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γ-Secretase regulates the α-secretase cleavage of the Alzheimer's disease, amyloid precursor protein

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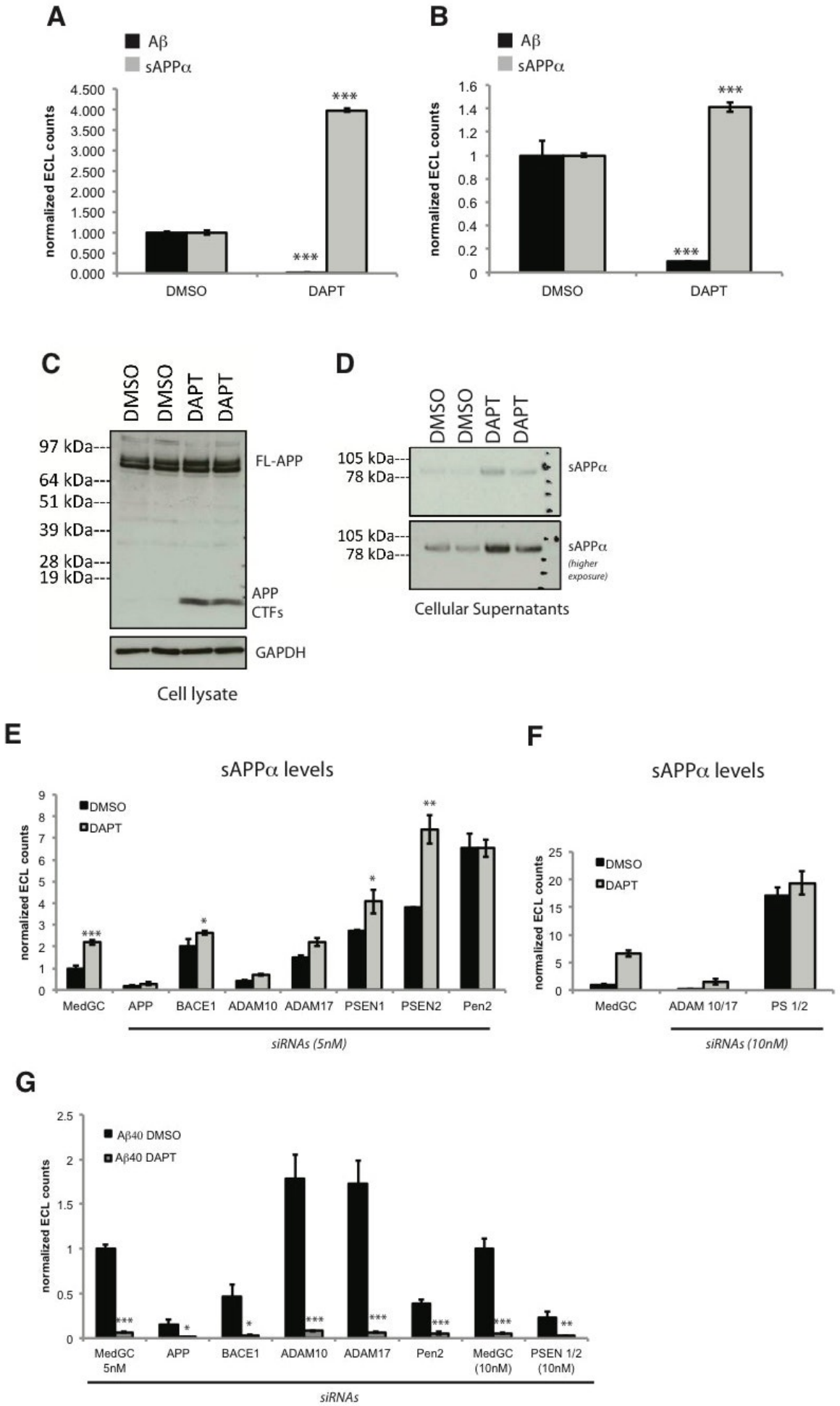
Abstract
Cerebral accumulation of β-amyloid (Aβ) peptides is causatively linked to Alzheimer's disease (AD). Aβ peptides are generated by the proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretases. α-Secretase, another membrane protein, cleaves APP but in the middle of the Aβ peptide domain, thus preventing the formation of the toxic peptide. Only after α- or β-secretase cleaves APP, γ-secretase can cleave the C-terminal membrane-bound fragments, thus making γ-cleavage downstream of α/β-cleavage. Here we uncover a feedback regulation from γ-secretase to α-secretase and show that γ-secretase inhibition either pharmacologically or by silencing γ-secretase components increases α-secretase cleavage of APP. Since γ-secretase inhibitors are in clinical trials, our results show an unexpected consequence on α-cleavage of APP. Our results have important implications on γ-secretase inhibitors for AD diagnosis and therapy.

Introduction
Alzheimer's disease (AD) is divided into two main subtypes, early-oneset AD and late-onset AD, which are defined according to the patient's age at disease onset. Early-onset AD or familial AD (FAD) comprises less than 2% of the AD cases and affects the patients before they reach the age of 65 years. The remaining 98% of the patients suffer from late-onset AD, which occurs at older ages [1]. Several genetic mutations that promote FAD have been identified and helped to characterize the molecular processes that contribute to the development of AD [2] [3]. Most of these mutations can be directly associated with the processing of amyloid precursor protein (APP), either directly affecting the molecular characteristics of APP itself or affecting the presenilin subunits of the γ-secretase complex (Presenilin 1 or 2) [2] [4]. The amyloidogenic peptide Aβ, which is causally linked to AD, is released from the APP through sequential proteolytic cleavage by β- and γ-secretases [1] [5]. The APP is a type I transmembrane protein that can undergo sequential proteolytic cleavage in two distinct routes [6]. In the non-amyloidogenic pathway, APP is cleaved by α-secretase, giving rise to the N-terminal soluble-APP alpha fragment (sAPPα) and the 83-amino-acid-long C-terminal fragment (C83). C83 can undergo further proteolytic cleavage by γ-secretase releasing the short transmembrane domain p3 and the APP intracellular domain (AICD or C59) [7]. In the amyloidogenic pathway, APP is first cleaved by β-secretase giving rise to the N-terminal-soluble APP beta fragment (sAPPβ) and the 99-amino-acid-long C-terminal fragment (C99). C99 can undergo further proteolytic cleavage by γ-secretase releasing the amyloid beta peptide (Aβ) and C59 (AICD). α-Secretase cleaves APP within the Aβ domain, hence precluding the production of Aβ and is, therefore, termed as the non-amyloidogenic pathway [8]. α-Secretase activity on APP has been shown to be conferred mainly by ADAM-10, a member of the protein family of disintegrin and metalloproteases (ADAM) [9]. Members of the ADAM family are transmembrane proteins locating to the plasma membrane, where α-cleavage of APP occurs [10]. They are implicated in ectodomain shedding of several substrates. Because α-secretase cleavage of APP evades Aβ formation, enhancing its activity was considered for therapeutic intervention in AD. But the resulting side effects include metastasis formation in tumor patients. Hence α-secretase was abandoned as a potential therapeutic target [11]. The generation of Aβ from APP is initiated through cleavage by a transmembrane aspartyl protease termed as BACE1 (β-site APP-cleaving enzyme 1) [12] [13]. BACE1/β-secretase is a type I transmembrane protein whose active site is located in the ectodomain that cleaves APP between the amino acids Met-671 and Asp-672 [14]

[15]. This cleavage results in the release of the extracellular APP domain (sAPPβ) and a membrane-bound, C99. BACE1 is mainly localized to the TGN, endosomes, and lysosomes [6], but β-secretase cleavage of APP occurs predominantly in endosomes [16] [17] where the acidic pH (4.0–5.0) is optimal for β-secretase activity. Agents that disrupt the intracellular pH will, therefore, also inhibit β-secretase activity [18]. Trafficking of BACE1 to the endosomes, which is necessary for Aβ production, can happen either via internalization from the plasma membrane or by direct sorting from the TGN. Furthermore, interference of this trafficking in order to prevent BACE1 to reach the endosomes might be a potential therapeutic strategy to reduce β-cleavage of APP [19]. Alternatively, accelerating its trafficking away from endosomes reduces β-cleavage of APP and, therefore, Aβ release [20] [21] [22]. γ-Secretase is a multimeric protein complex that is composed of four different transmembrane components: Presenilin-1(PS1)/Presenilin-2(PS2), Anterior pharynx defective-1 (in humans, Aph-1a or Aph-1b), Nicastrin, and Presenilin enhancer2 (Pen-2) [3] [23] [24]. In addition, numerous other proteins have been shown to bind and interact with this protein complex to modulate its function, but whether they also affect γ-secretase-mediated processing of APP remains to be resolved [24]. The γ-secretase complex is the last of the proteolytic enzymes in APP processing and, therefore, directly contributes to Aβ levels. γ-Secretase components are synthesized in the endoplasmatic reticulum (ER), but the assembly of the mature and functional complex requires the coordinated regulation of the ER-Golgi recycling circuit [25]. Not only at the synthesis level but also in order to access its substrate APP, the γ-secretase complex relies on intracellular trafficking as cleavage of C-terminal APP occurs in post-Golgi compartments, i.e., in endosomes [26]. APP cleavage by γ-secretase happens at different positions within the APP sequence, leading to the release of amyloid peptides of various lengths (Aβ1-38, Aβ1-40, Aβ1-42) [27]. The reason why γ-secretase acts after α-or β-cleavage has been attributed to the fact that the large ectodomain of the substrates probably sterically hinders the substrate binding to γ-secretase and needs to be shedded. This is probably one reason for ectodomain shedding so that the C-terminal fragments can now be accommodated in the active site of γ-secretase complex. Thus, ectodomain shedding by α-/β-secretases is a prerequisite for γ-cleavage and γ-secretase activity is needed only after the cleavages by α-/β-secretases have occurred. However, here we report that γ-secretase inhibition, either by pharmacological inhibition or by silencing γ-secretase components, increases α-secretase cleavage of APP. Our results uncover a novel feedback regulation of α-secretase via γ-secretase. Since γ-secretase inhibitors are considered for AD therapy, and since sAPPα plays a role in neuroprotection, our study reveals an important side effect of γ-secretase inhibitor therapy and also suggests the elevation of sAPPa levels to be a theragnostic marker for γ-secretase inhibition.

Objective

To examine whether the involvement of Aβ-generating enzymes in the biochemical pathway of amyloid generation is linear and unidirectional or if there are feedback regulations by the downstream components (such as γ-secretase) on the upstream players (α- or β-secretases), we studied how γ-secretase inhibition affected α-secretase cleavages of APP.


A
Aβ
sAPPα
normalized ECL counts
4.500
4.000
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DMSO
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normalized ECL counts
1.6
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DMSO
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97 kDa
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MedGC
APP
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PSEN1
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Pen2
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MedGC
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PS 1/2
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MedGC 5nM
APP
BACE1
ADAM10
ADAM17
Pen2
MedGC (10nM)
PSEN 1/2 (10nM)
siRNAs

a

Figure Legend

Figure 1. γ-Secretase inhibition using the small molecule inhibitor DAPT increases sAPPα levels.

HeLa swAPP cells **(A)** and HEK wtAPP cells **(B)** were treated with DAPT (10 μM) and the vehicle control (DMSO) for 4 and 24 h, respectively. Cell viability was measured and the cell culture supernatant was analyzed by the electrochemiluminescence assay to analyze the levels of the α-cleaved ectodomain (sAPPα) and levels of Aβ (HeLa swAPP Aβ $p<0.0005$, sAPPα $p<0.0005$, HEK wtAPP Aβ $p<0.0005$, sAPPα $p<0.0005$).

(C) Cell lysates of HeLa swAPP cells that were treated with DAPT and DMSO as control were subjected to Western Blotting, and levels of APP and its cleavage products were detected using an antibody against the C-terminal region of APP. GAPDH serves as loading control.

(D) Cell culture supernatant from the DAPT- and DMSO- treated cells was subjected to immunoblotting with 6E10 to detect the soluble APPα. Lower panel shows longer exposure.

(E) siRNA-mediated inhibition of γ-secretase leads to increased sAPPα levels. HeLa swAPP cells were revese-transfected with **(E)** 5 nM and **(F)** 10 nM total amount of siRNA directed against different genes. MedGC is a scrambled siRNA and serves as treatment control. 68 h post transfection, the cells were treated with DAPT (10 μM) and control DMSO, respectively. 72 h post transfection, sAPPα from the cell culture supernatant was analyzed in the electrochemiluminescence assay. **(E)** 5 nM: MedGC $p<0.0005$, BACE1 $p=0.035$, ADAM17 $p=0.0052$, PSEN1 $p=0.013$, PSEN2 $p=0.00067$. **(F)** 10 nM: MedGC $p=0.0021$, ADAM10/17 $p=0.017$, PSEN1/2 $p=0.038$.

(G) HeLa swAPP cells were transfected with the indicated siRNA. 68 h post transfection, the cells were treated with DAPT (10 μM) and control DMSO respectively. 72 h post transfection, the cell culture supernatant was subjected to electrochemiluminescence analysis to measure Aβ40 levels. MedGC (5 nM) $p<0.0005$, APP $p=0.014$, BACE1 $p=0.0063$, ADAM10 $p<0.0005$, ADAM17 $p<0.0005$, Pen2 $p<0.0005$, MedGC (10 nM) $p<0.0005$, PSEN1/2 $p=0.0044$.

Cell Culture

Hela cells expressing the Swedish mutant of APP were cultured in DMEM (1 g/l Glucose, Invitrogen) at 37℃ with 5% (vol/vol) CO_2 in a humidified incubator. DMEM media was supplemented with 10% FCS (vol/vol, Invitrogen), 1% Penicillin/streptomycin (vol/vol, Gibco), 0.1% G418 (vol/vol, Carl Roth) and 0.1% Zeocin (vol/vol) (Invitrogen). HEK cells expressing wt APP were cultured in DMEM (4.5 g/l Glucose, Invitrogen) at 37℃ with constant CO_2 supply in a humidified incubator. DMEM media was supplemented with 10% FCS (vol/vol, Invitrogen), 1% Penicillin/streptomycin (vol/vol, Gibco), 1% Glutamic acid (vol/vol) and G418 (4.1 ml per 500 ml, Carl Roth).

Inhibitor Treatment

Hela cells expressing the Swedish mutant of APP and HEK cells expressing wt APP were treated with DAPT (Sigma) for different incubation periods (ranging from 2 to 24 h) and the cell culture supernatant was assayed for Aβ and sAPPα levels.

siRNA Transfections

All siRNA are chemically synthesized and purchased from Invitrogen. Transfection complexes were prepared in 0.3 μl Oligofectamine as a transfection reagent mixed in Opti-MEM to 5 ml. 5 nM siRNA was mixed in Opti-MEM to a volume of 5 ml. Both mixes were incubated at RT for 5 min and then combined and incubated for 20 min at RT. The 10 ml transfection mix was transferred to one well of a 96 well plate and 100 μl of cell suspension with 3500 cells in DMEM (10% FCS, P/S) was added to each well. Cells were incubated at 37℃ with a constant supply of 5% CO_2. Medium was changed after 20 h to 100 ml of fresh culture medium.

Western Blotting

Cells were lysed using lysis buffer (0.1% SDS and 1% NP 40 in PBS) supplemented with protease inhibitors (complete by Roche). Cell lysates were run on SDS PAGE 4–12% Bis Tris Pre cast Gels (Invitrogen). Proteins were transferred on a nitrocellulose membrane (Bio-RAD), which were then blocked using PBS containing 5% (wt/vol) dry milk powder for 1 h at RT. The membranes were then incubated with primary antibodies: APP- CFT (Sigma), 6E10 (Covance), PSEN1, PSEN2 (Epitomics), Pen2 (Abcam) and

GAPDH (Meredin) followed by the appropriate HRP conjugated secondary antibody. Both primary and secondary antibodies were diluted in PBS containing 5% (wt/vol) dry milk powder and 0.05% tween20. Proteins were detected using chemiluminescence kit (Pierce).

Electrochemiluminescence assay

Detection of the Aβ and sAPPα peptides from cell culture supernatant was performed using MSD Human multiplex kits. Briefly conditioned medium from the cells was collected, cleared by centrifugation and processed for measurment according to manufacturer's instructions. Aβ and sAPPα peptides were detected with monoclnal antibody and quantified by electrochemiluminescence assay using sector imager 6000 reader (Meso Scale Discovery). Electrochemiluminescence readings were normalized to cell viability and control (DMSO or MED GC).

Results & Discussion

We perturbed the function of γ-secretase and first assayed for its effect on the levels of sAPPα (as a readout for α-cleavage) in HeLa cells expressing Swedish mutant APP. As expected, inhibition of γ-secretase activity by DAPT dramatically inhibited Aβ levels, showing the efficient inhibition of γ-secretase enzymatic activity (Fig. 1A). Surprisingly, when we assayed for sAPPα levels, we observed a large increase in the secreted levels of sAPPα in treated but not in vehicle- treated control cells (Fig. 1A). Similar data was obtained with another cell line, HEK cells expressing the wild-type APP, thereby ruling out any cell type or Swedish mutation-specific effects (Fig. 1B). Western blotting with anti-APP C-terminus antibodies confirmed the inhibition of γ-secretase with DAPT through the accumulation of the C-terminal fragment of APP (Fig. 1C). Finally, to independently validate our observations using a different detection methodology, we performed Western blotting analysis on the cell culture supernatants from the control and DAPT-treated cells using the 6E10 antibody that recognizes sAPPa. Indeed we observed a prominent increase in the sAPPα levels, specifically in supernatants from DAPT-treated cells (Fig. 1D).

We reasoned that if inhibition of γ-secretase activity by small molecules enhanced sAPPα levels, then this effect should be phenocopied by genetic perturbation of γ-secretase function. To this end, using specific siRNAs, we silenced PSEN1 and PSEN2, the two catalytically active subunits of the γ-secretase complex, and measured sAPPα levels in the cell culture supernatant (Fig. 1E). RNAi-mediated silencing of PSEN1 and PSEN2 dramatically increased sAPPa levels consistent with the previous observations after chemical inhibition of γ-secretase (Fig. 1E). Similarly, silencing of Pen-2, an essential subunit for γ-secretase complex maturation, also increased sAPPα levels (Fig. 1E). As positive controls for our assay, we silenced APP or ADAM10 and found that silencing them drastically decreased sAPPα levels whereas silencing BACE1 increased sAPPα levels as expected because more full-length APP remains as substrate for α-secretase when β-cleavage is abolished. In order to test whether enzymatic inhibition and genetic perturbation of γ-secretase activity act on sAPPa via identical or separate regulatory pathways, we combined both approaches in our cell system. Since Pen-2 silencing completely abrogates γ-secretase complex as it is needed for both Psen1 and Psen2 complexes, DAPT treatment in addition to silencing of Pen-2 did not increase sAPPα levels, clearly demonstrating that γ-secretase activity negatively regulates sAPPα levels (Fig. 1E). On the other hand, we found that DAPT treatment in addition to silencing either Psen1 or Psen2 increased sAPPα levels, as silencing of PSEN1 will still have the γ-secretase complex formed by PSEN2 and vice versa. Silencing of PSEN1 and PSEN2 together also dramatically increased sAPPα levels, consistent with our previous observations (Fig. 1F). As a proof of principle to check the influence of siRNA against γ-secretase components on Aβ levels, we silenced Pen2, PSEN1, and PSEN2. As expected, Aβ levels were drastically reduced in the presence or absence of DAPT (Fig. 1G). Silencing of ADAM10 and ADAM17 increased Aβ levels as more APP substrate was available for recessing by BACE1. However, as expected, silencing of ADAM10 and ADAM17 in the presence of DAPT reduced Aβ levels (Fig 1G). The efficient enzymatic inhibition of γ-secretase through DAPT is shown to have almost complete abolishment of Aβ production.

Conclusions

Here we show the regulation of α-secretase cleavage of APP by γ-secretase. γ-Secretase has been shown to process only transmembrane proteins that have been pre-shedded. One reason why γ-secretase is hypothesized to act after α-or β-cleavage is due to the fact that the large ectodomains of the substrates probably sterically hinder the substrate binding and hence these ectodomains need to be shedded in order to be incorporated in the γ-secretase complex. We find that γ-secretase inhibition enhanced the processing of APP by α-secretase. We performed pharmacological inhibition and silencing of γ-secretase in different cell lines and observed an increase in sAPPα levels.

Limitations

The experiments are performed in model cell lines. Neurons and/or *in vivo* validation is needed.

This is an exciting finding that we originally observed during a control experiment but we later found out that this has been shown previously shown as a side finding in the Sharples et al., paper. We believe that the effect on sAPPa could be due to a) γ-secretase activity could influence α-secretase levels or activity or b) trafficking of APP to the cell surface where α-cleavage occurs could be altered.

Additional Information

Methods

Cell Culture

Hela cells expressing the Swedish mutant of APP were cultured in DMEM (1 g/l Glucose, Invitrogen) at 37℃ with 5% (vol/vol) CO_2 in a humidified incubator. DMEM media was supplemented with 10% FCS (vol/vol, Invitrogen), 1% Penicillin/streptomycin (vol/vol, Gibco), 0.1% G418 (vol/vol, Carl Roth) and 0.1% Zeocin (vol/vol) (Invitrogen). HEK cells expressing wt APP were cultured in DMEM (4.5 g/l Glucose, Invitrogen) at 37℃ with constant CO_2 supply in a humidified incubator. DMEM media was supplemented with 10% FCS (vol/vol, Invitrogen), 1% Penicillin/streptomycin (vol/vol, Gibco), 1% Glutamic acid (vol/vol) and G418 (4.1 ml per 500 ml, Carl Roth).

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Supplementary Material

Please see https://sciencematters.io/articles/201601000003.

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Ethics Statement

Not applicable.

Citations

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