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ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons

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The amyloid precursor protein (APP) undergoes constitutive shedding by a protease activity called α-secretase. This is considered an important mechanism preventing the generation of the Alzheimer's disease amyloid-β peptide (Aβ). α-Secretase appears to be a metalloprotease of the ADAM family, but its identity remains to be established. Using a novel α-secretase-cleavage site-specific antibody, we found that RNAi-mediated knockdown of ADAM10, but surprisingly not of ADAM9 or 17, completely suppressed APP α-secretase cleavage in different cell lines and in primary murine neurons. Other proteases were not able to compensate for this loss of α-cleavage. This finding was further confirmed by mass-spectrometric detection of APP-cleavage fragments. Surprisingly, in different cell lines, the reduction of α-secretase cleavage was not paralleled by a corresponding increase in the Aβ-generating β-secretase cleavage, revealing that both proteases do not always compete for APP as a substrate. Instead, our data suggest a novel pathway for APP processing, in which ADAM10 can partially compete with γ-secretase for the cleavage of a C-terminal APP fragment generated by β-secretase. We conclude that ADAM10 is the physiologically relevant, constitutive α-secretase of APP.

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Introduction

The amyloid precursor protein (APP) is one of a large number of membrane proteins that are proteolytically converted to their soluble counterparts. This process is referred to as ectodomain shedding and is an important way of regulating the biological activity of membrane proteins (Pruessmeyer and Ludwig, 2009; Reiss and Saftig, 2009). APP shedding occurs constitutively by two different protease activities, called α- and β-secretases, and leads to the secretion of soluble APP (APPs) (Figure 1A). Both proteolytic cleavages are central regulatory events in the generation of the amyloid-β peptide (Aβ), which has an important function in the pathogenesis of Alzheimer's disease (AD) (Selkoe and Schenk, 2003; Haass, 2004). The α- and β-secretases are assumed to compete for APP as a substrate (Selkoe and Schenk, 2003; Postina *et al*, 2004), but have opposite effects on Aβ generation. The β-secretase is the aspartyl protease BACE1 and cleaves APP at the N-terminus of the Aβ domain, thus catalysing the first step in Aβ generation (Vassar *et al*, 1999). In contrast, α-secretase cleaves within the Aβ sequence of APP (Esch *et al*, 1990), thereby precluding Aβ generation. In addition, α-secretase cleavage generates a secreted form of APP (APPsα), which has been reported to have neurotrophic and neuroprotective properties (Furukawa *et al*, 1996; Meziane *et al*, 1998; Stein *et al*, 2004), whereas the slightly shorter form (APPsβ) generated by β-secretase seems to have a proapoptotic function (Nikolaev *et al*, 2009).

An increase of APP α-secretase cleavage is considered a therapeutic approach for AD (Fahrenholz, 2007), as it is assumed to reduce Aβ generation. However, the molecular identity of α-secretase is controversially discussed and remains to be fully established. Different metalloproteases were suggested as potential α-secretases, because their overexpression increased APP cleavage. The most frequently named ones are three members of the ADAM (a disintegrin and metalloprotease) family: ADAM9, 10 and 17 (Koike *et al*, 1999; Lammich *et al*, 1999; Slack *et al*, 2001). However, because the overexpression of a protease may artificially or indirectly increase APP α-secretase cleavage, the physiological relevance of a candidate protease needs to be shown using the corresponding protease knockdown or knockout cells. In fact, cells derived from ADAM9-, 10- or 17-deficient mice showed either no or a variable degree of reduction of APP shedding (Buxbaum *et al*, 1998; Hartmann *et al*, 2002; Weskamp *et al*, 2002). Likewise, RNAi-mediated knockdown of the individual proteases in cultured cells reduced APP shedding to different extents (Asai *et al*, 2003; Allinson *et al*, 2004; Camden *et al*, 2005; Freese *et al*, 2009; Taylor *et al*, 2009). The finding that APP shedding was never fully suppressed has led to the conclusion that ADAM9, 10 and 17 may all together contribute to α-secretase activity and that in the absence of one of them, the other proteases can still mediate APP α-secretase cleavage. This assumption is in clear contrast

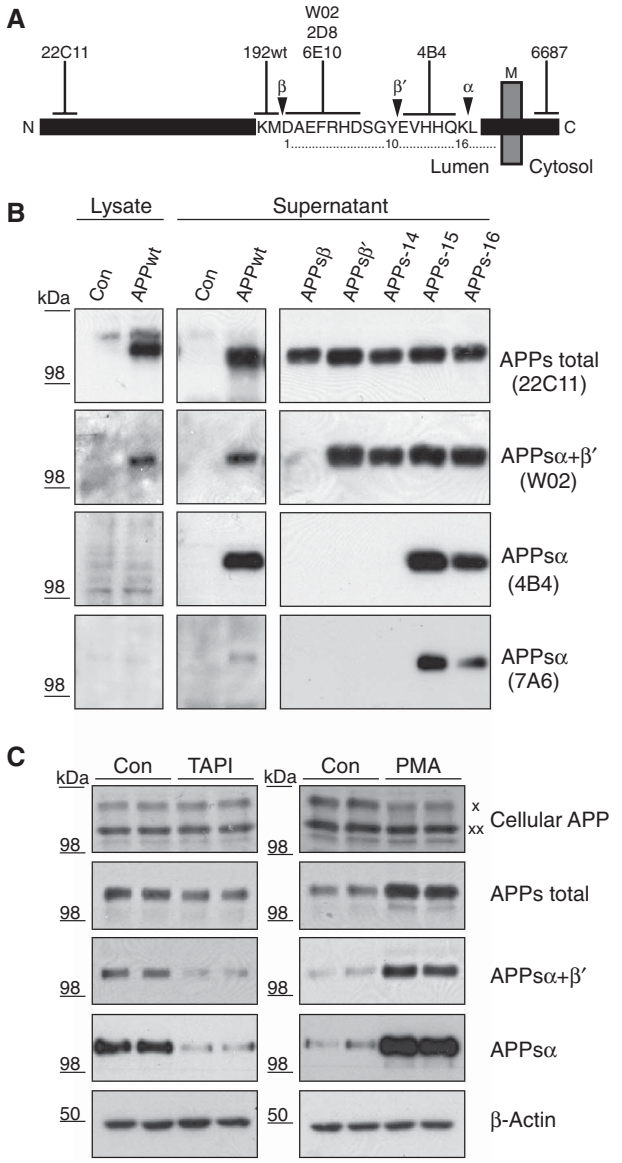


Figure 1 Characterization of newly generated APPsα-specific antibodies 4B4 and 7A6. (**A**) Schematic representation of APP. Indicated are the antibodies used in this study as well as antibody 6E10 and the APP-cleavage sites (with arrow heads) at the β-, β′- and α-secretases sites. Numbers below the sequence indicate the amino acids of the Aβ sequence. M, membrane. (**B**) Immunoblot of supernatants and cell lysates of cells expressing endogenous APP (Con) or transfected with the indicated constructs (APPwt, full-length APP; APPs, soluble APP lacking the transmembrane and cytoplasmic domain). Antibody 22C11 detects all secreted APP species, antibody W02 detects APPsα and APPsβ′, whereas antibodies 4B4 and 7A6 specifically detect APPsα (APPs-15 and APPs-16). (**C**) HEK293 cells were treated with the metalloprotease inhibitor TAPI-1 or the phorbol ester PMA. Immunoblots of conditioned media and cell lysates were probed with antibody 22C11 (APPs total), W02 (APPsα + β′) and the APPsα-specific antibody 4B4. Cellular APP is present in a lower molecular weight immature form (xx) and a higher molecular weight mature form (x) and was detected with 22C11. The β-actin blot serves as a loading control. The reduction by TAPI-1 and the increase in shedding by PMA are more pronounced when analysed with the α-cleavage-specific antibody 4B4, compared with the other antibodies.

to other ADAM protease substrates, many of which are predominantly cleaved by a single ADAM protease, such as transforming growth factor α, epidermal growth factor (EGF), the low-affinity immunoglobulin E receptor CD23 and N-cadherin (Sahin *et al*, 2004; Reiss *et al*, 2005; Weskamp *et al*, 2006; Le Gall *et al*, 2009). One aspect that makes it difficult to study APP α-secretase cleavage is the fact that APP is cleaved by distinct proteases at different peptide bonds in close proximity. For example, β-secretase has the main cleavage site at the N-terminus of the Aβ sequence, but a secondary cleavage site (termed β′-site) within the Aβ sequence close to the α-secretase-cleavage site (Figure 1A). Antibodies used in previous studies have not only specifically detected APPsα, but also the alternative β-secretase-cleavage product APPsβ′ (Figure 1A), which may have confounded the study of α-secretase cleavage. Here, we systematically address the identity of the physiologically relevant α-secretase. We generated two new monoclonal antibodies specific for the APP α-secretase-cleavage product APPsα. Using these antibodies as well as mass spectrometry, we found that ADAM10, but not ADAM9 or 17, is essential for the constitutive α-secretase cleavage of APP.

Results

Generation of an APPsα-specific antibody

To specifically detect the APP α-secretase-cleavage product APPsα, a new monoclonal antibody (4B4) was generated against a peptide comprising amino acids 11–16 of the Aβ sequence (Figure 1A). The peptide had a free C-terminus, mimicking the neoepitope generated upon α-secretase cleavage. Indeed, antibody 4B4 does not detect full-length APP in the cell lysate (Figure 1B). It specifically detects APPsα ending in amino acids 15 and 16 (APPs-15 and APPs-16), but does not detect shorter APPs species, including APPsβ′ and APPsβ (Figure 1B). In contrast, antibody W02 binds an epitope between the β- and the β′-cleavage sites and correspondingly detects both APPsα and APPsβ′, but not APPsβ. This antibody detects a similar epitope as antibody 6E10 that is frequently used for the detection of APPs (Miles *et al*, 2008). Antibody 22C11 binds to an N-terminal APP epitope and detects all APPs species tested (Figure 1B). All antibodies used specifically detect APP, because the antibodies do not detect a signal in APP knockdown cells (Supplementary Figure S1).

To further validate antibody 4B4, we tested whether conditions, which increase or decrease APPsα generation, lead to a corresponding change in the 4B4 signal. To this aim, human embryonic kidney 293 cells (HEK293) expressing endogenous APP were treated with the metalloprotease inhibitor TAPI-1 to reduce APP shedding or with the phorbol ester PMA (also known as TPA) to increase APP shedding. Both compounds did not alter the expression of APP or actin in the cell lysate (Figure 1C). TAPI-1 inhibited nearly completely APPsα generation (4B4 blot). In contrast, total APPs shedding was not as strongly reduced (22C11 blot), consistent with the fact that this antibody detects all APPs species and not only APPsα. PMA strongly stimulated total APP shedding (22C11), but the extent of the increase was much more pronounced when specifically detecting APPsα (4B4). The strong increase in APP shedding was paralleled by a reduction of the mature APP in the cell lysate (Figure 1C, marked with x). Antibody W02, which detects APPsα + APPsβ′, detected intermediate changes between 22C11 and 4B4, in agreement with the antibody detecting both APPsβ′ and APPsα. In an additional

control experiment, the β-secretase BACE1 was overexpressed, which is expected to increase β-secretase cleavage and to reduce α-secretase cleavage. BACE1 expression increased total APP shedding (22C11) in agreement with previous publications (Neumann *et al*, 2006; Schobel *et al*, 2006), but reduced as expected APPsα (4B4) (Supplementary Figure S2). Antibody W02 was not suited to detect the decrease in APPsα, because it also detects the alternative β-secretase-cleavage product APPsβ′, which was strongly enhanced upon overexpression of BACE1 (Supplementary Figure S2). Taken together, these experiments show that antibody 4B4 specifically detects APPsα, in contrast to other commonly used APP antibodies.

Knockdown of ADAM9, 10 and 17 in HEK293 and SH-SY5Y cells

Next, the three-candidate α-secretases ADAM9, 10 and 17 were transiently knocked down to evaluate their contribution to the constitutive α-secretase cleavage of endogenous APP in HEK293 and in human neuroblastoma SH-SY5Y cells. Compared with control-treated cells, the siRNA pools knocked down all three proteases with an efficiency of 75–90% (Figure 2A for HEK293; Figure 2D for SH-SY5Y; quantifications in Figure 2C and F). Levels of cellular APP as well as of the control membrane protein calnexin were not affected (Figure 2A and D). Knockdown of ADAM10 in HEK293 and SH-SY5Y cells reduced total APP shedding (22C11, normalized to cellular APP levels) to about 40% (Figure 2A, B, D and E), similar to the use of the metalloprotease inhibitor TAPI-1 (Figure 1C). In contrast, APPsα (4B4) was reduced to 10%, which corresponds to the remaining 10% of ADAM10 protease expressed in the knockdown cells. In contrast to ADAM10, the knockdown of ADAM9 did not significantly reduce total APPs or APPsα levels (Figure 2A, normalized to cellular APP levels). Knockdown of ADAM17 mildly reduced total APPs and APPsα in HEK293 cells (Figure 2A and B), but had no significant effect in SH-SY5Y cells (Figure 2C and D). The knockdown of either protease did not affect the expression level of the other proteases (Supplementary Figure S3). Taken together, expression of ADAM10, but not of ADAM9 or 17, is required for APP α-secretase cleavage. Moreover, ADAM9 and 17 did not compensate for the loss of ADAM10.

To further validate the results from the transient knockdown of ADAM10, HEK293 cells (Figure 3) and SH-SY5Y cells (Figure 4) with a stable knockdown of ADAM10 were generated using lentiviruses expressing two different shRNA sequences against ADAM10 or a negative control shRNA. In HEK293 cells, both ADAM10 shRNA sequences (sh7, sh9)

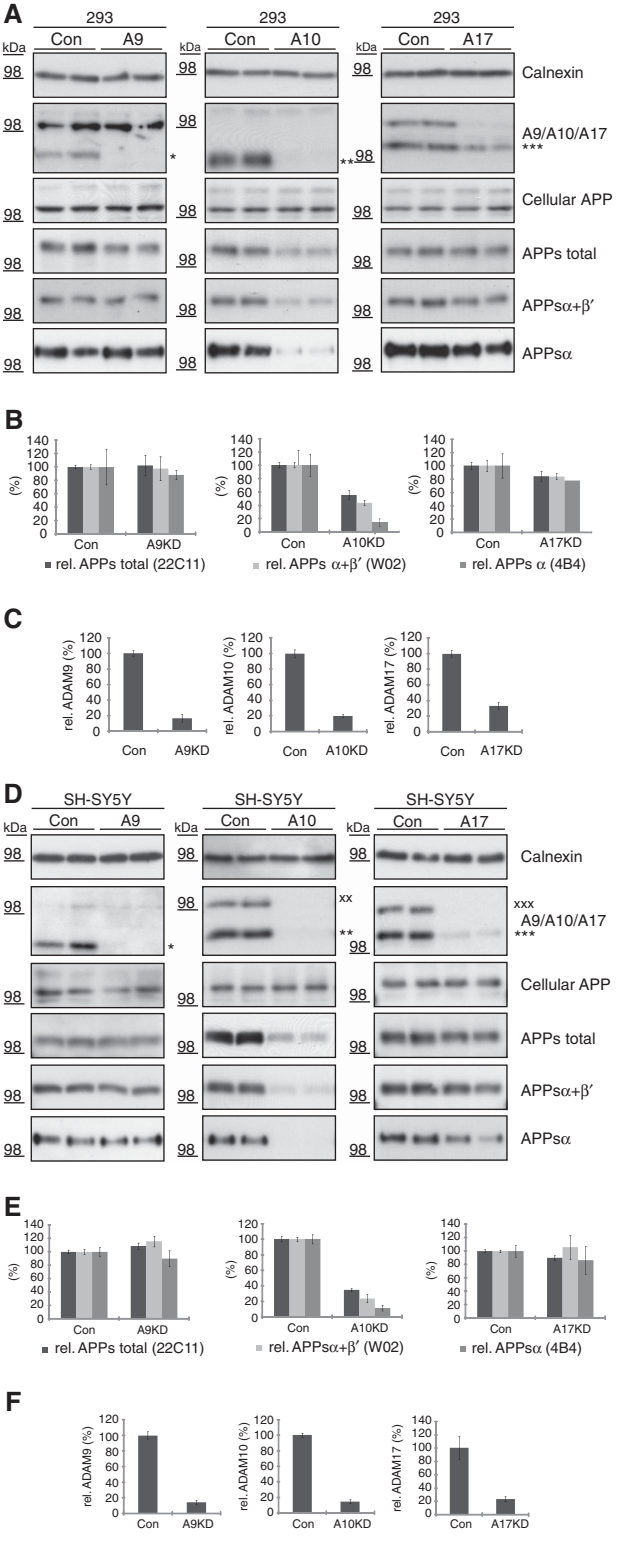
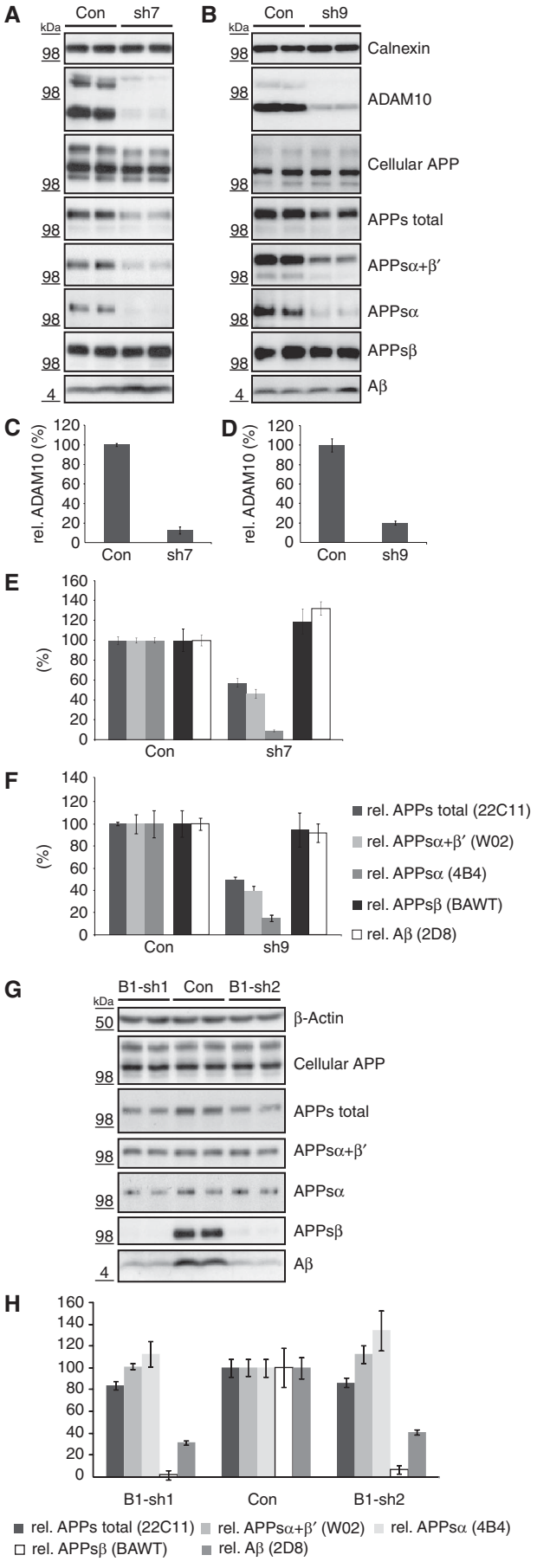


Figure 2 Transient knockdown of ADAM10 suppresses α-secretase cleavage of endogenous APP. (**A**) HEK293 cells (293) were transfected with siRNA pools directed against the proteases ADAM9 (A9), ADAM10 (A10) or ADAM17 (A17) or with control siRNA. All three proteases were detected by western blot in membrane preparations. The mature active form is indicated with * (ADAM9), ** (ADAM10) and *** (ADAM17). The immature form of ADAM17 was also detected in HEK293 cells at a higher molecular weight. Calnexin was detected as a loading control. Cell lysates were analysed for cellular APP and conditioned media for total secreted APP (APPs total, 22C11, α + β′-cleaved APP (APPsα + β′, W02) and α-cleaved APP (APPsα, 4B4). (**B**, **C**) Quantification of experiments in (**A**). APPs total, APPsα + β′ and APPsα levels were normalized to cellular APP (**B**). Quantification of the protease knockdown (KD) efficiency is in (**C**). Given are mean and standard error of eight independent experiments relative (rel.) to control. (**D**) SH-SY5Y cells were treated as in (**A**). APP fragments were detected as in (**A**). The immature protease form was not visible for ADAM9, but is indicated with xx for ADAM10 and xxx for ADAM17, whereas the mature active form is indicated with * (ADAM9), ** (ADAM10) and *** (ADAM17). (**E**, **F**) Quantification of experiments in (**D**). Given are mean and standard error of eight independent experiments.



reduced ADAM10 protease levels and APPsα levels to 10–20% of controls (Figure 3A and B; quantification in Figure 3C–F), respectively, which is similar to the transient knockdown experiments. Similar results were obtained for the stable knockdown of ADAM10 in SH-SY5Y cells (Figure 4A and B). The remaining APPsα from the SH-SY5Y cells could be fully inhibited by addition of the metalloprotease inhibitor TAPI-1 (Figure 4A), which is in agreement with the remaining ~10% of ADAM10 protease in the knockdown cells. The experiments in both cell lines show that also under stable knockdown conditions, ADAM10 is essential for APP α-secretase cleavage.

α- and β-secretase cleavage do not compete for each other in HEK293 and SH-SY5Y cells

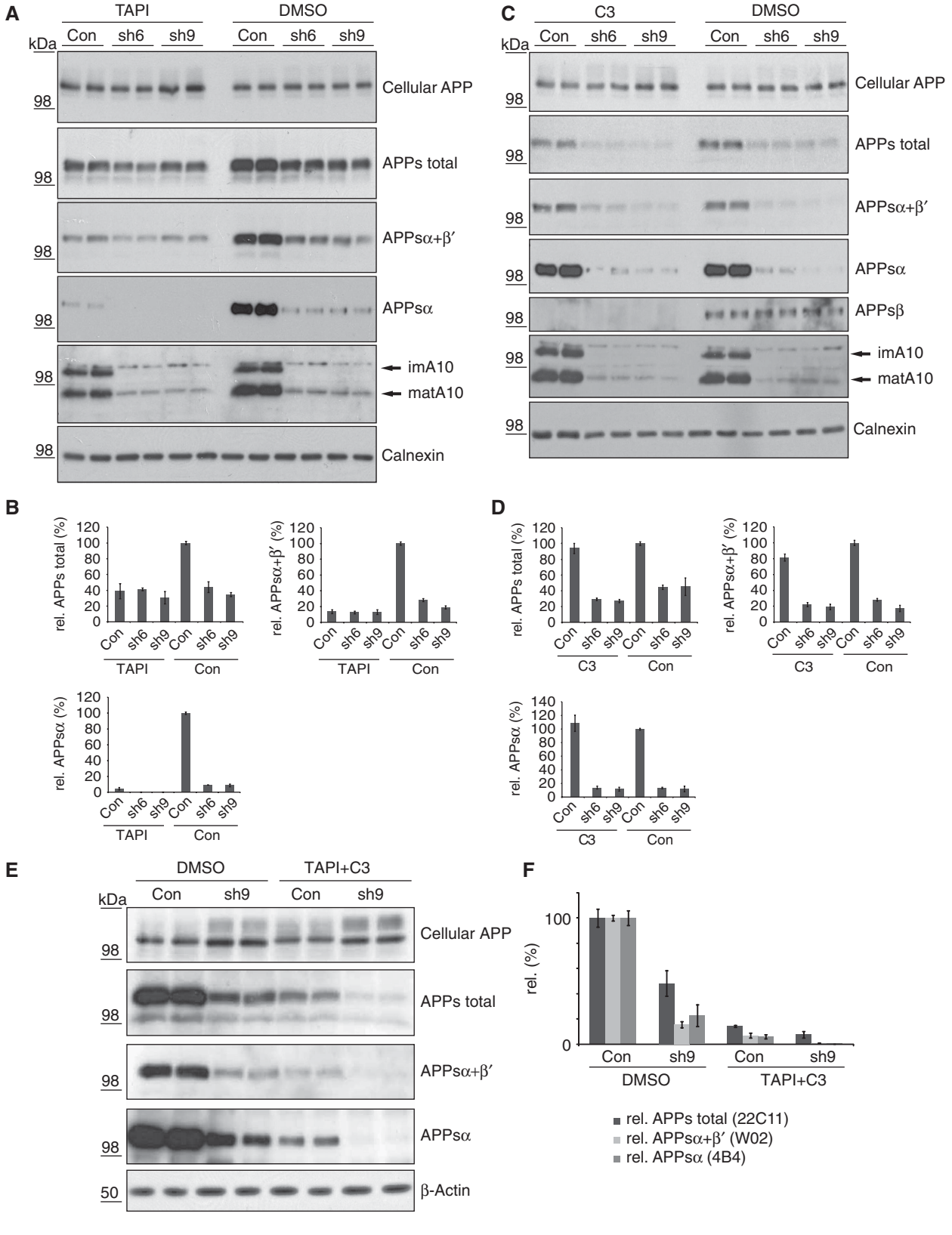
Next, we analysed whether the reduction of APPsα was paralleled by an increase in APPsβ and Aβ generation. Surprisingly, however, this was not the case (Figure 3A and B; quantification in Figure 3E and F). Compared with control cells, endogenous APPsβ and Aβ levels in HEK293 cells were unchanged for one ADAM10 knockdown construct (sh9), whereas a mild, but not significant, increase was observed for the other shRNA construct (sh7) (Figure 3E and F). These results show that the strong reduction of α-cleavage does not yield a correspondingly increased cleavage by β-secretase. To further validate this finding, the opposite experiment was carried out. Expression of the β-secretase BACE1 was reduced by lentiviral knockdown constructs (sh1 and sh2). This resulted in a strong inhibition of APPsβ and Aβ generation, but not in a significant increase in APPsα generation (Figure 3G and H). Similar results were obtained for SH-SY5Y cells. The stable knockdown of ADAM10 did not increase APPsβ levels (Figure 4C and D) and conversely, the pharmacological inhibition of BACE1 with the specific inhibitor C3 (Stachel *et al*, 2004) did not increase APPsα levels (Figure 4C and D). Thus, we conclude that under constitutive cleavage conditions, α- and β-secretases do not significantly compete for APP as a substrate in HEK293 and SH-SY5Y cells.

Contribution of α- and β-secretase cleavage to total APP secretion

Total APP shedding (22C11) was reduced to ~40% in the stable ADAM10 knockdown SH-SY5Y cells compared with

Figure 3 Analysis of APP processing in HEK293 cells with a stable ADAM10 knockdown. (**A**, **B**) HEK293 cells were infected with lentiviral vectors carrying GFP and different shRNAs: a non-targeting shRNA (Con) and two different ADAM10-targeting shRNAs (sh7 and sh9). Conditioned media of these cells were analysed with antibody 22C11 (APPs total), W02 (APPsα + β′), 4B4 (APPsα), 192wt (APPsβ) or 2D8 (Aβ), whereas cell lysates were analysed for cellular APP (22C11). Membranes were analysed for ADAM10 protein. Calnexin was detected as a loading control. (**C**, **D**) Quantification of ADAM10 knockdown from experiments in (**A**, **B**) relative (rel.) to control. (**E**, **F**) Quantification of APP fragments in (**A**, **B**). Given are mean and standard error of four independent experiments. (**G**) HEK293 cells were lentivirally infected as in (**A**). with a non-targeting shRNA (Con) or two different BACE1-targeting shRNAs (B1-sh1, B1-sh2). APP and its processing products were analysed as in (**A**). Actin was used as a loading control. (**H**) Quantification of experiments in (**G**), carried out as in (**E**) and (**F**). Given are mean and standard error of three independent experiments.

controls (Figure 4C and D). Additional inhibition of BACE1 with the specific inhibitor C3 further reduced total APP secretion to ~20%, revealing that BACE1 contributes about 20% to total APP secretion in the SH-SY5Y cells. This result also suggests that the remaining low level (<20%) of total APP secretion may result from proteases other than ADAM10 and BACE1. This was further confirmed by a combined pharmacological inhibition of α- and β-secretase cleavage in SH-SY5Y cells, which resulted in a remaining total APP secretion of 10–15% (Figure 4E and F).



ADAM10 truncates APP C-terminal fragments C99 and C89 to C83

α- and β-secretases not only generate APPsα and APPsβ, but also the C-terminal fragments C83 and C99, respectively. Both fragments are further processed by γ-secretase, leading to a short half-life of the fragments, which makes it difficult to detect them at endogenous levels. Thus, we treated SH-SY5Y cells with the γ-secretase inhibitor DAPT, which stabilizes the endogenous C-terminal fragments of APP (Figure 5A). In control cells, the α-secretase fragment C83 was clearly detected (marked with ***) and was strongly reduced in the ADAM10 knockdown cells in parallel to APPsα (quantification in Figure 5B). To our surprise, the β-secretase-cleavage product C99 was increased more than two-fold upon ADAM10 knockdown (marked with *). In addition, a mild increase of C89 was observed (marked with **), which is the C-terminal fragment arising through BACE1 at its alternative β′-cleavage site (see Figure 1A for schematic drawing). The increase in C99 and C89 is in contrast to the APPsβ levels, which were unchanged upon ADAM10 knockdown (Figure 5B). We interpret this result in the following way. C99 and C89 can be processed in two pathways. The first one consists of cleavage by γ-secretase leading to Aβ generation, the second one occurs by α-secretase, leading to C83 generation. Upon ADAM10 knockdown, the latter pathway is blocked, leading to an increase in C99 and C89 and leaving APPsβ levels unchanged.

ADAM10 is required for α-secretase cleavage in primary neurons

APP processing occurs in all cells and tissues analysed to date. However, in AD, APP processing is particularly relevant in the neurons of the central nervous system. Thus, we next investigated the contribution of ADAM9, 10 and 17 in α-secretase cleavage in primary murine cortical E16 neurons from C57/BL6 mice, expressing endogenous APP. Murine and human APP differ by three amino acids within the N-terminal half of the Aβ sequence. One of these amino-acid changes is within the peptide sequence used to generate the antibody 4B4. For this reason, we generated an additional antibody, called 7A6, which also detects the murine APPsα, but not APPsβ and APPsβ′ (Figure 1B). Using two different shRNA sequences, the lentiviral knockdown of ADAM10 reduced murine ADAM10 expression as well as murine APPsα to about 10–15% of the control (Figure 6A–C). This reveals that also in primary neurons, ADAM10 activity is required for APP α-secretase cleavage. As a control ADAM9 and 17 were also knocked down in the primary neurons. As both proteins could not be detected by immunoblot in the neuronal lysates, their expression was measured by quantitative RT–PCR (Figure 6F). Similar to the HEK293 and SH-SY5Y cells, knockdown of ADAM9 or 17 did not affect APP α-secretase cleavage (Figure 6D and E). Likewise expression of ADAM10 was not affected, as determined by both immunoblot (Figure 6D) and

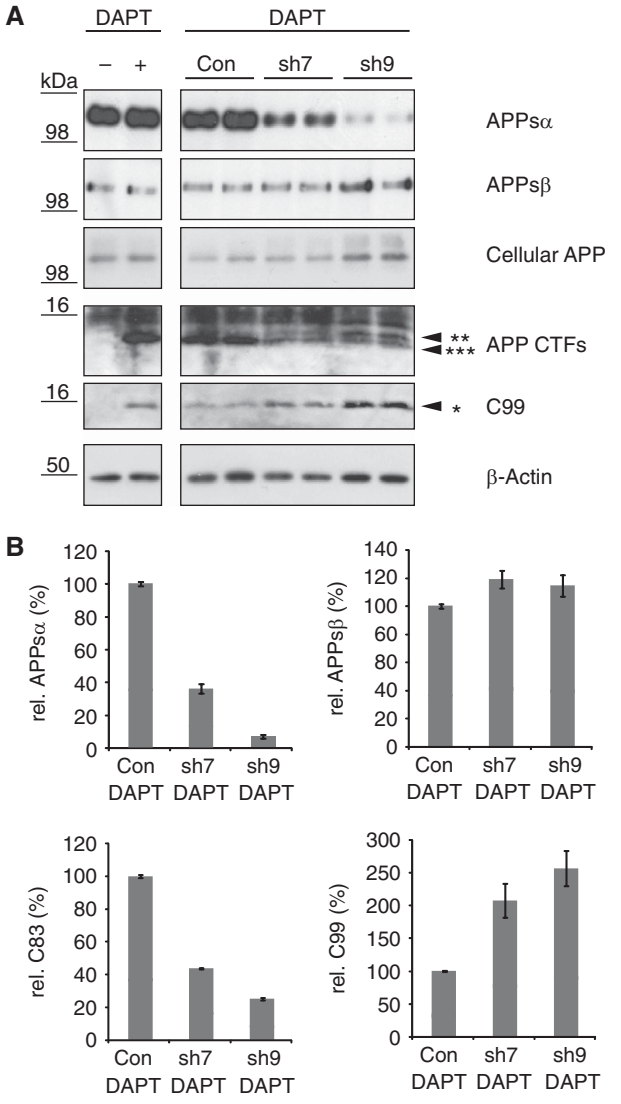
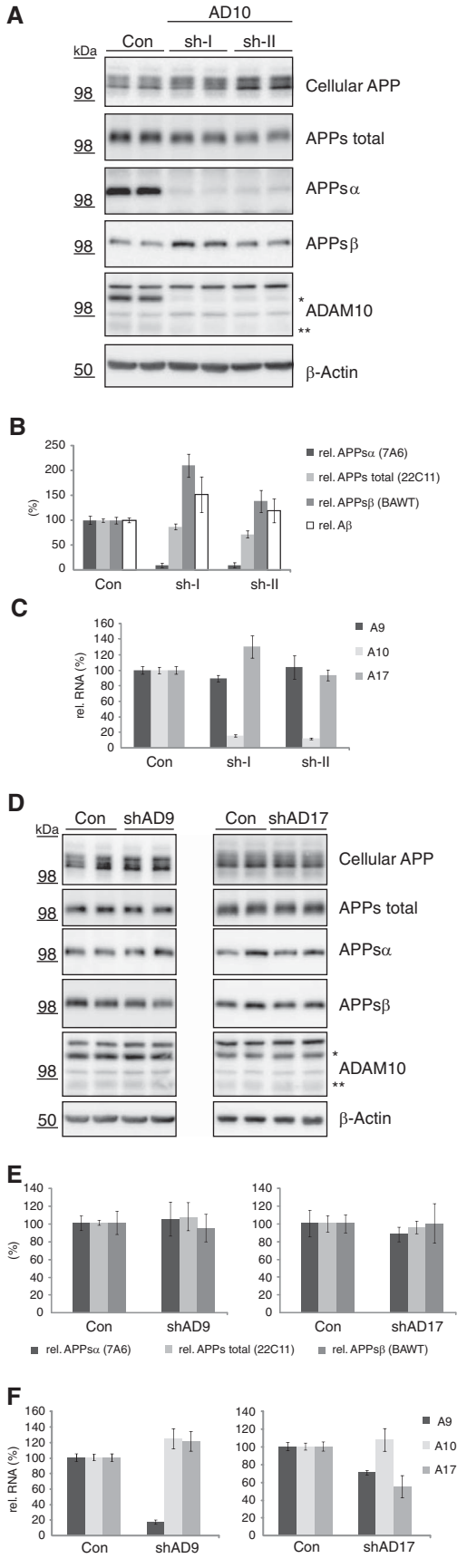


Figure 5 Absence of ADAM10 reduces C83. (**A**) Left panels: SH-SY5Y cells were treated (+) or not (−) with the γ-secretase inhibitor DAPT to stabilize APP C-terminal fragments (CTFs). Endogenous CTFs were only visible upon DAPT treatment. Right panels: SH-SY5Y cells stably expressing ADAM10 shRNAs (sh7, sh9) or control shRNA (Con) were treated with DAPT. Conditioned media were analysed for APPsα (4B4) and APPsβ (192wt). Cell lysates were analysed for cellular APP, all C-terminal fragments (6687) and specifically C99 (2D8). C99 is marked with (*), C89 with (**) and C83 with (***). (**B**) Quantification of APPsα, APPsβ, C83 and C99 normalized to cellular APP relative (rel.) to control. Given are mean and standard error of four independent experiments.

Figure 4 Analysis of APP processing in SH-SY5Y cells with a stable ADAM10 knockdown. (**A**) SH-SY5Y cells were infected with lentiviral vectors carrying GFP and different shRNAs: a non-targeting shRNA (Con) and two different ADAM10-targeting shRNAs (sh6 and sh9). Cells (Con, sh6 and sh9) were either treated with DMSO as solvent control or the metalloprotease inhibitor TAPI-1. Conditioned media of these cells were analysed with antibody 22C11 (APPs total), W02 (APPsα + β′) or 4B4 (APPsα), whereas cell lysates were analysed for cellular APP (22C11). Compared with HEK293 cells, the mature APP form is less well visible in SH-SY5Y cells. Membranes were analysed for ADAM10 protein. Calnexin was detected as a loading control. (**B**) Quantification of experiments in (**A**). APPs total, APPsα + β′ and APPsα were normalized to calnexin. Given are mean and standard error of four independent experiments. (**C**) Cells (Con, sh6 and sh9) were either treated with DMSO as solvent control or with the β-secretase inhibitor C3. Conditioned media of these cells were analysed as in (**A**). Antibody 192wt was used for the detection of APPsβ. (**D**) Quantification of experiments in (**C**), carried out as in (**B**) relative (rel.) to control. (**E**) Con and sh9 SH-SY5Y cells were treated with DMSO as a control or co-treated with the metalloprotease inhibitor TAPI-1 and the β-secretase inhibitor C3. (**F**) Quantification of experiments in (**E**), carried out as in (**B**). Given are mean and standard error of three independent experiments.

quantitative RT–PCR (Figure 6F). Together, these results show that both ADAM9 and 17 are not required for constitutive APP α-secretase cleavage in primary neurons.

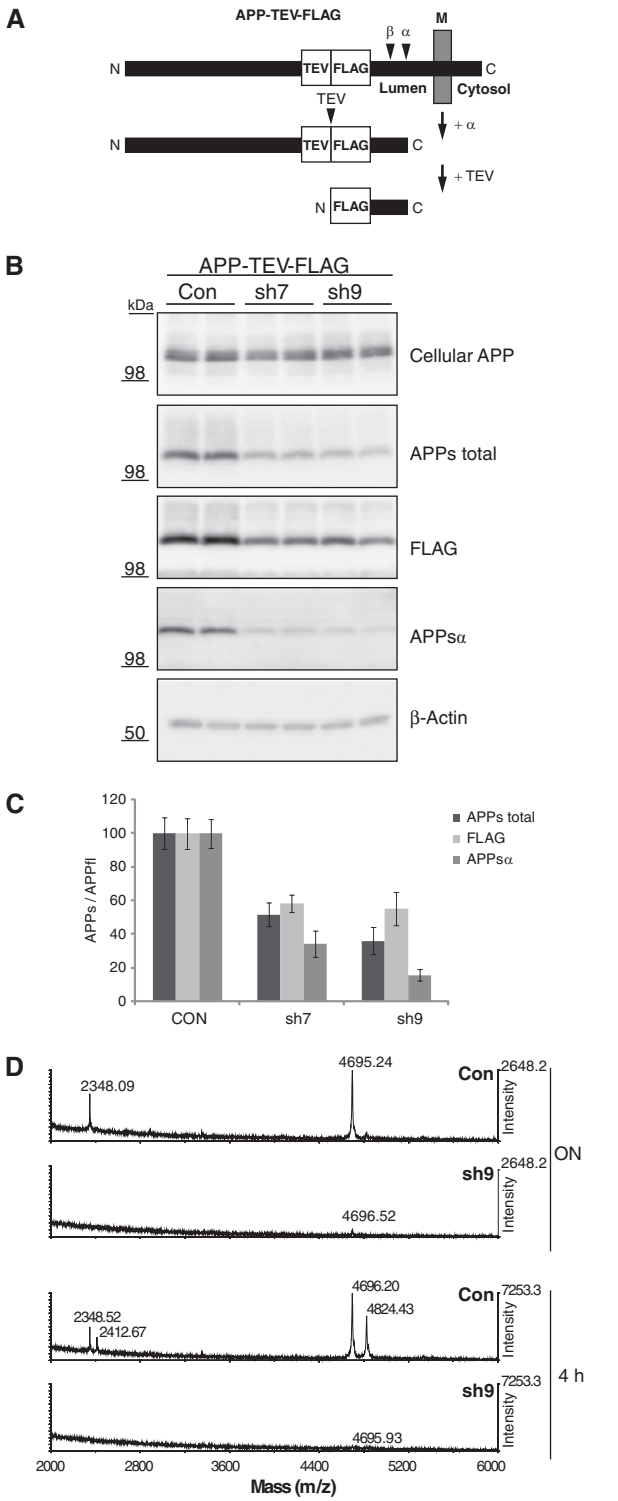


Interestingly, total APP secretion (22C11) was only mildly reduced in the ADAM10 knockdown neurons, which is most likely due to the fact that in primary embryonic neurons, α-secretase cleavage contributes only to a smaller extent to total APP secretion, compared with cell lines (Simons *et al*, 1996) (Figure 6A). Indeed, β-secretase expression is particularly high during embryonic development and in the first 2 weeks after birth, but then drops sharply (Willem *et al*, 2006). In contrast to the HEK293 and SH-SY5Y cells, the ADAM10 knockdown mildly increased APPsβ generation (1.4–2-fold) for both shRNAs tested. Aβ followed a similar trend. However, only one of the shRNA sequences (sh-I) significantly increased Aβ, whereas the other sequence (sh-II) did not. This suggests that at least in embryonic neurons with their high expression of β-secretase, a reduction of ADAM10 may increase Aβ levels.

Mass-spectrometric analysis of APP α-secretase-cleavage products

The experiments above relied on the use of cleavage site-specific antibodies for the detection of APPs and in particular of APPsα. Next, we used mass spectrometry as an independent method to detect APPsα and to investigate the reduction of APPsα in ADAM10 knockdown cells. To this aim, the following strategy was used. APP is *N*- and *O*-glycosylated at different positions within its ectodomain, which leads to a broadening of the peaks obtained for APPs by mass-spectrometric analysis, making identification of specific cleavage sites difficult. To avoid this situation, two short peptide tags were included into the APP ectodomain between the most C-terminal glycosylation site and the N-terminus of the Aβ sequence (Figure 7A). One of the two peptide sequences encodes a TEV protease-cleavage site, the other one encodes a FLAG tag. This mutant APP construct (APP-TEV-FLAG) was stably expressed in SH-SY5Y cells. The secreted form of APP-TEV-FLAG was immunoprecipitated from the conditioned medium with an anti-FLAG antibody and then digested *in vitro* with TEV protease. This leads to the removal

Figure 6 ADAM10 is essential for α-secretase cleavage of APP in primary cortical neurons. (**A**) Primary cortical neurons were prepared at E16 from C57/BL6 mice and infected with purified lentiviral particles carrying GFP and either a non-targeting control shRNA (Con) or two distinct murine ADAM10-targeting shRNAs (sh-I, sh-II). Four days before harvest, the medium was changed. Media were analysed for total secreted APP (antibody 22C11), APPsα (7A6) and APPsβ (BAWT). Cell lysates were analysed for cellular APP, ADAM10 and β-actin (loading control). Immature ADAM10 is indicated with (*) and mature ADAM10 with (**). Protein levels of ADAM10 were reduced by both shRNAs to about 10% of control (not shown). (**B**) Quantification of total secreted APP, APPsβ and APPsα, relative (rel.) to control. Aβ was measured by ELISA. Given are mean and standard error of eight independent experiments. (**C**) Quantitative RT–PCR shows that ADAM10 mRNA levels are strongly reduced, whereas levels of ADAM9 and 17 mRNA were not affected. Given are mean and standard error of four independent experiments. (**D**) Lentiviral knockdown of ADAM9 and 17 in primary neurons was carried out as in (**A**). (**E**) Quantification of results in (**D**) was performed as in (**B**). Both knockdowns did not affect APP processing. Given are mean and standard error of five independent experiments. (**F**) Quantitative RT–PCR shows efficient knockdown of ADAM9 and 17, whereas ADAM10 levels were not affected. Knockdown of ADAM17 partially reduced ADAM9 mRNA levels. Given are mean and standard error of four independent experiments.



E

Peptide	Charge	Measured Mass (Da)	Calculated Mass (Da)	Deviation (Da)	Sample
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQ	+1	4695.24	4695.11	+0.13	ON Con
" "	+2	2348.09	2349.06	−0.97	ON Con
" "	+1	4696.52	4695.11	+1.41	ON sh9
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQK	+1	4824.43	4823.21	+1.22	4 h Con
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQ	+1	4696.20	4695.11	+1.09	4 h Con
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQK	+2	2412.67	2413.11	−0.44	4 h Con
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQ	+2	2348.52	2349.06	−0.54	4 h Con
" "	+1	4695.93	4695.11	+0.82	4 h sh9

of the glycosylated part of the APP ectodomain, resulting in ~5 kDa peptides having the FLAG tag at their new N-terminus and C-terminally ending at the peptide bond, where APP shedding occurs by the secretases (Figure 7A). First, we verified that APP-TEV-FLAG was processed in a manner similar to the wild-type, endogenous APP. Stable knockdown of ADAM10 in APP-TEV-FLAG-expressing SH-SY5Y cells reduced APP-TEV-FLAG shedding to a similar extent (Figure 7B; quantification in Figure 7C) as observed for the endogenous, wild-type APP in SH-SY5Y cells (compare with Figure 4). Total APPs (22C11) was reduced to 40–50%, whereas APPsα (4B4) was reduced to 15–30% (Figure 7C). Taken together, APP-TEV-FLAG is processed similar to wild-type APP.

For the mass-spectrometric measurements, APP-TEV-FLAG cells were incubated overnight. Secreted APP-TEV-FLAG was immunoprecipitated from the conditioned medium and processed by TEV protease. A major peak at 4695.24 Da was identified, which corresponds to a peptide having glutamine 15 of the Aβ sequence as its C-terminal amino acid (Figure 7D and E). This is in agreement with the C-terminus of APPsα isolated from human brain (Pasternack *et al*, 1992). In addition, a less intensive peak was observed at 2348.09 Da. This is exactly half the molecular weight of the more prominent peak and corresponds to the same peptide, but with a double-positive charge instead of a single-positive charge. Importantly, upon ADAM10 knockdown, both peptide mass peaks were nearly completely suppressed, which is consistent with the reduction in α-secretase cleavage observed in the immunoblots (Figure 7C). Peptides with a C-terminus at the β- or β′-cleavage sites were not detected. As these cleavages make up a small proportion of total APP shedding (see C3-inhibitor treatment in Figure 4C), it is likely that these peptides were below the detection limit in our analysis or were not stable enough during the isolation procedure for the mass-spectrometric analysis. When the APP-TEV-FLAG-expressing cells were incubated for 4 h instead of overnight, a second mass peak at 4824.43 Da was observed (Figure 7D). This corresponds to the peptide having amino-acid lysine 16 of the Aβ sequence as its C-terminal amino acid (Figure 7E). The heterogeneity of one amino acid at the C-terminus is in agreement with the finding that *in vitro* ADAM10 cleaves between lysine 16 and leucine 17 (Lammich *et al*, 1999) and may then be followed by an as yet unidentified carboxypeptidase cleavage, removing lysine 16 (Esch *et al*, 1990). The knockdown of

Figure 7 Mass-spectrometric analysis of APP α-secretase cleavage in stable ADAM10 knockdown SH-SY5Y cells. (**A**) Schematic representation of the APP-TEV-FLAG construct, its cleavage by α-secretase and the further processing with TEV protease to generate small peptides for mass-spectrometric analysis. M, membrane. (**B**) SH-SY5Y cells stably expressing APP-TEV-FLAG were analysed by immunoblot for APPs total (22C11), APPsα (4B4) and FLAG immunoreactivity in the conditioned medium and for cellular APP and β-actin in the cell lysate. (**C**) Quantification of APPs total, FLAG reactivity and APPsα of three independent experiments. Given are mean and standard error. (**D**) APP-TEV-FLAG was immunoprecipitated from medium after overnight (ON) or 4 h culture, digested with TEV-protease and analysed by mass spectrometry. Medium was used from control (Con) or ADAM10 knockdown cells (sh9). (**E**) Table containing peptide sequence, charge, measured masses (from **D**) and calculated masses and deviation of the measured peptides in Da.

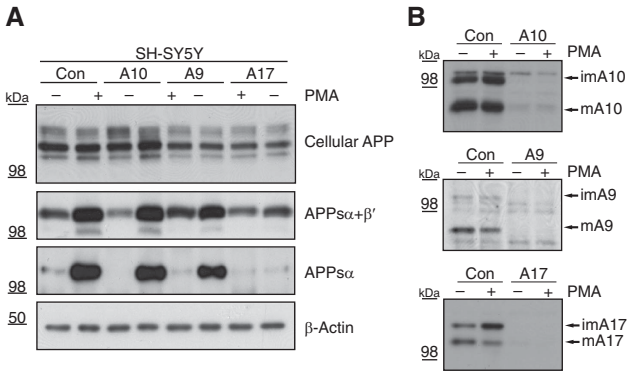


Figure 8 PMA-induced stimulation of APP shedding is independent of ADAM10, but requires ADAM17. SH-SY5Y cells were either transfected with control siRNA pool (Con) or siRNA pools against ADAM9 (A9), ADAM10 (A10) and ADAM17 (A17); 2 days after transfection, cells were treated with 1 μM PMA (+) or ethanol as solvent control (−) for 4 h. (**A**) Conditioned media were analysed for APPsα (4B4), APPsα + β′ (W02) and cell lysates were analysed for cellular APP (22C11). (**B**) Knockdown efficiency was analysed by blotting against the different proteases ADAM9, 10 and 17 in membrane preparations of the respective experiments.

ADAM10 also suppressed the generation of the longer peptide (Figure 7D).

Taken together, the mass-spectrometric analysis—in addition to the use of the cleavage site-specific antibody 4B4—provides a second, independent method to show that ADAM10 is essential for α-secretase cleavage of APP.

PMA stimulates ADAM17 cleavage of APP independently of ADAM10

The phorbol ester PMA stimulates the metalloprotease cleavage of many cell surface membrane proteins, including APPs (Figure 1; Buxbaum *et al*, 1998). PMA-induced shedding of APP requires ADAM17 activity, because this stimulation is lost in mouse embryonic fibroblasts deficient in ADAM17 (Buxbaum *et al*, 1998). Thus, we next investigated, whether the PMA stimulation of APP shedding also required ADAM10. To test this, SH-SY5Y cells expressing endogenous APP were transiently transfected with control siRNAs or siRNAs against ADAM9, 10 or 17 and then treated with or without PMA (Figure 8A and B). In control-transfected cells, PMA strongly increased APPsα (4B4) (Figure 8A), in agreement with Figure 1. knockdown of ADAM17 suppressed the PMA-induced increase in APPsα production. However, when ADAM10 or 9 were knocked down, PMA-stimulated APPsα generation occurred as in wild-type cells (Figure 8A). This shows that ADAM10 is not required for PMA induction of APP shedding and suggests that under these conditions ADAM17 can directly cleave APP.

Discussion

The α-secretase is an important proteolytic activity with the ability to prevent Aβ generation. In this study, we systematically evaluated the contribution of ADAM9, 10 and 17 to α-secretase cleavage of APP. Using a new, APPsα-specific antibody and two different cell lines as well as primary neurons expressing endogenous APP, we found that ADAM10, but not ADAM9 or 17, is essential for α-secretase cleavage. The requirement for ADAM10 was further validated by mass-spectrometric determination of APP-cleavage products. From this we conclude that ADAM10 is the physiologically relevant, constitutive α-secretase of APP and that ADAM9 and 17 are not redundant for this cleavage. This is particularly remarkable, because ADAM10 and 17 appear to have a broad substrate specificity and can cleave similar peptides *in vitro* at the same peptide bonds (Caescu *et al*, 2009). The clear specificity in cells suggests the existence of additional, as yet unknown factors, which control the protease specificity in the cellular environment.

Different metalloproteases, most notably ADAM9, 10 and 17 have previously been suggested as candidate α-secretases, because they cleave APP-derived synthetic peptides *in vitro* and because their overexpression in cells or mice increases APP shedding (Koike *et al*, 1999; Lammich *et al*, 1999; Roghani *et al*, 1999; Slack *et al*, 2001; Postina *et al*, 2004). However, data resulting from overexpression studies do not prove that a particular protease is the physiologically relevant protease for a given substrate. In fact, experiments using cells with a knockout or a knockdown of the corresponding proteases gave less clear results about their involvement in APP α-secretase cleavage. RNAi-mediated knockdown of ADAM9, 10 or 17 reduced APP shedding by 20 to 60% (Asai *et al*, 2003; Allinson *et al*, 2004; Camden *et al*, 2005; Freese *et al*, 2009; Taylor *et al*, 2009). In contrast, knockout cells deficient in ADAM9, 10 or 17 showed no change in APP shedding (Buxbaum *et al*, 1998; Hartmann *et al*, 2002; Weskamp *et al*, 2002). Only in a subset of ADAM10-deficient fibroblasts, APP α-secretase cleavage was altered to a variable degree (Hartmann *et al*, 2002). The reason for this variability is not yet clear. Importantly, because ADAM10 knockout mice die embryonically and ADAM17 knockout mice die perinatally, only embryonic fibroblasts, but not neurons, from these animals have been analysed for APP shedding. Taken together, the finding, that APP shedding was never fully abolished, was taken as evidence that all three proteases may have redundant functions with regard to APP α-secretase cleavage. In contrast to the previous studies, we used the novel antibody 4B4 that specifically detects the α-secretase cleaved APP (APPsα) without a contribution of other APP-cleavage products, such as APPsβ or APPsβ′. Using this new antibody, siRNAs and shRNAs against ADAM10 or treatment with the metalloprotease inhibitor TAPI almost completely blocked APPsα generation in HEK293 and SH-SY5Y cells and in primary neurons. ADAM9 and 17 were not required for APPsα formation. Only in HEK293 cells, the ADAM17 knockdown led to a very modest decrease in total APP secretion, raising the possibility that in specific cell lines ADAM17 may have a modulatory function in APPsα generation. Our knockdown data for ADAM9 and 17 complement the previous finding that ADAM9-deficient primary hippocampal neurons and ADAM17-deficient mouse embryonic fibroblasts do not show evidence of an altered α-secretase cleavage compared with their corresponding wild-type control cells (Buxbaum *et al*, 1998; Weskamp *et al*, 2002). At present, it is unclear, why additional studies using siRNAs against ADAM9 or 17 reported a moderate reduction of APP α-secretase cleavage. However, because those previous studies used only one siRNA per target gene or no control siRNA or relatively high siRNA concentrations (Asai *et al*, 2003; Allinson *et al*, 2004; Camden *et al*, 2005; Taylor

et al, 2009), it seems possible that earlier conclusions about ADAM9 and 17 as APP α-secretase may have been due to off-target effects (Jackson *et al*, 2003), but not due to the specific reduction of ADAM protease expression.

Interestingly, a cleavage by both ADAM10 and 17 was also suggested for the Notch1 receptor. Proteolytic Notch cleavage and signal transduction are required for cell differentiation processes. Recent work established that ADAM10 is the relevant protease for the physiological ligand-induced Notch1 cleavage and signalling, but that under certain ligand-independent conditions, including disease-linked Notch1 mutations, Notch1 cleavage can also be mediated by ADAM17 (Cagavi Bozkulak and Weinmaster, 2009; van Tetering *et al*, 2009).

APP α-secretase cleavage occurs constitutively, which requires ADAM10, as shown in this study. In addition, a heterogeneous group of molecules can stimulate APP α-secretase shedding (Bandyopadhyay *et al*, 2007), which is referred to as regulated α-secretase cleavage. Two stimuli activating APP α-cleavage are the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and the phorbol ester PMA. The PACAP peptide appears to stimulate the ADAM10 cleavage of APP (Kojro *et al*, 2006), suggesting that ADAM10 is not only the constitutive α-secretase, but also contributes to the regulated α-secretase activity. In addition, ADAM17 can act as regulated APP α-secretase activity, at least upon stimulation with the phorbol ester PMA. We found that this activation does not require ADAM10, but ADAM17, which is in agreement with a previous publication using ADAM17-deficient MEF cells (Buxbaum *et al*, 1998). Future studies need to address whether ADAM17 cleavage of APP also occurs under physiologically or pathophysiologically relevant conditions other than upon treatment with the synthetic phorbol ester PMA.

Previous studies, which will be discussed below, suggested that α- and β-secretases compete for APP as a substrate, such that a change in β-secretase cleavage results in the corresponding opposite change in α-cleavage and vice versa. On the basis of this assumption, an activation of α-secretase cleavage is considered as a therapeutic approach to reduce β-secretase cleavage and Aβ generation. Although this competition seems to be clearly the case for the regulated component of the α-secretase cleavage (see below), we show here that this is not always the case for the constitutive α-secretase cleavage of APP. The knockdown of ADAM10 did not significantly increase APPsβ levels in HEK293 and SH-SY5Y cells. Likewise the β-secretase inhibitor C3 blocked APPsβ generation in SH-SY5Y cells, but did not increase APPsα levels, which is in agreement with a recent study using a different β-secretase inhibitor in CHO cells (Kim *et al*, 2008). The reason for this uncoupling of α- and β-secretase cleavage under constitutive conditions is not yet clear. The cellular APP levels may not be rate limiting for α- and β-secretase cleavage, such that a reduction of one cleavage does not increase the other cleavage. Alternatively, it may reflect that α- and β-secretase cleavage occur in different cellular compartments (described below), such that a reduced α-secretase cleavage would not necessarily increase the endosomal APP levels available for β-secretase cleavage. In contrast to the cell lines, the knockdown of ADAM10 induced a mild increase in β-secretase cleavage in the primary neurons. This effect was more pronounced for APPsβ than for Aβ. The difference between the neurons and the cell lines may result from the different β-secretase expression levels in both cell types. Although the β-secretase BACE1 is ubiquitously expressed, its expression is particularly high in neurons during embryonic development (such as the E16 neurons used here) and in the first 2 weeks after birth and then drops sharply (Willem *et al*, 2006). Future studies need to address whether a knockdown of ADAM10 still increases β-secretase cleavage in adult neurons, where BACE1 levels are reduced. In fact, a lack of competition between constitutive α- and β-secretase cleavage in the adult brain comes from a study, where a dominant-negative ADAM10 mutant decreased APPsα, but did not alter APPsβ levels in a transgenic mouse brain (Postina *et al*, 2004).

The α-secretase cleavage predominantly occurs at the plasma membrane (Sisodia, 1992), but also in the *trans*-Golgi network (TGN), at least upon stimulation with PMA (Skovronsky *et al*, 2000). In contrast, β-secretase cleavage of wild-type APP occurs mainly in the endosome and to a lower extent in the TGN (Koo and Squazzo, 1994; Vassar *et al*, 1999). Previous studies reported a competition between α- and β-secretases for APP as a substrate. This was typically the case when the corresponding protease was overexpressed or when an APP mutant was used or when α-secretase cleavage was activated above its constitutive level. Under these conditions, α- or β-secretase cleavage mostly occurred in a cellular compartment where the constitutive cleavage does not take place to the same extent. For example, overexpression of the β-secretase BACE1 strongly reduced α-secretase cleavage (this study and Vassar *et al*, 1999), presumably because overexpressed BACE1 artificially cleaves APP in early compartments of the secretory pathway before APP has access to α-secretase at the plasma membrane. A second condition, where a competition between α- and β-secretase cleavage was observed, is the Swedish mutant form of APP (SweAPP), which is linked to a familial form of AD. Compared with wild-type APP, the SweAPP is more efficiently cleaved by β-secretase and is processed to more Aβ and less APPsα, presumably because the SweAPP is already cleaved by β-secretase in the TGN before it has access to α-secretase (Haass *et al*, 1995). As another example, PMA increased APPsα and reduced APPsβ and Aβ in APP-transfected CHO cells (Skovronsky *et al*, 2000). The authors argued that PMA shifts APP α-secretase cleavage away from the plasma membrane towards the Golgi/TGN, such that APP is cleaved earlier in the secretory pathway and less APP is available for β-secretase cleavage. This shows that the regulated component of α-secretase (i.e. the increase of α-secretase cleavage above its constitutive level) can compete with β-secretase and consequently reduces Aβ generation, in agreement with the idea that a pharmacological activation of α-secretase may be a therapeutic approach to AD (Fahrenholz, 2007).

Another outcome of our study is that the ADAM10 knockdown increased the levels of C99 in SH-SY5Y cells, although the amount of β-secretase cleavage (measured by APPsβ levels) was unchanged. From this finding, we conclude that C99 can principally be processed in two pathways. In the first one, C99 is directly cleaved by γ-secretase leading to Aβ generation. In the competing pathway, C99 is first cleaved by α-secretase, leading to C83 generation, which may prevent Aβ generation. Upon ADAM10 knockdown, the latter

pathway is blocked, leading to an increase in C99, while leaving APPsβ levels unchanged. As C99 is short-lived, we used a γ-secretase inhibitor to visualize it. The increase in C99 upon ADAM10 knockdown is unlikely to occur to the same extent in the absence of a γ-secretase inhibitor. If that were the case, we would expect an increase in Aβ, as it is the direct cleavage product of C99. This, however, was not the case. From this we conclude that the direct C99 cleavage by γ-secretase is the predominant pathway for C99 processing under normal conditions. However, when γ-secretase cleavage is blocked, the competing pathway by α-secretase becomes more prominent resulting in C99 turnover to C83. A cleavage of C99 by α-secretase is consistent with a previous study, showing that overexpressed C99 can be converted to C83 in neurons (Cupers *et al*, 2001). A possible competition between α- and γ-secretases for C99—even under conditions, where γ-secretase is not inhibited—is supported by a recent study, which detected N-terminal Aβ fragments, such as Aβ1–15 and Aβ1–16, that seem to result from α-secretase cleavage of C99 (Portelius *et al*, 2009).

Our new antibodies, which are specific for APPsα and do not detect APPsβ′, may be helpful in the search for biomarkers of AD. Previous studies reported that APPsα levels are significantly decreased in the CSF of sporadic AD patients compared with controls, but there was not a complete separation between both groups (Sennvik *et al*, 2000; Fellgiebel *et al*, 2009). Potentially, the use of APPsα-specific antibodies, such as 4B4 may help to separate AD and control subjects more clearly. In addition, changes in APPsα levels may help to identify individuals with mutations in ADAM10. Mutations in the prodomain of ADAM10, which reduce ADAM10 protease activity, have recently been genetically linked to an increased risk for late-onset AD in seven distinct families (Kim *et al*, 2009).

In summary, our study defines ADAM10 as the physiologically relevant, constitutive α-secretase for APP and will allow to further explore the function of ADAM10 in AD.

Materials and methods

Reagents, plasmids and shRNA

The following antibodies were used: FLAG M2 (Sigma), ADAM10 (Calbiochem-422751), ADAM17 (Chemicon), ADAM17 (Oncogene), ADAM9 (Cell Signaling), HRP-coupled anti-rabbit, anti-mouse (DAKO), HRP-coupled anti-rat (Santa Cruz), Calnexin (Stressgen), β-actin (Sigma), monoclonal antibody (mAb) 22C11 (anti-APP ectodomain) and mAb W02 (against amino acids 5–8 of Aβ) from Konrad Beyreuther; polyclonal antibody (pAb) 5313 (anti-APP ectodomain), pAb 6687 (against APP C-terminus), pAb 3552 (against Aβ) and mAb 2D8 (against Aβ1–16) from Christian Haass and pAb 192Wt from Dale Schenk. Rat mAb 4B4 (IgG2a, APPsα specific), mAb 7A6 (IgG2a, APPsα specific; detects also murine APPsα) and rat mAb BAWT (IgG2a, APPsβ specific, used for immunoprecipitation) were generated against peptides EVHHQK-COOH (amino acids 11–16 of Aβ), YEVHHQ-COOH (amino acids 10–15 of Aβ) and ISEVKM-COOH (amino acids directly preceding the β-secretase-cleavage site), respectively (Ullrich *et al*, 2010). The following reagents were used: metalloprotease inhibitor TAPI-1, BACE inhibitor C3 and Dodecyl maltoside (DDM) from Calbiochem; siRNA pools siGenome against ADAM9, 10 and 17 and siRNA pool on target plus against ADAM17 and corresponding controls from Dharmacon. Lipofectamine 2000, RNAimaxx and TEV protease from Invitrogen. Cloning of plasmids is described in the Supplementary data section. shRNA sequences are listed in Supplementary Table 1.

Cell culture, transfections, RNAi, sample preparation, immunoblot, Aβ measurements

HEK293-T cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% foetal calf serum (FCS/Gibco) and G418 (Invitrogen) to maintain the large-T antigen. SH-SY5Y cells were cultured in F12/DMEM (Lonza) supplemented with 15% FCS (Gibco) and non-essential amino acids (PAA). knockdown of ADAM9, 10 and 17 in HEK293T and SH-SY5Y cells was performed transfecting 10 nM of siGenome pool targeting ADAM9, 10, 17 and corresponding controls or transfecting 10 nM of OnTarget plus pool targeting ADAM17 and corresponding controls (in SH-SY5Y). One day after transfection, medium was replaced. After overnight incubation, conditioned medium and cell lysate (in 150 mM NaCl, 50 mM Tris pH 7.5, 1% Nonidet P-40) were collected. For detection of ADAM9, 10, 17 and calnexin, cell membranes were prepared as described (Sastre *et al*, 2001). Detection of secreted and cellular APP was as described (Schobel *et al*, 2008). For precipitation of APPsβ, 500 μl of conditioned medium were incubated with 30 μl protein G sepharose and 50 μl of BAWT antibody for 2 h on a rotary shaker. APPsβ was detected with 192wt antibody. For precipitation of APPsα, 500 μl of conditioned medium were incubated with 30 μl protein A sepharose and 3.3 μl of 5313 antibody. APPsα was detected with 4B4 antibody. Inhibition of α- and β-secretases was performed with 50 μM TAPI-1 and 1 μM C3, respectively, for 24 h. Endogenous human Aβ was immunoprecipitated with antibody 3552 and detected with rat mAb 2D8 as described (Page *et al*, 2008). Murine Aβ40 was measured in conditioned media of murine primary cortical neurons, which were diluted 25-fold before analysis with an ELISA-kit (IBL, JP27720) according to the instructions of the manufacturer.

Lentivirus production and transduction

Lentiviruses were generated by transient cotransfection of HEK293T cells with the plasmids psPAX2, pCDNA3.1 (−)-VSV-G and as transfer vector pLVTHMmod or pLKO2mod-EGFP-WPRE for gene knockdown or FU-ΔZeo for gene overexpression using Lipofectamine 2000. For transduction of cell lines, medium was replaced by fresh antibiotic-free medium 1 day after transfection. Overnight conditioned medium was filtered through 0.45 μm sterile filters and directly added to the target cells. After 6 h, incubation medium was exchanged against cell type-specific growth medium of the target cells. Lentiviral particles for infection of murine primary cortical neurons were concentrated by one run of ultracentrifugation for 2 h at 22 000 r.p.m. in a SW28 rotor (Beckman) of the overnight conditioned medium 48 h after transfection. Following ultracentrifugation, the supernatant was removed and the viral pellet carefully resuspended in TBS-5 (50 mM Tris, 130 mM NaCl, 10 mM KCL, 5 mM $MgCl_2$, 5% (w/v) BSA) after a 4 h incubation period at 4°C. Lentiviral stocks were stored at −80°C until use.

Preparation and lentiviral transduction of neurons

Primary neuronal cultures were obtained from the cerebral cortex of E16 C57/BL6 mouse embryos, incubated with 200 U of papain (Sigma Aldrich) (30 min at 34°C) and subsequently mechanically dissociated. All experimental procedures on animals were performed in accordance with the European Communities Council Directive (86/609/EEC). Neurons were plated in six-well plates ($\sim 1.5 \times 10^6$ cells/well) pre-coated with 25 μg/ml poly-D-lysine (Sigma Aldrich). Plating medium was B27/neurobasal (Gibco) supplemented with 0.5 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Neurons were infected with lentiviruses at 3 DIV. Cell lysates and supernatant were collected at 8 DIV.

Mass spectrometry of APP-cleavage sites

SH-SY5Y cells were infected with FUΔ-Zeo-DsRed-UAS-APP-TEV-FLAG and FUΔ-Zeo-Gal4-VP16. Infected cells were subsequently FACS sorted according to their DsRed expression to obtain a homogenous-expressing population. Afterwards, cells were infected with PLVTHM encoding shRNAs against ADAM10 sh7 and sh9 or a control shRNA. Secreted APP-TEV-FLAG was immunoprecipitated with FLAG-M2 agarose. Immunoprecipitation was followed by three washes with STED (NaCl 150, Tris 50 mM, EDTA 2 mM, 0,5% DDM), three washes with STE and three washes with ddH_2O. Precipitated APP-TEV-FLAG ectodomain was eluted with 40 μl 100 mM glycine pH 2.5. The eluate was subsequently neutralized with 200 μl 100 mM Tris pH 8.0 and supplemented with 0.5 mM EDTA and 1 mM DTT and addition of 0.5 μl TEV protease. Protease

digest was incubated at 4°C overnight on a rotary shaker. The digest was diluted with 15 ml of PBS. To precipitate the digested FLAG peptide, FLAG M2 agarose was added and incubated at 4°C for 2 h on a rotary shaker. Agarose was washed three times with PBS and three times with ddH_2O. Afterwards, peptides were eluted in a 1/20/20 mixture of trifluoracetic acid/acetonitrile/ddH_2O saturated with α-Cyano matrix. A total of 1 µl was spotted on a hydrophobic target and measured with a Voyager DestR in linear mode. The MALDI-TOF mass spectrometer was externally calibrated with a peptide standard mixture (Sequazyme calibration mixture III).

Quantitative real-time PCR

Total RNA was extracted using RNeasy Mini kit (Qiagen) from primary neurons following the manufacturer's instructions. Concentrations and purities of total RNA were spectrophotometrically assessed at 260 and 280 nm. Total RNA was reverse transcribed into cDNA in a 20 µl reaction volume, using high-capacity cDNA Reverse Transcription kit (Applied Biosystems/ABI). Real-time PCR reaction was carried out on a 7500 Fast Real-Time PCR machine (ABI) with the POWER SYBR®-Green PCR Master Mix (ABI) based on a modification of the manufacturer's recommended protocol. Reactions were performed in duplicate in 96-well plates (ABI) according to the following protocol: pre-incubation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Used primers including ADAM9, 10 and 17 and the three reference genes Actb, GapDH and Tbp are listed in Supplementary Table 2. Validation experiments were performed to verify the amplification efficiency of each gene, which consistently ranged from 1.8 to 2.2. The statistic analysis was performed by the ΔCt value method. The relative expression of ADAM9, 10 and 17 was normalized to all three reference genes.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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