ from the relatively neutral alanine (A) residue to the hydrophilic threonine (T) or hydrophobic valine (V), result in structural differences that either gain protective or detrimental potentials, respectively, signifying the importance of the N-terminal region of Aβ. The aforementioned protective mutation at the 2nd residue (described as A2T) has exhibited a preference toward the non-amyloidogenic metabolism of APP, proposed to impede the BACE1 cleavage site of APP (Jonsson et al., 2012). The amino acid variants at position 2 display distinctive aggregation propensities, oligomeric morphology, and distinct LTP inhibition (Benilova et al., 2014; Maloney et al., 2014). Furthermore, N-terminal interactions for the protective A2T mutation are significantly diminished as result of separated and dynamic inter-peptide associations leading to unstable oligomeric structures. In contrast, its toxic counterpart i.e., the A2V mutation, strengthened inter-peptide interactions reflected in the close proximity of its N-terminus that contributes to aggregative interactions with the hydrophobic central domain and the hydrophobic C-terminus of Aβ (Zheng et al., 2015; Das et al., 2017; Sharma et al., 2018). These recent discoveries propose that the flexible hydrophilic N-terminal domain (1-16) of Aβ orchestrate a distinctive role in disease susceptibility, advocating further pursuit of this underrated region to provide new perspectives.

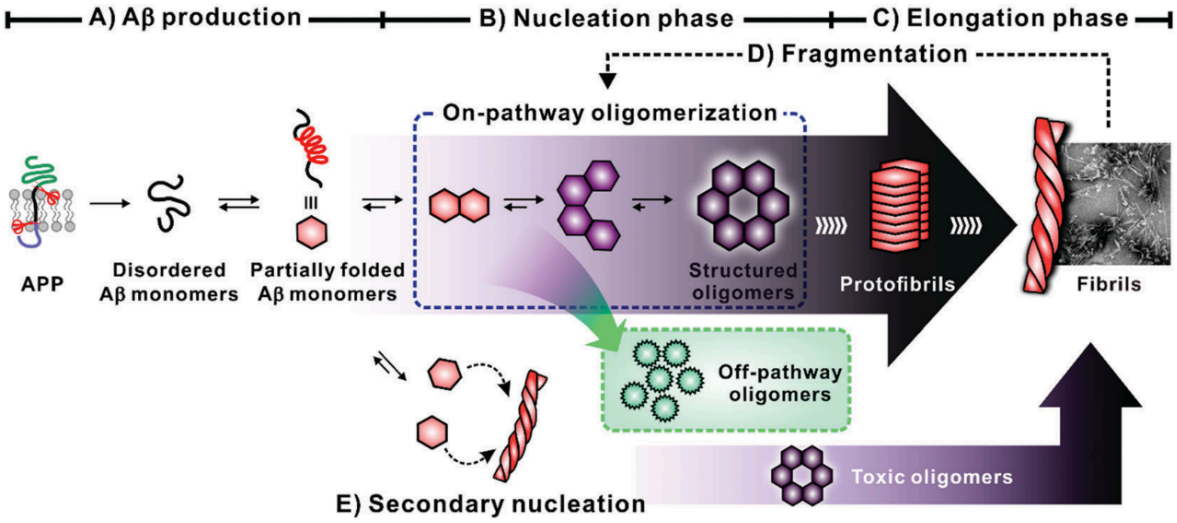

Figure 3.1. Aβ production, nucleation, elongation, fragmentation, and off pathway (adapted from Lee et al. (2017) *Chem. Soc. Rev.* 46:310-323).

3.4 Mechanisms of mutations affecting Aβ oligomer toxicity on AD pathology

The pathogenesis of various amyloids links protein-folding dynamics to toxic mechanisms that execute unique functions in different age-related human diseases (Knowles et al., 2014). Currently little is known how aggregation of Aβ leads to the formation of neuritic plaques, which is one of the characteristic features of AD pathology. Excessive Aβ levels, possibly due to increased production and/or decreased clearance as a consequence of impaired proteostasis, triggers aggregation and propagation of Aβ peptide. Individuals destined for AD remain clinically asymptomatic, but Aβ accumulation over the years result in the degeneration of neurons and development of pathology along with the onset of clinical symptoms (Musiek and Holtzman, 2015).

Initially, investigations into the pathogenic properties of Aβ suggested that the toxicity of the Aβ peptide were attributed to its ability to aggregate into insoluble plaques. However, experimental data over last two decades indicate that the most detrimental forms of Aβ peptides are the soluble oligomers, whereas the insoluble amorphous or fibrillar deposits represent a less harmful dormant form (Lambert et al., 1998; Walsh et al., 2002; Walsh and Teplow, 2012). Aβ oligomers can disrupt neuronal membranes by directly targeting the lipid bilayers through pore formation resulting in the loss of cell membrane integrity/potential. This is believed to be caused by the β-barrel structure of Aβ oligomers, allowing influx of Ca^{2+} and other ions into the cells that subsequently affect cell viability (Serra-Batiste et al., 2016). Oligomeric Aβ has been shown to induces cytotoxicity through a combination of mechanisms such as calcium influx, production of toxic free radicals, phosphorylation of tau protein, and activation of caspase-3 that disturb cellular homeostasis and initiate the development of disease pathology (De Strooper and Karran, 2016). The diversity of intracellular signalling pathways that mediate Aβ toxicity insinuate that a unique mechanism induced by a specific oligomeric structure binding to a specific receptor, is highly improbable to be solely responsible for AD pathogenesis (Smith and Strittmatter et al., 2017). Furthermore, the absence of conclusive conformational information regarding a specific disease-causing Aβ oligomer from AD brains supports the observation that Aβ can interact with a wide range of proteins/receptors. The dynamic nature of Aβ that could exist in multiple configurations, may permit a particular region to act as a ligand for certain molecules/proteins

such as NMDA receptor, AMPA receptor, α_7 nicotinic acetylcholine receptor, p75, amylin receptor and cellular prion protein (PrPc) to mediate synaptic plasticity, neurotransmission, synaptotoxicity and eventual loss of neurons (Benilova et al., 2012; Smith and Strittmatter et al., 2017). A general summary of toxicity pathways associated with different Aβ structures is depicted in Fig. 3.2.

Although a significant amount of Aβ is secreted into the extracellular space, the intracellular accumulation of the peptide has long been suggested to play a critical role in the degeneration of neurons (LaFerla et al., 2007; Mohamed and Posse De Chaves, 2011). It is likely that intracellular aggregation of Aβ mediates its effects by altering the function of ER (triggering the unfolded protein response), mitochondria (promoting Ca^{2+} release and production of toxic free radicals) and the autophagic-lysosomal pathway (reducing of clearance of proteins leading to their accumulations, lysosomal leakage etc.). These effects may be dependent on the sequence, structure, and location of the Aβ conformers. It is of interest to note that the Osaka mutation (E22Δ) results in a unique FAD associated with increased intraneuronal oligomerization of Aβ with a low propensity to form fibrils (Tomiyama et al., 2008; Umeda et al., 2011). This perpetual oligomeric form of Aβ that scarcely aggregates into fibril form is proven to be responsible for neuronal toxicity and AD (Umeda et al., 2011). The Osaka mutation is one of the several examples to support that intracellular Aβ accumulation may be sufficient under certain conditions to trigger AD pathogenesis (Gouras et al., 2010; Takahashi et al., 2017; Iadanza et al., 2018).

Despite the possibility for multiple oligomeric conformations, there is also evidence for a common oligomeric structure, as measured by antibodies that specifically recognizes soluble oligomeric Aβ. Interestingly, the oligomer specific Aβ antibodies, such as A11 or KW1, recognized soluble amyloid oligomers and demonstrated that there are universal conformation properties to the toxic structured oligomers (KW1 selectivity for surface-exposed hydrophobic motifs only present in oligomeric species – these are hidden in fibrils) (Kayed et al., 2003; Morgado et al., 2012). Furthermore, oligomer specific antibodies are able to distinguish soluble oligomers (but not monomeric or fibril forms) of other types of amyloids (Aβ40, Aβ42, α-

synuclein, human insulin, prion 106-126, lysozyme and polyglutamine) and significantly neutralized their toxicity as measured in human neuroblastoma SH-SY5Y cells using established MTT and LDH assays (Kayed et al., 2003). The fact that oligomer-specific antibodies can recognize such a diverse group of soluble disease associated amyloidogenic peptides indicates that there is a shared structure of the polypeptide backbone of the soluble amyloid oligomers that is independent of the peptide sequence. Thus, a common oligomer structure may possibly be responsible for a common pathogenic mechanism that may underlie various neurodegenerative diseases.

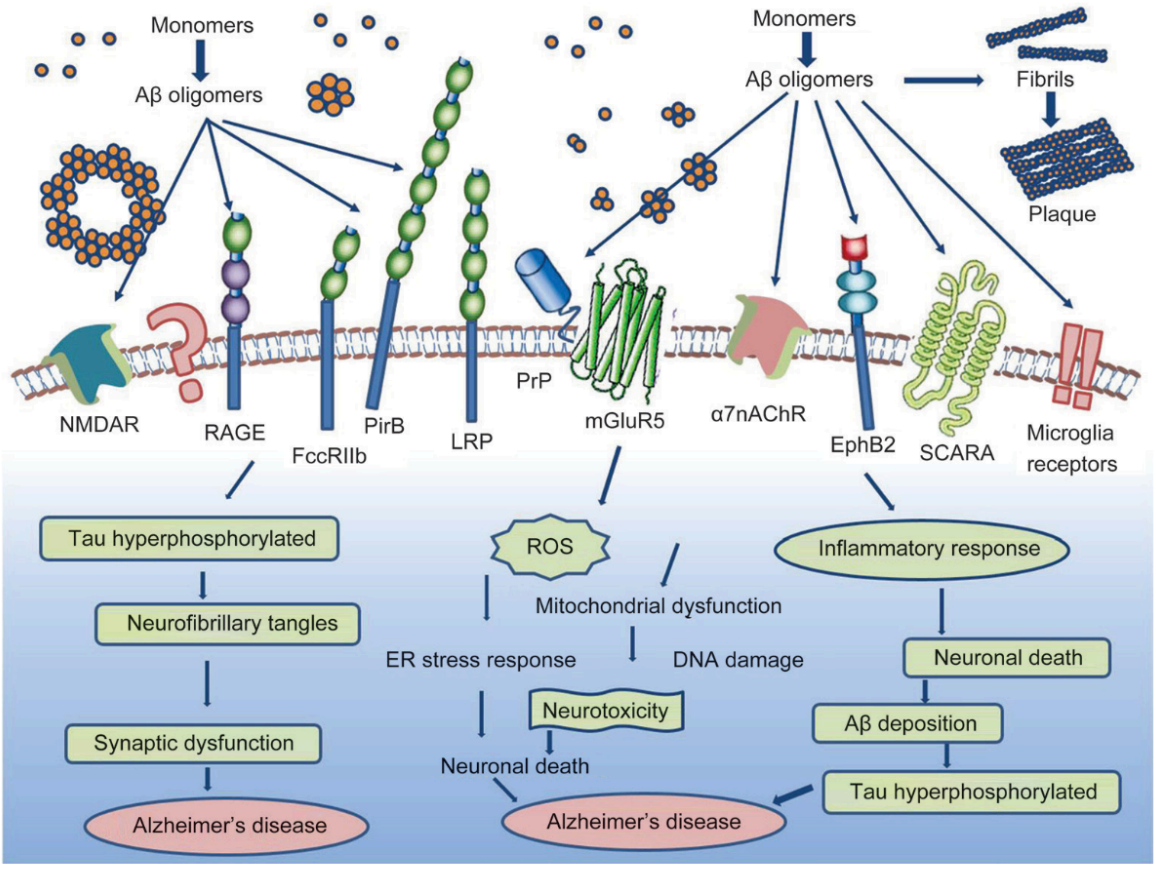

Figure 3.2. Different Aβ structures interact with various binding partners to induce a diversity of toxicity pathways believed to contribute to AD (adapted from Chen et al. (2017) *Acta Pharmacol Sin* 38:1205-1235).

3.5 Implications of inhibiting Aβ oligomerization as treatment strategies for AD

Multiple therapeutic strategies are being investigated to intervene in AD disease progression by inhibiting Aβ oligomerization at any step of its aggregation or nucleation pathway (Linse et al., 2017). At present the prospective approaches to structure focused therapy are peptide-based aggregation inhibitors and immunotherapy (monoclonal antibodies – designed to facilitate Aβ clearance and/or folding into specific conformations), both of which target soluble forms of Aβ. Peptide motifs within the Aβ self-association binding region sequence (KLVFF – between residues 16-20) and the self-recognition sequence within the central hydrophobic core (31-42) are identified as candidate target sites for peptide based aggregation inhibitors (Nie et al., 2011). However, finding a specific pathogenic conformation culpable for AD pathogenesis proves to be complex and convoluted when taking into account varying peptide lengths, mutations, and the heterogeneity of transient intermediates.

The unstructured N-terminal region of Aβ has also been considered as a potential therapeutic target. Natural compounds found in turmeric, olive oil, red wine, and green tea contained polyphenols that have been reported to inhibit Aβ aggregation and toxicity. The main polyphenol in tea is epigallocatechin gallate (EGCG), has been shown to have neuroprotective effects against Aβ oligomer-mediated toxicity through the inhibition of amyloid formation (Ehrnhoefer et al., 2008). Curcumin, present in turmeric, binds to and perturbs the formation of β-sheet structures (Yang et al., 2005; Thapa et al., 2016). Although the mechanisms as to how most of these natural compounds exhibit their disease modifying effects have yet to be established, both EGCG and curcumin have metal chelating capabilities that may contribute to the reduction of metal-ion induced Aβ aggregation (Velander et al., 2017). Furthermore, curcumin, EGCG, and various other polyphenols have been shown to interact with $A\beta_{1-42}$ through hydrogen bonds at its N-terminus (1-16) and hydrophobic regions (17-42), generating unstructured off-pathway $A\beta_{1-42}$ oligomers negating Aβ toxicity (Ehrnhoefer et al., 2008; Nedumpully-Govindan et al., 2016). Thus, diversion of Aβ towards stable off-pathways conformations by natural products has potential therapeutic implication in AD pathology as observed in some other neurodegenerative diseases.

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