BACE1 over-expression reduces SH-SY5Y cell viability through a mechanism distinct from Aβ-peptide accumulation; beta prime-mediated competitive depletion of sAβPPα

Lauren **Owens**[a,¶], Joshua **Bracewell**[a,¶], Alexandre **Benedetto**[a], Neil **Dawson**[a], Christopher **Gaffney**[b], Edward **Parkin**[a,*]

[a]Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, United Kingdom.

[b]Lancaster Medical School, Faculty of Health and Medicine, Lancaster University, Lancaster, United Kingdom.

[¶]These authors contributed equally to the research

Running title: Cytotoxic β-prime-mediated sAβPPα depletion

*Correspondence to: Edward Parkin, Division of Biomedical and Life Sciences, School of Health and Medicine, Lancaster University, Lancaster, United Kingdom. Telephone: +44 1524 592246. E-mail: e.parkin@lancaster.ac.uk

Abstract

Background: The Alzheimer's disease (AD)-associated amyloid-beta protein precursor (AβPP) can be cleaved by β-site AβPP cleaving enzyme 1 (BACE1) and the γ-secretase complex to yield neurotoxic amyloid beta (Aβ)-peptides. However, AβPP can also be cleaved in a 'non-amyloidogenic' manner either by α-secretase to produce soluble AβPP alpha (sAβPPα) (a fragment with neuroprotective/neurogenic functions) or through alternative BACE1-mediated 'beta prime' activity yielding soluble AβPP beta prime (sAβPPβ').

Objective: To determine whether sAβPPα depletion, as opposed to Aβ-peptide accumulation, contributes to cytotoxicity in AD-relevant SH-SY5Y neuroblastoma cell models.

Methods: AβPP proteolysis was characterized by immunoblotting in mock-, wild-type AβPP (wtAβPP)-, BACE1- and Swedish mutant AβPP (SweAβPP)-transfected cells. AβPP beta prime cleavage was confirmed through secretase inhibitor studies and C-terminal fragment analysis. The roles of sAβPPα and sAβPPβ' in cell viability were confirmed by over-expression studies.

Results: Despite producing enhanced Aβ-peptide levels, wtAβPP- and SweAβPP-transfected cells did not exhibit reduced viability whereas BACE1-transfected cells did. sAβPPα generation in SH-SY5Y-BACE1 cells was virtually ablated in lieu of BACE1-mediated sAβPPβ' production. sAβPPα over-expression in SH-SY5Y-BACE1 cells restored viability whereas sAβPPβ' over-expression decreased viability further. The anti-AβPP 6E10 antibody was shown to cross-react with sAβPPβ'.

Conclusion: sAβPPα depletion and/or sAβPPβ' accumulation but not elevated Aβ-peptide levels represents the cytotoxic mechanism following BACE1 over-expression in SH-SY5Y cells. These data support the novel concept that competitive sAβPPα depletion by BACE1 beta prime activity might contribute to AD. The cross-reactivity of 6E10 with AβPPβ'also

questions whether previous studies assessing sAβPPα as a biomarker using this antibody should be revisited.

Keywords: Alpha-secretase, Alzheimer's disease, amyloid-beta protein precursor, beta prime, beta-secretase.

INTRODUCTION

Alzheimer's disease (AD), the leading neurodegenerative disorder globally, is characterised clinically by a progressive decline in memory and cognitive function, ultimately leading to death. The disease is characterized pathologically by the presence of extracellular senile plaques and intracellular neurofibrillary tangles within the afflicted brain. The major constituents of senile plaques are amyloid beta (Aβ)-peptides derived through proteolysis of the amyloid-beta protein precursor (AβPP) [1]. In the amyloidogenic pathway, AβPP is sequentially cleaved by β-secretase (β-site AβPP cleaving enzyme 1; BACE1) (EC 3.4.23.46) and a multi-subunit protease complex known as γ-secretase (EC 3.4.23) to produce Aβ-peptides [2, 3]. In the alternative non-amyloidogenic pathway, an α-secretase of the A Disintegrin And Metalloprotease (ADAM) family cleaves AβPP at the Lys16-Leu17 bond within the Aβ region [4]. The predominant physiological α-secretase has been shown to be ADAM10 (EC 3.4.24.81) [5]. This pathway precludes the formation of Aβ-peptides instead releasing a soluble, neuroprotective, N-terminal ectodomain termed soluble AβPP alpha (sAβPPα), in addition to the C-terminal fragment (CTF) C83 [6].

Most experimental AD therapeutics targeting aspects of AβPP cell biology, such as β- and γ-secretase inhibitors and anti-amyloid targeting therapies [7-9], have focused on Aβ-peptide accumulation as the toxic event in disease pathology. The possible depletion of sAβPPα as a contributing factor has received less attention which is perhaps surprising given the numerous roles of this non-amyloidgenically derived proteolytic fragment in neuroprotection, neurogenesis and synaptic plasticity [10]. For example, *in vivo* studies have implicated sAβPPα in learning and memory, and in neuronal protection against both reactive oxygen species and glutamate-mediated excitotoxicity, while *in vitro* studies have described roles in differentiation, proliferation and neuronal survival [11-19]. Furthermore, sAβPPα interacts

with BACE1, causing its inhibition and a subsequent decrease in Aβ-peptide production [20, 21].

In contrast to the well-established functional roles of sAβPPα, whether the fragment is actually depleted in AD is more of a moot point. Early studies of this ilk employed antibodies that cross-reacted with all forms of soluble AβPP [22, 23]. Later studies employed more specific antibodies recognising epitopes between the β- and α-secretase cleavage sites within AβPP (e.g. anti-AβPP 6E10) [24, 25] but the accuracy of these results too is brought into question by advances in our knowledge surrounding the intricacies of AβPP proteolysis. For example, in addition to the canonical BACE1 cleavage site, the enzyme can also cleave between Tyr10 and Glu11 of the Aβ-peptide region to form sAβPPβ' and C89 [26, 27]. Notably, the sAβPPβ' generated by this 'beta prime' processing might retain epitopes detected by antibodies used in some of the afore-mentioned studies. Certainly, one might predict AD-associated depletion of sAβPPα generated via the non-amyloidogenic pathway under conditions where the 'reciprocal' amyloidogenic pathway is enhanced. This supposition is supported by the decreased levels of sAβPPα detected in the cerebrospinal fluid (CSF) of patients carrying the AβPP Swedish double mutation (KM670/671NL) which dramatically enhances processing of the protein by β-secretase [28]. Additionally, significant reductions in sAβPPα have been reported in the case of ADAM10 mutations associated with familial late-onset AD [29].

Human neuroblastoma, SH-SY5Y, cells are often employed as an *in vitro* model of AD due to their expression of some neuronal markers [30, 31]. Furthermore, the cells are rapidly dividing and can be used for the facile stable introduction of transgenes lending them effectively to the study of proteolytic events such as AβPP proteolysis. In particular, 'AD-

relevant' SH-SY5Y cell lines over-expressing BACE1, wild-type $A\beta PP_{695}$ (wtAβPP) or Swedish mutant $A\beta PP_{695}$ (SweAβPP) have previously been widely utilised to study AβPP proteolysis [32-36]. In the current study we have performed the first direct comparison of AβPP expression and proteolysis amongst these 'AD-relevant' cell lines and a mock-transfected control. During these studies we surreptitiously demonstrated that, uniquely amongst the cell lines employed, the non-amyloidogenic production of sAβPP (as detected using anti-AβPP 6E10 antibody) by SH-SY5Y-BACE1 cells was not inhibited by the classic α-secretase inhibitor, batimastat. We subsequently demonstrate that, when BACE1 is over-expressed in SH-SY5Y cells, non-amyloidogenic AβPP processing occurs nearly entirely via BACE1 'beta prime' activity and competitively ablates the production of sAβPPα. Additionally, we demonstrate that, whilst SH-SY5Y cells over-expressing BACE1, wtAβPP or SweAβPP all exhibit enhanced Aβ-peptide production relative to mock-transfected cells, only SH-SY5Y-BACE1 cells exhibit decreased viability. Furthermore, we utilise stable over-expression of sAβPPα and sAβPPβ' constructs to show that the viability of SH-SY5Y-BACE1 cells can be completely restored by the former fragment (and reduced by the latter) and that anti-AβPP 6E10 antibody cross-reacts with sAβPPβ'. Collectively, these data indicate that, under conditions of elevated BACE1 expression, competitive depletion of sAβPPα may well contribute to AD pathogenesis. Furthermore, the near complete replacement of sAβPPα with sAβPPβ' in the current study along with the cross-reactivity of anti-AβPP 6E10 with the latter fragment calls into question many previous biomarker studies purporting to quantify sAβPPα levels in AD patients and raises the question as to whether we may have dramatically under-estimated depletion of the fragment in at least a subset of AD patients in whom elevated BACE1 expression is evident.

MATERIALS AND METHODS

Materials

The generation of the wtAβPP$_{695}$ and BACE1 constructs in the mammalian expression vector pIREShyg (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) along with their expression and characterisation have been reported previously [36]. SH-SY5Y cells stably expressing Swedish mutant AβPP$_{695}$ (SweAβPP) in pIREShyg were a gift from Prof. Nigel Hooper (University of Manchester, Manchester, U.K.). Anti-actin monoclonal, anti-AβPP C-terminal (AβPP-CT) polyclonal, anti-AβPP N-terminal (AβPP-NT) monoclonal (22C11), anti-ADAM10 polyclonal and anti-BACE1 polyclonal antibodies were purchased from Merck Life Science (Gillingham, U.K.). Anti-AβPP 6E10 monoclonal and anti-sAβPPβ polyclonal antibodies were from Biolegend (San Diego, U.S.A.) and monoclonal anti-sAβPPβsw antibody (6A1) was from IBL America (Minneapolis, U.S.A.). Unless otherwise stated, all other reagents were from Merck Life Science (Gillingham, U.K.).

Cell culture

SH-SY5Y human neuroblastoma were cultured in Dulbecco's modified Eagle medium (DMEM) (Scientific Laboratory Supplies, Nottingham, U.K.) supplemented with 25 mM glucose, 4 mM L-glutamine, 10% (vol/vol) foetal bovine serum, penicillin (50 U/mL) and streptomycin (50 μg/mL). Cells were maintained at 37ºC in 5% CO_2 in air. For the detection of protein fragments in conditioned medium, cells were grown to confluence in complete growth medium, washed with 10 mL UltraMEM™ reduced serum medium (Fisher Scientific, Loughborough, UK) and then cultured for a further 24 h in a fresh 10 mL of the same medium. Batimastat (Merck Life Science, Gillingham, U.K.) and β-secretase inhibitor IV (Cayman Chemical, Ann Arbor, U.S.A.) were prepared as concentrated stocks in dimethylsulfoxide (DMSO) and added to UltraMEM™ to achieve the final concentrations

described up to a maximum carrier concentration of 0.05% (vol/vol). All control cultures contained the equivalent carrier concentration.

Generation of pIRESneo-sAβPPα and pIRESneo-sAβPPβ' plasmids

pIREShyg-wtAβPP$_{695}$ [36] was employed as a polymerase chain reaction (PCR) template for the generation of both pIRESneo-sAβPPα and pIRESneo-sAβPPβ'. PCR fragments were amplified using a Q5 high-fidelity PCR kit (New England Biolabs, Hitchin, U.K.). For sAβPPα a forward primer (5'-AGCTAGATATCGCCACCATGCTGCCCGGTTTGG-3') containing an *EcoRV* restriction site and Kozak sequence was employed along with a reverse primer (5'-ATAGCGCGGCCGCCTATTTTTGATGATGAACTTCATATCCTGAG-3') containing a *NotI* restriction site and a stop codon downstream of the codon encoding Lys16 of the Aβ sequence within the AβPP coding DNA. sAβPPβ' was amplified using the same forward primer and a reverse primer (5'-ACGTAGCGGCCGCTAGTATCCTGAGTCATGTCGGAATTCTG-3') containing a *NotI* restriction site and a stop codon downstream of the codon encoding Tyr10 of the Aβ sequence within the AβPP coding DNA. Both PCR products were then double digested with *EcoRV* and *NotI* before ligation into the mammalian expression vector pIRESneo (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). Plasmid sequences were confirmed by the MRC DNA Sequencing Service (University of Dundee, Dundee, U.K.).

Stable transfections

Plasmids (20-30 μg) or empty expression vector (control cells) were linearized using *AhdI* before being subjected to ethanol precipitation and subsequent introduction into SH-SY5Y cells by electroporation. Recombinant cells were selected using 0.5 mg/mL neomycin sulphate (Melford, Ipswich, U.K.). Note that, in the sAβPPα and sAβPPβ' construct

experiments, all the initial cells were stably transfected with either empty pIREShyg expression vector or pIREShyg-BACE1. These cells were then double stably transfected with empty pIRESneo, pIRESneo-sAβPPα or pIRESneo-sAβPPβ' in order to ensure that all the cells contained the same vector backbones and were, therefore, correctly controlled.

Preparation of cell lysates and conditioned medium samples

Conditioned cell culture medium was harvested, centrifuged at 10,000 *g* for 10 min to remove cell debris, and concentrated 40-fold using Amicon Ultra-4 Centrifugal Filters (Merck Millipore, Watford, U.K.). For analysis of cell-associated proteins, cells were washed with phosphate-buffered saline (PBS; 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.15 M NaCl, pH 7.4) and scraped from the flasks into fresh PBS (10 mL). Following centrifugation at 500 *g* for 5 min, cell pellets were lysed in 50 mM Tris, 150 mM NaCl, 1% (vol/vol) IGEPAL, 0.1 % (wt/vol) sodium deoxycholate, 5 mM (ethylenedinitrilo)tetraacetic acid (EDTA) at pH 7.4 containing 1% (vol/vol) protease inhibitor cocktail (Merck Life Science, Gillingham U.K.). Protein levels in cell lysates were quantified using bicinchoninic acid [37] in a microtitre plate with bovine serum albumin as a standard before equalizing the amount of protein in each sample by the addition of the required volumes of lysis buffer.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Equal quantities of lysate protein and equal volumes of concentrated conditioned medium samples were resolved by SDS-PAGE using 7-17% polyacrylamide gradient gels and transferred to Immobilon P polyvinylidene difluoride membranes [38] before incubating with primary antibody at a dilution of 1:5000 (except for anti-ADAM10, anti-BACE1 and anti-sAβPPβSwe (6A1) antibodies which were used at 1:1000, 1:2000 and 1:4000, respectively).

Bound primary antibody was detected using peroxidase-conjugated secondary antibodies (Merck Life Science, Gillingham U.K.) in conjunction with enhanced chemiluminescence detection reagents (Fisher Scientific, Loughborough, U.K.). Immunoblots were quantified using ImageJ.

For the resolution of AβPP C-terminal fragments, samples were run on 16% Tris/tricine gels before transferring to 0.2 micron nitrocellulose (Fisher Scientific, Loughborough, U.K.) and subsequently boiling for 5 min in PBS. Membranes were then further processed as described above.

Aβ-peptide quantification

Aβ-peptides in unconcentrated conditioned medium samples were quantified using the Mesoscale Discovery (MSD) platform. $A\beta_{40}$ and $A\beta_{42}$ were measured using the V-Plex Aβ peptide panel (6E10) kit according to the manufacturer's instructions (MSD, Maryland, U.S.A.).

Cell viability assays

For trypan blue assays, cells were seeded at a density of $2 \times 10^4/cm^2$. At the relevant time points, cells were harvested by trypsinisation and resuspended in PBS. An aliquot of cell suspension was mixed with an equal volume of 0.4% (wt/vol) trypan blue solution (Merck Life Science, Gillingham U.K.) and loaded onto a haemocytometer. Average live cell count across four squares was scaled up to obtain the total number of cells in each flask. For methanethiosulfonate (MTS) assays, cells were seeded at a density of $9.38 \times 10^4/cm^2$ in 96-well culture plates. At the relevant time points, cells were incubated with CellTiter 96® AQueous One Cell Proliferation Assay solution (Promega, Wisconsin, U.S.A) for 35 min at

37°C. Absorbance at 490 nm was measured using a Victor^2 1420 microplate reader (Perkin Elmer, Waltham, U.S.A.).

Statistical analysis

Results are presented as means ± standard deviation (S.D.). Data were analysed using either Student's t-test or a one-way analysis of variance (ANOVA). For cell viability assays, area under the curves were calculated, on which one-way ANOVAs were subsequently conducted. Additionally, one-way ANOVAs were conducted between cell lines at each time point in cell viability assays. All one-way ANOVA tests were followed by Tukey post-hoc analysis. Graphpad Prism 9.0.0 was used for all statistical analysis. Levels of significance are indicated in figure legends.

RESULTS

Soluble AβPP production via non-amyloidogenic pathways is not impaired by batimastat in SH-SY5Y-BACE1 cells

To our knowledge, no study has previously compared AβPP proteolysis between BACE1-, wtAβPP- and SweAβPP-transfected SH-SY5Y cells. To this end, we grew all of these AD-relevant cell lines, along with mock-transfected controls, to confluence and then transferred them for an additional 24 h into UltraMEM™ reduced serum medium in the absence/presence of the broad spectrum ADAM-inhibitor, batimastat [39]. Cell lysate and conditioned medium samples were then prepared as described in the Materials and Methods section. Subsequent immunoblotting of lysates, as expected, demonstrated high levels of BACE1 expression only in the SH-SY5Y-BACE1 stable transfectants; equal protein loading was confirmed by immunoblotting for actin (Fig. 1A). Immunoblotting of the same lysate samples with the anti-AβPP CT antibody (Fig. 1B) revealed, interestingly, that AβPP expression was elevated 3.55

± 0.75-fold in SHSY-BACE1 cells relative to the mock-transfected SH-SY5Y cells. More expected were the 13.83 ± 6.05- and 16.04 ± 7.00-fold increases in AβPP expression observed, respectively, in the SH-SY5Y-wtAβPP and SH-SY5Y-SweAβPP cells. Inhibition of α-secretase activity using batimastat only resulted in a reciprocal accumulation of full-length AβPP in mock-transfected cell lysates (154.71 ± 43.64 % of untreated controls) (Fig. 1C).

In order to compare non-amyloidogenic AβPP shedding between the cell lines, 24 h conditioned medium was immunoblotted with the anti-AβPP 6E10 antibody. Although this antibody is reactive to residues 1-16 of the Aβ region within AβPP, the epitope lies within amino acids 3-8 of the same region (EFRHDS) [40]. The results (Fig. 1D) showed that $sA\beta PP_{695}$ levels detected using 6E10 were increased significantly in the conditioned medium of both wtAβPP- and SweAβPP-transfected cells (3.58 ± 0.74- and 2.78 ± 0.19-fold, respectively, relative to mock transfectants). Given that both transfected forms of AβPP were the 695 isoform, this was entirely expected and, furthermore, no differences were observed in non-amyloidogenic sAβPP derived from the 751 and 770 amino acid AβPP isoforms (Fig. 1D). Batimastat treatment (Fig. 1E) resulted in 92.57 ± 2.99, 37.18 ± 6.22 and 52.31 ± 1.11 % reductions (relative to untreated controls) in the non-amyloidogenic shedding of $sA\beta PP_{695}$ from the mock-, wtAβPP and SweAβPP cells, respectively. Similarly, the levels of non-amyloidogenic $sA\beta PP_{751/770}$ produced by the same cell lines were reduced by 79.51 ± 3.56, 67.45 ± 17.86 and 95.30 ± 2.68 %. However, notably, the non-amyloidogenic shedding of all AβPP isoforms from SH-SY5Y-BACE1 cells remained entirely unchanged following batimastat treatment (Fig. 1E). Furthermore, the expression and proteolytic maturation of the AβPP α-secretase, ADAM10, did not differ between any of the cell lines studied (Supplementary Figure 1).

We also examined the β-secretase-mediated generation of sAβPPβ by the four different cell lines by immunoblotting the conditioned medium samples with the anti-sAβPPβ antibody. The results (Fig. 2A) revealed a significant increase in sAβPPβ$_{751/770}$ and sAβPPβ$_{695}$ levels in medium from the SH-SY5Y-BACE1 cells (3.44 ± 14.30- and 16.96 ± 6.52-fold increases, respectively, relative to mock-transfected cells). In the case of the SH-SY5Y-wtAβPP cells, as expected, only sAβPPβ$_{695}$ was significantly increased (33.88 ± 20.15-fold relative to mock transfectants). Notably, the epitope generated following the cleavage of SweAβPP by BACE1 differs from that generated from wtAβPP due to the nature of the Swedish mutation and, as such, the anti-sAβPPβ antibody initially employed did not detect enhanced generation of this fragment in conditioned medium from the SH-SY5Y-SweAβPP cells (Fig. 2A). However, we did verify production of sAβPPβSwe using the anti-sAβPPβSwe (6A1) antibody (Fig. 2B). Inhibition of the 'reciprocal' non-amyloidogenic pathway using batimastat had little effect on sAβPPβ production by any of the cell lines other than a small but significant increase in sAβPPβ$_{695}$ in the medium from SH-SY5Y-BACE1 cells (an 11.19 ± 0.06% increase relative to untreated cells) (Fig. 2C).

Soluble AβPP production via non-amyloidogenic pathways is predominantly mediated by BACE1 beta prime activity in SH-SY5Y-BACE1 cells

Given that batimastat did not reduce the production of sAβPP via non-amyloidogenic pathways in SH-SY5Y-BACE1 cells (Fig. 1E) we hypothesized that the BACE1 over-expressed in this cell line might be cleaving AβPP in a 'beta prime' fashion C-terminal to Tyr10 of the Aβ region [26, 27] in direct competition with canonical α-secretase-mediated processing of the protein. This would leave intact the minimum 6E10 epitope between amino acids 3-8 of the Aβ region [40] and, at the same time, explain the resistance of non-

amyloidogenic sAβPP production to batimastat. Given that no antibody can specifically detect the N-terminal sAβPPβ' fragment in conditioned medium, we sought to examine the reciprocal production of AβPP C-terminal fragments (CTFs). As such, the cell lysates used in Fig.1. were also subjected to Tris/tricine gel electrophoresis (see Materials and Methods) and immunoblotted with the anti-AβPP CT antibody. The results (Figs. 3A and 3B) demonstrated significant increases in the levels of β-secretase-derived C99 in SH-SY5Y-wtAβPP and SH-SY5Y-SweAβPP cell lysates (2.82 ± 0.86- and 4.01 ± 0.58-fold enhanced relative to mock transfectants, respectively) but not, curiously, in the BACE1-transfected cells (despite the previously observed increase in sAβPPβ production in medium from the latter cells; Fig. 2A). In partial contrast, the levels of C83 derived from canonical α-secretase processing of AβPP were enhanced 1.98 ± 0.20- and 2.26 ± 0.34-fold, respectively (relative to mock transfectants) in SH-SY5Y-wtAβPP and SH-SY5Y-SweAβPP cells (Fig. 3C), this time in line with the enhanced non-amyloidogenic sAβPP production previously observed in these cells (Fig. 1D). Notably, however, consistent with a lack of canonical α-secretase AβPP processing, there was virtually no C83 detected in SH-SY5Y-BACE1 cells (Figs. 3A and 3C). Instead, a slightly larger C-terminal fragment consistent with C89 generated following beta prime AβPP cleavage was detected (Figs. 3A and 3C).

In order to further confirm that the non-amyloidogenically derived sAβPP produced by SH-SY5Y-BACE1 cells was generated via BACE1 activity, we examined the effect of β-secretase inhibitor IV [41] on production of the fragment. Initially mock-transfected cells were cultured for 24 h in the absence or presence of batimastat and/or β-secretase inhibitor IV and the conditioned medium was immunoblotted with the anti-AβPP 6E10 antibody. The results (Fig. 4A) demonstrated a near complete ablation of sAβPP derived via non-amyloidogenic AβPP processing (94.02 ± 7.08 and 96.30 ± 2.77 % reductions in $sA\beta PP_{751/770}$ and $sA\beta PP_{695}$,

respectively, relative to untreated cells) following batimastat treatment. Conversely, β-secretase inhibitor IV treatment alone had no impact on the production of these fragments unless batimastat was also present in which case levels were again reduced (97.80 ± 1.70 and 99.62 ± 0.50 % reductions in $sA\beta PP_{751/770}$ and $sA\beta PP_{695}$, respectively, relative to untreated controls). When the same conditioned medium from inhibitor-treated mock-transfected SH-SY5Y cells was immunoblotted with the anti-sAβPPβ antibody, as would be expected, β-secretase inhibitor IV nearly completely ablated sAβPPβ production either singularly or in combination with batimastat (Fig. 4B). We next repeated the inhibitor experiments but using SH-SY5Y-BACE1 cells. This time, when conditioned medium was immunoblotted with the anti-AβPP 6E10 antibody, as observed previously (Fig. 1E), batimastat did not significantly inhibit production of sAβPP derived via non-amyloidogenic processing (Fig. 4C). Conversely, β-secretase inhibitor IV, reduced the production of these fragments to 21.53 ± 19.00 % ($sA\beta PP_{695}$) and 30.98 ± 22.62 % ($sA\beta PP_{751/770}$) relative to untreated cells but only when used in combination with batimastat.

The fact that β-secretase inhibitor IV alone did not reduce sAβPP levels indicated that α-secretase was in direct competition with BACE1 beta prime AβPP processing in SH-SY5Y-BACE1 cells such that, unless the former was also inhibited, it could compensate when BACE1 was inhibited. In an attempt to validate this hypothesis we also examined AβPP CTF levels in the lysates of the various inhibitor treated cells. The results showed that, in mock-transfected SH-SY5Y cells, β-secretase-derived C99 levels were, as expected, reduced by β-secretase inhibitor IV used singularly or in combination with batimastat (27.79 ± 17.94 % and 19.84 ± 7.18 % of untreated controls, respectively) (Figs. 5A and 5B). C83 derived from canonical α-secretase AβPP processing was significantly reduced in mock-transfected cells treated with batimastat alone (72.19 ± 7.56 % of untreated controls) (Fig. 5C). However,

when cells were treated with β-secretase inhibitor IV alone or in combination with batimastat, C83 levels were unchanged relative to the untreated controls. In the case of SH-SY5Y-BACE1 cells, C83 was not detected in untreated cells or those treated with batimastat alone but was entirely replaced with BACE1 beta prime-generated C89 (Fig. 5D). However, rather unexpectedly, whether used singularly or in combination with batimastat, β-secretase inhibitor IV caused large increases in both C99 (2.08 ± 0.19- and 2.15 ± 0.23-fold, respectively, relative to untreated controls) (Figs. 5D and 5E) and C83 levels to an extent that the latter fragment was not resolved effectively from C89. As such the C83/C89 band was quantified as a singular entity in SH-SY5Y-BACE1 cells treated with β-secretase inhibitor IV. This band was increased 1.53 ± 0.06- and 1.46 ± 0.14-fold (relative to C89 levels in untreated controls) in cells treated with β-secretase inhibitor IV alone or in combination with batimastat, respectively (Fig. 5F). This increase was also significant compared to C89 levels in SH-SY5Y-BACE1 cells treated with batimastat alone. Levels of C89 were unchanged relative to untreated controls in cells treated with batimastat alone (Fig. 5F). The unexpected and seemingly global increase in AβPP CTF levels in β-secretase inhibitor IV (singular and combined with batimastat)-treated SH-SY5Y-BACE1 cells is considered further in the Discussion of the current study.

BACE1-, wtAβPP- and SweAβPP-transfected cells all exhibit enhanced Aβ-peptide generation but only SH-SY5Y-BACE1 cells exhibit reduced viability

In the preceding experiments we had demonstrated that BACE1 beta prime activity competitively depletes sAβPPα production when the enzyme is over-expressed in SH-SY5Y cells. Given this AβPP fragment has roles in cell proliferation and protection [13, 15, 16, 18], we hypothesized that the lack of sAβPPα (and/or enhanced sAβPPβ') in SH-SY5Y-BACE1 cell cultures might result in a reduction in cell viability relative to mock-transfected cells.

However, we first needed to eliminate the enhanced production of Aβ-peptides by the former cells as a confounding factor which necessitated a global comparison of levels of these peptides produced by all four cell lines used in the current study.

In order to determine Aβ-peptide levels, unconcentrated conditioned medium samples were analysed for $A\beta_{40}$ and $A\beta_{42}$ levels as described in the Materials and Methods section. The results (Fig. 6A) showed that $A\beta_{42}$ concentrations were significantly increased in medium from BACE1- (25.62 ± 3.66 pg/mL), wtAβPP- (37.74 ± 2.97 pg/mL) and SweAβPP-transfected (299.43 ± 17.00 pg/mL) cells compared to mock-transfected cells (6.35 ± 0.20 pg/mL). $A\beta_{42}$ levels in SH-SY5Y-SweAβPP conditioned medium were also significantly increased compared to both BACE1- and wtAβPP-transfected cells. Similarly, $A\beta_{40}$ levels were also significantly higher in medium from BACE1- (285.00 ± 42.51 pg/mL), wtAβPP-(431.37 ± 70.68 pg/mL) and SweAβPP-transfected cells (2636.63 ± 293.28 pg/mL) compared to mock-transfected cells (76.74 ± 10.24 pg/mL). Again, levels in SH-SY5Y-SweAβPP cells were significantly increased compared to both BACE1- and wtAβPP-transfected cells (Fig. 6A).

Having determined that Aβ-peptide levels were elevated in the medium from all three AD-relevant cell lines (relative to the mock transfectants), we next examined the viability of the various cell lines over a 12 day culture period. Initially, cells were seeded and viability was monitored using trypan blue (see Materials and Methods) and the results (Fig. 6B) showed that the live cell counts for SH-SY5Y-BACE1 cells were significantly lower than the other three cell lines at every time point from day five onwards. Note that, due to the number and extent of significant differences between the four cell lines analysed by ANOVA in Fig. 6B, it was not feasible to annotate them on the growth curves. Instead, we adopted an area under the

curve (AUC) analysis to more effectively demonstrate the differences (Fig. 6C). Here, the results demonstrated a significant reduction in the case of SH-SY5Y-BACE1 cells relative to all the other three cell lines; whilst this difference was small it was highly significant. Furthermore, we had remarked that SH-SY5Y-BACE1 cells, when examined under the light microscope, generally looked, morphologically, less healthy than the other three cell lines. We, therefore, examined cell viability over the same 12 day culture period using an assay that might more accurately reflect changes in biochemical cell viability i.e. the MTS assay. These latter data (Figs. 6D and 6E) demonstrated more clearly the decreased viability of SH-SY5Y-BACE1 cells throughout the majority of the culture period and these cells exhibited an AUC value of (21.16 ± 1.44) which was significantly lower than the value for mock- (32.19 ± 1.55), wtAβPP- (39.34 ± 1.78) and SweAβPP-transfected (37.07 ± 1.48) cells (Fig. 6E).

Collectively, these data show that, despite BACE1-, wtAβPP- and SweAβPP-transfected SH-SY5Y cells all generating far more Aβ-peptides than mock-transfected cells, only the SH-SY5Y-BACE1 cells exhibited a reduced cell viability. In combination with the fact that the latter cell line also produced a lower quantity of Aβ than the wtAβPP- and SweAβPP-transfected cells and yet was less viable, these results indicated that it might be sAβPPα depletion (or an alteration of the sAβPPα/sAβPPβ' axis) that was responsible for the reductions in viability observed in SH-SY5Y-BACE1 cells.

Generation and characterization of sAβPPα- and sAβPPβ'-over-expressing SH-SY5Y-BACE1 cells

In order to examine further the impact of an altered sAβPPα/sAβPPβ' axis on SH-SY5Y cell viability, we sought to develop mock- and BACE1-transfected cell lines over-expressing one or the other of these non-amyloidogenically derived fragments. To this end we used the

original pIRES-wtAβPP$_{695}$ plasmid as a PCR template employing a reverse primer incorporating a stop codon downstream of the AAA codon encoding Lys16 of the Aβ sequence within the AβPP coding DNA. The PCR product was then ligated into pIRESneo and the correct incorporation of the TAG stop codon following the nucleotide sequence encoding HHQK in the Aβ region was verified through plasmid sequencing of pIRESneo-sAβPPα (Fig. 7A). Similarly, a sAβPPβ' construct was generated using a reverse primer incorporating a stop codon downstream of the codon encoding Tyr10 of the Aβ region. The correct incorporation of the stop codon downstream of the nucleotide sequence encoding DSGY in the subsequently generated pIRESneo-sAβPPβ' was also verified by sequencing (Fig. 7A). Note that the TAT codon encoding tyrosine in the AβPP coding DNA was altered to TAC (a non-coding change) in the primer in order to facilitate a suitable primer melting temperature.

We then stably transfected empty pIRESneo, pIRESneo-sAβPPα and pIRESneo-sAβPPβ' into previously pIREShyg mock-transfected SH-SY5Y cells and characterised levels of sAβPP fragments in the conditioned medium of the resultant cell lines (note that none of the transfections altered full-length AβPP expression in cell lysates; Supplementary Figure 2). Immunoblotting with the anti-AβPP 6E10 antibody revealed 5.94 ± 1.58- and 4.35 ± 0.93-fold (relative to the pIRESneo/hyg mock transfectants) increases in the levels of apparently non-amyloidogenically processed AβPP$_{695}$ in the medium from cells transfected with the pIRESneo-sAβPPα and pIRESneo-sAβPPβ', respectively (Fig. 7B) confirming that the antibody did indeed cross react with sAβPPβ'. No significant changes in sAβPPβ production were observed in medium from any of the transfectants (Fig. 7C). The fact that the newly transfected sAβPPα and sAβPPβ' fragments constituted the major soluble AβPP fragments in cells was confirmed using the anti-AβPP NT (22C11) antibody which showed 3.04 ± 0.32-

and 3.05 ± 0.19-fold increases in total $sA\beta PP_{695}$ in the medium from cells transfected with the pIRESneo-sAβPPα and pIRESneo-sAβPPβ', respectively (Fig. 7D).

We then stably transfected empty pIRESneo, pIRESneo-sAβPPα and pIRESneo-sAβPPβ' into previously pIREShyg-BACE1-transfected SH-SY5Y cells and, once more, examined the levels of sAβPP in conditioned medium (again none of the transfections altered full-length AβPP expression in cell lysates; Supplementary Figure 2). As expected, the anti-AβPP 6E10 antibody detected large increases in the amount of apparently non-amyloidogenically processed $A\beta PP_{695}$ in conditioned medium from pIRESneo-sAβPPα and pIRESneo-sAβPPβ'-transfected cells (4.69 ± 0.45- and 3.56 ± 0.61-fold, respectively, relative to the pIRESneo-transfected SH-SY5Y-BACE1 cells) (Fig. 7E). Again, there was little significant change in the levels of sAβPPβ produced by any of the transfectants (Fig. 7F). Finally, immunoblotting with the anti-AβPP NT (22C11) antibody, once more, verified that the newly transfected sAβPPα and sAβPPβ' fragments constituted the major soluble AβPP fragments in their cognately transfected SH-SY5Y-BACE1 cells (Fig. 7G).

sAβPPα but not sAβPPβ' completely restores viability deficits in SH-SY5Y-BACE1 cells

Having verified correct sAβPPα and sAβPPβ' over-expression, we sought to examine the potential ability of sAβPPα to restore the decreased cell viability brought about by BACE1 transfection and whether sAβPPβ' might contribute to this decreased viability. To this end we compared the viability over a twelve day growth period of both pIREShyg-mock- and pIREShyg-BACE1-transfected cells double-transfected with either empty pIRESneo expression vector or the pIRESneo-sAβPPα and pIRESneo-sAβPPβ' constructs. Using the trypan blue assay the resultant growth curves (Fig. 8A) demonstrated that sAβPPα significantly enhanced the viability of the SH-SY5Y-mock cells relative to their pIRESneo-

transfected counterparts and, furthermore, sAβPPβ' decreased the viability of these cells. The same pattern but to an exaggerated extent was observed in the SH-SY5Y-BACE1 cells in which sAβPPα notably restored the viability of these cells to the level of the sAβPPα-transfected mock cells. Similarly, sAβPPβ' over-expression seemed to have an additive effect with the 'endogenously' generated sAβPPβ' in SH-SY5Y-BACE1 cells resulting in these cells having a significantly lower viability than any of the other cell lines employed. These effects can be seen more clearly in the AUC analysis (Fig. 8B) where sAβPPα over-expression in the SH-SY5Y-mock cells and in the SH-SY5Y-BACE1 cells enhanced viability relative to the cognate pIRESneo expression vector controls but also to a level indistinguishable between the sAβPPα-transfected SH-SY5Y-mock and SH-SY5Y-BACE1 cells. Similarly, sAβPPβ' caused a slight reduction in cell viability in both the SH-SY5Y-mock and SH-SY5Y-BACE1 cells relative to their cognate controls.

We then repeated the viability studies but using the MTS assay. Here, the growth curves (Fig. 8C) again demonstrated that sAβPPα transfection enhanced viability in both the SH-SY5Y-mock and SH-SY5Y-BACE1 cells. What was particularly notable, however, was that the detrimental effect of sAβPPβ' on cell viability in both the SH-SY5Y-mock and SH-SY5Y-BACE1 cells was even more pronounced when quantified using the MTS assay (Fig. 8C) compared to the previous trypan blue analysis (Fig. 8A). Similarly, the AUC analysis (Fig. 8D) also reflected this exaggerated effect. As with the trypan blue analysis, sAβPPα transfection was, again, shown to enhance the viability of both SH-SY5Y-mock and SH-SY5Y-BACE1 cells.

DISCUSSION

In the current study we initially compared AβPP proteolysis in three basic cell models commonly employed in AD research (SH-SY5Y-BACE1, SH-SY5Y-wtAβPP and SH-SY5Y-SweAβPP) [32-36]. In terms of AβPP holoprotein expression we showed that the stable over-expression of wtAβPP$_{695}$ and SweAβPP$_{695}$ in SH-SY5Y cells resulted in 13.83 ± 6.05- and 16.04 ± 7.00-fold increases of FL-AβPP in cell lysates (Fig. 1B). What was less expected was the 3.55 ± 0.75-fold increase observed in endogenous FL-AβPP expression in SH-SY5Y-BACE1 cells. One explanation for this might be enhanced AβPP intracellular domain (AICD) generation via the amyloidogenic pathway as a consequence of increased BACE1 expression and the transcriptional activity of this fragment which can target the *APP* gene leading to elevated FL-AβPP expression [42]. Whilst this process would also be enhanced in the wtAβPP- and SweAβPP-transfected cells, the transfected coding DNA would not be under the control of the endogenous *APP* promoter and, therefore, smaller increases in the expression of endogenous AβPP as a consequence of enhanced AICD transcriptional activity are likely to be masked by the much greater magnitude of transfected protein expression. Another unexpected observation in the SH-SY5Y-BACE1 cells was the lack of a significant increase in C99 levels relative to the mock-transfected controls (Figs. 3A and 3B). However, this could result either from the preferential beta-prime cleavage of endogenous AβPP by over-expressed BACE1 (to generate the C89 fragment) or possibly through a direct physical interaction between the over-expressed BACE1 and the γ-secretase complex mediating enhanced C99 processing to form Aβ-peptides (as discussed later).

Prima facie, it was not unexpected that the anti-AβPP 6E10 antibody detected 3.58 ± 0.74- and 2.78 ± 0.19-fold increases in the production of non-amyloidogenically derived sAβPP by SH-SY5Y-wtAβPP and SH-SY5Y-SweAβPP cells (Fig. 1D). However, it has previously been reported [43] that the transfection of SweAβPP in SH-SY5Y cells resulted in a decreased

production of sAβPP as detected using anti-AβPP 6E10 antibody relative to mock-transfected controls. There appears to be little logic in this latter observation as, whereas one might expect the transfected SweAβPP to be processed less via the non-amyloidogenic pathway (relative to wtAβPP) due to enhanced BACE1 cleavage of the protein, the non-amyloidogenic processing of the endogenous protein would have to be reduced following SweAβPP transfection in order to decrease levels relative to mock-transfected controls. Notably, the authors corrected conditioned medium samples on the basis of protein levels which is not advisable as changes in the levels of any protein in medium following SweAβPP transfection would have artefactually impacted on the results (in the current study we analysed medium samples on an equal volume basis; only lysates were equalized in terms of protein concentrations). Notably, the level of non-amyloidogenically derived sAβPP generated from SweAβPP in the current study was, in fact, actually the same as that generated from over-expressed wtAβPP (Fig. 1D) and both events were inhibited by batimastat suggesting the involvement of a common α-secretase-like activity. We suggest that, whilst SweAβPP is irrefutably more efficiently processed by BACE1 than its wild-type counterpart [44], the high levels of the protein expressed in our cells ensure that BACE1 effectively reaches substrate saturation leaving sufficient excess substrate available for α-secretase processing.

One of the particularly novel observations in the current study is that, in terms of sAβPP generation, whilst batimastat clearly inhibited non-amyloidogenic processing in SH-SY5Y-mock, -wtAβPP and –SweAβPP cells, it had absolutely no impact in cells over-expressing BACE1 (Fig. 1E). We have presented mechanistic data that indicate this phenomenon is due to enhanced BACE1-mediated beta prime processing of AβPP. Notably, in the SH-SY5Y-BACE1 cells, the α-secretase-generated CTF (C83) was completely replaced with a fragment of a size consistent with C89 generated by BACE1 beta prime activity (Figs. 3A and 3C) [26,

27]. That beta prime activity was responsible for non-amyloidogenic processing in SH-SY5Y-BACE1 cells was further indicated by our experiments showing that β-secretase inhibitor IV largely ablated the phenomenon in these cells but not in mock-transfected cells (Fig. 4). This clearly implied that the soluble fragment generated in SH-SY5Y-BACE1 cells was not produced by canonical α-secretase activity yet must retain the 6E10 epitope. Notably, whilst the antibody is raised against the immunogen $A\beta_{1-17}$, the epitope lies within residues 3-8 (EFRHDS) of the peptide [40]. Beta prime cleavage of AβPP occurs at the Tyr10-Glu11 bond within the Aβ region [26, 27], leaving the minimum 6E10 epitope intact in sAβPPβ'.

A direct competition between α-secretase and BACE1 beta prime activity is demonstrated by the fact that only combination treatment of SH-SY5Y-BACE1 cells with batimastat and inhibitor IV ablated non-amyloidogenic processing; treatment with either compound alone had no effect (Fig. 4C). *Prima facie*, this hypothesis is also supported by the fact that the treatment of SH-SY5Y-BACE1 cells with β-secretase inhibitor IV resulted in a seemingly reciprocal increase in the production of α-secretase derived C83 (Figs. 5D and 5E). However, this observation is confounded by the fact that combination treatment with both inhibitors also resulted in an accumulation of this fragment. Notably, the increase in CTF levels following inhibitor IV treatment was not restricted to C83; it also, unexpectedly, resulted in enhanced β-secretase derived C99 (Figs. 5D and 5E). Such more global accumulation of AβPP CTFs can only really be explained by the inhibition of γ-secretase activity. It has previously been reported that BACE1 can physically interact with γ-secretase [45], and that inhibition of such interaction can slow down the sequential processing of AβPP [46] but whether catalytic inhibition of the former enzyme rather than the physical disruption of a complex between the two enzymes could inhibit γ-secretase activity is a moot point. We have previously observed that, in ARPE-19 human retinal pigment epithelial cells, β-secretase inhibitor IV, at

concentrations as low as 25 nM, causes a net increase in AβPP CTF levels (Sultan & Parkin, in submission) yet others have suggested that the compound does not alter γ-secretase activity [46].

Another very important and novel element to the current study stems from the fact that, through our sAβPPβ' over-expression studies, we have highlighted the fact that the anti-AβPP 6E10 antibody does indeed cross react with this soluble AβPP fragment (Fig. 7). This observation, together with the fact that, under conditions of elevated BACE1 expression, beta prime activity can competitively ablate canonical sAβPPα production questions the accuracy of some previous studies that have sought to test the potential of this proteolytic fragment as an AD biomarker. Certainly, several studies have employed the anti-AβPP 6E10 antibody to report that sAβPPα levels in the CSF of AD patients are not depleted compared to cognitively healthy controls [24, 25, 47, 48]. Given that elevated BACE1 expression has previously been described in the brains and CSF of AD patients [49-54], and sAβPPα biomarker studies do not correct for BACE1 activity in patients, it is possible that levels of this non-amyloidogenic AβPP fragment may well have been overestimated in such studies.

In addition to potential implications for sAβPPα biomarker studies our research also raises novel questions in relation to AD causation. Clearly, Aβ-peptides, according to the amyloid cascade hypothesis, are central to AD pathogenesis and yet, despite all three AD-relevant SH-SY5Y cell lines employed in the current study exhibiting greatly enhanced production of these peptides relative to mock transfectant controls (Fig. 6), only the BACE1-transfected cells exhibited reduced cell viability. There may be several explanations for this, not least the fact that these cells represent an *in vitro* system which may well lack other factors necessary for Aβ-peptide aggregation and/or toxicity. Additionally, sAβPPα production was enhanced

in both the wtAβPP- and SweAβPP-SH-SY5Y cells and this neuroprotective fragment [10] may well have countered the enhanced Aβ-peptide levels. Nonetheless, the fact that sAβPPα transfection was able to restore SH-SY5Y-BACE1 cell viability to at least the level of control cells (in the case of the MTS assays) and indeed to the level of sAβPPα-transfected normal SH-SY5Y cells (i.e. those that were not transfected with BACE1) (in the case of the trypan blue assays) (Fig. 8) suggests that, at least in this cell type, reductions in cell viability were more down to beta prime-mediated depletion of this fragment than to Aβ-peptide toxicity. Similarly, if BACE1 toxicity was being mediated through the enhanced cleavage of an alternative BACE1 substrate, it is highly unlikely that the restoration of sAβPPα levels alone would restore the viability of SH-SY5Y-BACE1 levels, not just to the level of mock-transfected cells but to that of the sAβPPα-transfected control SH-SY5Y cells. These findings may provide rationale for the use of sAβPPα-enhancing therapies to treat AD at least in a subset of patients with elevated BACE1 expression.

Interestingly, our experiments also demonstrated the novel concept that sAβPPβ' decreases the viability of SH-SY5Y-mock and SH-SY5Y-BACE1 cells (Fig. 8) perhaps suggesting that, in the latter cells, a dramatic change in the sAβPPα/sAβPPβ' axis rather than a simple depletion of the former fragment might be conducive to cytotoxicity. That sAβPPβ' might be neurotoxic seems at odds with the fact that the A673T Icelandic *APP* mutation protects against the development of AD [55] and has been shown to change the cleavage site selection of BACE1 from the canonical β-secretase cleavage site to the beta prime site [56] which one would expect to enhance sAβPPβ' production. However, the main protective effect of the A673T mutation is thought to be derived from the preclusion of intact Aβ-peptide formation due to enhanced cleavage of the Aβ sequence at Tyr10-Glu11 to generate $Aβ_{(11-40/42)}$ [55] and this might outweigh the possible detrimental production of sAβPPβ' in those individuals

carrying the A673T mutation. Furthermore, it has been suggested that canonical β-secretase generated C99 can be cleaved at the beta prime site to ultimately form $A\beta_{(11\text{-}40/42)}$ [56] such that patients carrying the Icelandic mutation may well not exhibit enhanced sAβPPβ' production.

To conclude, in the current study we have demonstrated that, under conditions of elevated BACE1 expression, beta prime activity of the enzyme can compete directly with α-secretase activity such that sAβPPα production is virtually ablated. Furthermore, the reductions in cell viability in SH-SY5Y-BACE1 cells can be completely reversed by sAβPPα. These data raise several novel concepts not least the fact that, in a subset of AD patients exhibiting enhanced BACE1 activity, the depletion of sAβPPα or, at least an alteration in the sAβPPα/sAβPPβ' axis, might contribute to neurotoxicity. Furthermore, the fact that the anti-AβPP 6E10 antibody has been shown here to cross-react with sAβPPβ' suggests that we may need to reassess sAβPPα depletion as a possible biomarker for AD factoring in levels of BACE1 activity in patients as we may have previously considerably under-estimated the depletion of this proteolytic fragment in the disease.

ACKNOWLEDGEMENTS

This work was supported by the fund raising efforts of 'Defying Dementia' (Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, United Kingdom). We would also like to thank Prof. Nigel Hooper and Dr. Katherine Kellett (Faculty of Biology, Medicine and Health, University of Manchester, Manchester, United Kingdom) for their assistance with the Aβ-peptide analysis.

CONFLICT OF INTEREST/DISCLOSURE STATEMENT

The authors have no conflict of interest to report.

REFERENCES

[1] Gorevic PD, Goñi F, Pons-Estel B, Alvarez F, Peress NS, Frangione B (1986) Isolation and Partial Characterization of Neurofibrillary Tangles and Amyloid Plaque Core in Alzheimer's Disease: Immunohistological Studies. *Journal of Neuropathology & Experimental Neurology* **45**, 647-664.

[2] Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735-741.

[3] Haass C (2004) Take five--BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. *The EMBO journal* **23**, 483-488.

[4] Anderson JP, Esch FS, Keim PS, Sambamurti K, Lieberburg I, Robakis NK (1991) Exact cleavage site of Alzheimer amyloid precursor in neuronal PC-12 cells. *Neurosci Lett* **128**, 126-128.

[5] Postina R, Schroeder A, Dewachter I, Bohl J, Schmitt U, Kojro E, Prinzen C, Endres K, Hiemke C, Blessing M, Flamez P, Dequenne A, Godaux E, van Leuven F, Fahrenholz F (2004) A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer disease mouse model. *J Clin Invest* **113**, 1456-1464.

[6] Selkoe DJ (2001) Alzheimer's Disease: Genes, Proteins, and Therapy. *Physiological Reviews* **81**, 741-766.

[7] Dhillon S (2021) Aducanumab: First Approval. *Drugs* **81**, 1437-1443.

[8] Kumar D, Ganeshpurkar A, Kumar D, Modi G, Gupta SK, Singh SK (2018) Secretase inhibitors for the treatment of Alzheimer's disease: Long road ahead. *Eur J Med Chem* **148**, 436-452.

[9] Lozupone M, Solfrizzi V, D'Urso F, Di Gioia I, Sardone R, Dibello V, Stallone R, Liguori A, Ciritella C, Daniele A, Bellomo A, Seripa D, Panza F (2020) Anti-amyloid-beta protein agents for the treatment of Alzheimer's disease: an update on emerging drugs. *Expert Opin Emerg Drugs* **25**, 319-335.

[10] Dar NJ, Glazner GW (2020) Deciphering the neuroprotective and neurogenic potential of soluble amyloid precursor protein alpha (sAPPalpha). *Cell Mol Life Sci* **77**, 2315-2330.

[11] Mattson MP, Cheng B, Culwell AR, Esch FS, Lieberburg I, Rydel RE (1993) Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the beta-amyloid precursor protein. *Neuron* **10**, 243-254.

[12] Goodman Y, Mattson MP (1994) Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury. *Exp Neurol* **128**, 1-12.

[13] Hayashi Y, Kashiwagi K, Ohta J, Nakajima M, Kawashima T, Yoshikawa K (1994) Alzheimer amyloid protein precursor enhances proliferation of neural stem cells from fetal rat brain. *Biochem Biophys Res Commun* **205**, 936-943.

[14] Ohsawa I, Takamura C, Kohsaka S (1997) The amino-terminal region of amyloid precursor protein is responsible for neurite outgrowth in rat neocortical explant culture. *Biochem Biophys Res Commun* **236**, 59-65.

[15] Ohsawa I, Takamura C, Morimoto T, Ishiguro M, Kohsaka S (1999) Amino-terminal region of secreted form of amyloid precursor protein stimulates proliferation of neural stem cells. *Eur J Neurosci* **11**, 1907-1913.
[16] Caille I, Allinquant B, Dupont E, Bouillot C, Langer A, Muller U, Prochiantz A (2004) Soluble form of amyloid precursor protein regulates proliferation of progenitors in the adult subventricular zone. *Development* **131**, 2173-2181.
[17] Kwak YD, Marutle A, Dantuma E, Merchant S, Bushnev S, Sugaya K (2011) Involvement of notch signaling pathway in amyloid precursor protein induced glial differentiation. *Eur J Pharmacol* **650**, 18-27.
[18] Demars MP, Hollands C, Zhao Kda T, Lazarov O (2013) Soluble amyloid precursor protein-α rescues age-linked decline in neural progenitor cell proliferation. *Neurobiol Aging* **34**, 2431-2440.
[19] Tan VTY, Mockett BG, Ohline SM, Parfitt KD, Wicky HE, Peppercorn K, Schoderboeck L, Yahaya MFB, Tate WP, Hughes SM, Abraham WC (2018) Lentivirus-mediated expression of human secreted amyloid precursor protein-alpha prevents development of memory and plasticity deficits in a mouse model of Alzheimer's disease. *Mol Brain* **11**, 7.
[20] Obregon D, Hou H, Deng J, Giunta B, Tian J, Darlington D, Shahaduzzaman M, Zhu Y, Mori T, Mattson MP, Tan J (2012) Soluble amyloid precursor protein-α modulates β-secretase activity and amyloid-β generation. *Nat Commun* **3**, 777.
[21] Peters-Libeu C, Campagna J, Mitsumori M, Poksay KS, Spilman P, Sabogal A, Bredesen DE, John V (2015) sAβPPα is a Potent Endogenous Inhibitor of BACE1. *J Alzheimers Dis* **47**, 545-555.
[22] Ghiso J, Tagliavini F, Timmers WF, Frangione B (1989) Alzheimer's disease amyloid precursor protein is present in senile plaques and cerebrospinal fluid: immunohistochemical and biochemical characterization. *Biochem Biophys Res Commun* **163**, 430-437.
[23] Weidemann A, Konig G, Bunke D, Fischer P, Salbaum JM, Masters CL, Beyreuther K (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57**, 115-126.
[24] Lewczuk P, Popp J, Lelental N, Kolsch H, Maier W, Kornhuber J, Jessen F (2012) Cerebrospinal fluid soluble amyloid-beta protein precursor as a potential novel biomarkers of Alzheimer's disease. *J Alzheimers Dis* **28**, 119-125.
[25] Rosen C, Andreasson U, Mattsson N, Marcusson J, Minthon L, Andreasen N, Blennow K, Zetterberg H (2012) Cerebrospinal fluid profiles of amyloid beta-related biomarkers in Alzheimer's disease. *Neuromolecular Med* **14**, 65-73.
[26] Liu K, Doms RW, Lee VMY (2002) Glu11 Site Cleavage and N-Terminally Truncated Aβ Production upon BACE Overexpression. *Biochemistry* **41**, 3128-3136.
[27] Huse JT, Liu K, Pijak DS, Carlin D, Lee VMY, Doms RW (2002) Beta-Secretase Processing in the Trans-Golgi Network Preferentially Generates Truncated Amyloid Species That Accumulate in Alzheimer's Disease Brain *. *Journal of Biological Chemistry* **277**, 16278-16284.
[28] Lannfelt L, Basun H, Wahlund LO, Rowe BA, Wagner SL (1995) Decreased alpha-secretase-cleaved amyloid precursor protein as a diagnostic marker for Alzheimer's disease. *Nat Med* **1**, 829-832.
[29] Kim M, Suh J, Romano D, Truong MH, Mullin K, Hooli B, Norton D, Tesco G, Elliott K, Wagner SL, Moir RD, Becker KD, Tanzi RE (2009) Potential late-onset Alzheimer's disease-associated mutations in the ADAM10 gene attenuate {alpha}-secretase activity. *Hum Mol Genet* **18**, 3987-3996.

[30] Kovalevich J, Langford D (2013) Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods Mol Biol* **1078**, 9-21.
[31] Påhlman S, Ruusala A-I, Abrahamsson L, Mattsson MEK, Esscher T (1984) Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. *Cell Differentiation* **14**, 135-144.
[32] Findlay JA, Hamilton DL, Ashford ML (2015) BACE1 activity impairs neuronal glucose oxidation: rescue by beta-hydroxybutyrate and lipoic acid. *Front Cell Neurosci* **9**, 382.
[33] Kong J, Wan L, Wang Y, Zhang H, Zhang W (2020) Liraglutide Attenuates Abeta42 Generation in APPswe/SH-SY5Y Cells Through the Regulation of Autophagy. *Neuropsychiatr Dis Treat* **16**, 1817-1825.
[34] Lee TH, Park S, You MH, Lim JH, Min SH, Kim BM (2016) A potential therapeutic effect of saikosaponin C as a novel dual-target anti-Alzheimer agent. *J Neurochem* **136**, 1232-1245.
[35] Mattsson N, Rajendran L, Zetterberg H, Gustavsson M, Andreasson U, Olsson M, Brinkmalm G, Lundkvist J, Jacobson LH, Perrot L, Neumann U, Borghys H, Mercken M, Dhuyvetter D, Jeppsson F, Blennow K, Portelius E (2012) BACE1 inhibition induces a specific cerebrospinal fluid beta-amyloid pattern that identifies drug effects in the central nervous system. *PLoS One* **7**, e31084.
[36] Parkin ET, Watt NT, Hussain I, Eckman EA, Eckman CB, Manson JC, Baybutt HN, Turner AJ, Hooper NM (2007) Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. *Proc Natl Acad Sci U S A* **104**, 11062-11067.
[37] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**, 76-85.
[38] Hooper NM, Turner AJ (1987) Isolation of two differentially glycosylated forms of peptidyl-dipeptidase A (angiotensin converting enzyme) from pig brain: a re-evaluation of their role in neuropeptide metabolism. *Biochem J* **241**, 625-633.
[39] Brown PD, Giavazzi R (1995) Matrix metalloproteinase inhibition: a review of anti-tumour activity. *Ann Oncol* **6**, 967-974.
[40] Grant MKO, Handoko M, Rozga M, Brinkmalm G, Portelius E, Blennow K, Ashe KH, Zahs KR, Liu P (2019) Human cerebrospinal fluid 6E10-immunoreactive protein species contain amyloid precursor protein fragments. *PLoS One* **14**, e0212815.
[41] Stachel SJ, Coburn CA, Steele TG, Jones KG, Loutzenhiser EF, Gregro AR, Rajapakse HA, Lai MT, Crouthamel MC, Xu M, Tugusheva K, Lineberger JE, Pietrak BL, Espeseth AS, Shi XP, Chen-Dodson E, Holloway MK, Munshi S, Simon AJ, Kuo L, Vacca JP (2004) Structure-based design of potent and selective cell-permeable inhibitors of human beta-secretase (BACE-1). *J Med Chem* **47**, 6447-6450.
[42] von Rotz RC, Kohli BM, Bosset J, Meier M, Suzuki T, Nitsch RM, Konietzko U (2004) The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor. *Journal of Cell Science* **117**, 4435.
[43] An Y, Qi XL, Pei JJ, Tang Z, Xiao Y, Guan ZZ (2010) Amyloid precursor protein gene mutated at Swedish 670/671 sites in vitro induces changed expression of nicotinic acetylcholine receptors and neurotoxicity. *Neurochem Int* **57**, 647-654.
[44] Johnston J, O'Neill C, Lannfelt L, Winblad B, Cowburn RF (1994) The significance of the Swedish APP670/671 mutation for the development of Alzheimer's disease amyloidosis. *Neurochem Int* **25**, 73-80.

[45] Hébert SS, Bourdages V, Godin C, Ferland M, Carreau M, Lévesque G (2003) Presenilin-1 interacts directly with the beta-site amyloid protein precursor cleaving enzyme (BACE1). *Neurobiol Dis* **13**, 238-245.
[46] Cui J, Wang X, Li X, Wang X, Zhang C, Li W, Zhang Y, Gu H, Xie X, Nan F, Zhao J, Pei G (2015) Targeting the gamma-/beta-secretase interaction reduces beta-amyloid generation and ameliorates Alzheimer's disease-related pathogenesis. *Cell Discov* **1**, 15021.
[47] Lewczuk P, Kamrowski-Kruck H, Peters O, Heuser I, Jessen F, Popp J, Burger K, Hampel H, Frolich L, Wolf S, Prinz B, Jahn H, Luckhaus C, Perneczky R, Hull M, Schroder J, Kessler H, Pantel J, Gertz HJ, Klafki HW, Kolsch H, Reulbach U, Esselmann H, Maler JM, Bibl M, Kornhuber J, Wiltfang J (2010) Soluble amyloid precursor proteins in the cerebrospinal fluid as novel potential biomarkers of Alzheimer's disease: a multicenter study. *Mol Psychiatry* **15**, 138-145.
[48] Cuchillo-Ibañez I, Lopez-Font I, Boix-Amorós A, Brinkmalm G, Blennow K, Molinuevo J-L, Sáez-Valero J (2015) Heteromers of amyloid precursor protein in cerebrospinal fluid. *Molecular neurodegeneration* **10**, 2-2.
[49] Holsinger RM, McLean CA, Beyreuther K, Masters CL, Evin G (2002) Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. *Ann Neurol* **51**, 783-786.
[50] Fukumoto H, Cheung BS, Hyman BT, Irizarry MC (2002) β-Secretase Protein and Activity Are Increased in the Neocortex in Alzheimer Disease. *Archives of Neurology* **59**, 1381-1389.
[51] Yang L-B, Lindholm K, Yan R, Citron M, Xia W, Yang X-L, Beach T, Sue L, Wong P, Price D, Li R, Shen Y (2003) Elevated β-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nature Medicine* **9**, 3-4.
[52] Li R, Lindholm K, Yang L-B, Yue X, Citron M, Yan R, Beach T, Sue L, Sabbagh M, Cai H, Wong P, Price D, Shen Y (2004) Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3632-3637.
[53] Coulson DT, Beyer N, Quinn JG, Brockbank S, Hellemans J, Irvine GB, Ravid R, Johnston JA (2010) BACE1 mRNA expression in Alzheimer's disease postmortem brain tissue. *J Alzheimers Dis* **22**, 1111-1122.
[54] Alexopoulos P, Thierjung N, Grimmer T, Ortner M, Economou P, Assimakopoulos K, Gourzis P, Politis A, Perneczky R (2018) Cerebrospinal Fluid BACE1 Activity and sAβPPβ as Biomarker Candidates of Alzheimer's Disease. *Dementia and Geriatric Cognitive Disorders* **45**, 152-161.
[55] Xia Q, Yang X, Shi J, Liu Z, Peng Y, Wang W, Li B, Zhao Y, Xiao J, Huang L, Wang D, Gao X (2021) The Protective A673T Mutation of Amyloid Precursor Protein (APP) in Alzheimer's Disease. *Mol Neurobiol* **58**, 4038-4050.
[56] Kimura A, Hata S, Suzuki T (2016) Alternative Selection of beta-Site APP-Cleaving Enzyme 1 (BACE1) Cleavage Sites in Amyloid beta-Protein Precursor (APP) Harboring Protective and Pathogenic Mutations within the Abeta Sequence. *J Biol Chem* **291**, 24041-24053.

FIGURE LEGENDS

Figure 1. Amyloid-beta protein precursor expression and non-amyloidogenic processing in AD-relevant SH-SY5Y cell lines. Mock-, BACE1-, wtAβPP- and SweAβPP-transfected SH-SY5Y cells were grown to confluence and then incubated in the absence/presence of batimastat (5 μM) for 24 h before harvesting and preparing lysates and conditioned medium samples (Materials and Methods section). **(A)** Lysates were immunoblotted with anti-BACE1 (upper panel) and anti-actin (lower panel) antibodies. **(B)** Detection of full-length AβPP (FL-AβPP) in cell lysates using the anti-AβPP C-terminal (AβPP-CT) antibody. Multiple immunoblots were then quantified and the results expressed relative to mock-transfectant controls. **(C)** The effect of batimastat on FL-AβPP levels in lysates. Results are expressed relative to the no inhibitor controls for each cell line. **(D)** Detection of non-amyloidogenically derived soluble AβPP (sAβPP) in medium using anti-AβPP 6E10 antibody. Multiple immunoblots were then quantified and the results expressed relative to mock-transfectant controls. **(E)** The effect of batimastat on non-amyloidogenically derived sAβPP in conditioned medium. Results are expressed relative to the no inhibitor controls for each cell line. Results are means ± S.D. (n=3, independent cultures). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Hash symbols (#) indicate the same levels of significance but relative to the cognate experimental controls.

Figure 2. Soluble amyloid-beta protein precursor beta (sAβPPβ) generation by AD-relevant SH-SY5Y cell lines. Mock-, BACE1-, wtAβPP- and SweAβPP-transfected SH-SY5Y cells were grown to confluence and then incubated in the absence/presence of batimastat (5 μM) for 24 h before harvesting and preparing conditioned medium samples (Materials and Methods section). **(A)** Detection of wtAβPP-derived sAβPPβ in conditioned medium. Multiple immunoblots were then quantified and the results expressed relative to

mock-transfectant controls. **(B)** Detection of SweAβPP-derived sAβPPβ in conditioned medium. **(C)** The effect of batimastat on wtAβPP-derived sAβPPβ in conditioned medium. Results are expressed relative to the no inhibitor controls for each cell line. Results are means ± S.D. (n=3, independent cultures). *, $p < 0.05$; **, $p < 0.01$. Hash symbols (#) indicate the same levels of significance but relative to the cognate experimental controls.

Figure 3. Amyloid-beta protein precursor C-terminal fragment generation in AD-relevant SH-SY5Y cell lines. Mock-, BACE1-, wtAβPP- and SweAβPP-transfected SH-SY5Y cells were grown to confluence before harvesting, preparing lysate samples and resolving proteins on Tris/Tricine gels (Materials and Methods section). **(A)** Detection of AβPP C-terminal fragments (CTFs) in cell lysates using the anti-AβPP C-terminal (AβPP-CT) antibody. **(B)** Quantification of C99 levels in lysates. Results are expressed relative to mock-transfectant controls. **(C)** Quantification of C83 and C89 levels in lysates. Results for C83 are expressed relative to mock-transfectant controls. C89 could only be detected in SH-SY5Y-BACE1 cells and, therefore, is expressed relative to mock-transfectant C83 levels in order to demonstrate the complete replacement of the latter fragment in SH-SY5Y-BACE1 cells with C89. Results are means ± S.D. (n=3, independent cultures). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Hash symbols (#) indicate the same levels of significance but relative to the cognate experimental controls.

Figure 4. The effect of α- and β-secretase inhibitors on the non-amyloidogenic production of soluble amyloid-beta protein precursor by mock- and BACE1-transfected SH-SY5Y cells. Mock- **(A, B)** and BACE1- **(C, D)** transfected SH-SY5Y cells were grown to confluence and then incubated in the absence/presence of batimastat (5 μM) and/or β-secretase inhibitor IV (5 μM) for 24 h before harvesting and preparing conditioned medium

samples (Materials and Methods section). **(A, C)** Detection of non-amyloidogenically derived sAβPP using anti-AβPP 6E10 antibody in medium from mock- and BACE1-transfected cells, respectively. **(B, D)** Detection of wtAβPP-derived sAβPPβ in conditioned medium from mock- and BACE-1 transfected cells, respectively. Multiple immunoblots were quantified and the results expressed relative to the no inhibitor controls for each cell line. Results are means ± S.D. (n=3, independent cultures). **, $p < 0.01$; ***, $p < 0.001$. Hash symbols (#) indicate the same levels of significance but relative to the cognate experimental controls.

Figure 5. The effect of α- and β-secretase inhibitors on amyloid-beta protein precursor C-terminal fragment generation in mock- and BACE1-transfected SH-SY5Y cells. Mock- **(A-C)** and BACE1- **(D-F)** transfected SH-SY5Y cells were grown to confluence and then incubated in the absence/presence of batimastat (5 μM) and/or β-secretase inhibitor IV (5 μM) for 24 h before harvesting, preparing lysate samples and resolving proteins on Tris/Tricine gels (Materials and Methods section). **(A, D)** Detection of AβPP C-terminal fragments (CTFs) using the anti-AβPP C-terminal (AβPP-CT) antibody in lysates from mock- and BACE-1 transfected cells, respectively. **(B, E)** Quantification of C99 levels in lysates from mock- and BACE1-transfected cells, respectively. Results are expressed relative to the no inhibitor controls for each cell line. **(C)** Quantification of C83 levels in lysates from mock-transfected cells. Results are expressed relative to the no inhibitor controls. **(F)** Quantification of C89/C83 levels in lysates from BACE1-transfected cells. C89 in the batimastat-treated cells is expressed relative to the no inhibitor controls. In the β-secretase inhibitor IV and β-secretase inhibitor IV + batimastat samples the C83 and C89 bands were not effectively resolved and so are combined and expressed relative to C89 in the no inhibitor controls. Results are means ± S.D. (n=3, independent cultures). *, $p < 0.05$; **, $p < 0.01$; ***, $p <$

0.001. Hash symbols (#) indicate the same levels of significance but relative to the cognate experimental controls.

Figure 6. Amyloid beta-peptide generation by AD-relevant SH-SY5Y cell lines does not relate to changes in cell viability. (A) Mock-, BACE1-, wtAβPP- and SweAβPP-transfected SH-SY5Y cells were grown to confluence and then incubated for a further 24 h in UltraMEM™ reduced serum medium before quantifying Aβ-peptide levels in the conditioned medium (Materials and Methods section). Note that $A\beta_{40}$ levels are presented on the left-hand Y-axis and $A\beta_{42}$ levels on the right-hand Y-axis. Results are the means ± S.D. (n=3, independent cultures) of absolute Aβ-peptide concentrations (pg/ml). **(B, C)** Trypan blue and **(D, E)** Methanethiosulfonate (MTS) analyses of cell viability. All four cell lines were seeded and viability assays conducted at the indicated time points as described in the Materials and Methods section **(B and D)**. The area under the curve (AUC) was subsequently determined for each cell line (**C, E**). Results for the viability assays are means ± S.D. (n=3 (Trypan blue) and n=6 (MTS), independent cultures). **, $p < 0.01$; ***, $p < 0.001$. Hash symbols (#) indicate the same levels of significance but relative to the cognate experimental controls.

Figure 7. Generation and characterization of sAβPPα- and sAβPPβ'-over-expressing SH-SY5Y cells. The 3' coding region sequencing results for the pIRESneo-sAβPPα and pIRESneo-sAβPPβ' plasmids are shown in **(A)**. Both plasmids, along with empty pIRESneo expression vector, were then stably transfected into SH-SY5Y-mock cells (previously stably transfected with empty pIREShyg) **(B-D)** and SH-SY5Y-BACE1 cells (previously stably transfected with pIREShyg-BACE1) **(E-G)**. All transfectants were then grown to confluence and incubated for a further 24 h in UltraMEM™ reduced serum medium before harvesting and preparing conditioned medium samples (Materials and Methods section). **(B, E)**

Detection of non-amyloidogenically derived sAβPP in medium using anti-AβPP 6E10 antibody. **(C, F)** Detection of wtAβPP-derived sAβPPβ in conditioned medium. **(D, G)** Detection of total sAβPP in conditioned medium using anti-AβPP N-terminal (AβPP-NT) 22C11 antibody. Multiple immunoblots were quantified and the results expressed relative to the cognate mock transfectant controls for the SH-SY5Y-mock and SH-SY5Y-BACE1 cells. Results are means ± S.D. (n=3, independent cultures). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Hash symbols (#) indicate the same levels of significance but relative to the cognate experimental controls.

Figure 8. The effect of sAβPPα and sAβPPβ' over-expression on the viability of mock- and BACE1-transfected SH-SY5Y cells. (A, B) Trypan blue and **(C, D)** Methanethiosulfonate (MTS) analyses of cell viability. All cell lines were seeded and viability assays conducted at the indicated time points as described in the Materials and Methods section **(A and C)**. The area under the curve (AUC) was subsequently determined for each cell line (**B, D**). Results for all viability assays are means ± S.D. (n=3 (Trypan blue) and n=6 (MTS), independent cultures). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Hash symbols (#) indicate the same levels of significance but relative to the cognate experimental controls.

Figure 1

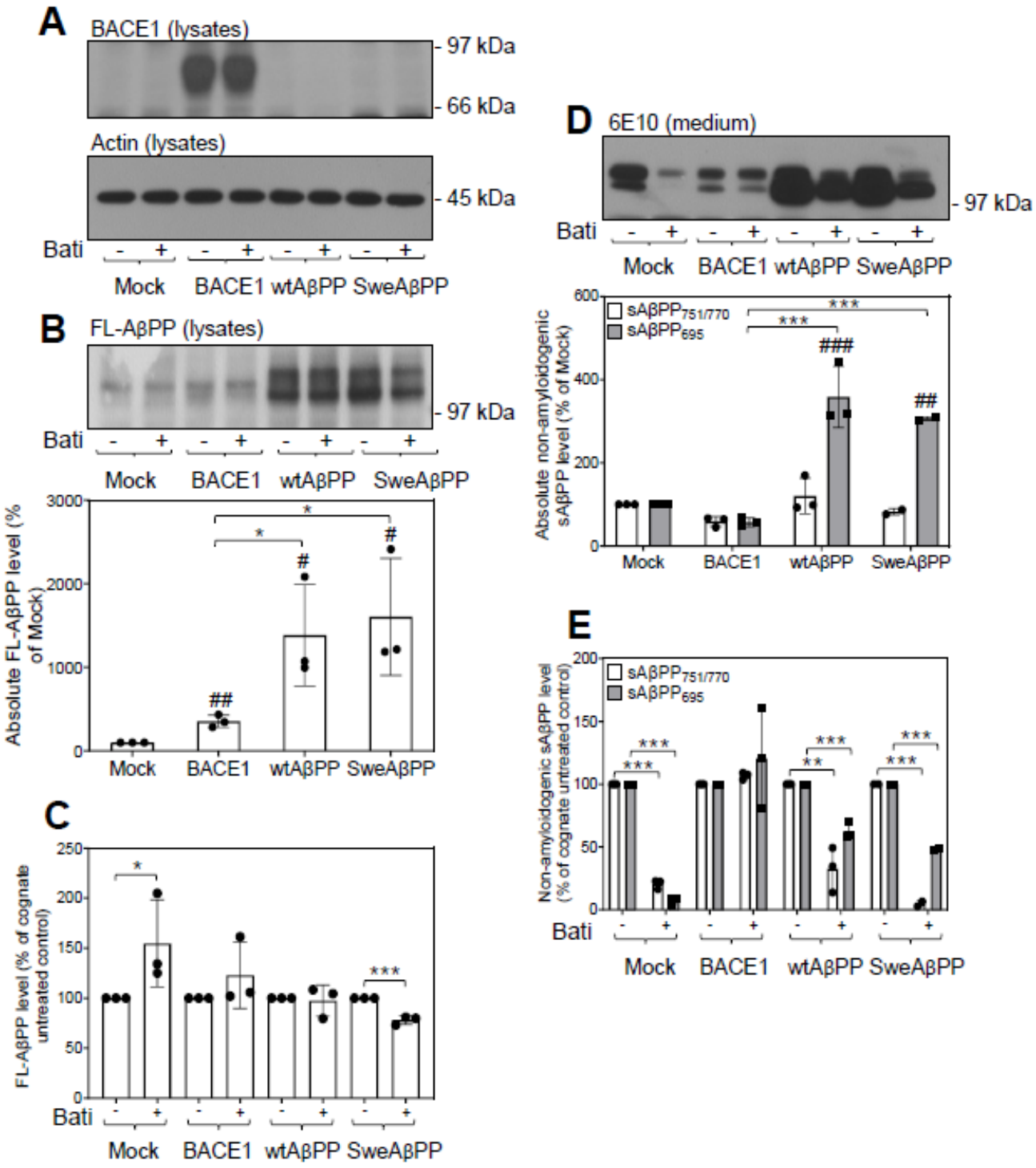

A
BACE1 (lysates)
- 97 kDa
- 66 kDa
Actin (lysates)
- 45 kDa
Bati
Mock
BACE1
wtAβPP
SweAβPP
B
FL-AβPP (lysates)
- 97 kDa
Absolute FL-AβPP level (% of Mock)
C
FL-AβPP level (% of cognate untreated control)
D
6E10 (medium)
- 97 kDa
Absolute non-amyloidogenic sAβPP level (% of Mock)
sAβPP751/770
sAβPP695
E
Non-amyloidogenic sAβPP level (% of cognate untreated control)

Figure 2

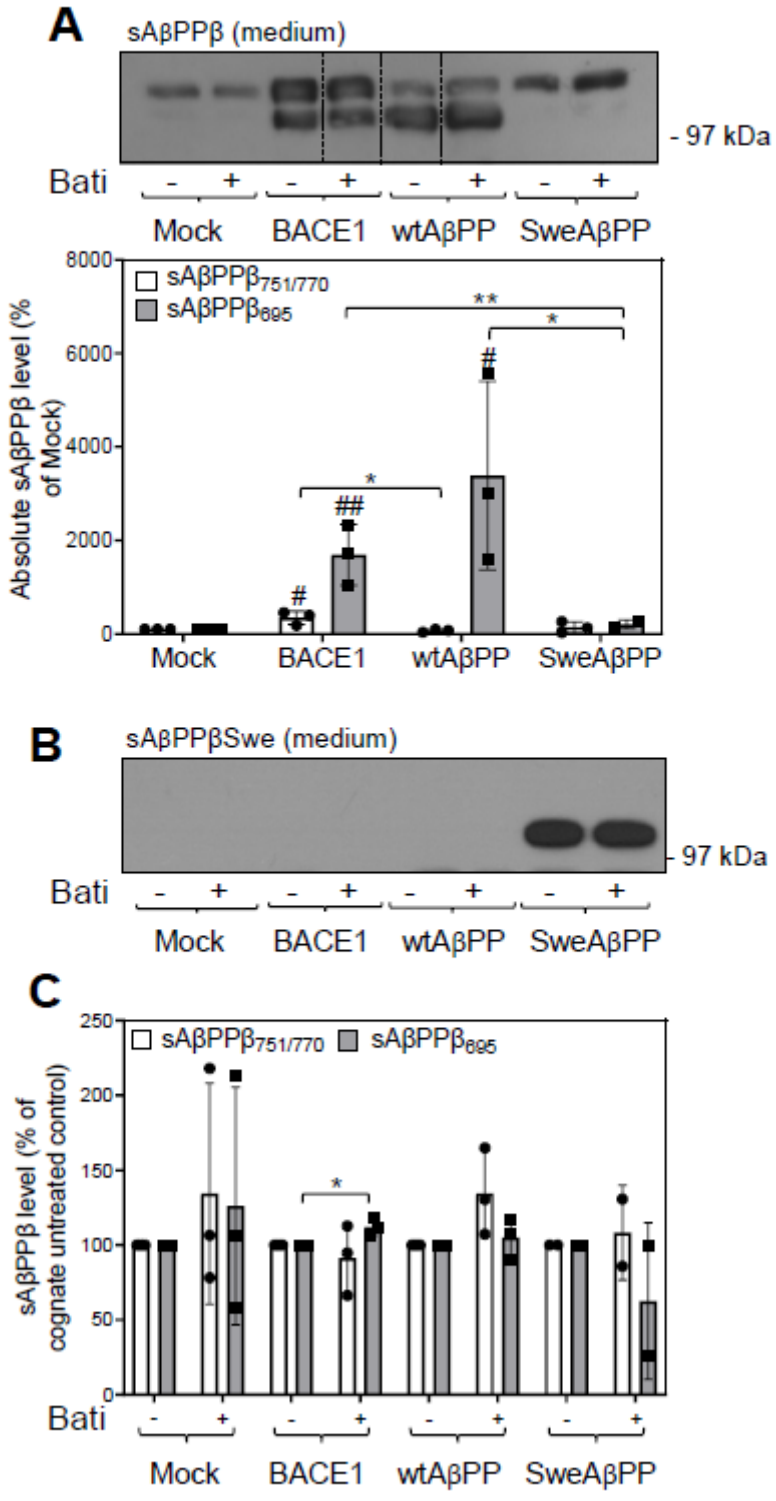

A
sAβPPβ (medium)
- 97 kDa
Bati
- + - + - + - +
Mock
BACE1
wtAβPP
SweAβPP
8000
6000
4000
2000
0
sAβPPβ751/770
sAβPPβ695
Absolute sAβPPβ level (% of Mock)
**
*
*
#
##
#
Mock
BACE1
wtAβPP
SweAβPP
B
sAβPPβSwe (medium)
- 97 kDa
Bati
- + - + - + - +
Mock
BACE1
wtAβPP
SweAβPP
C
250
200
150
100
50
0
sAβPPβ751/770
sAβPPβ695
sAβPPβ level (% of cognate untreated control)
*
Bati
- + - + - + - +
Mock
BACE1
wtAβPP
SweAβPP

Figure 3

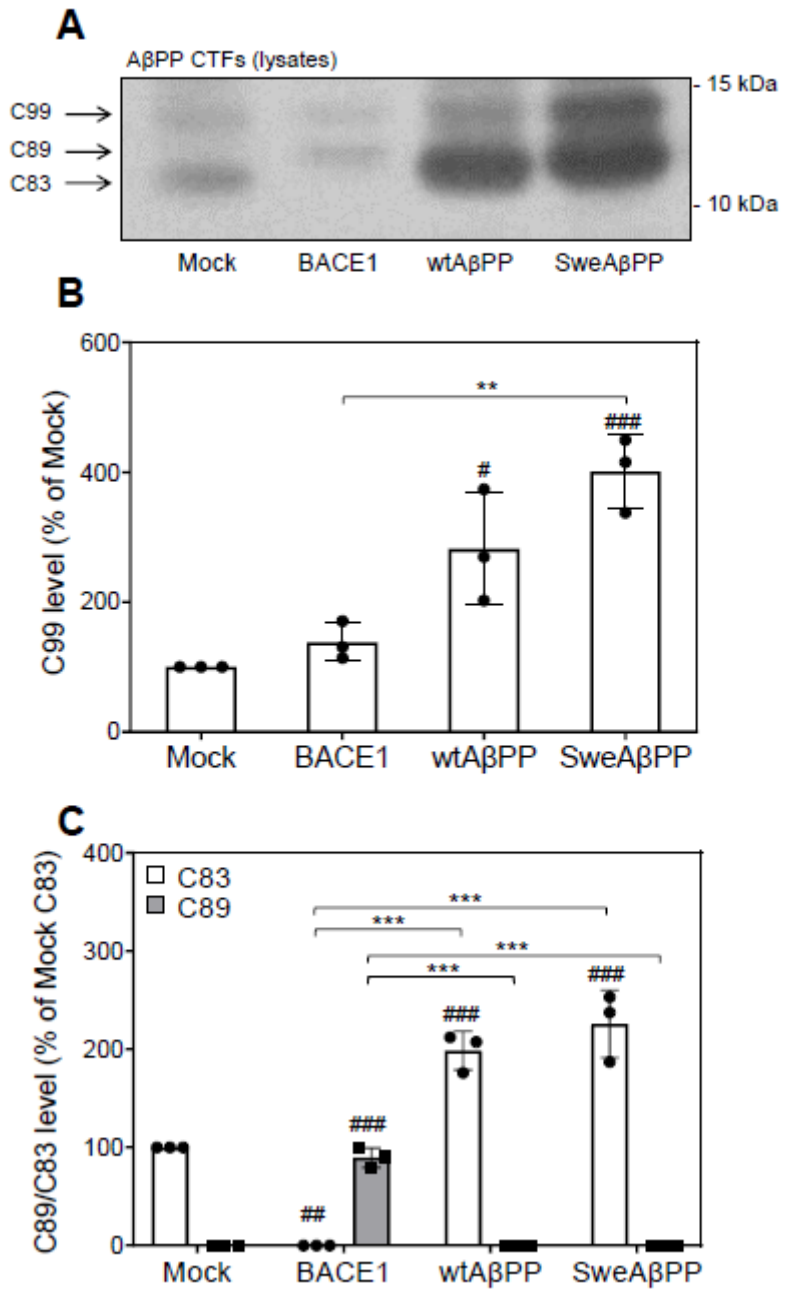

A
AβPP CTFs (lysates)
C99
C89
C83
15 kDa
10 kDa
Mock
BACE1
wtAβPP
SweAβPP
B
C99 level (% of Mock)
600
400
200
0
**
###
#
Mock
BACE1
wtAβPP
SweAβPP
C
C89/C83 level (% of Mock C83)
400
300
200
100
0
C83
C89
***
***
***
***
###
###
###
##
Mock
BACE1
wtAβPP
SweAβPP

Figure 4

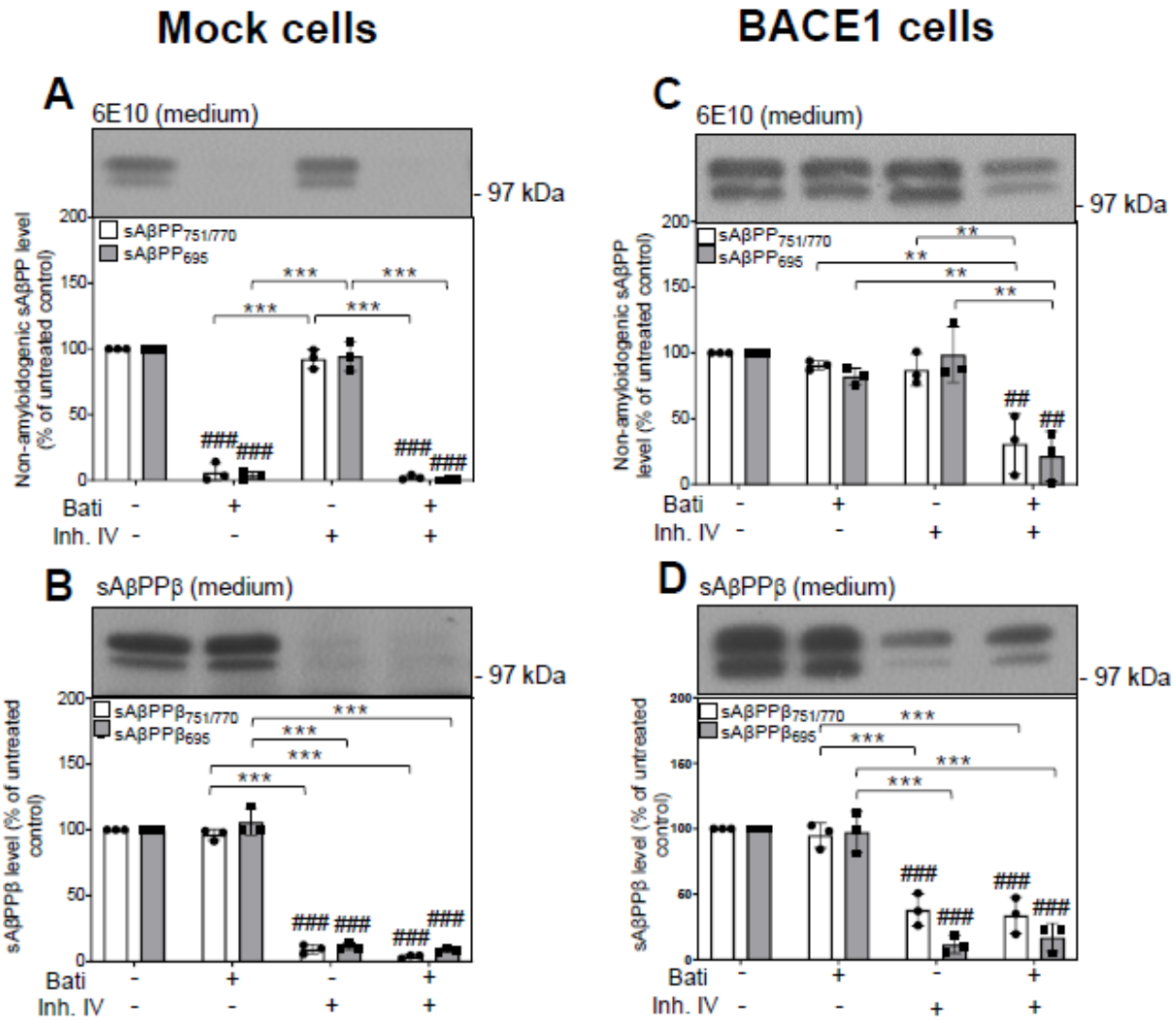

Mock cells
BACE1 cells
A
6E10 (medium)
- 97 kDa
sAβPP751/770
sAβPP695
Non-amyloidogenic sAβPP level (% of untreated control)
Bati
Inh. IV
B
sAβPPβ (medium)
sAβPPβ751/770
sAβPPβ695
sAβPPβ level (% of untreated control)
C
6E10 (medium)
D
sAβPPβ (medium)

Figure 5

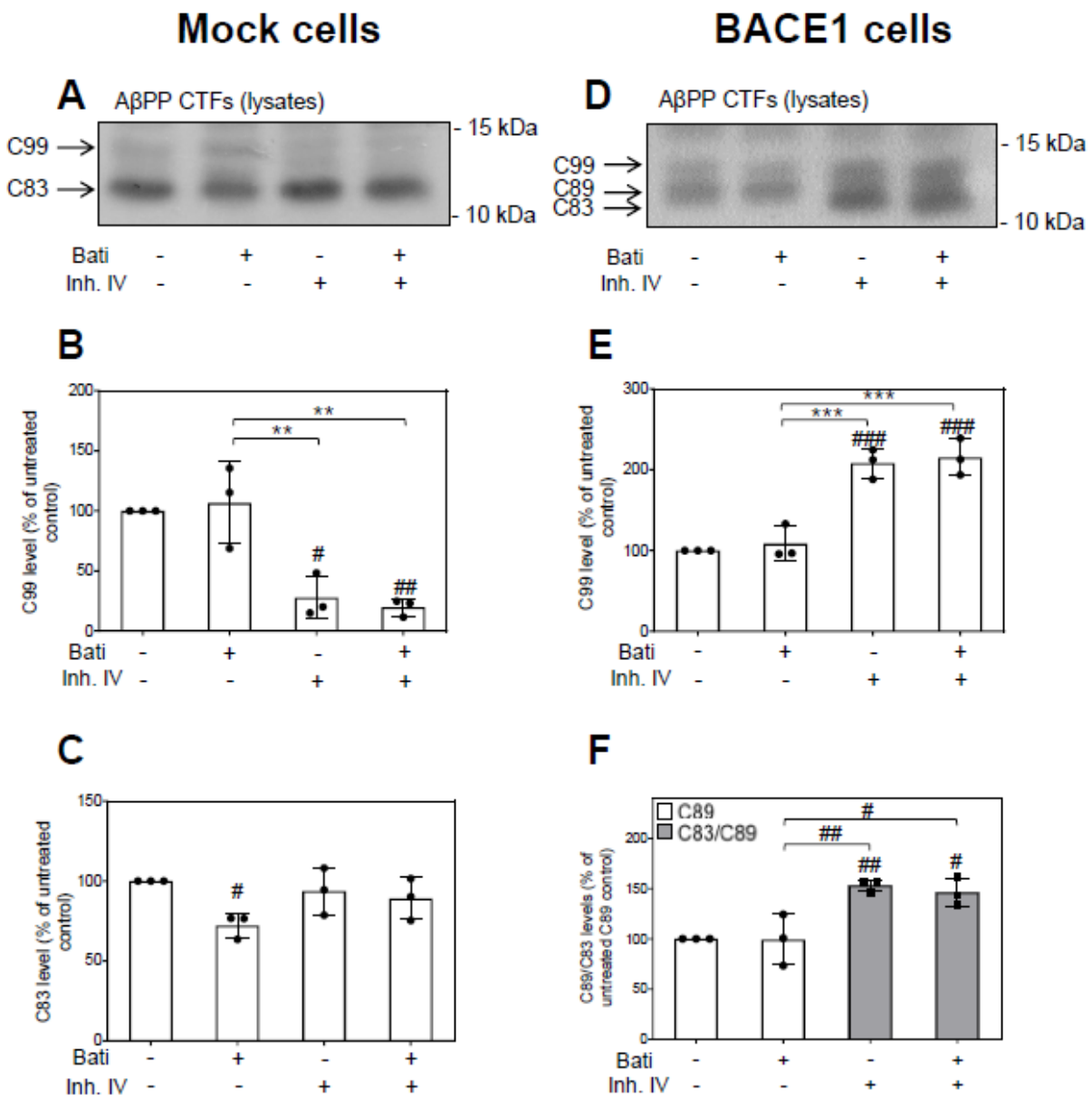

Mock cells
BACE1 cells
A
AβPP CTFs (lysates)
C99
C83
- 15 kDa
- 10 kDa
Bati - + - +
Inh. IV - - + +
D
AβPP CTFs (lysates)
C99
C89
C83
- 15 kDa
- 10 kDa
Bati - + - +
Inh. IV - - + +
B
C99 level (% of untreated control)
200
150
100
50
0
**
**
#
##
Bati - + - +
Inh. IV - - + +
E
C99 level (% of untreated control)
300
200
100
0
***
***
###
###
Bati - + - +
Inh. IV - - + +
C
C83 level (% of untreated control)
150
100
50
0
#
Bati - + - +
Inh. IV - - + +
F
C89
C83/C89
C89/C83 levels (% of untreated C89 control)
200
150
100
50
0
#
##
##
#
Bati - + - +
Inh. IV - - + +

Figure 6

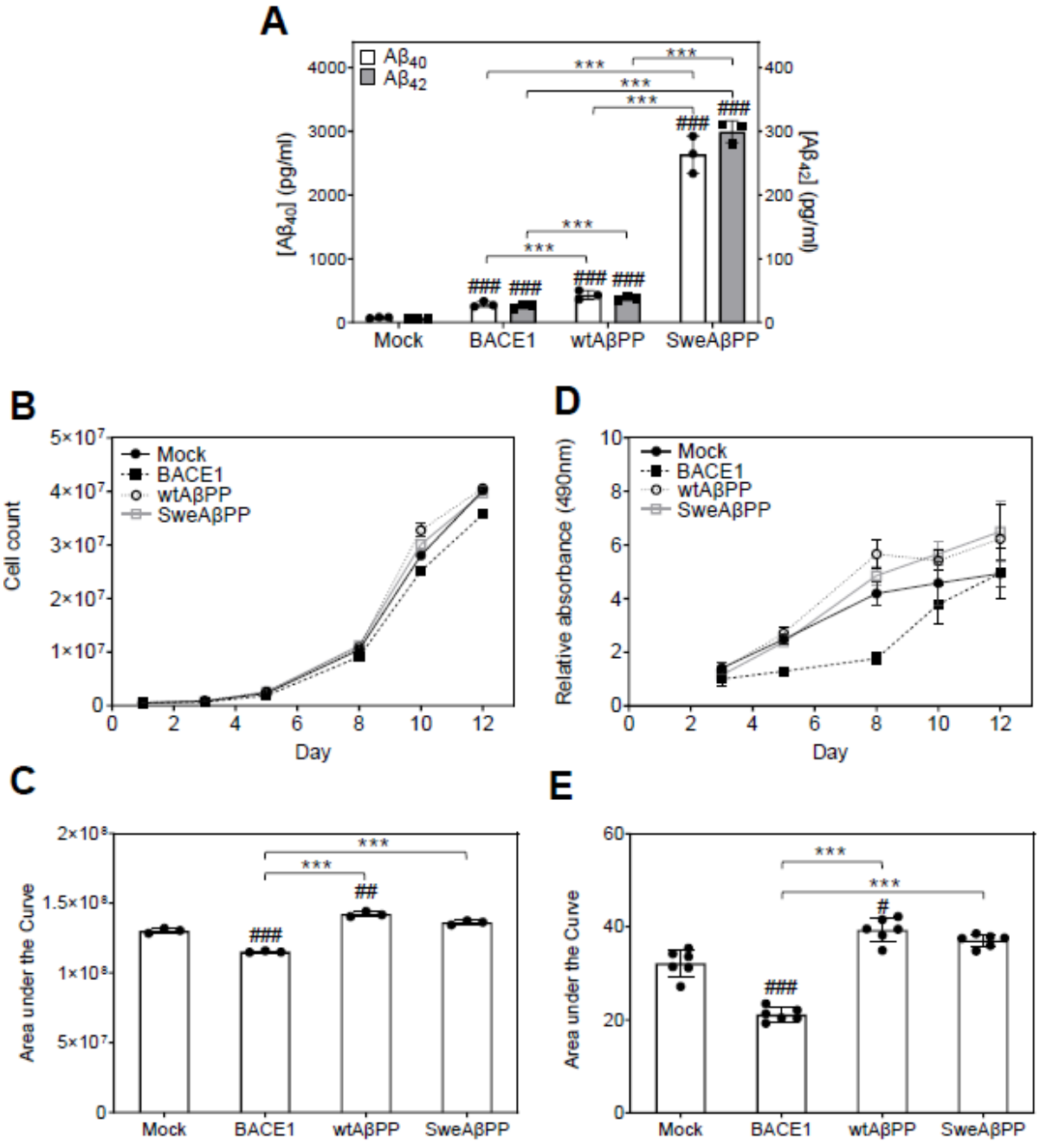

A
Aβ40
Aβ42
[Aβ40] (pg/ml)
[Aβ42] (pg/ml)
Mock
BACE1
wtAβPP
SweAβPP
B
Cell count
Day
C
Area under the Curve
D
Relative absorbance (490nm)
E
Area under the Curve

Figure 7

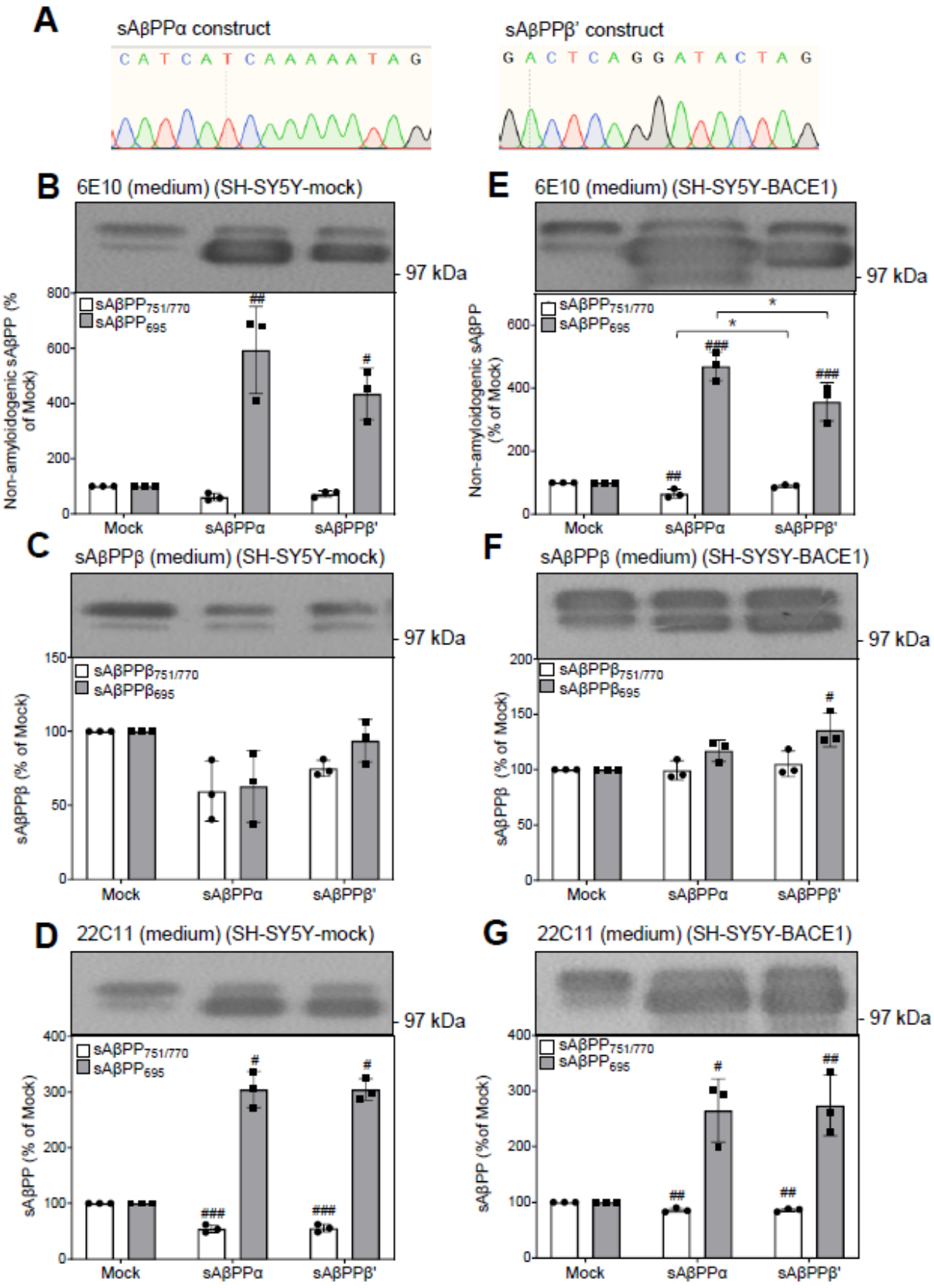

A
sAβPPα construct
C A T C A T C A A A A A T A G
sAβPPβ' construct
G A C T C A G G A T A C T A G
B
6E10 (medium) (SH-SY5Y-mock)
97 kDa
sAβPP751/770
sAβPP695
Non-amyloidogenic sAβPP (% of Mock)
Mock
sAβPPα
sAβPPβ'
E
6E10 (medium) (SH-SY5Y-BACE1)
97 kDa
Non-amyloidogenic sAβPP (% of Mock)
C
sAβPPβ (medium) (SH-SY5Y-mock)
97 kDa
sAβPPβ751/770
sAβPPβ695
sAβPPβ (% of Mock)
F
sAβPPβ (medium) (SH-SYSY-BACE1)
97 kDa
D
22C11 (medium) (SH-SY5Y-mock)
97 kDa
sAβPP (% of Mock)
G
22C11 (medium) (SH-SY5Y-BACE1)
97 kDa
sAβPP (%of Mock)

Figure 8

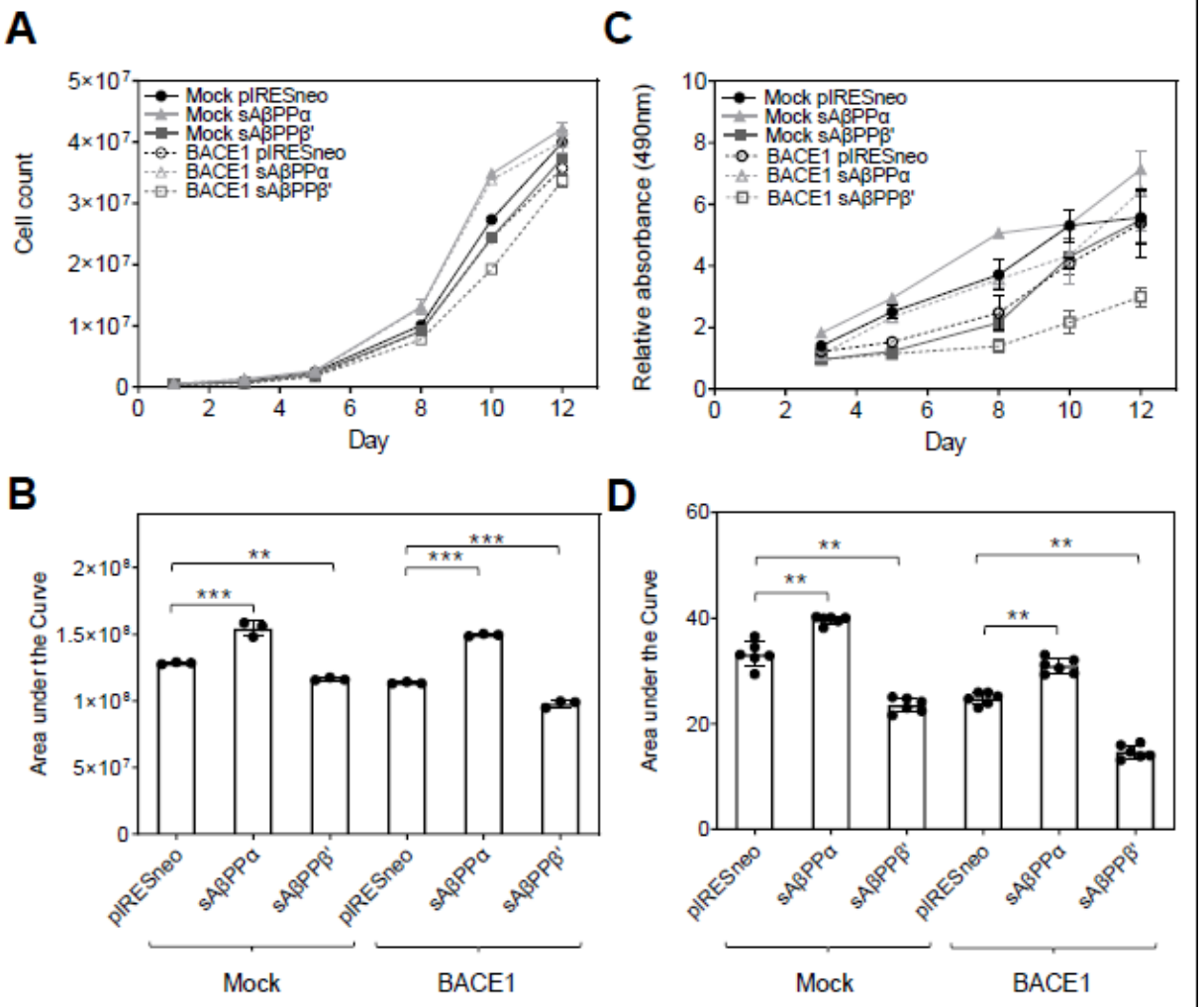
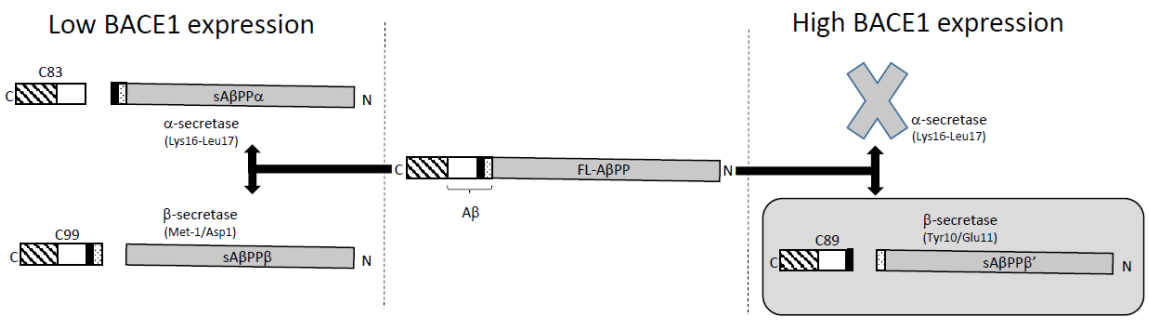

A
Cell count
Day
Mock pIRESneo
Mock sAβPPα
Mock sAβPPβ'
BACE1 pIRESneo
BACE1 sAβPPα
BACE1 sAβPPβ'
C
Relative absorbance (490nm)
Day
B
Area under the Curve
pIRESneo
sAβPPα
sAβPPβ'
Mock
BACE1
D
Area under the Curve
Mock
BACE1

Figure 9


Low BACE1 expression
High BACE1 expression
C83
C
sAβPPα
N
α-secretase
(Lys16-Leu17)
C
FL-AβPP
N
Aβ
α-secretase
(Lys16-Leu17)
β-secretase
(Met-1/Asp1)
C99
C
sAβPPβ
N
β-secretase
(Tyr10/Glu11)
C89
C
sAβPPβ'
N

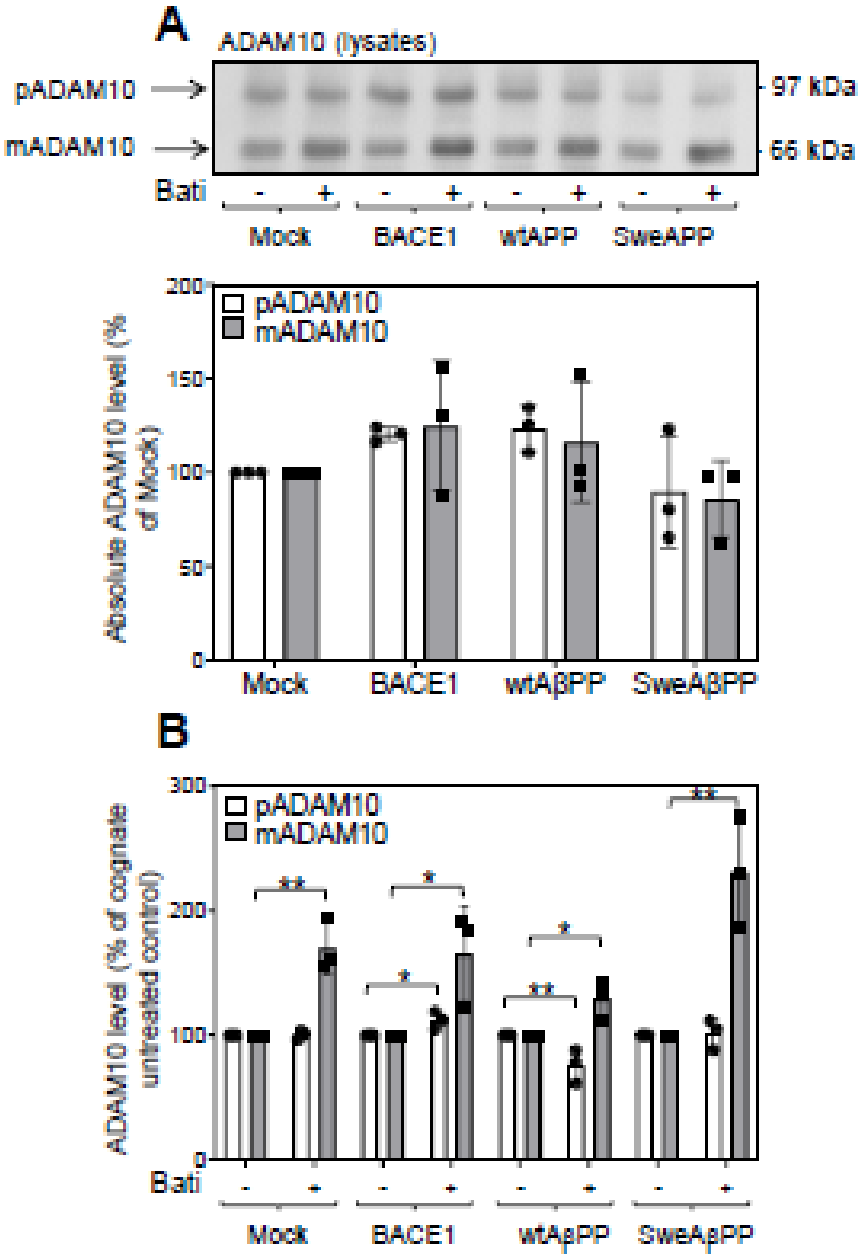


Figure S1. A Disintegrin And Metalloproteinase (ADAM) 10 expression and proteolytic maturation in Alzheimer's disease (AD)-relevant SH-SY5Y cell lines. Mock-, β-site AβPP cleaving enzyme 1 (BACE1)-, wild-type $AβPP_{695}$ (wtAβPP)- and Swedish mutant $AβPP_{695}$ (SweAβPP)-SH-SY5Y cells were grown to confluence and then incubated in the absence or presence of batimastat (5 μM) for 24 h before harvesting and preparing lysates (Materials and Methods section). **(A)** Lysates were immunoblotted with anti-ADAM10 antibody. Multiple immunoblots were then quantified and the results expressed relative to mock-transfectant controls for both prodomain-containing ADAM10 (pADAM10) and the proteolytically mature form of the enzyme (mADAM10). **(B)** The effect of batimastat on ADAM10 levels in lysates. Results are expressed relative to the no inhibitor controls for each cell line. Results are means ± standard deviation (S.D.) (n=3, independent cultures). *, $p < 0.05$; **, $p < 0.01$.

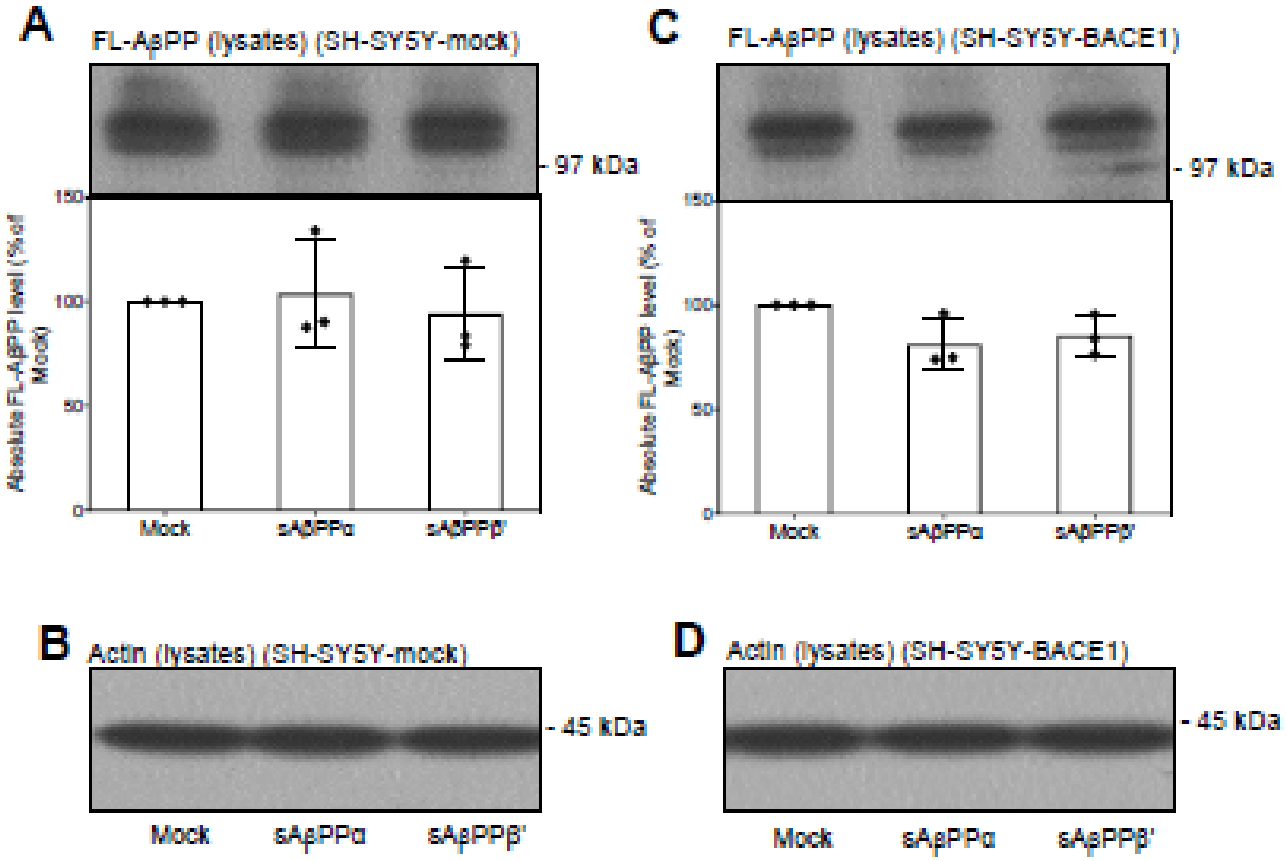


Figure S2. The effect of soluble amyloid-beta precursor protein (sAβPP) α and sAβPPβ' over-expression on endogenous full-length AβPP (FL-AβPP) expression in mock- and β-site AβPP cleaving enzyme 1 (BACE1)-transfected SH-SY5Y cells. pIRESneo-sAβPPα and pIRESneo-sAβPPβ' plasmids, along with empty pIRESneo expression vector, were stably transfected into SH-SY5Y-mock (previously stably transfected with empty pIREShyg) (**A, B**) and SH-SY5Y-BACE1 (previously stably transfected with pIREShyg-BACE1) (**C, D**) cells. All transfectants were then grown to confluence and incubated for a further 24 h in UltraMEM™ reduced serum medium before harvesting and preparing lysate samples (Materials and Methods section). (**A, C**) Detection of FL-AβPP in cell lysates using the anti-AβPP C-terminal (AβPP-CT) antibody. Multiple immunoblots were then quantified and the results expressed relative to the cognate mock transfectant controls for the SH-SY5Y-mock and SH-SY5Y-BACE1 cells. (**B, D**) Lysates were immunoblotted with anti-actin antibody. Results are means ± standard deviation (S.D.) (n=3, independent cultures).