The structure and function of Alzheimer's gamma secretase enzyme complex

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ABSTRACT

The production and accumulation of the beta amyloid protein (Aβ) is a key event in the cascade of oxidative and inflammatory processes that characterises Alzheimer's disease. A multi-subunit enzyme complex, referred to as gamma secretase, plays a pivotal role in the generation of Aβ from its parent molecule, the amyloid precursor protein (APP). Four core components (presenilin, nicastrin aph-1 and pen-2) interact in a high molecular weight complex to perform intramembrane proteolysis on a number of membrane bound proteins, including APP and Notch. Inhibitors and modulators of this enzyme have been assessed for their therapeutic benefit in AD. However, though these agents reduce Aβ levels, the majority have been shown to have severe side-effects in pre-clinical animal studies, most likely due to its role at processing other proteins involved in normal cellular function. Current research is directed at a greater understanding of this enzyme in particular elucidating the roles that each of the core proteins play in its function. In addition, a number of interacting proteins that are not components of γ-secretase also appear to play important roles in modulating enzyme activity. This review will discuss the structural and functional complexity of the gamma secretase enzyme and the effects of inhibiting its activity.

Keywords: Alzheimer's disease, amyloid precursor protein, Anterior pharynx defective homolog 1, beta amyloid, gamma secretase, Notch receptor, presenilins, nicastrin, , presenilin enhnacer-2, Regulated intramembrane proteolysis

Abbreviations: AD, Alzheimer's disease; **FAD,** Familial Alzheimer's disease; **EOAD,** Early onset Alzheimer's disease; **Aβ** Amyloid beta; **APP,** Amyloid precursor protein; **APLP,** Amyloid precursor like protein; **PS1** Presenilin 1; **PS2** – Presenilin 2; **NCT** Nicastrin; **Aph-1,** Anterior pharynx defective homolog 1; **Pen-2,** Presenilin enhancer 2; **BACE,** Beta-site APP-cleaving enzyme; **ICD,** Intra cellular domain; **AICD**, Amyloid precursor protein intra cellular domain; **NICD**, Notch intra cellular domain; **NECD**, Notch extracellular domain; **CICD**, Cadheren intracellular domain; **RIP**, Regulated intramembrane proteolysis; **LRP**, Lipo protein receptor related protein; **SREBP**, Sterol regulatory element binding protein; **IRE**, Interferon response element; **ATF**, Activated transcription factor; **CTF**, C-terminal fragment; **TMD**, Transmembrane domain; **NTF**, N-terminal fragment; **SPP**, Signal peptide peptidase; **NSAIDs**, Non-steroidal anti-inflammatory drugs; **NMDA**, N-methyl D-Aspartate; **GSI**, Gamma secretase inhibitors; **GSM**, Gamma secretase modulators; **LDL**, Low density lipoprotein; **VLDL**, Very low density lipoprotein; **IGF** – Insulin like growth factor

INTRODUCTION

Alzheimer's disease (AD) is a complex, progressive neurodegenerative disorder that is neuropathologically characterised by extensive neuronal loss and the presence of neurofibrillary tangles and senile plaques. While the majority of AD cases are sporadic, ~5% of AD cases are familial (FAD) with mutations in three genes, amyloid precursor protein, presenilin 1 (PS1) and presenilin 2 (PS2) accounting for the majority of cases. A major feature of both sporadic and familial forms of AD, is the accumulation and deposition of a small peptide referred to as beta amyloid (Aβ) within brain tissue of AD sufferers. The mechanisms that underlie the disease processes are poorly understood. However, the accumulation of Aβ is thought to play a pivotal role in neuronal loss or dysfunction through a cascade of events that include the generation of free radicals, mitochondrial oxidative damage and inflammatory processes (reviewed in Refs. [1,2]). One of the primary events that results in the abnormal accumulation of Aβ is thought to be the dysregulated proteolytic processing of its parent molecule, the amyloid precursor protein (APP).

The APP molecule is a transmembrane glycoprotein that is proteolytically processed by two competing pathways, the non-amyloidogenic and amyloidogenic (Aβ forming) pathways (Figure 1). How these pathways are regulated remains unclear. However, there are many factors including diet, hormonal status, and genetic mutations that influence the processing of APP to generate Aβ (reviewed in Refs. [3,4]). Three major secretases are postulated to be involved in the proteolytic cleavage of APP. These include α-secretase [of which the metalloproteases ADAM17/ TACE and ADAM 10 are likely candidates], beta APP cleaving enzyme [BACE, formally known as β-secretase] and the γ-secretase. The α-secretase cleaves within the Aβ domain of APP thus precluding the formation Aβ and generating non-amyloidogenic fragments and a secreted form of APP (α-APPs). In the amyloidogenic pathway, BACE cleaves near the N-terminus of the Aβ domain on the APP molecule, liberating another soluble form of APP, β-APPs and a C-terminal fragment (C99) containing the whole Aβ domain. The final step in the amyloidogenic pathway is the intramembranous cleavage of the C99 fragment by γ-secretase, to liberate the Aβ peptide (reviewed in Ref. [1]).

Two additional cleavage sites on the APP-C terminal fragment have been identified and termed the ε-cleavage site and ζ-cleavage site. The ε-cleavage site occurs 7-9 residues distal to the γ-secretase cleavage site at Aβ40/42, generating the APP intracellular domain (AICD) [5-8]. The AICD has been reported to bind to different proteins and may be involved in several intracellular pathways, including apoptosis, neuronal growth and regulation of gene expression (reviewed in [9]). It is interesting that the ε-secretase site is equivalent to the S3 site of the Notch receptor [10] liberating the intracellular domain of Notch (NICD), which like AICD translocates to the nucleus and activates target gene expression (see below for more discussion of Notch processing). These findings suggest that cleavage at the ε-site may be a common event for the processing of type I transmembrane proteins cleaved by γ-secretase, to liberate fragments involved in cell signalling. Recently, an additional Aβ fragment, referred to as Aβ46 has been identified [11-13]. This fragment, unlike Aβ40/42 is exclusively intracellular and has not been shown to be secreted from the cell. The pathological relevance of this longer form of Aβ is unknown, however known inhibitors of γ-secretase that reduce Aβ40/42 production, lead to an intracellular accumulation of the potentially pathological Aβ46 [11,13] which is important to consider when developing agents that target Aβ42 production.

The finding of additional Aβ fragments (Aβ37, 38 or 39) in cells and brain homogenates from humans and transgenic mice and the identification of additional cleavage sites may suggest that there are multiple γ-secretase enzymes (further discussion below). However, one model proposed by Zhao et al., [13] suggests a single γ-secretase enzyme with a broad range of activity, performing multiple cleavages sequentially along the APP-C99 fragment. In this model, (Figure 2) the APP-C99 fragment is first cleaved at the ε-site (Aβ49) and then undergoes further cleavage at the ζ-site to generate Aβ46. Additional cleavage of this fragment into Aβ43 can occur, which is then processed further into Aβ40 followed by additional cleavage liberating Aβ37. Alternatively, Aβ46 can be cleaved into Aβ42, which is processed further into Aβ38/39. Evidence for a single γ-secretase moiety is provided by the observation that a number of inhibitors of this enzyme reduce the levels of all Aβ species. However, the single catalytic site model inadequately explains the concurrent production of the major Aβ40 and Aβ42 species. In addition, this model doesn't explain the observations that Non-steroid anti-inflammatory drugs, target Aβ42 specifically without altering NICD or

AICD production, and that some γ-secretase inhibitors reduce Aβ40 or Aβ42 production but not Aβ46 levels. Although it has been suggested that a single enzyme could possess two catalytic sites [13] it however doesn't rule out multiple γ-secretase enzymes or other proteases with γ-secretase activity existing.

THE γ-SECRETASE ENZYME: STRUCTURE, ASSEMBLY AND POSTULATED FUNCTION OF ITS COMPONENTS.

Structure

The γ-secretase enzyme is thought to be an aspartyl protease that has the unusual ability to regulate intramembrane proteolysis (RIP) for a growing list of type 1 integral membrane proteins which include, APP, APP like proteins (APLPs), E-Cadherin, CD44, lipoprotein receptor related protein (LRP), Notch, sterol regulatory element –binding protein (SREBP), interferon response element (IRE1) and activated transcription factor 6 (ATF-6) (for recent review see [14]). The mechanism of RIP and γ-secretase activity is unknown. However a transient hydrophilic environment for catalysis within the lipid membrane must be created. Furthermore, the enzyme (or an enzyme domain) must have the ability to bend and unwind the α-helical substrates, exposing their amide bonds to hydrolysis. Therefore it is conceivable that the γ-secretase enzyme is an integral protein of the lipid bi-layer and contains a number of proteins that may have different functions within an enzyme complex. The exact conformation or molecular architecture of the γ-secretase enzyme remains unclear. However, large molecular mass complexes of ~250 kDa [15,16] ~500 kDa [17-21] and ~2000 kDa [17,22] have been identified.

Over the last few years biochemical and genetic approaches have identified four components of the γ-secretase complex, presenilins, nicastrin, anterior pharynx defective (aph-1) and presenilin enhancer 2 (pen-2). Over-expression and expression knockdown studies have provided strong evidence that these proteins are essential for γ-secretase activity [19,23-28]. Subsequent reconstitution studies in non-mammalian cells have provided evidence that these are the only components responsible for γ-secretase catalytic activity [29-31]. Presenilins are nine-pass transmembrane proteins and considered to possess enzyme catalytic activity. Nicastrin is a single pass membrane protein with a large ectodomain that is heavily

glycosylated which plays a vital role in γ-secretase assembly. Aph-1 occurs as a seven-pass transmembrane protein that exists in two homologous forms, located on chromosome 1 and chromosome 15 (aph-1a & aph-1b) respectively; [23]. Aph-1a undergoes further splicing to generate a long and short isoform of aph-1a, with the short isoform more abundantly expressed in most tissues [25]. Aph-1 shares its function with nicastrin in forming the stable complex. The smallest component Pen-2 is a two-pass transmembrane protein that is thought to activate presenilin endoproteolysis [27,32]. Thus, overall the enzyme possesses 18 transmembrane domains making it difficult to elucidate the crystalline structure of the enzyme.

Structural analysis of the gamma secretase enzyme has mainly been through protein purification, visualisation using electron microscopy and analysis of particle images to provide a 3D- reconstruction of the purified complex [33,34]. These studies have revealed a globular structure for γ-secretase. The low-resolution 3D images of purified γ-secretase has revealed a central cavity with two low density regions and two openings indicating a possible existence of a substrate gating and releasing mechanism. How this may exist in a lipid environment remains to be determined. However, the active component and the active catalytic site of this enzymatic complex are yet to be identified. A clue as to how this may occur comes from successful crystallisation of a bacterial intramemebrane protease (GlpG). GlpG activity actually takes place in a V-shaped cavity separated from the lipid environment by six transmembrane domains [35]. More recently, the crystal structure of bacterial signal peptide peptidase (SPP) was determined [36]. The highly conserved SPP protease, performs intramembranous cleavage of type 2 transmembrane proteins and shares some similar characteristics to presenilins (discussed further below). The crystalline structure of the bacterial SPP showed a tetrameric structure, which is bowled shaped with an opening at its base of approximately 96Å in diameter, predicted to be the membrane association surface. The ridge inside the bowel is restricted to 40Å. A concave surface creates the substrate binding pockets for four catalytic active sites within the tetramer structure. These structural studies with bacterial proteases have provided insight into high resolution structure and catalytic site of enzymes that perform intramembrane cleavage. Similar high resolution studies with the mammalian γ-secretase complex, although more difficult, are required to provide detail of the catalytic core of the γ-secretase complex. In addition, structure of the complex in the presence of lipids, to simulate the lipid membrane has not been established.

Although several techniques can be used to provide structural information of membrane bound proteins, all of them suffer from notorious problems associated with handling membrane proteins. The proteins are adapted to the lipid bi-layer and tend to denature when extracted from the environment. The region of hydrophobic residues will need to be stabilised by detergents during preparation for structural analysis. High resolution X- ray crystallography methods have improved in which 3D crystallisation methods can be used. However, establishing the atomic structure of membrane protein is still risky as high resolution 3D crystals are required [37]. Nuclear magnetic resonance (NMR) is a structural analysis that doesn't require 3D crystals. However, this requires the membrane proteins to be solubilised, causing issues with protein stability [37]. An alternative technique is 2D electron crystallography, in which the protein is reconstituted into 2D crystals in the presence of lipids, thus providing the native environment for membrane bound proteins or complexes, such as γ-secretase.

Assembly And Postulated Function Of The γ-Secretase Components.

Assembly Of The γ-Secretase Complex.

The core subunits of the γ-secretase enzyme are essential for the maturation and trafficking of the enzyme [38,39]. Interactions between the transmembrane domain of NCT and Aph1 play a significant role in the formation of the first stable sub-complex during the process of the active enzyme formation [40]. Evidence suggests that this interaction may occur very soon after APH-1 synthesis [41] with the conserved GXXXG motif within transmembrane 4 of APH-1 important for this interaction [42,43]. This interaction has been shown to be independent of PS1 or PEN-2 as nicastrin mutants have been shown to restore APH-1 expression but not expression of the other components [44]. More recent evidence suggests that nicastrin is critical for the correct assembly of the γ-secretase complex within the endoplasmic reticulum and the intracellular trafficking of the complex to the cell surface [45,46]. It is thought that the presenilin holoprotein is then incorporated into the aph-1: NCT sub-complex, nicastrin undergoes post-translational modifications and the complex is transported to the cell surface (or other Aβ generating compartments, i.e. TGN) as a trimeric complex. Pen-2 is then incorporated into the complex possibly through an interaction with presenilins with the "DYLSF" domain of pen-2 and a "NF" motif on transmembrane 4 of PS1 shown to

be critical for the interaction between these proteins [47-49]. Ablation of pen-2 results in significantly reduced PS1 endoproteolysis and Aβ production, suggesting that pen-2 is essential for the proteolytic cleavage of presenilins into the active components [19,23,26-28]. However, it is unclear whether pen-2 is the elusive protease responsible for this cleavage event. Once the presenilins are cleaved an active γ-secretase complex is formed.

Function Of The Enzyme Components

Evidence to date has suggested that the presenilins (PS1 and PS2) are the most critical component of the γ-secretase complex and maybe the catalytic component of this enzyme. Findings that mutations in PS1 account for the majority of inherited early onset forms of AD and result in the overproduction of the highly amyloidogenic Aβ42 [50-52] provided the initial evidence that PS1 facilitates γ-secretase activity. Subsequent studies provided evidence that presenilins may be the elusive γ-secretase enzyme. Many reports have shown that presenilin ablation or mutagenesis of two highly conserved aspartate residues within transmembrane domains 6 and 7 result in a reduction in Aβ levels in vitro and in vivo[53-56]. Furthermore, aspartyl protease inhibitors and transition state analogue inhibitors designed to target the active site of the protease, all reduce Aβ40 and Aβ42 levels and have been shown to affinity label and bind to PS1 [22,56,57]. In addition, physical interactions between presenilins and γ-secretase substrates have been identified (reviewed in [58]). Although initial evidence strongly implicated presenilins in γ-secretase catalytic activity they do not exhibit typical aspartyl protease structural characteristics, in particular they lack the typical D(T/S)G motif required for the active site of an aspartyl protease. However, presenilins do contain the two aspartyl residues (eg: D257 and D385 for PS1) which are either critical for the active site on the γ-secretase complex or constitute the active site. The formation of this aspartyl catalytic site could result from one or multiple presenilin molecules. The full length PS1 protein is rapidly endoproteolytically cleaved within its characteristic large hydrophilic loop into amino- and carboxy-terminal fragments (NTF/CTF) of ~27 and ~17 kDa, respectively [59,60]. These fragments are thought to interact with each other to form the catalytic component of γ-secretase [61,62]. The stoichiometry and the nature of the interaction between these fragments remain unclear. It has been shown by many studies that the NTF:CTF form a heterodimer in mammalian cells [16,63-66] leading to suggestions that this heterodimer is the active γ-secretase [67]. However, Cervantes and colleagues 2001 [68] provided evidence that the presenilin fragments can form a tetramer by identifying heterodimers as well as NTF and CTF

homodimers in yeast. Evidence for heterodimer and NTF homodimer (but not CTF homodimer) formation has been provided by photoaffinity labelled crosslinking studies [69]. This formation provides a core of aspartyl residues required for aspartyl protease activity. However, it has yet to be established wether the hypothetical “core” is formed between fragments from one PS1 molecule or multiple molecules within the complex.

Other proteases also share the unusual aspartyl motif GxGD and perform intramembranous cleavage of type I membrane proteins (reviewed in [70]). One such protease that has been widely studied due to its similar characteristics to presenilins are the signal peptide peptidases (SPPs). These proteases are members of a larger group of intramemebrane cleaving proteases (I-Clips) which have only a few endogenous substrates. They exist as monomers [71] and homodimers [72], however, the form that is actively involved in the cleavage process is still unclear. Although presenilins are to some extent similar to the SPPs, differences do occur. Presenilins mediate the cleavage of type I transmembrane proteins whereas SPPs process type 2 membrane proteins [71]. In contrast to presenilins, SPPs do not require interactions with other co-factors or undergo endoproteolysis for functional activity. Moreover, the topology of SPP is opposite to that of PS, such that, SPP cleaves transmembrane substrates with a membrane orientation opposite that of γ-secretase substrates [73]. Despite these differences the activities of presenilins and SPPs are similar.

The SPPs are evolutionarily conserved as they have been identified in Archea bacteria, Yeast plants and animals [74]. The amino acid sequence identity between human presenilin and SPPs is very low (20%) [74], however, they possess identical active site motifs YD, PAL and GXGD. Aspartate residues in the YD and GXGD sites are highly conserved and mutation of these residues abolishes the catalytic activity of both SPPs and presenilins [71,75]. γ-Secretase inhibitors that target presenilin- mediated activity also alter SPP activity, likewise some of the SPP inhibitors also suppress presenilin- mediated enzyme activity [75,76]. By showing that active site-directed γ-secretase inhibitors label the SPP homodimer, Nyborg and colleagues[72] showed that the homodimer, rather than the monomer contains the active site. This is similar to presenilins where it has been proposed that dimerisation of the presenilin N and C-terminal fragments form the catalytic active GxGD site [16,63-66]. Isolating SPPs and presenilins from cell membranes and solubilising with detergent, Sato and colleagues [75] showed that these proteins share certain biochemical properties. Both proteases have loose sequence specificity for substrates and recognise α-helical regions of substrates

and cleave within these regions. Inhibition of a substrate-based helical peptide that binds to a site distinct from the active site, suggests that binding of the substrate to the outer surface of the protease is an important initial step before entry into the water-containing active site. This is a likely mechanism of other membrane embedded proteases such as the S2P metalloproteases and the rhomboid family of serine protease and also a mechanism suggested for γ-secretase [77]. Recent evidence has suggested that like γ-secretase activity, SPPs cleaves the substrate at multiple sites within the TMD [75,78] although the exact mechanism by which this occurs remains unknown for both SPP and γ-sectretase. Most surprisingly, the cleavage site specificity of SPP can be altered by certain non-steroidal anti-inflammatory drugs (NSAIDs) which are known to alter the cleavage of γ-secretase activity on APP [79]. The similarities and differences exhibited by SPP and γ-secretase could offer considerable insight into determining enzyme structure and developing novel inhibitors that target one enzyme over the other.

Apart from functioning in the assembly process of the γ-secretase complex, the core enzyme components have also other postulated functions. Evidence has shown that nicastrin is essential for the interaction between the complex and APP-C99 and thus may act as a receptor for γ-secretase substrates [46,80]. This suggests that nicastrin maybe a substrate docking site in addition to its role as a scaffold for building the active complex. Three dimensional electron microscopy studies have suggested similar conformation where nicastrin ectodomain and other regions (TM or HL) of the components may act as a plug that regulates the opening of the catalytic pore [reviewed in [81]].

Although aph-1 shares its function with nicastrin in the assembly and stabilisation of the γ-secretase complex, it may also be critical for the activity of the fully constructed γ-secretase complex. As well as binding to immature components of γ-secretase in early stages of complex formation, APH-1 also interacts with the mature forms of PS1, nicastrin and PEN-2 [42,82]. Furthermore, recent evidence suggests that this interaction occurs on the cell surface where it also binds the γ-secretase substrate, Notch and facilitates its cleavage [82]. Structural and functional similarities between aph-1 and other proteases that possess the ability for intramembranous cleavage (ie PS1, rhomboid), [42,83] would suggest that this transmembrane protein might have an enzymatic function within the complex. However, aph-1 sequence homology to known proteases and evidence for a role as a protease is currently lacking.

Evidence suggests additional roles for pen-2 within the γ-secretase complex. The C-terminal end of pen-2 has been shown to be important for γ-secretase activity since altering the length of the pen-2 C-terminus by addition or deletion of residues has been shown to reduce Aβ40 and 42 generation without altering the binding of pen-2 to the complex [47] suggesting that pen-2 may have an alternative function within the complex. One suggestion put forward by Hasegawa and colleagues [47] is that the C-terminus of pen-2 may be a linker/space molecule that maintains the spatial interactions between proteins within the complex. However, recent evidence using pen-2 C-terminal loss of function mutations suggest that the C-terminus acts as a "molecular clamp" holding together the presenilin fragments and the whole γ-secretase complex [84]. If this is the case, then pen-2 is an integral part of the catalytic process holding the complex together whilst the γ-secretase products are generated.

Two additional proteins, TMP21 and CD147 have been shown to co-purify with the γ-scretase complex and modulate γ-secretase activity. TMP21 is a type 1 transmembrane protein and a member of a p24 cargo-family which may have a signalling role in the sorting and transport of proteins from the endoplasmic reticulum to the Golgi [85,86]. Chen and colleagues showed that TMP21 is a member of the complex as it was isolated in a high molecular weight presenilin complex, interacted with all of the known components of the γ-secretase complex, co-localised with the complex components in the ER, Golgi and cell surface and destabilised from the complex in the absence of the presenilins and pen-2. Although the over-expression of TMP21 did not alter γ-secretase activity, its suppression resulted in and increase in Aβ40 and Aβ42. However, suppression of TMP21 did not alter the production of AICD, Notch cleavage to generate NICD or cleavage of E-cadherin to generate CICD. These results are consistent with the notion that γ- and ε- secretase cleavage activities are independently regulated and indicate a role for TMP21 in modulating γ-secretase activity to generate Aβ. This role for TMP21 appears to be independent of its role in protein transport since the suppression of both TMP21and p24a (a member of the p24 cargo family that interacts with TMP21) does not result in additional increases in Aβ production to that observed following the suppression of TMP21 only [87]. This finding led the authors to postulate that there are two pools of TMP21, a major pool that is stabilised by, p24a and has no role in Aβ production and a minor pool that modulates Aβ production, independent of

p24a. Although, further investigation is required to determine the precise mechanism of action, it appears that TMP21 may function to regulate intramembrane proteolysis controlling γ-secretase activity and thus preventing the over-production of Aβ. This modulator role for TMP21 is important to consider when elucidating mechanisms on how γ-secretase activity is altered (for example by presenilin mutations) resulting in enhanced Aβ40 and Aβ42 production.

CD147 is a member of the immunoglobin superfamily that is involved in fetal development and retinal function as well as many other neurological processes such as development of the nervous system, involvement in spatial learning and recently in modulating beta amyloid accumulation [88,89]. The mechanism by which CD147 modulates Aβ accumulation is unclear, with two studies proposing different mechanisms of action. Zhou and colleagues (2005) [88] showed that CD147 is possibly a regulatory component of the γ-secretase complex. In their study it was observed that CD147 depletion using RNA interference resulted in elevated Aβ40/42 production, without affecting expression levels of NCT, PEN-2, APH-1 and PS1 [88]. Vetrivel and colleagues however, showed that CD147 did not interact with the other γ-secretase complex components and concluded that it was not an integral part of the complex. Instead, the authors showed that CD147 mediates the degradation of Aβ possibly via stimulating the production of the metalloproteases (MMPs). The discrepancies between these two studies could be due to methodological differences, and it is possible that CD147 may have varying functions in different cell lines. Vetrivel and colleagues used HEK-293 (compared to CHO cells used the Zhou et al study) over-expressing CD147, which is known to contain an abundance of proteases that degrade Aβ. Although CD147, along with TMP21, co-purified in a high molecular complex with endogenous γ-secretase, these proteins could not be affinity captured with γ-secretase inhibitors, indicating that they are not part of the active γ-secretase complex [90]. These findings support that both CD147 and TMP21 may act to control Aβ levels within the cell but they most likely are not part of the core components of the γ-secretase complex, affecting Aβ levels indirectly.

Presenilin Mutations

In the early 90's genetic linkage studies, mapped a new AD gene to the AD3 locus on chromosome 14q24.3 [91,92]. Sherrington and colleagues in 1995 [93] located the presenilin 1

PSEN1 gene in two clusters of the AD3 locus. A second gene was mapped to the AD4 locus on chromosome 1q31.42, termed presenilin 2 (*PSEN2*) in which mutations were first identified in a Volga German kindred containing seven related families, clinically diagnosed with autosomal dominant EOAD [94,95]. The protein products PS1 and PS2 share almost 67% sequence similarity but may have distinct functions (see below). Mutations in PS1 and PS2 account for the majority of early onset familial Alzheimer's disease cases. To date 176 mutations in PS1 and 14 mutations in PS2 have been reported in 390 and 23 families, respectively (for list of mutations refer to http://www.molgen.ua.ac.be/ADMutations/). Cases with PS2 mutations typically have later ages of onset of AD with the majority associated with an average age of onset considered to be late onset AD (70-73yrs) [94-97]. In contrast, mutations in PS1 are associated with earlier ages of onset ranging from 24-59 yrs old. Although the mutations are spread throughout the presenilin molecules, they appear to be concentrated around or within the transmembrane regions of the molecule, suggesting that these regions may be critical for protein function. Indeed, the conserved aspartate residues (D257 and D385) in transmembrane domain (TMD) 6 and 7 are critical for γ-secretase activity and may constitute the catalytic core of the complex [21,54]. Other residues such as R389, the C-terminal PAL motif and Cys residues in transmembrane domains 1 and 8 also appear to have important roles in catalytic activities, and in the formation of the postulated ring-like structure for the presenilins [reviewed in [81]].

For many years it was thought that presenilin mutations led to an increase in the more pathogenic Aβ42 or an increase in the Aβ42/40 ratio *in vitro* and *in vivo* [50-52,98-102]. However, this notion is changing with a loss of function explaining an increase in Aβ42 production [103,104]. Some evidence for presenilin mutations causing a loss of function has come from studies that have showed that presenilin mutations result in the loss of Notch signaling [105]. Recent experiments have lead us to re-evaluate the effects of clinical presenilin mutations on APP proteolytic processing in cells deficient of wild-type PS1 or in stably transfected cell lines [106,107]. These studies have measured the absolute levels of Aβ40 and Aβ42 generated from these cells and confirmed that the majority of the PS1/PS2 mutations resulted in an increase in Aβ42/Aβ40 ratio. However, these studies also showed that some mutations led to a reduction in the levels of Aβ40, together with the two additional fragments generated from γ-secretase enzyme activity [Notch and APP intracellular domains, (NICD and AICD,

respectively)] and the accumulation of APP-C99 fragments, similar to that observed in cells lacking presenilins [106,107]. These studies suggest that these mutations maybe altering the "normal" function of presenilins to generate Aβ40, NICD or AICD, favoring the production of Aβ42.

TARGETING γ-SECRETASE AS A POTENTIAL THERAPEUTIC FOR ALZHEIMER'S DISEASE.

Currently, acetyl cholinesterase inhibitors (donepezil, rivastigmine and galantamine) and the N-methyl-D-aspartate receptor antagonist, memantine are the only available treatments for AD. Acetylcholine esterase inhibitors (AChI) prevent the breakdown of the neurotransmitter, acetylcholine, thereby conserving this neurotransmitter at synaptic junctions and compensating for loss of cholinergic circuits [108]. However, in clinical trials (and in practice) the cognitive benefits of these inhibitors are minimal in which more than half the subjects show no measurable improvement. The window of efficacy in those patients in which AChI's show benefit averages 6-12 months and then rapidly reduce as brain deterioration worsens (reviewed in [109]). Another prevalent neurotransmitter is glutamate, which when released pre-synaptically is essential in learning and memory via facilitation of the n-methyl-d- aspartate (NMDA) receptors allowing small influxes of calcium into stimulated nerve cells. This in-turn triggers changes required for long term potentiation, culminating in formation of memory trace [110]. Excess glutamate can overstimulate NMDA receptors, allowing too much calcium into nerve cells, resulting in functional disruption and cell death. Memantine is an NMDA-receptor antagonist that although shows no benefit in mild-to-moderate AD, it is FDA approved for the treatment of moderate-severe AD [111]. Limited, but statistically significant benefits for improved cognition, behaviour, and activities of daily living in AD patients over a six-month trial period has been observed for Memantine [112].

The drugs mentioned above however only treat disease symptoms without targeting the underlying pathology or neurodegeneration. The majority of therapeutic strategies currently being developed and validated target the accumulation of Aβ or its associated neuro-toxicity. One approach being pursued is modulating Aβ production through the use of γ-secretase inhibitors (GSI's) or modulators (GSMs). However, the innate ability of the γ-secretase

complex to cleave other substrates, has raised concerns about the specificity, selectivity and toxicity of agents aimed at inhibiting or modulating γ-secretase activity.

γ-Secretase: The Multi-Substrate Enzyme

Currently it is thought that the γ-secretase enzyme cleaves more than 50 different transmembrane proteins [113-115], this has dubbed the enzyme with the name "the proteasome of the membrane". The list of substrates for γ-secretase include epithelial (E)- and neural (N)-cadherins that are thought to play a major role in cell adhesion [116], CD44 (role) [117] and Erb-B4 which plays a significant role in neuronal development by regulating cell proliferation and differentiation [118]. The intracellular domain released from the cleavage of Erb-B4 has been shown to mediate apoptosis. A more comprehensive list of substrates are shown in table 1.

Apart from APP, the second most studied substrate for the γ-secretase, and possibly the most pharmacologically relevant, is the Notch receptor [119-121]. Notch receptors play a vital role in cell signalling events and not only during embryogenesis but also in the adulthood [122,123]. The signalling pathway is activated with one of five DSL (Delta and Serrate) ligands. During maturation, the Notch receptor is first cleaved by a furin like protease, at the S1 site of the extracellular domain resulting in two fragments, the Notch extracellular domain (NECD) and the transmembrane domain (NTM) which are held together by a heterodimerisation domain (HD) [124]. Upon interaction with its ligand, the metalloprotease, ADAM cleaves at the S2 site of extracellular domain of the Notch receptor (Figure 3). Two ADAMs have been implicated in the cleavage of Notch at the S2 site, ADAM10 and ADAM17. Interestingly, these enzymes also perform α-secretase cleavage of APP, providing additional evidence that Notch undergoes similar proteolytic processing to APP. ADAM10 itself has also recently been shown to undergo γ-secretase cleavage to liberate an ICD which translocates to the nucleus and thought to be involved in gene regulation [125]. This may also be a process by which ADAM10 activity is regulated.

Following cleavage at the S2 site, the NTM then undergoes proteolysis at the S3/S4 sites by γ-secretase, liberating the Notch intracellular domain (NICD) which then translocates to the nucleus and activates target gene expression [10,126,127]. The intramembrane cleavage of Notch is referred to as the dual-intramembrane proteolysis since there are two γ-secretase cleavage sites (S3 and S4). The S3 cleavage site occurs at the interface between the cytosol

and membrane liberating the NICD [10,126]. The S4 cleavage site near the middle of the transmembrane domain has been shown to liberate a fragment referred to as Nβ [128,129]. The C-terminus of Nβ has a similar sequence to that of Aβ. Further, as with Aβ42 levels, presenilin mutations have been associated with an increase in Nβ25, the longer version of Nβ [128]. The function of this fragment within the cell still remains unknown.

Notch and APP have been shown to compete for γ-secretase activity. Using an *in vitro* γ-secretase activity assay that cleaves Notch and APP-based substrates, Kimberly et al., [130] showed that these substrates prevented each other's cleavage, suggesting that the same γ-secretase complex is responsible for the processing of both substrates. Berezovska et al., [131] showed that treatment of neurons with the Notch ligand delta led to a dose dependent reduction in Aβ levels and over-expression of APP led to a reduction in Notch signalling. Further, over-expressing either of the two direct substrates for γ-secretase, C99 and the NΔEC (truncated version of NECD that requires a PS1-dependent cleavage event), led to a reduction in generated products (NICD and Aβ, respectively) [132]. Further, transfection with NICD also down-regulated Aβ production and was associated with a reduction in PS1 transcription and protein levels. These results suggest that competition may exist for the γ-secretase catalytic site between different substrates where generation of a product from one substrate may regulate the proteolytic processing of other γ-secretase substrates. Further, the work of Lleo et al.[132] suggests that Notch signalling may exert some control on PS1 expression which may ultimately impact on APP processing to generate Aβ. Although these studies suggest that the same enzyme complex performs the majority of the processing of the substrates, there is also strong evidence that multiple enzyme complexes with varied functions may also exist.

The existence of two presenilin molecules (PS1 and PS2) as possible catalytic components of the γ-secretase enzyme would suggest that multiple complexes could possibly occur. Although PS1 and PS2 share an overall protein sequence similarity of 67%, several lines of evidence suggest that these proteins may have quite distinct biological functions. Mice lacking PS1 die before birth and the embryos display severe skeletal and brain deformities, whilst mice lacking PS2 develop a mild pulmonary fibrosis and haemorrhages with age [55,133-135]. Neuronal cultures isolated from PS1 ablated mice when compared to those isolated from PS2 knockout mice exhibit lower Aβ production [134,136]. Lai and colleagues also provided evidence suggesting the existence of distinct PS1 containing and PS2 containing

complexes [136]. *In vivo,* PS2 and PS1 transgenic mice have differential effects on γ-secretase activity [137]. There is evidence to suggest that two Aβ generating complexes may exist, whereby PS1 containing complexes produce both Aβ40 and Aβ42, and PS2 containing complexes are involved in Aβ42 (but not Aβ40) production. Recently, Placanica and colleagues 2009 [138] provided further evidence for distinct PS1 and PS2 γ-secretase complexes in the generation of Aβ. The authors described that the two complexes are in dynamic equilibrium, possibly under control of Pen-2 expression.

As there are 2 presenilin genes (*PS1* and *PS2*) and 3 Aph-1 genes (*Aph-1aS*, *Aph-1aL*, and *Aph-1b*), at least 6 different γ-secretase complexes can theoretically occur within a cell. Evidence suggests that the aph1a containing complexes are crucial for Notch signalling during embryogenesis [139,140]. *Aph-1a* knockout mice are embryonic lethal compared to *Aph-1b* knockout mice, which appear to be phenotypically normal, and the absence of *Aph-1a* disrupts the formation of an active γ-secretase complex. [140]. Collectively, these data suggests that complexes containing aph-1a are active and those containing aph-1b are possibly redundant. However, aph-1b complexes have recently been shown to have a role in a neurregulin-1 (*Nrg-1*) signalling. Aph-1b knockout mice show signs of schizophrenia including hypersensitivity to psychiatric drugs, sensorimotor gating abnormalities and working memory deficits [141]. This finding is to be expected given the enrichment of aph-1b in the mouse pre-frontal cortex [141]. In summary, there is sufficient evidence to suggest the presence of multiple γ-secretase complexes with different functional activities and substrates. These characteristics are an important consideration when developing appropriate agents aimed at inhibiting Aβ production.

Inhibiting Or Modulating γ-Secretase Activity

A number of compounds that inhibit or modulate γ-secretase activity have been identified. These include transition state analogues (e.g: L-685,458, WPE-II-31C), dipeptidic inhibitors (DAPT, LY450139), sulphonamide (BMS-299897), kinase inhibitor (imatinib) and NSAIDs (R-Flurbiprofen). Transition state analogues are compounds with a chemical structure, resembling the transition state of a substrate molecule in an enzymatic reaction. Of these, L-685,458 has been extensively studied. This inhibitor was originally shown to inhibit

γ-secretase activity on APP to reduce Aβ generation [142,143] providing some initial evidence that this agent could be a suitable amyloid lowering drug. However, any promise that this agent may be a disease modifying drug was soon dispelled as it was also shown to inihbit the cleavage and subsequent signalling of a number of other substrates including, Notch [144,145], ErbB-4 [121,146] and gamma-protocadherins [147], resulting in the detrimental effects on a number of cellular processes such as cell development, proliferation and adhesion. The ability of this inhibitor to bind PS1 [142,148] provided evidence that it may be used as a detection agent for γ-secretase. Indeed radiolabelled [H3] L-685, 458 was shown as an excellent tracer for γ-secretase activity in vivo [149]. This agent could also show some promise for treatment of cancers. The inhibitors effects on Notch processing has revealed that this inhibitor exerts anti-tumour activity [150]. The chronic treatment of lymphoblastic leukemia cell lines with L-685, 458 has been shown to reversibly inhibited cell proliferation and caused cell block in sensitive T-cell acute lymphoblastic leukemia cell lines [151]. Despite showing some promise as a tumour suppressor, the *in*-vitro effects of this inhibitor on APP and other substrates indicate that that this would not be a suitable treatment for AD.

Amongst the first dipetide inhibitor reported to be an active agent *in-vivo*, where it inhibited Aβ production in the plasma and brain in an APP transgenic mouse model was DAPT [152,153]. Modifications to DAPT led to the development of more potent inhibitors including compound E [57] and related analogues that have shown better efficacy in vivo, including LY-411,575 [154] and LY-450,139 [155,156]. Although Ly-411,575 chronically reduced Aβ levels in plasma and brain, elevated doses resulted in severe gastrointestinal toxicity and interfered with maturation of B- and T-lymphocytes [157,158], presumably due to its effects at inhibiting cleavage of other substrates such as Notch. Clinical studies have been reported for only one γ-secretase inhibitor, LY-4150,139 [155,156]. These reports showed that the inhibitor reduced plasma Aβ levels but had no effect on CSF Aβ, reflecting the low doses being used. Although increasing the dose of this inhibitor may show more beneficial results in the CNS, caution must be taken considering the toxic side effects of its analogues.

A recent clinical trial with the NSAID, R-flurbiprofen (also called Tarenflurbil and Flurizan™) as a treatment for AD failed at phase 3, despite showing promising results in phase 2 [159]. In phase 2 clinical trials [160] compared to placebo treated patients with mild-AD, those administered R- flurbiprofen showed improvements on ADCS-ADL scale (an outcome

measure of drug efficacy that is commonly used in AD clinical trials). However, in a large phase III clinical trial in mild AD patients, those receiving R-flurbiprofen significantly deteriorated more than the placebo treated patients on the Clinical dementia rating scores [159]. A simple explanation of the failure of R- flurbiprofen could be that γ-secretase enzyme is not a suitable target. If this is the case than perhaps the same can be said for other drug agents that target the Aβ molecule and therefore questioning the validity of the amyloid hypothesis. However, the failure of the trial could simply be due to the weak pharmacological activity in the CNS and the poor pharmokinetic profile (as shown by the high doses (-800 mg) of this drug that were required to show benefits in phase II trials. In addition, anti-cytochrome oxidase (COX) activity at these high doses, was most likely an explanation for severe gastro-intestinal side effects (peptic ulcers) in several of those patients treated with R-flurbiprofen (8 compared to only 1 in placebo group) [159]. The inhibitory effects of NSAIDs on microglia may also contribute to the failure of this agent. Microglial, surround plaques and activate phagocytosis and subsequent clearance of Aβ and also stimulate compensatory neurogenesis in the hippocampus [161]. Inhibiting microglia activation could thereby compromise clearance of Aβ and neuronal homeostasis.

Despite the apparent failure of gamma secretase inhibitors and modulators, the enzyme is still pursued as target for developing appropriate therapies. Assessing the efficacy of a number of kinase inhibitors at reducing Aβ production without altering Notch cleavage showed that the Ab1 kinase inhibitor imatinib (GleevecTM) exhibited the desired effects [162]. Similar results were shown with an inhibitor of Janus kinase 3 (Jak3) inhibitor [163]. Screening of large drug libraries for those agents that reduce Aβ with no effect on Notch processing are also currently being pursued [164]. Although, APP and Notch appear to be currently the most physiological and pharmacological substrates, the other substrates also play important roles in maintaining cell homeostasis and are also important to consider when developing therapies aimed at selectively targeting Aβ.

CONCLUSION

There is no doubt that the complexity of this unique enzyme makes it a very difficult target for developing appropriate and effective treatments for AD. Its ability to proteolytically cleave a plethora of membrane bound substrates highlights the importance of this enzyme in normal cell function. Although, intensive research in the past 10 years has revealed a wealth

of information, the exact structure and function of each of its components and where within the enzyme to target and how it is active within a hydrophobic environment with the cell membrane still remain unclear. More detailed insight into the molecular workings of this enzyme is still required not only to develop more effective drug agents, but identify cellular pathways in which gamma secretase is such a critical component.

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Figure Legends

Figure 1: Proteolytic processing of APP: A schematic of the **(A)** non-amyloidogenic and **(B)** amyloidogenic proteolytic processing pathways. (A) In the non-amyloidogenic pathway APP is cleaved by a-secretase (ADAM10/TACE) liberating an APP-C-terminal fragment (C83) that remains embedded in the membrane and a soluble α-APPs fragment that is secreted. The C83 fragment is then cleaved into a non-amyloidogenic fragment p3. An APP intracellular domain (AICD) is also formed which translocates to the nucleus and activating gene transcription. (B) In the amyloidogenic pathway, the APP molecule is cleaved by BACE liberating the APP-C99 fragment that remains embedded in the membrane and a secreted truncated form of APPs called β-APPs. The C99 fragment is cleaved by γ-secretase generating Aβ fragments, of which Aβ40 and Aβ42 are the major species, and the AICD. (Modified from [58])

Figure 2: Sequential cleavage model of APP: In this model, APP-C99 undergoes a number of sequential cleavage events to generate Aβ40 and Aβ42. It is still unclear on whether a single γ-secretase enzyme or multiple enzymes cleaves the fragments at the particular cleavage sites. The C99 fragment is first cleaved at the e-site to generate an Aβ49 fragment and liberating the AICD. The Aβ49 fragment then undergoes cleavage at the z-site to generate Aβ46. This is the parent fragment which then undergoes cleavage at the g-sites; Aβ43 which can undergo further processing to Aβ40 or Aβ42, which can undergo further cleavage to Aβ38/39. (Modified from [13]).

Figure 3: Proteolytic processing of Notch: During maturation, the Notch receptor is first cleaved by a furin like protease, at the S1 site of the extracellular domain resulting in two fragments, the Notch extracellular domain (NECD) and the transmembrane domain (NTM) which are held together by a hetero-dimerisation domain (HD). Upon interaction with its ligand, the metalloprotease, ADAM cleaves at the S2 site of extracellular domain of the Notch receptor. Following cleavage at the S2 site, the NTM then undergoes proteolysis at the

S3/S4 sites by γ-secretase, liberating the Notch intracellular domain (NICD), which then translocates to the nucleus and activates target gene expression. The S3 cleavage site occurs at the interface between the cytosol and membrane liberating the NICD. The S4 cleavage site near the middle of the transmembrane domain has been shown to liberate the Nβ fragment.