Alzheimer's disease is a serious public health problem, and a dramatic increase in Alzheimer's disease patients is apparent in our rapidly aging society. Pathologically, Alzheimer's disease is characterized by the accumulation of tangles and senile plaques (1). Senile plaques are composed of amyloid β-peptide (Aβ), which is generated by proteolytic processing from the β-amyloid precursor protein (APP) (2). Secretases (named after the secretion of some of their cleavage products) cut APP within and around the Aβ domain. β-Secretase mediates the N-terminal amyloidogenic cut and competes with the non-amyloidogenic α-secretase, which prevents Aβ production by cleaving in the middle of the Aβ domain (2). The cleavage products of β- and α-secretases (99- and 83-amino acid C-terminal fragments) are the direct substrates for the γ-secretase enzyme. This cleavage occurs within the transmembrane domain (TMD) and is exerted by an aspartyl protease complex composed of the four core components presenilin (PS)-1 or -2, nicastrin (NCT), APH-1a/b (anterior pharynx-defective-1), and PEN-2 (presenilin enhancer-2) (reviewed in Ref. 2). PSs are most likely the catalytic subunit of this complex and provide the two critical aspartyl residues. This is now strongly supported by a number of independent observations. In the absence of PS1 and PS2, no authentic Aβ is produced (4, 5). Moreover, numerous familial Alzheimer's disease-linked mutations in the PS genes shift the ratio from the predominant 40-amino acid Aβ species to the more aggregation-prone 42-amino acid variant (2). Furthermore, active-site γ-secretase inhibitors can be cross-linked to PSs (6, 7), and mutagenesis of the two critical aspartyl residues inhibits γ-secretase activity (8). PSs belong to the novel family of polytopic aspartyl proteases of the GXGD type (9, 10). These proteases have a highly conserved C-terminal active-site motif composed of a GXGD motif containing the catalytically critical aspartate. This family of proteases, which includes the signal peptide peptidases and their homologs (11) and the type 4 preprolin peptidases (10, 12), may have evolved independently to cleave hydrophobic sequences within or close to TMDs (13).

In contrast to all known putative GXGD proteases, which are active either on their own or as homodimers (11, 12, 14), PSs must associate with three other components to gain their proteolytic activity (15–18). Coordinated regulation of the expression level of the four complex components severely complicates functional analysis of individual components (reviewed in Ref. 2). Down-regulation of PS1, NCT, APH-1, or PEN-2 decreases the levels of the other components and prevents maturation of the complex.

γ-Secretase is an aspartyl protease complex composed of the four core components APH-1, nicastrin (NCT), presenilin (PS), and PEN-2. It catalyzes the final intramembraneous cleavage of the β-secretase-processed β-amyloid precursor protein to liberate the neurotoxic amyloid β-peptide. Whereas unassembled complex components appear to be unstable and/or to be retained within the endoplasmic reticulum (ER), the fully assembled complex is known to exert its biological function in late secretory compartments, including the plasma membrane. We thus hypothesized that the γ-secretase complex undergoes a stepwise assembly within the ER. We demonstrate that γ-secretase-associated NCT can be actively retained within the ER by the addition of a retention signal. Under these conditions, complex assembly occurred in the absence of maturation of NCT, and ER-retained immature NCT associated with APH-1, PEN-2, and PS fragments. Moreover, a biotinylated transition state γ-secretase inhibitor allowed the preferential isolation of the fully assembled complex containing immature NCT. Furthermore, we observed a conformational change in immature NCT, which is known to be selectively associated with complete γ-secretase complex assembly. This was also observed for a small amount of immature endogenous NCT. ER-retained NCT also rescued the biochemical phenotype observed upon RNA interference-mediated NCT knockdown, viz. reduced amyloid β-peptide production; instability of PS, PEN-2, and APH-1; and accumulation of β-amyloid precursor protein C-terminal fragments. Finally, we demonstrate that dimeric (NCT/APH-1) and trimeric (NCT/APH-1/PS) intermediates of γ-secretase complex assembly containing endogenous NCT are retained within the ER and that the incorporation of the fourth and last binding partner (PEN-2) also occurs on immature NCT, suggesting a complete assembly of the γ-secretase complex within the ER.

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Anja Capell‡§, Dirk Beher*, Stefan Prokop¶, Harald Steiner‡, Christoph Kaether‡, Mark S. Shearman†, and Christian Haass‡‡

From the ‡Adolf-Butenandt-Institut, Department of Biochemistry, Laboratory for Alzheimer’s and Parkinson’s Disease Research, Ludwig-Maximilians-Universität, Schillerstrasse 44, 80336 Munich, Germany and the ¶Department of Molecular and Cellular Neuroscience, Merck Sharp & Dohme Research Laboratories, Neuroscience Research Center, Terling Park, Harlow, Essex CM20 2QR, United Kingdom

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† To whom correspondence may be addressed. Tel.: 49-89-2180-75-484; Fax: 49-89-2180-75-451; E-mail: acapell@med.uni-muenchen.de.

‡ To whom correspondence may be addressed. Tel.: 49-89-2180-75-415; E-mail: chaas@med.uni-muenchen.de.

The abbreviations used are: Aβ, amyloid β-peptide; APP, β-amyloid precursor protein; swAPP, Swedish mutant β-amyloid precursor protein; TMD, transmembrane domain; PS, presenilin; NCT, nicastrin; NCTwt, wild-type nicastrin; ER, endoplasmic reticulum; HEK, human embryonic kidney; RNAi, RNA interference; CT, C terminus; NT, N terminus; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-1-hydroxy-1-propanesulfonic acid; CTP, C-terminal fragment; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Bicine, N,N-bis[2-hydroxyethyl]glycine.
NCT (2). Once assembled, the complex is, however, extraordinarily stable (19). It appears that a dimeric complex composed of APH-1 and immature NCT (15, 20–22) provides the scaffold for the addition of the remaining components. The PS holoprotein may be added to this putative precomplex to form a trimeric intermediate (15, 23). Finally, PEN-2 apparently associates with the precomplex (21–25), resulting in a conformational change in NCT (20, 26) and the induction of PS endoproteolysis (Refs. 15, 18, 23, and 25; reviewed in Ref. 27).

At the moment, it is unclear where within the secretory pathway γ-secretase complex assembly occurs. We hypothesized that γ-secretase complex components may be retained within the endoplasmic reticulum (ER) as long as the complex is not fully assembled. To identify the cellular compartment where γ-secretase complex assembly occurs, we attached an ER retention signal to the cytoplasmic domain of NCT. By forcing the assembly of the γ-secretase complex on immature NCT, we demonstrate that a fully functional γ-secretase complex can be generated within the ER. Moreover, endogenously assembled complex intermediates containing only two or three components were retained within the ER, but were released after the missing γ-secretase subunits were incorporated.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Lines**—Human embryonic kidney (HEK) 293 cells stably expressing Swedish mutant APP (swAPP) and the NCT knockout cell line were described previously (26). The swAPP-expressing cell line and the NCT knockout cell line were stably transfected with the wild-type NCT (NCTwt/V5) (28) or NCT-ER-V5 constructs using FuGENE 6 (Roche Diagnostics). For NCT expression, cells were selected with blasticidin (50 μg/ml). The PEN-2 knockout cell line and cell lines expressing PEN-2 derivatives have been described previously (25).

**cDNA Constructs of NCT**—All NCT constructs expressed in the NCT knockout cell line contained a cluster of silent mutations conferring RNA interference (RNAi) resistance (26). The NCT-ER-V5 construct, containing the sequence motif for ER retention and a stop codon after the V5 tag, was generated by PCR-mediated mutagenesis. For the CD4-NCT-CT-V5 and CD4-NCT-CT-V5-ER fusion constructs, the C termini of NCT were amplified by PCR using the appropriate NCT construct as template and cloned via an artificial NotI site after the last CD4 codon and the XhoI site of the vector pcDNA3 (Invitrogen). The CD4-NCT-CT-V5 construct was generated by PCR. All cDNA constructs were sequenced to verify successful mutagenesis.

**Antibodies**—The anti-PS1 loop (3027) (29), anti-PEN-2 (1638) (30), anti-APH-1aL (O2C2 or 434) (31, 32), anti-APP C terminus (CT) (6687) (33), and anti-Aβ (6E10) (34) polyclonal antibodies have been described previously.

**RESULTS**

NCT that is not associated with the γ-secretase complex remains as an immature species within the ER (39–42). This suggests that NCT contains an ER retention motif, which may be masked during complex formation. To investigate whether NCT contains such active retention motifs, we used CD4 as a reporter protein (43). First, the C-terminal domain of NCT including a V5 tag (note that the V5 tag does not interfere with the physiological function of NCT, see below) was fused to CD4, creating CD4-NCT-CT-V5 (Fig. 1A). Second, the TMD of CD4 was exchanged with the TMD of NCT to create the CD4 variant CD4-NCT-CT-TM V5 (Fig. 1A). Upon transfection of COS-7 cells with CD4-NCT-CT-TM V5 or CD4-NCT-CT-V5, intracellular and plasma membrane immunostaining was carried out (Fig. 1B). Both fusion proteins showed punctuate intracellular staining as well as strong labeling of the plasma membrane (Fig. 1B). A similar pattern was found when CD4 alone (data not shown) or a cDNA construct encoding CD4 fused to 38 amino acids of the C terminus of a KATP channel with a mutagenized inactive ER retention signal (CD4-AAA_surface) (43) was investigated (Fig. 1B). This indicates that the cytoplasmic tail and TMD of NCT do not contain an active ER retention signal. To further address the question of whether γ-secretase complex assembly occurs within the ER, NCT was forced to be retained within the ER by the addition of an ER retention signal. We first confirmed the function of the well established KKKK motif for ER retention (44) fused to the C terminus of a V5 tag variant of NCT using the CD4 reporter system (Fig. 1A). CD4-NCT-CT-
V5-ER was retained within the ER, and only very little surface staining could be detected (Fig. 1B). Similarly, the CD4-RKR ER variant (Fig. 1A), in which the last 36 amino acids of the C terminus of a KATP channel with an active ER retention signal (RKR) (43) are fused to CD4, was also efficiently retained within the ER (Fig. 1B).

To analyze whether NCT that is actively retained within the ER influences /H9253-secretase complex formation and function, we transfected an NCT variant with a functionally active ER retention signal (for ER retention signal, see Fig. 1) in an NCT knockdown HEK 293 cell line stably expressing swAPP (26, 28, 39). Consistent with our previous results (26, 28, 39), NCT expression was strongly decreased by RNAi (Fig. 2A). As expected, this was accompanied by reduced PS1, APH-1aL, and PEN-2 expression and a significant increase in the APP C-terminal fragments (CTFs), followed by reduced Aβ generation (Fig. 2B). When this cell line was stably transfected with the RNAi-insensitive NCTwt-V5 construct (26, 28, 39), the biochemical phenotype of the NCT knockdown was rescued (Fig. 2B). The APP CTFs did not accumulate anymore, and Aβ production was fully restored (Fig. 2B). Maturation of NCTwt-V5 was observed (Fig. 2A), which is consistent with the functional restoration of γ-secretase activity (39, 41). In contrast, NCT maturation was reduced upon expression of NCT-ER-V5 (Fig. 2A). Although maturation of NCT was significantly reduced by the addition of the ER retention signal, NCT-ER-V5 rescued the loss of γ-secretase function caused by the RNAi-mediated knockdown of endogenous NCT. Like NCTwt, ER-retained NCT rescued PS1, APH-1aL, and PEN-2 expression; reduced APP CTF levels; and restored Aβ generation (Fig. 2B). No significant change in the Aβ40/Aβ42 ratio was observed upon ER retention of NCT (Fig. 2B). To exclude that remaining small amounts of endogenous NCT were responsible for the full rescue, we performed in vitro γ-secretase assays using γ-secretase complexes selectively immunoisolated via the V5 tag of ectopically expressed NCT. This revealed that γ-secretase complexes containing NCT-ER-V5 efficiently produced Aβ and APP intracellular domain-like complexes composed of NCTwt (Fig. 2C).

NCT with and without an ER retention signal co-immunoprecipitated with both PS1 fragments (Fig. 2D), suggesting insertion into a complete γ-secretase complex. In contrast to NCTwt-V5, where mainly mature NCT associated with PS fragments, significant levels of immature NCT-ER-V5 co-immunoprecipitated with PS (Fig. 2, D and E). Both variants

![Fig. 1. The C terminus and TMD of NCT do not contain an ER retention signal. A, schematic representation of NCT and the fusion constructs of the reporter protein CD4. The NCT C terminus without (CD4-NCT-CT-V5) or with (CD4-NCT-CT-V5-ER) an ER retention signal was fused to the C terminus of CD4, or the TMD of CD4 was exchanged with the TMD of NCT (CD4-NCT-TM-V5). As controls, two CD4 constructs containing either an ER retention signal (CD4-RKR ER) or a mutated retention signal (CD4-AAA surf) were used. TM, the transmembrane region; hatched boxes, the NCT TMD; striped boxes, the NCT C terminus; black bars, V5 epitope. B, subcellular localization of the CD4-NCT-CT-V5 and CD4-NCT-TM-V5 fusion constructs and the ER-retained CD4-NCT-CT-V5-ER construct, including the surface-located control (CD4-AAA surf) and the ER-retained control (CD4-RKR ER). COS-7 cells were transiently transfected with the indicated cDNAs. 48 h after transfection, intracellular staining (left panels) or surface staining (right panels) was carried out using the anti-CD4 monoclonal antibody.](image)
(NCTwt and ER-retained NCT) efficiently assembled into a γ-secretase complex. This suggests that γ-secretase complex formation occurs within an early secretory compartment and that γ-secretase complexes containing immature NCT can be biologically active. Indeed, quantification of co-immunoprecipitation experiments showed a decreased interaction of mature NCT with PS1 in the case of the ER-retained variant (Fig. 2E). Although significantly less mature NCT was observed, Aβ generation was unchanged (Fig. 2E), indicating proteolytic activity of an ER-retained fully assembled γ-secretase complex.

To further investigate whether ER-retained immature NCT indeed assembles into a functional γ-secretase complex, we investigated the conformational switch of NCT known to be associated with γ-secretase activity (20, 26). As expected from our previous findings (20, 26), mature NCTwt-V5 was selectively trypsin-resistant, whereas the immature variant was degraded under the same conditions (Fig. 3A). In contrast, substantial amounts of immature NCT-ER-V5 were found to be resistant to trypsin (Fig. 3A). This suggests that a significant portion of immature NCT-ER-V5 is incorporated into a func-
trypsin-sensitive (Fig. 4, whereas immature NCT in low molecular weight fractions was located immature NCT becomes part of a fully assembled high molecular weight complex. Importantly, a substantial amount of immature NCT co-fractionating with the endogenous PS1 CTFs (Fig. 4B, fractions 9–12), immature NCT-ER-V5 co-fractionating with endogenous PS1 CTFs became trypsin-resistant (Fig. 4B, fractions 9–12), whereas immature NCT in low molecular weight fractions was trypsin-sensitive (Fig. 4B, fractions 9–12), immature NCT in low molecular weight fractions was almost completely digested in all fractions (Fig. 4B), whereas mature NCT comigrating with PS1 CTFs was selectively stable (fractions 9–12). This again supports the observation that ER-located immature NCT becomes part of a fully assembled high molecular weight γ-secretase complex.

To analyze whether fully assembled γ-secretase complexes containing immature NCT are proteolytically active, we selectively isolated fully functional γ-secretase using a biotinylated affinity ligand. The biotinylated Merck C inhibitor selectively binds biologically active γ-secretase as described previously (6, 37, 45). Membranes of NCT-ER-V5- or NCTwt-V5-expressing cells were solubilized in CHAPSO and incubated with the biotinylated Merck C affinity ligand, followed by precipitation of enzyme-inhibitor complexes with streptavidin-Sepharose beads. Upon expression of NCTwt-V5, the inhibitor precipitated almost exclusively mature NCT, although a robust amount of immature NCT was present in the membrane lysate (Fig. 5). In contrast, when NCT-ER-V5 was expressed, immature NCT was also precipitated by the biotinylated Merck C affinity ligand (data not shown).
Therefore, we sought to stabilize the complex by blocking the proteasomal degradation of PS N- and C-terminal fragments and PEN-2ΔC using MG132. Under these conditions, co-immunoprecipitation with the anti-APH-1a antibody indeed resulted in the co-purification of the complete γ-secretase quartet (Fig. 6D). Moreover, we selectively co-purified immature NCT (Fig. 6D), demonstrating the association of all four components before complex glycosylation in the late secretory pathway occurred. Note that even without stabilization by proteasomal inhibition, small amounts of PS1 CTFs and PEN-2ΔC associated with APH-1 and immature NCT.

**DISCUSSION**

The so-called spatial paradox claimed that PS is located mostly within the ER, whereas γ-secretase activity is observed within the late secretory pathway, the plasma membrane, and endosomes (48, 49). Indeed, only small amounts of PS associated with mature NCT are located on the plasma membrane (50). This suggested that unincorporated or excess amounts of γ-secretase components may reside within the ER and are released only upon their assembly into the (complete) native γ-secretase complex. This prediction was supported by the observation that down-regulation of PS results in the accumulation of immature NCT (39, 41, 42, 50). These observations prompted us to investigate whether γ-secretase complex assembly can occur within the ER. Such a mechanism would indicate that complex formation and correct folding of components of the complex could be controlled by ER-located chaperones. Misfolded complexes or unincorporated components would be degraded within the ER before reaching later compartments of γ-secretase function (51, 52).

Indeed, we recently identified an ER retention signal within PS1 (53), which holds unassembled PS back in the early secretory pathway. For NCT, we could detect an ER retention motif neither in the TMD nor in the cytoplasmic domain. However, NCT that is not incorporated into the γ-secretase complex resides within the ER; therefore, accumulation of immature NCT may be explained by unsuccessful folding. This is consistent with our previous observation that γ-secretase complex formation/activity is closely associated with a major conformational change in NCT (20, 26). Thus, forward transport of NCT apparently depends on its interaction with the other γ-secretase complex components.

Consistent with previous reports (15, 22, 27), we have demonstrated a sequential assembly of the complete γ-secretase complex. Immature NCT and APH-1 appear to be a dimeric ER-located scaffold for further complex assembly. Next, the PS holoprotein binds and forms a trimeric intermediate, which still contains immature NCT. Subsequently, PEN-2 may come into play (27). After PEN-2 association with the trimeric complex, PS is endoproteolysed; NCT undergoes a conformational switch; and the complete complex is then apparently released from the ER as monitored by its trypsin resistance and maturation. A rapid release of γ-secretase from the ER may make it difficult to investigate the cellular site of its assembly. We therefore used several independent approaches to study the assembly of the γ-secretase complex. First, we forced NCT to be retained within the ER by the addition of a functionally active ER retention motif. Under these conditions, NCT maturation is significantly reduced, although small amounts of NCT do escape retention. Upon assembly into the complex, the retention signal may thus be partially covered, and consequently, γ-secretase complexes containing NCT variants with the ER retention motif can escape the ER and undergo maturation. Additional evidence for formation of a high molecular weight γ-secretase complex containing immature NCT was obtained by velocity gradient centrifugation and trypsin digestion. ER-

![Fig. 5. Affinity capture of immature NCT by the biotinylated Merck C inhibitor.](image-url)

The findings described above suggest that γ-secretase assembly occurs within the ER. However, most of these experiments were conducted with ER-retained NCT variants. To obtain further evidence for ER assembly of the γ-secretase complex under native conditions, we investigated the stepwise assembly of the endogenous complex components. First, the dimeric pre-complex consisting of NCT and APH-1 (15, 20, 23) was investigated. To do so, fibroblasts from PS1/2 double knockout mice (42) were investigated for NCT maturation. Cell lysates were immunoprecipitated with the anti-APH-1 antibody, and co-immunoprecipitated proteins were detected by Western blotting. As observed previously (15, 20, 23), immature NCT co-immunoprecipitated with APH-1, whereas no PEN-2 was detected (Fig. 6A). Thus, a dimeric NCT/APH-1 intermediate appears to be assembled and retained within the ER. To investigate the trimeric intermediate of the assembly (15, 27), we used a cell line in which PEN-2 was stably knocked down by RNAi (25). Under these conditions, PS fails to be endoproteolysed, but still associates with APH-1 and NCT (15, 27). Co-immunoprecipitations using the anti-APH-1 antibody revealed that such a trimeric complex could indeed be isolated (Fig. 6B). Moreover, this complex also contained preferentially immature NCT (Fig. 6B), indicating that this intermediate complex was still located within the ER. Furthermore, immature NCT that co-immunoprecipitated with APH-1 or PS1 was trypsin-sensitive (Fig. 6C). However, upon expression of an RNAi-insensitive PEN-2 variant, the complex left the ER. Under these conditions, all four γ-secretase complex components were co-immunoprecipitated (Fig. 6D), and mature NCT was preferentially co-immunoprecipitated (Fig. 6B).

To search for a fully assembled γ-secretase complex containing immature endogenous NCT, we made use of the observation that PEN-2 is required for endoproteolysis of PS holoprotein and for stabilization of PS fragments (25, 46, 47). We (25) and others (46, 47) have previously shown that PEN-2 containing a C-terminal deletion (PEN-2ΔC) associates with the PS holoprotein and triggers its endoproteolysis, but that the resulting PS N- and C-terminal fragments, as well as PEN-2ΔC itself, are unstable and degraded by the proteasome. Thus, PEN-2ΔC inefficiently rescues PEN-2 knockdown (25, 46, 47).
Fig. 6. γ-Secretase complex assembly within the ER. CHAPS lysates were immunoprecipitated (IP) with the anti-APH-1a antibody (434) or preimmune serum (PIS), and the coprecipitated γ-secretase components were analyzed by Western blotting using anti-NCT-CT, anti-APH-1a (434), anti-PS1-NT, anti-PS1 loop, and anti-PEN-2 (1638) antibodies. A, in mouse embryonic fibroblasts (MEF) derived from PS1/2 knockout cells, immature NCT but no PEN-2 co-immunoprecipitated with APH-1a. In control mouse embryonic fibroblasts, mature (NCT\textsubscript{m}) and immature (NCT\textsubscript{im}) NCT, PS1 CTFs, and PEN-2 co-immunoprecipitated with APH-1a. B, in PEN-2 knockdown cells, mainly immature NCT and PS holoprotein (PS\textsubscript{holo}) co-immunoprecipitated with APH-1a (second lane). The rescue of the PEN-2 knockdown cell line with RNAi-stable PEN-2 resulted in NCT maturation and immunoprecipitation of all γ-secretase complex components with the anti-APH-1a antibody (third lane). The γ-secretase complex components were not precipitated with the preimmune serum of the anti-APH-1a antibody (first lane). C, the trimeric γ-secretase complex present in PEN-2 knockdown cells was immunoprecipitated with the anti-APH-1 or anti-PS1 loop antibody and subjected to trypsin digestion. As a control, lysates of swAPP-expressing cells (sw) were immunoprecipitated with the anti-PS1 loop antibody and used for trypsin digestion (lower panel). The immunoprecipitates incubated with (+) and without (−) trypsin were analyzed by Western blotting using the anti-NCT-CT antibody. D, rescue of the PEN-2 knockdown cell line with unstable PEN-2ΔC (ΔC) resulted in no maturation of NCT. After its stabilization with the proteasomal inhibitor MG132, NCT still failed to mature, but immature NCT, PS N (NTF) and C-terminal fragments, and PEN-2ΔC co-immunoprecipitated with APH-1a.

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retained immature NCT co-purified with PS fragments in high molecular weight fractions. Moreover, upon digestion with trypsin, we detected substantial amounts of trypsin-resistant immature NCT in high molecular weight fractions. The appearance of trypsin-resistant NCT is associated with the formation of a complete γ-secretase complex (29, 46). Thus, the identification of trypsin-resistant immature NCT suggests ER assembly of the complete γ-secretase complex. Furthermore, by selective trypsin resistance, we have demonstrated that immature endogenous NCT assembles into a γ-secretase complex under physiological conditions. In addition, the biotinylated Merck C inhibitor, which selectively binds to biologically active γ-secretase (37), also preferentially purified γ-secretase complexes containing immature NCT from cells expressing NCT with the ER retention motif. Finally, we isolated the γ-secretase complex containing all four components, including immature wild-type (endogenous) NCT, upon expression and stabilization of an unstable PEN-2 variant (25, 46, 47). This suggests that even PEN-2, which is the last component to enter the complex, associates with the trimeric intermediate already within the ER, before NCT maturation occurs. An immediate release of fully assembled complexes from the ER may have made this observation so far impossible, and consequently, it was expected that the final complex assembly, refolding, and activation occur in late compartments (54).

Taken together, our data indicate a sequential assembly of the γ-secretase complex within the ER/early secretory compartments. Immediately after full assembly, PS is endoproteolyzed; NCT undergoes the conformational switch; and the complex may be released from the ER and targeted to its sites of biological activity in late secretory/endocytic compartments.
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