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Minor contribution of presenilin 2 for γ-secretase activity in mouse embryonic fibroblasts and adult mouse brain

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ABSTRACT

γ-Secretase plays an important function in the development of Alzheimer disease, since it participates in the production of the toxic amyloid β-peptide (Aβ) from the amyloid precursor protein (APP). Besides APP, γ-secretase cleaves many other substrates resulting in adverse side effects when γ-secretase inhibitors are used in clinical trials. γ-Secretase is a membrane bound protein complex consisting of at least four subunits, presenilin (PS), nicastrin, Aph-1 and Pen-2. PS and Aph-1 exist as different homologs (PS1/PS2 and Aph-1a/Aph-1b, respectively), which generates a variation in complex composition. PS1 and PS2 appears to have distinct roles since PS1 is essential during embryonic development whereas PS2 deficient mice are viable with a mild phenotype. The molecular mechanism behind this diversity is, however, largely unknown.

In order to investigate whether PS1 and PS2 show different substrate specificity, we used PS1 or PS2 deficient mouse embryonic fibroblasts to study the processing on the γ-secretase substrates APP, Notch, N-cadherin, and ephrinB. We found that whereas depletion of PS1 severely affected the cleavage of all substrates, the effect of PS2 depletion was minor. In addition, less PS2 was found in active γ-secretase complexes. We also studied the effect of PS2 depletion in adult mouse brain and, in concordance with the results from the mouse embryonic fibroblasts, PS2 deficiency did not alter the cleavage of the two most important substrates, APP and Notch.

In summary, this study shows that the contribution of PS2 on γ-secretase activity is of less importance, explaining the mild phenotype of PS2-deficient mice.

1. Introduction

As the catalytic core of the γ-secretase complex, presenilins (PSs) are involved in the processing of a multitude of type I transmembrane proteins including the amyloid precursor protein (APP) and the Notch receptor [1]. γ-Secretase cleavage of APP results in the generation of the APP intracellular domain (AICD) as well as the amyloid β-peptide (Aβ), widely believed to play a central role in the pathogenesis of Alzheimer disease [2]. γ-Secretase cleavage of Notch liberates the Notch intracellular domain (NICD) which in turn translocates to the nucleus and regulates gene transcription [3]. Notch signaling is crucial for cell differentiation and proliferation during development but is also of importance in adulthood [4]. Inhibition of the Notch pathway, following administration of non-selective γ-secretase inhibitors, have been shown to interfere with B- and T-lymphocyte maturation and differentiation processes of the gastrointestinal tract in adult mice as well as in patients [5,6]. Prior to γ-secretase cleavage, the ectodomain of the substrates is shedded, generating membrane bound C-terminal fragments (CTFs). These CTFs are further processed by γ-secretase within the hydrophobic environment of the membrane [7], yielding secreted peptides and intracellular domains (ICDs). For some of the substrates the produced ICDs have been shown to affect gene transcription, while others seem to have non-nuclear functions. Apart from PS, the γ-secretase complex is dependent on three other proteins for its activity: nicastrin, anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2) [8]. In humans, there are two homologs of PS (PS1 and PS2) and two of Aph-1 (Aph-1a and Aph-1b), resulting in complexes with different composition. Whereas PS1 as well as Aph-1a deficient mice show severe developmental defects and embryonic or perinatal mortality [9–11], PS2 and Aph-1b deficient mice have very mild phenotypes [10,12]. While the developmental effects seen in PS1 and Aph-1a deficient mice most likely depend on decreased Notch signaling, the contribution of the different homologs on other

Abbreviations: Ab, amyloid β-peptide; APP, amyloid precursor protein; PS, presenilin; Aph-1, anterior pharynx defective-1; Pen-2, presenilin enhancer-2; ICD, intracellular domain; AICD, APP intracellular domain; NICD, Notch intracellular domain; CTF, C-terminal fragment; MEF, mouse embryonic fibroblast; dKO, double knock-out; PIC, protease inhibitor cocktail; GCB, γ-secretase inhibitor coupled to biotin via a cleavable linker.

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substrates and of Notch cleavage in the adult brain remains elusive. The possible difference in substrate specificity between isoforms could be of importance for the design of novel specific γ-secretase inhibitors. Therefore, we here study the effect of depleting mice embryonic fibroblasts of PS1 or PS2 on processing of a number of γ-secretase substrates. In addition, we studied the effect of PS2 depletion in adult mice on APP and Notch processing.

2. Materials and methods

2.1. Antibodies

The following antibodies were used for immunoblotting: nicastrin: N1660 (Sigma) raised against the C-terminal residues 693–709, PS1-CTF: MAB5232 (Chemicon) recognizing the C-terminal loop region; PS1-NTF: (Calbiochem) raised against amino acids 1–65, PS2-CTF: Ab2 (Calbiochem) raised against residues 324–335, Pen-2: UD1 (a gift from Dr. Jan Näslund, Karolinska Institutet, Sweden) raised against residues 1–11; Aph-1aL: 433 (a gift from Dr. Harald Steiner, Ludwig Maximilians University, Germany) raised against the C-terminus (residues 245–265), APP: C1/6.1 (a gift from Dr. Paul M. Mathews, Nathan Kline Institute, NY, USA) raised against the C-terminus of APP, ephrinB1: C-18 (Santa Cruz Biotechnology) raised against the C-terminus of human ephrinB1 but cross-reacts with ephrinB2, N-cadherin: (BD Biosciences) raised against residues 802–819, cleaved Notch1: Val1744 (Cell Signaling) recognizing the intracellular C-terminal domain of Notch1 only when cleaved between Gly1743 and Val1744, Notch1: C-20 (Santa Cruz Biotechnology) raised against the C-terminus.

2.2. Cell lines

Mouse embryonic fibroblasts (MEFs) generated from $PS1^{+/+}PS2^{+/+}$ (wild-type), $PS1^{-/-}PS2^{+/+}$, $PS1^{+/+}PS2^{-/-}$ and $PS1^{-/-}PS2^{-/-}$ (dKO) mice (kindly provided by Prof. Bart De Strooper, Flanders Interuniversity Institute for Biotechnology, K.U. Leuven, Belgium) [13] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen). Cells were washed and harvested in phosphate-buffered saline (PBS) and either lysed in cell lysis buffer (10 mM Tris, pH 8.1, 1 mM EDTA, 150 mM NaCl and 0.65% Igepal CA-630) supplemented with complete protease inhibitor cocktail (PIC) (Roche, Basel Switzerland) or subjected to membrane preparation.

2.3. Mouse brain tissue

PS2 deficient female C57BL mice were kindly provided by Prof. Bart De Strooper, K.U. Leuven, Belgium [14]. Age matched wild-type C57BL female mice were bred at the Karolinska University Hospital, Huddinge animal facility. The mice were sacrificed by cervical dislocation at the age of 12–14 months. The brains were isolated and dissected to remove blood vessels and cerebellum. Ethical approval was received from the Animal Trial Committee of Southern Stockholm (S30-09).

2.4. Membrane preparations

MEFs and brain tissue were homogenized in homogenization buffer (10 mM KCl and 10 mM MOPS, pH 7.0, supplemented with PIC) by 25 or 50 strokes, respectively at 1500 rpm using a pestle homogenizer. The homogenates were centrifuged at 1000*g* for 10 min at 4 °C and the obtained post-nuclear supernatants were centrifuged further at 100,000*g* for 1 h at 4 °C. The resultant membrane pellets were suspended in homogenization buffer supplemented with 20% glycerol, flash frozen in liquid nitrogen and stored at −80 °C prior to use.

2.5. Activity assay

Membrane preparations were thawed and membranes were recollected by centrifugation at 100,000*g* for 1 h at 4 °C. The membranes were suspended in buffer H (20 mM Hepes pH 7.0, 150 mM NaCl, 5 mM EDTA) supplemented with PIC and the protein concentration was measured by BCA protein assay (Pierce). In the case of measuring Aβ40 production, buffer H was also supplemented with 5 mM 1,10-phenanthroline and 10 μM thiorphan, inhibitors of the Aβ degrading enzymes insulin degrading enzyme and neprilysin, respectively. Equal amounts of protein from the different samples were incubated in the absence or presence of the γ-secretase inhibitor L-685,458 for 16 h at 37 °C or at 4 °C as a negative control. During incubation the protein concentration in the samples was kept at 3–4 μg/μl for studies of endogenous ICD production, at 1 μg/μl for AICD-FLAG detection, and at 1.25 μg/μl for Aβ40 measurements. For studies of γ-secretase cleavage of C99-FLAG, the activity assay was performed as above with the addition of 0.4% CHAPSO. In order to avoid aggregates of C99-FLAG and enhance its α-helical conformation, the peptide was pre-treated with trifluoroethanol prior to the addition to the samples (20–100 ng/sample).

2.6. Affinity capture of γ-secretase

This method has previously been characterized and described by Teranishi et al. [15]. Briefly, membranes prepared from MEFs were re-suspended in buffer H containing 0.5% CHAPSO and PIC and pre-absorbed by incubation with magnetic streptavidin beads (Invitrogen). The samples were thereafter incubated for 20 min at 37 °C with 200 nM of an affinity probe (GCB), which is based on L-685,458 and coupled to biotin via a cleavable linker. As a negative control, the samples were incubated with 1 μM of L-685,458 for 10 min at 37 °C prior to the addition of GCB. Magnetic streptavidin beads were added and samples were incubated for 1 h at 4 °C. The beads were washed and bound proteins were eluted with Laemmli sample buffer at room temperature (RT) for 30 min and subjected to SDS–PAGE and western blotting.

2.7. SDS–PAGE and western blot

Equal amounts of protein from membrane samples or total cell lysates were mixed with 2× Laemmli sample buffer. For activity measurements, the samples were incubated at 95 °C for 5 min and for detection of the γ-secretase components, the samples were kept at room temperature for 20 min. The samples were separated by SDS–PAGE on 4–12% BisTris or 16% Tricine gels (Invitrogen) and transferred to either nitrocellulose or PVDF membranes. The membranes were probed with primary antibodies and horseradish-coupled secondary antibodies (GE-Healthcare) and visualized by enhanced chemiluminescence (Pierce and Millipore).

2.8. ELISA

Aβ40 production was measured in duplicates by ELISA, Human/Rat β Amyloid (40) ELISA Kit Wako II (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions. Following the activity assay, the reaction was stopped by adding RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8.0) and boiling for 5 min. The samples were centrifuged at 10,000*g* for 5 min and the supernatants were dispensed into the ELISA plate.

3. Results and discussion

PS1 and PS2 form distinct γ-secretase complexes and show differential tissue distribution [16] making it plausible that they have different substrate specificity. Previous studies in cell lines have shown that PS2 complexes are less efficient in producing Aβ [17–19]. However, the contribution of endogenous PS1 and PS2 complexes for γ-secretase processing of other substrates have not been studied, although Bentahir et al. [18] studied processing of endogenous Notch, syndecan 3, and N-cadherin in PS1 and PS2 transfected PS-deficient MEFs indirectly by analyzing the levels of CTFs. To study the substrate specificity of PS1 and PS2 complexes, we compared the production of ICDs from Notch1, APP, N-cadherin, and ephrinB in wild-type (WT), PS1 deficient ($PS1^{-/-}$), and PS2 deficient ($PS2^{-/-}$) MEFs. MEFs devoid of both PS1 and PS2 (dKO) were used as a negative control. In addition, we studied the effect of depleting PS2 on APP and Notch processing in adult mouse brain.

3.1. Depletion of PS1 or PS2 differentially affects protein levels of Aph-1a and Pen-2 and maturation of nicastrin, as well as processing of γ-secretase substrates

Initially, WT, $PS1^{-/-}$, $PS2^{-/-}$, and dKO MEF cells lines were characterized with respect to γ-secretase components. Cell lysates were analyzed by SDS–PAGE and western blot. It was confirmed that the $PS1^{-/-}$ cells were indeed deficient of PS1, the $PS2^{-/-}$ cells were deficient of PS2, and neither of the PS homologs were detected in the dKO cell line (Fig. 1A). The Pen-2 levels were reduced in $PS1^{-/-}$ and dKO cells (Fig. 1A) indicating that PS1 is more important than PS2 for the expression or stability of Pen-2. In contrast, the Aph-1a levels were reduced in the PS2 deficient MEFs but not in $PS1^{-/-}$ or dKO cells (Fig. 1A). The down-regulation of Aph-1a in $PS2^{-/-}$ cells was not only confined to cell lysates but also to membrane preparations (data not shown) and is in line with the results by Bentahir and colleagues [18]. Together, these results suggest a negative role of PS1 and/or a positive regulatory role of PS2 on Aph-1a expression. In accordance with previous studies [20], the levels of mature nicastrin were significantly reduced in the cells deficient of PS1 (Fig. 1A), suggesting that PS2 is less efficient in promoting nicastrin maturation.

To study the capability of PS1 and PS2 to form active γ-secretase complexes, we used affinity capture with an active site-directed γ-secretase inhibitor linked to a biotin group via a cleavable linker (GCB) [15]. As a negative control the non-labeled γ-secretase inhibitor L-685,458 was added to the samples prior to GCB. We found that whereas PS1-complexes were readily detected in both wild-type and $PS2^{-/-}$ MEFs, barely detectable levels of captured PS2-complexes were found in the $PS1^{-/-}$ MEFs and WT MEFs (Fig. 1B). In accordance, we have earlier noted that a smaller proportion of PS2 compared to PS1 is incorporated in active complexes in rat brain [21]. In addition, the high amount of Aph-1a detected in lysates from $PS1^{-/-}$ cells (Fig. 1A) could not be pulled down by GCB and nor could the lower, immature form of nicastrin (Fig. 1B). Together with the notion that less mature nicastrin is

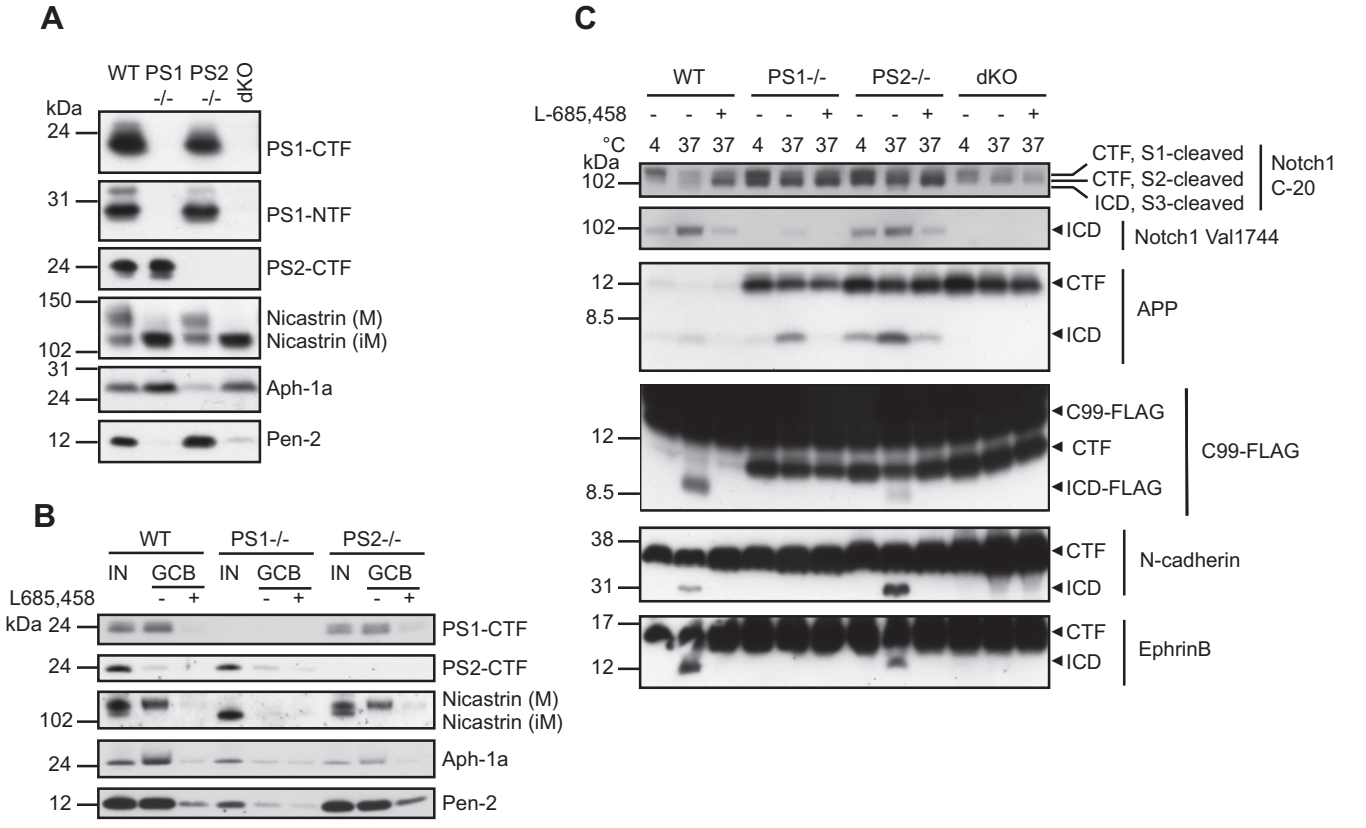

Fig. 1. PS1 and PS2 differentially affect expression and maturation of other γ-secretase components and processing of γ-secretase substrates. (A) Equal amounts of protein (15 μg) extracted from wild-type (WT), PS1 deficient ($PS1^{-/-}$), PS2 deficient ($PS2^{-/-}$), and PS1 and PS2 deficient (dKO) MEFs were analyzed by western blotting for the indicated proteins. M, Mature; iM, immature. (B) Active γ-secretase complexes were captured from membrane preparations of wild-type (WT), PS1 deficient ($PS1^{-/-}$), and PS2 deficient ($PS2^{-/-}$) MEFs with 200 nM of GCB in the presence or absence of 1 μM L-685,458. The bound complexes and 10% of the input (IN) were analyzed by western blotting using antibodies directed to the indicated γ-secretase components. (C) Membrane fractions prepared from wild-type (WT), PS1 deficient ($PS1^{-/-}$), PS2 deficient ($PS2^{-/-}$), and PS1 and PS2 deficient (dKO) MEFs were incubated at 4 °C or at 37 °C with or without L-685,458. The samples were analyzed by western blotting using antibodies directed to the C-terminal of the indicated γ-secretase substrate. NICD was also detected by the antibody Val1744, which only recognizes the free N-terminal of NICD.

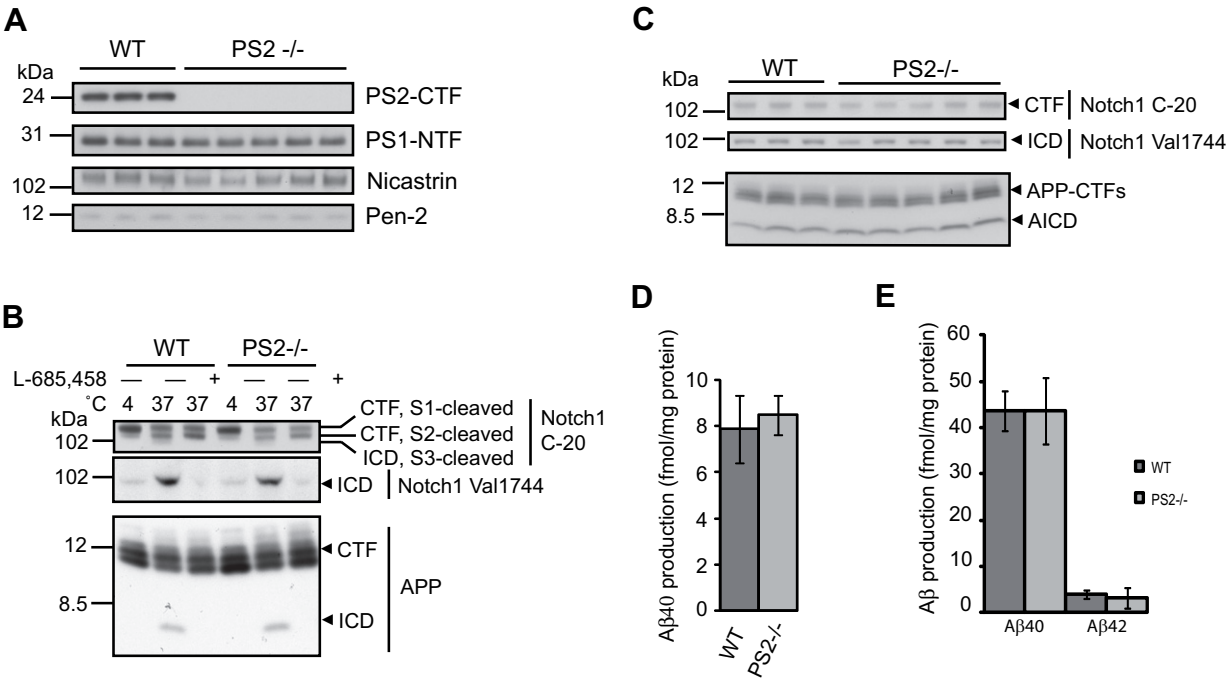

Fig. 2. Membrane fractions prepared from wild-type and PS2$^{-/-}$ mouse brains show similar production of NICD, AICD, and Aβ. (A) Equal amounts of protein from membrane fractions prepared from three wild-type (WT) and five PS2 deficient (PS2$^{-/-}$) adult mouse brains were analyzed by western blotting using antibodies for the γ-secretase components. (B) Membrane fractions prepared from wild-type (WT) and PS2 deficient (PS2$^{-/-}$) adult mouse brains were incubated at 4 °C or at 37 °C with or without L-685,458. The samples were analyzed by western blotting using antibodies directed to the C-terminal of Notch1 or APP. NICD was also detected by the antibody Val1744, which only recognizes the free N-terminal of NICD. (C) NICD and AICD production in three wild-type (WT) and five PS2 deficient (PS2$^{-/-}$) adult mouse brains. Incubation and analysis was performed as above. (D) Endogenous Aβ40 production was measured by sandwich ELISA. Membrane fractions from wild-type (WT) and PS2 deficient (PS2$^{-/-}$) adult mouse brains were incubated at 37 °C with or without L-685,458. Aβ production was determined by subtracting Aβ40 values in the samples with L-685,458 from the values in the samples without L-685,458. Data are presented as mean value ± SD, $n = 5$. (E) Wild-type (WT) and PS2 deficient (PS2$^{-/-}$) adult mouse brains were assayed for Aβ40 or Aβ42 production as in (D) but the activity assay was performed in the presence of C99-FLAG. Data are presented as mean value ± SD, $n = 5$.

detected in PS1$^{-/-}$ cell lysates, these results suggest that PS2 is less prone to form active complexes, although we cannot rule out the possibility that GCB is less effective in capturing PS2 complexes.

Next, we wanted to examine the contribution of PS1 and PS2 to γ-secretase dependent production of ICDs from endogenous Notch1, APP, N-cadherin, and ephrinB; four γ-secretase substrates important for neuronal function. Membranes prepared from WT, PS1$^{-/-}$, PS2$^{-/-}$, and dKO MEFs were incubated at 4 °C or at 37 °C in the absence or presence of L-685,458. The production of ICDs from the different substrates was examined by western blot analysis using C-terminal antibodies recognizing the indicated substrates and a Notch1 antibody recognizing the neoepitope of NICD. Production of ICDs from Notch and ephrinB was similar in the WT and in the PS2$^{-/-}$ cell line (Fig. 1C). This suggests that PS1 is responsible for the majority of these substrates' processing in MEFs, even if trace amounts of NICD was observed in PS1 deficient cells (Fig. 1C). The levels of APP and N-cadherin CTFs were lower in the WT cell line compared to the other cell lines and consequently the ICD production was lower. This is probably due to that the immediate substrates are consumed in the cells before the membrane preparation. Some AICD production was seen in PS1$^{-/-}$ cells. Hence, PS2 can also cleave APP although not as effectively as PS1. This is in accordance with previous work studying Aβ production [17–19]. The difference in processing efficacy between PS1 and PS2 appears less pronounced for APP than for other substrates, suggesting that PS2 complexes have a preference for APP as a substrate. To study the γ-secretase processing independent of APP substrate levels, we added an exogenous APP substrate, C99-FLAG, and detected the production of AICD-FLAG. As in the case of endogenous processing APP, AICD-FLAG could be detected in the wild-type and PS2$^{-/-}$ cell line (Fig. 1C). In agreement with our earlier studies [22], endogenous APP appears to be a more efficient substrate than C99-FLAG and thus less AICD-FLAG is produced in the PS2$^{-/-}$ cells where high amounts of endogenous substrates are present. For all substrates that we examined, the ICD generation was inhibited by L-685,458 and absent in the dKO cell line demonstrating that the processing is indeed γ-secretase dependent.

The low level of ICD generation in the PS1$^{-/-}$ MEFs, from both endogenous and exogenous substrates, is likely due to the low levels of active PS2 containing γ-secretase complexes. Thus, the activity per active complex could still be as high for PS2 as for PS1 complexes.

Together, our pull-down experiments and activity assays indicate that low amounts of active γ-secretase complexes are formed by PS2 and that these complexes do not contribute significantly to the processing of γ-secretase substrates in MEF cells.

3.2. Membrane fractions prepared from wild-type and PS2$^{-/-}$ mouse brains show similar production of NICD, AICD and Aβ

Next, we investigated the effect of PS2 depletion in adult mouse brain. Unfortunately, PS1 deficient mice are not viable to adulthood and could not be analyzed. Initially, membrane fractions from wild-type and PS2 deficient adult mouse brain were prepared and analyzed by western blotting using antibodies recognizing the different γ-secretase components. In line with our observations made in the MEFs, the levels of PS1, nicastrin, and Pen-2 were similar in the wild-type and PS2 deficient membrane fractions (Fig. 2A). The membrane fractions were then subjected to activity assay and analyzed for production of ICDs as described above. In accordance with our earlier studies in rat brain [21], no ICD production from endog-

enous N-cadherin and ephrinB could be detected in adult mouse brain (data not shown). The levels of NICD and AICD produced as well as Notch1 and APP substrate levels were similar in wild-type and PS2 deficient membrane fractions (Fig. 2B) suggesting that PS2 do not contribute significantly to the processing of these substrates. We also investigated the effect of PS2 depletion on Aβ production both from endogenous APP (Fig. 2D) as well as from C99-FLAG (Fig. 2E). The Aβ production was determined by subtracting the Aβ levels of the inhibited samples (+L-685,458) from the Aβ levels of the samples without inhibitor (−L-685,458). Unfortunately, the Aβ42 levels produced from endogenous substrates were below the detection limit. The endogenous production of Aβ40 (Fig. 2D), as well as the production from Aβ40 and Aβ42 from C99-FLAG (Fig. 2E), were similar between wild-type and PS2 deficient mice. The relative amount of PS2-containing complexes has earlier been shown to correlate with the Aβ42/40 ratio [23]. Our data do not support increased Aβ42 production by PS2-complexes even though we cannot rule out minor changes in Aβ42 production, since the levels were close to the detection limits and standard deviations were rather large. Taken together, our results from mouse brain indicate that the majority of the NICD, AICD, and Aβ produced in the adult mouse brain are generated by PS1 containing γ-secretase complexes.

To summarize, our MEF and mouse brain results suggest that PS1 forms more active complexes than PS2 and are more important for the cleavage of endogenous substrates. These results explain the severe $PS1^{-/-}$ phenotype and the inability for PS2 to compensate for this phenotype.

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