

Nicastrin Interacts with γ -Secretase Complex Components via the N-terminal Part of Its Transmembrane Domain*

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Two secretases are involved in the generation of amyloid β -peptide, the principal component of amyloid plaques in the brains of Alzheimer's disease patients. While β -secretase is a classical aspartyl protease, γ -secretase activity is associated with a high molecular weight complex. One of the complex components, which is critically required for γ -secretase activity is nicastrin (NCT). Here we investigate the assembly of NCT into the γ -secretase complex. NCT mutants either lacking the entire cytoplasmic tail, the cytoplasmic tail, and the transmembrane domain (TMD), or containing a set of heterologous TMDs were expressed in cells with strongly reduced levels of endogenous NCT. Maturation of exogenous NCT, γ -secretase complex formation and proteolytic function was then investigated. This revealed that the cytoplasmic tail of NCT is dispensable for γ -secretase complex assembly and function. In contrast, the authentic TMD of NCT is critically required for the interaction with γ -secretase complex components and for formation of an active γ -secretase complex. Neither soluble NCT lacking any membrane anchor nor NCT containing a heterologous TMD were inserted into the γ -secretase complex. We identified the N-terminal region of the NCT TMD as a functionally important entity of NCT. These data thus demonstrate that NCT interacts with other γ -secretase complex components via its TMD.

Secretases are protease activities known to be required for proteolytic processing of the β -amyloid precursor protein (BAPP).¹ BAPP is the precursor for the neurotoxic amyloid β -peptide ($A\beta$), the principal component of amyloid plaques invariably found in all Alzheimer's Disease (AD) brains (1, 2). Apparently the highly insoluble $A\beta$ aggregates immediately after its proteolytic generation by secretases and forms oligomers, which are the main pathological players in the disease

(3). Thus understanding secretase function is not only of great academic interest but will also help to develop therapeutic strategies against AD.

Two secretases are involved in $A\beta$ generation. The first cut is mediated at the Met/Asp bond at amino acid one of the $A\beta$ peptide by β -site APP cleaving enzyme. β -Site APP cleaving enzyme is a membrane-bound protease, which contains the classical active sites of aspartyl proteases (4). In contrast γ -secretase activity is associated with a high molecular weight protein complex (5–8). The first proteins identified within these complexes were the two homologous presenilins (PS1/PS2) (5, 9). PSs were originally found by their genetic linkage to early onset familial AD (10). PS mutations were shown to affect $A\beta$ generation in a pathological manner by increasing the production of the 42 amino acid $A\beta_{42}$, which tends to oligomerize much faster than the regular 40 amino acid $A\beta_{40}$ (7, 8). PSs contain two conserved aspartyl residues, which are critically required for γ -secretase activity and their endoproteolysis (11). PSs must associate with a number of additional proteins to gain proteolytic activity (7, 8). Most likely all functionally essential components have now been identified (12). Besides PS1 and PS2 these include nicastrin (NCT), PEN-2, and APH-1a/APH-1b (12–14). These components are required and sufficient to reconstitute γ -secretase activity in yeast, an organism lacking any endogenous γ -secretase activity (15). Complex formation and generation of a proteolytically active γ -secretase complex is coordinately regulated and removing any one of the four components results in the lack of γ -secretase activity and expression or maturation of the remaining partners (12, 16–22). APH-1 and NCT are apparently required for the stabilization of full-length PS, whereas PEN-2 stimulates endoproteolysis of PS and/or stabilizes the resulting PS fragments (17, 20, 23). The complex glycosylation of NCT indicates transport to distal compartments (18, 22), and components of the γ -secretase complex have been detected at the plasma membrane (19, 24, 25).

Although probably all components of the γ -secretase complex have now been identified, little is known about the individual interactions of components, their assembly into the complex, and trafficking of the complex to its sites of biological activity. To identify domains of NCT required for complex assembly we investigated NCT variants, which either lack the cytoplasmic tail, the complete transmembrane domain (TMD) and the cytoplasmic tail thus generating a soluble NCT variant, or containing a heterologous TMD. We demonstrate that NCT preferentially interacts via the N-terminal half of its TMD with other γ -secretase complex components and does not require the cytoplasmic domain.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Lines—Human embryonic kidney 293 cells (HEK 293) stably expressing swAPP and the NCT knockdown cell line

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¹ The abbreviations used are: BAPP, β -amyloid precursor protein; $A\beta$, amyloid β -peptide; AD, Alzheimer's Disease; PS, presenilin; NCT, nicastrin; TMD, transmembrane domain; HEK, human embryonic kidney; RNAi, RNA interference; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid; AICD, APP intracellular domain; wt, wild type; siRNA, small interfering RNA; CTF, C-terminal fragment.

were described previously (26). The NCT knockdown cell lines were stably transfected with the indicated NCT constructs by using FuGENE (Roche Diagnostics). For NCT expression cells were selected with blasticidin (50 μ g/ml).

cDNA Constructs of NCT—For down-regulation of endogenous NCT the pSUPER/NCT-1045 construct was used (26). All NCT constructs expressed in the NCT knockdown cell line contained a cluster of silent mutations conferring RNA interference (RNAi) resistance (26). All cDNA constructs were generated by PCR and sequenced to verify successful mutagenesis.

Antibodies—The polyclonal antibodies α PS1-loop (3027) (27), α APH-1aL (O2C2) (17), α PEN-2 (1638) (21), α APP-CT (6687) (28), and α A β (3926) (29) were described previously. The polyclonal antibody against the N terminus of NCT (α NCT-NT, 1658) was raised against a MBP-NCT (amino acids 320–420) fusion protein, and the C-terminal NCT antibody was obtained from Sigma (N1660). The monoclonal antibodies against A β (6E10), the V5 tag, and the penta-His tag were obtained from Senetek and Invitrogen, respectively.

Protein Analysis—Cell lysates were prepared using STEN lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA). For co-immunoprecipitations CHAPS lysis buffer was used (2% CHAPS, 20 mM HEPES, pH 7.2, 100 mM KCl, 2 mM EDTA, 2 mM EGTA). Co-immunoprecipitations were carried out as described before (5). For analysis of secreted A β , medium was collected after 5 h of incubation and subjected to a combined immunoprecipitation/immunoblotting protocol, using antibody 3926/6E10 (30).

In Vitro γ -Secretase Activity Assay—The assays were carried out as described previously (15, 31). Briefly, CHAPSO cell lysates (2% CHAPSO, 150 mM sodium citrate, pH 6.4) were immunoprecipitated with α V5 antibody or α PS1-loop antibody (3027), the beads were incubated for 14 h with 0.75 μ g of purified recombinant APP-C100, C-terminally histidine-tagged (C100-His₆) in 20 μ l of assay buffer (150 mM sodium citrate, pH 6.4, 0.25% CHAPSO, 0.1 mg/ml bovine serum albumin, 0.5 mg/ml phosphatidylcholine, 10 mM dithiothreitol, protease inhibitor (Roche Diagnostics, as recommended)). A β and APP intracellular domain (AICD) were detected by Western blot analysis.

RESULTS

To determine whether the cytoplasmic tail or the TMD of NCT plays a critical role for γ -secretase complex assembly and function, we generated the deletion constructs Δ C NCT-V5 (lacking the cytoplasmic domain) and sNCT-V5 (a soluble NCT variant, which lacks the cytoplasmic domain and the TMD). In addition the TMD of wt NCT was also replaced by the TMD of ADAM-10 to create the NCT variant TM_{A10}NCT-V5 (Fig. 1A). These constructs as well as wt NCT-V5 were stably transfected into a HEK 293 cell line stably expressing Swedish mutant APP (swAPP) and a pSUPER-based NCT-1045 small interfering RNA (siRNA)-encoding vector, which stably knocks down endogenous NCT expression by RNAi (16, 26). All constructs are RNAi-insensitive. Consistent with our previous results (26) endogenous NCT expression was dramatically decreased by RNAi (Fig. 1B, lane 2). This is accompanied by reduced PS1, APH-1aL, and PEN-2 expression and a significant increase of the β APP C-terminal fragments (CTFs) followed by reduced A β generation (Fig. 1C, lane 2). In cells expressing wt NCT-V5 (26) the biochemical phenotype of the NCT knockdown was rescued (Fig. 1C). The β APP CTFs were reduced to normal levels and A β production was restored demonstrating normal γ -secretase function (Fig. 1C). In total lysates maturation of wt NCT-V5 was observed (Fig. 1B), which is consistent with the functional restoration of γ -secretase activity (16, 19, 24). NCT maturation was also observed in cells expressing the Δ C NCT-V5 variant, although to a slightly lesser extent than wt NCT (Fig. 1B). Δ C NCT-V5 rescued the loss of γ -secretase function caused by the RNAi mediated knockdown of endogenous NCT. Similar to wt NCT, Δ C NCT-V5 rescued PS1, APH-1aL, and PEN-2 expression, reduced APP CTF levels, and restored A β generation (Fig. 1C) demonstrating efficient γ -secretase complex assembly and function. In contrast, the NCT variants TM_{A10}NCT-V5 and sNCT-V5 entirely failed to mature (Fig. 1B, panel α V5) and to restore expression of PS1 fragments, AHP-1aL and PEN-2

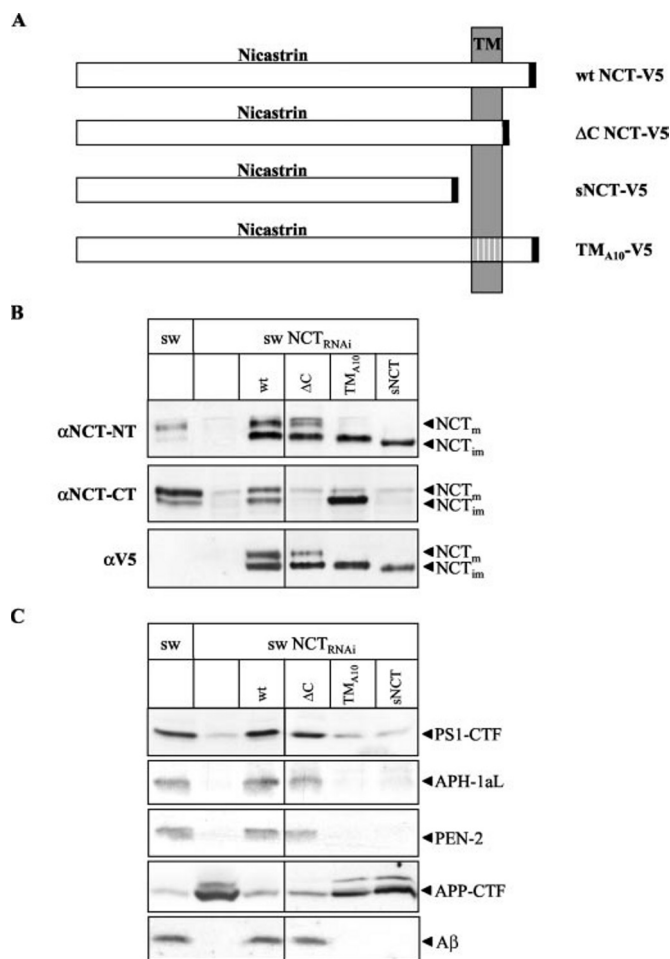


FIG. 1. The TMD but not the cytoplasmic domain of NCT is required for γ -secretase complex formation. A, schematic representation of the wild type construct (wt NCT-V5), the construct with the deleted C terminus (Δ C NCT-V5), the soluble NCT (sNCT-V5) and the construct with the exchanged TMD of NCT (TM_{A10}NCT-V5). The striped box indicates the ADAM-10 TMD and the black box the V5 tag. B, HEK 293 cells expressing swAPP and Nct-1045 siRNA were stably transfected with wt NCT-V5, Δ C NCT-V5, TM_{A10}NCT-V5, or sNCT-V5 cDNA constructs. All constructs harbor a cluster of silent mutations to escape RNAi mediated destruction. Cell lysates were analyzed for NCT expression by Western blot with antibodies α NCT-NT, α NCT-CT, or α V5. Note that low levels of endogenous NCT are detected with antibodies α NCT-NT and α NCT-CT but not with α V5 antibody. C, wt NCT-V5 as well as Δ C NCT-V5 restores the expression of the components of the γ -secretase complex, prevents APP-CTF accumulation, and rescues A β production, whereas TM_{A10}NCT-V5 and sNCT-V5 have no rescuing activity. Cell lysates were analyzed for levels of the PS1-CTFs, APH-1aL, PEN-2, and APP-CTFs by Western blot. A β was analyzed in conditioned media by a combined immunoprecipitation/Western blotting protocol with α A β antibodies 3926/6E10.

(Fig. 1C). Consequently, they did not reduce APP CTF accumulation and failed to restore A β production (Fig. 1C).

Thus the authentic TMD of NCT is apparently necessary for NCT function and γ -secretase complex formation whereas the cytoplasmic tail of NCT is dispensable. To identify a critical region within the NCT TMD required for complex formation, short domains of the NCT TMD were sequentially replaced with that of ADAM-10 (Fig. 2A). Chimeras replacing one-third (TM-NNA) or two-thirds (TM-NAA) of the C-terminal region of the authentic NCT TMD with the corresponding transmembrane sequences of ADAM-10 were able to mature (Fig. 2B). To further prove if the N-terminal region of the NCT TMD is required for maturation of NCT and γ -secretase complex assembly, three additional chimeras (TM-ANA, TM-ANN, TM-AAN; Fig. 2A) were expressed in the NCT knockdown cell line

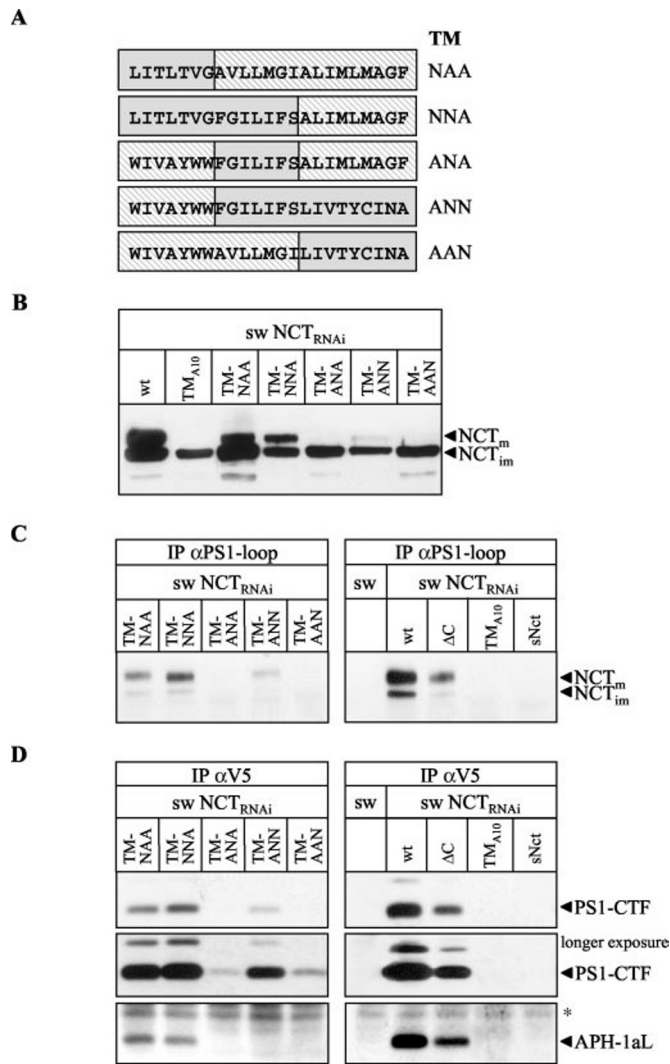


FIG. 2. The N-terminal one-third of the TMD is sufficient for complex incorporation of NCT. *A*, sequence of the NCT (gray box) and ADAM-10 TMD (hatched box) chimeras. *B*, HEK 293 cells expressing swAPP and Nct-1045 siRNA were stably transfected with the indicated cDNA constructs. Cell lysates were analyzed for exogenous NCT expression by Western blot using α V5 antibody. Lysates of wt NCT-V5 or TM_{A10}NCT-V5 expressing cells were loaded as control. Note the difference in the level of maturation of the NCT variants. *C*, CHAPS lysates were analyzed by immunoprecipitation with the α PS1-loop antibody, and co-precipitated NCT was detected by Western blotting with α V5 antibody. *D*, CHAPS lysates were analyzed by immunoprecipitation with α V5 antibody and Western blotting with α PS1-loop, α AHP-1aL antibodies. Asterisks indicate nonspecific bands.

described above. No maturation was observed when the NCT variants TM-ANA or TM-AAN were expressed. In contrast some mature NCT could be detected in cells expressing NCT TM-ANN chimera (Fig. 2*B*). These data suggest that the N-terminal third of the NCT TMD is sufficient for maturation of NCT. However, the middle part of the TM domain also contributes to maturation to some extent, since TM-ANN clearly restored maturation although at low levels. In addition TM-NNA appears to mature to a higher extent than TM-NAA. Although the Nct variant TM-NNA matured most efficiently of all constructs tested it is still unable to mature as efficiently as wt NCT (Fig. 2*B*). To further prove assembly of these NCT variants into a γ -secretase complex co-immunoprecipitation experiments were performed. α PS1-loop antibody co-immunoprecipitated all NCT chimeras that undergo maturation (Fig. 2*C*). These include the NCT variants wt, Δ C, TM-NAA, and TM-NNA. Consistent with the results shown above small

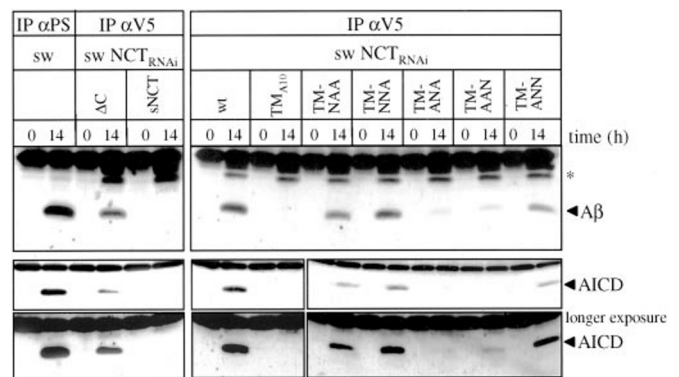


FIG. 3. All NCT constructs, which interact with γ -secretase complex components, are functionally active in the *in vitro* γ -secretase assay. CHAPS cell lysates were prepared and γ -secretase complexes containing exogenous NCT were selectively immunoprecipitated with α V5 antibody. As a control, endogenous γ -secretase was precipitated from CHAPS lysates of HEK cells expressing swAPP, using the α PS1-loop antibody (lanes 1 and 2). The precipitated material was incubated with recombinant, purified APP-C100 and analyzed for A β and AICD generation in Western blot using antibodies 6E10 (upper panel) and α -penta-His (lower panel). Asterisks indicate nonspecific bands.

amounts of the mature NCT variant TM-ANN were also co-immunoprecipitated (Fig. 2*C*). In all these cases the mature species of NCT variants were preferentially co-precipitated (Fig. 2*C*) as expected (16, 18, 19, 24). As discussed above, the NCT variants Δ C, TM-NAA, and TM-NNA showed less co-immunoprecipitation with PS1 as compared with wt NCT (Fig. 2*C*). These data were supported by co-immunoprecipitation experiments using the α V5 antibody to exogenous NCT. The NCT variants wt, Δ C, TM-NAA, TM-NNA, and to a weaker extent TM-ANN co-immunoprecipitated the PS1 C-terminal fragment (Fig. 2*D*). Again, NCT TM-NNA co-immunoprecipitated the PS1 fragment to a lesser extent than wt NCT (Fig. 2*D*). Co-immunoprecipitation of AHP-1aL occurred with wt NCT, Δ C, TM-NAA, and TM-NNA, confirming assembly into a functional γ -secretase complex (see also Fig. 3). Since TM-ANN is the chimera with the weakest PS interaction, the AHP-1aL interaction is apparently below detection limit (Fig. 2*D*). This is supported by our finding that NCT-ANN does exhibit γ -secretase activity in a highly sensitive *in vitro* assay (Fig. 3; see below).

To determine whether the NCT chimeras, which restored γ -secretase-complex formation allow generation of A β and the AICD we performed *in vitro* γ -secretase activity assays (15, 31). To rule out any γ -secretase activity contributed by remaining small amounts of endogenous complexes, CHAPS lysates of the indicated cell lines were immunoprecipitated using the α V5 antibody. All constructs, which allowed NCT maturation and incorporation into the complex (wt, Δ C, TM-NAA, TM-NNA, TM-ANN), efficiently restored A β and AICD generation (Fig. 3). The highly sensitive γ -secretase assay revealed very low levels of activity upon expression of TM-AAN or TM-ANA (Fig. 3). This is consistent with our finding that very minor amounts of co-immunoprecipitating PS1 fragments could be observed (Fig. 2*D*; longer exposure). Thus the N-terminal region of the NCT TMD is sufficient for assembly of NCT into the γ -secretase complex. However, the remaining middle part together with the N-terminal or C-terminal third of the TMD also contributes the NCT incorporation, suggesting structural requirements within the authentic TMD (see Fig. 4 and "Discussion").

DISCUSSION

Assembly of a proteolytically active γ -secretase complex is essential for A β generation (7, 8). Identifying the interacting

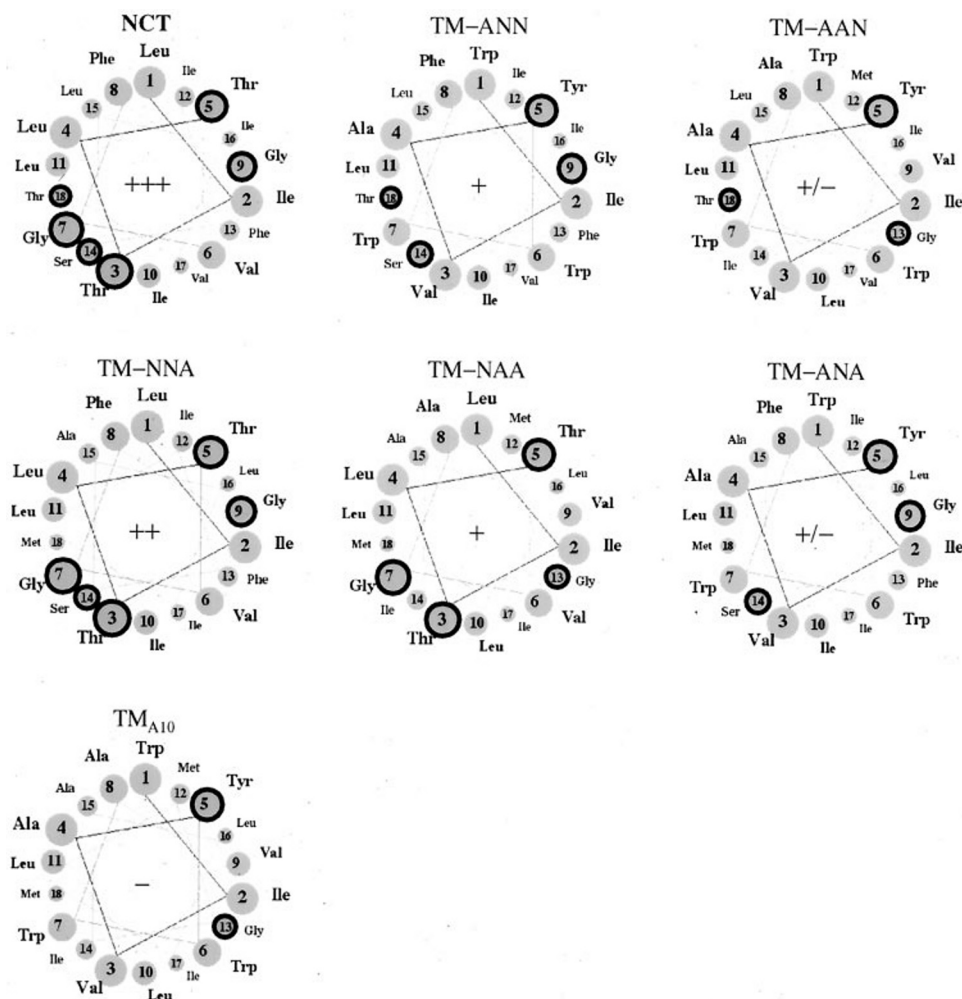


FIG. 4. A helical wheel projection containing the 18 N-terminal amino acids of the TMD of wt NCT and the TMD swap constructs, using the following program: cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html. Polar, uncharged amino acids are marked with bold circles. + and - reflect the ability of the respective fusion protein to reconstitute a functional γ -secretase complex.

domains of the complex components will provide insights into the sequential assembly of the γ -secretase complex and will also signify novel targets for therapeutic intervention. Here we investigate the association of NCT with the γ -secretase complex via a deletion analysis followed by co-immunoprecipitations studies and *in vitro* assays for γ -secretase activity. Based on the finding that down-regulation of PS does not eliminate NCT expression, but rather affects its maturation, causing NCT to remain as a free molecule within the ER, evidence exists that NCT may be the initial scaffold for the assembling process (16, 18, 19, 24). Our findings are consistent with the previous findings suggesting that NCT can only mature and leave the ER upon its incorporation into the γ -secretase complex (16, 18, 19, 24, 25). Assembly of NCT into the γ -secretase complex requires the TMD whereas the cytoplasmic domain is largely dispensable. A sequential deletion analysis then indicated that the N-terminal portion of the NCT TMD is sufficient for this interaction. However, the remaining C-terminal portion of the NCT TM domain also contributes to some extent to γ -secretase complex formation. Therefore the overall structure of the NCT TMD appears to play a critical role for interaction with other complex components. Indeed a helical wheel projection of the wt NCT TMD revealed a hydrophilic patch comprised of Thr-3, Gly-7, Ser-14, and Thr-18 (Fig. 4). Changes within this hydrophilic patch appear to affect the capability of the mutant NCT variants to assemble into the γ -secretase complex. Non-functional TMD mutants such as TM-AAN, TM-

ANA, or TM_{A10} contain only one remaining hydrophilic amino acid or lack this patch entirely (Fig. 4). In contrast TM-NNA largely preserves this hydrophilic domain (Fig. 4), and consequently this construct efficiently assembles into the γ -secretase complex. Similarly, TM-NAA and TM-ANN also preserve the hydrophilic patch to some extent and are assembled into a functional γ -secretase complex. Thus the hydrophilic TMD may be involved in the interaction with other γ -secretase complex components. Our data do not exclude the possibility that additional luminal sequences may be involved in the interaction as well. However, these would not be sufficient by themselves since expression of sNCT did not rescue the phenotype caused by the knockdown of endogenous NCT. While this manuscript was in preparation Morais *et al.* (32) presented evidence by using artificial variants of human and *Caenorhabditis elegans* NCT suggesting that the TMD of these fusion proteins may be required for the assembly of the γ -secretase complex. Our data are consistent with this finding and extend the evidence of the importance of the TMD by functional assays using authentic NCT proteins. Furthermore, our deletion analysis allowed us to identify the N-terminal third of the NCT TMD as an important interacting domain. In addition we performed our investigations in a NCT knockdown background. In our opinion this is the only way to prove or disprove functional activity of a γ -secretase complex component. Overexpression by itself may not be sufficient. In the case of NCT little or no replacement is observed (13), thus still allowing normal γ -secretase function

upon overexpression of non-functional NCT. Therefore, the function of mutant NCT variants can only be conclusively analyzed in a NCT knock-out/down background.

Taken together, our data demonstrate that NCT incorporation into the γ -secretase complex occurs predominantly via the N-terminal third of its TMD. In addition the middle part of the TMD facilitates incorporation and maturation. Thus our data suggest that the authentic TMD in its correct folding is required for fully functionally active NCT. This includes the presence of a hydrophilic patch. The cytoplasmic tail seems to be largely dispensable for function and may primarily be required to assure appropriate membrane anchoring of NCT. Previously a pre-complex of APH-1 and NCT has been reported (17, 23, 32–34). Our data suggest that NCT interacts with APH-1 via its TMD, because expression of TM_{A10} NCT is not able to rescue APH-1 expression in the NCT knockdown cell line. Moreover APH-1 did not co-precipitate with TM_{A10} NCT. Additional work will be required to identify the TMD of APH-1 interacting with NCT. However, multiple interactions of NCT with other γ -secretase complex components can certainly not yet be excluded.

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