Characterization of Intermediate Steps in Amyloid Beta (Aβ)
Production under Near-native Conditions*

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Background: The 42 amino acid long amyloid beta peptide (Aβ42) plays a pivotal role in Alzheimer disease.

Results: A novel assay was developed and enabled us to describe the process of Aβ42 production and modulation.

Conclusion: Aβ42 is generated through different pathways, which are differently affected by disease causing mutations and anti-amyloidogenic drugs.

Significance: The data provide key information for therapeutic development in Alzheimer disease.

Processing of the amyloid precursor protein (APP) by γ-secretase results in generation of Aβ peptides of different lengths ranging from 51 to 30 residues. Accumulation of Aβ and in particular Aβ42 is enhanced by familial Alzheimer disease (FAD) causing mutations in APP and is believed to play a pivotal role in AD pathogenesis (1, 2). The molecular mechanism underlying normal Aβ production, the impact of FAD mutations on this process and how anti-amyloidogenic γ-secretase modulators (GSMs) cause a selective decrease in Aβ40 and Aβ42 and an increase in shorter Aβ peptides, however, is poorly understood. By using a combined immuno- and LC-MS-based assay we identify several major intermediates, i.e. 3- and 4-peptides that line up head to head across the entire APP transmembrane sequence from Aβ51 to Aβ31/Aβ30 and from Aβ49 to Aβ30/31. FAD APP mutations displayed a relative increase in 3- and 4-peptides from Aβ48 to Aβ38 compared with Aβ49 to Aβ37. These findings correlate with an increase in the Aβ42/40 ratio. GSMs caused a decrease in Aβ40 and Aβ42 and an increase in Aβ37 and Aβ38 paralleled by an increase of the intermediates Aβ40–38 and Aβ42–39. Collectively, these data provide a thorough characterization of all intermediate steps in Aβ production in native cell membranes and provide key mechanistic insights to genetic and pharmacological modulation of Aβ generation.

Alzheimer disease (AD) is the most common form of dementia in the elderly and still lacks a disease-modifying treatment. A growing body of data strongly suggests that early aggregation of the amyloid beta peptide (Aβ) plays a key role in AD pathogenesis (1, 2). Aβ is a post-proteolytic product generated as the result of the sequential cleavage of amyloid precursor protein (APP) by BACE and γ-secretase (3). The initial cut position of the γ-secretase in the transmembrane domain of APP is rather imprecise, which explains why Aβ comes in different lengths: the longest Aβ peptide identified is Aβ51 while the shortest is Aβ30 as we describe herein. Aβ40 is the most abundant form and Aβ42 has received increasing attention in AD research, as it is very prone to aggregation and the major species deposited in the center of senile plaques, which are neuropathological hallmarks for AD. Furthermore, familial forms of AD (FAD) caused by mutations in the transmembrane sequence of APP frequently result in an increased Aβ42/40 ratio (4). A high Aβ42/40 ratio positively correlates with the age of disease onset and is the key determinant of amyloidogenesis rather than total Aβ (5–7).

To inhibit Aβ production, in particular of Aβ42, is therefore a prioritized strategy for therapy development against AD. γ-Secretase inhibitors (GSIs), which completely abrogate γ-secretase cleavage, have encountered several problems in clinical trials, because they block cleavage also of other γ-secretase–processed proteins, notably Notch receptors (8). So-called γ-secretase modulators (GSMs), which modulate Aβ production without affecting overall γ-secretase activity, have therefore emerged as an interesting alternative. In contrast to GSIs, GSMs do not affect overall enzyme activity but cause a parallel decrease in Aβ40 and Aβ42 accompanied by increase in Aβ37 and Aβ38 (and sometimes Aβ39) (9, 10). The majority of GSMs exhibit a slightly higher potency for Aβ42 reduction compared with Aβ40. The first generation GSMs appeared to target APP directly by affecting dimerization (11, 12). More recently, and chemically very different, second generation GSMs instead appear to preferentially interact with γ-secretase (10, 13, 14).

The mechanism by which GSMs or FAD mutations modulate levels of specific Aβ forms however remains elusive, and reflects an underlying lack of understanding of the distinct steps in the sequential Aβ processing cascade, where longer Aβ forms are converted into shorter peptides. Takami et al. have proposed a stepwise cleavage model where γ-secretase processing of the APP transmembrane sequence begins at either Aβ49, Aβ51, or Aβ52 (the so called epsilon cut) followed by process-

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¶ The abbreviations used are: AD, Alzheimer disease; GSM, γ-secretase modulators; APP, amyloid precursor protein; FAD, familial Alzheimer disease; Aβ, amyloid beta.
ing at every third or fourth amino acid leading to the release of progressively shortened Aβ peptides (15). Depending on the exact initial epsilon cleavage site, either Aβ40 or Aβ42 will be the preferred form produced (15–17). More recently, it was shown that not only membrane bound stubs but also Aβ peptides are direct substrates for γ-secretase, which are converted into shortened Aβ peptides (16). It is however important to note that in all these studies, data were derived from biochemical experiments with solubilized membrane extracts and *E. coli*-produced truncated APP molecules (beta-stub or C99) or recombinant Aβ peptides (Aβ43 and Aβ42), rather than membrane bound native substrates expressed in mammalian cells. There are reasons to believe that not all aspects of γ-secretase-mediated APP processing and GSM functions are fully recapitulated in reconstituted systems (10, 16, 18). There is therefore a need to examine Aβ processing in more native systems.

In our study we combined immuno- and LC-MS-based assays to analyze γ-secretase mediated APP processing in native membranes in the absence of detergents and without recombinant substrates. We delineate γ-secretase mediated processing of the entire APP transmembrane sequence which ranges from the longest (Aβ51) to the shortest (Aβ30) form of Aβ. Our data identifies novel γ-secretase cleavage events and show that the entire APP transmembrane sequence is subjected to γ-secretase processing. The data suggest that Aβ42 could be generated through different cleavage reactions and that the Aβ42/40 ratio could be regulated at several steps of γ-secretase-mediated APP processing (Fig. 1A). Our data also provide novel insights into how amyloidogenic Aβ production is promoted and changed by FAD APP mutations and by GSMs, respectively; information that is highly relevant for therapeutic development in AD.

**MATERIALS AND METHODS**

**Reagents**

*Compounds*—R-flurbiprofen and Sulindac sulfide were from Sigma, Semagacestat from Selleck Chemicals, AZ4800 was prepared according to WO2010053438, AZ4126 according to WO2010132015, E2012 according to US20060004013 and AZ1136 as previously described (10).

*Immunoassay*—Mesoscale discovery assay (MSD): Aβ38, 40, and 42 triplex plates and antibodies 6E10 and 4G8 were from the supplier, rabbit polyclonal antibodies specific for Aβ34, 37, and 39 were custom made.

*LC-MS*—Peptide standards were from Polypeptide Laboratories. Internal standard, leucine, enkephalin, and formic acid were purchased from Sigma, acetonitrile (Gradient grade) was from Applichem. Water of 18-MΩ quality was prepared from Milli-Q system (Millipore).

**Cellular Assay of Aβ Production**

HEK293 cells overexpressing the APPSwe mutant (HEK/APPswe) were split and seeded into 384-well tissue culture plates at day 1. At day 2, medium were removed and replaced with medium with or without compound. 5 h later, the conditioned media was assayed for Aβ production using the MesoScale Discovery (MSD) platform. Briefly, the conditioned media was transferred to MSD assay plates coated with either Aβ34, Aβ37, or Aβ39 specific antibodies, or MSD triplex plates coated with Aβ38, 40, 42 specific antibodies, and incubated overnight at 4°C. The following day, the plates were washed three times following the addition of 4G8 as detection antibody (recognizing Aβ17–24) and the plates were incubated for 1 h at room temperature before 3 repeated washes and measurement of the electrochemiluminescence signal according to the manufacturer’s instructions.

**Cell Membrane Preparations**

Membranes from HEK/APPswe cells and SH-SY5Y cells stably over expressing APP, APP714, APP717, or APP723 were prepared as described before (10). The wells were exposed to 0.5 μM of the reversible GSI Semagacestat for 19 h prior to cell harvesting to accumulate non-γ-secretase processed APP metabolites (i.e. C99 and C83) and thereby enhance the amount of γ-secretase products generated in the subsequent membrane based γ-secretase activity assay (see below). P2 membranes were re-suspended in assay buffer (50 mM MES, pH 6.4, 1.5 mM MgCl₂, 75 mM sodium citrate, and Complete protease inhibitor (Roche Applied Science)) and stored at −80°C.

**Membrane Assay of γ-Secretase-mediated APP Processing**

Membranes were thawed on ice and diluted in assay buffer (see above) to a final concentration of 1.5 mg/mL. In each reaction, 150 μl of ice-cold membranes were treated with compounds diluted in 100% DMSO (final DMSO concentration 4%) and incubated at 37°C for 2 h. The reaction mixtures were thereafter kept on ice and centrifuged at 1000 × g for 1 min to remove any cell debris. 70 μl of the reaction mixture were then subjected to LC-MS analysis (see below), and 50 μl of the reaction mixture were added to MSD assay plates coated with either Aβ34, Aβ37, Aβ39, Aβ42 specific antibodies or MSD triplex plates (Aβ38, Aβ40, and Aβ42). After incubation overnight at 4°C, 4G8 or 6E10 (recognizing Aβ1–10) antibodies were added and analyzed for specific Aβ peptides in accordance with the assay described under “Cellular Assay of Aβ Production.”

**Cellular Assay of P3 Production**

Mouse embryonic fibroblasts (MEFs) were prepared from BACE deficient mice, which lack the ability to generate Aβ but that has an intact non-amyloidogenic pathway where α-secretase and subsequently γ-secretase cleavage of APP results in sAPPα and P3 peptide production, respectively. The MEFs were seeded in 96-well tissue culture plates and grown to ~80% confluency. The cells were subsequently exposed to either AZ4126 or R-Flurbiprofen for 24 h. The conditioned media was subsequently removed for P3 peptide analysis. P3 levels were analyzed using Aβ38/40/42 triplex plates (MSD) and 4G8 (epitope 17–24 of N-terminally intact Aβ peptide) as detection antibody according to the manufacturer’s instructions.

**LC-MS Analysis**

Standard peptides were diluted in water, typically 0.4 mg to 1 ml of water except for LVML, which was diluted in water and 16% acetonitrile. The prepared stock solution was divided into aliquots in polypropylene tubes and stored at −20°C. 70 μl of
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sample were mixed (in a 96-well propylene plate, Thermo-Fast 96 Skirted, Thermo Scientific) with 20 μl of internal standard (571 nM). The samples were then transferred to a filter plate (MultiScreen HTS, HV, 0.45 μm, low protein binding, Millipore) and centrifuged at 2600 × g for 10 min at room temperature. The collection plate was of the same type as the plate used for mixing before the centrifugation step.

Analysis of peptides was performed by the use of a triple quadrupole mass spectrometer, Quantum Ultra and a ultrahigh pressure (UPLC) pump, Accela, (ThermoFisher, CA) an auto sampler (CTC Analytics AG, Switzerland) equipped with a 100 μl syringe (Hamilton Bonaduz AG, Switzerland), a chilled (5 °C) 96-well plate holder a six-port injection valve with a 50 μl sample loop VICI (Valco Instruments, TX). The chromatographic column was a Hypersil gold 2.1 × 150 mm, 1.9 μm particles (ThermoFisher) kept at 40 °C by a column heater, Model 7955 (Gracevycad, CA).

The mobile phase consisted of solvent A: 0.05% formic acid and solvent B: 95% acetonitrile, 5% water, 0.05% formic acid. The gradient (350 μl/min) conditions were as follows: 0–10 min 2–15% B; 10–15 min 15–70% B; 15–20 min 2% B.

The electro spray ionization was in the positive ion mode with multiple reaction monitoring. The vaporizer temperature, capillary voltage, and temperature were set to 150 °C, 4500 volts, and 300 °C, respectively. The collision pressure was 1.5 mTorr.

Settings of the mass spectrometer for the detection of specific peptides are shown in Fig. 2B. The standard curve was analyzed by a dilution series of a mixture of all the peptides (70 μl) in water (200, 40, 8, 1.6, 0.32, and 0.064 nM) and treated in the same procedure as the samples. Quantification was performed by weighting the response of each peptide divided by the response of the internal standard (area/area) 1/1 (ignore origo). The response in matrix was tested by adding 5 μl of a mixture of all the peptides at different concentrations (standard curve) to 65 μl of an aliquot of sample previously exposed to a large excess of the γ-secretase inhibitor semagacestat. The response of the added peptides was found to be linear. Typically variation of the method was 10% (rel.std. deviation, n = 6). The limit of quantification was set to 0.2 nM.

RESULTS

A Combined Immuno/LC-MS-based Assay to Monitor Aβ Production in Near-native Membranes—Through conventional immuno-based methods with Aβ species-specific antibodies it has been possible to identify a range of Aβ species, but not the cleaved smaller peptides, and information about those would be needed to conclusively establish the internal order in the Aβ processing cascade. To overcome this limitation, we established an assay combining an immuno-based electroluminescence assay (Mesoscale discovery technology, MSD), and liquid chromatography-mass spectrometry (LC-MS) to detect longer Aβ peptides and to identify all smaller (3-, 4-, 5-, 6-, and 7-) peptides derived from APP processing (Fig. 1 and 2). Importantly, analysis of de novo Aβ peptide generation is carried out in the absence of detergents and recombinant substrate. We used HEK cells expressing APPswe (HEK/APPswe cells) that we incubated with the reversible γ-secretase inhibitor semagacestat for 19 h prior to cell harvesting in order to accumulate non-γ-secretase processed APP and thereby increase the signal by subsequent MSD and LC-MS analysis (Fig. 1B). We observed that the cell membrane preparation contained relatively more α-secretase-generated APP CTFs (C83) than BACE-catalyzed beta-stub C99 (Fig. 1C), presumably as the incubation of the cells with semagacestat prior to cell harvesting allowed α-secretase processing of C99 to C83. Both, APP intracellular domain (AICD) and Aβ42 were readily detected in the membrane assay and their production was γ-secretase activity dependent, since the addition of semagacestat blocked the production of both peptides (Fig. 1, B and C).

We next compared the membrane-assay derived Aβ profile to that obtained from conditioned medium from HEK/APPswe cells. The profiles were similar and peptides of different lengths (Aβ34, 37, 38, 39, 40, and 42) were detected in the conditioned medium (Fig. 1D) and in the membrane assay indicating the near-native condition. Some differences were also observed, as the relative amounts of Aβ34 was higher and of Aβ37 and Aβ42 lower in the membrane preparation as compared with conditioned medium (Fig. 1D) and as calculated from the raw data obtained by MSD analysis of the membrane de novo Aβ assay (Fig. 1E). The reasons for these differences are unknown but may stem from the fact that we compare total Aβ production with the secreted pool of Aβ (conditioned media, non-membrane associated Aβ) or that the clearance rate of selected Aβ peptides are different in the two systems.

Several shorter peptides were detected by LC-MS, using the same membrane preparation from the HEK/APPswe cells (Fig. 2). By analyzing the short peptide spectrum we identified 3- and 4 peptides as well as a 6 peptide (Aβ40–35) resulting from γ-secretase cleavage (Fig. 2, A–E). In some experiments the sensitivity of the assay enabled us to identify γ-secretase dependent generation of 3- and 4 peptides across the entire transmembrane sequence, including reactions that results in Aβ34–32 and Aβ34–31 (data not shown). We identified some non-γ-secretase-dependent impurities, exhibiting the same retention time as Aβ51–49, Aβ42–40, and Aβ41–39, which precluded determination of their absolute levels (peptides marked with an asterisk in Fig. 2E). In sum, we have developed a membrane assay for de novo Aβ detection to monitor all major Aβ peptides as well as a number of shorter 3-, 4-, and 6-mer peptides derived from γ-secretase mediated processing of the APP transmembrane sequence.

Complete Processing of the APP Transmembrane Sequence and Regulatory Steps in Aβ42 Production—By combining the data from the immuno and LC-MS analyses on the longer and shorter peptides, respectively, we identified major cleavage events that aligned along two distinct routes across the APP transmembrane helices (Fig. 2E). One processing route spans from Aβ51 via Aβ48, Aβ45, Aβ42, Aβ39, or Aβ38 and then further to Aβ34. Such processing events will from herein be denoted as the Aβ42 product line. The other processing route started from Aβ49 via Aβ46, Aβ43, Aβ40, to Aβ36 or Aβ37, and then further to Aβ34 and will from herein be denoted the Aβ40 product line (Fig. 2E). In some experiments we could also identify γ-secretase-dependent Aβ34–32 and Aβ34–31 peptides, which result in Aβ31 and Aβ30 production (data not
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FIGURE 1. De novo Aβ production in membrane preparations from HEK/APPswe cells mimics cellular Aβ production. A, schematic depiction of the APP transmembrane sequence with γ-secretase cleavage sites (γ, ζ, ε) and the FAD APP mutations (T714I, V717F, L723P) explored in this study highlighted. B, HEK/APPswe cells were either left non-treated (−) or exposed to the γ-secretase inhibitor (GSI) semagacestat (+) for 19 h prior to cell harvesting and membrane preparation (substrate accumulation). Exposure of cells with GSI prior to membrane preparation results in higher Aβ1–42 and Aβx-42 signals as compared with membranes derived from non-GSI-treated cells. Direct addition of semagacestat to the membranes in the novo Aβ production assay inhibited Aβ production (semagacestat 1 μM). The amount of Aβx-42 is higher than Aβ1–42 suggesting that C83 is the major substrate for the Aβ42 signal detected. C, Western blot analysis of APP derivatives in the same assay reveals AICD production (lane Vehicle). The addition of GSI (semagacestat, 1 μM) to the reactions prevents the formation of AICD (lane GSI). C83 is the dominating APP species in the membranes in accordance with the data obtained in B. Recombinant C99 was loaded as reference (lane C99). D, MSD analysis of different Aβ peptides in HEK/APPswe cell culture supernatants (dark bars) and in the de novo Aβ production assay (light bars), respectively. Data are presented as the relative amount specific Aβ peptides compared with total Aβ and the data are based on three independent experiments. The graph shows the average ± S.D., and statistics were calculated with Student’s unpaired t test. ns, non significant, **, p < 0.01, ***, p < 0.001. E, raw data from MSD analysis of Aβ peptides from the membrane de novo Aβ assay. Max, no treatment; Min, in presence of 1 μM semagacestat. Data shows the mean of duplicates from three independent experiments (Signal) and the standard deviation (Stdev).

shown). γ-Secretase cleavages at every third amino acid thus dominated the production of Aβ peptides. Based on the abundance of each 3- or 4-peptide, our data suggest that the reactions resulting in Aβ49–47, Aβ46–44, Aβ43–41, and Aβ40–37 are the most prominent (Fig. 2D). Our experiments did not address whether Aβ production is a stepwise cascade of proteolytic reactions, starting at the epsilon cleavage site and continues in a precursor-product manner resulting in shorter Aβ peptides, or reflects single independent cleavage events of the APP transmembrane sequence. However, the results obtained are in large compatible with both of these potential mechanisms. It is worth to mention that the amount of the Aβ43–41 peptide was almost twice as high as the upstream Aβ46–44 peptide, suggesting that either other precursors of Aβ43 exists, that the quantification is less precise for this particular Aβ species or that the precursor-product hypothesis could not fully explain γ-secretase-mediated APP processing.

The data also indicate that γ-secretase processing is not limited to the two routes and a switch from the Aβ40 product line to the Aβ42 product line can happen at two cleavage steps. The first switch point occurs at Aβ49, which besides forming Aβ49–47 produces the 4-peptide Aβ49–46. The second switch point occurs at Aβ46, which besides forming Aβ46–44 also generates Aβ46–43 (Fig. 2E). We also identified additional positions within the APP transmembrane sequence where γ-secretase processing in a similar manner resulted in either 3- or 4-peptides: Aβ48–45 and Aβ48–46; Aβ45–42 and Aβ45–41; and Aβ34–32 and Aβ34–31 (Fig. 2E and data not shown). In addition to all 3- and 4-peptides identified, we also discovered Aβ40-Aβ35, the only 6-peptide identified in our analysis. Together, these data suggest that γ-secretase mediated APP processing occurs across the entire APP transmembrane sequence and that the major cleavage reactions occur along two major routes, denoted the Aβ40 and 42 product lines, respectively, which both converge at Aβ34. The data also define proteolytic events that result in switch points between the major routes revealing alternative reactions to Aβ42 production and to the regulation of the Aβ42/40 production ratio.

FAD APP Mutations Switch the Cleavage Preference of γ-Secretase from the Aβ40 to the Aβ42 Product Line—We next used the immuno/LC-MS assay to study the impact of FAD mutations on APP processing, and to explore why the mutations cause elevated Aβ42 levels. We selected three different mutants, (T714I, V717F, and L723P), which correspond to Aβ numbering 43, 46, and 52, localizing the mutations to the Aβ40 product line. To assess the effect of the mutations, we
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analyzed membrane preparations of SH-SY5Y cells stably transfected with the different FAD APP constructs. All three FAD mutants showed an increased or retained relative production of Δβ38, Δβ39, and Δβ42, as compared with wild-type APP (Fig. 3A). In contrast, the T714I and L723P mutants caused a decrease in Δβ40 production relative to wild-type APP (Fig. 3A). Three all FAD mutations caused an increase in the Δβ42/40 ratio as compared with wild-type APP (Fig. 3B), in agreement with previous data from tissue culture studies (20).

From the same SH-SY5Y/APP membrane preparation we established a small peptide profile very similar to that obtained from HEK293/APPsw-derived membranes except that the level of each peptide was lower and that we could not detect Δβ46–43 and Δβ38–35 (compare Figs. 2D and 3C). The three FAD APP mutations however caused a shift in the distribution of the small peptides, such that the production of Δβ48–46, Δβ45–43, and Δβ42–39 peptides were increased or retained, whereas the level of Δβ49–47, Δβ46–44, Δβ43–41, and Δβ40–37 peptides was reduced or retained (Fig. 3C), indicating that the Δβ42 product line was enhanced at the expense of products from the Δβ40 line. The difference in abundance was observed already at the epsilon cleavage event, as judged by the increase in Δβ48–46 peptide and as compared with the decrease in Δβ49–47 (Fig. 3, C and D). None of the mutants gave rise to product line switching, as we could neither detect the Δβ49–46 nor the Δβ46–43 peptide (Fig. 3C), which is indicative of an Δβ40 to Δβ42 product line conversion (see above). Collectively, these data suggest that the three FAD APP mutations favor the Δβ42 over the Δβ40 product line but that product line switching does not occur later in the processing cascades.

**Modulation of the Aβ Profile by the Type and Concentration of GSM**—In contrast to GSIs, which produce unwanted side effects on other γ-secretase targets, GSMs have been developed to reduce Δβ42 levels. To gain insights into the mode of action of GSMs, we assessed whether first and second generation GSMs differed in their effect on APP processing as first generation GSMs target APP while second generation GSMs appear to preferentially interact with γ-secretase (11, 13). Since the membrane assay mainly monitors γ-secretase mediated processing of C83 (Fig. 1, B and C), we first wanted to make sure that GSMs also modulate ADAM10 involving P3 peptide production. To test this we exposed mouse embryonic fibroblasts, derived from RACE-deficient mice and lacking BACE activity (Fig. 3A), and Δβx-42, suggesting that GSMs also modulate P3 production in cellular systems (Fig. 4, A and B). We then studied the pharmacology of the NSAID compounds R-Flurbiprofen and Sulindac Sulﬁde as first generation GSMs and AZ4800, AZ4126, E-2012, and AZ1136 as second generation GSMs in the de novo Aβ production assays using HEK/APPsw cells membranes (Fig. 4C). Importantly, all compounds were tested at concentrations not affecting total Aβ production in the membrane assay, ensuring that we studied Aβ modulation and not inhibition (Fig. 4D). At a higher concentration AZ4800 (25 μM) derived total Aβ was slightly reduced suggesting that this GSM becomes inhibitory at higher concentrations (Fig. 4D).

Both the first and second generation GSMs reduced Δβ42 more efficiently than Δβ40 and increased the levels of Δβ37 and Δβ38 (Fig. 4C). Using the LC-MS assay on the same sample membrane incubations, the spectrum of short peptides was identical to that observed in non-treated cells or the FAD APP mutants, indicating that the basic processing pattern was not altered by GSMs. The observed Aβ modulation was paralleled by an increase in the Δβ42/39, Δβ40–38, and Δβ38–35 peptides (Fig. 4E) suggesting that GSMs stimulate multiple cleavage reactions. Interestingly, all GSMs except R-Flurbiprofen and Sulindac Sulﬁde caused a decrease in the Δβ40–35 peptide, suggesting that GSMs also cause a reduction in specific reactions (Fig. 4E). By using the LC-MS data to calculate the Δβ40 and Δβ42 production/turnover ratio we found that all GSMs explored affected Δβ42 turnover to a larger extent (Fig. 4F).

The first and second generation GSMs however differed to some extent in their processing patterns. R-Flurbiprofen and Sulindac Sulﬁde showed an induction of Δβ34 whereas neither of the other GSMs showed this effect (Fig. 4C). Processing from Δβ46 to Δβ42 (Δβ46–43) was greatly enhanced by all second generation GSMs, except AZ1136, whereas R-Flurbiprofen in contrast caused a profound decrease in Δβ46–43 and Sulindac Sulﬁde showed a strong tendency, too (Fig. 4E).

We also found a dose-dependent Aβ modulatory effect, which was paralleled by an alteration in specific short Aβ-deprived peptides (see Δβ46–43, Δβ42–39, Δβ40–38) (Fig. 4, C and E). Combined, these data show that first and second generation GSMs influence the APP processing pattern and specific reaction steps in the two product lines dose-dependently, but do not induce novel cleavage reactions.

**DISCUSSION**

To better understand how APP is processed to various Aβ forms, and in particular to Δβ42, is central for Alzheimer research and drug development. Progress in elucidating the mechanism of Aβ production has been made, but not all aspects of the Aβ processing are known. With the combined

![FIGURE 2. Aβ42 is generated from different APP-derived intermediates. LC-MS analyses of native APP processing in the de novo Aβ production assay in HEK/APPsw-derived membranes. A, total ion chromatogram from LC-MS analysis of APP transmembrane sequence derived peptides in the de novo Aβ production assay. B, settings of the mass spectrometer for the specific detection of the peptides analyzed. C, illustration of γ-secretase dependence of selected peptides. Upper trace: Max signal, no treatment; Lower trace: membrane assay performed in presence of 1 μM of the GSI semagacestat. The scale on the upper and lower traces is the same for each pair of chromatograms and the max signal was set to 100%. Note that the LVM signal (bottom right graph) was only partially inhibited by semagacestat, which precluded absolute quantification of this peptide. D, graph shows the average amount of γ-secretase activity-dependent peptides and stem from the same three experiments as in Fig 1D. The data are presented in 3 sets: the Aβ40 and 42 product lines and other peptides, respectively. E, summary of the data presented in an illustration of the APP transmembrane sequence presented as an α-helix in a two-dimensional lay-out. The numbering is according to Aβ1-x. Each peptide is illustrated as an arrow, where black arrows are 3-mer; white arrows are 4-mer; gray arrow is 6-mer. The arrows are written in C→N-terminal direction. Impurities were migrating at the same retention time as peptides LVM, VIA, and VVI and excluded their direct quantification (marked with an asterisk). Statistics were calculated with Student’s unpaired t test. ns, nonsignificant, **, p < 0.01, ***, p < 0.001. Some peptides were clearly γ-secretase dependent but we could not perform statistics (na, not applicable), since their concentration was below the limit of quantification (0.2 nmol) in the presence of semagacestat. Peptides analyzed but not detected are illustrated as hatched arrows (light gray is 3- and 4-mers; dark gray, 5- to 7-mers).
immuno- and LC-MS assay to study de novo Aβ production, we can simultaneously analyze both the long Aβ peptides and the APP/Aβ-derived short peptides under near-physiological conditions with cell autonomous synthesis, trafficking, and interaction between γ-secretase and APP. Using this assay we provide novel data that explain how the generation of major Aβ peptides occurs in vivo and how generation of these peptides is affected by FAD APP mutations and GSMs. A full set of Aβ

FIGURE 3. APP FAD mutants affect APP transmembrane sequence product-line preferences resulting in an increased Aβ42/40 production ratio. A, membranes were prepared from SHSY5Y cells expressing either wild type or the APP FAD mutants illustrated in Fig. 1A. The graph shows the relative level of different Aβ peptides generated in the Immuno/LC-MS assay as determined by MSD analysis. Bars show the average from three independent experiments. B, each APP FAD mutant give rise to an increased Aβ42/40 production ratio as compared with wild-type APP. Note the large effect of the T714I mutant. C, LC-MS analysis of the effect of the APP FAD mutations on APP TMS processing. γ-Secretase-mediated processing of wild-type APP results in a similar pattern of APP transmembrane sequence derivatives as APP processing in membranes derived from non-neuronal membranes (i.e. Fig. 2, D–E). All mutations cause a decrease in the Aβ40 product line and a concomitant retained or increase in the Aβ42 product line. D, two-dimensional illustration of the APP transmembrane sequence with the major reactions affected by the APP FAD mutants highlighted with circles: black circle, retained or increased, gray circle, decreased or retained. All data are from three independent experiments, and the figure shows the average ± S.D. Statistics were calculated with Student’s unpaired t test. ns, nonsignificant, *, p < 0.05, **, p < 0.01, ***, p < 0.001.
**FIGURE 4.** **GSMs affect distinct γ-secretase reactions resulting in Aβ modulation.** MSD analysis of different P3 peptides in conditioned media from BACE-deficient MEFs cells, exposed to the GSMs AZ4126 (A) or R-Flurbiprofen (B) for 24 h prior to analyzes. Both compounds show a GSM characteristic modulation of P3 peptides as analyzed by the MSD triplex (Aβ38/40/42) assay using 4G8 as detection antibody. C—F, membranes from HEK/APPswe cells were used in the de novo Aβ production assay and incubated with GSMs R-Flurbiprofen (1 mM), Sulindac Sulfide (125 μM), AZ4800 (50 nM and 25 μM), AZ4126 (50 nM), AZ1136 (25 μM), and E-2012 (5 μM). C, all GSMs cause an Aβ modulation that mimic their effect in cellular assays. D, total Aβ was not affected by any treatment or compound besides AZ4800 at 25 μM, which caused a 25% decrease in Aβ suggesting that the compound becomes inhibitory at higher concentrations. E, same reactions were analyzed by LC-MS analysis. Note the GSM-induced effect on the Aβ40–42, Aβ40–35, Aβ42–39, Aβ38–35, and Aβ46–43 peptides, as well as the opposite effect of first generation GSMs (R-Flurbiprofen and Sulindac Sulfide) and second generation GSMs on Aβ46–43 and Aβ40–35, respectively. The basal level of Aβ49–46 is very low resulting in large experimental variations. The inset shows a chromatogram for Aβ46–43 in the presence of increasing concentrations of AZ4800. AZ4800 (25 μM) was set to 100%. F, ratio of peptides resulting in Aβ42 production/turnover and Aβ40 production/turnover is decreased in presence of the GSMs, and the difference in ratio is highest for Aβ42 for all compounds tested. The figure shows the average result from three independent experiments and the statistics were calculated with Student’s unpaired t test. ns, nonsignificant, *, p < 0.05, **, p < 0.01, ***, p < 0.001.
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peptides was produced, such as Aβ34, Aβ37, Aβ38, Aβ39, Aβ40, and Aβ42. Such cleavage products were captured from membrane preparations suggesting that the assay reflects near-native γ-secretase-mediated APP processing.

The data demonstrate the existence of several major cleavage reactions that take place at every third or fourth amino acid residue and line up along two separate routes across the APP transmembrane sequence: the Aβ42 and the Aβ40 product line, respectively. The Aβ42 product line denotes reactions resulting in the 3-peptides Aβ31 – 49, Aβ48 – 46, and Aβ45 – 43 and the corresponding Aβ peptides Aβ48, Aβ45, and Aβ42. Similarly, the Aβ40 product line represents reactions resulting in Aβ49 – 47, Aβ46 – 44, and Aβ43 – 41, where the latter cleavage reaction results in Aβ40 production. Importantly, we show that the two processing routes continue beyond Aβ40 and Aβ42. The extension of the Aβ42 product line results in Aβ39, Aβ38, Aβ34, Aβ31, and Aβ30, respectively whereas the Aβ40 line produces Aβ37, Aβ36, Aβ34, Aβ31, and Aβ30. It is interesting that Aβ34 represents a convergence point for the two product lines, as it can be produced both via reactions of the Aβ40 line (Aβ37 – 35) and the Aβ42 line (Aβ38 – 35). Interestingly, γ-secretase also generates the hexameric peptide Aβ40 – 35, which represents the only exception to the cleavages at every third and fourth amino acid residue in our study and which provides an alternative mechanism to Aβ34 production. A summary of all γ-secretase catalyzed APP processing events observed is shown in Fig. 5.

Our data reveal a certain complexity and flexibility in the process of Aβ production, which suggests that the generation of amyloidogenic Aβ peptides could be regulated at several levels of γ-secretase-mediated APP processing. Accordingly, we identify specific processing events that form “cross-talks” between the two product lines and impact the balance of Aβ40 and Aβ42 generation. These cross-talks occur at positions Aβ49 and Aβ46 of the Aβ40 product line and results in the 4-peptides Aβ49 – 46 and Aβ46 – 43 and the full length Aβ peptides Aβ45 and Aβ42 of the Aβ42 product line. Similarly, we identify reactions that “steer away” from the Aβ42 product line. These reactions take place at positions Aβ48 and Aβ45 and generate the 4-peptides Aβ48 – 45 and Aβ45 – 42 and the full-length peptides Aβ44 and Aβ41, respectively. Moreover, our data identify another five reactions that directly impact the Aβ40 and Aβ42 levels and which result in the following peptides: Aβ40 – 38 and Aβ37, Aβ40 – 37 and Aβ36, Aβ40 – 35 and Aβ34, Aβ42 – 40 and Aβ39, and Aβ42 – 39 and Aβ38. These findings may be of significant interest in AD drug discovery efforts since they suggest that the modulation Aβ40 and Aβ42 production and the Aβ42/40 ratio could be modulated at different levels of γ-secretase-mediated APP processing.

The data in this report significantly extend previous observations from reconstituted systems, where Aβ production has been explored using recombinant C100, the immediate APP-derived substrate for γ-secretase, and solubilized membrane extracts as the source of enzyme (15, 16). Such data concur with our findings that APP is processed along two major product lines, the Aβ40 product line resulting in the release of Aβ40 and the Aβ42 product line resulting in Aβ42 and Aβ38 as major forms. The reconstituted systems however fail to generate the shorter Aβ peptides, such as Aβ30-Aβ37, which are naturally occurring Aβ peptides present both in CSF and in cell culture media (22).

Several important findings considerably contribute and complement our understanding of Aβ generation and distinguish our work from previous data delineating the Aβ processing pathway (15, 16). First, we measure true de novo APP processing in near-native cell membranes under conditions of the full spectrum of in vivo relevant Aβ species. Second, for the first time we show that γ-secretase catalyzed processing at every third or fourth amino acid continues beyond Aβ40 and Aβ38 and we show how this mechanism results in major shorter Aβ peptides including Aβ30, Aβ31, Aβ34, Aβ37, and Aβ39. Third, we identify Aβ34 as a major convergence point for the product lines, which may also explain the particular sensitivity of this Aβ species in recent human trials with two different γ-secretase inhibitors (19, 24). Fourth, and perhaps most importantly, we identify cross-talks between the major Aβ product lines, and several novel reactions resulting in Aβ46 – 43, Aβ42 – 40, Aβ40 – 38, and Aβ40 – 37 and that directly impact Aβ40 and Aβ42 production opening up new opportunities for modulation of Aβ42 production and the Aβ42/40 ratio. It is important to emphasize that our work has assessed γ-secretase-mediated APP processing in membrane preparations from cells express-

**FIGURE 5. Summary of major APP transmembrane sequence processing events and GSM pharmacology.** APP is subjected to two major γ-secretase-catalyzed processing routes resulting in Aβ40 and 42, respectively. Minor cleavage events result in cross-talks between the major routes and alternative pathways to modulate Aβ42 production and the Aβ42/40 production ratio (hatched arrows). The major processing routes converge at Aβ34, which is further hydrolyzed by γ-secretase resulting in Aβ30, which is the shortest BACE and γ-secretase-dependent Aβ1-x peptide identified in human CSF (19). All peptides (arrows) are written in C-N terminal direction. Each Aβ peptide may stem from a 3- or 4- amino acid longer Aβ peptide in a precursor-product cascade. Alternatively, each Aβ peptide is not necessarily derived from a 3- or 4- amino acid longer Aβ peptide. Our data did not directly assess these two alternatives, but the data obtained with GSMs support the former mechanism: GSMs cause an increased turnover (+) of Aβ40 to Aβ37, Aβ42 to Aβ38, and Aβ38 to Aβ34 without affecting either Aβ40 or Aβ42 production to a major extent. All potent non-acid second generation GSMs display a robust increase (+) in Aβ46 – 43 and a decrease (–) in Aβ40 to Aβ34, whereas two GSMs of the NSAID class display a reduction in Aβ46 – 43 and a tendency toward increased Aβ40 – 35 production. Other cleavage events were also affected by some of the GSMs, but to a lesser extent and/or less consistent to the reactions outlined above.
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ing full length APP but the data does not prove whether the different 3- and 4-peptides as well as the 6-peptide identified stem from processing of longer Aβ peptides into shorter Aβ peptides in a precursor-product relationship, which was originally proposed by Takami and colleagues (15). An alternative mechanism to the precursor-product mechanism is that the 3-, 4- and 6-peptides are formed as individual products as a result of γ-secretase-mediated processing of C83 or C99 as direct substrates. In our analysis we find that the stoichiometry of 3- and 4-peptides generated does not always match up, such as the levels of Aβ43–41 of the Aβ40 product line were twice the amount of its “precursors” Aβ49 – 47 and Aβ46 – 44. Whether these data support the latter hypothesis for alternative mechanisms to the precursor-product hypothesis or reflects a lack of precision and difficulties associated with LC-MS quantification of certain peptides or is related to cellular clearance activity of small peptides is not clear. Recent reports from Ihara et al., however, have shown that recombinant Aβ43 and Aβ42 could indeed be processed into Aβ40 and Aβ37 and Aβ38 and Aβ39, respectively, when added to solubilized and reconstituted membrane preparations. These findings would rather support the precursor-product hypothesis but, as discussed above, with the caveat that they were generated in an experimental system failing to recapitulate central aspects of Aβ production and need to be confirmed during more physiological relevant conditions.

FAD APP mutations frequently cause an accumulation of Aβ42 via an increased Aβ42/40 production ratio, but how this is accomplished is not well understood. Our data show that the membrane assay indeed retained this phenotype and all three different FAD APP mutations studied, T714I, V717F, and L723P, caused a significant increase in the Aβ42/40 ratio. This effect was in particular evident for the T714I mutation, which has been reported to cause an even earlier disease onset versus the other two mutations (disease onset in the third versus fifth decade of life). These observations are in consensus with the theory that the Aβ42/40 ratio plays an important role for disease onset (6). Despite affecting the Aβ ratio with different magnitudes, our data show that all three mutants affected APP processing in a very similar way. Each of the mutants displayed a clear reduction in the Aβ40 product line. As the level of all intermediates was increased in the Aβ42 product line, we interpret it as the primary effect of all three mutations possessing a preference for the Aβ42 product line. A likely explanation is that the FAD APP mutations result in a conformational change of the APP transmembrane sequence, which makes it a more amenable substrate for the Aβ42 processing pathway. This view is in agreement with a recent report, which suggested that several APP FAD mutants increase the Aβ42/40 ratio via an enhanced preference for the Aβ42 product line (17).

GSMs cause a decrease in Aβ42 and Aβ40, which is accompanied by an increase in shorter Aβ peptides, such as Aβ37 and Aβ38 (9, 10, 23). In accordance with studies performed with recombinant Aβ peptides as substrate (16), our data suggest that GSMs increase the turnover of Aβ40 and Aβ42 into Aβ37 and Aβ38 rather than altering the preference for γ-secretase for these specific reactions on the expense of reactions generating Aβ40 and Aβ42. These data directly suggest that γ-secretase could process longer Aβ peptides into shorter Aβ species. Indeed, the GSMs caused a net increase in the turnover of Aβ40 and Aβ42 into Aβ37 and Aβ38, and the induced net effect of Aβ42 production/turnover was stronger than of Aβ40 production/turnover. This finding explains why GSMs in general are more potent in inhibiting Aβ42 compared with Aβ40 production (10, 13). In addition to the increased levels of specific 3- and 4-peptides, we also found the opposite, i.e. that GSMs cause the reduction in specific products. Accordingly, the Aβ40–35 levels were significantly reduced by many of the GSMs explored. We also show that GSMs regulate several other processing steps that either impact Aβ40 and Aβ42 production or other Aβ peptides. For second generation GSMs, there was a robust relative increase in the Aβ46 – 43 peptide, and more moderate increases in Aβ42–39 and Aβ38 – 35 levels. These findings suggest that these GSMs stimulate γ-secretase processing at every fourth amino acid from the level of Aβ46 to Aβ34. In contrast to the second generation GSMs, the first generation NSAID class of GSMs caused a very clear reduction in Aβ46 – 43 while they also caused an increase in Aβ42 to Aβ34 turnover. A possible explanation for this GSM generation-specific difference is that APP-targeting first generation GSMs and the presenilin-targeting second generation GSMs affect the APP/γ-secretase interaction through different mechanisms, e.g. differing in their affinities to multiple binding sites either on the target or the enzyme. We have earlier proposed a model suggesting that GSMs interfere with the dimerization of the APP homodimer, and thereby facilitate a more efficient processing of Aβ40 and Aβ42 into shorter Aβ peptides (12). The data presented here indicate a more complex situation, where GSMs appear to be associated not only with activation of cleavage reactions but also reduction of other reactions.

In conclusion, we have established conditions under which APP processing can be monitored in native cells from full-length proteins. We provide new insights into the Aβ peptide spectrum and hierarchy of processing under normal conditions and during genetic and pharmacological modulation, thereby opening new avenues to modulate the Aβ42/40 ratio by targeting specific processing steps.

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