Reduced Alzheimer's Disease β -Amyloid Deposition in Transgenic Mice Expressing S-Palmitoylation-Deficient A β 1aL and Nicastrin

Xavier Meckler,¹ Jelita Roseman,¹ Pritam Das,⁴ Haipeng Cheng,¹ Susan Pei,¹ Marcia Keat,¹ Breanne Kassarian,¹ Todd E. Golde,⁵ Angèle T. Parent,¹ and Gopal Thinakaran^{1,2,3}

Departments of ¹Neurobiology, ²Neurology, and ³Pathology, The University of Chicago, Chicago, Illinois 60637, ⁴Department of Neurosciences, Mayo Clinic College of Medicine, Jacksonville, Florida 32224, and ⁵Department of Neurosciences, Center for Translational Research in Neurodegenerative Disease, College of Medicine, Gainesville, Florida 32610

Sequential cleavage of amyloid precursor protein by β - and γ -secretases generates β -amyloid peptides (A β), which accumulate in the brains of patients with Alzheimer's disease. We recently identified S-palmitoylation of two γ -secretase subunits, A β 1 and nicastrin. S-Palmitoylation is an essential posttranslational modification for the proper trafficking and function of many neuronal proteins. In cultured cell lines, lack of S-palmitoylation causes instability of nascent A β 1 and nicastrin but does not affect γ -secretase processing of amyloid precursor protein. To determine the importance of γ -secretase S-palmitoylation for A β deposition in the brain, we generated transgenic mice coexpressing human wild-type or S-palmitoylation-deficient A β 1aL and nicastrin in neurons in the forebrain. We found that lack of S-palmitoylation did not impair the ability of A β 1aL and nicastrin to form enzymatically active protein complexes with endogenous presenilin 1 and PEN2 or affect the localization of γ -secretase subunits in dendrites and axons of cortical neurons. When we crossed these mice with 85Db0 transgenic mice, which coexpress familial Alzheimer's disease-causing amyloid precursor protein and presenilin 1 variants, we found that coexpression of wild-type or mutant A β 1aL and nicastrin led to marked stabilization of transgenic presenilin 1 in the brains of double-transgenic mice. Interestingly, we observed a moderate, but significant, reduction in amyloid deposits in the forebrain of mice expressing S-palmitoylation-deficient γ -secretase subunits compared with mice overexpressing wild-type subunits, as well as a reduction in the levels of insoluble A β _{40–42}. These results indicate that γ -secretase S-palmitoylation modulates A β deposition in the brain.

Introduction

Alzheimer's disease (AD)-associated β -amyloid peptides (A β) are produced by the sequential cleavage of the β -amyloid precursor protein (APP) by β - and γ -secretases. γ -Secretase is a multiprotein complex made of four integral subunits, namely presenilins (PS1 or PS2), nicastrin, A β 1, and PEN2 (Spasic and Annaert, 2008). PS1 and PS2 are synthesized as $\sim$ 43 kDa full-length protein with nine predicted transmembrane domains that undergo endoproteolysis (Thinakaran et al., 1996), generating stable N- and C-terminal fragments (NTF and CTF, respectively) that remain associated with each other (Thinakaran et al., 1998). The

PS1 (or PS2) NTF/CTF assembly is thought to be the catalytic subunit of γ -secretase. The manner in which the highly unstable nascent γ -secretase subunits assemble and mature into stable enzyme complexes is not completely understood. However, it appears that formation of a trimeric complex consisting of PS1 holoprotein, the type I transmembrane protein nicastrin, and the seven transmembrane domain protein A β 1 is an important step that confers stability to these three subunits (LaVoie et al., 2003; Niimura et al., 2005). The two transmembrane protein PEN2 is thought to be important for PS1 endoproteolysis and stabilization of PS1 NTF and CTF (Francis et al., 2002; Kim and Sisodia, 2005). In addition to proteolysis of APP, γ -secretase is responsible for intramembrane proteolysis of a variety of type I membrane proteins (Vetrivel et al., 2006).

Recently, we identified S-palmitoylation of A β 1 and nicastrin within two cytosolic cysteine residues and a transmembrane cysteine residue, respectively (Cheng et al., 2009). S-Palmitoylation refers to the attachment of the 16-carbon lipid palmitate to cysteine residues of proteins by thioester linkage (Linder and Deschenes, 2007). Using several cell lines stably expressing S-palmitoylation-deficient subunits (C/S mutants), we found that S-palmitoylation is essential for the stability and lipid raft association of nascent nicastrin and A β 1 (Cheng et al., 2009).

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Correspondence should be addressed to Gopal Thinakaran, Department of Neurobiology, The University of Chicago, 924 East 57th Street, Knapp R212, Chicago, IL 60637. E-mail: gopal@uchicago.edu.

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Figure 1. Expression of transgenic wild-type and S-palmitoylation-deficient APh1aL and nicastrin in the brain. **A**, Schematic representation of the transgenic constructs. Human APh1aL and nicastrin were expressed under the control of mouse Thy1.2 promoter. Genotyping was performed by PCR of tail DNA using a common Thy1 reverse primer (TR) and three forward primers: one specific for each of the transgenic constructs (APH1aL, AF; nicastrin, NF), and a third specific for the mouse *Thy1* intron 3 (GTF). The panel on the right shows the PCR products amplified from the human (hu) APh1aL and nicastrin transgenes as well as the endogenous mouse (mo) *Thy1* gene in DNA isolated from double-transgenic (Nic/APH tg) and nontransgenic (Non-tg) mice. UTR, Untranslated region. **B**, The distribution of transgene-derived APh1aL and nicastrin. Immunoperoxidase staining was performed using SP718 (nicastrin) and A2tag (APH1aL) on sagittal brain sections from nontransgenic animals (NTG) and transgenic mice expressing wild-type (dWT) or S-palmitoylation-deficient APh1aL and nicastrin (dMut). In the dWT line, transgenes are broadly expressed in cortex (Cx), diencephalon (D), midbrain (Mb), cerebellum (Cb), and brainstem (BS). In the dMut line, transgene expression is high in cortex, midbrain, and brainstem.

However, γ -secretase complexes containing S-palmitoylation-deficient subunits were still able to process APP-CTFs as efficiently as its wild-type counterpart in cultured fibroblasts (Cheng et al., 2009).

S-Palmitoylation is a reversible posttranslational modification implicated in mediating trafficking, raft association, synaptic localization, and function of many neuronal proteins (Huang and El-Husseini, 2005). For example, S-palmitoylation of the scaffolding protein PSD-95 is important for postsynaptic targeting and clustering of glutamate receptors and is required for certain forms of synaptic plasticity (Huang and El-Husseini, 2005). Similarly, γ -secretase S-palmitoylation might be important for its neuronal localization, function, and ability to catalyze specific substrates. We generated and characterized transgenic mice expressing wild-type or S-palmitoylation-deficient APh1aL and nicastrin in the brain. By crossing these animals to mice that coexpresses APP_{Swe} and PS1 Δ E9 (85Dbo) (Jankowsky et al., 2004), we report that overexpression of wild-type or mutant APh1aL and nicastrin stabilizes transgene-derived PS1 Δ E9. We also report lower levels of insoluble A β and significant reduction of amyloid deposits in the frontal cortex of mice expressing S-palmitoylation-deficient subunits, revealing a potential role for γ -secretase S-palmitoylation in the modulation of A β burden in the brain.

Materials and Methods

Generation of transgenic mice. The cDNAs encoding human nicastrin (wild-type or C689S mutant) and human APh1aL (wild-type or C182/245S mutant) (Cheng et al., 2009) followed by a C-terminal tag (SSRGPSSAEVLLLPVS) were subcloned into the Thy-1.2 genomic expression cassette (provided by Dr. P. Caroni, University of Basel, Basel, Switzerland) (Aigner et al., 1995). Linearized and gel-purified wild-type

APH1aL and nicastrin plasmids (for double wild type, referred to as dWT) or C182/245S APh1aL and C689S nicastrin plasmids (for double mutant, referred to as dMut) were coinjected into C57BL/6 embryos, and several lines of transgenic founders were generated. The presence of both APh1aL and nicastrin transgenes in the germ line was determined by PCR analysis of tail DNA (Fig. 1A). The PCR reactions contained four primers: one antisense primer matching the 3' untranslated sequence within the Thy-1.2 vector and the mouse genomic *Thy1* locus (Thy1 reverse, 5'-ATTT-GAAGGACTTGGGGAGGGA-3'), two sense primers specific for the transgene cDNAs (APH1aL forward, 5'-TGTGGTCTGGTTCATCTTGGTC-3'; nicastrin forward, 5'-GC-CAGGATCCAAGTAAACTC-3'), and a third sense primer specific for the mouse *Thy1* gene intron 3 (forward, 5'-TGGAGCCGCTAAGATGAGAAAG-3'). We established five lines of dWT and nine lines of dMut mice in which the two transgenes have coinherited and cosegregate as a single locus. These mice were bred to C57BL/6 $\times$ C3H F1 animals and maintained on this mixed background for our present investigation. Immunoblot and immunohistochemical analyses confirmed the overexpression of APh1aL and nicastrin in the cortex and hippocampus of F2 offspring analyzed at 3 months of age. For the present study, female mice at 6 or 9 months of age were analyzed along with littermate controls.

Immunohistochemistry. Mice were anesthetized by isoflurane inhalation, before perfusion with ice-cold PBS. Brains were cut sagittally in half, one hemisphere was fixed overnight in 4% paraformaldehyde at 4°C and then cryoprotected in PBS containing 30% sucrose. Sagittal sections, 30 μ m, were cut on a cryostat and processed following a free-floating procedure for either enzyme-linked immunoperoxidase or double-immunofluorescence staining as described (Vetrivel et al., 2008). The following antibodies were used for immunohistochemistry: mouse monoclonal anti-A β ₁₋₅ (3D6, 83 ng/ml; Elan Pharmaceutical); polyclonal anti-nicastrin [SP718, 1:2000 (Kodam et al., 2008)]; rabbit polyclonal antibody A2tag (1:4000), which reacts with transgenic human APh1aL, and endogenous mouse APh1 was raised against a synthetic peptide corresponding to the C-terminal 15 aa of APh1aL, followed by residues SSRGPSSAEVLLLPVS; rat monoclonal anti-human PS1 N-terminal antibody (1:500; generously provided by Allan Levey, Emory University, Atlanta, GA) (Lah et al., 1997); monoclonal anti-phosphorylated neurofilament H antibody (1:500, SM131; Sternberger Monoclonals/Covance); monoclonal anti-MAP2 (1:10,000, clone HM-2, ascites) and anti-synaptophysin (1:500, clone SVP-38, ascites) antibodies were both from Sigma; and rabbit polyclonal antibody anti-Giantin (1:5000, PRB-114C; Covance). Alexa Fluor 555-conjugated goat anti-rabbit or donkey anti-mouse antibodies and Alexa Fluor 647-conjugated donkey anti-rabbit (Invitrogen), cyanine 5-conjugated donkey anti-mouse or donkey anti-rat secondary antibodies, and cyanine 3-conjugated donkey anti-rat antibody (Jackson ImmunoResearch) were used as secondary antibodies for immunofluorescence labeling. Images from immunoperoxidase staining were acquired with an Axioplan 2 microscope equipped with an AxioCam (Carl Zeiss). Images from immunofluorescence staining were acquired on a Nikon TE2000 microscope equipped with a Cascade II:512 CCD camera (Photometrics) or on Leica SP2 confocal laser scanning microscope. For confocal microscopy, z-stacks of the regions of interest were acquired and then deconvolved using Huygens software (Scientific Volume Imaging).

Coimmunoprecipitation and Western blotting. Coimmunoprecipitation analysis of PS1 complexes was performed as described previously (Thinakaran et al., 1998; Vetrivel et al., 2008), with few modifications: cortical tissue was lysed on ice in CHAPSO [3-[(3-cholamidopropyl)dimethylammonio]-

2-hydroxy-1-propanesulfonate) buffer [1% CHAPSO, 150 mM NaCl, 50 mM HEPES, pH 7.4, 2 μ M EDTA, and protease inhibitor cocktail (Sigma)] by 15 strokes in a glass/Teflon homogenizer. The lysates were then passed five times through a 25 gauge needle and clarified by centrifugation at $960 \times g$ for 10 min. The resulting pellet was extracted with CHAPSO buffer by the same procedure. Total proteins at 750 μ g from the pooled supernatants were diluted with CHAPSO buffer to 500 μ l and used for coimmunoprecipitation. The lysates were precleared with 40 μ l of protein-A agarose beads for 2 h and then incubated at 4°C overnight with 3 μ l of PS1_{NT} [raised against residues 1–65 of PS1 (Thinakaran et al., 1998)] or unrelated rabbit antiserum as negative control. Immunoprecipitates were collected with 50 μ l of protein-A agarose beads, and bound proteins were analyzed by sequentially blotting with antibodies against each γ -secretase subunit (see below).

Detergent lysates used for Western blot analysis were prepared as described previously (Thinakaran et al., 1996). Fifty to 75 μ g of total proteins were fractionated on 4–20 or 7% Tris-glycine gel (APP full length) or 16.5% Tris-tricine gel (APP-CTFs). The following rabbit polyclonal antibodies were used: PS1_{NT} (described above); α PS1Loop was raised against residues 263–407 of PS1 (Thinakaran et al., 1996); PNT2 was raised against residues 1–26 of PEN2 (Vetrivel et al., 2004); A1tag was generated with the same method as A2tag (see above) (Cheng et al., 2009); CTM1 was raised against a synthetic peptide corresponding to the C-terminal 15 aa of APP, followed by the c-Myc epitope (MEQKLISEEDLN) (Cheng et al., 2009); and anti-Flotillin 2 antiserum was elicited in rabbit by immunization with glutathione S-transferase–human Flotillin 2 fusion protein. Signal intensities were quantified by the Odyssey Infrared Imaging System (Li-COR).

In vitro γ -secretase activity assay. Total membranes were isolated from frontal cortex of 6-month-old dWT or dMut female animals as described previously (Placanica et al., 2009). Briefly, frozen brain tissue were allowed to thaw on ice, minced with a razor blade, and then homogenized with a tissue tearor in ice-cold buffer A (50 mM MES, pH 6.0, 150 mM KCl, 5 mM CaCl₂, 5 mM MgCl₂, 1 mM benzamide, 2.9 μ M leupeptin, 5 μ M antipain, and 0.1 mM PMSF). Nuclear debris was cleared by low-speed centrifugation, and the resulting supernatant was ultracentrifuged at $110,000 \times g$ for 1 h at 4°C. The resulting pellet was resuspended in buffer A and ultracentrifuged again at $110,000 \times g$ for 1 h at 4°C. The final pellet representing the total membrane fraction was resuspended in buffer A.

In vitro γ -secretase activity was quantified using the previously described Sb4 substrate (Shelton et al., 2009; Tian et al., 2010). Brain membranes (4 μ g in 100 μ l reaction) were incubated with buffer B (50 mM PIPES, pH 7.0, 150 mM KCl, 5 mM CaCl₂, 5 mM MgCl₂, and protease inhibitors) with 0.25% CHAPSO (v/v), 1 μ M Sb4 substrate, and 0.1% bovine serum albumin (v/v) in the absence or presence of compound E (1 μ M) or DMSO for 2.5 h at 37°C. The reaction mixture was incubated with antibody G2–10 for the detection of A β ₄₀-site cleavage. Brain γ -secretase activity was measured from two independent membrane preparations ($n = 6$ per genotype), and the results from two independent assays were averaged.

ELISA quantification of A β peptides. Frozen hemibrains were sequentially extracted in a two-step procedure described previously (Levites et al.,

Figure 2. Characterization of neuronal expression of transgenic wild-type and S-palmitoylation-deficient APh1aL and nicastrin. **A, B**, Immunoperoxidase staining was performed on sagittal brain sections from dWT and dMut transgenic mice using SP718 and A2tag antibodies. The expression of APh1aL (**A**) and nicastrin (**B**) in the cerebral cortex (Cx, far left panel), hippocampus (middle left; Sb, subiculum; DG, dentate gyrus), or entorhinal cortex (EC; middle right; H, hippocampus) is depicted. The panel on the far right is a higher magnification of the cerebral cortex. **C**, Confocal microscopy analysis of APh1aL and nicastrin localization in axonal and dendritic processes of cortical neurons. Immunofluorescence staining was performed on sagittal sections, and confocal z-stacks were acquired and processed for deconvolution. Stack arithmetic was applied in MetaMorph using best-focus algorithm to project neuronal processes on one plane. Arrows indicate apical dendrites, and arrowheads indicate thin axonal processes.

2006). Briefly, each hemibrain (150 mg/ml wet weight) was sonicated in 2% SDS with protease inhibitors and centrifuged at $100,000 \times g$ for 1 h at 4°C. After centrifugation, the resultant supernatant was collected, representing the SDS-soluble fraction. The pellet was then extracted in 70% formic acid and centrifuged, and the resultant supernatant was collected as the formic acid extracted fraction. The following monoclonal antibodies against A β were used in the sandwich capture ELISA (Levites et al., 2006): for A β ₄₀, Ab9 capture and Ab40.1–HRP detection; for A β ₄₂, Ab42.2 capture and Ab9–HRP detection.

Quantification of amyloid deposits. For each animal, a series of five brain sections (360 μ m apart) with a starting point close to the inter-hemispheric line was processed for A β immunoperoxidase staining using monoclonal antibody 3D6. Captured images were thresholded to delineate amyloid deposits and quantified (pixel area of deposit relative to total area of region of interest) using Integrated Morphometry Analysis tools in MetaMorph 7.5 Software (Molecular Dynamics).

Results

Characterization of APh1aL and nicastrin transgenes expression

To investigate the importance of γ -secretase S-palmitoylation *in vivo*, we generated transgenic mice expressing either wild-type or

Figure 3. Analysis of transgene expression, association with endogenous γ -secretase subunits and γ -secretase activity. **A**, The levels of γ -secretase subunits in the brains of dWT and dMut mice and nontransgenic littermates (NTG) were assessed by Western blot analysis. **B**, Endogenous PS1 was immunoprecipitated with an antibody against its N-terminal fragment and the coimmunoprecipitation (coIP) of PS1–CTF and γ -secretase subunits were assessed by Western blot. Transgenic APH1aL (tg) migrates slower on gels relative to endogenous APH1aL (end) because of the presence of an epitope tag. Note that nicastrin coimmunoprecipitated with PS1 exhibits retarded migration on gels (relative to the input band), consistent with the incorporation of complex-glycosylated mature (mat) nicastrin into γ -secretase complexes; the majority of overexpressed nicastrin found in the input lysate remains in the immature core-glycosylated form (imm). Ctrl, Control. **C**, *In vitro* γ -secretase activity was measured from CHAPS-solubilized membranes prepared from the frontal cortex of dWT and dMut mice ($n = 6$ per genotype). Enzyme activity is depicted as relative light units per microgram membrane measured after 2.5 h of incubation with 1 μ M substrate at 37°C.

S-palmitoylation-deficient human APH1aL and nicastrin under the control of the *Thy-1.2* promoter, which restricts transgene expression to neurons in the forebrain (Aigner et al., 1995) (Fig. 1A). For convenience and clarity, we will refer hereafter to the transgenic mice coexpressing human wild-type APH1aL and nicastrin as dWT mice and the ones expressing S-palmitoylation-deficient mutant proteins as dMut mice. Several mouse lines were generated that were all viable and fertile. We performed an initial screening of APH1aL and nicastrin expression in several dWT and dMut transgenic lines by immunoblot and immunohistochemistry and, for the present study, chose to use dWT (line 1) and dMut (line 47), which revealed the closest and therefore the most comparable levels and patterns of expression of APH1aL and nicastrin transgenes in the forebrain. Specifically, these two lines show robust expression of APH1aL and nicastrin throughout the cortex and especially high levels in layer 5 (Figs. 1B, 2A, B, left panels). In the hippocampus however, both lines show robust expression mainly in the subiculum (Fig. 2A, B, middle left panels). Lower levels of expression were also detected in the CA1 region of the pyramidal cell layer. Outside the cortex and hippocampus, which are the regions of prime interest for the characterization of A β deposition in this study, dWT and dMut mice displayed variable expression of APH1aL and nicastrin (Fig. 1B). dWT mice had strong expression of both transgenes in multiple nuclei in the diencephalon, midbrain, and brainstem (Fig. 1B). In contrast, dMut mice did not show expression in the diencephalon but had also several nuclei labeled in the midbrain and brainstem. We also noticed that neurons in the entorhinal cortex of dMut mice were labeled (Fig. 2A, B, middle right panels). APH1aL and nicastrin immunolabeling in the cell body and processes of neurons in the cortex appear very similar between dWT and dMut mice (Fig. 2A, B, far right panels). Indeed, confocal microscopy analysis reveals the localization of wild-type and S-palmitoylation-deficient transgenic APH1aL and nicastrin in both axons and dendrites of cortical neurons (Fig. 2C).

We further characterized transgenic expression of APH1aL and nicastrin by Western blot analysis of cortical lysates (Fig. 3A). Transgenic overexpression was observed in both lines, but dWT mice showed higher levels of overexpression of APH1aL and nicastrin in total lysate compared with dMut mice. The lower levels of mutant polypeptides could partially be explained by the lower stability of S-palmitoylation-deficient APH1aL and nicastrin as we reported previously in cultured cell lines (Cheng et al.,

2009). The levels of other endogenous γ -secretase subunits (PS1 NTF/CTF and PEN2) did not appear to be affected by APH1aL and nicastrin overexpression (Fig. 3A).

We performed a series of double-immunofluorescence staining experiments using neuronal markers such as the neuronal-specific nuclear protein NeuN, MAP2, and synaptophysin to assess whether overexpression of S-palmitoylation-deficient APH1aL and nicastrin had any deleterious effects on neurons in the forebrain. dWT and dMut mice did not show apparent signs of neurodegeneration and had similar dendritic and synaptic density in frontal cortex in which the transgenes are particularly expressed at high levels (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Wild-type and S-palmitoylation-deficient APH1aL and nicastrin associate with endogenous PS1 and PEN2 to form γ -secretase complexes with comparable activity

We next performed coimmunoprecipitation studies using PS1_{NT} antibody to examine whether transgenic human γ -secretase subunits successfully assembled with endogenous PS1 and PEN2 to form a protein complex. Transgene-encoded human APH1aL is C-terminally epitope tagged, which allowed us to electrophoretically distinguish it from endogenous APH1 attributable to the retarded mobility on gels. Transgenic wild-type and S-palmitoylation-deficient APH1aL were efficiently coimmunoprecipitated from brain lysates with PS1_{NT} antibody, which was raised against the N terminus of PS1 (Fig. 3B). In addition, endogenous APH1 was also coimmunoprecipitated from dWT and dMut lysates, as efficiently as coimmunoprecipitation from nontransgenic lysates. This observation reflects the fact that transgenic APH1aL is strongly expressed only in a subset of neurons in the cortex, whereas endogenous APH1 and PS1 are expressed in virtually all neurons and glial cells, thus contributing to a large fraction of γ -secretase complexes recovered by coimmunoprecipitation with PS1_{NT} antibody. Reprobing the blots with nicastrin antibody showed comparable levels of coimmunoprecipitated mature glycosylated nicastrin in nontransgenic and transgenic mice. Unlike APH1aL, transgene-derived human and endogenous mouse nicastrin could not be distinguished based on electrophoretic mobility. Finally, similar levels of PS1 CTF and PEN2 were coimmunoprecipitated, despite overexpression of APH1aL and nicastrin. This observation is consistent with data from cultured cell lines, which showed that subunits of PS1 complex are

normally expressed at limiting levels; thus, overexpression of only two subunits (APH1aL and nicastrin) is insufficient to affect the steady-state levels of PS1 and PEN2 (Thinakaran et al., 1997; Kim et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003).

Although based on the coimmunoprecipitation assay, it appears that S-palmitoylation-deficient transgenic APH1aL and nicastrin associate as efficiently as wild-type transgenic subunits with endogenous γ -secretase subunits PS1 and PEN2; formation of the protein complex in itself does not provide any information whether the complex is enzymatically active. To address this point, we measured the γ -secretase activity in CHAPSO-solubilized membranes prepared from the frontal cortex of dWT and dMut mice using a well established *in vitro* γ -secretase assay (Placanica et al., 2009; Shelton et al., 2009; Tian et al., 2010). The results showed that membranes prepared from dMut mice had slightly higher (non-significant) γ -secretase activity compared with dWT mice (dWT, 5734 ± 494.4 and dMut, 6797 ± 951.7 relative light units/ μ g membrane). Together, these results show that, despite the somewhat lower steady-state levels of transgene expression in dMut line as observed by immunohistochemistry, the cortex of dWT and dMut animals have very similar levels of active γ -secretase complexes.

APH1aL and nicastrin overexpression stabilizes PS1 Δ E9

Next, we crossed these mice to the AD mouse model 85Dbo (Jankowsky et al., 2004) to study how lack of γ -secretase S-palmitoylation affects cerebral A β deposition. 85Dbo mice overexpress chimeric mouse/human APP695 (only A β sequence is human) harboring the familial “Swedish” mutation (APP_{Swc}) and human PS1 lacking exon-9 encoded residues (PS1 Δ E9). 85Dbo mice develop amyloid deposits as early as 4 months (Jankowsky et al., 2004) and are widely used to study AD-related pathogenesis.

Mouse PS1 NTF and CTF are replaced by human PS1 Δ E9 in 85Dbo mice as the overexpressed PS1 Δ E9 outnumbers endogenous PS1 for assembly with endogenous APH1, nicastrin, and PEN2 (Fig. 4A). By crossing 85Dbo mice to dWT or dMut that overexpress APH1aL and nicastrin, we have overexpressed three of the four γ -secretase subunits in 85Dbo/dWT and 85Dbo/dMut mice. Current models of γ -secretase assembly agree on the formation of an initial nicastrin/APH1 subcomplex that is stabilized during interaction with PS1 (Spasic and Annaert, 2008). Consistent with this model, Western blot analysis revealed the stabilization of PS1 Δ E9 by the co-overexpression of APH1aL and nicastrin in double-transgenic mice (Fig. 4A). Compared with 85Dbo, the levels of PS1 Δ E9 were $146 \pm 0.9\%$ higher in 85Dbo/dWT mice and $137 \pm 8.1\%$ higher in 85Dbo/dMut mice (Fig. 4A,B). Although 85Dbo/dWT

Figure 4. Co-overexpression of wild-type or S-palmitoylation-deficient APH1aL and nicastrin stabilizes PS1 Δ E9. **A**, Detergent lysates from brains of 6-month-old 85Dbo, 85Dbo/dWT, and 85Dbo/dMut transgenic mice and nontransgenic mice (NTG) were analyzed by immunoblotting using antibodies against each of the γ -secretase subunits and the loading control Flotillin-2. Arrowheads indicate endogenous PS1-NTF and endogenous APH1. **B**, Quantification of PS1 Δ E9 stabilization. The signal intensity of PS1 Δ E9 from multiple animals (normalized to Flotillin 2 signal) was quantified by the Odyssey Infrared Imaging System (Li-COR) and plotted. Note the stabilization of PS1 Δ E9 in 85Dbo/dWT and 85Dbo/dMut mice relative to 85Dbo littermates (Student's *t* test, $*p < 0.05$). **C**, Immunohistochemical analysis of PS1 Δ E9 stabilization. Sagittal brain sections from 6-month-old 85Dbo, 85Dbo/dWT, and 85Dbo/dMut mice were stained using a rat monoclonal antibody that specifically reacts with human PS1. Very little cellular staining for PS1 Δ E9 was detectable in 85Dbo mice when monoclonal anti-human PS1 was applied at higher dilutions. Co-overexpression of wild-type or mutant APH1aL and nicastrin lead to a detectable cellular staining of PS1 Δ E9 in cortex. **D**, Double-immunofluorescence analysis of APH1aL (red) and PS1 Δ E9 (green) coexpression in 9-month-old mice. High levels of PS1 Δ E9 were detected in cells coexpressing transgene-derived APH1aL, whereas only weak cellular staining could be detected in 85Dbo, which expresses only endogenous APH1.

mice and 85Dbo/dMut mice overexpressed three γ -secretase subunits (PS1 Δ E9, APH1aL, and nicastrin), PEN2 is expressed at endogenous levels, similar to nontransgenic animals (Fig. 4A).

We further characterized PS1 Δ E9 stabilization by performing immunohistochemistry using a rat monoclonal antibody that reacts specifically with human PS1 (Lah et al., 1997). At the dilution of the rat monoclonal antibody used in this experiment, cellular staining of PS1 Δ E9 was barely detectable in the cortex of 85Dbo mice (Fig. 4C). However, the antibody labeled numerous cells in the cortex of 85Dbo/dWT and 85Dbo/dMut cells (Fig. 4C), with a pattern reminiscent of APH1aL and nicastrin transgene expression (Fig. 2). To confirm that transgenic PS1 stabilization was indeed related to APH1aL and nicastrin transgene expression, we performed double-immunofluorescence staining for APH1aL and human PS1 (Fig. 4D). As expected, human PS1-labeled cells in the frontal cortex of 85Dbo/dWT or 85Dbo/dMut mice were colabeled by APH1aL. Double-immunofluorescence

Figure 5. Characterization of PS1 Δ E9 localization in the molecular layer of the dentate gyrus in 85Dbo/dMut mice. **A**, Immunohistochemical analysis of PS1 Δ E9 expression in sagittal brain sections from 9-month-old mice. PS1 Δ E9 immunoreactivity in entorhinal cortex (EC) neurons and a diffuse staining in the dentate gyrus (DG) are detected in 85Dbo/dMut mice but not in 85Dbo mice. **B**, Double-immunofluorescence analysis of APh1aL (red) and human PS1 (green) reveals coexpression in the neurons in granule cell layer (GCL) and diffuse staining in the molecular layer (ML) in 85Dbo/dMut. Only very weak staining was detected in these regions in 85Dbo mice. **C**, Confocal microscopy analysis of APh1aL (red) and human-PS1 (green) coexpression in the granule cells of 85Dbo/dMut mice. The panels on the side show a neuron with the projecting dendrite at higher magnification. Scale bar, 20 μ m. **D**, Confocal microscopy analysis of PS1 Δ E9 expression in the molecular layer of the dentate gyrus. PS1 Δ E9 staining in 85Dbo/dMut (same as middle row in **E**) is compared with the one in 85Dbo to show its specificity. **E**, Double-immunofluorescence analysis of PS1 Δ E9 (green, left column) and neuronal markers (red, middle column) in 85Dbo/dMut mice. Inset shows higher magnification of the boxed area on the main image. Note that PS1 Δ E9 staining partially overlaps with the dendritic marker MAP2 but not with the axonal marker phosphoneurofilament H or the presynaptic marker synaptophysin.

staining for nicastrin and human PS1 also revealed a similar overlapping expression of both subunits (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

In 85Dbo/dMut mice, anti-human PS1 antibody also labeled a group of neurons in entorhinal cortex (Fig. 5A) in which APh1aL and nicastrin are also expressed (Fig. 2A, B, middle right panels). Interestingly, neurons from entorhinal cortex project to the dentate gyrus of the hippocampus in which a diffuse staining was observed only in 85Dbo/dMut animals by immunohistochemistry (Fig. 5A). To better characterize this diffuse staining, we analyzed double-immunofluorescence staining of APh1aL and human PS1 in the dentate gyrus of 85Dbo/dMut mice (Fig. 5B).

Diffuse staining was observed for APh1aL and human PS1 in the molecular layer together with strong staining of neurons of the granule cell layer in 85Dbo/dMut mice but not in 85Dbo brains, confirming that diffuse human PS1 staining was related to coexpression of APh1aL. By confocal microscopy, coexpression of APh1aL and human PS1 is clearly visible in granule cells and dendritic extensions in the molecular layer (Fig. 5C). Thus, human PS1 diffuse staining in the molecular layer could correspond to dendritic projections from granule cells, axonal projections from the entorhinal cortex, or both. To further clarify this point, we performed double-immunofluorescence staining and confocal microscopy to localize human PS1 and neuronal markers in the dentate gyrus of 85Dbo/dMut mice (Fig. 5E). Human PS1 staining in the molecular layer did not overlap with the axonal marker phosphoneurofilament H or with the presynaptic marker synaptophysin but overlapped partially with the dendritic marker MAP2. Therefore, the diffuse human PS1 staining is unlikely to represent PS1 in the projections that originate from the entorhinal cortex neurons but rather the dendritic extensions from the granule cells. This observation indicates that PS1 Δ E9 stabilized by coexpression of S-palmitoylation-deficient APh1aL and nicastrin in granule cells is transported along dendritic processes.

A β deposition is reduced in mice expressing S-palmitoylation-deficient APh1aL and nicastrin

We performed Western blot analysis of SDS lysates to assess the levels of APP and metabolites in the brain of 85Dbo/dWT and 85Dbo/dMut mice and their 85Dbo littermates. Full-length APP as well as APP-CTFs were present at comparable levels in these animals, without discernable differences in the relative abundance of α CTFs and β CTFs (Fig. 6A) (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). When compared with 85Dbo mice, both 85Dbo/dWT and 85Dbo/dMut animals had slightly higher levels of APP-CTFs, although all three genotypes expressed similar levels of full-length APP. To compare the levels of A β peptides in the brain, we first measured levels of insoluble A β species recovered by formic acid extraction of forebrain tissue from 6-month-old animals using ELISA (Fig. 6B). The levels of formic acid-extracted A β_{40} and A β_{42} levels were both slightly lower in 85Dbo/dMut mice (63.3 ± 9.9 and 171.9 ± 36.8 pmol/g, respectively) compared with 85Dbo/dWT (75.3 ± 11.7 and 207 ± 31.4 pmol/g, respectively); these reductions did not reach statistical significance. However, we observed a significant reduction of amyloid deposition in the frontal cortex of 6-month-old 85Dbo/d

dMut mice compared with 85Dbo or 85Dbo/dWT, as quantified by immunohistochemistry using monoclonal 3D6 antibody staining (Fig. 6C,D). A β load reached up to $1.21 \pm 0.08\%$ of total cortex area in 6-month-old 85Dbo littermates, $1.23 \pm 0.11\%$ in 85Dbo/dWT mice, and only $0.86 \pm 0.10\%$ in 85Dbo/dMut mice. A β load was also significantly reduced in the hippocampus of 6-month-old 85Dbo/dMut mice compared with 85Dbo littermates; 85Dbo/dWT mice also showed a nonsignificant reduction in amyloid deposition relative to 85Dbo littermates [$1.04 \pm 0.08\%$ in 85Dbo, $0.82 \pm 0.10\%$ in 85Dbo/dWT, and $0.73 \pm 0.12\%$ in 85Dbo/dMut] (Fig. 6E,F). Together, these results suggest that S-palmitoylation status of A β 1aL and nicastrin can influence A β deposition in the brain.

PS1 Δ E9 subcellular localization in neurons of the frontal cortex is very similar between 85Dbo/dWT and 85Dbo/dMut mice

As shown in Figure 3C, γ -secretase *in vitro* activity is equivalent in the cortex of dWT and dMut mice. It is therefore unlikely that the reduction of amyloid deposition observed in 85Dbo/dMut mice can be simply attributed to reduced amount of active γ -secretase in the frontal cortex of these animals. We then considered the possibility that lack of S-palmitoylation could affect the subcellular localization of γ -secretase in dMut animals. Indeed, S-palmitoylation is a critical modification that is known to dynamically regulate membrane trafficking and function of neuronal transmembrane protein complexes (AMPA and NMDA receptors) and cytosolic proteins (PSD-95) (El-Husseini et al., 2000; Hayashi et al., 2005, 2009; Lin et al., 2009). We performed double-immunofluorescence staining of PS1 Δ E9 and neuronal markers to gain insights into neuronal localization of γ -secretase in the frontal cortex of 85Dbo and double-transgenic mice. As described above (Fig. 4C,D), PS1 Δ E9 cellular staining is only weakly detectable in the frontal cortex of 85Dbo. Using higher magnification and confocal microscopy, we were able to detect intracellular staining of PS1 Δ E9 in organelles that are not positive for Giantin, an integral Golgi membrane protein (Fig. 7A). In contrast, PS1 Δ E9 staining in neurons of the frontal cortex of 85Dbo/dWT and 85Dbo/dMut mice was detected in the entire soma without particular accumulation in the Golgi or other discernible organelles (Fig. 7A). In addition, PS1 Δ E9 was also detected in neuronal processes in the cortex of 85Dbo/dWT and 85Dbo/dMut mice (Fig. 7B–F). However, there was no marked difference in the localization of PS1 Δ E9 between these mice. PS1 Δ E9 was detected mainly in apical dendrites, characterized by an overlapping staining with MAP2 (Fig. 7B,C). Lateral processes of smaller caliber were also positive for PS1 Δ E9 in both 85Dbo/dWT and 85Dbo/dMut mice (Fig. 7B,D,E). These processes colabeled with the dendritic marker MAP2 (Fig. 7D) but not with the axonal marker phos-

Figure 6. Analysis of amyloid deposition in 85Dbo/dWT and 85Dbo/dMut mice. **A**, Western blot analysis of APP full length (APP FL) and APP-CTFs in 85Dbo, 85Dbo/dWT, and 85Dbo/dMut mice using CTM1 antibody. Flotillin 2 was used as the loading control. A schematic of the different APP-CTFs species detected in the brain samples is indicated below the blot (Buxbaum et al., 1998). **B**, Half of forebrains from 6-month-old animals were extracted with formic acid, and A β peptides were quantified by ELISA. 85Dbo/dMut mice had slightly less (nonsignificant) insoluble (formic acid extractable) A β ₄₀ and A β ₄₂ compared with 85Dbo/dWT mice ($n = 7$ per genotype). **C**, **E**, Sagittal sections from 6-month-old 85Dbo, 85Dbo/dWT, or 85Dbo/dMut mice were processed for 3D6 immunohistochemistry to reveal amyloid peptide deposition. **D**, **F**, Five sagittal sections from each animal sampling a half-brain were used to quantify amyloid deposits. Amyloid deposition was significantly reduced in the frontal cortex (**D**) of 85Dbo/dMut mice compared with 85Dbo/dWT mice or 85Dbo littermates ($n = 3$ – 7 per genotype). In the hippocampus (**F**), both 85Dbo/dWT and 85Dbo/dMut had lower deposition compared with 85Dbo littermates; however, these differences reached statistical significance only between 85Dbo and 85Dbo/dMut mice. Statistical differences were calculated by Student's *t* test, * $p < 0.05$, ** $p < 0.01$.

phoneurofilament H (Fig. 7E). As in the hippocampus, we did not observe any accumulation of PS1 Δ E9 within neurites, in particular in the vicinity of presynaptic terminals labeled by synaptophysin (Fig. 7F). Together, these results indicate that S-palmitoylation of γ -secretase subunits does not appear to regulate steady-state localization of PS1.

Discussion

Recently, we characterized posttranslational S-palmitoylation of γ -secretase in two of its integral subunits, nicastrin and A β 1aL (Cheng et al., 2009). In the present study, we generated transgenic mice overexpressing wild-type or S-palmitoylation-deficient A β 1aL and nicastrin in the brain under the control of Thy1.2 promoter. By crossing them with 85Dbo transgenic mice, we report two major findings. First, our study demonstrates stabilization of overexpressed human PS1 *in vivo* by coexpression of A β 1aL and nicastrin in 85Dbo/dWT and 85Dbo/dMut animals. The levels of endogenous PEN2 remained unchanged, suggesting that stable nicastrin/A β 1aL/PS1 Δ E9 subcomplexes could accumulate in the brain. Second, mice expressing S-palmitoylation-deficient γ -secretase subunits have decreased A β deposition compared with the ones expressing wild-type subunits. These results show that S-palmitoylation of A β 1aL and nicastrin is important for A β accumulation and/or deposition in the brain. Thus, the transgenic mice we have developed represent

Figure 7. Immunolocalization of PS1 Δ E9 neurons of the frontal cortex of 85Dbo, 85Dbo/dWT, and 85Dbo/dMut mice. Double-immunofluorescence analysis of PS1 Δ E9 (green) and neuronal markers (red) was performed on sagittal sections of frontal cortex from 9-month-old mice using confocal scanning microscopy. **A**, In 85Dbo, PS1 Δ E9 staining was concentrated in intracellular organelles that did not overlap with the Golgi marker Giantin. In 85Dbo/dWT and 85Dbo/dMut, PS1 Δ E9 staining filled the entire soma and proximal dendritic area. **B–F**, PS1 Δ E9 has a very similar distribution in 85Dbo/dWT and 85Dbo/dMut mice with prominent staining of the apical dendrite (arrows) and, to a lower extent, of thinner lateral processes (arrowheads). **B**, A z-stack of PS1 Δ E9 staining was processed using the best-focus algorithm in MetaMorph to project neuronal processes on one plane. **C–F**, Single-plane confocal images were used to analyze PS1 Δ E9 colocalization with the indicated neuronal markers. PS1 Δ E9 in apical dendrites colabeled with the dendritic marker MAP2 (**C**). PS1 Δ E9 containing lateral processes also overlapped with MAP2 staining (**D**) but not with the axonal marker phosphoneurofilament H (**E**). PS1 Δ E9 was not present in the proximity of presynaptic sites (synaptophysin; **F**).

useful tools to dissect the details of γ -secretase complex formation *in vivo*, as well as modulation of A β deposition by posttranslational lipid modification of γ -secretase.

The details regarding the sequence of events in the assembly of four integral subunits into mature γ -secretase complexes are only beginning to unravel at present (Spasic and Annaert, 2008). Available evidence supports the formation of an early intermediate subcomplex made of APH1 and nicastrin (LaVoie et al., 2003). The proximal C-terminal region of PS1 holoprotein is then thought to bind to this subcomplex by interacting with the transmembrane segment of nicastrin, to generate a stable trimeric assembly (Luo et al., 2003; Takasugi et al., 2003; Kaether et al., 2004). Although in our 85Dbo/dWT and 85Dbo/dMut transgenic mice expression of APH1aL and nicastrin is controlled by a Thy1.2 promoter and PS1 Δ E9 expression is under the control of a mouse

Prion promoter (PrP) (Jankowsky et al., 2004), immunofluorescence staining clearly demonstrates the coexpression of nicastrin, APH1aL, and PS1 Δ E9 in a substantial number of neurons in the cortex (Fig. 4D) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Consequently, coexpression of nicastrin and APH1aL led to marked stabilization of human PS1 Δ E9 in the brains of 85Dbo/dWT and 85Dbo/dMut mice, as observed by immunoblot and immunohistochemistry (Fig. 4A–C). This stabilization occurs in the absence of discernible compensatory change in the levels of endogenous PEN2. Finally, judging from the similar extent of stabilization of human PS1 Δ E9 in the brains of 85Dbo/dWT and 85Dbo/dMut mice despite the observable differences in the levels of overexpressed APH1aL and nicastrin at steady state, the abundance of PEN2 is very likely limiting in this experimental setting (Thinakaran et al., 1997).

Concomitant with the stabilization of PS1 Δ E9, we note only a minor increase in the levels of mature nicastrin in 85Dbo/dWT and 85Dbo/dMut mice; instead, the majority of overexpressed nicastrin is present in the immature form (Fig. 4A), indicating that stable nicastrin/APH1aL/PS1 Δ E9 subcomplexes did not traffic beyond *cis*-Golgi for complex glycosylation to occur. This observation is consistent with previous studies, which used PEN2 stable RNA interference knockdown cells to demonstrate the presence of PS1/nicastrin/APH1 trimeric complexes in which most of the nicastrin remained in the core-glycosylated, immature form (Capell et al., 2005). It should be also noted that not all immature nicastrin expressed in 85Dbo/dWT and 85Dbo/dMut mice are engaged in trimeric complex formation with APH1 and PS1 Δ E9, because there is a clear difference in the relative abundance of APH1aL and nicastrin when we compare their levels using total brain lysates

from 85Dbo/dWT and 85Dbo/dMut mice (Fig. 4A). As we observed in previous studies using cultured cell lines, S-palmitoylation contributes to nascent polypeptide stability, thus leading to an overall decrease in the steady-state accumulation of (presumably unassembled) S-palmitoylation-deficient APH1aL and nicastrin in dMut mice. Nevertheless, stabilization of PS1 Δ E9 to similar extent in the brains of 85Dbo/dWT and 85Dbo/dMut mice is consistent with our conclusion from cell culture studies that the stability of S-palmitoylation-deficient APH1aL and nicastrin that have assembled into γ -secretase complexes is indistinguishable from that of the assembled wild-type subunits (Cheng et al., 2009).

Endogenous γ -secretase subunits are widely expressed in all cell types including neurons and astrocytes, whereas Thy1.2 promoter-directed expression of human APH1aL and nicastrin is

restricted to a subset of neurons in the brain. Therefore, it is not expected that endogenous A β 1 expression will be completely replaced by epitope-tagged human A β 1aL, which migrates slower on SDS gels. Nevertheless, the results from coimmunoprecipitation experiments demonstrate incorporation of wild-type and S-palmitoylation-deficient human A β 1aL into PS1 complexes (Fig. 3B). Thus, the *in vivo* data presented here and the *in vitro* results in our previous report (Cheng et al., 2009) reveal that the assembly of nicastrin and A β 1 into the γ -secretase complexes does not require S-palmitoylation of either subunit. Moreover, with the availability of excess human PS1 Δ E9, trimeric complex formation could proceed with the incorporation of transgenic or endogenous A β 1 and nicastrin in the subset of neurons in which both Thy1.2 and PrP promoters drive expression of their respective transgenes (Fig. 4) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

When we first characterized S-palmitoylation of nicastrin and A β 1 in cultured cells, we observed similar levels of A β secretion by fibroblasts and N2a neuroblastoma cells stably expressing wild-type or S-palmitoylation-deficient γ -secretase (Cheng et al., 2009). However, neuronal expression of S-palmitoylation-deficient A β 1aL and nicastrin in transgenic mice resulted in a significant decrease in A β deposits in the frontal cortex by 6 months of age compared with transgenic mice expressing the wild-type subunits or 85Dbo littermates. In addition, we found decreased levels of insoluble A β ₄₀ and A β ₄₂ by ELISA. Results of immunoblot and immunostaining analyses clearly show that the levels of PS1, the catalytic subunit of the γ -secretase, are essentially identical between 85Dbo/dWT and 85Dbo/dMut mice. Therefore, the observed difference cannot be attributed to differences in the levels of transgene expression. In agreement, there is no difference in the steady-state levels of α CTFs and β CTFs between 85Dbo/dWT and 85Dbo/dMut mice, which rules out significant differences in the levels of active γ -secretase that is capable of processing APP-CTFs. Finally, *in vitro* γ -secretase assays confirmed that the frontal cortex of dWT and dMut mice have comparable levels of active γ -secretase (Fig. 3C). Thus, if production of A β per se is not different, γ -secretase S-palmitoylation must influence other aspects important for A β accumulation and deposition in the brain. Thus, these results demonstrate that γ -secretase S-palmitoylation is one of the many factors that determine the extent of cerebral A β deposition.

Subunit S-palmitoylation may modulate γ -secretase localization in the brain in response to physiological stimuli, thus influencing where and when A β is produced in neurons and subsequently deposited. Indeed, S-palmitoylation is important for the trafficking and localization of several neuronal proteins, including glutamate receptors. S-Palmitoylation at cytosolic or transmembrane residues modulates Golgi retention as well as surface expression of the NMDA receptor subunits NR2A and NR2B and AMPA receptor subunits GluR1–GluR4 (Hayashi et al., 2005, 2009; Lin et al., 2009). Moreover, the physiological function of dynamic S-palmitoylation is best characterized in the case of PSD-95, a cytosolic scaffolding protein that participates in the postsynaptic clustering of AMPA receptors as well as cell adhesion molecules (Han and Kim, 2008). Postsynaptic targeting of PSD-95 depends on S-palmitoylation status, and glutamate receptor activation stimulates the depalmitoylation of PSD-95 and its subsequent removal from the synapse (Topinka and Brecht, 1998; El-Husseini et al., 2002). In turn, this removal causes AMPA receptors endocytosis and provides a mechanism to regulate synaptic strength. Conversely, blocking synaptic activity stimulates PSD-95 S-palmitoylation and synaptic clustering of

AMPA receptors (Noritake et al., 2009). Similar to glutamate receptors and PSD-95, S-palmitoylation may regulate the subcellular distribution of active γ -secretase in neurons, and neuronal activity might modulate γ -secretase S-palmitoylation and its dynamic localization in membrane microdomains.

Neuronal localization of active γ -secretase and the precise sites of A β production (e.g., axon/dendrites/synapses/endosomes/transport vesicles/cell surface) still remain elusive. In the present study, we observed that overexpressed wild-type and S-palmitoylation-deficient A β 1aL and nicastrin are present in apical dendrites and axons of cortical neurons (Fig. 2C). Somatodendritic distribution of PS1 Δ E9 staining in cortical neurons also appears indistinguishable between 85Dbo/dWT and 85Dbo/dMut mice (Fig. 7). Nevertheless, because S-palmitoylation is a reversible modification that can be modulated by neuronal/synaptic activity, immunofluorescence staining of the entire pool of PS1 Δ E9 (regardless of the palmitoylation status of the γ -secretase complex) on fixed tissue is insufficient to reveal subtle but functionally critical differences. Thus, it still remains to be determined whether potential functions of S-palmitoylation on γ -secretase neuronal localization could affect A β deposition by bringing a small pool of active γ -secretase close to release sites of A β (e.g., synapses). These are important issues we plan to address in our future studies. Because increasing number of reports have shown that neuronal activity modulates A β production and synaptic release (Kamenetz et al., 2003; Cirrito et al., 2005; Marcello et al., 2008), it is equally important to determine whether neuronal activity can affect γ -secretase activity or localization and whether S-palmitoylation has any role in this process. The dWT and dMut mice characterized in this work will be useful experimental models to address these and other important unresolved issues related to the biology and functions of γ -secretase *in vivo*.

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