Independent Inhibition of Alzheimer Disease β- and γ-Secretase Cleavage by Lowered Cholesterol Levels*

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The major molecular risk factor for Alzheimer disease so far identified is the amyloidogenic peptide Aβ1-42. In addition, growing evidence suggests a role of cholesterol in Alzheimer disease pathology and Aβ generation. However, the cellular mechanism of lipid-dependent Aβ production remains unclear. Here we describe that the two enzymatic activities responsible for Aβ production, β-secretase and γ-secretase, are inhibited in parallel by cholesterol reduction. Importantly, our data indicate that cholesterol depletion within the cellular context inhibits both secretases additively and independently from each other. This is unexpected because the β-secretase β-site amyloid precursor protein cleaving enzyme and the presenilin-containing γ-secretase complex are structurally different from each other, and these enzymes are apparently located in different subcellular compartments. The parallel and additive inhibition has obvious consequences for therapeutic research and may indicate an intrinsic cross-talk between Alzheimer disease-related amyloid precursor protein processing, amyloid precursor protein function, and lipid biology.

Aβ peptides are the main proteinaceous component of Alzheimer disease amyloid plaques. Aβ is derived from post-translational cleavage of the amyloid precursor protein (APP). Cleavage of APP by BACE I (1) at the N terminus of the Aβ sequence generates a C-terminal fragment (C99) that includes the entire Aβ sequence. In mouse cortical neurons BACE I is essential for APP β-cleavage (2). A second proteolytic activity termed γ-secretase cleaves APP at the C-terminal end of the Aβ sequence, releasing Aβ40 and Aβ42 during normal cellular metabolism of APP (3, 4). A fraction of APP is processed by the α-secretase pathway in which APP is cleaved within the Aβ region thus precluding Aβ formation. However, neurons predominantly use the β-secretory pathway at the expense of the α-secretory pathway to process APP (5). Moreover, neurons produce significant amounts of intracellular Aβ in vivo and in vitro (6–8). A specific feature of γ-secretase is that it is capable of cleaving APP only after a major part of the APP luminal domain is removed. Under normal circumstances it is therefore not possible to assay γ-secretase activity directly.

Analyses of APP-FAD mutations (9) as well as of PS-FAD mutations (10) have corroborated the assumption that a small increase in Aβ42 levels causes AD (11). The subcellular activities of both β- and γ-secretase have been extensively studied. Processing of APP to Aβ differs for different intracellular compartments (12) and depends among others on the interaction of membrane composition and the APP transmembrane domain (13). Variable amounts of β-secretase activity were found along the secretory pathway starting in the ER/intermediate compartment, post-Golgi vesicles, TGN, and endosomes (14, 15). In contrast, γ-secretase activity was found to be prominent in the ER, TGN, and plasma membrane and to produce different Aβ isoforms in different compartments (reviewed by Hartmann (16) and in Ref. 74). APP processing by the ER/intermediate compartment pathway in neurons generated only Aβ42 (17, 18), whereas Aβ40 was produced in TGN (17, 19). There is a strong connection between AD and lipid metabolism. There are a number of findings that indicate that cholesterol lowering might be of use in AD therapy. Several epidemiological studies showed a strongly decreased prevalence of AD and other dementias in patients that underwent treatment with statins (20, 21). Two high dosage pilot intention-to-treat clinical trials using atorvastatin or simvastatin in patients with mild to moderate Alzheimer disease showed a slower cognitive decline in the treatment group (23, 24) and statin treatment may favor the non-amyloidogenic pathway of APP processing in AD patients treated with simvastatin (25). Although these findings indicate a molecular link between cholesterol and AD too little is still known about the molecular mechanisms involved and it currently remains unclear whether cholesterol lowering is sufficiently effective for AD therapy or prevention. Statins are inhibitors of the key enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). They are widely used and well tolerated drugs, active in various cell types including brain cells (26), but the first
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Potential link between lipids and AD was established with apolipoprotein E (apoE), a protein involved in cholesterol transport between tissues (27). In AD the apoE ε-4 allele reduces the age of disease onset (28) and cholesterol and apoE genotype interact to influence progression of Alzheimer disease (29). The link between lipids and AD has recently been significantly extended with findings connecting elevated cholesterol levels with increased amyloid plaque formation in animal models (30–32) and Aβ aggregation in vitro (33). On a cellular level cholesterol has been identified to be a relevant factor regulating Aβ production in vitro (34–37) and in vivo (32, 38), whereas amyloid oligomers can change membrane function (75).

β-Secretase activity can be stimulated by cholesterol (39), the γ-secretase complex and BACE I have been found associated with lipid microdomains (40, 41). Moreover, γ-secretase activity is increased in cellular compartments in which cholesterol accumulates (42). The enzyme cascade of γ-secretase, APP processing, and Aβ is mechanistically linked to lipid homeostasis because Aβ regulates cholesterol and sphingomyelin homeostasis via HMG-CoA reductase inhibition and sphingomyelinase activation (43).

Here we analyzed the cellular mechanism by which cellular cholesterol levels influence intracellular Aβ production. For this purpose we utilized a set of APP deletion mutants that allowed us also to analyze the influence of cholesterol on each of the Aβ generating enzymes independently of each other.

Our results demonstrate that γ-secretase activity is regulated by cellular cholesterol levels and corroborate that cellular cholesterol levels also regulate β-secretase activity. Importantly, our results show that regulation of β- and γ-secretase activities by cellular cholesterol levels are, at least in part, independent of each other. This indicates that cellular cholesterol levels regulate the enzymatic activity of both secretases by different mechanisms and may indicate ways to specifically target β-secretase activity.

**EXPERIMENTAL PROCEDURES**

Constructs—To clarify the modulation of cellular cholesterol levels on Aβ generation we took advantage of comparisons between the processing of APP (34) and of two truncated APP constructs termed C99 and C111 (Fig. 1). Whereas β-secretase is able to cleave full-length APP, γ-secretase requires previous APP cleavage by another secretase. Therefore, we used a construct termed SP-C99, which mimics β-cleaved APP, a substrate for γ-secretase, which directly yields Aβ. Thus, in comparison to full-length APP, this construct allowed us to examine Aβ generation independent of β-secretase. It was previously shown that directly expressed SP-C99 protein is processed to Aβ40 and Aβ42 in the same way as full-length APP (48). To facilitate efficient removal of the signal peptide by SP pepitidase, the dipeptide LE was introduced directly between the original APP signal peptide and C99 (49).

To further address the mechanisms involved in cholesterol-dependent regulation of Aβ production we used another construct termed SP-C111, which has the structure of δ-secretase-cleaved APP. This δ cleavage is a naturally occurring secretase cleavage event 12 amino acids N-terminal of the β-cleavage (56). Therefore C111 is identical to C99 except that it contains the 12 amino acids preceding the Aβ domain. This construct resembles APP as it requires β- and γ-secretase cleavage for Aβ production. However, due to the small luminal domain the β-cleavage is not an essential prerequisite for γ-secretase cleavage. Without β-cleavage, γ-cleavage of Sp-C111 produces a 5.5-kDa product, also allowing Aβ generation. The table indicates which secretase activity or combination of activities are needed to result into the product indicated at the right. For C99 and C111 p3 is a minor product only. SP, signal peptide, α, β, γ, δ; APP secretases.

![FIGURE 1. Schematic outline of constructs APP695 and truncated forms C99 and C111. C99 mimics β-cleaved APP, therefore allowing direct assessment of γ-secretase cleavage for Aβ production. C111 mimics δ-cleaved APP, simultaneously allowing γ-secretase cleavage, yielding a 5.5-kDa product, and also allowing Aβ generation. The table indicates which secretase activity or combination of activities are needed to result into the product indicated at the right. For C99 and C111 p3 is a minor product only. SP, signal peptide, α, β, γ, δ; APP secretases.](image-url)
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cription mixture of both pSFV1/APP and pSFV1/C99 or pSFV1/C111 and pSFVhelper was co-transfected into baby hamster kidney cells using electroporation (77). The baby hamster cells were cultured in Glasgow’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 2 mM glutamate, and 10% tryptose phosphate broth. The culture supernatant containing infective recombinant SFV particles was collected 36 h after electroporation (77).

Cell Culture and Infections—Hippocampal neurons were prepared as described elsewhere (44). The gial glial feeding layer was replaced by addition of the B27 supplement (45). Primary mixed cortical neurons were prepared from 14-day-old fetal rats as described earlier with slight modifications (46). Briefly pregnant Sprague-Dawley rats were sacrificed by etherization at gestation day E14, brains were dissected, cells were plated on 10 µg/ml poly-1-lysine (Sigma)-coated dishes and kept in minimal essential medium supplemented with 0.2% ovalbumin, 1% N2 supplement, and 1% B27 supplement at 5% CO2 and 36.5 °C. In the absence of a glial feeding layer, but with B27 supplement, neurons survived for 30 days or more. Experiments were performed at the age of 6–10 days. At this time neurons were mature and fully polarized (47). Cell culture agents were purchased from Invitrogen (Karlsruhe).

Infections were performed by diluting the recombinant SFV particles with serum-free N2 medium. To allow viral entry infection was continued for 1 h, the virus solution was then removed and incubation continued for 4–6 h to allow SFV-driven protein synthesis (56).

Antibodies—Monoclonal antibodies G2-10 and G2-11 specific for Aβ40 and Aβ42, respectively, were used for immunoprecipitation. Antibody W0-2 directed against amino acids 4–10 of human Aβ was used for detection (50). W0-2 antibody (1 µg/ml) was used for detection of G2-10 and G2-11 precipitates to facilitate a greater linear range, better detection levels, and to avoid detection of endogenous rat Aβ. Aβ40, Aβ42, and APP. W0-2 is specific for the human Aβ sequence. P3 was detected with G2-10. Polyclonal antibody 22/13 raised against the C-terminal 13 residues of C99 (13) was used for immunoprecipitation and detection of C-terminal fragments.

Cholesterol Depletion—For cholesterol depletion of primary neurons we used two different strategies. De novo cholesterol synthesis was inhibited by lovastatin, an inhibitor of HMG-CoA reductase. A basic level of mevalonate was maintained by adding mevalonate to the cell culture media to avoid toxicity due to inhibition of non-steroloidal pathways (51, 52). Plasma membrane cholesterol was extracted by methyl-β-cyclodextrin. Methyl-β-cyclodextrin is a well established tool that selectively and quickly extracts cholesterol from plasma membranes in preference to other lipids (53). These treatments reduce intracellular cholesterol without affecting cell viability and polarity within the time window selected for the experiments (54, 55). After 4–8 days in culture, 4 µM lovastatin (Calbiochem) and 0.25 mM mevalonate (Sigma) were added for 48 h. Control cells were left untreated. Recombinant SFV encoding SP-APP695, SP-C99, or SP-C111 were prepared as described elsewhere (56). Neurons between 6 and 10 days of age were infected for 90 min at 36.5 °C and 5% CO2 with recombinant SFV. This solution was replaced by maintenance medium and cells incubated for 2 h. Cells were then treated with 5 mM methyl-β-cyclodextrin (Sigma) for 10 min and further incubated for 4.5 h. These extraction times did not completely abolish Aβ production, therefore allowing quantification of the effects of cholesterol depletion on the production of Aβ40/Aβ42 and their ratio. For some experiments different amounts of extraction times were used as indicated.

Immunoprecipitation and Quantification—Cell extracts were prepared in 1% Nonidet P-40, 1% Triton X-100, 10 mM Tris, 2 mM EDTA, supplemented with 2× Complete protease inhibitor (Roche Diagnostics). Because Aβ42 constitutes only a minor amount of total Aβ, different amounts of cell lysate were used for immunoprecipitation to obtain similar signal intensity. Cell lysates were divided for Aβ42 (80%) and Aβ40 immunoprecipitation (20%). Immunoprecipitates were recovered on protein G-Sepharose (Sigma) and analyzed on 10–20% Tris-Tricine polyacrylamide gels (Novex, Frankfurt/Main). Quantitative Western blotting was performed as described (57), except that 3% milk powder instead of bovine serum albumin was used for blocking. For detection of CTFs cell lysates were immunoprecipitated with polyclonal antibody 22/13 raised against the C-terminal 13 residues of C99 (13) and recovered on protein A-Sepharose (Amersham Biosciences). For detection, antibody 22/13 was used in a dilution of 1:5000. For detection of p3, conditioned medium was collected and centrifuged at 10,000 × g for 10 min. The supernatant was then immunoprecipitated with G2-10 and Western blot analysis was performed as described above with the exception that bionylated G2-10 (50) and streptavidin-coated horseradish peroxidase (Pierce) were used for detection. Quantification was performed using Macbas-2 software with densitometric scans from Western blots standardized against control Aβ protein.

Staining of Hippocampal Neurons with Filipin—Neurons grown on coverslips for 8 days were treated for 48 h with lovastatin and mevalonate and for 10 min with 5 mM methyl-β-cyclodextrin. After fixation on ice with 4% paraformaldehyde and permeabilization with 0.1% saponin (Sigma) neurons were stained with 125 µg/ml filipin (Sigma) in phosphate-buffered saline. Filipin is a fluorescent polyene antibiotic that forms complexes with cholesterol that can be visualized with ultraviolet light (58). For Golgi58 co-staining, filipin staining was followed by blocking with 5% goat serum (Sigma) and 3% bovine serum albumin (Biomol, Hamburg). Afterward cells were incubated with monoclonal antibody Golgi58 (Sigma) and rhodamine isothiocyanate-conjugated second antibody (Sigma). Cells were mounted in Mowiol (Hoechst, Frankfurt/Main). Digital images were taken with a Zeiss aixioskop microscope equipped with a three-chip color camera (Micromax, Princeton Instruments, Trenton, NJ).

β-Secretase Activity Assay—Confluent grown SH-SY5Y cells were incubated with 5 mM methyl-β-cyclodextrin or solvent as a control for 10 min and afterward cultivated at 37 °C in growth medium without fetal calf serum for 2 h.

The cells were washed 3 times with phosphate-buffered saline and scraped off and after homogenization the protein amount of the samples were adjusted to 0.2 mg. The commercially available assay was performed as described in the protocol provided by Calbiochem. Briefly this assay utilizes a secretase-
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specific peptide, which is conjugated to a fluorophor (EDANS) and a quencher (DABCYL). The cleavage of the β-secretase separates the quencher from the fluorophor. The fluorescence was measured continuously at an excitation wavelength of 345 nm (bandwidth 5 nm) and emission wavelength of 500 nm (bandwidth 5 nm) for 3 h at 37 °C with a fluorometer (Tecan safire 2). The slope of the linear range correlates with β-secretase activity.

γ-Secretase Activity Assay—The γ-secretase assay is in principle comparable with the β-secretase assay with minor changes. After methyl-β-cyclodextrin extraction and protein adjustment to an amount of 1 mg/sample the membrane was prepared according to a method described elsewhere (78). The assay was performed in 50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 0.25% CHAPSO (w/v), and 8 μM fluorogenic γ-secretase substrate (Calbiochem)/sample. The fluorescence was measured continuously at an excitation wavelength of 355 nm (bandwidth 10 nm) and emission wavelength of 440 nm (bandwidth 10 nm) for 3 h at 37 °C with a fluorometer (Tecan safire 2). The slope of the linear range correlates with γ-secretase activity after the nonspecific signal was subtracted and analyzed using 10 μM of the specific γ-secretase L-658,458.

BACE-1, PS1, sAPPα, sAPPβ, Na+/K+-ATPase, and Cadherin Protein Quantification—The human SH-SY5Y neuroblastoma cell line was maintained in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal calf serum and 1% nonessential amino acid solution (Sigma). 90% confluent cells were incubated for 10 min with 5 mM cyclodextrin followed by a 4-h incubation period without cyclodextrin in culture medium. For the detection of sAPPα equal volumes of conditioned media were immunoprecipitated with 20 μl of protein G-Sepharose (Sigma) and the antibody WO2. The immunoprecipitated proteins were separated on 10–20% Tricine gels (Invitrogen). Western blot analysis was performed with the antibody WO2 (1 μg/ml). For the detection of endogenous BACE-1 and PS1, cells were scraped in 200 μl of phosphate-buffered saline containing protease inhibitor mixture (Roche Diagnostics GmbH) and centrifuged for 10 min at 13,000 × g at 4 °C. Sedimented cells were lysed in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, and 2 mM EDTA) supplemented with protease inhibitor mixture and homogenized using a 27-gauge needle. Cell homogenates were centrifuged for 10 min at 13,000 × g for 4 °C and the supernatant was used for protein determination. Equal amounts of protein were separated on 10–20% Tricine gels. For the detection of BACE1, a commercially available polyclonal antibody was used (B0806, Sigma), for the detection of presenilin 1, antibody sc-7860 was used (Santa Cruz Biotechnology Inc). sAPPβ was detected by using antibody 18591 from IBL Inc. Na+/K+-ATPase and cadherin were detected after plasma membrane preparation using the commercial cadherin antibody ab6528 purchased from Abcam and the Na+/K+-ATPase sc-21712 from Santa Cruz Biotechnology Inc.

Membrane Preparation—Membrane preparation was done as follows, a postnuclear fraction was made. For this cells were homogenized in 1 ml of homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 200 mM sucrose, Complete protease inhibitor mixture (Roche, 1 tablet/10 ml)). The nuclei and cell debris from organelles were removed from the homogenate by centrifugation at 1,000 × g for 10 min at 4 °C. To separate the residual membrane from cytosolic contaminations the resulting supernatant was centrifuged at 100,000 × g for 90 min at 4 °C (SW40 rotor, Beckman ultracentrifuge). The membrane pellet was solubilized in buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100) at 4 °C. If required the membranes were further purified by adding dextrin/polyethylene glycol buffer (16% dextran (Sigma average mol weight 425,000–575,000), 30.8 g for 20% polyethylene glycol 3350 (Sigma), 6.6 ml of 0.2 M phosphate buffer, pH 6.5, and 50 ml of H2O2). After centrifugation at 2,000 × g for 15 min phase separation occurred. For further experiments the above mentioned solubilized membrane pellet or upper polyethylene glycol phase was used.

Cholesterol Determination—Cholesterol levels were determined using the Amplex Red cholesterol assay (Molecular Probes) in accordance to the manufacturers protocol with a slight modification. Cell homogenates, which were adjusted to the same protein or cell amount, were preincubated with cholesterol oxidase and horseradish peroxidase and Amplex red (all obtained from Molecular Probes in amounts described by the manufacturer) for 20 min in the absence of light at 37 °C. Fluorescence was measured using an excitation wavelength of 530 ± 10 nm and emission wavelength of 590 ± 10 nm. As a negative control, lysis buffer, and for positive control, cholesterol (Sigma) at different concentrations were used.

Caspase and Lactate Dehydrogenase Assay—The caspase 3 assay was performed as described by the manufacturer (Calbiochem) with slight modifications. In principal the specificity of caspase 3 for cleavage after aspartate residues in a particular peptide sequence (DEVDD) was used to monitor the enzyme activity. The activity was monitored by a blue to green shift in fluorescence upon cleavage of the DEVD substrate labeled with the 7-amino-4-trifluoromethyl coumarin (AFC) fluorophore. Therefore an adjusted amount of cells (106 cells) was collected in 60 μl of sample buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1% Triton X-100, 0.1% CHAPS, 2 mM EDTA, and 10 mM dithiothreitol). After a 15-min lysis at 4 °C, 50 μl were transferred to a 96-well plate and the reaction was started by adding 50 μl of assay buffer (provided by the manufacturer), which contains the fluorophor-labeled caspase 3 substrate. Activity was measured by detecting the fluorescence (excitation 400 ± 10 nm; emission 505 ± 10 nm) for 30 min. As a positive control recombinant caspase 3 was used, for a negative control recombinant caspase 3 activity in the presence of a specific caspase 3 inhibitor was measured. The fluorescence was monitored for 30 min and the slope of the increasing fluorescence was calculated representing the caspase 3 activity. Lactate dehydrogenase assay was exactly performed as described by the manufacturer (Calbiochem).

RESULTS

β-Secretase—In a previous study (34) we postulated that reduced cholesterol levels inhibited β-secretase cleavage. To clarify the exact mechanism of cholesterol-dependent Aβ generation we compared APP with two truncated APP constructs, C99 and C111. Cholesterol lowering treatment with methyl-β-
FIGURE 2. A, protein and activity levels of PS-1/γ-secretase and BACE-1/β-secretase. Cyclodextrin treatment did not affect the protein levels of the enzymatically active center proteins of both proteases. However, cleavage of the respective fluorogenic secretase substrate peptides in a cell-free assay declined. Inset (right) shows representative Western blots for BACE-1 and PS-1. B, CTFs of APP695, C99, and C111 upon cholesterol depletion. Immunoprecipitation with antibody 2214 from cell lysates of primary mixed cortical neurons that were cholesterol depleted or left untreated and expressed APP695, C99, or C111. The upper bands at C99 and C111 represent the uncleaved precursor forms of these constructs. For APP695 the upper band, which corresponds to β-cleaved APP-CTF, is strongly reduced upon cholesterol depletion, whereas the lower band, which most likely represents α-cleaved APP-CTFs, is largely unaffected. C99 migrates slower than β-CTF due to the presence of an additional charged residue (LE) at the N terminus. C99 and C111 produce very small amounts of CTFs corresponding to α-secretase cleavage and some intermediate CTFs that are either not or only slightly increased. Migrating just below the major uncleaved C111 is a strong band (indicated by *) apparently representing the β-cleaved CTF, which is strongly reduced upon cholesterol depletion. C, wash out. SH-SYSY cells (1% fetal calf serum) were treated with methyl-β-cyclodextrin (CDX) for 10 min. Cells were rested for 48 h and then cholesterol was replenished by culturing for 24 h with 5% fetal calf serum. This resulted in almost full recovery of cellular cholesterol levels and a parallel full recovery of Aβ secretion. D, CDX and statin-dependent cytotoxicity. SH-SYSY cells or rat primary hippocampal neurons (data not shown) were treated with CDX for 10 min followed by lovastatin treatment for 24 h and subsequently assayed for lactate dehydrogenase release and caspase 3-mediated substrate cleavage. No influence of the cholesterol depletion treatment was found.
cyclodextrin did not affect the protein levels of BACE I or presenilin 1, but the proteolytic activities of β- and γ-secretase declined to a similar extent in a cell-free assay using fluorophoric peptides for activity determination (Fig. 2A). Moreover, by using an antibody directed against CTFs we find a cholesterol-dependent reduction of a 12-kDa CTF in cells expressing the full-length APP (Fig. 2B) that by apparent molecular weight corresponds to β-cleaved APP CTF (7).

The lower CTF with an apparent molecular mass of 10 kDa, which most likely represents α-cleaved APP-CTFs, was not affected. For SP-C99 and SP-C111 constructs CTFs with an apparent molecular mass of 11 kDa are visible. The 11-kDa CTFs may represent secondary β-secretase cleavages in neurons (2, 7, 59, 60) and were not or only slightly increased by cholesterol depletion. Small amounts of this 11-kDa cleavage product can be found with APP (data not shown) (5), but were below the detection limit in those experiments. As expected, SP-C111 expression like full-length APP expression, resulted in reduced β-CTF levels upon cholesterol depletion. Washout of methyl-β-cyclodextrin recovered cellular cholesterol levels almost entirely with a parallel full recovery of Aβ production (Fig. 2C). The experimental conditions used here apparently did not impair membrane integrity or the apoptosis rate (Fig. 2D). A previous report (60) indicated under somewhat different experimental conditions increased α-secretase activity. However, as expected (7), α-secretase activity was weak with hippocampal neurons and accordingly α-secretase-generated CTFs were found in small amounts only. No significant differences in α-CTF levels were observed. To verify that indeed the reduced Aβ levels were not caused by increased production of p3, we analyzed production of this peptide directly. For this we used APP695 as a precursor, because it gives rise to higher levels of p3 than SP-C99 (61). Because p3 is not detected by W0-2 we used G2-10, a C-terminal-specific antibody to Aβ40 that is able to detect all N-terminal-truncated Aβ species including p3 (57). In agreement with the low α-secretase activity, p3 levels secreted by primary neurons were very low (Fig. 3A) (5, 7, 62) and absent intracellularly (data not shown). Importantly, cholesterol depletion reduced secretion of Aβ and p3, but not sAPPα levels (Fig. 3B). Therefore increased α-cleavage cannot account for decreased Aβ levels after cholesterol reduction within this experimental setup. This is in agreement with reduced sAPPβ levels (Fig. 3B). The latter is in agreement with the reduced BACE I activity. Alternatively, reduced precursor levels of either APP or the C111 on the plasma membrane could be considered, this, however, was not the case (Fig. 3C).

**γ-Secretase**—Reduced levels of p3 cannot be explained by β-secretase inhibition, indicating reduced γ-secretase activity. Additionally, as p3 is not found intracellularly (7, 63), analysis of intracellular Aβ production allows us to analyze directly
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levels decreased to $63.0 \pm 27.7\%$ as compared with control cells ($p < 0.001$ as compared with untreated control cells and $p < 0.01$ for $\beta_{40}$ versus $\beta_{42}$, Fig. 3D). But in comparison with our results obtained with C99, $\beta$ production from APP following cholesterol depletion was more strongly reduced (Fig. 3D and Ref. 54). The lower inhibition of $\beta$ production from C99 as compared with APP695 can be explained by an additive effect of β-secretase and γ-secretase inhibition by cholesterol reduction.

Cholesterol-dependent Effects on $\beta_{40}$- and $\beta_{42}$-specific Intracellular Secretase Activity—We have previously shown that treatment with lovastatin alone or in combination with methyl-β-cyclodextrin reduces APP695-derived Aβ production (54). To investigate further the different sensitivity of intracellular $\beta_{40}$ and $\beta_{42}$ to extracellular cholesterol depletion, neurons were treated with methyl-β-cyclodextrin for different lengths of time (5, 10, and 20 min) without the addition of lovastatin. This treatment leaves de novo cholesterol synthesis unperturbed in the ER (65), whereas cholesterol content in the plasma membrane and late secretory pathway gradually decreases depending on the extent of external cholesterol depletion (65).

Interestingly, we found that $\beta_{40}$ is very sensitive to treatment with methyl-β-cyclodextrin, whereas $\beta_{42}$ is largely resistant to peripheral cholesterol depletion (Fig. 4). Because cyclodextrin removes cholesterol from the plasma membrane and late compartments (65, 53) these results are in line with findings that $\beta_{40}$, unlike $\beta_{42}$, is generated in the late secretory pathway (17–19, 63).

To confirm the extent and location of cholesterol depletion with our treatment procedures we stained cells with filipin, which form complexes with cholesterol that can be detected with ultraviolet light. Our results show that untreated neurons displayed the typical plasma membrane cholesterol staining profile (Fig. 5), whereas treatment with lovastatin showed decreased intensity in cholesterol staining (data not shown). Combined treatment withLovastatin and methyl-β-cyclodextrin displayed highly reduced staining intensity and abolished plasma membrane staining. Cholesterol was localized in structures resembling the Golgi. Co-staining with filipin and an antibody against Golgi network protein Golgi58 showed a matching colocalization of the cholesterol-containing remnants and Golgi58 protein after cholesterol depletion, whereas untreated cells displayed normal cholesterol concentration at the plasma membrane.

γ-secretase activity in the absence of p3 and possibly in the absence of any relevant α-secretase activity.

In our previous study we showed that intracellular $\beta_{40}$ and $\beta_{42}$ derived from APP695 were both diminished upon cholesterol depletion (54). We now used the C99 construct that mimics β-cleaved APP for directly assessing γ-secretase activity. In cell lysates of neurons infected with SFV SP-C99, $\beta_{40}$ decreased upon cholesterol depletion by combined usage of lovastatin and methyl-β-cyclodextrin to $59.3 \pm 14.7\%$ as compared with non-treated cells. Similar to $\beta_{40}$, intracellular $\beta_{42}$
The effect of cholesterol depletion on γ-secretase activity was first examined in cells infected with C99. This is the major β-cleaved product of APP and the ultimate substrate for γ-secretase as the precursor for Aβ generation. Cholesterol depletion significantly reduced intracellular Aβ levels, showing that γ-secretase activity is sensitive to cholesterol depletion. In this case a role of β- or α-secretase can be ruled out, because C99 is not a substrate for β-secretase and increased α-secretase activity can be ruled out by the absence of increased p3 levels (7, 63). Other studies have shown increased α-secretase activity as a result of cholesterol depletion. We observed this only when we applied their more stringent extraction protocols, but not under the conditions used here (data not shown) (60).

Reduced intracellular Aβ levels are also unlikely to be caused by unspecific toxicity of cholesterol depletion because we have used a relatively mild depletion protocol (5 and 10 min) not known to have any significant toxic effects in vivo and in vitro. In a previous study we determined the total protein biosynthesis rate, cell viability, and SFV-neuronal infection rate and found all these unaffected by cholesterol depletion (54). In addition, APP biosynthesis and secretion are not affected by our cholesterol depletion protocol and Aβ production was fully restored after cholesterol repletion (34). Even with the more harsh conditions of 20 min, methyl-β-cyclodextrin treatment of the majority of Aβ42, but not other Aβ peptides, remained arguing against a generalized toxicity effect inhibiting γ-secretase. Our experiments have also shown BACE-1 and PS-1 protein levels not to be altered by treatment with cyclodextrin. A recent study has shown that statins cause intracellular accumulation of Aβ via a non-steroidal isoprenoid-dependent mechanism (67). In our study, however, we added mevalonate to avoid toxicity due to inhibition of these non-steroidal pathways (51, 52, 54).

The effects of cholesterol depletion on both the β- and γ-secretases were examined in cells infected with C111, which can be cleaved, like APP, by β- and γ-secretase in a sequential process (56) and resulting in the release of Aβ. Alternatively C111 is cleaved like C99, by γ-secretase alone resulting in the release of p5.5. With C111, cholesterol depletion markedly inhibited all peptides produced by γ-secretase activity, as seen by the decrease in the p5.540 and Aβ40 products. Similar to this all peptides produced by β-secretase activity markedly declined (Aβ40 and Aβ42). Only the peptide p5.5, which is produced by the γ-secretase activity 42 alone, remained fairly stable. Overall the peptides ranked within the order AB42 > Aβ42 > p5.540 > p5.542, with Aβ40 almost entirely gone and p5.542 almost unaffected by cholesterol depletion. Surface labeling with antibodies directed against Aβ and APP done in a

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**Independent Mechanisms for β- and γ-Secretase Inhibition**—To further address the mechanism involved in cholesterol-dependent regulation of Aβ generation we used SP-C111, because this construct may be either cleaved by γ-secretase alone, yielding a 5.5-kDa fragment, or by β- and γ-secretase, generating Aβ.

After peripheral cholesterol depletion we found (Fig. 6) C111 with strongly reduced p5.540 and Aβ40 levels. Notably, Aβ40 was more pronounced, which is in agreement with additive effects of β- and γ-secretase (approximately 94 and 75% reduction). Equally, the β- and γ-secretase product Aβ42 was strongly reduced as well (approximately 87% reduction). However, the parallel β-secretase-independent but γ-secretase-dependent p5.542 levels were only moderately reduced (approximately 40% reduction). Therefore, the strongly reduced Aβ42 levels cannot be explained by γ-secretase inhibition. This argues for a second enzyme, β-secretase, independently inhibited by peripheral cholesterol depletion.

**DISCUSSION**

Aβ production is sensitive to cellular cholesterol levels in vitro and in vivo (34, 35, 54). To define the enzymatic requirements of cholesterol-dependent Aβ production we applied a set of APP and truncated APP constructs designed to reveal consecutive and independent activities of β- and γ-secretase with modulated cholesterol levels.

As indicated by the increased accumulation of APP C-terminal fragments, cholesterol depletion resulted in decreased β-secretase activity corroborating earlier results (34). However, accumulation of APP C-terminal fragments may also indicate reduced γ-secretase activity. Our data now indicate that cholesterol depletion results in a parallel and additive inhibition of β- and γ-secretase activities. Even in the same cell, inhibition of β-secretase and γ-secretase activities can be partly separated from each other and therefore appears to be independent of each other.

**FIGURE 6. Independent of β- and γ-secretase cleavage inhibition by reduced cholesterol levels.** Cell lysates from mixed cortical neurons were immunoprecipitated with antibodies G2-10 and G2-11 and detected with antibody W0-2. To assess simultaneously β- and γ-cleavage independently of one another and in sequential order, construct C111 was analyzed. The upper band of an apparent molecular mass of 5.5 kDa termed p5.5 represents γ-cleavage activity only, whereas the lower band represents Aβ generated by β- and γ-secretase activity as indicated. The β- and γ-secretase products, Aβ40 and Aβ42, are both very sensitive to peripheral cholesterol depletion by methyl-β-cyclodextrin. The same holds true for the γ-secretase only product, p5.540, whereas p5.542 is much less affected by reduced cholesterol levels. CDX, methyl-β-cyclodextrin.

**Independent Effect of Cholesterol on β- and γ-Secretase**

**Independent Mechanisms for β- and γ-Secretase Inhibition**—To further address the mechanism involved in cholesterol-dependent regulation of Aβ generation we used SP-C111, because this construct may be either cleaved by γ-secretase alone, yielding a 5.5-kDa fragment, or by β- and γ-secretase, generating Aβ.

After peripheral cholesterol depletion we found (Fig. 6) C111 with strongly reduced p5.540 and Aβ40 levels. Notably, Aβ40 was more pronounced, which is in agreement with additive effects of β- and γ-secretase (approximately 94 and 75% reduction). Equally, the β- and γ-secretase product Aβ42 was strongly reduced as well (approximately 87% reduction). However, the parallel β-secretase-independent but γ-secretase-dependent p5.542 levels were only moderately reduced (approximately 40% reduction). Therefore, the strongly reduced Aβ42 levels cannot be explained by γ-secretase inhibition. This argues for a second enzyme, β-secretase, independently inhibited by peripheral cholesterol depletion.

**DISCUSSION**

Aβ production is sensitive to cellular cholesterol levels in vitro and in vivo (34, 35, 54). To define the enzymatic requirements of cholesterol-dependent Aβ production we applied a set of APP and truncated APP constructs designed to reveal consecutive and independent activities of β- and γ-secretase with modulated cholesterol levels.

As indicated by the increased accumulation of APP C-terminal fragments, cholesterol depletion resulted in decreased β-secretase activity corroborating earlier results (34). However, accumulation of APP C-terminal fragments may also indicate reduced γ-secretase activity. Our data now indicate that cholesterol depletion results in a parallel and additive inhibition of β- and γ-secretase activities. Even in the same cell, inhibition of β-secretase and γ-secretase activities can be partly separated from each other and therefore appears to be independent of each other.
Independent Effect of Cholesterol on β- and γ-Secretase

previous study from our group (56) demonstrated that truncated APP proteins such as C111 reached the plasma membrane suggesting that the transmembrane topology of APP was maintained in these constructs. In the same study sorting and expression of C99, C111, and full-length APP were carefully studied. Full-length APP was sorted axonally, C111 displayed mixed axonal and somatodendritic sorting, whereas C99 was sorted somatodendritically. However, our results clearly show the inhibition of β- and γ-secretase-mediated cleavage to be present with all three constructs making different sorting properties of the constructs an unlikely explanation.

Taken together, these results strongly indicate that β- and γ-secretase activities are inhibited by separate mechanisms. Moreover, this inhibition may occur in parallel and in that case is additive. The additive nature of β- and γ-secretase inhibition is evident from our findings that Aβ generation is more strongly inhibited in APP expressing cells as compared with C99 expressing cells (Fig. 3) and the more pronounced effects that inhibited in APP expressing cells as compared with C99 present with all three constructs making different sorting properties of the inhibitions indicate that the majority of Aβ42 production in hippocampal neurons is sensitive to cholesterol reduction in the early secretory pathway and corroborate earlier results indicating an independent production of Aβ40 and Aβ42 in hippocampal neurons (69).

The small decrease in Aβ42 levels observed as a result of peripheral cholesterol depletion may be due to depletion of the fraction of Aβ42 that is produced beyond the early secretory pathway (19, 70, 71). Although the peripheral cholesterol extraction protocol mainly affected the plasma membrane we cannot exclude that a limited depletion of cholesterol had occurred from the early secretory pathways. Because Aβ42 reduction was small, limited cholesterol reduction here may contribute to the small reduction observed for Aβ42 as well.

Interestingly, we found that the β-secretase activity that ultimately results in Aβ42 release is inhibited by peripheral cholesterol depletion. This result has important implications. It indicates that cholesterol-dependent β-secretase activity may be inhibited by a mechanism distinct from γ-secretase inhibition. BACE I has been shown to recycle between the TGN and the plasma membrane via endosomes (72), and cyclohexrin is known to reduce endocytosis (73). Therefore, the observed β-secretase inhibition may be due to inhibited endocytosis, whereas normal secretion remains intact (34, 35, 54), leaving BACE at the plasma membrane, a compartment of unfavorable pH for β-secretase cleavage. However, Kalvodova et al. (39) showed that BACE activity is sensitive to cholesterol levels even in the absence of trafficking in vitro, which may indicate that removal of cholesterol from the membrane microenvironment of BACE I in the late secretory pathway may suffice to reduce its activity. The result on cell-free BACE I activity and the similar reduction in sAPPβ levels would favor the latter interpretation of the data.

In this study two well characterized pharmaceutical agents were used (22, 26). The parallel and additive inhibition of both enzymatic activities responsible for Aβ production by a single intervention may have consequences for the development of Aβ-lowering therapeutic strategies and may offer advantages over strategies targeted to individual secretases alone.

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