# Supplementary methods

*Co-localization of CERTs and APP/Aβ in 5xFAD brain*

Immunofluorescent staining was performed in 10 µm thin sagittal brain section of 3 months old males 5xFAD. The sections were fixed with acetone 10 minutes, blocked with 3% BSA for 1h and incubated overnight at 4 oC with rabbit polyclonal anti-CERTS (epitope 300-350, Bethyl Laboratories) diluted 1:500 and anti APP/Aβ (clone 6E10 Covance) diluted 1:500. Next day sections were washed 3 times and the corresponding secondary antibodies conjugated to Cy2 or Cy3 (1:500) were applied for 1h at RT. After washing, the NeuroTrace 640/660 (ThermoFisher) was used following manufacturer instruction. Hoechst staining was performed for nuclei visualization. The slices were mounted with 80% glycerol and imaged.

*Lipidomics in CERTL transfected neuroblastoma cells*

Neuro-2a (N2a) obtained from ATCC (CCL-131™)were cultured in DMEM supplemented with fetal bovine serum (FBS), Pen/Strep, and L-glutamine. Cells were seeded in 75 cm2 flasks and maintained in complete DMEM for 24 hours prior to the transfection. Cells were transfected with 2 µg pcDNA3.1-CERTL (NM\_005713.3) by Effectene Reagent (Qiagen) following the manufacturer’s instructions. Cells transfected with 2 µg of plasmid expressing GFP were used as control. After 48 hours, cells were washed three times with PBS, trypsinized, and centrifuged at 300 x g for 5 min. Pellet was stored at -80 before the lipid analysis. Sphingolipid analysis was performed as explained in Material and Methods section.

Far Western blot

For Far Western experiments, human recombinant CERTL (hCERTL, 1875bp NP\_005704.1) (80ng), produced as previously described ([29](#_ENREF_29)), and Aβ peptide 1-42 (80ng) (Anaspec) were analyzed by SDSPAGE under reducing conditions. A 17kDa Lama antibody fragment (H6) with myc and hist6 tags (a kind gift of Dr A.J. Groot), was used as a negative control (80ng). Proteins were transferred to nitrocellulose membranes renatured in Tris-buffered saline (TBS) in the presence of Tween20 (0.05%) and probed for 1 hour at 37°C with 30µg/mL of CERTL or Aβ peptide in the same buffer. Bound complexes were detected using polyclonal rabbit anti-CERTs (epitope 1-50 of human CERTs, Bethyl Laboratories), mouse anti-Aβ mAb 6E10 (Covance) and mouse mAb anti-human c-Myc (clone 9E10, Invitrogen). Finally, incubation with secondary goat anti-rabbit IRdye 800 and donkey anti-mouse IRdye 680 (Rockland Immunochemicals) was followed as described above.

*Neuronal primary cell culture*.

Rat hippocampal neuronal cultures were prepared as reported elsewhere ([86](#_ENREF_86)). In brief, embryos from pregnant Wistar rats (Charles River) were removed on embryonic day 18. After removing the meninges, the hippocampi were insolated under the lupa preserved in the hibernate medium. Subsequently, they were incubated with trypsin for 20 minutes at 37 ºC. Hippocampus were then transferred to a tube with DMEM complete medium. With a sterile glass pipette, the cells were disbanded doing up and down until the tissue was disaggregated/disintegrated and homogeneous. Cells were plated at ~80,000 cell/ 35mm Ø plate containing coverslips and incubated in Neurobasal medium (supplemented with B27) (Thermo Fisher Scientific) for 2-3 weeks. The medium was partially exchanged 24 hours after and then once a week.

*Immunoassay for CERTs detection.*

CERTs were quantified by enzyme-linked immunoassay or Western blot. In brief, microplates were coated with 100 μL of polyclonal rabbit 01 (2,46 mg/mL) diluted 1:1000 using coating buffer (50 mM carbonate pH 9.6), sealed with a plastic sticker and incubated for 1 h at 37ºC. To build up the standard curve serial dilutions of recombinant CERTs, produced as previously explained ([29](#_ENREF_29)), were diluted in 10% BSA, 0.02% Tween-20 in PBS. Biotinylated polyclonal rabbit anti-CERTs 02, was used as a detection antibody diluted 1:500 in 1% BSA and 0.02% Tween-20 in PBS and incubated for 1 h at 37 ºC. Blocking, washes, and absorbance were performed as described in method sections immunoassays. Cortex sample were lysed in composed by 0.1% SDS, 0.1% Triton X-100, 1% glycerol, 1 mM EDTA, 1 mM EGTA, PhosSTOP and protein inhibitors (Roche). 40µg of protein extracted separated in SDS-PAGE electrophoresis and blotted onto nitrocellulose membrane. The membrane was probed with anti-GAPDH antibody (10R-G109A, Fitzgerald) as internal control and anti CERTs (epitope 300-350 of human CERTs, Bethyl Laboratories). After incubation with donkey anti-mouse, IRdye680, and anti-rabbit IRdye800 (Rockland Immunochemicals) diluted 1:10,000 in Odyssey blocking buffer, the membrane was scanned and analyzed with Odyssey imager Li-Cor. The intensities were measured with Odyssey imager Li-Cor.

CERTs inhibitor administration

HPA-12 was prepared in control solution PEG400/PBS 1:4 (V/V). Before re-suspension vehicle was sterilized by filtration and pH was adjusted to 7.4. PEG400 / PBS was warmed up to 37°C and vortexed to completely dissolve the compound. HPA-12 was administered subcutaneously (SC) for 4-weeks at the dose 4 µg / g animals every 48 hours. The volume injected per animal was of 0.15 mL with insulin syringes. HPA-12 was tested on 8-14 months old mice. The AD transgenic (E4FAD and E3FAD) mice were purchased from Dr. Mary Jo LaDu (University of Illinois at Chicago) and bred in house as described elsewhere ([87](#_ENREF_87)). The mice were housed socially on a 12-hour day-night cycle and had *ad libitum* access to food and water. All experiments were approved by the Animal Welfare Committee of Maastricht University and followed the laws, rules, and guidelines of the Netherlands.

## *Cytokines analysis*

Cytokines were measured in total protein homogenate extracted from the cortex in lysis buffer containing 0.1% SDS, 0.1% Triton X-100, 1% glycerol, 1 mM EDTA, 1 mM EGTA, phosphatase and protease inhibitors. Samples were diluted to 0.1mg / mL total protein and cytokines (IFN-γ, IL-1β, TNF-α, IL-6, IL-4, IL-10, and IL-33) were measured on Meso Scale Discovery V-PLEX custom mice (K152A0H-1) and read on a Meso Scale Discovery SECTOR 600 (Meso Scale Discovery).

# Supplementary figures

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**Supplementary figure 1. Immunostaining of CERTL in the brain and analysis of CERTL Aβ interaction by far Western blot.** A) Photomicrographs of a 5xFAD brain section showing CERT and APP/Aβ colocalization intra and extra neurons. CERT is shown in red, APP/Aβ in green, neurons in magenta and nuclei in blue. Arrows point to intra and extra-neuronal colocalization B) Image showing detection of recombinant CERTL separated by SDS-PAGE, transferred to membrane and detected with anti-CERTL antibody without Aβ1-42 peptide pre-incubation. CERTL was detected with anti-Aβantibody after Aβ1-42 peptide pre-incubation. IgG remained negative control. C) Image showing Aβ1-42 peptide separated by SDS-PAGE, transferred to membrane and detected with anti-Aβantibody (6E10). After recombinant CERTL incubation, Aβ1-42 peptide was immunolabeled with anti-CERTL antibody but not with a 17 kDa Lama antibody fragment (H6) (negative control). D) Percentage of Thioflavin T (ThT) fluorescence intensity to detect aggregation of Aβ1-42, concentrated 20 µM, alone or in presence of recombinant CERTL 2.5 µM, anti- Aβ epitope 1-16 (6E10) or 17-24 (4G8), dosed 0.1mg/mL, at 20 hours. Each data point represents the percentage of mean fluorescent intensity of three wells. (ANOVA; Dunnett's multiple comparisons test \*p< 0.05, \*\*\*p<0.001)

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**Supplementary figure 2. CERTs concentration in WT compared to 5xFAD and evidence of CERTL transduction *in vitro* and *in vivo*.** A) AAVs transduction on neuronal primary cell culture. Neonatal rat neuronal cells were isolated from the cortex of pups at embryonic day 18. After 2-3 weeks in Neurobasal medium were incubated with AAVs particles at the dose of 100 multiplicity of infection (MOI) for 6 days. CERTL was visualized in red and nuclei in blue. B) A timeline describing the experimental design to assess transduction efficiency *in vivo*. C) AAVs transduction in WT animals 1, 2, 6- and 12-weeks post-injection by immunofluorescence. CERTs were visualized in red and nuclei in blue (scale bar 50 μm). D) Immunofluorescence staining on brain sections showing injections site (CERT**L** in red and nuclei in blue). Scale bars 200 and 50 μm. **M1**: motor sensory cortex 1; **M2**: motor sensory cortex 2; **LV**: lateral ventricle; **I, II, III, IV,** and **V** cortical layers. E) AAVs were tested for brain transduction in WT animals 1, 2, 6- and 12-weeks post-injection by qPCR. Bars represent the mean ± S.E.M 3/per group. A significant increase in CERTL transduction was measured independently from time point (ANOVA; \*p< 0.05).

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**Supplementary figure 3. Sphingolipid changes in the hippocampus region of AAV-control or AAV-CERTL treated WT and 5xFAD mice.** Sphingolipids levels were measured in the hippocampus by HPLC-MS/MS. Ceramides were classified based on acyl chain number of carbons (Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:1). as well as sphingomyelin (SM d18:1/16:0, SM d18:1/18:0, SM d18:1/18:1, SM d18:1/20:0, SM d18:1/22:0 and SM d18:1/24:1). Ceramides levels were expressed as pg / mg tissue, while sphingomyelins were expressed as pmol/mg tissue. Bars represent the mean ± S.E.M per group (Two-way ANOVA, LSD, significant effects, \*p< 0.05; \*\*p<0.01; \*\*\*p<0.001).

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**Supplementary figure 4. Sphingolipid changes in plasma of AAV-control or AAV-CERTL treated WT and 5xFAD mice.** Plasma was collected 1 week before AAV injection (time1), 2 (time 2), and 6 (time 3) weeks after injection, and when the experiment was ended (time 4). Sphingolipids levels were measured at each time point by HPLC-MS/MS. Ceramides were classified based on acyl chain number of carbons (Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:1). Ceramides concentration is expressed in µ Molarity. Bars represent the mean ± S.E.M per group (Two-way ANOVA, Bonferroni correction, significant effects, WT AAV-control vs 5xFAD AAV-CERTL §p<0.05; 5xFAD AAV-control vs 5xFAD AAV-CERTL \*p<0.05).

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**Supplementary figure 5. Western blot analysis of APP fragments to study APP processing.** TBS cortex homogenate was analyzed by Western blot FL-APP, CTFβ, and Aβ bands were detected with 6E10 antibody at the band corresponding to respectively of 100, 15, and 6-4kDa.

Diagram, engineering drawing

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**Supplementary figure 6. Cytokines and apoptotic markers Bcl-2 and Caspase 3 were unaffected by AAV-CERTL treatment.** A) Cytokines measurements in brain homogenate with multiplex system IFN-γ, IL-1β, TNF-α, IL-6, IL-33, IL-4, and IL-10 (5-10 number of animals per group). **B)** Analysis of gene expression of apoptotic markers Bcl-2 and Caspase-3 (4-5 number of animals per group, two-way ANOVA, Bonferroni correction).

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Cortex** | | | | | **Hippocampus** | | | | | **Cerebellum** | | | | |
| **WT (N=11)** | | **5xFAD (N=12)** | |  | **WT (N=11)** | | **5xFAD (N=12)** | |  | **WT (N=11)** | | **5xFAD (N=12)** | |  |
| **Sphingolipid species** | **Mean** | **STDEV** | **Mean** | **STDEV** | **p-value** | **Mean** | **STDEV** | **Mean** | **STDEV** | **p-value** | **Mean** | **STDEV** | **Mean** | **STDEV** | **p-value** |
| **SPH** | 0.3 | 0.2 | 0.3 | 0.1 | *0.9475* | 0.2 | 0.1 | 0.3 | 0.1 | *0.2347* | 0.4 | 0.1 | 0.3 | 0.1 | *0.1823* |
| **SPA** | 0.5 | 0.2 | 0.5 | 0.2 | *0.5042* | 0.3 | 0.1 | 0.5 | 0.2 | **↑0.0216\*** | 0.4 | 0.2 | 0.4 | 0.1 | *0.9206* |
| **S1P** | 2.8 | 1.6 | 3.0 | 1.6 | *0.8084* | 2.0 | 0.8 | 3.0 | 1.8 | **↑****0.0038\*** | 3.2 | 1.5 | 3.6 | 2.3 | **↑0.0123\*** |
| **Cer d18:1/14:0** | 0.1 | 0.1 | 0.1 | 0.0 | *0.8960* | 0.0 | 0.0 | 0.0 | 0.0 | *0.4043* | 0.0 | 0.0 | 0.0 | 0.0 | *0.5884* |
| **Cer d18:1/16:0** | 0.8 | 0.3 | 1.1 | 0.3 | **↑0.0080\*** | 0.6 | 0.3 | 0.8 | 0.2 | **↑0.0400\*** | 0.5 | 0.3 | 0.6 | 0.2 | *0.6812* |
| **Cer d18:1/18:1** | 0.2 | 0.1 | 0.2 | 0.1 | *0.0531* | 0.1 | 0.1 | 0.2 | 0.1 | **↑0.0050\*** | 0.1 | 0.0 | 0.1 | 0.0 | *0.9309* |
| **Cer d18:1/18:0** | 40.1 | 14.0 | 43.9 | 10.5 | *0.4833* | 36.8 | 10.8 | 43.7 | 15.8 | *0.0959* | 14.6 | 4.7 | 14.3 | 3.9 | *0.8843* |
| **Cer d18:1/20:0** | 1.4 | 0.4 | 1.7 | 0.4 | *0.1361* | 1.0 | 0.3 | 1.4 | 0.3 | **↑0.0041\*** | 0.8 | 0.2 | 0.9 | 0.2 | *0.6595* |
| **Cer d18:1/22:0** | 0.6 | 0.2 | 0.7 | 0.2 | *0.1888* | 0.4 | 0.2 | 0.6 | 0.2 | **↑0.0222\*** | 0.6 | 0.2 | 0.6 | 0.2 | *0.9508* |
| **Cer d18:1/24:1** | 50.5 | 25.0 | 55.9 | 20.6 | *0.5752* | 31.3 | 11.1 | 32.1 | 9.6 | *0.7817* | 101.7 | 57.9 | 90.7 | 36.9 | *0.5888* |
| **Cer d18:1/24:0** | 0.4 | 0.2 | 0.5 | 0.2 | *0.5214* | 0.2 | 0.1 | 0.4 | 0.2 | *0.0666* | 0.5 | 0.2 | 0.5 | 0.2 | *0.9824* |
| **Total SM** | 2592.1 | 1221.9 | 2895.1 | 888.2 | *0.2631* | 4041.1 | 1926.9 | 5050.3 | 3730.6 | *0.4308* | - | - | - | - | - |

**Supplementary Table 1** Sphingolipid levels on cortex, hippocampus, and cerebellum of WT and 5xFAD treated with AAV-control

(student′s t‐test\*p<0.05)

↑ 5xFAD mean significantly bigger than WT animals

|  |  |  |  |
| --- | --- | --- | --- |
| Gene | GenBank Accession No. | Sequence (5'->3') |  |
| Bax | [XM\_011250780.2](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=1039777052) | CACCTGAGCTGACCTTGGAG | Forward |
|  |  | CCACGTCAGCAATCATCCTCT | Reverse |
| Caspase 3 | NM\_009810 | TGCAGCATGCTAAGCTGTA | Forward |
|  |  | GAGCATGGACAATACACG | Reverse |
| Bcl-2 | NM\_009741 | TGGGATGCCTTTGTGGAACT | Forward |
|  |  | GAGACAGCCAGGAGAAATCA | Reverse |
| CERTL | [NM\_023420.2](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=255982522) | ATGTTCACAGATTCAGCTCCC | Forward |
|  |  | CTTCTTCAACAACCAGTTGCC | Reverse |
| CERT | [XM\_011244694.2](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=1039744052) | CAGGATGTAGGTGGTGATGC | Forward |
|  |  | CACCTTTAACTGCATGAGTAGC | Reverse |
| GAPDH | XM\_017321385.1 | CTCATGACCACAGTCCATGC | Forward |
|  |  | TTCAGCTCTGGGATGACCTT | Reverse |
| Actin | [NM\_007393.5](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=930945786) | CTCTCAGCTGTGGTGGTGAA | Forward |
|  |  | AGCCATGTACGTAGCCATCC | Reverse |
| CD86 | NM\_019388.3 | GGGCTTGGCAATCCTTATCT | Forward |
|  |  | ACCAACTTTTGCTGGTCCTG | Reverse |
| Fizz-1 | NM\_020509.3 | GGAACTTCTTGCCAATCCAG | Forward |
|  |  | ACACCCAGTAGCAGTCATCCC | Reverse |

**Supplementary Table 2. RT-PCR primer sequences**