Stem Cell Reports, Volume *1*

Supplemental Information

APP Processing in Human Pluripotent Stem Cell-Derived Neurons Is Resistant to NSAID-Based γ-Secretase Modulation

Jerome Mertens, Kathrin Stüber, Patrick Wunderlich, Julia Ladewig, Jaideep C. Kesavan, Rik Vandenberghe, Mathieu Vandenbulcke, Philip van Damme, Jochen Walter, Oliver Brüstle, and Philipp Koch

Inventory of Supplemental Material

Supplementary Figure S1: Characterization of Alzheimer patient-derived iPSC lines (related to Figure 1).
Relates to main figure 1 and shows additional characterization of AD patient-derived iPSC lines and their neuronal differentiation.

Supplementary Figure S2: Primary GSM screen on hESC-derived neurons (related to Figure 2).
Shows the initial screen for GSMs in high concentrations on hESC-derived neurons and thus relates to the main figure 2.

Supplementary Figure S3: Low concentrations of NSAID-based GSMs are ineffective in human neurons (related to Figure 3).
Shows detailed information on the concentration dependency of GSMs in different cell systems as well as control experiments that all relate to the main figure 3.

Supplementary Experimental Procedures.

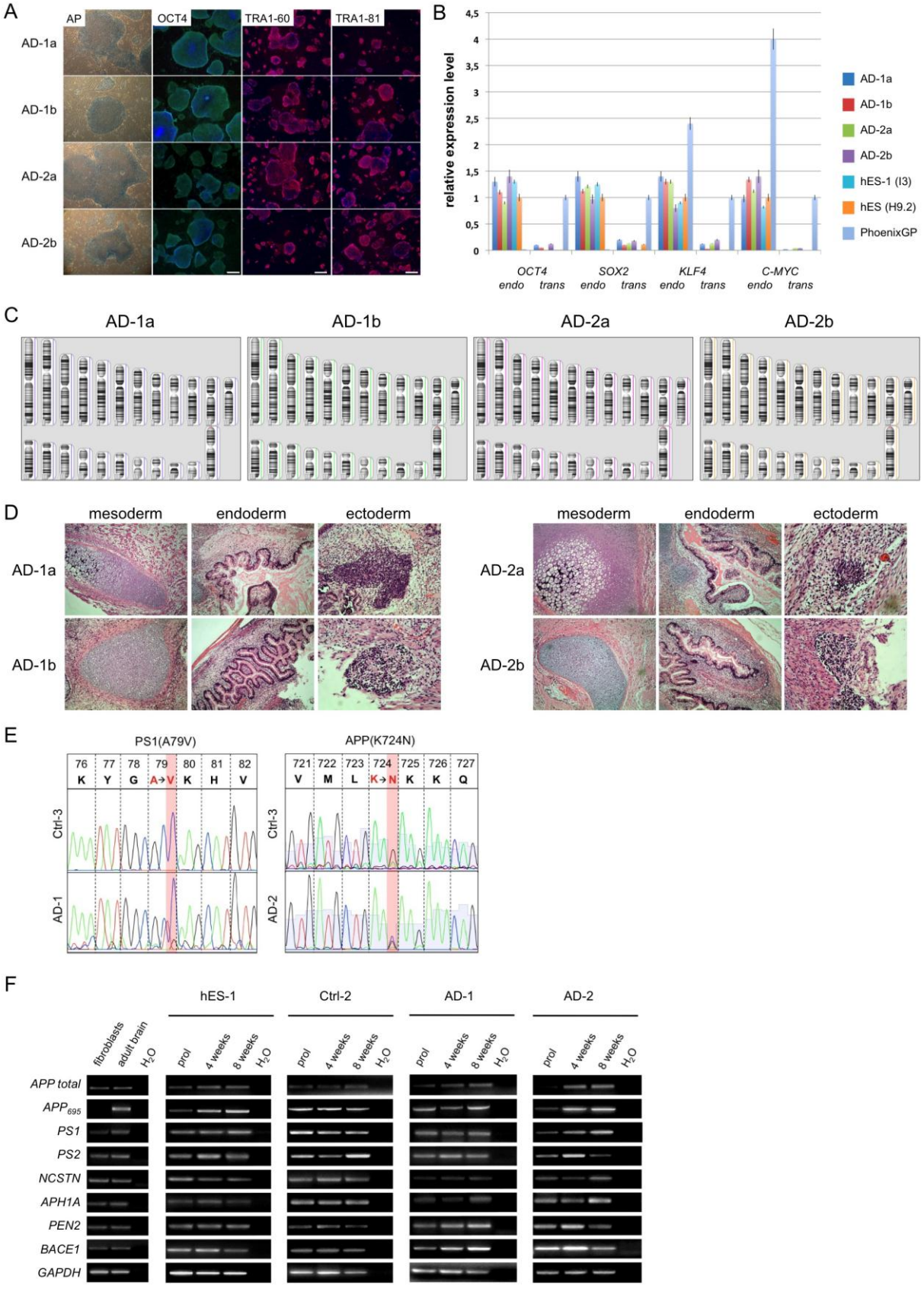
- Generation and culture of human iPSC lines.
- Generation of lt-NES cells
- Teratoma formation
- Karyotype evaluation by SNP analysis
- Transgene silencing of iPSCs
- Reverse-Transcriptase (RT)-PCR analysis for genes involved in APP processing
- Immunocytochemical analysis

- Electrophysiological recordings
- ELISA measurements of Aβ40 and Aβ42
- Conditional overexpression of APP in CHO cells and lt-NES cells and neurons
- Cell culture of APP-transgenic CHO and HEK cells
- Western immunoblotting
- Statistical analyses

Supplemental Table 1. Shows detailed information on patients and controls.

Supplemental References.

Supplemental Figure S1



Supplemental Figure S1: Characterization of Alzheimer patient-derived iPSC lines (related to Figure 1).

(A) IPSCs express the pluripotency-associated markers alkaline phophatase (AP), OCT4, TRA1-60 and TRA1-81. Scale bars 200 µm.

(B) Detection of endogenous (endo) and transgene (trans) expression of *OCT4*, *SOX2*, *KLF4* and *C-MYC* via RT-qPCR. RNA from two hESC lines (H9.2 and I3) and virus-producing PhoenixGP cells served as controls. Histogram shows relative expression levels. Expression of hESC line H9.2 was set to 1 for endogenous gene expression. Expression of virus producing PhoenixGP cells was set to 1 for transgene expression. Error bars show SD of triplicates.

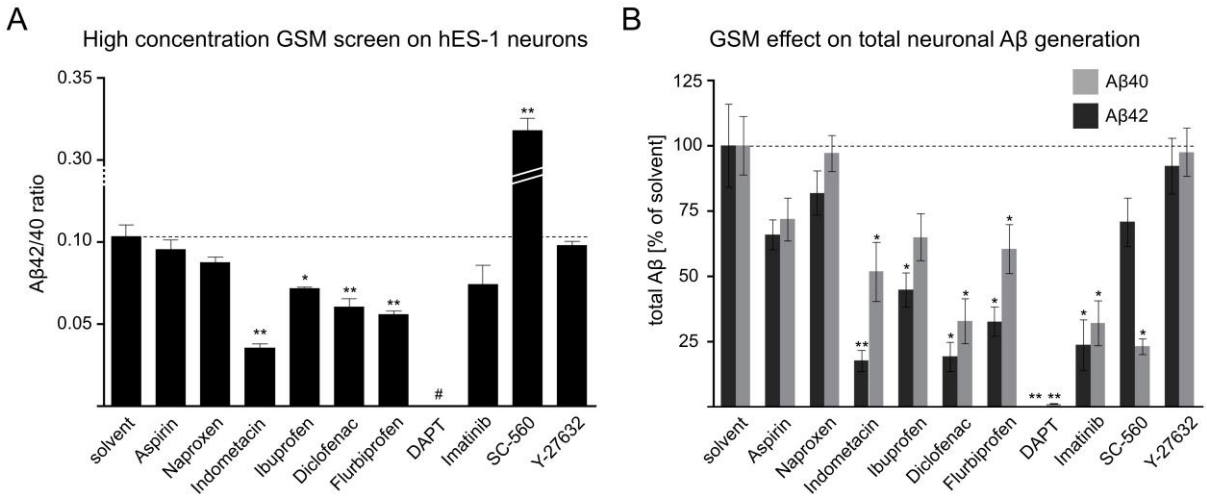
(C) DNA of iPSCs was subjected to SNP analyses. No significant chromosomal aberrations were detectable.

(D) IPSCs transplanted into the testes of immunosuppressed mice give rise to teratomas containing derivatives of all 3 germ layers such as neuroepithelium (ectoderm), smooth muscle and cartilage (mesoderm) as well as glandular structures (endoderm). Pictures were taken by a digital camera attached to a light microscope using a 10x (meso- and endoderm) or 20x (ectoderm) objective.

(E) DNA sequencing results of genomic DNA from Ctrl-3, AD-1 PS1(A79V) and AD-2 APP(K724N) lt-NES cells. Presented are regions of the PS1 and APP genes as indicated. The color code for the sequencing is as follows: A (green), C (blue), G (black), T (red) and N (pink)

(F) RT-PCR analysis for total *APP*, APP_{695}, γ-secretase genes and *BACE-1* in proliferating lt-NES cells as well as 4- and 8-week-differentiated cultures. RT-PCR was repeated 3 times; total RNA samples from human adult brain and fibroblasts served as controls.

Supplemental Figure S2

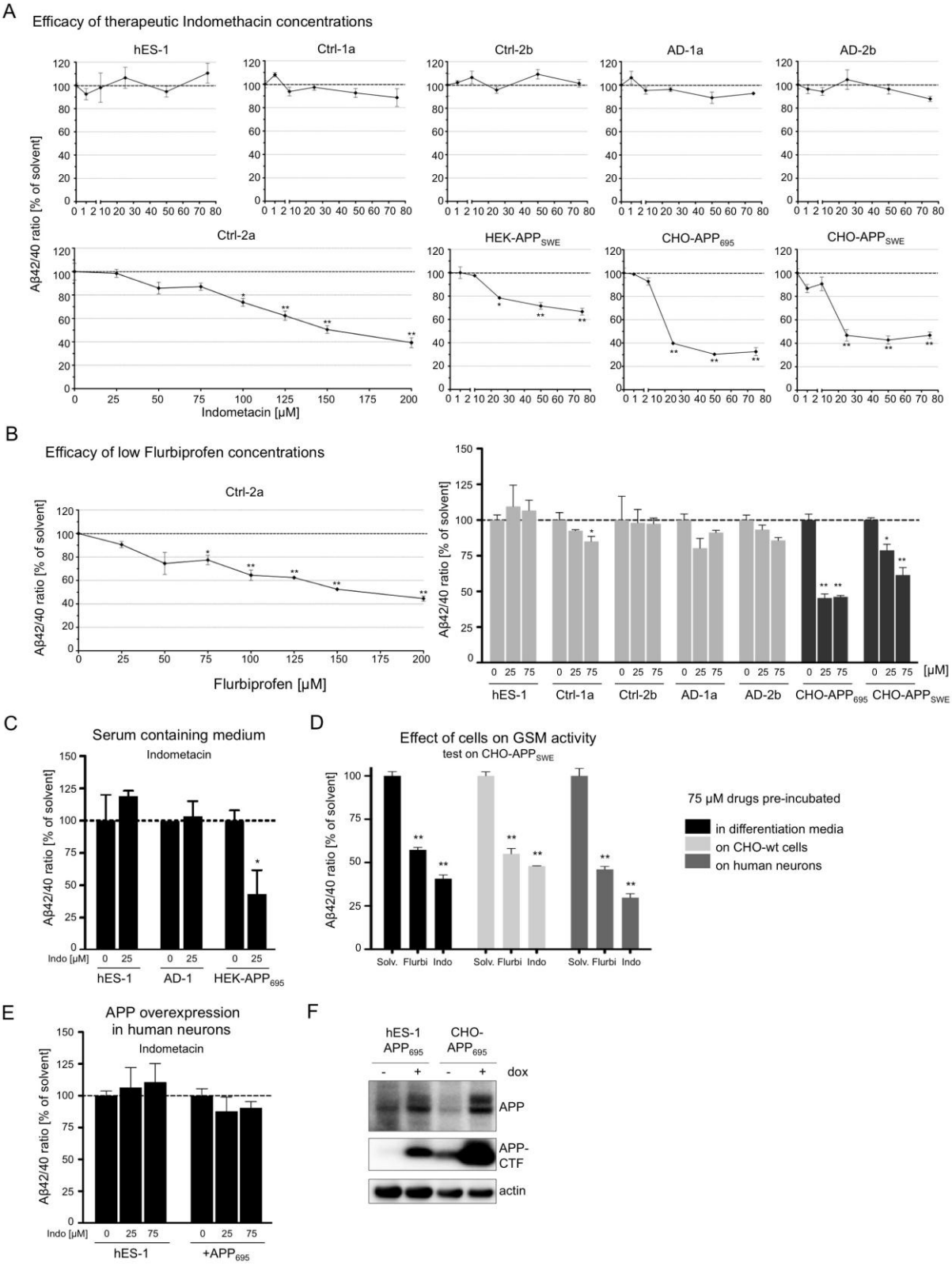


Supplemental Figure S2: Primary GSM screen on hESC-derived neurons (related to Figure 2).

(A) Differentiated neuronal cultures (4 weeks from hESC-derived lt-NES cells) were treated for 36 h with Aspirin (250 µM), Naproxen, Indometacin, Ibuprofen, Diclofenac, Flurbiprofen, SC-560 (all 200 µM) DAPT, Imatinib (all 10 µM), Y-27632 (5 µM) or solvent only. Graph shows calculated Aβ42/40 ratios.

(B) Diagram shows total Aβ40 and Aβ42 levels in conditioned media normalized to solvent treated neurons. All ELISA measurements were at least performed as biological triplicates and technical duplicates. #: below the detection limit of the assay. Bar graphs show mean ± SD and significance values were determined by ANOVA: *, P ≤0.01; **, P ≤0.001.

Supplemental Figure S3



Supplemental Figure S3: Low concentrations of NSAID-based GSMs are ineffective in human neurons (related to Figure 3).

(A) Effect of different Indometacin concentrations on the Aβ42/40 ratio of human neurons from 6 individuals and APP-transgenic HEK and CHO cells. Depicted are single graphs from main Figure 3 A as well as an additional full dose-response curve for Ctrl-2a up to 200 µM. Points mark mean ± SEM.

(B) Effect of Flurbiprofen on Aβ42/40 ratio. Left: Dose-response curve for Ctrl-2a treated with Flurbiprofen. Points mark mean ± SEM. Right: Bar graphs showing mean ± SD of Aβ42/40 ratios in human neurons, HEK and CHO cells in response to 25 µM and 75 µM Flurbiprofen.

(C) Human neurons generated from hESC- and iPSC-derived neurons and HEK-APP_{SWE} cells were cultured in serum-containing medium (MEF) and treated with 25 µM and 75 µM Indometacin for 36 h. Conditioned media were analyzed for Aβ40 and Aβ42 by ELISA.

(D) To detect a possible GSM-inactivating activity of human neurons, Flurbiprofen or Indometacin (both 75 µM) were pre-incubated at 37° for 16 h either in medium alone, on CHO_{WT} cells or on human neurons. Conditioned media were transferred to CHO-APP_{SWE} cells for 24h, and Aβ40 and Aβ42 were subsequently measured by ELISA. Bar graphs show mean + SD.

(E) Aβ42/40 ratios of APP_{695}-overexpressing hESC-derived neurons treated with different concentrations of Indometacin. All ELISA measurements were at least generated in biological triplicates and bar graphs show mean ± SD. Significance values were determined by ANOVA: *, $P \leq 0.01$; **, $P \leq 0.001$.

(F) Western immunoblotting of hES-1-APP_{695} and CHO-APP_{695} cells with and without doxycycline-mediated transgene induction for full-length APP, APP-CTFs and actin.

Supplemental Experimental Procedures

Generation and culture of human iPSC lines. For production of retroviral particles, PhoenixGP cells (ATTC #3514) were transfected with plasmids encoding the viral glycoprotein VSV-G and the reprogramming factors (OCT4, SOX2, KLF4 and C-MYC). Dermal fibroblasts were transduced twice with ultracentrifuge-concentrated virus in DMEM high glucose media containing 10% fetal calf serum (FCS), 1% sodium pyruvate (stock: 100 mM), 1% non-essential amino acids (stock: 10 mM) and 1% L-glutamine (stock: 200 mM) (all Life Techologies) in the presence of 10 ng/ml FGF2. Four days post transduction, cells were split into plates pre-seeded with mouse embryonic fibroblasts (MEFs). Medium was switched to human iPS cell culture medium containing KnockOut DMEM supplemented with 20% KnockOut serum Replacement, 0.1 mM non-essential amino acids (all from Life Techologies), 1 mM L-glutamine, 0.1 mM ß-mercaptoethanol and 4 ng/ml bFGF six days post-transduction and changed every other day. Three to four weeks after transduction, colonies with human ES cell morphology were manually picked, carefully triturated and expanded to establish human iPS cell lines.

Generation of lt-NES cells. For neural differentiation, iPSC colonies were detached and transferred to non-adhesive plastic dishes containing iPSC medium without ß-mercaptoethanol and without bFGF to form embryoid bodies (EBs). After 5-7 days, EBs were plated onto poly-L-ornithine/laminin-coated dishes. Emerging neural rosette structures were manually isolated at day 8-12 and cultured in suspension for 2 days in DMEM/F12, 2 mM L-glutamine, 1.6 g/l glucose, 0.1 mg/ml penicillin/streptomycin, N2 supplement (1:100; high transferrin, PAA), then dissociated with trypsin, plated onto PO/Ln-coated dishes and propagated in N2 medium supplemented with B27 (1 µl/ml, Life Technologies), 10 ng/ml FGF2 and 10 ng/ml EGF (both from R&D systems) as stable lt-NES cell lines.

Teratoma formation. For teratoma formation, 1 x 10^6 cells were resuspended as clumps in iPSC medium and injected into testes of immune-compromised SCID beige mice. After 5 - 8 weeks, teratomas were isolated, fixed in 4% PFA over-night, stained with hematoxylin and eosin and subjected to histological examination.

Karyotype evaluation by SNP analysis. Genomic DNA was prepared using the DNeasy Blood & Tissue Kit (Qiagen), diluted to 50 ng/µl and subjected to whole genome amplification. Amplified DNA was fragmented and hybridized to sequence-specific oligomers bound to beads on an Affimetrix Cytogenetics 2.7M chip. Data was analyzed using Affimetrix Chromosome Analysis Suite 1.0.

Transgene silencing of iPSCs. Total RNA was extracted using TRIzol reagent (Life Technologies), reversely transcribed into cDNA using the BioRad iScript Kit (BioRad). Quantitative RT-PCR was performed using the iCycler (BioRad) platform and data was normalized to GAPDH. Primers used for the analysis of transgene silencing and expression of pluripotency-associated genes were OCT4 endo (GACAGGGGGAGGGGAGGAGCTAG; GTTCCCTCCAACCAGTTGCCCCAAAC), OCT4 total (GTGGAGGAAGCTGACAACAA; ATTCTCCAGGTTGCCTCTCA), SOX2 endo (GTATCAGGAGTTGTCAAGGCAGAG; TCCTAGTCTTAAAGAGGCAGCAAAC), SOX2 total (GCCGAGTGGAAACTTTTGTCG; GCAGCGTGTACTTATCCTTCTT), C-MYC endo (TTCGGGTAGTGGAAAACCAG; CCTCCTCGTCGCAGTAGAAA), C-MYC total (AAGACTCCAGCGCCTTCTCT; TCTTGTTCCTCCTCAGAGTCG), KLF4 endo (GACCAGGCACTACCGTAAACA; CTGGCAGTGTGGGTCATATC), KLF4 total (CCCAATTACCCATCCTTCCT; ACGATCGTCTTCCCCTCTTT) and GAPDH (TGACAACTTTGGTATCGTGGA; CCAGTAGAGGCAGGGATGAT)

Reverse-Transcriptase (RT)-PCR analysis for genes involved in APP processing.
Cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). RT-PCR was performed using TaqDNA Polymerase (Life Technologies). As a negative control cDNA was omitted. cDNA from RNA extracts of human adult brain or fibroblasts (Stratagene, Agilent Technologies, Santa Clara, CA) and human fibroblasts was used as control. Primers were: APP total (TGGCCCTGGAGAACTACATC; AATCACACGGAGGTGTGTCA), APP_{695} (GAGGTGGTTCGAGTTCCTACAACAGC; AGGGCGGGCATCAACAGGCTCAA), PS1 (TGGTTGGTGAATATGGCAGA; GCGAGGATACTGCTGGAAAG), PS2 (CATCTGAGGGACATGGTGTG; AAACCTGCTGTGCTTCCTGT), Nicastrin (GGGACATTAAAGCCTGACGA; CGAGCTGCCAATGTAGTCAA), APH1a (GCATTTTTCTGGCTGGTCTC, AACCCCTCATCTGCCTTCTT), Pen2(CACCTCCTGGATCACCATCT; GGTCCTTTATTGGGGGATGT), BACE1(AGGCCATTCCCTGTAGGAGT; TTCCTGTCCTGGGAAAAATG), GAPDH (CTGCTTTTAACTCTGGTAAAGT; GCGCCAGCATCGCCCCA).

Immunocytochemical analysis. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA, 10 min, RT). For detection of the pluripotency-associated surface markers TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4, cells were blocked with 10% FCS in PBS, incubated with the primary antibodies (2 h, RT), washed with PBS, incubated with secondary antibody (45 min, RT) counterstained with DAPI (Sigma) and mounted with Mowiol 4-88 mounting solution (Carl Roth). For all other antibodies cells were blocked in

0.1% Triton X-100 (Sigma) and 10% FCS in PBS, incubated with the primary antibodies (16 hours, 4°C), washed with PBS, counterstained with DAPI and mounted with Mowiol 4-88. Primary antibodies and concentrations were as follows: Tra-1-60 (1:500, Life Technologies), Tra-1-81 (1:500, Life Technologies), SOX2 (1:500, R&D Systems), Nestin (1:600, R&D Systems), DACH1 (1:100, Proteintech), PLZF (1:50, Calbiochem), ZO-1 (1:100, Zymed), β-III tubulin (1:2000, Covance), MAP2ab (1:250, Chemicon), GFAP (1:1000, DakoCytomation), APP (1:1000, 4G8, Chemicon), PS1 (1:300, GeneTex), PHF1 (1:1000, gift from Peter Davies). Secondary antibodies were Alexa488 anti-ms, Alexa555 anti-ms, Alexa488 anti-rb and Alexa555 anti-rb (all 1:1000, Life Technologies).

Electrophysiological recordings. Whole cell current-clamp and voltage-clamp recording was carried out with an Axopatch- 200B amplifier (Molecular Devices, USA) interfaced by an A/D-converter (Digidata 1440, Molecular Devices, USA) to a PC running PClamp software (Version 10, Molecular Devices, USA). Pipette electrodes (GB150F-8P, Science products, Germany) were fabricated using a vertical puller (Narishige PC-10, Japan) and filled with a solution containing (in mM): 120 potassium gluconate ($C_6H_{11}O_7K$), 20 KCl, 10 NaCl, 10 EGTA, 1 $CaCl_2$, 4 Mg ATP, 0.4 Na GTP and 10 Hepes (pH 7.2; 280–290 mOsm). The signals were low-pass filtered at 2 kHz or 10 kHz and sampled at 10 or 50 kHz, respectively. All recordings were performed at room temperature (22 – 25°C) in a bath solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes and 25 D-glucose (pH 7.2; 310–320 mOsm). Electrophysiological recordings were performed at 3-5 months of in vitro differentiation. Action potentials were evoked by current injection steps from -50 pA to +140 pA in 10 pA increments. Neuronal membrane potentials were maintained at approximately -60 mV. Traces of whole-cell currents were elicited by 10 mV depolarizing voltage steps from a holding potential of −80 mV to +60 mV. Depolarizing voltage steps were further performed in the presence of 300 nM TTx and 100 µM Cd^{2+} and the K^+ channel blocker 4-aminopyridine (4-AP; 5 mM).

ELISA measurements of Aβ40 and Aβ42. Secreted Aβ40 and Aβ42 peptides in conditioned medium were quantified by a sandwich immunoassay using the Meso Scale Discovery Sector Imager 2400 as described previously (Page et al., 2010). Following incubation of Streptavidin-coated 96-well Multi-Array plates with biotinylated 2D8 capture antibody, media samples and Aβ peptide standards (Bachem) were added to the mixture. Ruthenylated C-terminal-specific anti-Aβ40 or anti-Aβ42 antibodies were used as detection antibodies. For detection, Meso Scale Discovery Read buffer was added, and the light emission at 620 nm after electrochemical stimulation was measured using the Meso Scale Discovery Sector

Imager 2400 reader. The corresponding concentrations of Aβ peptides were calculated using the Meso Scale Discovery Workbench software.

Conditional overexpression of APP in CHO cells, lt-NES cells and neurons. For conditional APP overexpression we used our previously described lentiviral tetOn system (Ladewig et al., 2012). The coding DNA sequences for human APP_{695} and APP_{SWE} were amplified by PCR from respective image clones and cloned into the lentiviral transfer vector. Production of lentiviral particles was performed as described previously (Koch et al., 2012). Briefly, HEK-293FT cells were co-transfected with the packaging plasmid psPAX2, the envelope plasmid pMD2.G and the respective lentiviral vector plasmid. Viral particles were enriched by centrifugation. CHO or lt-NES cells were first transduced with the virus carrying the rtTA protein and secondly with the respective transfer vector. Cells were cultured in the presence of G418 (200 µg/ml; PAA) and puromycin (1 µg/ml; Sigma Aldrich). For overexpression of APP_{695} or APP_{SWE}, CHO cells were treated with doxycycline (3 µg/ml; Sigma Aldrich). To overexpress APP_{695} in differentiated neurons, lt-NES cells were differentiated for 4 weeks before overexpression was induced by doxycycline treatment (3 µg/ml).

Cell culture of APP-transgenic CHO and HEK cells. Cell lines were cultured in MEF medium (DMEM-high glucose supplemented with 10% fetal bovine serum, non-essential amino acids 0.1 mg/ml penicillin/streptomycin (all from Gibco)). HEK cells were cultured in the presence of G418 (200 µg/ml; PAA) and transgenic CHO cells in the presence of G418 (200 µg/ml; PAA) and puromycin (1 µg/ml; Sigma Aldrich).

Western immunoblotting. APP was detected by Western immunoblotting in isolated membrane extracts. Cells were isolated and incubated in hypotonic buffer (10 mM Tris, pH 7.3, 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA) for 15 min on ice and subsequently homogenized by a 21 gauche needle. Nuclei were separated by centrifugation (3400 rpm; 10 min) and supernatants were centrifuged again at 13200 rpm for 60 min to obtain membrane fractions. Membrane protein isolations were separated by SDS-PAGE, transferred to nitrocellulose membrane and stained with the AB140 antibody. Blots were stained with HRP-conjugated secondary antibodies and visualized using the enhanced chemiluminescence reagent ECL (Amersham Pharmacia Biotech).

Statistical analyses. Quantitative data was generated at least in biological triplicates. Means and standard deviations were computed. Results presented as graphs show mean + SD for bar graphs and mean ± SEM for line graphs. ANOVA was performed to determine

whether a significant difference exists between groups (*, $P < 0.01$; **, $P < 0.001$).

Supplemental Table 1

<table>
<tr>
<td>Patient AD-1

Patient AD-1 (female; AD3/2) was evaluated for the first time in the memory clinic of the University hospital in Leuven, when she presented with an advanced stage of dementia. The diagnosis of AD was made in another hospital several years earlier. At presentation, she had memory loss, apathy, loss of insight. General neurological examination was unremarkable. Cognitive testing revealed an MMSE of 9/30. The patient was treated with cholinesterase inhibitors and showed a progressive disease course, but is still alive. A skin biopsy was performed at the age of 65.

The patient’s brother was known with a mutation in the presenilin 1 gene. On genetic testing an A79V mutation was confirmed in the patient as well. The mother died of dementia at the age of 78 years.</td>
<td rowspan="4">Patient AD-2

Patient AD-2 (female; AD1/1) developed a progressive memory deficit with onset at the age of 55. This interfered with her daily activities and made her job impossible. She did not have hallucinations or behavioral changes. General neurological examination was unremarkable. No extrapyramidal features were noted. She was on a treatment with cholinesterase inhibitors. On cognitive examination a memory deficit was present, with perseveration and a mild executive dysfunction (MMSE 18/30, Katz-scale 9, IADL 21, GDS 4). An FDG-PET showed hypometabolism in the parietal and temporal lobe with a left-sided predominance. Her father had developed a rapidly progressive dementia at the age of 52. He died 4 years later. No autopsy was performed.

Genetic testing of the patient revealed an APP gene mutation (Lysine 724 ASN). The disease course was progressive (MMSE dropped to 12 in two years time). A skin biopsy was performed at the age of 64. She died at the age of 66. An autopsy was performed and confirmed the diagnosis of Alzheimer’s disease with amyloid and tau pathology, Braak stadium V-VI.

This patient has also been described in Theuns et al., 2006, Human Mutation.</td>
</tr>
<tr>
<td>Control-1
male, healthy, age of biopsy: 34
(Pka in Falk et al., 2012)</td>
</tr>
<tr>
<td>Control-2
male, healthy, age of biopsy: 33
(PKb in Falk et al., 2012)</td>
</tr>
<tr>
<td>Control-3
male, suffers from ataxia, no dementia, age of biopsy: 38 (MJD-1 in Koch et al., 2011)</td>
</tr>
</table>

Abbreviations: MMSE: Mini-Mental-State-Examination; IADL: Instrumental Activities of Daily Living; GDS: Global Detoriation Skala.

Supplemental References

Koch, P., Tamboli, I., Mertens, J., Wunderlich, P., Ladewig, J., Stüber, K., Esselmann, H., Wiltfang, J., Brüstle, O., and Walter, J. (2012). Presenilin-1 L166P Mutant Human Pluripotent Stem Cell-Derived Neurons Exhibit Partial Loss of γ-Secretase Activity in Endogenous Amyloid-ß Generation. Am J Pathol. 180(6):2404-16.

Ladewig, J., Mertens, J., Kesavan, J., Doerr, J., Poppe, D., Glaue, F., Herms, S., Wernet, P., Kogler, G., Muller, F.J*., et al.* (2012). Small molecules enable highly efficient neuronal conversion of human fibroblasts. Nat Methods *9*, 575-578.

Page, R.M., Gutsmiedl, A., Fukumori, A., Winkler, E., Haass, C., and Steiner, H. (2010). Beta-amyloid precursor protein mutants respond to gamma-secretase modulators. J Biol Chem *285*, 17798-17810.

Theuns, J., Marjaux, E., Vandenbulcke, M., Van Laere, K., Kumar-Singh, S., Bormans, G., Brouwers, N., Van Den Broeck, M., Vennekens, K., Corsmit, E., *et al.* (2006). Alzheimer dementia caused by a novel mutation located in the APP C-terminal intracytosolic fragment. Hum. Mutat. 27:888-896.

Koch P, Breuer P, Peitz M, Jungverdorben J, Kesavan J, Poppe D, Doerr J, Ladewig J, Mertens J, Tüting T, et al. (2011) Excitation induced ataxin-3 aggregation in neurons from patients with Machado–Joseph disease. Nature 2011; 480: 543–6.

Falk, A., Koch, P., Kesavan, J., Takashima, Y., Ladewig, J., Alexander, M., Wiskow, O., Tailor, J., Trotter, M., Pollard, S., et al. (2012). Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons. PLoS One 7, e29597.