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ASSEMBLY, MATURATION, AND TRAFFICKING OF THE γ-SECRETASE COMPLEX IN ALZHEIMER'S DISEASE

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Abstract

In this review, we discuss the biology of γ-secretase, an enigmatic enzyme complex that is responsible for the generation of the amyloid-β peptide that constitutes the amyloid plaques of Alzheimer's disease. We begin with a brief review on the processing of the amyloid precursor protein and a brief discussion on the family of enzymes involved in regulated intramembrane proteolysis, of which γ-secretase is a member. We then identify the four major components of the γ-secretase complex – presenilin, nicastrin, Aph-1, and Pen-2 – with a focus on the identification of each and the role that each plays in the maturation and activity of the complex. We also discuss two new proteins that may play a role in modulating the assembly and activity of the γ-secretase complex. Next, we summarize the known subcellular locations of each γ-secretase component and the sites of γ-secretase activity, as defined by the production of Aβ. Finally, we close by synthesizing all of the included topics into an overarching model for the assembly and trafficking of the γ-secretase complex, which serves as a launching point for further questions into the biology and function of γ-secretase in Alzheimer's disease.

Keywords

Alzheimer's disease; amyloid precursor protein; Aph-1; γ-secretase; nicastrin; Pen-2; presenilin; protease

Introduction

In 1906, Dr. Alois Alzheimer first described the amyloid plaques and neurofibrillary tangles (NFTs) that represent the definitive pathological features of the disease that bears his name. Yet after more than a century of research, these hallmarks remain two of the best criteria on which the diagnosis of *A*lzheimer's *d*isease (AD[2]) lies. The vague nature of its diagnosis is indicative of the myriad of unanswered questions that plague the AD field. We now know that

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Conflict of interest
The authors declare no conflicts of interest.

[2]List of Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; AICD, APP intracellular domain; Aph-1, anterior pharynx-defective-1; APP, amyloid precursor protein; C83 and C99, the C-terminal fragment of APP resulting from cleavage of APP by α- and β-secretases, respectively; CTF, C-terminal fragment; ER, endoplasmic reticulum; FAD, familial Alzheimer's disease; HMW, high molecular weight; Nct, nicastrin (imNct: immature, partially glycosylated Nct; mNct: fully mature Nct); NTF, N-terminal fragment; Pen-2, presenilin enhancer-2; PS, presenilin; RIP, regulated intramembrane proteolysis; sAPPα and sAPPβ, the soluble ectodomain resulting from cleavage of APP by α- and β-secretases, respectively; TMD, transmembrane domain;

these two hallmark pathological features of AD brains – amyloid plaques and NFTs – are composed of the *a*myloid-*β* (Aβ) peptide and the tau protein, respectively. Though still contentious as to which is the more important pathological marker and therapeutic target of the two, it is now generally accepted that the formation of amyloid plaques precedes NFT formation in AD and is more intimately and directly associated with the mental decline associated with AD [1]. Here, we describe the components, assembly, and localization of γ-secretase, the enzyme complex that is directly responsible for the final step in the formation of the neurotoxic Aβ peptide.

Proteolytic Processing of the Amyloid Precursor Protein to Aβ

The advent of modern molecular biology techniques made possible the ability to pin a genetic and molecular origin on AD, which had eluded detection since the identification of AD nearly 80 years previous. The first definitive genetic studies began with two papers that identified an amyloid peptide in the cerebrovasculature of Down's syndrome individuals that was identical to that found in AD brains [2,3]. Shortly thereafter, the cloning of the amyloid precursor protein (APP) gene provided the beginning of a series of mutational analyses that pinned APP as the cause of several inherited forms of AD [4–8]. APP is a type I integral membrane protein, with a large, N-terminal extracellular domain and a short, C-terminal cytoplasmic domain (Fig. (1)). The APP gene is alternatively spliced to yield isoforms of various lengths: the 751- and 770- (longest) amino acid isoforms predominate non-neuronal tissues, while the 695-amino acid form (APP695) is by far the most predominant isoform in neurons [9]. APP and the *AP*P-*l*ike *p*rotein (APLP) has orthologues across nearly all vertebrate and invertebrate animals, and possible roles for APP and its proteolytic products range from axonal transport to transcriptional control and from cell adhesion to apoptosis [10–12].

While very little is known about its biological function in the cell, very much is known about APP genetics and proteolytic processing with respect to AD [13,14]. APP can undergo a series of cleavages to generate a wide array of proteolytic products (Figs. (1) and (2)). APP is first cleaved by either α- or β-secretase at the α- or β-sites, respectively. These two sites lie close to (within ~10–30 amino acids of) the extracellular/luminal side of APP's *t*rans*m*embrane *d*omain (TMD), and cleavage at these sites results in a process known as "ectodomain shedding" [15]. These proteases compete for APP cleavage to give two products: a soluble APP (sAPPα or sAPPβ) which is released into the extracellular space and a membrane-anchored C-terminal stub (C83 or C99, for α- and β-secretase cleavage, respectively). It is this stub upon which γ-secretase acts, with peptide bond cleavage occurring at a most unlikely location: within the lipid bilayer. Cleavage of C83 (the α-secretase product) generates the 6-kDa *A*PP-*i*ntra*c*ellular *d*omain (AICD) and releases the N-terminal ~3 kDa peptide (p3) into the extracellular space; cleavage of C99 (the β-secretase product) generates AICD and the nefarious Aβ peptide. Because the α- and β-secretases compete for APP, cleavage of APP by α-secretase to generate C83 precludes formation of Aβ; therefore, the two main culprits in Aβ genesis and AD are the β- and γ-secretases (Fig. (1)).

γ-secretase cleavage is promiscuous, as the growing list of γ-secretase substrates show little sign of any sequence similarity [16,17]. Interestingly, however, these substrates are all type I transmembrane proteins which require ectodomain shedding as a prerequisite to γ-secretase cleavage [17,18]. In addition, a second heavily studied substrate of γ-secretase is the Notch receptor, which, upon binding to ligand, undergoes sequential proteolysis by an α-secretase-like protease and γ-secretase to analogously produce a soluble Notch receptor fragment and the *N*otch *i*ntra*c*ellular *d*omain (NICD). The proteolytic activity of γ-secretase on Notch is crucial for developmental pathways and, thus, represents a possible undesirable "off-target" effect for AD therapies based on γ-secretase inhibition [19]. Similarly, ErbB4, the low-density lipoprotein (*L*DL)-*r*eceptor-related *p*rotein, the neurotrophin receptor ($p75^{NTR}$), and Sortilin

are just a few of the many seemingly-unrelated type I membrane proteins that serve as substrates for γ-secretase [16,17,19].

The promiscuity of γ-secretase further persists within substrates, as γ-secretase cleavage of APP occurs at any number of sites to generate Aβ peptides from 37 to 49 residues long; these resulting peptides are termed Aβ37 through Aβ49, numbered according to the site of cleavage from the N-terminus of C99 ([20] and Fig. (2)). While a thorough discussion of "subsite" selectivity with respect to γ-secretase enzymology is beyond the scope of this review (for an example, the reader is referred to [21]), it is important to note that two forms are most pertinent to AD etiology: Aβ40, which is considered "normal" and accounts for ~90% of all non-AD Aβ peptides, and the more aggregation-prone Aβ42, which accounts for 5–10% of Aβ peptides in the unaffected, normal brain [22]. Moreover, the Aβ42 peptide is the first and major peptide to form amyloid plaques [23]. Thus, the Aβ42:Aβ40 ratio has been assimilated as a diagnostic tool for the aberrant processing of APP that leads to AD; this progression from Aβ to AD pathology, termed "the amyloid cascade hypothesis", is reviewed extensively elsewhere [24]. Indeed, the majority of AD-linked mutations in APP occur within the Aβ sequence, and most increase the Aβ42:Aβ40 ratio [13]. Moreover, as outlined in the introduction, individuals with Down's syndrome (trisomy 21) often display early signs of AD-like behavior, presumably due to a gene-dosing effect, as APP resides on chromosome 21 [25]. Finally, the vast number of missense mutations found in the presenilin-1 and -2 genes either increase the total Aβ peptide load and/or switch the specificity to produce more Aβ42 and, therefore, increase the Aβ42/Aβ40 ratio *in vitro* and *in vivo* [13,26]. Therefore, understanding the molecular nature of the γ-secretase complex and its biological function with respect to processing of the amyloid precursor protein is critical for a thorough understanding of AD pathology.

RIPping with Intramembrane Proteases

The turn of the 21st century brought a new appreciation for a novel class of proteases, the intramembrane proteases, which catalyze the unique chemistry of hydrolysis within a hydrophobic environment in a process now known as *r*egulated *i*ntramembrane *p*roteolysis (RIP) [27–30]. These intramembrane proteases can be further classified into four families, all of which have orthologues through eukaryotes and, with the exception of γ-secretase, prokaryotes. The metalloprotease-like S2P family is typified by the S2P protease, which regulates sterol and fatty acid homeostasis through proteolysis of its substrate, the *s*terol *r*egulatory *e*lement *b*inding *p*rotein (SREBP). These proteases contain an HExxH motif that is characteristic of metalloproteases. The second family – the Rhomboids – appears to function in mitochondrial maintenance and cellular signaling, as the *Drosophila* orthologue cleaves the *e*pidermal *g*rowth *f*actor (EGF)-like substrate, Spitz. These intramembrane proteases, which cleave type I membrane proteins, are serine proteases, with a conserved serine-histidine catalytic dyad.

The last two families of intramembrane proteases are both membrane-embedded aspartyl proteases whose catalytic residues appear to lie at the same relative positions within the lipid bilayer [27–30]. The first of these is named for its prototype: the *s*ignal *p*eptide *p*eptidase (SPP) family. These proteases are unique in that they cleave type II membrane proteins, and, as such, SPP is responsible for the clearance of signal peptides produced after cleavage of the insertion sequence of membrane proteins by signal peptidase [27]. The second family of these membrane-embedded aspartyl proteases is represented by γ-secretase, which is responsible for the cleavage of a long and growing list of type I membrane proteins [16,17]. The two most heavily studied γ-secretase substrates are the Notch receptor, whose ligand-dependent proteolysis is critical in developmental pathways, and APP, whose cellular role is varied and unclear (described above). Interestingly, the substrates of the first three of these intramembrane proteases (S2P, Rhomboid, and SPP) families contain helix-breaking residues, implying that

metastability is critical for hydrolysis. γ-secretase substrates, on the other hand, do not appear to share this feature, suggesting that either instability is not required or that γ-secretase has an intrinsic ability to destabilize α-helices [27]. While the superfamily of intramembrane proteases is reviewed extensively elsewhere [27–30], this review will focus on the last of these, γ-secretase, and its role in the processing of APP in AD.

γ-Secretase: A Complex Complex

The first indications for the protein(s) responsible for γ-secretase activity came from genetic studies that linked early-onset *f*amilial *AD* (FAD) to a variety of mutations in two novel genes on chromosomes 1 and 14, termed the presenilins [31–34]. Early studies were filled with debate over whether the presenilins themselves were the elusive γ-secretase or whether they simply supported γ-secretase activity. Indeed, by the end of the 20th century, the presenilins were accepted as only one component of a *h*igh *m*olecular *w*eight (HMW) complex that exhibited γ-secretase activity [35–39]. Through the years that followed, several other components have been identified that modulate or co-purify with the γ-secretase complex. However, protein expression in yeast and reconstitution assays with recombinant proteins have defined the minimal active γ-secretase complex as consisting of four core components: endoproteolyzed presenilin (a heterodimer of N- and C-terminal fragments), nicastrin, Aph-1, and Pen-2 [40–45]. Only very recently, however, has functional information been elucidated for these components, as described in the sections that follow. Due to its size and complexity, specific structural information for the γ-secretase complex has eluded the AD field, although efforts using electron microscopy, molecular modeling, and solvent accessibility have provided glimpses into the potential structure of the heterogeneous complex [46–50]. With structure being beyond the scope of this review, we present here our current understanding of the function and biology of the four core components that comprise the complex.

The Presenilins – The Active Site

Although APP was the first gene to be causally linked to AD, it soon became apparent that other genetic loci were responsible for the majority of FAD cases [51,52]. Indeed, the floodgates opened with the initial characterization of the *p*resenilin genes (PS1 and PS2) [31–34], and the next 10 years exhibited an onslaught of papers showing more than 150 mutations linked to FAD in either of these two genes [53]. Many of these mutations are not scattered haphazardly throughout the primary sequence; rather, many PS mutations in both isoforms cluster into defined regions, particularly within the transmembrane domains and along a stretch just C-terminal to the sixth TMD in PS's large intracellular loop [50,53–55]. While recent studies have suggested alternative roles for PS from apoptosis to cellular adhesion to calcium homeostasis [56,57], this review describes the biology of PS with respect to its role in the proteolytic processing of APP in the context of AD.

PS1 and PS2 are 467- and 448-amino acid membrane proteins, respectively, whose transmembrane topology has been debated [58]. Using prediction programs and epitope mapping, models of 6 to 9 TMDs have been proposed, with the 8 TMD model being the most popular [59–65]. Recently, however, the 9 TMD model has been gaining acceptance, with the very C-terminus embedded within the lipid bilayer (Fig. (2)) [61,63–65]. Regardless of topology, TMDs 6 and 7 contain the catalytic aspartic acid residues that participate in the proteolytic cleavage of the peptide bond of substrates (Asp257 and Asp385 on TMD6 and 7 of PS1, respectively). Early studies debated whether PS itself was the elusive γ-secretase, or whether γ-secretase was a separate enzymatic activity [66]. However, by the turn of the century, evidence mounted showing that PS was indeed the aspartyl protease responsible for γ-secretase activity, with two aspartic acid residues functioning in the catalysis of APP cleavage (reviewed in [67,68]). Whether PS alone could support γ-secretase activity, however, was unclear.

Shortly after their cloning, it became apparent that the presenilins themselves undergo autocatalytic endoproteolysis between TMD6 and TMD7 to generate presenilin *N*- and *C*-*t*erminal *f*ragments (PS-NTF and PS-CTF, respectively, Figs. (2) and (3)). PS was shown to be proteolyzed *in vivo*, and a PS1 deletion mutant in which the large intracellular loop between TMD6 and TMD7 is partially missing does not get processed to these derivatives [69]. Later reports demonstrated that only a small portion of PS undergoes this proteolysis by competition for limiting factors [70]. Uncleaved PS holoprotein (holo-PS) is rapidly degraded, whereas PS-NTF and PS-CTF exist as a stable 1:1 heterodimer in the HMW γ-secretase complex [35–37, 71, 72]. The limiting and stabilizing factors responsible for PS endoproteolysis and γ-secretase complex formation were later identified (see the subsections that follow), but it was quickly shown that it is these fragments that constitute the active form of presenilin, as PS-NTF and -CTF could rescue a *sel-12* (the *C. elegans* PS homologue)-deficient phenotype. Moreover, these same PS fragments with mutations in the putative catalytic aspartic acid residues could not rescue the phenotype, demonstrating that these aspartic acids truly participated in the γ-secretase activity and that the loss in Aβ production is directly due to a loss in catalysis, rather than indirectly through a decrease in PS endoproteolysis. These same studies suggested that PS does not fulfill γ-secretase activity alone, as overexpression of PS-NTF and -CTF does not result in more active complex, despite their stable association [73]. Moreover, active site and aspartyl protease transition state inhibitors bind directly to both PS-NTF and -CTF, further demonstrating that the PS fragments represent the active form of PS within the γ-secretase complex [74, 75]. Finally, a recent report showing that coexpression of wild-type PS1 cannot rescue endoproteolysis of catalytically-inactive PS1 demonstrated that PS endoproteolysis to generate PS-NTF and -CTF is truly intramolecular and occurs in *cis*, without any evidence of PS/γ-secretase dimers [76]. Taken together, then, it became apparent that a small portion of newly-synthesized PS holoprotein is stabilized and intramolecularly cleaved to N- and C-terminal fragments by limiting cellular factors and that these fragments are stably associated to form the γ-secretase active site, whereas unincorporated holoprotein is rapidly degraded (Fig. (3)) [69–73, 76].

Several important functional determinants have been found in the presenilins. One conserved motif is the GxGD motif, which contains the second catalytic Asp from TMD7. The Gly residues are critical for γ-secretase function, with Ala being a reasonable substitution. Moreover, this GxGD motif is conserved throughout the SPP and γ-secretase families, as well as within the bacterial type-4 prepilin peptidases, further emphasizing its importance [77]. Curiously, using distant conservation as a guide, it was shown that the identity of the "x" residue within the GxGD motif can discriminate between substrates: Leu at position 383 in PS1 allows efficient cleavage of both APP and Notch, whereas Phe at the same position eliminates γ-secretase cleavage of Notch [78]. A second functional motif was found in the C-terminus: the conserved PALP motif that follows the eighth TMD in PS1 and PS2. Mutation of this motif, particularly the first proline residue (P414 in PS2), leads to a loss-of-function (Notch) phenotype, and P414 mutations lead to rapid degradation of PS holoprotein, with no formation of PS fragments [79,80]. While the initial report suggested that the PALP motif was required for stabilization and formation of HMW complexes, a later report showed that PS1-P433L (analogous to P414 in PS2) can still enter HMW complexes and are, in fact, stable, but they fail to undergo endoproteolysis due to catalytic inactivity [81]. These data were later complemented with the identification of the PALP motif as an ER-retention signal; exposure of the PALP motif could result in retrieval of PS to the ER to ensure proper folding and incorporation into the γ-secretase, whereas masking this motif by insertion into the membrane may allow release from the ER for trafficking to the plasma membrane ([82] and Fig. (3)). A third functional determinant was recently identified in the TMD1 of PS: the N-terminal portion of TMD1 affects only γ-secretase activity, while only the C-terminal portion of TMD1 is responsible for PS endoproteolysis [83]. Finally, a second APP-binding site separate from, but near to, the γ-secretase active site was recently identified [84–89]. This docking site may serve

as a means of allosteric regulation or simply as an anteroom where substrate binds before entering the active site.

Nicastrin – The Substrate Receptor

With its FAD-linked mutations and its involvement in the γ-secretase-mediated cleavage of APP and Notch, PS was proposed to directly participate in the catalysis of peptide bond hydrolysis within the lipid bilayer. However, it appeared that PS required interactions with other proteins to fulfill this function. One argument for accessory proteins came from the observation that the active PS fragments functioned in HMW complexes, and several groups sought to identify novel proteins involved in γ-secretase activity in these HMW complexes. Although several proteins had previously been shown to bind to PS (e.g. β-catenin), none appeared to be involved in the processing of APP [35,90–92]. In 2000, however, a novel protein, *ni*c*a*s*t*rin (Nct), was identified by using immunoprecipitation of PS1 coupled with mass spectrometry. Nicastrin, named after the Italian village of Nicastro, where a PS-linked case of FAD was identified 40 years previous, is a large, 709-amino acid type I transmembrane glycoprotein, with a large N-terminal extracellular domain and a short C-terminal intracellular domain ([93] and Fig. (2)). At the time, no sequence homology or motif similarity was found with any other protein, but subsequent reports have identified distant conservation with aminopeptidases [43,94]. Despite a lack of homologues, the initial report identified orthologues in *M. musculus*, *D. melanogaster*, *C. elegans*, and *Arabidopsis*, thus implying a deeply conserved function(s), even across non-neuronal tissues. Endogenous Nct was shown to interact directly and specifically with both PS1 and PS2 in the human brain, and exogenous Nct could interact with various PS mutants, suggesting that PS mutant-dependent alterations in Aβ production were not due to the inability of Nct to bind PS [93]. Further evidence for a conserved function throughout vertebrates and invertebrates came from studies in which the Nct homolog was necessary for Notch signaling in *C. elegans* [93,95], *Drosophila* [96–98], and mice [99].

The first glimpse into the function of Nct came from mutagenesis studies. The original report on Nct identified a motif conserved across all species, the DYIGS motif (so named for the amino acid identity, residues 336–340), which when mutated or deleted results in a gain- or loss-of-function in the generation of Aβ peptides, respectively. Another early indication of function came from studies on the interaction between Nct and γ-secretase substrates, where in the original study, Nct was found to co-immunoprecipitate the products of α- and β-secretase cleavage of APP (C83 and C99, respectively) [93]. It took another five years, however, to elucidate the specific function of Nct as the substrate receptor, thereby providing an explanation for specificity in γ-secretase proteolysis in the absence of sequence similarity. In the first concerted effort to pin a function on Nct, Shah and coworkers demonstrated that the large extracellular domain of Nct, and not its TMD, specifically and stoichiometrically binds to C99, the product of APP cleavage by β-secretase. Moreover, both the soluble extracellular domain of Nct and a TMD-swapped Nct chimaera (which does not bind active PS as efficiently as does wild-type Nct) inhibit γ-secretase activity by sequestering substrate away from the active site. Finally, the substrate-binding residues of Nct were mapped to the *D*YIGS *a*nd *p*eptidase (DAP) domain, with the carboxylate side chain of Glu333 binding the α-amino group of the free N-terminus of substrates that have undergone ectodomain shedding (Fig. (2)). Thus, these data, taken with the studies of others, defined Nct as the substrate-binding subunit of γ-secretase, with Nct's extracellular domain providing the determinants for binding substrates and the first third of the N-terminal portion of its TMD necessary for its efficient corporation into the γ-secretase complex by binding to the C-terminus of PS [43,82,100]. Most importantly, the nature of substrate recognition – by requiring spatial and steric barriers to be overcome for Nct's recognition of N-terminal stubs for proteolysis by γ-secretase – provides specificity in the absence of sequence similarity (Fig. (2)).

Similar to PS, Nct undergoes a highly-regulated maturation process that is necessary for γ-secretase activity [101]. The 78-kDa holoprotein is rapidly *N*-glycosylated in the ER to yield an endo-H-sensitive ~110-kDa "immature" form (imNct). This imNct is rapidly degraded (half-life < 6 hours) unless it is further glycosylated to a highly stable (half-life > 24 hours), ~130-kDa "mature" form (mNct), which appears to be the only form that endogenously incorporates into the γ-secretase complex by binding to PS [102–104]. Moreover, PS is necessary for efficient maturation to mNct, as $PS1^{-/-}PS2^{-/-}$ mouse embryonic fibroblasts trap Nct in the immature form in the endoplasmic reticulum (ER) [104,105]. Interestingly, however, maturation of imNct to mNct, which is heavily *N*-glycosylated with complex mannose and sialic acid sugars but devoid of *O*-glycosylation, does not appear to be required for γ-secretase activity [106]. Furthermore, maturation of Nct does not require PS endoproteolysis [107], although mNct is increased when PS is found in its heterodimeric form, presumably due to formation of a more stable γ-secretase complex that contains mNct and PS-NTF and -CTF. Finally, mature Nct was found to adopt a compact, trypsin-resistant conformation that is not simply due to addition of complex sugars to Nct's ectodomain, as imNct incorporated into γ-secretase complexes can also adopt this trypsin-resistant conformation [108,109]. Thus, newly-synthesized Nct is rapidly *N*-glycosylated in the ER to imNct, after which it is transported to the Golgi for further complex glycosylation to form the highly stable mNct, which preferentially interacts with PS; furthermore, Nct undergoes a conformational change in its large ectodomain during maturation, with trypsin-resistant mNct representing the active species in the mature γ-secretase complex. Interestingly, Nct matures independent of PS endoproteolysis and is not required for γ-secretase activity (Fig. (3)).

Aph-1 – The Stabilizer

While PS and Nct were found in the HMW complexes that contained γ-secretase activity, overexpression of these two components alone did not lead to an increase in the assembly of active γ-secretase complex, suggesting that another limiting factor(s) must be required for the stabilization and/or assembly of the γ-secretase complex [101]. In the midst of a flurry of research on the biology, trafficking, and biochemistry of PS and Nct, two separate groups in 2002 looking for loci that genetically interact with Notch, PS, and Nct in *C. elegans* identified *aph-1* and *pen-2* (see the next subsection) as candidate genes for the maturation of the γ-secretase complex. *aph-1* deficiency gave a similar phenotype (*a*nterior *ph*arynx-defective) as was seen earlier for PS (*sel-12* and *hop-1* in *C. elegans*) and Nct mutants (*aph-2* in *C. elegans*), and although the precise role of Aph-1 in the γ-secretase story was unclear, its function appeared to precede γ-secretase activity [110,111].

One of the first clues to Aph-1 function came from its original identification, where *aph-1* mutant embryos showed mislocalization of the Nct homologue in a manner similar to that seen in PS-defective embryos [111]. This phenomenon, coupled with the observation that depletion of Aph-1 decreased PS processing without a concomitant increase in PS holoprotein levels [112], suggested that Aph-1 may be important for processing of PS and/or stabilization and trafficking of the mature γ-secretase complex. Indeed, in mammalian cells, the seven-transmembrane-spanning, ~30-kDa Aph-1 was found to associate with Nct and PS as an integral regulator for the assembly of the γ-secretase complex [113]. Stable transfection of cells with Aph-1 increased PS holoprotein levels, and co-expression with Nct enhanced this effect [112]. Despite its effect on stabilizing PS, Aβ secretion did not change, and this result was reconciled later by several groups that showed that while Aph-1 stabilizes the PS holoprotein, maturation of the γ-secretase complex requires Aph-1 and another limiting factor (see Pen-2 below) [112,114–117].

More detailed analyses extended the function of Aph-1 away from being an inert scaffold in the assembly of γ-secretase complex. First, Aph-1 was found to physically interact with Nct

and PS holoprotein and PS-NTF and -CTF, regulate maturation of imNct to mNct and PS to NTF and CTF, and be required for γ-secretase activity [113,114]. Shortly afterwards, Aph-1 was found to preferentially interact with imNct in a stable, 1:1, ~140-kDa sub-complex [118]. Moreover, a GxxxG motif was identified in the fourth TMD of Aph-1 that appears to mediate interactions within the γ-secretase complex [119]: Aph-1 binds to imNct in the ER through other interactions, but the GxxxG motif is critical for either the direct or indirect binding of the Aph-1/imNct complex to PS holoprotein and subsequent trafficking to the Golgi [120]. (It should be noted, however, that upon assembly of the γ-secretase complex, this motif appears to be dispensable for activity [121].). On the other hand, imNct is mainly associated with immature PS holoprotein, whereas mNct is associated with mature PS (i.e. PS-NTF and -CTF) [103,122]. These observations are consistent with a model whereby Aph-1 binds imNct in the ER/*cis*-Golgi to form a "pre-complex" that then binds to the C-terminus of the immature holo-PS. Binding is either direct or indirect via the GxxxG motif in TMD4 of Aph-1 [82, 119,120,123–125]. This imNct·Aph-1·holo-PS ternary complex is then trafficked to the *trans*-Golgi, where Nct can fully mature to its heavily *N*-glycosylated, ~140-kDa, trypsin-resistant form (Figs. (2) and (3)).

Pen-2 – The Linchpin

The *p*resenilin *en*hancer-*2* gene, or *pen-2*, was identified side-by-side with *aph-1* in a *C. elegans* screen, as discussed above (*aph-1* was the first presenilin enhancer, or "*pen-1*"). The 101-amino acid Pen-2 protein represented a novel protein family with no shared domains or motifs, and its topology revealed two transmembrane domains, with luminal N- and C-termini (Fig. (2)). Pen-2 is encoded by a single gene on chromosome 19, and orthologues were identified in *M. musculus*, *D. melanogaster*, *D. rerio*, *C. elegans*, and *Arabidopsis*. *pen-2* was found to genetically interact with PS and Nct homologues, with activity upstream of γ-secretase activity. Moreover, *pen-2* was necessary for processing of PS to NTF and CTF and for γ-secretase activity [110].

Over the next few years, a number of studies revealed that Pen-2 was the long-sought-after regulator of PS endoproteolysis and, subsequently, γ-secretase activity [112,115,126–128]. Pen-2 was found to co-immunoprecipitate with Nct and PS (both holoprotein and NTF/CTF heterodimer), with Pen-2 apparently tightly packed within the γ-secretase complex [125,127]. Moreover, Pen-2 protein was stabilized by expression of PS and Nct, further lending credence to the idea that formation of the γ-secretase complex is a tightly regulated process [127]. In a second study, depletion of Pen-2 by RNA-mediated interference (RNAi) led to a loss in PS-NTF and -CTF with a commensurate accumulation of PS holoprotein [128]. Moreover, since combinations of Pen-2 RNAi with Nct and Aph-1 RNAi *decreased* PS holoproteins similar to Nct and Aph-1 RNAi treatments alone, Pen-2's role was placed downstream of those of Nct and Aph-1 as the final step before PS endoproteolysis [112]. Furthermore, Pen-2 was implicated in the full maturation of Nct, as imNct accumulated when Pen-2 was depleted in a PS1Δexon9 background (where PS1 cannot undergo endoproteolysis) [128]. Thus, it appeared that, as the last component to be added, Pen-2's incorporation into the γ-secretase complex allows for exit from the ER to the Golgi, at which point Nct can be fully matured (Fig. (3)).

Further evidence that Pen-2 is the last component to enter the immature γ-secretase complex to facilitate its maturation came from studies that showed the presence of a Nct·Aph-1·PS intermediate complex [112,118]. Moreover, it was shown that Pen-2 not only facilitates endoproteolysis, but also further enhances γ-secretase activity, as Pen-2 expression 1) enhances the level of Aβ generation when co-expressed with PS-NTF and PS-CTF in a PS-null background (thus bypassing its role in endoproteolysis) and 2) decreases the IC_{50} of a transition-state analogue. In these studies, Pen-2 did not enhance activity by enhancing the association of PS-NTF and -CTF, suggesting that Pen-2 further optimizes the γ-secretase active

site for catalysis by some other means [129]. Thus, these two functions of Pen-2 – facilitation of PS endoproteolysis and optimization of the γ-secretase active site – appear to be functionally coupled.

The first biochemical experiments to dissect the determinants necessary for Pen-2's role in the maturation of γ-secretase came in 2004, where solubilization of cell membranes with various detergents revealed a major sub-complex of Pen-2 and PS1-NTF, even though Pen-2 also associated with PS holoprotein [125,126,130]. The absolute length and sequence of the C-terminus of Pen-2 was a prime candidate for interactions, as C-terminal fusion proteins and deletions led to its loss of function [110,128,131,132]. Indeed, a conserved DYLSF motif was found in Pen-2's C-terminus that, when deleted or mutated to alanine in its entirety, led to its inability to incorporate into HMW complexes and, thus, its rapid degradation. As these effects did not result from mislocalization of Pen-2, it was suggested that these residues – and the total length of the C-terminus – are critical for 1) its interaction with PS, 2) the subsequent maturation of PS and Nct, and 3) stabilization of the γ-secretase complex [128,131,132]. Moreover, the length and sequence of the C-terminus are critical for stabilizing the mature γ-secretase complex that is otherwise rapidly degraded by the proteasome; however, the Pen-2 C-terminus does not appear to regulate endoproteolysis [128,132]. Finally, a second point of interaction was found, where the N-terminal (luminal) two thirds of the first TMD of Pen-2 are necessary for interaction with PS [130]. Further studies with PS revealed that the "NF" motif at the cytoplasmic end of the PS's fourth TMD is involved in interactions with Pen-2, although the exact corresponding site on Pen-2 remains unidentified ([107,133]Fig. (2) and).

A recent report found that a short extension of Pen-2's N-terminus can modulate γ-secretase activity by altering solvent accessibility around the catalytic pore. The exact mechanism is unknown, although given the nature of the fusion tags used, all of which varied in sequence yet had a net negative charge close to the membrane, it is possible – and even likely – that the tags have electrostatic effects on active site residues and/or channeling of water molecules [134]. As a last note, Pen-2 can bind to PS even when the imNct·Aph-1 complex cannot, and yet under normal conditions, endogenous Pen-2 only appears to exist in complexes with the remaining three components, thereby suggesting that other means, such as localization, control when and where Pen-2 is added to the γ-secretase complex [107]. Finally, two studies have demonstrated that the assembly of all four components can occur stepwise in the ER; moreover, the ER-localized, four-component γ-secretase can, in fact, undergo endoproteolysis in the absence of Nct maturation [109,135]. Taken together, then, the above observations with localization data suggest that Pen-2 is incorporated into the imNct·Aph-1·PS heterotrimeric pre-complex in the ER by binding to the fourth TMD of PS in a highly compact manner. Binding of Pen-2 allows for PS endoproteolysis and exit of the γ-secretase complex from the ER to the Golgi, where Nct can fully mature before trafficking to other locations (Figs. (2) and (3)).

Other Putative γ-Secretase Members and Modifiers

Genetics and cell biology have demonstrated the necessity of PS, Nct, Aph-1, and Pen-2 for γ-secretase activity *in vivo*. Moreover, reconstitution assays with these four proteins have demonstrated that these four components are necessary and sufficient for γ-secretase activity *in vitro* [40–45]. Still, it is plausible that other proteins can bind to and modulate the activity of the γ-secretase complex. The gold standard for finding a *bona fide* component of the γ-secretase complex would be 1) direct interactions with one or more of the four components 2) in HMW complexes to 3) directly modulate activity (best shown *in vitro*). Several additional proteins have been proposed to fulfill a modulatory role, including CD147, phospholipase D, calsenilin, and the X11/Mint family [136]; however, to date, these have not yet been shown to directly modulate γ-secretase activity *in vitro*. Here we single out two of the more recently described regulators of the γ-secretase complex.

*T*rans*m*embrane trafficking *p*rotein 21 (Tmp21) was identified in a screen for other components of the HMW complex that coimmunoprecipitate with PS [137]. Tmp21 is a 219-residue, type I transmembrane protein that is found at the plasma membrane. Moreover, Tmp21 is a member of the p24 cargo-protein family, which functions in quality control and protein transport in the ER and Golgi [138,139]. Interestingly, a bioinformatics analysis revealed that Tmp21 is co-transcribed with PS and APP, suggesting that these three proteins share a similar biological process [140]. Tmp21 could immunoprecipitate all four γ-secretase components, could be found in the HMW complexes identified by glycerol velocity gradients, and was destabilized in the absence of PS or Pen-2 in a manner reminiscent of Nct and Aph-1 instability under the same conditions. Finally, loss of Tmp21 appeared to directly modulate γ-secretase activity to produce more Aβ; this effect was independent of expression of the remaining γ-secretase components or substrate and was independent of any role in complex maturation or trafficking, since such modulation occurred even in cell-free assays [137]. A recent study, however, showed that Tmp21 also regulates trafficking of APP to the cell surface, thus highlighting the complex nature between Tmp21's role in direct modulation of γ-secretase activity and trafficking of APP and γ-secretase [141].

A second new γ-secretase-interacting protein is *r*etrieval to *ER 1 p*rotein (Rer1p), which was identified in a screen for membrane proteins that assist in retrieval of proteins from the ER-Golgi intermediate compartment and *cis*-Golgi to the ER. The role of Rer1p in the retention of γ-secretase components has been described independently by two groups. In the first study, the authors found that Rer1p binds to the TMD of Nct and apparently functions by retrieving unincorporated Nct from the *cis*-Golgi for retrograde trafficking to the ER. Since binding of Rer1p and PS1/Aph-1 to Nct were mutually exclusive, and since depletion of Rer1p resulted in more γ-secretase activity, it was suggested that Rer1p functions as a negative regulator of γ-secretase complex formation by competing for Nct through its TMD [142]. Recently, a second study described a role of Rer1p in retrieving Pen-2 for incorporation into the γ-secretase complex. This report identified an ER retention signal in the C-terminal half of Pen-2's first TMD and demonstrated that Rer1p selectively retrieves unincorporated Pen-2 to the ER. Furthermore, overexpression of Rer1p leads to further maturation of Nct, thus indicating elevated trafficking of the complex through the Golgi and, presumably, higher γ-secretase activity [143]. Both studies would place Rer1p as a critical protein involved in quality control: since γ-secretase assembly has been shown to occur in the ER [135], retrieval of Nct or Pen-2 from the Golgi by Rer1p ensures efficient incorporation into maturing γ-secretase complexes. Once assembled, the γ-secretase complex exits the ER for transport through the Golgi network, whereupon Nct is fully matured (Fig. (3)). The data for binding of Rer1p to imNct and Pen-2 are equally compelling, and binding of Rer1p to these two components may be intimately related and not mutually exclusive, thus making Rer1p an exciting new target for future γ-secretase research.

Heterogeneity, Redundancy, and Non-Redundancy Within the γ-Secretase Complex

The PS1 and PS2 isoforms share ~67% identity [136], with the largest variations occurring in the N-terminus and in the large intracellular loop between TMDs 6 and 7 [144]. Several lines of evidence suggest that the two PS isoforms share non-redundant functions. First, although both are relatively ubiquitously expressed on both mRNA and protein levels, the two isoforms do show some tissue specificity [145]. Second, PS knockout mice show very different phenotypes: knockout of PS1 in mice is embryonic lethal, while PS2 knockout mice are viable and fertile and show only mild pulmonary fibrosis [146]. Third, cellular studies have shown that while PS1 and PS2 may share the same activity towards the same set of substrates, redundancy is only partial with each isoform having an as-yet-identified distinct function [147]. Fourth, PS transgenic mice revealed isoform- and mutant-specific alterations in both APP processing and in skeletal morphology [148]. On the other hand, PS1 and PS2 seem to

be at least partially redundant at a cellular level. For example, PS1 knockout cells show a sharp reduction in Aβ production [149], with complete reduction of Aβ generation only when both PS1 and PS2 are knocked out [146,150,151], and PS2 can compensate for loss of PS1 function in Notch signaling [152]. Moreover, full-length chimaeras, in which the NTF of one isoform is fused to the CTF of the other and *vice-versa*, undergo normal endoproteolysis and result in fully active protein, suggesting that the essential biochemistry of PS1 and PS2 – from assembly of the γ-secretase complex to its catalysis – has been conserved throughout evolution [144]. Taken together, then, PS1 appears to be the main component in γ-secretase activity with respect to Notch processing, with PS2 playing a minor "safety valve" role.

Although partial redundancy between PS1 and PS2 first suggested that all γ-secretase complexes are functionally similar, if not identical, the discovery of the *aph-1* gene renewed the notion of distinct γ-secretase complexes with distinct functions. The human *aph-1* gene has two family members (*aph-1a* and *aph-1b*), with a single gene in *C. elegans*, *D. melanogaster*, and *D. rerio* [110]. Moreover, the *aph-1a* allele can undergo splicing to generate a long (Aph-1a^{L}) and short (Aph-1a^{S}) isoform, thus yielding several distinct γ-secretase complexes with the possible combinations of Aph-1 and PS isoforms [123,145,153]. Furthermore, Aph-1 knockout mice revealed that the Aph-1a isoform is the only murine isoform required for proper development, solidifying the idea that distinct γ-secretase complexes without functional redundancy exist and are vital for proper cellular function [154]. Indeed, coimmunoprecipitation experiments reveal that each PS isoform (−1 and −2) can independently interact with each Aph-1 variant (−1a^{L}, −1a^{S}, and −1b) to theoretically yield six distinct complexes within a given cell. Moreover, between the multiple isoforms and splice variants of PS and Aph-1 and the multiple glycosylated forms of Nct, distinct γ-secretase complexes with distinct functions are not only possible but are indeed likely [123,145,153, 155].

Reconciling the "Spatial Paradox" of γ-Secretase Localization: Components vs. Activity

Early studies revealed that while PS was largely found in the ER and Golgi compartments, γ-secretase activity resided at the cell surface. Such a discrepancy was first described by Annaert and de Strooper who coined the term "spatial paradox" to describe this phenomenon [156]. Many groups have used a combination of immunofluorescence, *f*luorescence *r*esonance *e*nergy *t*ransfer (FRET), and subcellular fractionation with marker proteins to demonstrate the extra- and subcellular localization of PS, Nct, Aph-1, Pen-2, APP, C83, C99, sAPP, Aβ peptides, and AICD. To document and cite all of these studies would require more space than this review permits, but here, we will summarize the known distribution of the four γ-secretase components and the sites of γ-secretase activity. We conclude this section with arguments from several studies in order to attempt to reconcile the resulting spatial paradox between γ-secretase complex localization and activity.

Endogenous PS has largely been localized to intracellular membranes, particularly the ER and the intermediate compartment [157]. However, biotinylation, immunofluorescence, and immunoelectron microscopy have shown populations of PS at the plasma membrane [158]. The original identification of Nct showed localization to the ER and Golgi [93], and further biochemical and immunofluorescence experiments showed that Nct undergoes extensive glycosylation in its transit from the ER through the Golgi [103,106]. However, like PS, fully-mature Nct has also been detected at the plasma membrane by biotinylation [105,106,159]. Endogenous Aph-1 shows strong localization to the ER and *cis*-Golgi [114], and yet later studies showed Aph-1 at the plasma membrane [158,160]. Similarly, Pen-2 was initially found in the ER and Golgi [109,110,126,131,135,161], although later studies revealed its presence at the plasma membrane [158,160].

Based on the above data, then, it would appear that two pools of γ-secretase exist: one at the plasma membrane and one in intracellular membranes. But which pool represents the active pool? The first half of the equation is Aβ generation, and recent data have shown that Aβ is generated at the plasma membrane, endosomes near the cell membrane, and/or exosomes [162,163]. As a recent example, in a clever set of experiments, Kaether and colleagues tracked the cleavage of a C-terminally-GFP-tagged C99 construct while inhibiting various steps along the exocytic pathway. In their study, it was shown that γ-secretase has activity towards C99 no sooner than upon its arrival at the plasma membrane (due to limitations in the technique, however, the authors could not address the possibility of endosomal or lysosomal cleavage of substrate). That is, despite the majority of γ-secretases at intracellular membranes, C99 does not appear to be cleaved at the ER, Golgi, or post-*trans*-Golgi-network/pre-exocytosed vesicles [164]. The second half of the equation – *active* γ-secretase complexes – has also been shown to reside at the plasma membrane. Indeed, detailed analyses have shown that inactive γ-secretase, which represents the majority of γ-secretase, does not reside at the plasma membrane; instead, a minor pool of γ-secretase complexes, which represent ~6–7% of total γ-secretase, is active and sits at the plasma membrane, thus reconciling the earlier "spatial paradox" [158, 160]. Such a mechanism, taken together with the rapid degradation of unincorporated components, further reiterates how tightly controlled the assembly and activation of γ-secretase complexes are (Fig. (3)).

Finally, there is quite a bit of evidence demonstrating that Aβ is produced in the endosomal/lysosomal system [165]. Early in APP research, C99 was found to be endocytosed from the plasma membrane [166], an observation that has been repeatedly seen by several independent groups [162,165,167]. Also, this internalization followed by Aβ production is reduced by alteration of the C99 endocytosis signal, blocking of vesicle fission by expressing dominant mutations in dynamin II, or expression of a *G*TPase *a*ctivating *p*rotein (GAP) for Rab5, a GTP-dependent protein involved in endocytosis [165]. Moreover, Aβ appears to be produced in the lysosome, as ammonium chloride and bafilomycin (both of which prevent acidification of the lysosome) reduces Aβ formation [168–170] and as PS1 knockout mice accumulate C83 and C99 in lysosomes [171]. One study demonstrated the presence of ~5% of total PS and ~30% of total Nct in the lysosome, and this group also found a pH optimum of 4.5, with little activity at pH 7.0, further evidence suggesting that these proteins were not simply targeted for lysosomal degradation [165,172]. Moreover, it appears that acidic pH promotes Aβ oligomerization, that Aβ oligomers accumulate in intracellular compartments, and that exogenous Aβ42 is endocytosed to form insoluble aggregates in the lysosome [165]. Another interesting possibility is that trafficking of γ-secretase to acidic organelles facilitates release of the Aβ product from Glu333 of Nct in a manner analogous to the transferrin and mannose-6-phosphate receptors. Further evidence for the location of APP processing comes from the other secretases: α-secretase activity appears to reside at the plasma membrane, whereas β-secretase activity appears to reside within endosomes and lysosomes, with β-secretase demonstrating an acidic optimum pH for activity [15]. Perhaps the most compelling evidence for the role of lysosomal Aβ production comes from a recent study on Aβ immunotherapy; in this study, the Aβ-lowering effect of anti-Aβ antibodies required both APP endocytosis and endosomal function [173]. Indeed, mounting evidence points to intracellular Aβ load as an important therapeutic target [174], and the specific site of γ-secretase-mediated ε cleavage within APP appears to differ between the plasma membrane and endosomes [175]. Still, while the small amounts of γ-secretase at the plasma membrane appear to reconcile the "spatial paradox," where γ-secretase activity is spatially separated from the majority of γ-secretase components, the precise location of Aβ generation – be it at the plasma membrane or in endosomes and/or lysosomes – remains controversial.

Conclusion and Perspective: A Model for the Assembly and Trafficking of the γ-Secretase Complex

The localization data, taken together with the long half-life of those components that are incorporated into stable γ-secretase complexes *versus* the relatively short half-life of free components, suggest that two pools of γ-secretase exist: the large majority of γ-secretase complex (~95%) shuttles between the ER and Golgi, whereas a small fraction of γ-secretase that displays activity (~5%) resides at the plasma membrane and in endosomal compartments (Fig. (3)). The recent identification of Rer1p as a potential modulator of γ-secretase assembly seems a likely candidate to explain the existence of these two pools (see above) [142]. Moreover, it has been suggested that the C-terminus of PS1 contains a hydrophobic ER retention signal to retrieve unincorporated PS1 back to the ER [82].

Taken together, then, we present the following model for the assembly, maturation, and trafficking of the γ-secretase complex (Fig. (3)). All four components – PS holoprotein, Nct, Aph-1, and Pen-2 – are synthesized in the ER, where they await incorporation into stable sub-complexes. Rer1p retrieves any imNct and/or Pen-2 that escapes to the Golgi and returns it/them to the ER for incorporation into γ-secretase complexes (*center right*). Any protein that is not incorporated into more stable complexes at any of the following steps is rapidly degraded, primarily through the proteasome. Shortly after its synthesis, Nct is rapidly *N*-glycosylated in the ER to go from a ~80 kDa holoprotein to the partially glycosylated, ~110 kDa, endo-H-sensitive immature nicastrin (imNct; *bottom right*). Complex formation begins with the binding of Aph-1 to the TMD of imNct in the ER to form a stable, 1:1, ~140-kDa *sub-complex I* (*bottom left*). This imNct·Aph-1 heterodimer then binds to PS holoprotein in the ER to form the ternary *sub-complex II* through interactions that include 1) the GxxxG motif of Aph-1's fourth TMD, 2) the luminal portion of the TMD of Nct, and 3) the C-terminus of the PS holoprotein. The final step in assembly occurs in the ER, where Pen-2 binds to the NF motif in the fourth TMD of PS; the exact corresponding site of interaction on Pen-2 is unknown, although the first two thirds of TMD1 and the C-terminus are required for the stable formation of *sub-complex III*, in which Pen-2 is tightly buried within the γ-secretase complex. Sub-complex III is very transient, as incorporation of Pen-2 into the complex results in the rapid endoproteolysis of PS in its large intracellular loop between TMD6 and TMD7; endoproteolysis is dependent on the luminal portion of TMD1 in PS. The resulting PS fragments, PS-NTF and PS-CTF, represent the active form of PS within *sub-complex IV* in the Golgi and/or ER. Finally, while most (~95%) of the mature γ-secretase complexes cycle between the ER and Golgi, a minority (~5%) of the γ-secretase complexes is trafficked through the Golgi on their way to their final destination(s). In its transit through the *trans*-Golgi, Nct is further matured to form the heavily *N*-glycosylated, endo-H-insensitive, ~140 kDa, mature form (mNct); maturation of Nct also leads to a conformational change to render the extracellular domain trypsin-resistant. It is this final *sub-complex V* (PS-NTF and -CTF, mNct, Aph-1, and Pen-2) that represents the active form of γ-secretase in the cell. However, it should be noted that imNct can also adopt this trypsin-resistant conformation within a "pseudo-mature" γ-secretase complex in the ER [109].

Once stabilized and active, the γ-secretase complex moves to the plasma membrane by an as-yet poorly understood mechanism. One possibility is that the formation of sub-complex III buries within the protein(s) or membrane any ER retention signals that may exist within the four components, thus permitting its trafficking to the plasma membrane for activity; these ER retention signals may include the PALP motif of PS, the TMD of imNct, and/or the first TMD of Pen-2. At the plasma membrane, a portion may remain or a portion may be endocytosed to endosomes, late endosomes/multivesicular bodies (MVBs), and/or the lysosome for activity in these compartments. Regardless of the site of activity, Nct binds to the N-terminal amine of C83 or C99 substrates through Glu333 in its DAP domain, thus positioning substrates for cleavage by γ-secretase (Fig. (2)). The catalytic aspartic acid residues, including the GxGD

motif in TMD7, participate in catalysis, but γ-secretase activity is also dependent on the cytosolic-facing portion of PS's first TMD. A second substrate docking site may exist outside of, but near to, the active site for "pre-loading" the complex. Finally, the products of γ-secretase activity are released – Aβ is released into the lumen or extracellular space, and AICD is released into the cytosol, where it may translocate to the nucleus or interact with other cytosolic proteins. Admittedly, this model is by no means complete, and several pieces of data do not fit within this model. Neither does this model account for other putative modulators, such as Tmp21. Nevertheless, the details for this scheme are provided in the text above, and Fig. (3) serves as a model from which predictions on the biology and biochemistry of the γ-secretase complex can be made.

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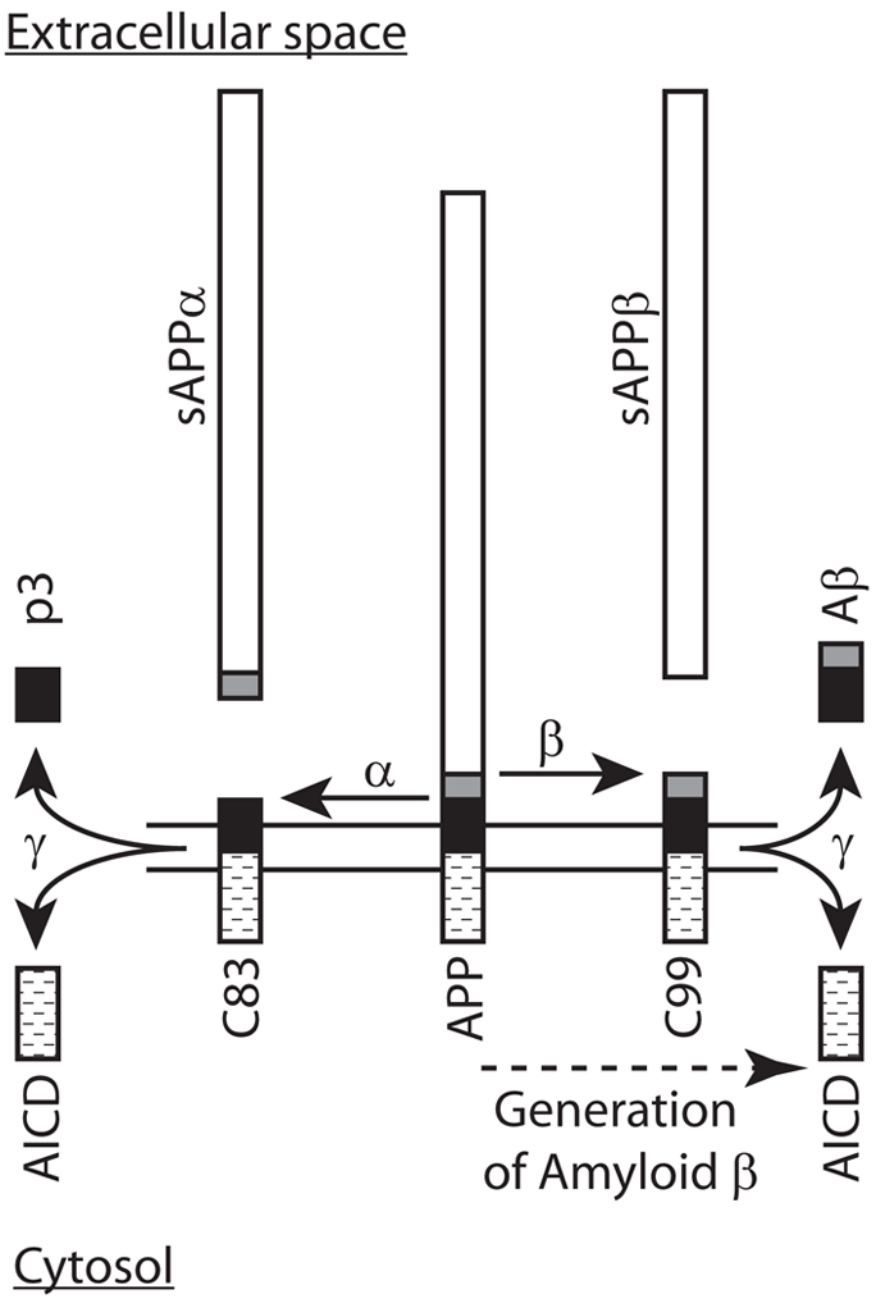

Figure 1. Proteolytic processing of the amyloid precursor protein (APP)

APP is processed by either of two pathways. First, the large extracellular domain is removed by either α-(*left*) or β-secretase (*right*) in a processed termed "ectodomain shedding" to release the soluble APP ectodomain (sAPPα and sAPPβ, respectively) into the extracellular space. The resulting APP C-terminal stubs (C83 and C99, respectively) can then serve as substrates for intramembranous cleavage by γ-secretase. Cleavage of C83 generates the p3 peptide and the APP intracellular domain (AICD), whereas cleavage of C99 generates the Aβ peptide and AICD. p3 and Aβ are ultimately released into the extracellular space, whereas AICD remains in the cytosol. Note that cleavage of APP by α-secretase precludes formation of Aβ.

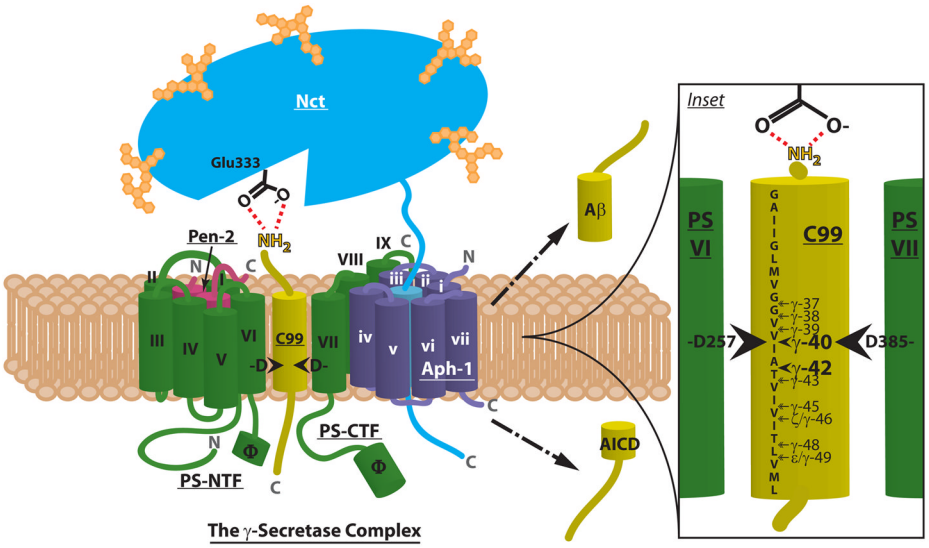

Figure 2. Composition of the active γ-secretase complex and sites of γ-secretase cleavage in APP

The active γ-secretase complex (*left*) is composed of four components: presenilin (PS, *green*), nicastrin (Nct, *light blue*), Aph-1 (*dark blue*), and Pen-2 (*pink*). PS is cleaved in the hydrophobic region (*Φ*) to yield the PS N- and C-terminal fragments (PS-NTF and -CTF, respectively). The catalytic aspartic acid residues, Asp257 and Asp385, are found on TMDs 6 (VI) and 7 (VII), respectively. Glu333 of the DAP domain of Nct binds the N-terminal amine of substrates for positioning in the γ-secretase active site. Endogenous Nct is heavily glycosylated (*orange*) in active γ-secretase complexes. The Nct·Aph-1 heterodimer binds to PS-CTF through TMD4 (iv) of Aph-1 and both the TMD and extracellular domain of Nct. Pen-2 is buried in interactions with PS-NTF, and both Pen-2 and the first TMD of PS appear to modulate the active site. A "docking site" for substrates may also exist near the active site. *Inset*, γ-secretase cleaves APP (*yellow*) at multiple sites within its transmembrane domain. The resulting Aβ peptides are named according to their length from the N-terminus. Aβ40 and Aβ42 (*large*, *bold*) represent the majority of the Aβ species produced.

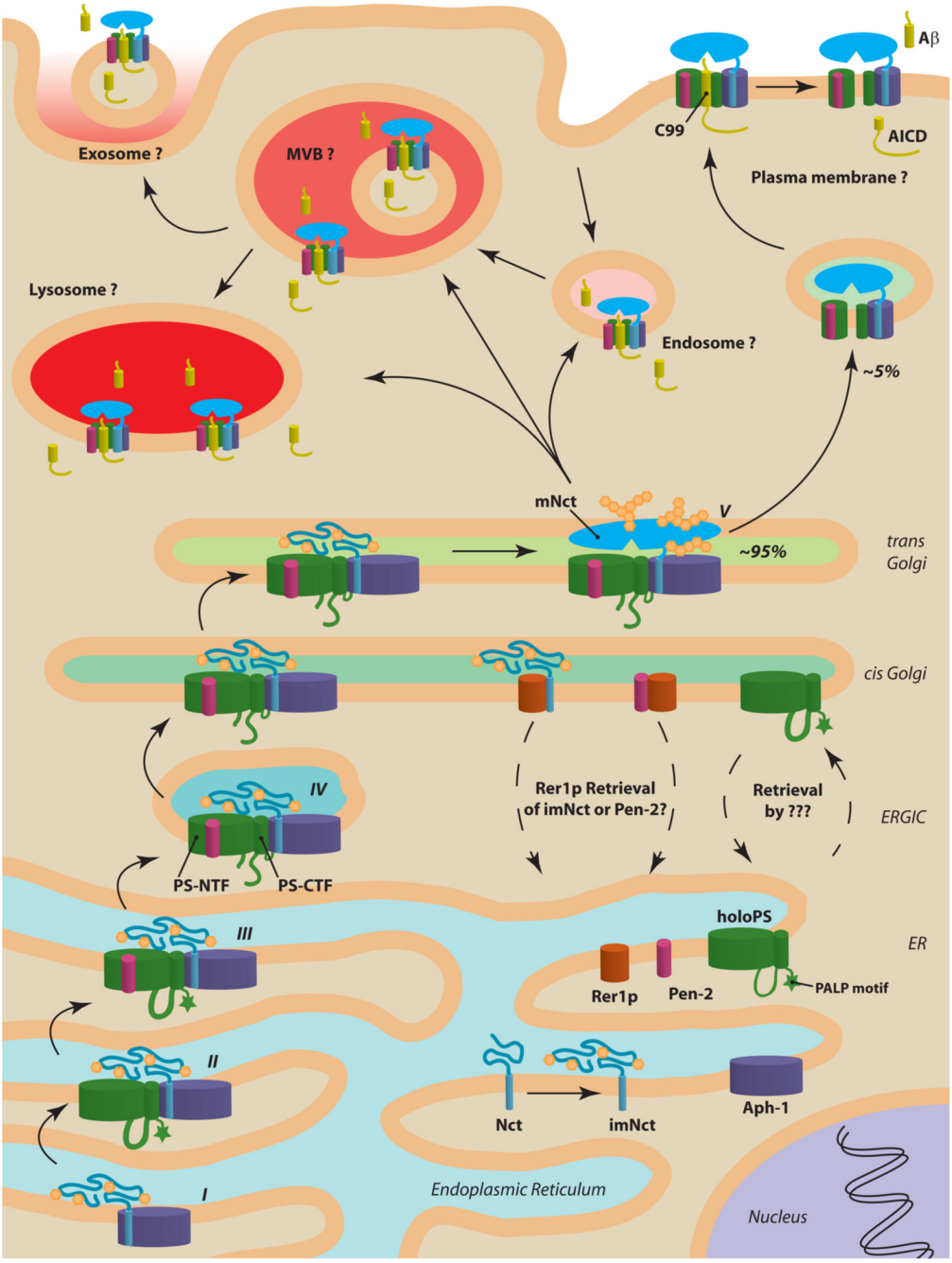

Figure 3. Assembly and trafficking of the γ-secretase complex

The four γ-secretase components (colored as in Fig. (**2**)) are synthesized in the endoplasmic reticulum (ER), where they await incorporation into stable complexes (*bottom right*). Unincorporated components are degraded with a half-life ($t_{1/2}$) ~ < 6 h; the half-life for components in stable sub-complexes is ~ > 24 h. Rer1p can retrieve unincorporated imNct or Pen-2 that escapes from the ER to the Golgi (*center right*). The PALP motif of unincorporated PS is exposed (*star*), such that PS that escapes to the Golgi is rapidly retrieved by an unknown mechanism to return PS to the ER (*center right*). Shortly after its synthesis, Nct is rapidly *N*-glycosylated to form the immature, partially-glycosylated form (imNct, *bottom right*). It is this imNct that binds to Aph-1 in the ER to form a stable 1:1 complex (*sub-complex I, bottom*

left). This stable heterodimer then binds the PS holoprotein (holoPS) in the ER to form the imNct·Aph-1·holoPS heterotrimer (*sub-complex II*). The mNct·Aph-1·holoPS trimer can now bind Pen-2 to form *sub-complex III*. PS within this heterotetramer rapidly undergoes endoproteolysis to form N- and C-terminal fragments (PS-NTF and -CTF, respectively), yielding *sub-complex IV*. Assembly of all four components and the resulting endoproteolysis hides any ER retention signals (e.g. the PALP motif of PS1 and the TMDs of Nct and Pen-2) to allow for exit of the complex from the ER for transit to the Golgi via the ER-Golgi intermediate compartment (ERGIC). These ER retention signals may be buried between the proteins and/or within the membrane. While endoproteolysis may occur in the ER, the ERGIC, and/or the Golgi, endorproteolysis in the ERGIC is represented here. Upon reaching the *trans*-Golgi, Nct is fully matured to mNct (*sub-complex V*). It is this complex – PS NTF and CTF, mNct, Aph-1, and Pen-2 – that represents the typical active form of γ-secretase. The majority (~95%) of mature γ-secretase complexes cycles between the ER and the Golgi, with a minority (~5%) that is further trafficked to the plasma membrane or to other membranous components (e.g. endosomes, multivesicular bodies (MVBs), or lysosomes). Once it reaches these compartments, γ-secretase can catalyze the intramembranous cleavage of substrates, such as C99, which is the product of β-secretase cleavage of APP (*upper half of figure*). Cleavage generates the extracellular/luminal Aβ peptide and the APP intracellular domain (AICD), which can bind to cytosolic effector proteins or translocate to the nucleus to participate in transcriptional regulation. The exact site of Aβ generation is controversial, but several studies have shown γ-secretase activity in the following locations: the cell surface, the endosome, within late endosomes/MVBs, in the lysosome, or in extracellular vesicles after exosome release. For simplicity, glycosylation (*orange*) was omitted from the upper half of the figure. Please see “Conclusion and Perspective” and Fig. (2) for more details.