Supplementary data

Figure S1. In vitro generation of ICD products from APPC99, Notch, ErB4 and N-Cadherin is γ-secretase dependent. *In vitro* activity assays using CHAPSO extracted membranes from **A)** *Psen1/2*$^{-/-}$ mEFs stably transduced with human wt or FAD *PSEN1* mutants and purified substrates-3XFlag. Substrate concentrations: 1,125 μM APP-C99 or 2 μM for the other substrates**. B**) *Psen1/2*$^{-/-}$ mEFs stably transduced with human wt PSEN1 and purified wt- or FAD-APP-C99-3XFlag. Addition of the transition state analog inhibitor L-685,458 demonstrates that ICD products are generated in a γ-secretase dependent manner.

Figure S2. Wt or FAD *PSEN1* mutant Aβ profiles evidence accumulation of Aβ intermediary products. *In vitro* activity assays are performed with CHAPSO extracted membranes from *Psen1/2*$^{-/-}$ mEFs stably expressing human wt or FAD *PSEN1* mutants and saturating concentrations of APP-C99 substrate (1.125 μM). **A).** Treatment with 10μM inhibitor L-685,458 excludes γ-secretase independent de novo Aβ generation. **Lanes 1 and 2.** Control reactions: no membranes or no substrate added. **Lanes 3-5, 7-8 and 16:** Aβ standards containing the indicated peptides. **Lane 6:** wt γ-secretase Aβ product profile. **Lane 9-15:** wt or FAD *PSEN1* γ-secretase Aβ product profiles in the presence of 10μM inhibitor X. *** Arrow** indicates a non specific band present in the C99 substrate reaction (lane 1) **B)** FAD *PSEN1* Y115H, L166P, G384A and DeltaExon9 mutants accumulate long Aβ species (≥Aβ46). 1:2, 1:4 or 1:8 dilutions of the activity reactions were loaded in urea-denaturing gels.

Figure S3. $AICD_{50-99}$ neo-antibody specificity. $AICD_{50}$ neo-antibody was tested against 10 or 100ng $AICD_{50-62}$ and $AICD_{49-62}$ synthetic peptides in western blot analysis. The neo-epitope antibody is able to interact with the $AICD_{50-62}$ but not with the $AICD_{49-62}$ peptide (lanes 1-2 vs. 3-4). Neo-epitope and FLAG antibodies were detected with secondary antibodies coupled to different fluorophores. Signals in red and green for the neo and anti-Flag antibodies; respectively. As expected, the neo-epitope antibody does not recognize C99-FLAG substrate (lane 5). **Arrow** indicates the neo-antibody specific signals**. *** Dye front.

Figure S4. Aβ products in the conditioned media of HEK293 cells transfected with wt or FAD-C99 mutants. HEK293 cells transiently transfected with human wt C99 or mutants were maintained in DMEM supplemented with 2% serum. Media were collected at 48h post-transfection and Aβ levels determined by Elisa. Secreted Aβ43 was at undetectable levels in the media. Plot shows mean ± s.d. Notice the same shifts in profiles (increase of the Aβ38 pathway, decrease of the Aβ 40 pathway) as measured with the cell free assays discussed in the paper

Figure S5. Aβ38 drives amyloid aggregation and elicits acute synaptotoxicity. A) Aggregation kinetics of 10μM, 30 μM, 45μM and 90 μM Aβ ratios in 50 mM Tris, 1 mM EDTA at 25°C followed by Thioflavin T (ThT) fluorescence shows the influence of short Aβ peptides (Aβ38 or Aβ40) on the

aggregation kinetics of the Aβ42:Aβ38 (1:9) and Aβ42:Aβ40 (1:9) mixes. **B)** Aβ synaptotoxicity on primary neurons. Primary mouse hippocampal neurons were cultured on MicroElectrode Array (MEA) substrate for 7-10 days. Synaptic activity was recorded as a function of increasing Aβ concentration. Aβ42 (dark grey) and Aβ43 (black) are significantly synaptotoxic at a concentration of 0.5 μM. At a concentration of 1 μM Aβ38 (white) inhibits activity at 15 %. Values are percent of initial firing rate ± SEM; culture response was first normalized to the initial basal firing rate. Recorded values were averaged over all active electrodes from different chips. Different buffer treatments were each normalized to 100 %. Statistical significance (unpaired 2-tailed t-test) is indicated by *** P<0.0001 or ** P<0.0005. (5 independent cultures, n=3 MEA substrates for Aβ38 and Aβ42, n=4 MEA substrates for Aβ40 and Aβ43). For further details on method see (Kuperstein et al, 2010).

Figure S6. Dose-response inhibitory assays for semagacestat, begacestat and avagacestat. IC_{50} curves for GSI using CHAPSO extracted membranes from *Psen1/2*$^{-/-}$ MEFs stably expressing human wt PSEN1 mutants and 400 nM APP-C99 substrate (1X Km concentration). All GSI inhibit Aβ38, Aβ40, Aβ42 and Aβ43 production to similar extents. Aβ products are plotted as percentage of control reaction (DMSO). Error bars indicate S.D. (n=3); except for semagacestat plot (S.E., n=5).

Figure S7. Effects of increasing concentrations of GSM 1-3 on γ-secretase activity. A). *In vitro* activity assays using CHAPSO extracted membranes from *Psen1/2*$^{-/-}$ mEFs stably expressing human wt PSEN1 and 0.4 or 1.75 μM APP-C99 substrate (1X Km or saturating concentrations, respectively). The three GSM (at $1X10^{-5}$, $1X10^{-6}$, $1X10^{-7}$ M) do not affect the endopeptidese activity of the γ-secretase (no effect on AICD production). AICD production is plotted as percentage of control reaction (DMSO). **B)**. Increasing concentrations of GSM 1-3 on Aβ38/Aβ42 and Aβ40/Aβ43 ratios show that GSM 1-3 activate the 4th catalytic cycle of the γ-secretase; in particular, the Aβ48>Aβ38 product line. Panels A&B show mean ± s.d.; statistical significance of the data (n=3) tested with ANOVA and Dunnett's post test, vehicle (DMSO) as control group; * P<0.05.

Material & Methods

ThT fluorescence assay

Aβ peptides were prepared as described in (Kuperstein et al, 2010). Briefly, Aβ42/Aβ40 or Aβ42/Aβ38 were mixed in the molar ratio (1:9) at a final concentration of 1 mg ml^{-1}. The peptide mixes were separated from DMSO by means of a 5-ml HiTrapTM desalting column (GE Healthcare, Sweden) and eluted into a 50-mM Tris, 1 mM EDTA buffer, pH 7.5. Peptide concentrations were then measured by Bradford assay. Aβ peptides were further diluted to 50 μM in 50 mM Tris, 1 mM EDTA buffer. The fibrillation kinetics were followed in situ using a Fluostar OPTIMA fluorescence plate

reader at an excitation wavelength of 440 nm and an emission wavelength of 480 nm in presence of 12 μM thioflavine T. Readings were recorded in triplicate every 10 min for a period of 6 h.

Synaptotoxicity on primary neurons

Trypsinized brain from 17-days-old FVB mice embryos was used to generate hippocampal neurons. Cultures were plated at 1,000 cells/mm^2 on a MEA substrate (Multichannel Systems GmbH, Germany) and maintained in neurobasal medium supplemented with B27 for 3 days at 37 °C in a humidified 5 % CO_2 atmosphere. Then, medium was changed to neurobasal medium supplemented with B27, without $_L$-glutamate and cells were cultured for 8-10 days prior to further experiments. During the recording experiment (Kuperstein et al, 2010), a temperature controller (Multichannel Systems) was used to maintain the MEA platform temperature at 37 °C. The basal firing rate was recorded during 500 sec. Subsequently, cultures were titrated with pre-aggregated Aβ (1.5 h, 25 °C) at final concentrations of 0.5 and 1μM at 500 sec intervals. The spontaneous synaptic activity was continuously recorded during these 500 sec. Raw signals from MEA electrodes were amplified by MEA1060 amplifier (gain 1200) (Multichannel Systems) and digitized by the A/D MC_Card at a sampling rate of 25 kHz. MC_Rack 3.5.10 software (Multichannel Systems) was used for data recording and processing. The raw data stream was high-pass filtered at 200 Hz, and the threshold for spike detection was set to 5 standard deviations of the average noise amplitude computed during the first 1000 ms of recording. The firing rates recorded by all electrodes were averaged over the 500-sec time bins and were normalized to buffer treatment. Experiments were performed using 5 independent cultures, n=3 MEA-chips for Aβ38 and Aβ42, and n=4 MEA-chips for Aβ40 and Aβ43. Results were analyzed using two-tailed unpaired t-test for significance. Significance is indicated by *** $P<0.0001$ and ** $P<0.0005$.

Quantification of total Aβ using Sandwich ELISA

Sandwich ELISA were done as describe in Materials and Methods using the 4G8 (Convance Signet) as capture antibody at final concentrations of 1.5μg/mL in a final volume of 50μL coating buffer (10mMTris HCl, 10mMNaCl, 10mMNaN3, pH8.5). Synthetic human Aβ40 peptide was used for the preparation of the standard curve.

References

Kuperstein I, Broersen K, Benilova I, Rozenski J, Jonckheere W, Debulpaep M, Vandersteen A, Segers-Nolten I, Van Der Werf K, Subramaniam V, Braeken D, Callewaert G, Bartic C, D'Hooge R, Martins IC, Rousseau F, Schymkowitz J, De Strooper B (2010) Neurotoxicity of Alzheimer's disease Abeta peptides is induced by small changes in the Abeta42 to Abeta40 ratio. *EMBO J* 29(19): 3408-3420