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Cell Reports, Volume *19*

Supplemental Information

The Alzheimer's Disease γ-Secretase Generates Higher 42:40 Ratios for β-Amyloid Than for p3 Peptides

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Supplemental experimental procedures

Mouse primary neuronal cultures

Mixed cortical/hippocampal primary neuronal cultures were prepared from E16 ICR (CD-1®) outbred mice (Harlan Laboratories, Horst, Netherlands). Cortices with adjacent hippocampi were dissected in ice-cold HBSS, incubated in 7ml TrypLe Express for 10min at 37°C and triturated in Dulbecco's modified Eagle's medium (DMEM) (1g/l glucose) containing 5% FCS (all from Life-Technologies) through repeated pipetting with a 5ml serological pipette and passed through a 70µM cell strainer. Neurons were cultured on poly-D-lysine (Sigma Aldrich, St. Gallen, Switzerland) coated 96-well plates. Cells were cultured in DMEM containing 5% FCS for the initial 12h, thereafter the medium was changed to Neurobasal medium supplemented with B27 (1:50) and 1mM GlutaMax (all from Life Technologies, Zug, Switzerland).

Human iPSC-derived neurons

Human induced pluripotent stem cells (iPSC) from two healthy male donors (33-years and 34-years old) were maintained in DMEM/F12, 2 mM L-glutamine, 1.6 g/l glucose, 0.1 mg/ml penicillin/streptomycin, N2 supplement, B27 (1:1000) (all from Life-Technologies), and 10 ng/ml fibroblast growth factor 2 and 10 ng/ml epidermal growth factor (both from Cell-Guidance-Systems) on poly-L-ornithine/laminin coated plates (both from Sigma-Aldrich). Neurons were differentiated by growth factor withdrawal for 4 weeks in differentiation medium (MACS Neuro Medium supplemented with MACS NeuroBrew-21 (1:50) (both from Miltenyi-Biotec)) and DMEM/ F12 supplemented with N2 (mixed at 1:1 ratio) that was exchanged every other day.

HEK-BACE1 cells

HEK cells stably expressing BACE1 (kind gift from Prof. D. Selkoe) were cultured in high-glucose DMEM (Life Technologies) containing 10% FCS. All experiments were performed between passage 5 and 15.

siRNA transfection

4DIV primary neurons were transfected with 100nM of siRNA (stealth siRNA, Life Technologies, Zug, Switzerland) using Lipofectamine RNAiMax (Life Technologies, Zug, Switzerland) as transfection reagent. A pool of four different siRNAs was used for each gene. A pool of three different non-targeting siRNAs served as negative control. For each well of a 96well plate, 0.45µl of RNAiMax were mixed with siRNA and Neurobasal in a final volume of 20µl and incubated for 20min at room temperature. Each transfection mix was filled up to 100µl with culture medium (Neurobasal supplemented with B27 and GlutaMax). The medium on the primary neuronal cultures was replaced with the transfection mix and incubated for 6h in the cell culture incubator, after which cells were again placed in fresh culture medium.

siRNA sequences:

negative controls		Catalog number (Life Technologies)
	medGC duplex #1	12935-111
	medGC duplex #2	12935-112
	medGC duplex #3	12935-113

target gene		
APP	Sense sequence 1	GCGGAUGGAUGUUUGUGAGACCCAU
	Sense sequence 2	UCAGGAUUUGAAGUCCGCCAUCAAA

	Sense sequence 3	GACCAGGUUCUGGGCUGACAAACAU
	Sense sequence 4	CACACACCCACAUCGUGAUUCCUUA
BACE1	Sense sequence 1	GAGCCCUUCUUUGACUCCCUGGUGA
	Sense sequence 2	GAGGGAGCAUGAUCAUUGGUGGUAU
	Sense sequence 3	CAUCCGGCGGGAGUGGUAUUAUGAA
	Sense sequence 4	GGCAGUGCAAGAGUCACAUUGUUAU
PSEN1	Sense sequence 1	ACUCUCUUUCCAGCUCUUAUCUAUU
	Sense sequence 2	GCACCUUUGUCCUACUUCCAGAAUG
	Sense sequence 3	UCGACUGCAGCAGGCGUAUCUCAUU
	Sense sequence 4	ACCAUCAAAUCAGUCAGCUUCUAUA
PSEN2	Sense sequence 1	AGUUCAUCCAUGGCUGGCUGAUCAU
	Sense sequence 2	CCACUAUCAAGUCUGUGCGUUUCUA
	Sense sequence 3	GAGAUGGAAGAAGACUCCUACGACA
	Sense sequence 4	CCCAGGAGAGAAAUGAGCCCAUAUU
ADAM10	Sense sequence 1	GCAUCUGAUCCCAAGCCCAACUUUA
	Sense sequence 2	CCUGAUCAUGUUAAUGGCUGGAUUU
	Sense sequence 3	CCUGCCAUUUCACUCUGUCAUUUAU
	Sense sequence 4	ACGCACAACUCUGGCUGAAAGAAAU
APH1C	Sense sequence 1	CCCAUCUAUGCAGUCACCGUUUCCA
	Sense sequence 2	GACUCACCCUAUUACUUCCUGACUU
	Sense sequence 3	AAGAAGGCAGAUGAGGGCUUAGCAU
	Sense sequence 4	CACCUUCUGACAUCGGGACUGACAU
APH1B	Sense sequence 1	GACCCUUUGCGAGUCAUCUUCCUCA
	Sense sequence 2	GGUGUCUCUCCUGCUGUCAUCCGUU
	Sense sequence 3	GCGCUCGCUCUUUAUGUCUUCACCA
	Sense sequence 4	CAGUACAGAAUUACCUGCUCAUCUU
APH1C	Sense sequence 1	GGUUCCUAGUGAGAGUCAUUACUAA
	Sense sequence 2	CAACAGAGAUGAAUCAGUACAGAAU

Sense sequence 3	GAGCUGUUCAGGCUCGCAUAUUAUA
Sense sequence 4	GCGUUGCUCUCUGUCUGUAUCCAAG

Plasmid transfection

HEK-BACE1 cells were seeded at ~30% confluency in DMEM containing 5% FCS on PDL-coated 48well plates at ~3h before transfection. For each well of a 48well plate 50ng plasmid DNA and 0.3µl of Lipofectamine2000 transfection reagent (Life Technologies, Zug, Switzerland) were mixed in OPTI-MEM (Life Technologies, Zug, Switzerland) in a final volume of 10µl and incubated for 20min at room temperature. Each transfection mix was filled up to 30µl with DMEM containing 5% FCS and then added to the medium (250µl) on the cells. ~16h after addition of the transfection mix, 280µl of DMEM containing 15% FCS were added to each well with transfected cells. $APP^{695}_{wildtype}$ plasmid was kindly provided by Dr. Uwe Konietzko. Mutations were introduced with the Quik-Change Lightning Site Directed Mutagenesis Kit (Agilent, Basel, Switzerland) following manufacturer's instructions.

Alamar Blue assay

Alamar blue TM assay reagent (AbD Serotec Ltd, Bio-Rad, Cressier, Switzerland) was added at a final concentration of 10% to the cell culture medium for 3h before termination of the medium collection period. 544^{EX}nm/590^{EM}nm fluorescence measurements were taken with a Spectra MAX-GEMINI-XS spectrofluorometer (Molecular-Devices, Sunnyvale, CA, USA).

BCA assay

Human iPSC derived neurons were lysed in TBS containing 0.1% SDS, 1% NP40 supplemented with protease inhibitor cocktail (Complete™ Mini, EDTA-free; Sigma

Aldrich, Buchs, Switzerland). Lysates were cleared from genomic DNA by 5min centrifugation at 2000 *x* g and protein concentrations were determined by Pierce BCA assay (Thermo Scientific, Waltham, MA USA) following manufacturer's instructions.

RNA isolation

For RNA extraction adherent neurons were washed with PBS and lysed in TRI-Reagent (Sigma-Aldrich, Buchs, Switzerland). Technical quadruplicates of each experimental condition were pooled and isolation of RNA was performed according to manufacturer's instructions. All RNA samples were subjected to DNaseI treatment (Fermentas; Life Technologies, Zug, Switzerland).

cDNA synthesis

500ng of total RNA were reverse-transcribed using the iScript cDNA-synthesis-kit (Bio-Rad, Cressier, Switzerland) following the manufacturer's protocol.

Real-time PCR

Real-time PCR for relative quantification of cDNA levels was performed with the 7900HT Real-Time PCR System (Life-Technologies, Zug, Switzerland), using the iTaq-SybrGreen Supermix with ROX (Bio-Rad, Cressier, Switzerland). Relative gene expression levels were calculated with the $\Delta\Delta C_T$-method using GAPDH for normalization.

Primer sequences:

Gene		
GAPDH	Forward primer	5' AGGUCGGUGUGAACGGAUU 3'
	Reverse primer	5' GGGUCGUUGAUGGCAACA 3'
APP	Forward primer	5' ACCGUUGCCUAGUUGGUGAGU 3'
	Reverse primer	5' CGGUGUGCCAGUGAAGAUG 3'
BACE1	Forward primer	5' CGGCAGACAUGGAAGACUGU 3'
	Reverse primer	5' AGGCAGAGUGGCAACAUGAA 3'

PSEN1	Forward primer	5' CGUGGCCACCAUCAAAUC 3'
	Reverse primer	5' GAUCGAGUGCAGGGCUCUU 3'
PSEN2	Forward primer	5' GAUGGAAGAAGACUCCUACGACAG 3'
	Reverse primer	5' CUUCACGCCCCUUUCCUC 3'
ADAM10	Forward primer	5' GCUGGGAGGUCAGUAUGGAA 3'
	Reverse primer	5' UCAUGUGAGACUGCUCGUUUG 3'
APH1A	Forward primer	5' AAGCUCCUUAAGAAGGCAGAUGA 3'
	Reverse primer	5' GAUACCGAAGGACAGACCAGAA 3'
APH1B	Forward primer	5' AGUCAUUACUGACAACAGAGAUGGAC 3'
	Reverse primer	5' GAGGGUGCUGUCUCCUCA 3'
APH1C	Forward primer	5' UGAGAGUCAUUACUAACAACAGAGAUGAAU 3'
	Reverse primer	5' CGCAUCGAGGGUGCUAUG 3'

SDS-PAGE and Western blot

Inhibitor treated mouse primary neurons were lysed in PBS, 1% NP40, 0.1 % SDS supplemented with protease inhibitor cocktail (Complete™ Mini, EDTA-free; Sigma Aldrich, Buchs, Switzerland). Protein from siRNA transfected neurons was recovered from the organic/phenol phase that was obtained during RNA isolation with TRI-Reagent (Sigma-Aldrich, Buchs, Switzerland) following the manufacturer's protocol for protein precipitation with acetone. The obtained protein pellet was resuspended in 9.5M Urea (pH9.0), 2% CHAPS. HEK-BACE1 cells were lysed in PBS, 1% NP40 supplemented with protease inhibitor cocktail (Complete™ Mini, EDTA-free; Sigma Aldrich, Buchs, Switzerland). HEK-cell lysates were treated with λ-phosphatase (NEB, Hitchin, UK). For analysis of APP CTFs upon inhibitor treatment, proteins were separated by SDS-PAGE using 10-20% Tris-Tricine gels (Life Technologies, Zug, Switzerland). For all other conditions, proteins were separated on 4-20% Tris-Glycine gels (Bio-Rad, Cressier, Switzerland). Proteins were blotted on 0.2µm Nitrocellulose membranes (Bio-Rad, Cressier, Switzerland). Unspecific binding was blocked by preincubation of membranes with TBS-Tween(0.05%) containing 5% w/v nonfat milk

powder. Incubation with primary antibodies was performed over night at 4°C, incubation with secondary antibodies for 1h at room temperature. Infrared signal at 700nm and 800nm were acquired with an Odyssey CLx Imaging System (Li-COR Biosciences, Bad Homburg, Germany).

Primary antibodies	Provider and product number	Antibody dilution used
mouse anti-Aβ (rat/mouse) (M3.2 clone)	Covance # SIG39155	1:500
rabbit anti-APP (C-terminus) (Y188)	Abcam # ab 32136	1:1000
rabbit anti-APP (C-terminus) (CT15)	Sigma Aldrich # A8717	1:1000
rabbit anti-BACE1	Abcam # ab108394	1:1000
rabbit anti-PSEN1	Abcam # ab76803	1:1000
rabbit anti-PSEN2	Abcam # 51249	1:2000
rabbit anti-ADAM10	Calbiochem #732-748	1:1000
rabbit anti-APH1A	Kindly provided by Bart De Strooper B80.3 clone	1:1000
rabbit anti-APH1B/C	Kindly provided by Bart De Strooper B78.2 clone	1:250
mouse anti-cMyc (9E10)	Santa Cruz # sc-40	1:1000
mouse anti-β-Actin	Abcam #ab6276	1:10000
mouse anti-GAPDH	Life Technologies #AM4300	1:5000

Secondary antibodies	Provider and product number	Antibody dilution used
donkey anti-mouse IRDye 800CW	Li-COR Biosciences # 926-32212	1:5000
donkey anti-mouse IRDye 680RD	Li-COR Biosciences # 926-68072	1:5000
donkey anti-rabbit IRDye 800CW	Li-COR Biosciences # 926-32213	1:5000

SULFO-Tag conjugation of antibody

Anti mouse/rat-Aβ (M3.2 mAb) antibody (Covance SIG-39155) was conjugated to SULFO-Tag using SULFO-TAG NHS-Ester reagent (Meso-Scale-Discovery) at 1:20 ratio following the manufacturer's protocol. The tagged antibody was separated from unconjugated SULFO-TAG NHS-Ester reagent using a ZEBA Spin Column 0.5ml 40kDa MW cut-off (Thermo Scientific, Waltham, MA USA)

Electrochemiluminescence assay

24h conditioned medium of 8DIV mouse primary neurons or HEK-BACE1 cells (72h post-transfection) or 20h conditioned medium of human iPSC derived neurons was used for analysis. Mouse or human $[Aβ/Aβ'+p3]_{40}$ and $[Aβ/Aβ'+p3]_{40}$ were analyzed with the Aβ-Panel-1 Kit (4G8) (Meso-Scale-Discovery). Murine $Aβ_{40}$ and $Aβ_{42}$ were analyzed with the Aβ-Panel-1 Kit assay plate (Meso-Scale-Discovery) together with SULFO-TAG anti mouse/rat-Aβ (M3.2 mAb) antibody. sAPPα and sAPPβ were analyzed with the sAPPα/sAPPβ multiplex assay (Meso-Scale-Discovery). Conditioned media from pimary neuronal cultures were analyzed undiluted. Conditioned media of HEK-BACE1 cells were diluted 1:5 for sAPPα/sAPPβ measurements in order to reach analyte concentrations within the linear detection range. Undiluted conditioned media of HEK-BACE1 cells was used for Aβ multiplex assays. Samples from γ-secretase *in vitro* cleavage assay were diluted 1:15 to 1:30 in order to reach analyte concentrations within the linear detection range. Sample incubation was performed either over night at 4°C (for Aβ assays) or for 2h at room temperature (for sAPPα/sAPPβ assay). 4G8 mAb, 6E10 mAb and anti-APP detection antibodies were used at 1:50 dilutions, M3.2 mAb at a concentration of 1µg/ml. Measurements were taken on a Sector-Imager-6000 (Meso-Scale-Discovery). For primary neurons, ECL values were normalized to the

corresponding Alamar-Blue assay values. For human iPSC derived neurons, ECL values were normalized to BCA assay measurements. Human $A\beta_{40}$ and $A\beta_{42}$ and sAPPα and sAPPβ standards were supplied with the respective kits. Mouse $A\beta_{40}$ and $A\beta_{42}$ standards were obtained from rPeptide (Bogart, GA, USA).

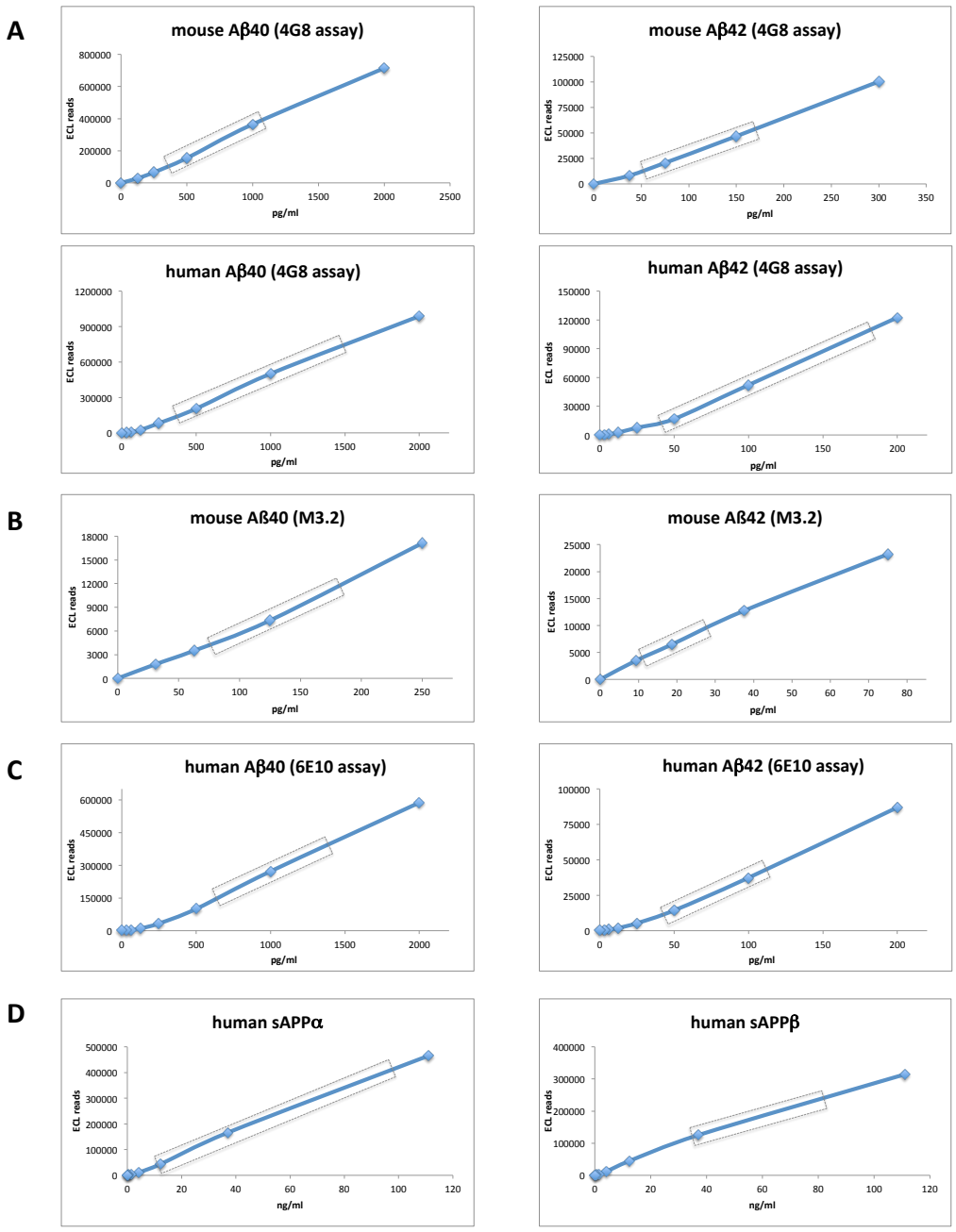
γ -Secretase activity assay

γ-Secretase assays using recombinant human APP C99-His, C89-His and C83-His were performed as previously reported (Cacquevel et al., 2008; Dimitrov et al., 2013; Wu et al., 2010). γ-Secretase purified from S20 CHO cells was solubilized in 0.2% (wt/vol) CHAPSO, 50 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$ and 5 mM $CaCl_2$ and incubated at 37°C for 4 h with 1 μM substrate, 0.1% (wt/vol) phosphatidylcholine and 0.025% (wt/vol) phosphatidylethanolamine. The generated product AICD-His was analyzed by Western Blot and immunoprecipitation-mass spectrometry (IP-MS) as previously described (Dimitrov et al., 2013). For details see supplementary material.

Immunoprecipitation-mass spectrometry (IP-MS) analysis of AICD-His

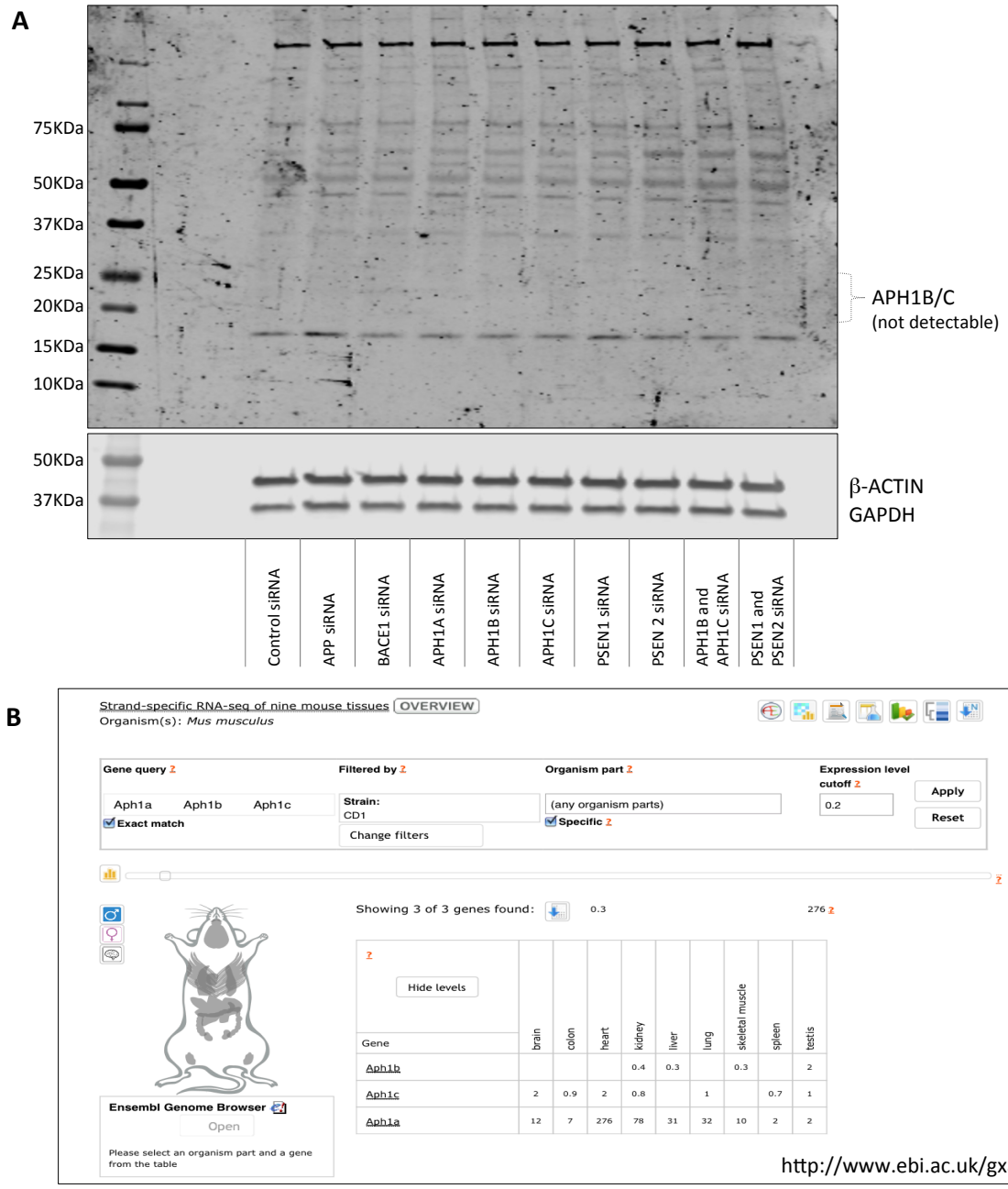
AICDs generated in γ-secretase *in vitro* assays with purified APP C99-His , C89-His and C83-His substrates were analyzed as previously described (Dimitrov et al., 2013). Briefly, Triton X-100 was added to a final concentration of 1% (vol/vol) after the enzymatic reaction and incubated for 20 min at 55°C prior to overnight immunoprecipitation at 4°C with rabbit anti-APP C-terminus antibody (CT15) (Sigma-Aldrich GmbH, Buchs, Switzerland) and protein A coupled to agarose resin (Roche Applied Science, Penzberg, Germany). AICD-His was eluted with 1:20:20 (v:v:v) 1% (vol/vol) trifluoroacetic acid:acetonitrile:H_2O, equally mixed with saturated sinapic acid and analysed by MALDI-TOF mass spectrometry in linear mode on an ABI 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Carlsbad, California, USA).

SUPPL. FIG.1



Suppl. Fig.1: *ECL multiplex assay standard curves*
The range of measured analyte concentrations for the control conditions of the cell-based experiments is indicated by the dashed box on each standard curve.
(A) Standard curves for human/mouse [Aβ/Aβ' + p3] assays (4G8 detection antibody) with mouse $A\beta_{40}$ and $A\beta_{42}$ standards (upper panels) and human $A\beta_{40}$ and $A\beta_{42}$ standards (lower panels).
(B) Standard curves for mouse Aβ assay (M3.2 detection antibody) with mouse $A\beta_{40}$ and $A\beta_{42}$ standards.
(C) Standard curves for human Aβ assay (6E10 detection antibody) with human $A\beta_{40}$ and $A\beta_{42}$ standards.
(D) Standard curves for human sAPPα/β assay with human sAPPα and sAPPβ standards.

SUPPL. FIG.2



Suppl. Fig.2: Low APH1B and APH1C expression in mouse primary neurons and mouse brain
(A) APH1B/C protein levels in lysates of siRNA-transfected mouse primary neurons were below detection limit by Western Blot. The rabbit anti-APH1B/C antibody (B78.2 clone) that was kindly provided by Bart de Strooper is a validated antibody that had been able to detect APH1B/C in different murine tissues and in a mouse embryonic fibroblast cell line (Hebert et al.. 2004; Serneels et al., 2005)
(B) The APH1B, APH1C and APH1A mRNA expression table for different tissues of CD1 mice (generated with the EMBL-EBI online gene expression atlas; http://www.ebi.ac.uk/gxa) shows very low mRNA expression of both APH1B and APH1C in the brain compared to APH1A.

Supplemental References:

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