Rat Brain γ-Secretase Activity Is Highly Influenced by Detergents†

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ABSTRACT: γ-Secretase is important for the development of Alzheimer's disease, since it is a crucial enzyme for the generation of the pathogenic amyloid β-peptide (Aβ). Most data on γ-secretase is derived from studies in cell lines overexpressing γ-secretase components or amyloid precursor protein (APP), and since γ-secretase is a transmembrane protein complex, detergents have been frequently used to facilitate the studies. However, no extensive comparison of the influence of different detergents at different concentrations on γ-secretase activity in preparations from brain has been made. Here, we establish the optimal conditions for γ-secretase activity in rat brain, using an activity assay detecting endogenous production of the APP intracellular domain, which is generated when γ-secretase cleaves the APP C-terminal fragments. We performed a subcellular fractionation and noted the highest γ-secretase activity in the 100000*g* pellet and that the optimal pH was around 7. We found that γ-secretase was active for at least 16 h at 37 °C and that the endogenous substrate levels were sufficient for activity measurements. The highest activity was obtained in 0.4% CHAPSO, which is slightly below the critical micelle concentration (0.5%) for this detergent, but the complex was not solubilized efficiently at this concentration. On the other hand, 1% CHAPSO solubilized a substantial amount of the γ-secretase components, but the activity was low. The activity was fully restored by diluting the sample to 0.4% CHAPSO. Therefore, using 1% CHAPSO for solubilization and subsequently diluting the sample to 0.4% is an appropriate procedure for obtaining a soluble, highly active γ-secretase from rat brain.

Alzheimer's disease (AD)[1] is the most common form of dementia and affects several millions of people, primarily in the Western World. The characteristics of the disease are memory impairment and other cognitive dysfunctions. The pathology of the diseased brain reveals neurofibrillar tangles as well as amyloid plaques composed of fibrils of polymerized amyloid β-peptide (Aβ). Aβ is generated from the 695−770 amino acid long amyloid precursor protein (APP). APP is cleaved by β-secretase forming 99 or 89 amino acid long carboxy-terminal fragments (CTFs) (*1*) or in a presumably nonpathogenic pathway by α-secretase generating a 83 amino acid long CTF. The membrane-bound CTFs are further cleaved in the middle of the transmembrane region by a transmembrane protein complex called γ-secretase to generate Aβ or the nontoxic p3 peptide (*2*). γ-Secretase consists of at least four transmembrane proteins: presenilin (PS), nicastrin, Aph-1, and Pen-2 (*3*). In mammals, two homologues of presenilin exist: PS1 and PS2. In addition to the Aβ-generating intramembrane cleavage site, γ-secretase cleaves APP at the ϵ-site, located close to the cytosolic border of the membrane (*4*, *5*). This cleavage presumably occurs in all CTFs and results in the release of the APP intracellular domain (AICD) (Figure 1). The physiological role of AICD is not fully understood, but it has been shown to activate transcription through the adaptor proteins Fe65 and Tip60 (*6*). A great number of other substrates are cleaved in a similar manner by γ-secretase (*7*). Notch is the most studied of these, and in this case it is clear that the intracellular domain induces transcription of many genes that are essential during development (*8*).

During the last years the knowledge about the mechanisms of γ-secretase has increased substantially. Most of the experimental data, however, come from cell lines overexpressing γ-secretase components or APP and do not truly reflect the situation in the brain. Since γ-secretase is a transmembrane protein complex, detergents are used for its purification. Hence, it would be of importance to use brain material and to study which detergent best preserves γ-secretase activity. Some studies have shown Tween-20, Tween-80, or Brij-35 to be the optimal detergent (*4*, *9*) whereas others found CHAPSO to be the optimal detergent (*10*, *11*) for γ-secretase activity. However, most of these studies used artificial systems with an exogenous substrate or overexpressed APP, and none of them include an extensive

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[1] Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; AICD, APP intracellular domain; APP, amyloid precursor protein; CMC, critical micelle concentration; CTFs, carboxy-terminal fragments; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; PS, presenilin; TFE, trifluoroethanol.

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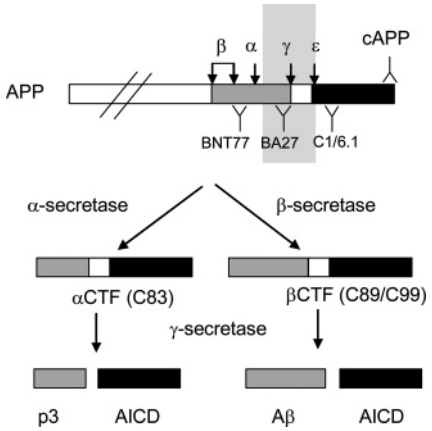


FIGURE 1: Processing of APP. APP is processed by either α- or *β*-secretase followed by the subsequent cleavage by *γ*-secretase to produce p3 or A*β*, respectively. Both pathways result in the release of AICD. The epitopes of the antibodies C1/6.1 and cAPP, which were used for detecting AICD and the BNT77 and BA27, which were used for the A*β* ELISA are denoted in the figure.

investigation of the effect of detergent concentration on endogenous APP processing.

Here, we report a detailed study on the *γ*-secretase cleavage of endogenous APP CTFs in rat brain, determining the optimal conditions for membrane preparation, incubation time, and pH as well as the effects of a range of different detergents at different concentrations on *γ*-secretase activity.

EXPERIMENTAL PROCEDURES

Materials. The detergents 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), polyoxyethylene lauryl ether **(**Brij-35), polyethylene glycol sorbitan monolaureate (Tween-20), and polyethylene glycol *tert*-octylphenyl ether (Triton X-100) were purchased from Sigma-Aldrich (St. Louis, MO). 3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) and *n*-dodecyl *β*-D-maltoside (DDM) were purchased from Calbiochem (Darmstadt, Germany). Lubrol was purchased from MP Biomedicals (Irvine, CA) and PreserveX QML polymeric micelles (PreserveX) was purchased from QBI Life Sciences (Madison, WI). APP CTF-50 peptide (Calbiochem) was reconstituted in MilliQ water (1 *μ*g/*μ*L), aliquoted, and stored at −20 °C before use. The *γ*-secretase inhibitor L-685,458 from Bachem (Bubendorf, Switzerland) was diluted from stock solutions in DMSO (10 mM) and added to the reactions (final concentration of 1 *μ*M). The FLAG-tagged APP-based substrate C99-FLAG was provided by Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan). The following antibodies were used for immunoblotting: C1/6.1 (a gift from Dr. Paul M. Mathews, Nathan Kline Institute) and cAPP (Chemicon, Temecula, CA) raised against the C-terminal end of APP, nicastrin (N1660, Sigma-Aldrich) raised against C-terminal residues 693−709 of human nicastrin, PS1-CTF (MAB5232, Chemicon) recognizing the C-terminal loop region of PS1, UD1 raised against residues 1−11 of Pen-2 (a gift from Dr. Jan Näslund, Karolinska Institutet), the mitochondrial marker Hsp 60, the endosomal marker syntaxin 13, and the endoplasmic reticulum (ER) marker calnexin (Stressgen Biotechnologies, Ann Arbor, MI), the Golgi marker GM130 and the plasma membrane marker N-cadherin (BD Biosciences, San Jose, CA), and the synaptic marker synaptophysin (Chemicon).

Preparation of Membrane Fractions from Rat Brain. Male Sprague-Dawley rats were obtained from B&K Universal (Sollentuna, Sweden). Ethical approval was received from the Animal Trial Committee of Southern Stockholm (No. S60-05). The rats were killed by carbon dioxide and decapitated. The brains were dissected to remove blood vessels and white matter and homogenized in 1 mL/0.2 g of buffer A (20 mM Hepes, pH 7.5, 50 mM KCl, 2 mM EGTA) containing Complete protease inhibitor mixture (PI) (Roche, Basel, Switzerland) with 25 strokes at 1500 rpm using a mechanical pestle−homogenizer (IKALaborteknik RW20). The brain homogenates were centrifuged at 1000*g* for 10 min (P1) to remove nuclei and cell debris. The postnuclear supernatant was further fractionated by sequential centrifugation at 10000*g* for 30 min (P2) and 100000*g* for 1 h (P3). The collected membrane fractions (P1, P2, and P3) were resuspended in buffer A supplemented with 20% glycerol, aliquoted, flash-frozen in liquid N_2, and stored at −80 °C until use. All centrifugation steps were carried out at 4 °C.

γ-Secretase Activity Assay. The membrane fractions were incubated in buffer S (20 mM Hepes, pH 7.0, 150 mM KCl, 2 mM EDTA, 2 mM EGTA, and PI) or buffer H (20 mM Hepes, pH 7.0, 150 mM NaCl, 5 mM EDTA, and PI) for samples including detergents, with or without detergent at 37 °C for the indicated time. The protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). In the experiments including detergents the protein concentration was kept at 1 mg/mL for solubilization. The reactions were stopped by cooling the samples on ice, and in most cases the samples were centrifuged for 1 h at 100000*g* to remove membranes. AICD in the supernatant (or the total sample for experiments including detergents) and APP CTFs in the membrane pellet were analyzed by Western blotting. For diluted samples (Figure 8), the total samples were subjected to PAGEprep advance kit (Pierce) according to the manufacturer's protocol before Western blotting in order to concentrate the sample and improve chromatography. To perform the pH optimum analysis, buffer S was adjusted to various pH levels by the addition of KOH or HCl. For the detergent experiments, the membranes were solubilized in the given detergent for 1 h on ice with occasional mixing and further incubated at 37 °C overnight together with the detergent at the given concentration (weight/volume) without prior separation of insoluble and soluble material. For the soluble-insoluble activity experiments, the membranes were solubilized in different concentrations of CHAPSO for 1 h on ice, soluble and insoluble material were separated by centrifugation at 100000*g* for 1 h, and the resultant supernatants and pellets were adjusted to 0.4% CHAPSO and further incubated at 37 °C for 16 h. For experiments including C99-FLAG, recombinant C99-FLAG prepared by Dr. Takeshi Nishimura, Dainippon Sumitomo Pharma, was diluted in trifluoroethanol (TFE) to a concentration of 20 ng/*μ*L, and 10 ng was added to each sample.

For turbidity measurement, 1 mg/mL P3 fraction was solubilized in buffer H containing the given detergent at the given concentration for 1 h on ice. Following solubilization the samples were transferred to a 96-well plate, and the turbidity was measured at 375 nm in a Safire^2 microplate reader (TECAN, Salzburg, Austria). Buffer with the same detergent composition without membranes served as a blank.

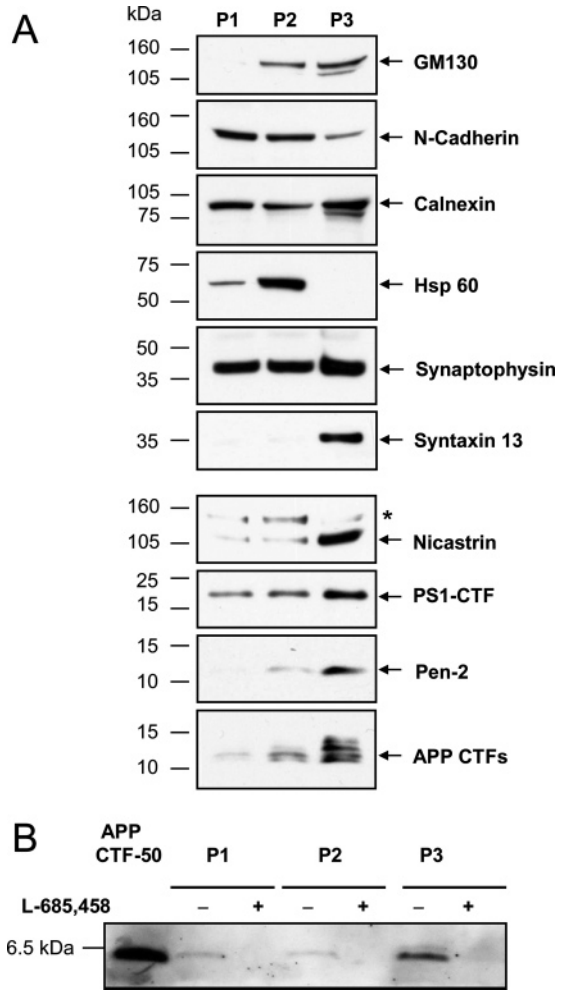


FIGURE 2: γ-Secretase components and activity are enriched in the microsomal fraction. (A) Rat brains were homogenized and centrifuged at 1000*g* for 10 min (P1). The supernatant was removed and centrifuged sequentially at 10000*g* for 30 min (P2) and at 100000*g* for 1 h (P3). Equal amounts of protein (20 μg) from the three pellets, P1, P2, and P3, were separated by SDS−PAGE. The distribution of organelles and γ-secretase components was analyzed using antibodies directed to the markers indicated on the right side of the panel: GM130, Golgi; N-cadherin, plasma membrane; calnexin, ER; HSP 60, mitochondria; synaptophysin, synaptic vesicles; syntaxin 13, endosomes. The mature form of nicastrin is observed above the 105 kDa marker. An unspecific band recognized by the nicastrin antibody is denoted with an asterisk in the figure. (B) Equal amounts of protein from P1, P2, and P3 were incubated at 37 °C for 16 h in the absence or presence of the γ-secretase inhibitor L-685,458. The samples were separated by SDS−PAGE, and AICD production was detected by Western blotting using C1/6.1, an antibody directed to the C-terminal of APP. The AICD fragment is comigrating with a 50 residue commercial peptide corresponding to the C-terminal of APP (APP CTF-50, 100 pg loaded).

SDS−PAGE and Western Blotting. Samples were heated at 95 °C for 5 min in Laemmli sample buffer (Sigma-Aldrich) and subsequently separated by SDS−PAGE on 10−20% or 16% Tricine gels (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose or PVDF membranes (Bio-Rad Laboratories, Hercules, CA) and probed with the indicated primary antibodies followed by incubation with horseradish peroxidase-coupled secondary antibody (Amersham Biosciences, San Francisco, CA). For visualization Supersignal West Pico enhanced chemiluminescence reagents (Pierce) and Hyperfilm ECL (Amersham Biosciences) were used. The signals were quantified using a FluorX-Max CCD camera (Bio-Rad Laboratories).

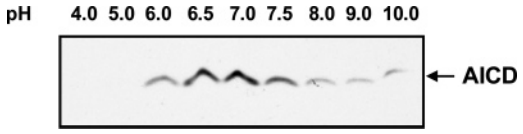


FIGURE 3: γ-Secretase activity is dependent on pH. The P3 pellet was incubated in buffer S, and AICD production was measured as described in Figure 1B. The blot is a representative of three independent experiments.

ELISA. De novo generation of Aβ40 was analyzed by a commercial sandwich enzyme-linked immunosorbent assay (ELISA) (Wako Chemicals, Osaka, Japan) according to the manufacturer's instructions. Briefly, the 100000*g* pellet was incubated for 16 h at 37 °C, and the reaction was stopped by adding RIPA (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) and boiling for 5 min. The samples were centrifuged, and the supernatants were dispensed into the wells (100 μg of microsomal protein/well) coated with BNT77 antibody directed against amino acids 11−28 of Aβ and incubated at 4 °C overnight. The bound Aβ40 was detected by TMB reaction using HRP-conjugated BA27 antibody directed against the C-terminus of Aβ. All of the measurements were performed in triplicate, and Aβ40 levels were calculated from the rodent synthetic Aβ(1−40) standard curve.

RESULTS

Generation of Endogenous AICD from Rat Brain Membranes. We studied the optimal conditions for γ-secretase activity by measuring production of endogenous AICD by purified membranes from rat brain. The rat brain was homogenized and fractionated by three sequential centrifugation steps, 1000*g*, 10000*g*, and 100000*g*, generating the corresponding pellets P1, P2, and P3. The P3 pellet was enriched in endosomes, ER, Golgi, and synaptic vesicles as well as in the γ-secretase components nicastrin, presenilin-1, and Pen-2 and the γ-secretase substrate APP CTFs (Figure 2A). Equal amounts of protein from each fraction were incubated for 16 h at 37 °C and centrifuged at 100000*g* for 1 h. γ-Secretase activity was assayed by measuring the formation of AICD by Western blotting analysis using the antibody C1/6.1, directed to the 20 most N-terminal amino acids of the AICD region of APP (*12*; Figure 1). A 6 kDa fragment comigrating with a 50 residue synthetic peptide corresponding to the C-terminus of APP was detected in all fractions, and the formation could be inhibited by the γ-secretase inhibitor L-685,458 (Figure 2B). The 6 kDa band was also detected by the cAPP antibody directed against the nine most C-terminal residues of APP, as well as by several other antibodies directed against the AICD region of APP. Therefore, we concluded that this band was indeed AICD. In concordance with the enrichment of γ-secretase components the highest relative activity (AICD/μg of protein) was observed in the P3 fraction (Figure 2B), and this fraction was used for further studies.

Second, we studied the pH dependence of γ-secretase activity and found that it was active from pH 6 to pH 10 with an optimum pH around 7 (Figure 3), which is in agreement with earlier studies (*9*−*11*).

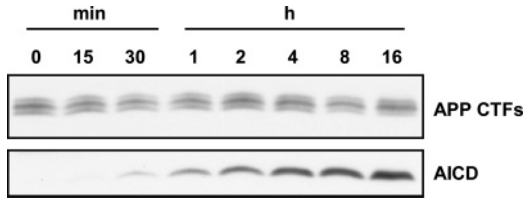
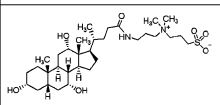
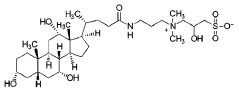
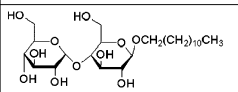
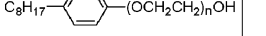
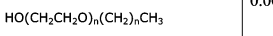
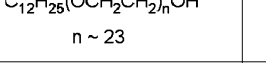
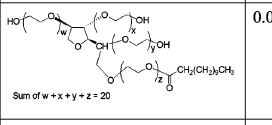


FIGURE 4: γ-Secretase is active for at least 16 h. The P3 fraction was incubated for 0, 15, and 30 min and 1, 2, 4, 8, or 16 h. The samples were centrifuged, and the pellets and supernatants were loaded onto 16% Tricine gels. Antibody C1/6.1 was used for Western blotting.

We next studied the effect of time on the generation of AICD by incubating the P3 fraction for 0, 15, and 30 min and 1, 2, 4, 8, or 16 h. We observed a time-dependent formation of the AICD fragment, and an incubation time of 16 h generated the highest AICD levels (Figure 4). The endogenous substrate levels were sufficient for generation of detectable amounts of AICD, and the APP CTFs were present in the preparation even after 16 h. After 16 h of incubation, one sample was centrifuged, the supernatant containing AICD was removed, and the pellet was further incubated. γ-Secretase was still active in this sample, indicating that it is relatively stable despite its complexity (data not shown). In cells, studies of AICD are confined by its rapid degradation by soluble proteases (*13*). Therefore, we also investigated the stability of AICD in our system. A sample was incubated for 4 h, and thereafter γ-secretase activity was inhibited by the addition of L-685,458. The incubation proceeded for 0, 1, 2, or 16 h. No significant difference in the AICD signal was observed (data not shown), indicating that AICD is stable in our system. On the basis of these findings, the subsequent incubations were performed at pH 7.0 and for a time period of 16 h.

Detergents Affect Endogenous γ-Secretase Activity. Once we had established the optimal fraction and pH for the assay, we investigated the effect of a range of detergents on γ-secretase activity. The P3 fraction from rat brain was incubated in buffer H containing protease inhibitors and 0.25% or 1% (w/v) of the following detergents: CHAPS, CHAPSO, DDM, Triton X-100, Lubrol, Brij-35, Tween-20, or the relatively new detergent PreserveX, consisting of a mixture of polymeric micelles (*14*) (Table 1). At a concentration of 1%, PreserveX retained all activity whereas all other detergents had a major negative effect on γ-secretase activity as compared to control without detergent (Figure 5A). Tween-20 and Brij-35 had the least negative effect of the other detergents. The activity was barely detectable in 1% CHAPSO or CHAPS and completely abolished in 1% Triton X-100 (data not shown), DDM, or Lubrol. At 0.25%, PreserveX, CHAPS, and Brij-35 had no significant effect on γ-secretase activity, and CHAPSO even increased the activity at this concentration (Figure 5A). We also studied the ability of the detergents CHAPSO, PreserveX, Brij-35, Tween-20, and Triton X-100 to solubilize the γ-secretase components and membranes. After solubilization the samples were centrifuged at 100000*g* for 1 h, and the proteins in the supernatant were defined (here and in the rest of the study) as soluble whereas the proteins in the pellet were defined as insoluble. The supernatants and pellets were assayed for the γ-secretase components PS1 and nicastrin using Western blotting. We found that PreserveX, Tween-20, and Brij-35 were unable to solubilize these components even at a concentration of 1% whereas Triton X-100 solubilized essentially all of the proteins both at 0.25% and 1%. CHAPSO did not solubilize the components at all at 0.25% but solubilized around 50% of PS1 and nicastrin at a concentration of 1% (Figure 5B). The ability to solubilize the γ-secretase components correlated with a decrease in turbidity (Figure 5C).

Table 1: Properties of the Detergents Used in This Study

Detergent	Type	Structure	CMC
CHAPS	Zwitterionic Steroid skeleton		0.4%
CHAPSO	Zwitterionic Steroid skeleton		0.5%
DDM	Non-ionic disaccharide Aliphatic tail		0.005-0.03%
Triton X-100	Non-ionic ether Aromatic and aliphatic tail		0.013-0.015%
Lubrol	Non-ionic ether Aliphatic tail		0.006%
Brij-35	Non-ionic ether Aliphatic tail		0.01%
Tween-20	Non-ionic glycol ether Aliphatic tail		0.007%
PreserveX	Mixture of polymeric amphiphiles		0.0003%

Since γ-secretase showed the highest activity in 0.25% CHAPSO, we focused on this detergent for the following studies. We determined the optimal CHAPSO concentration by incubating the P3 fraction with 0%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, or 1% CHAPSO. The optimal concentration of CHAPSO was found to be 0.4%, generating approximately 4 times more AICD compared to the amount generated in absence of detergent (Figure 6A). Above 0.4% the activity declined and was at 1% substantially lower than in the absence of detergent. The decline in activity starts close to the critical micelle concentration (CMC) for CHAPSO (0.5%). The steepest decrease in turbidity occurred between 0% and 0.4% CHAPSO (Figure 6B), and thus, at the CMC most of the membranes are probably dissociated into smaller entities.

Aβ Is Produced at the Optimal Conditions Established for AICD Production. Although AICD production is a good measurement of γ-secretase activity, it is also important to study the generation of Aβ, which has a pathological role in Alzheimer's disease. Thus we studied whether Aβ is also produced during the optimal conditions established for AICD production. The 100000*g* pellet was incubated for 16 h in buffer H, pH 7.0, and 0.4% CHAPSO and subjected to a sandwich ELISA. The BNT77 antibody, directed against amino acids 11−28 of Aβ, was used for coating, and the BA27 antibody, directed against the C-terminus of Aβ40,

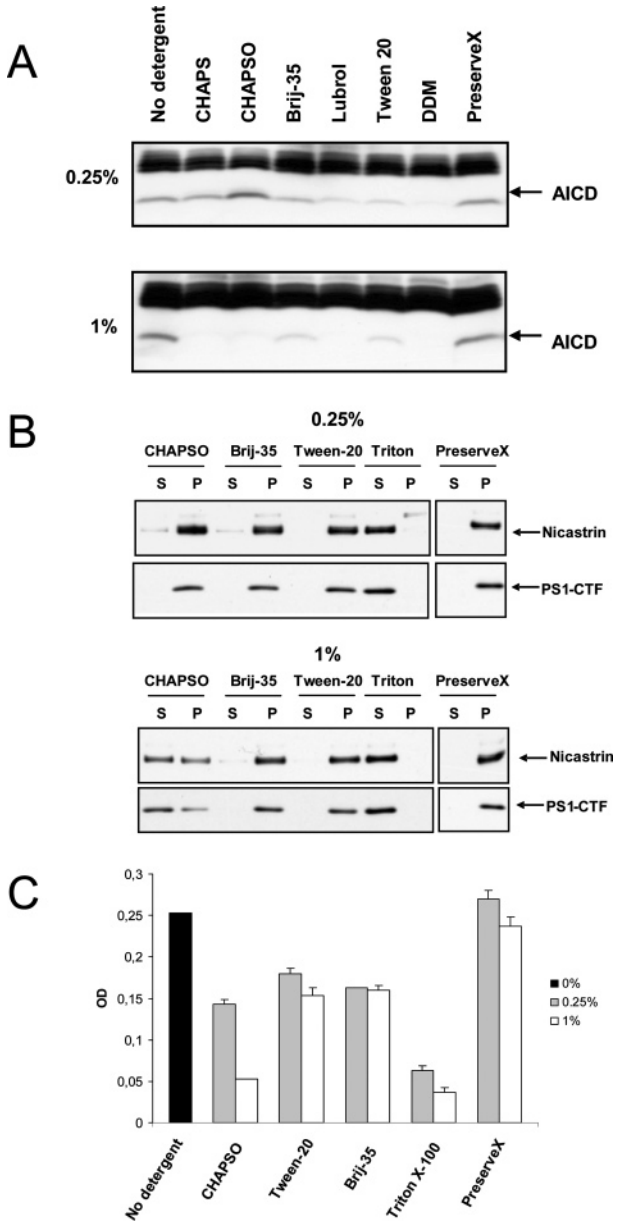


FIGURE 5: Effect of detergents on γ-secretase activity and solubility. (A) For activity measurements the P3 fraction was incubated in the presence of 0.25% (w/v) or 1% (w/v) of the indicated detergents, and AICD production was measured. (B) The ability of PreserveX, CHAPSO, Brij-35, Tween-20, and Triton X-100 to solubilize the γ-secretase complex was studied by treating the samples with 0.25% or 1% of the detergents for 1 h on ice. The samples were centrifuged at 100000*g* for 1 h, and the supernatants (S) and pellets (P) were subjected to SDS−PAGE/Western blot analysis of the γ-secretase components presenilin 1 C-terminal fragment (PS1-CTF) and nicastrin. (C) The turbidity was measured at 375 nm after incubation of the samples in the indicated detergents for 1 h. Note that the ability to solubilize the components (B) correlates with a decrease in turbidity.

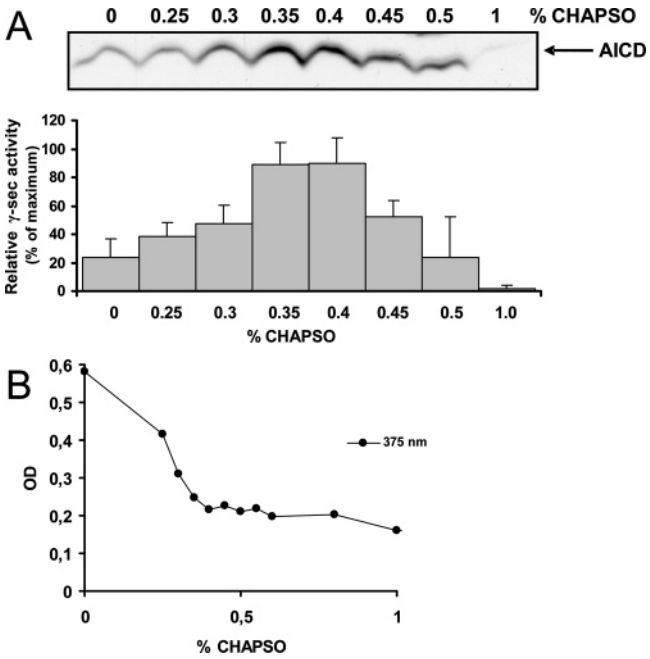


FIGURE 6: Determination of the optimal CHAPSO concentration for γ-secretase activity. (A) The P3 fraction was incubated with various concentrations of CHAPSO, and the AICD production was measured as in Figure 1B. The upper panel is a representative of three independent experiments. The values, obtained from quantification of Western blots, shown in the diagram are mean values ± SD of the three independent experiments, where the maximum signal is set to 100%. (B) The P3 fraction was solubilized in various concentrations of CHAPSO for 1 h, and the turbidity was measured at 375 nm.

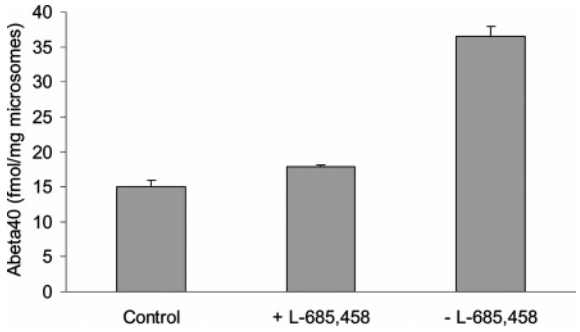


FIGURE 7: Production of Aβ40. Using the optimal conditions established for AICD production, we detected Aβ40 formation using ELISA. The samples were incubated at 37 °C before the ELISA. The production was inhibited by the addition of the γ-secretase inhibitor L-685,458. The control sample was not incubated.

was used for detection. We found that Aβ40 was indeed produced under these conditions (Figure 7).

γ-Secretase Is Soluble at CHAPSO Concentrations above the CMC. Many studies on γ-secretase have been performed in a soluble state (*11*), for example, in attempts to identify novel γ-secretase components (*15*). We therefore studied whether the activity we observed in our samples containing different concentrations of CHAPSO originated from the soluble or the insoluble fraction. Membranes at a concentration of 1 mg of protein/mL were solubilized in buffer H with 0%, 0.4%, 0.6%, or 1% CHAPSO at 4 °C for 1 h. The sample was then centrifuged at 100000*g*, and the supernatants and pellets were adjusted to 0.4% CHAPSO. The γ-secretase assay was then performed as above. The pellets and supernatants were also assayed for γ-secretase components. No soluble activity was found in the samples without CHAPSO. At CHAPSO concentrations above the CMC, most of the activity was found in the supernatant in contrast to the γ-secretase components, which were only soluble to around 50% (Figure 8A,B). Interestingly, when the supernatant of the sample solubilized in 1% CHAPSO was diluted to 0.4%, the activity was about as high as when the pellet of the sample without CHAPSO was incubated in 0.4% CHAPSO, although some variation between experiments was

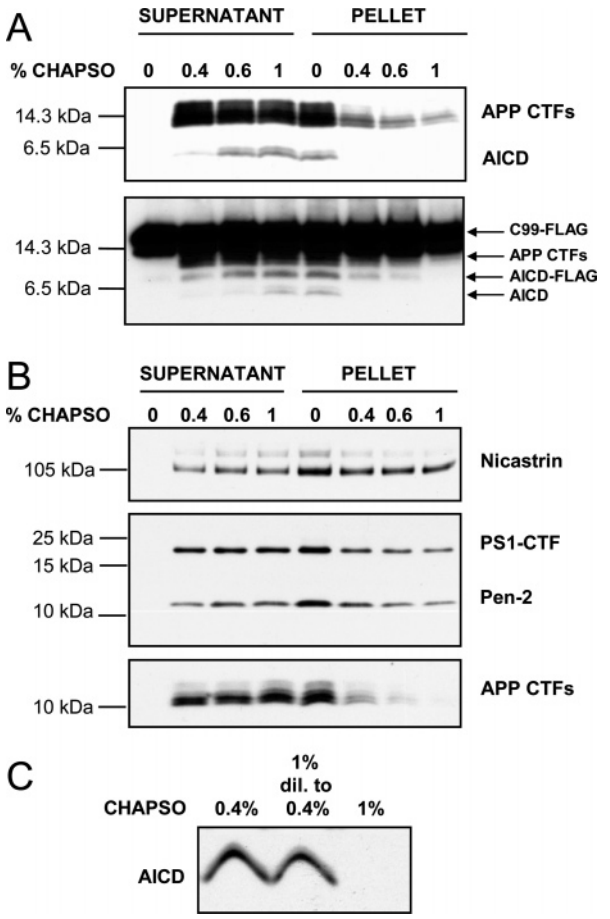


FIGURE 8: Ability of CHAPSO to solubilize γ-secretase activity and components. The P3 fraction was solubilized in buffer H containing 0%, 0.4%, 0.6%, and 1% CHAPSO on ice for 1 h. The samples were centrifuged for 1 h at 100000*g*. (A) The CHAPSO concentration of the supernatants and pellets was adjusted to 0.4% and incubated at 37 °C for 16 h. In the lower panel, C99-FLAG was added to the sample. Endogenous AICD formation could be detected in the supernatants of the CHAPSO-treated samples and in the pellet of the sample without detergent (upper panel). Activity could also be observed in the 0.4% pellet after longer exposure (not shown). (B) The supernatants and pellets were separated and analyzed by SDS−PAGE and Western blotting for the γ-secretase components and APP CTFs. (C) The P3 fraction was solubilized in either 0.4% or 1% CHAPSO for 1 h. One of the samples solubilized in 1% was then diluted to 0.4% CHAPSO. All samples were incubated directly without separation into soluble and insoluble fractions.

observed (Figure 8A). Thus, the activity can be restored by diluting the sample to a lower CHAPSO concentration. Indeed, comparison of a sample dissolved in 1% and diluted to 0.4% CHAPSO and a sample solubilized in 0.4% CHAPSO (the final sample volume and protein concentration were the same in both samples) showed that essentially all of the activity was restored (Figure 8C). At 0.4% CHAPSO, the soluble activity was substantially less than in 1%, and we also detected activity in the insoluble pellet at longer exposure times (Figure 8A).

The substrates, i.e., the APP CTFs, were found mainly in the supernatants at all CHAPSO concentrations (Figure 8B), and the low activity in the pellet could thus be due to lack of substrates. Therefore, we also studied the γ-secretase activity in the presence of the exogenous substrate C99-FLAG. This is a commonly used exogenous substrate and frequently used at very high concentrations (up to 1 μg per sample). The use of TFE for dissolving C99-FLAG enabled us to detect AICD production from this substrate at the concentration of 10 ng per sample. However, this concentration is still significantly higher than the endogenous substrate levels required for production of equal amounts of AICD (Figure 8A, lower panel; compare the AICD/APP CTF ratio with the AICD-FLAG/C99-FLAG ratio). We detected formation of AICD-FLAG in the 0.4%, 0.6%, and 1% CHAPSO supernatants as well as in the 0%, 0.4%, and 0.6% pellets (Figure 8A, lower panel). The formation of AICD-FLAG was inhibited by the γ-secretase inhibitor L-685,458 (data not shown). Thus, some of the active γ-secretase is unsoluble in 0.4% and 0.6% CHAPSO while essentially all of the activity was soluble in 1% CHAPSO.

The distribution of the components, substrates, and activity was highly dependent on the protein content, and the ratio between soluble and insoluble γ-secretase was decreased when higher protein concentrations than 1 mg/mL were used (data not shown). The activity was also protein concentration dependent. When 40 μg of membrane proteins was dissolved and incubated in 0.4% CHAPSO, the activity per microgram of protein was substantially lower at 0.25 mg of protein/mL than at 1 mg of protein/mL (data not shown).

DISCUSSION

Most of what we know today about γ-secretase comes from studies in transfected cell lines. These studies need to be complemented with data from studies using brain material. Since long post-mortem times have a negative effect on γ-secretase activity (Hur et al., unpublished results), we used rat brain instead of human brain in our studies. We determined the optimal subcellular fraction, pH, and incubation time for rat brain γ-secretase activity. In addition, we made an extensive study of the effect of different detergents on γ-secretase, and we will below discuss these effects in relation to the properties of the detergents.

To obtain a cellular fraction enriched in γ-secretase activity, we first performed a subcellular fractionation, centrifuging the brain homogenate at 1000*g*, 10000*g*, and 100000*g*. The highest relative γ-secretase activity was found in the 100000*g* pellet (P3), enriched in endosomes, Golgi, the ER, and synaptic vesicles. We concluded that this fraction was a useful source of active γ-secretase, in agreement with Gu et al. (*4*). On the other hand, Pinnix et al. (*10*) showed a higher activity in the 10000*g* pellet. Differences in buffer composition, homogenization procedures, and centrifugation times as well as species may partly account for this difference in results. However, we obtained the highest activity in the 100000*g* pellet also when using a different protocol including 0.32 M sucrose in the buffer instead of 50 mM KCl (data not shown).

The AICD production was time dependent, and the γ-secretase was still active after 16 h of incubation at 37 °C. AICD was not degraded during these 16 h (data not shown), which is an advantage of this in vitro assay compared to studies in cells where AICD is rapidly degraded (*13*). Interestingly, the APP CTFs did not decline significantly during the incubation period. Earlier studies have shown that the size of the extracellular domain is the determinant of γ-secretase cleavage (*16*), and thus γ-secretase should be able to cleave the substrate as soon as ectodomain

shedding has occurred. Our results, however, indicate either that the amount of γ-secretase is a limiting factor or that γ-secretase processing of APP is regulated. The latter could be accomplished either by separation of the complex and its substrate in space, by a negative regulator, by competition from other substrates, or by a combination of these. Another possibility is that the lipid environment could be of importance for activity; see below.

γ-Secretase is a transmembrane protein complex, and thus, detergents are required in order to solubilize the complex. Therefore, we decided to determine the influence of different detergents at different concentrations on the γ-secretase processing of endogenous APP CTFs in membranes from rat brain. We first tested a range of detergents at the concentrations 0.25% or 1% (w/v). At 1%, all detergents except PreserveX had a negative influence on γ-secretase activity. The low activity could be due to conformational changes or dissociation of the complex, dissociation of the substrate from the complex, changes in lipid environment, or a combination of these. 1% Tween-20 or Brij-35 showed a small reduction in activity, in accordance with Gu et al. (*4*). In contrast, McLendon et al. (*9*) showed an almost 5-fold increase in 1% Brij-35 using CHO cells overexpressing mutant APP. These are weak detergents, and most proteins and lipids have been shown to be resistant to treatment with 1% Tween-20 (*17*). Thus, the milieu for the γ-secretase complex is probably only slightly altered at this concentration. In line with this notion, there were no changes in turbidity in 1% PreserveX, Tween-20, or Brij-35, and all of the γ-secretase components PS1, nicastrin, and Pen-2 were found in the pellet. Although γ-secretase is very active at 1% PreserveX, Tween-20, or Brij-35, these detergents are thus not suitable for solubilizing the complex. Triton X-100 at 1% solubilized all of the γ-secretase components, but the activity was completely abolished. In 1% CHAPSO, on the other hand, a low amount of AICD could be detected, and around 50% of the components were solubilized.

At 0.25%, CHAPS and Brij-35 showed similar activity as samples without detergent while CHAPSO had a positive effect on the activity. We went on to study the optimal CHAPSO concentration and found this to be 0.4%, which is just below the CMC (0.5%). The positive effect on γ-secretase activity could be due to the ability of CHAPSO (and CHAPS) to form mixed micelles with membrane lipids below the CMC (*18*). It is plausible that γ-secretase is dependent on the lipid environment for its activity. Indeed, cholesterol and sphingolipids have a positive effect on γ-secretase activity (*19*, *20*), and γ-secretase has been found in detergent-resistant membranes rich in these lipids (*19*, *21*). CHAPSO has been found to be especially good at preserving interactions between cholesterol, sphingolipids, and proteins, while other lipids are solubilized (*22*), thereby maintaining a suitable environment for γ-secretase. CHAPSO and CHAPS could also have a positive influence on the activity per se since their structures resemble cholesterol. Alternatively, the positive effect of CHAPSO could be due to a better availability of substrate by increasing the diffusion of the complex and its substrate.

We also determined whether the γ-secretase activity was highest in the soluble state or in the insoluble membranes by centrifuging the sample at 100000*g* after treatment with CHAPSO and incubating the resulting supernatants and pellets in 0.4% CHAPSO. Below the CMC, the soluble activity was low and some activity could be detected in the insoluble pellet, while the activity was found in the supernatant at concentrations above the CMC. Since the γ-components to a large extent (around 50%) could be found in the pellet even at 1% CHAPSO, we hypothesized that the low activity in the pellet could be due to low substrate levels. To test this hypothesis, we added exogenous substrate in the form of C99-FLAG at a concentration of 10 ng per sample. Indeed, activity was observed in the pellets from samples in 0.4% and 0.6% CHAPSO, showing that active γ-secretase is partly insoluble at these CHAPSO concentrations. However, C99-FLAG appears to be a poor substrate since the amount needed for detection of AICD is clearly above the endogenous levels of APP CTFs. Kume et al. (*23*) have shown that C83 is a better substrate for γ-secretase than C99, and it is possible that the AICD we observe after cleavage of the endogenous substrates derives mainly from C83. Another possible explanation could be that only a fraction of the added substrate is correctly inserted into, and processed by, the γ-secretase complex. We noted that treatment of C99-FLAG with TFE had a positive effect on the production of AICD, possibly by enhancing the native α-helical conformation of the transmembrane region and reducing aggregation. In summary, our results indicate that the processing of endogenous substrate better reflects the in vivo activity of γ-secretase.

Not only the detergent concentration but also the protein concentration is of importance. At protein concentrations higher than 1 mg/mL, the relative amount of soluble active γ-secretase in 1% CHAPSO decreased, suggesting that a detergent:protein ratio of at least 10:1 should be used for efficient solubilization. For optimal *activity*, however, a 4:1 ratio seems optimal (1 mg of protein/mL in 0.4% CHAPSO; see above). Interestingly, all of the activity in a sample solubilized in 1% CHAPSO could be restored if the sample was later diluted to 0.4% CHAPSO. It is possible to co-immunoprecipitate the γ-secretase complex in 1% CHAPSO (*24*), indicating that the components are associated, and more subtle conformation changes and/or removal of the optimal lipid environment could be the explanation to the decreased activity in 1% CHAPSO. Too high protein concentrations are thus detrimental for efficient solubilization of the complex, but on the other hand, the γ-secretase activity per microgram of protein was lower at protein concentrations below 1 mg/mL. A possible explanation for this is that the complex and the substrate are present in different entities. Hence, 1 mg of protein/mL seems to be a good compromise for obtaining soluble active γ-secretase.

In many cases, it is of interest to measure not only AICD but also Aβ production. Many APP CTFs (for instance, C83) does not contain the full Aβ sequence, and cleavage of such substrates generates AICD but not Aβ. Hence, more sensitive methods are necessary for Aβ analysis, and using ELISA, it was possible to detect Aβ production in our system.

In summary, we have here optimized the conditions for studying γ-secretase activity in rat brain membranes in terms of membrane preparation, incubation time, pH, detergent, detergent concentration, and detergent:protein ratio. We conclude that solubilizing the 100000*g* pellet at 1 mg/mL in 1% CHAPSO at pH 7 and diluting the soluble sample to 0.4% CHAPSO is an appropriate procedure of obtaining a

soluble, highly active γ-secretase complex from rat brain. At these conditions, the endogenous substrate levels are sufficient for activity measurements using either AICD or Aβ production as an outcome.

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