S-Palmitoylation of γ-Secretase Subunits Nicastrin and APH-1**§

Received for publication, August 18, 2008, and in revised form, November 20, 2008. Published, JBC Papers in Press, November 20, 2008, DOI 10.1074/jbc.M806308200

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Proteolytic processing of amyloid precursor protein (APP) by β- and γ-secretases generates β-amyloid (Aβ) peptides, which accumulate in the brains of individuals affected by Alzheimer disease. Detergent-resistant membrane microdomains (DRM) rich in cholesterol and sphingolipid, termed lipid rafts, have been implicated in Aβ production. Previously, we and others reported that the four integral subunits of the γ-secretase associate with DRM. In this study we investigated the mechanisms underlying DRM association of γ-secretase subunits. We report that in cultured cells and in brain the γ-secretase subunits nicastrin and APH-1 undergo S-palmitoylation, the post-translational covalent attachment of the long chain fatty acid palmitate common in lipid raft-associated proteins. By mutagenesis we show that nicastrin is S-palmitoylated at Cys689, and APH-1 is S-palmitoylated at Cys382 and Cys425. S-Palmitoylation-defective nicastrin and APH-1 form stable γ-secretase complexes when expressed in knock-out fibroblasts lacking wild type subunits, suggesting that S-palmitoylation is not essential for γ-secretase assembly. Nevertheless, fractionation studies show that S-palmitoylation contributes to DRM association of nicastrin and APH-1. Moreover, pulse-chase analyses reveal that S-palmitoylation is important for nascent polypeptide stability of both proteins. Co-expression of S-palmitoylation-deficient nicastrin and APH-1 in cultured cells neither affects Aβ40, Aβ42, and AICD production, nor intramembrane processing of Notch and N-cadherin. Our findings suggest that S-palmitoylation plays a role in stability and raft localization of nicastrin and APH-1, but does not directly modulate γ-secretase processing of APP and other substrates.

Alzheimer disease is the most common among neurodegenerative diseases that cause dementia. This debilitating disorder is pathologically characterized by the cerebral deposition of 39–42 amino acid peptides termed Aβ, which are generated by proteolytic processing of amyloid precursor protein (APP)* by β- and γ-secretases (1, 2). The β-site APP cleavage enzyme 1 cleaves full-length APP within its luminal domain to generate a secreted ectodomain leaving behind a C-terminal fragment (β-CTF). γ-Secretase cleaves β-CTF within the transmembrane domain to release Aβ and APP intracellular C-terminal domain (AICD). γ-Secretase is a multiprotein complex, comprising at least four subunits: presenilins (PS1 and PS2), nicastrin, APH-1, and PEN-2 for its activity (3). PS1 is synthesized as a 42–43-kDa polypeptide and undergoes highly regulated endoproteolytic processing within the large cytoplasmic loop domain connecting putative transmembrane segments 6 and 7 to generate stable N-terminal (NTF) and C-terminal fragments (CTF) by an uncharacterized proteolytic activity (4). This endoproteolytic event has been identified as the activation step in the process of PS1 maturation as it assembles with other γ-secretase subunits (3). Nicastrin is a heavily glycosylated type I membrane protein with a large ectodomain that has been proposed to function in substrate recognition and binding (5), but this putative function has not been confirmed by others (6). APH-1 is a seven-transmembrane protein encoded by two human or three rodent genes that are alternatively spliced (7). Although PS1 (or PS2), nicastrin, APH-1, and PEN-2 are sufficient for γ-secretase processing of APP, a type I membrane protein, termed p23 (also referred to as TMP21), was recently identified as a γ-secretase component that modulates γ-secretase activity and regulates secretory trafficking of APP (8, 9).

A growing number of type I integral membrane proteins has been identified as γ-secretase substrates within the last few years, including Notch1 homologues, Notch ligands, Delta and...

* This work was supported, in whole or in part, by National Institutes of Health Grants AG021495 (to G. T.), AG019070 (to G. T.), NS45150 (to P. C. W.), and P01 NS047308 (to P. C. W.). This work was also supported by grants from the Alzheimer Association (to G. T. and K. S. V.) and American Health Assistance Foundation (to G. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 51–57.

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**The abbreviations used are: APP, amyloid precursor protein; Aβ, β-amyloid; AICD, APP intracellular domain; BFA, brefeldin A; CTF, C-terminal fragment; MEF, mouse embryonic fibroblasts; NCIID, Notch intracellular domain; PS, presenilin(s); NTF, N-terminal fragment; WT, wild-type; DRM, detergent-resistant membrane; HEK, human embryonic kidney; biotin-BMCC, 1-biotinamido-4-(4'-maleimidooctyl-cyclohexane-carboxamido)butane; ELISA, enzyme-linked immunosorbent assay; CHX, cycloheximide; ER, endoplasmic reticulum; mAb, monoclonal antibody; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio)-2-hydroxy-1-propanesulfonic acid.

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Jagged, cell adhesion receptors N- and E-cadherins, low density lipoprotein receptor-related protein, ErbB-4, netrin receptor DCC, and others (10). Mounting evidence suggests that APP processing occurs within cholesterol- and sphingolipid-enriched lipid rafts, which are biochemically defined as detergent-resistant membrane microdomains (DRM) (11, 12). Previously we reported that each of the $\gamma$-secretase subunits localizes in lipid rafts in post-Golgi and endosome membranes enriched in syntaxin 6 (13). Moreover, loss of $\gamma$-secretase activity by gene deletion or exposure to $\gamma$-secretase inhibitors results in the accumulation of APP CTFs in lipid rafts indicating that cleavage of APP CTFs likely occurs in raft microdomains (14). In contrast, CTFs derived from Notch1, Jagged2, N-cadherin, and DCC are processed by $\gamma$-secretase in non-raft membranes (14). The mechanisms underlying association of $\gamma$-secretase subunits with lipid rafts need further clarification to elucidate spatial segregation of amyloidogenic processing of APP in membrane microdomains.

Post-translational S-palmitoylation is increasingly recognized as a potential mechanism for regulating raft association, stability, intracellular trafficking, and function of several cytosolic and transmembrane proteins (15–17). S-palmitoylation refers to the addition of 16-carbon palmitoyl moiety to certain cysteine residues through thioester linkage. Cysteines close to transmembrane domains or membrane-associated domains in non-integral membrane proteins are preferred S-palmitoylation sites, although no conserved motif has been identified (18). Palmitoylation modifies numerous neuronal proteins, including postsynaptic density protein PSD-95 (19), a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (20), nictotinic $\alpha7$ receptors (21), neuronal t-SNAREs SNAP-25, synaptobrevin 2 and synaptogagmin (22, 23), neuronal growth-associated protein GAP-43 (24), protein kinase CLICK-III (CL3)/CaMKI$\gamma$ (25), $\beta$-secretase (26), and Huntingtin (27). Although palmitoylation can occur in vitro without the involvement of an enzyme, a family of palmitoyltransf erase s that specifically catalyze S-palmitoylation has been identified (28, 29).

In this study, we have identified S-palmitoylation of $\gamma$-secretase subunits nicastrin and APH-1, and characterized its role on DRM association, protein stability, and $\gamma$-secretase enzyme activities. We show that nicastrin is S-palmitoylated at Cys$^{689}$, and APH-1 at Cys$^{192}$ and Cys$^{245}$. Mutagenesis of palmitoylation sites results in increased degradation of nascent nicastrin and APH-1 polypeptides and reduced association with DRM. Nevertheless, in cultured cells overexpression of S-palmitoylation-deficient nicastrin and APH-1 does not modulate $\gamma$-secretase processing of APP or other substrates.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—cDNAs encoding nicastrin mutant C689S, and APH-1 mutants C182S, C245S, and C182S/C245S were generated by overlap PCR, and all PCR products were verified by sequencing. Expression plasmids containing PS1, nicastrin, APH-1aL-Myc-His, and PEN-2 were used for transient transfection studies. The cDNAs encoding nicastrin, APH-1aL-Myc-His, and PEN-2 were subcloned into retroviral expression vectors pLHCX, pMXs-puro (30), and pMXs-blasticidin, respectively, and used for the generation of stably transduced pools. APP695Swe, NotchEMV-6Myc (31), and APP C99-6Myc (32) were subcloned into pMXs retrovirus vector and used for transient infection. The plasmid encoding His-Myc-tagged ubiquitin was provided by Dr. Ron Kopito (Stanford University) (33).

**Cell Culture**—NCT$^{-/-}$ and APH-1ac$^{-/-}$ mouse embryonic fibroblasts (MEF), and HEK293 were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. Mouse N2a neuroblastoma cell lines were maintained in the medium above mixed with 1:1 Opti-MEM (Invitrogen). PS1$^{+/-}$/PS2$^{+/-}$ embryonic stem cells were cultured in embryonic stem cell medium supplemented with leukemia inhibitory factor (34). HEK293-based Plat-E packaging cells were transfected with cDNAs cloned in retroviral vectors. 48 h after transfection, media were collected and clarified by centrifugation at 800 × g for 5 min. Target cells were infected by adding virus-containing supernatant in the presence of 4 µl of Polybrene. Stable MEF pools were generated by infection of NCT$^{-/-}$ and APH-1ac$^{-/-}$ MEF with retroviruses encoding nicastrin or APH-1aL, respectively. NCT$^{-/-}$ dWt and dMut pools were generated by sequential infection of NCT$^{-/-}$ MEF with retroviruses encoding WT nicastrin and APH-1 (dWt) or nicastrin C689S and APH-1aL C182S/C245S (dMut), respectively. Similarly, N2aSwe dWt and dMut pools were generated by sequential infection of N2a cell line Swe10, which expresses human APP695 harboring the “Swedish” double mutants (35).

Stably transduced cells were selected as pools in 5 µg/ml puro mycin and/or 400 µg/ml hygromycin. N2a cells overexpressing PS1 and APPSwe were infected with PEN-2 retrovirus and N2aPAP cells were selected as a pool in 5 µg/ml blasticidin. N2aPAP dWt and dMut were generated by sequential transduction with nicastrin and APH-1aL retroviruses as described above.

**Antibodies**—The following antibodies were used in this study: PS1$^{NT}$ and $\alpha$ PS1Loop were raised against residues 1–65 and 263–407 of PS1, respectively (4, 36); SP716 was raised against residues 62–93 of nicastrin (37); PNT2 was raised against residues 1–26 of PEN-2 (13). Rabbit polyclonal antibody CTM1 was raised against a synthetic peptide corresponding to the C-terminal 15 amino acids of APP followed by the c-Myc epitope (MEQKLISEEDLN). Rabbit polyclonal antibody A1tag was raised against a synthetic peptide corresponding to the C-terminal 15 amino acids of APH-1aL followed by residues SRRGPSSAEVLPLLPS. This antibody reacts with mouse and human APH-1. Polyclonal APH-1aL antibody O2C2 was a gift of Drs. Paul Fraser and Peter St. George-Hyslop (The University of Toronto). Monoclonal antibody A5226 (38) specifically reacts with human nicastrin. The following antibodies were purchased from commercial sources: filotillin-2 and N-cadherin from BD Transduction Laboratories, 9E10 from ATCC, and 4G8 from Covance.

**S-Palmitoylation Assays**—Labeling of S-palmitoylated residues was carried out as described previously with some modifications (39). Briefly, transfected HEK293 cells were lysed in 1 ml of lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 3 T. Li and P. C. Wong, manuscript in preparation.

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pH 7.4, 1% Triton X-100, protease inhibitor mixture (Sigma), and 50 mM N-ethylmaleimide. Aliquots of lysates were incubated with specific antibodies to immunoprecipitate each γ-secretase subunit. Immunoprecipitated proteins bound to agarose beads were treated with 1 x hydroxylation (pH 7.4) at room temperature to cleave the thioester bond and expose a reactive cysteine by detaching the palmitic acid. Subsequently, the beads were incubated with 0.2 mM 1-biotinamido-4-(4'-maleimidobenzovesate)-carboxamido)butane (btiotin-BMCC) (pH 6.2) at 4 °C for 2 h to label reactive cysteine residues. Following SDS-PAGE and transfer to polyvinylidene difluoride membranes, the blots were reacted with streptavidin-horseradish peroxidase to detect biotin-BMCC-labeled proteins. For biotin-BMCC labeling of nicastrin, cells were lysed in methylation buffer (20 mM Tris- HCl pH 7.7, 2.5% SDS, 20 mM S-methylmethylsulfonic acid, 1 mM EDTA, and 0.1 mM neocupeine). After incubation at 50 °C for 30 min, proteins were precipitated from free S-methylmethylsulfonic acid by the addition of 2 volumes of cold acetone and centrifuged. The pellets were resuspended in lysis buffer containing 1% SDS, and neutralized by adding Triton X-100 to a final concentration of 5% before immunoprecipitating nicastrin. For acyl-biotin exchange labeling of subunits expressed in brain, the nicastrin and APH-1 were immunoprecipitated from 1-month-old mouse brain homogenates using antibodies SP716 and O2C2, respectively. For [3H]palmitic acid labeling, transfected 1-month-old mouse brain homogenates using antibodies SP716 and O2C2, respectively. For [3H]palmitic acid labeling, transfected HEK293 cells were starved for 30 min in Dulbecco’s modified Eagle’s medium containing 1% dialyzed fetal bovine serum and incubated with 100 μCi/ml of [10, 11-3H]palmitic acid (American Radiolabeled Chemicals) for 6 h. Proteins were immunoprecipitated from detergent lysates, fractionated by SDS-PAGE, and detected by phosphorimage analysis.

**Metabolic Labeling, Immunoprecipitation, and ELISA—**Cells were starved in methionine/cysteine (Met/Cys)-free medium. Conditioned medium from N2aSwe cells were assayed as described previously (40). NCTu cells overexpressing WT or mutant nicastrin and APH-1 in 60-mm dishes were infected with retrovirus encoding APPSwe and harvested 48 h later in buffer A (50 mM HEPES, 150 mM NaCl, 5 mM 1,10-phenanthroline monohydrate, pH 7.4). Cells were homogenized by passing 10 times through a 25-gauge needle and the homogenate was centrifuged at 10,000 x g for 15 min. The pellet containing membrane fraction (P10) was resuspended in 50 μl of buffer A supplemented with protease inhibitor mixture (Sigma). The samples were incubated for 2 h on ice (as negative control), or at 37 °C to induce the release of AICD. Then the samples were centrifuged at 10,000 x g for 15 min and the supernatant containing soluble proteins was collected and analyzed by Western blotting.

**RESULTS**

**Nicastrin and APH-1 Are S-Palmitoylated—**In our search for potential post-translational modifications that might regulate γ-secretase residence in cholesterol-rich membrane microdomains, we turned our attention to the protein S-palmitoylation of γ-secretase subunits. The thioester bond linking 16-carbon saturated fatty acid palmitate to cysteine residues of S-palmitoylated proteins can be cleaved by hydroxylation, leaving a free sulphydryl group, which can be subsequently labeled using a variety of reagents including [3H]N-ethylmaleimide and biotin-BMCC (39). Using this S-palmitoylation-specific acyl-biotin exchange strategy, we show that two of the four γ-secretase subunits, nicastrin and APH-1, can be specifically modified by biotin-BMCC (Fig. 1A). Labeling was successful only when the immunoprecipitates were pretreated with hydroxylation, which demonstrates that these two subunits undergo S-palmitoylation. More importantly, endogenous nicastrin and APH-1 immunoprecipitated from mouse brain can be labeled, indicating that endogenous nicastrin and APH-1 are S-palmitoylated in the brain (Fig. 1B).

We performed site-directed mutagenesis of cytosolic and transmembrane cysteine residues to identify the sites of S-palmitoylation. Nicastrin undergoes S-palmitoylation at a single site, CyS689 (Fig. 1, C and D). In vitro biotin-BMCC labeling of nicastrin is abolished when CyS689 was mutagenized to a serine residue (Fig. 1C). Moreover, WT but not Cys689S mutant nicastrin can be metabolically labeled with [3H]palmitic acid in transfected HEK293 cells (Fig. 1D). This S-palmitoylation site within the transmembrane domain is conserved in Caenorhabditis elegans and vertebrates (Fig. 1F). We also identified S-palmitoylation at two conserved cysteine residues in APH-1 (Fig. 1E). Mutation of either CyS182 or CyS245 reduced the extent of labeling with biotin-BMCC, whereas mutation of both CyS182 and CyS245 completely abolished labeling, identifying these two residues as S-palmitoylation sites. These two palmitoylation sites are conserved in all APH-1 isoforms expressed in mouse and human (Fig. 1F). We were unable to metabolically label APH-1 using [3H]palmitic acid, likely due to the relatively
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**A** Streptavidin-HRP Immunoblot

- + - + NH4OH Nicastrin imm APH-1 Myc PS1 FL NTF PEN-2

**C** Streptavidin-HRP Immunoblot

- + - - Nicastrin + - Nicastrin imm APH-1 Myc PS1 FL NTF PEN-2

**B** Streptavidin-HRP Immunoblot

- + - + NH4OH Nicastrin mat APH-1

**D** 3H-Palmitic acid labeling Immunoblot

- + - + 3H-Palmitic acid Nicastrin mat APH-1 Myc

**E** Streptavidin-HRP Immunoblot

- + - + Nicastrin NH4OH + - Nicastrin mat APH-1 Myc

**F** Nicastrin

- + - + Cys 689 Human Mouse Rat C. elegans APH-1

![ClustalW2 alignment of the nicastrin and APH-1 sequences](Image)

**FIGURE 1. S-Palmitoylation of nicastrin and APH-1.** A, acyl-biotin exchange reaction labeling of γ-secretase subunits. Nicastrin, APH-1, PS1, or PEN-2 were transiently expressed in HEK293 cells and immunoprecipitated using antibodies SP716, 9E10, PS1NT, and PNT2, respectively. Immunoprecipitates were treated or not with hydroxyamine (NH4OH), followed by incubation with biotin-BMCC to label S-palmitoylated cysteine residues. Labelled subunits were visualized by incubating with streptavidin-horseradish peroxidase (HRP). The blots were reprobed with specific antibodies to reveal all immunoprecipitated proteins. B, acyl-biotin exchange reveals palmitoylation of nicastrin and APH-1 in mouse brain. C, mutagenesis identifies Cys689 as the only palmitoylated residue in nicastrin. D, [3H]Palmitic acid labeling of nicastrin. E, [35S]Met/Cys labeling of nicastrin. F, alignment of the nicastrin and APH-1 sequences near the sites of S-palmitoylation was performed using ClustalW2. The transmembrane domain of human nicastrin (amino acids 670–692) is marked by a horizontal line.

low rate of palmitoylation of nascent APH-1 or the turnover of palmitate in this subunit. Together, the above results show that two γ-secretase subunits are S-palmitoylated in vitro and in the brain.

**S-Palmitoylation-deficient Nicastrin and APH-1 Assemble γ-Secretase Complex with Other Subunits**—The biogenesis, maturation, and stability of each of the γ-secretase components is co-regulated (3). Stable expression of WT or S-palmitoylation-deficient nicastrin (C689S) in NCT−/− MEF restored the defects in the accumulation of endoproteolytically derived mature PS1 fragments and PEN-2. Similarly, stable expression of C-terminal tagged WT or S-palmitoylation-deficient APH-1aL (C182S/C245S) in APH1a/c−/− MEF (with targeted disruption of mouse APH1a and APH1c loci) restored the levels of PS1, PEN-2, and nicastrin (Fig. 2A and supplemental Fig. S1). We then generated stable NCT−/− MEF pools stably co-expressing WT human nicastrin and WT human APH-1aL (dWt) or S-palmitoylation-deficient mutants (dMut). As expected, overexpressed nicastrin and APH-1 undergo S-palmitoylation in NCT−/− dWt but not in NCT−/− dMut cells (Fig. 2B). Immunoblot analysis revealed that overexpression of S-palmitoylation-deficient nicastrin and APH-1 in NCT−/− MEF does not affect endoproteolytic processing of PS1 (Fig. 2A), which suggested that S-palmitoylation of these subunits is not required for γ-secretase assembly.

We performed co-immunoprecipitation experiments to provide direct evidence for γ-secretase complex formation. Endogenous APH-1 in WT MEF can be readily co-immunoprecipitated by antibodies raised against PS1 NTF or CTF (Fig. 2C). Parallel co-immunoprecipitation analysis of NCT−/− dWt and dMut cells revealed that similar levels of Myc-tagged human WT or mutant APH-1 (with retarded mobility on gels compared with endogenous APH-1) and small levels of the remaining mouse APH-1 were complexed with endogenous mouse PS1 in these cells (Fig. 2C). Thus, exogenously expressed APH-1 can replace endogenous APH-1 and incorporate into γ-secretase complexes in dWt and dMut pools. Moreover, we found that similar levels of nicastrin, APH-1, PEN-2, and PS1 were obtained for palmitoylation-deficient APH-1 expressed in dMut cells using [35S]Met/Cys labeling. By pulse labeling, we noted relatively lower steady-state levels of [35S]Met/Cys labeling in WT NCT−/− dWt but not in NCT−/− dMut cells (Fig. 2B). Immunoblot analysis revealed that overexpression of S-palmitoylation-deficient nicastrin and APH-1 in NCT−/− MEF restored the levels of endoproteolytically derived mature PS1 fragments and PEN-2. Indeed, when NCT−/− cells expressing WT or C689S mutant nicastrin were treated with CHX, we can readily observe the relative instability of nascent nicastrin and APH-1 (Fig. 3A, see below).

**S-Palmitoylation Contributes to the Stability of Nascent Nicastrin and APH-1**—In several independent MEF pools we noted relatively lower steady-state levels of S-palmitoylation-deficient nicastrin and APH-1 as compared with WT subunits, raising the possibility that S-palmitoylation might regulate the stability of nascent nicastrin and APH-1. Indeed, when NCT−/− cells expressing WT or C689S mutant nicastrin were treated with CHX, we can readily observe the relative instability of palmitoylation-deficient nicastrin (Fig. 3A). Similar results were obtained for palmitoylation-deficient APH-1 expressed in APH1a/c−/− MEF (Fig. 3B). To confirm these findings, we performed pulse-chase experiments in NCT−/− dWt and NCT−/− dMut cells using [35S]Met/Cys labeling. By pulse labeling, we found that the level of synthesis of nicastrin and APH-1 was very similar in dWt and dMut cells. However, S-palmitoylation-
deficient nicastrin and APH-1 polypeptides degraded at a significantly faster rate as compared with WT polypeptides (Fig. 3, C and D). Nevertheless, maturation of nicastrin into a complex glycosylated, slower migrating species was not affected by -palmitoylation deficiency. To formally establish that endogenous immature nicastrin is indeed modified by S-palmitoylation, we carried out experiments in embryonic stem cell lines cultured from PS1 C689S and APH-1 C182S/C245S (that are likely not part of PS1 complex) are unstable as compared with WT subunits overexpressed at similar levels, suggesting one potential function for S-palmitoylation in stabilizing nascent nicastrin and APH-1 polypeptides.

Our observation that S-palmitoylation deficiency results in the degradation of immature but not mature nicastrin raised the possibility that the S-palmitoylation-deficient mutants degrade before they exit the ER. To test this idea, we used the fungal metabolite brefeldin A (BFA), which perturbs membrane traffic at the ER-Golgi intersection by inducing Golgi disassembly. NCT−/− dWt and NCT−/− dMut cells were treated with BFA, and then analyzed by pulse-chase labeling in the presence of BFA. Lack of S-palmitoylation significantly increased the degradation of both nicastrin and APH-1 during the 8-h chase period even in the presence of BFA (Fig. 3E). To investigate whether the proteasome pathway is involved in the enhanced degradation of S-palmitoylation-deficient nicastrin and APH-1 in the ER, NCT−/− dWt and NCT−/− dMut cells were treated with classic proteasome inhibitors. The steady-state levels of mutant APH-1 were markedly increased following 8 h treatment with lactacystin or MG132, whereas mutant immature nicastrin levels were only slightly increased (supplemental Fig. S4A). As expected, treatment of dWt and dMut with the lysosome inhibitor chloroquine exerted no effect on the steady-state levels of nicastrin and APH-1 mutants. Collectively, these data suggest that S-palmitoylation deficiency results in enhanced degradation of a subset of nascent APH-1 and nicastrin through the proteasome pathway.
The impaired stability of nicastrin and APH-1 mutants led us to investigate whether S-palmitoylation deficiency increases ubiquitination of nicastrin and APH-1. To test this idea, we cotransfected an N-terminal His/Myc epitope-tagged ubiquitin (H₆M-Ub) with nicastrin and APH-1αL (tagged at the C terminus with the sequence SSRGPSSAEVLLLPVS) in HEK293 cells. Cell lysates were immunoprecipitated with antibodies against nicastrin or APH-1 and immunoprecipitates were subjected to immunoblotting with 9E10 antibody. The presence of H₆M-Ub on nicastrin and APH-1 was observed as a smear of high molecular weight species in the immunoprecipitates, which was enhanced by treatment of cells with the proteasome inhibitor lactacystin. Moreover, we found considerably greater levels of polyubiquitinated S-palmitoylation-deficient mutants as compared with WT nicastrin and APH-1. When the blots were reprobed, however, the high molecular weight signal was not detectable by antibodies against nicastrin or APH-1, indicating that only a small subset of these polypeptides undergo polyubiquitination (supplemental Fig. S4B). These data indicate that ubiquitin-mediated and ubiquitin-independent proteasome degradation pathways are involved in the degradation of a subset of S-palmitoylation-deficient nicastrin and APH-1.

**S-Palmitoylation Is Required for DRM Association of Nicastrin and APH-1**—S-Palmitoylation is an essential signal for lipid raft association of a large number of soluble and integral membrane proteins (42). Lipid rafts are biochemically defined as detergent-resistant membrane domains that resist extraction with detergents such as Triton X-100 and Lubrol at 4 °C (43). Previously, we and others reported that all four γ-secretase subunits are present in DRM (13). Having established S-palmitoylation of two γ-secretase subunits, we examined the role of S-palmitoylation on DRM localization of γ-secretase subunits. We prepared membrane rafts from dWt and dMut NCT/MIT11002/MEF on the basis of detergent insolubility and low buoyant density on sucrose density gradients, essentially as described (13). Fractions enriched in DRM were identified by the enrichment of lipid raft marker, flotillin-2. We found a significant decrease (∼70%) in the levels of S-palmitoylation-deficient nicastrin and APH-1 in the DRM fractions (Fig. 4, A and B). However, the absence of S-palmitoylation in nicastrin and APH-1 has only a minor effect on DRM localization of endogenous PS1 and PEN-2 (Fig. 4A). Further analysis by PS1 coimmunoprecipitation revealed reduced levels of PS1-bound nicastrin and APH-1 in DRM prepared from NCT⁻/⁻ dMut.
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**FIGURE 4. S-Palmitoylation targets nicastrin and APH-1 to DRM.** A, NCT−/− dWt and dMut MEF were lysed in 0.5% Lubrol WX at 4 °C for 30 min. The lysates were then subjected to flotation centrifugation on discontinuous sucrose density gradients as described previously (13). Twelve 1-ml fractions were collected from the top and the distribution of γ-secretase subunits was determined by fractionating equal volume of fractions 4–12 followed by Western blotting using antibodies SP716, 9E10, PS1NT, PNT2, and flotillin-2. Fractions 4 and 5 represent the interface between 5 and 35% sucrose in the gradient, and are enriched in the lipid raft marker flotillin-2. An asterisk indicates a nonspecific band observed in blots probed with PNT2. B, signal intensities of nicastrin and APH-1 in DRM fractions were quantified and the ratio of DRM (fractions 4 and 5) to total (signal in all fractions) signal was calculated for each experiment. The relative fold difference between dWt and dMut is plotted by normalizing WT ratios to 1. *, p < 0.05; **, p < 0.01. C, PS1 complexes in DRM were analyzed by co-immunoprecipitation using PS1NT antibody followed by immunoblotting using nicastrin and 9E10 antibodies.

MEF (Fig. 4C). Our data demonstrate a requirement for palmitoylation to promote DRM association of nicastrin and APH-1.

**S-Palmitoylation Does Not Regulate Aβ Production—** Next, we examined the effects of S-palmitoylation on γ-secretase processing of APP. First, we investigated the effect of S-palmitoylation on Aβ production in independent NCT−/− MEF pools stably co-expressing WT or S-palmitoylation-deficient nicastrin and APH-1. We transiently overexpressed APPSwe by retroviral infection and examined APP processing by metabolic labeling. A short 15-min pulse labeling with [35S]Met/Cys confirmed that APP synthesis was comparable in each dWt and dMut pool examined (Fig. 5A). Full-length APP and APP CTFs were then immunoprecipitated from the lysates of cells labeled with [35S]Met/Cys for 3 h. We found that the levels of α- and β-CTFs were comparable between NCT−/− dWt and NCT−/− dMut cells (Fig. 5A). As expected, NCT−/− cells transduced with empty vectors accumulated much higher levels of APP CTFs because of the lack of γ-secretase activity (Fig. 5A, lane 1). To directly quantify the levels of Aβ secreted by dWt and dMut pools, we collected media conditioned by these cells and performed sandwich ELISA. The results show that media conditioned by NCT−/− Vector cells contain only negligible amounts of Aβ; the levels of both Aβ40 and Aβ42 were significantly higher in dWt and dMut pools. However, the results of ELISA analyses show no difference in the levels of Aβ40 or Aβ42 in the conditioned media, suggesting that lack of S-palmitoylation of γ-secretase subunits did not affect γ-secretase processing of APP (Fig. 5B).

To confirm the above findings we co-expressed S-palmitoylation-deficient nicastrin and APH-1 in the mouse N2a neuroblastoma cell line Swe10, which overexpresses human APP695 harboring the Swedish double mutation (35). We generated four independent N2aSwe pools each stably co-expressing WT or S-palmitoylation-deficient human nicastrin and APH-1aL. Co-immunoprecipitation analysis revealed that endogenous PS1 is associated with similar levels of nicastrin and APH-1 in dWt and dMut N2aSwe pools (Fig. 6A). Further analysis using a human nicastrin-specific mAb A5226 confirmed that endogenous mouse PS1 complexes with human WT or mutant nicastrin. Similarly, probing of PS1 co-immunoprecipitates with a polyclonal APH-1 antibody revealed that Myc-tagged human WT or mutant APH-1 co-immunoprecipitated with mouse PS1, replacing endogenous APH-1 (Fig. 6B). These results document that exogenously expressed nicastrin and APH-1 associated with endogenous γ-secretase subunits PS1 and PEN-2 in dWt and dMut N2aSwe pools. Moreover, these findings indicate that overexpressed S-palmitoylation-deficient human nicastrin and APH-1aL can replace endogenous WT subunits.

We then performed [35S]Met/Cys labeling experiments to examine APP metabolism and found that APP processing and Aβ production were not affected by S-palmitoylation deficiency in nicastrin and APH-1 (Fig. 6C). Furthermore, ELISA quantification confirmed similar levels of secreted Aβ40 and Aβ42 peptides in the media conditioned by dWt and dMut N2aSwe pools (Fig. 6D). To finally rule out the possibility that substoichiometric expression of the four γ-secretase subunits might potentially cause experimental artifacts in N2aSwe cells, we overexpressed all four γ-secretase subunits and generated N2aAPAP dWt and dMut pools (Fig. 7A). [35S]Met/Cys labeling studies revealed similar levels of APP CTFs and secreted Aβ peptides in control N2aAPAP (overexpressing PS1 and PEN-2) and N2aAPAP dWt and N2aAPAP dMut pools. Incubation of parallel dishes with 10 nM Com-
pound E resulted in the accumulation of APP CTFs to similar levels, and marked (but incomplete) inhibition of Aβ secretion (Fig. 7B). Similarly, transient overexpression of mutant nicastrin and/or APH-1 failed to alter Aβ production in HeLa cells stably overexpressing APPSwe (supplemental Fig. S5). Collectively, these results demonstrate that deficiency in S-palmitoylation of γ-secretase does not affect amyloidogenic processing of APP.

S-Palmitoylation Does Not Modulate e-Cleavage of APP, Notch, and N-cadherin—Next, we asked whether S-palmitoylation is necessary for γ-secretase cleavage of APP CTFs at the e-site, which generates AICD. In these experiments, we infected cells with a C-terminal Myc-tagged APP CTF (APP C99–6Myc) and analyzed AICD generation by Western blotting and pulse-chase labeling. Because of the presence of C-Myc tag, AICD generated by APP C99–6Myc has a longer half-life and is readily detectable in cell lysates. Western blot analysis of NCT/Vec and NCT/dMut MEF revealed comparable levels of AICD in cell lysates at steady-state (Fig. 8A). As expected, NCT−/− cells transduced with empty retrovirus vectors do not generate AICD. We then performed pulse-chase labeling studies to quantify AICD production and found no difference in the extent of APP C99 conversion to AICD between dWt and dMut cells (Fig. 8B). To confirm this finding using full-length APP, we prepared cell membranes from NCT−/−Vec, NCT−/−dWt, and NCT−/−dMut MEF transient overexpression of APPSwe, and performed in vitro AICD assays.

**FIGURE 5.** S-Palmitoylation of nicastrin and APH-1 does not regulate Aβ production in NCT−/− MEF. **A**, analysis of APP metabolism in stably transduced NCT−/− Vec, and three independent pools of dWt or dMut MEF. Cells were infected with APPSwe and labeled for 15 min or 3 h with 125 μCi/ml of [35S]Met/Cys. Full-length APP and APP CTFs were immunoprecipitated from cell lysates using CTM1 antiserum. **B**, secreted Aβ40 and Aβ42 in the media conditioned by NCT−/− MEF expressing APPSwe were quantified using two-site ELISAs and normalized to the levels of secreted sAPPα.

**FIGURE 6.** S-Palmitoylation of nicastrin and APH-1 does not regulate Aβ production in N2a neuroblastoma cells. **A and B**, co-immunoprecipitation (IP) analysis of PS1 complexes. A, independent stable pools of N2aSwe cells overexpressing WT nicastrin and APH-1 (N2aSwe dWt) or palmitoylation-defective nicastrin and APH-1 (N2aSwe dMut) were lysed in 1% CHAPSO buffer and analyzed by co-immunoprecipitation using PS1NT antibody. B, PS1 co-immunoprecipitates were analyzed by blotting with SP716 or A1tag antisera to detect endogenous and exogenous nicastrin and APH-1, respectively. The blots were reprobed with mAb A5226 or 9E10 to detect exogenous nicastrin and APH-1, respectively. C, analysis of APP metabolism. Stably transduced N2aSwe pools were labeled with [35S]Met/Cys for 15 min or 3 h. Full-length APP and APP CTFs were immunoprecipitated from cell lysates with antibody CTM1. Aβ and p3 were immunoprecipitated from conditioned medium using mAb 4G8. D, secreted Aβ40 and Aβ42 were quantified from the conditioned media and normalized to the levels of sAPPα.
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using established methods by incubating cell membranes prepared from these cells at 37 °C for 2 h to allow AICD generation (40). After centrifugation of the membranes, AICD released into supernatants were analyzed by Western blotting. We found that membranes from NCT−/− dWt and NCT−/− dMut MEF generate similar levels of AICD when incubated at 37 °C. As expected, membranes from cells NCT−/− Vector and NCT−/− dWt MEF reactions that were kept on ice failed to generate AICD (Fig. 8C). We also examined AICD production in intact cells. Overexpression of all γ-secretase subunits increased the levels of AICD in the lysates of N2aPAP dWt cells, as previously described (44). Consistent with the data from in vitro studies described above, we found similar levels of AICD were in lysates of N2aPAP dWt and dMut cells (Fig. 7B). Collectively, these results suggested that S-palmitoylation does not regulate e-site cleavage of APP.

In addition to APP processing, γ-secretase cleavage of Notch receptor at the “e-site” releases the Notch intracellular domain (NICD) (45–47). We examined whether S-palmitoylation of nicastrin and APH-1 affects Notch processing by γ-secretase, using the well-characterized substrate NotchΔEMV-6Myc (31). Stable NCT−/− MEF pools were infected with NotchΔEMV-6Myc and analyzed by immunoblotting. As expected, the NCT−/− Vector pool does not generate NICD (Fig. 8D, lane 1). Compared with the NCT−/− dWt and dMut cells processed NotchΔEMV-6Myc precursor to NICD with similar efficiency (Fig. 8D). We then pulse-labeled cells with [35S]Met/Cys for 15 min and chased the cells for 60 min to quantify NICD generation, and found that NCT−/− dWt and dMut cells processed NotchΔEMV-6Myc precursor to NICD with similar efficiency (Fig. 8E). Finally, we assessed γ-secretase-mediated e-cleavage of 40-kDa N-cadherin CTFl, which results in the production of a soluble intracellular

FIGURE 7. Overexpression of S-palmitoylation-defective γ-secretase. A, N2a cells stably overexpressing human WT PS1, APPSwe, and PEN-2 (PAP) were used to derive vector (Vec) or dWt and dMut pools (overexpressing nicastrin and APH-1). Expression of each γ-secretase subunit was analyzed by immunoblotting using antibodies SP716, 9E10, PS1NT, and PNT2. B, PAP dWt and dMut cells were metabolically labeled with 125 μCi/ml of [35S]Met/Cys for 3 h and analyzed as above. Parallel dishes were pretreated with 10 nM CompE overnight and labeled in the presence of CompE. Note that due to the overexpression of γ-secretase, treatment with 10 nM CompE resulted in partial inhibition of AICD production, despite the complete inhibition of AICD production and marked accumulation of APP CTFs.

FIGURE 8. S-Palmitoylation of nicastrin and APH-1 is not required for e-cleavage. A, the indicated NCT−/− pools were transiently infected with APP C99-6Myc and AICD generation was analyzed by immunoblotting with mAb 9E10. B, cells infected with C99-6Myc were pulse-labeled with 125 μCi/ml of [35S]Met/Cys for 15 min and chased for 60 min. C99-6Myc and AICD-6Myc were immunoprecipitated from lysates using mAb 9E10 and the levels of AICD were plotted as percentage of C99-6Myc synthesis. Shown are the mean ± S.E. of three independent experiments. C, membranes were prepared from the indicated NCT−/− MEF transiently infected with APPSwe and incubated at 0 or 37 °C for 2 h to allow AICD generation. Supernatants containing AICD were analyzed by immunoblotting with mAb 9E10. D, NCT−/− pools were transiently infected with NotchΔEMV-6Myc and NICD production was assessed by immunoblotting. E, NICD production was quantified from three independent experiments by pulse-chase labeling as described above for AICD. F, lysates of NCT−/− MEF pools were analyzed by immunoblotting to assess γ-secretase cleavage of endogenous N-cadherin CTFl.
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domain, termed N-cadherin CTF2 (48). In NCT−/− MEF endogenous N-cadherin CTF1 accumulates to high levels due to the absence of functional γ-secretase activity. We found that CTF1 generated from endogenous N-cadherin does not accumulate in either NCT−/− dWt or dMut MEF (Fig. 8F), indicating that lack of γ-secretase S-palmitoylation does not affect e-cleavage of N-cadherin. These findings were confirmed by incubating dWt and dMut cells with varying concentrations of L685,458 or CompE, two highly selective γ-secretase inhibitors (supplemental Fig. S7). Thus, we conclude that S-palmitoylation of nicastrin and APH-1 has no effect on e-site cleavage of γ-secretase substrates.

DISCUSSION

Over the past decade γ-secretase has been under greater scrutiny because of its essential role in the production of Aβ peptides and intramembrane proteolysis of numerous other transmembrane substrates. In the present study, we have identified and characterized a novel post-translational modification of two γ-secretase subunits. By direct metabolic labeling and a highly sensitive acyl thiol exchange labeling method (39), we demonstrate that nicastrin and APH-1 undergo S-palmitoylation in cultured cells and in the brain. The single S-palmitoylation site of nicastrin Cys689, is conserved form C. elegans to human, whereas the S-palmitoylated APH-1 residues Cys182 and Cys245 are conserved in all three APH-1 isoforms expressed in mouse and human. By expression of S-palmitoylation-deficient nicastrin and APH-1 in NCT−/− and APH-1ac−/− MEF, respectively, we show that this modification is not essential for γ-secretase complex formation. Nevertheless, S-palmitoylation contributes to nascent polypeptide stability and DRM association of nicastrin and APH-1. By detailed examination of MEF and N2a cells stably co-expressing S-palmitoylation-deficient nicastrin and APH-1, we rule out a functional role of S-palmitoylation in γ-secretase processing of APP, Notch, and N-cadherin. Based on these findings, we conclude that S-palmitoylation plays a role in stability and raft localization of nicastrin and APH-1, but does not directly modulate γ-secretase processing of substrates.

Several functions have been ascribed to protein S-palmitoylation. In many cases, the presence of palmitate on proteins affects intracellular trafficking, association with cholesterol-rich membrane rafts, protein-protein interaction, and signaling functions (42). We used NCT−/− and APH-1ac−/− MEF to assess the role of S-palmitoylation in the formation of stable multiprotein enzyme complexes. Lack of either nicastrin or APH-1 destabilizes the remaining γ-secretase subunits resulting in marked diminution of their steady-state levels (reviewed in Ref. 10). Re-expression of WT or S-palmitoylation-deficient nicastrin and APH-1 in the respective knock-out MEF restored this defect. Data from co-immunoprecipitation analysis using an antibody against PS1 demonstrates that deficiency in S-palmitoylation does not affect γ-secretase subunit interactions that lead to the formation of stable complexes. Furthermore, when overexpressed in MEF and N2a cells S-palmitoylation-deficient human nicastrin and APH-1al assembled with endogenous PS1 and PEN-2 to form γ-secretase complexes, thus acting as dominant mutants by replacing endogenous WT mouse nicastrin and APH-1 (Figs. 2 and 6).

Post-translational S-palmitoylation appears to confer stability to the target protein in many cases (reviewed in Ref. 17). In agreement, our pulse-chase labeling experiments reveal that S-palmitoylation contributes to the stability of nascent nicastrin and APH-1 polypeptides. One proposed mechanism by which S-palmitoylation alters protein stability involves shielding target proteins from proteasomal degradation, in some cases by blocking their ubiquitination. For example, palmitoylation contributes to the stability of cation-dependent mannose 6-phosphate receptor by masking a sorting signal; palmitoylation-defective mutant receptors are degraded by the proteasome following exit from the Golgi apparatus (49). Similarly, linker for activation of T cells mutant lacking palmitoylation is unstable and susceptible to degradation via the proteasome pathway (50). In the case of S. cerevisiae SNARE Tlg1 lack of palmitoylation results in ubiquitination and mislocalization to the vacuole for degradation (51). Consistent with the above idea, proteasomal inhibition by lactacystin and MG-132 partially stabilized S-palmitoylation-deficient APH-1al and nicastrin, whereas the lysosome inhibitor chloroquine had no protective effect (supplemental Fig. S4A). Interestingly, a lysine residue is located three residues away from the S-palmitoylated cysteine in nicastrin (689CINAK), and lysine residues are also located adjacent to Cys182 and Cys245 in human APH-1 isoforms 1aS, 1b, and 1c (Fig. 1F). In addition, a cluster of three lysine residues located within the second cytosolic loop (92KLLKK) is conserved in all APH-1 isoforms. The precise site(s) of ubiquitination in APH-1 has not been defined. Although these data implicate that a subset of palmitoylation-defective mutants are degraded via the proteasome pathway, experiments performed by co-expressing ubiquitin reveal only a minor role for polyubiquitination as the signal responsible for proteasomal targeting (supplemental Fig. S4B).

S-Palmitoylation of nicastrin can occur in the ER as evidenced by the modification of endogenous immature nicastrin PS1−/−/PS2−/− embryonic stem cells (supplemental Fig. S3). Pulse-chase studies conducted in the presence of BFA strongly suggests that S-palmitoylation-defective mutants are susceptible for rapid degradation in the absence of ER exit and transit through the secretory pathway (Fig. 3). It is therefore tempting to speculate that S-palmitoylation protects nascent nicastrin and APH-1 from ER-associated degradation. Interestingly, in pulse-chase studies mature complex glycosylated WT and S-palmitoylation-defective nicastrin were found to have comparable stability. Because maturation of nicastrin requires interaction with other γ-secretase subunits (reviewed in Ref. 10), the likely scenario is that complex formation with PS1 and PEN-2 stabilizes S-palmitoylation-deficient nicastrin and APH-1. In support of this notion, similar levels of WT or mutant nicastrin and APH-1 can be co-immunoprecipitated from stably transduced fibroblasts and N2a cells using antibodies against PS1. The above data are also in agreement with multiprotein complex formation as a means of escaping ubiquitin-independent “default” proteasome degradation of unstructured nascent polypeptides by the core 20 S proteasome (52, 53). Together, these findings suggest that one important function of S-palmitoylation is to protect nascent nicastrin and APH-1.
from proteasomal degradation until they interact with PS1 and PEN-2, during their assembly into γ-secretase complexes.

S-Palmitoylation has been demonstrated as essential modification for raft localization of numerous cytosolic and transmembrane proteins (25, 54, 55). Our DRM fractionation studies reveal that S-palmitoylation contributes to, but is not absolutely required for, raft association of nicastrin and APH-1 (Fig. 4). Lack of S-palmitoylation in either nicastrin or APH-1 alone does not affect raft association γ-secretase subunits (supplemental Fig. S6). Importantly, in knock-out MEF co-expression of S-palmitoylation-deficient nicastrin and APH-1 does not have a significant effect on DRM association of endogenous PS1 and PEN-2. Based on these findings, we suggest that S-palmitoylation might promote the targeting of the nicastrin-APH-1 subcomplex to lipid rafts. The presence of a stable nicastrin-APH-1 subcomplex has been demonstrated as an early intermediate formed during the assembly of γ-secretase (56, 57). We found that co-immunoprecipitation with antibodies against PS1 still isolates S-palmitoylation-defective nicastrin and APH-1 from lipid raft fractions (Fig. 4). These later findings imply the presence of additional dominant signals or interacting proteins that target the fully assembled γ-secretase complex to rafts. Alternatively, when PS1 and PEN-2 assemble with S-palmitoylation-defective nicastrin and APH-1 subcomplex in non-raft domains, the fully assembled γ-secretase complexes would attract cholesterol and sphingolipids to create de novo raft microdomains around them. Further investigations are necessary to gain more information on γ-secretase association with lipid rafts.

We conducted detailed investigations to determine the functional importance of subunit S-palmitoylation on γ-secretase processing of substrates. NCTN−/− MEF were initially used to readily assess gain of Aβ production upon expression of WT or mutant subunits. N2a neuroblastoma cells were used to accurately quantify Aβ40 and Aβ42 peptides, and to assess APP processing following overexpression of all four integral γ-secretase subunits. Results from metabolic labeling and ELISA analyses show that overexpression of S-palmitoylation-deficient nicastrin and APH-1 in NCTN−/− MEF and N2aSwe does not affect Aβ40 or Aβ42 production. Moreover, S-palmitoylation of nicastrin and APH-1 had no effect on AICD generation in cultured cells as well as in vitro γ-secretase assays performed using purified cell membranes. Thus, our results clearly demonstrate that S-palmitoylation is not required for amyloidogenic processing of APP by γ-secretase in cultured cells. In addition to APP, we also analyzed γ-secretase processing of two additional substrates, Notch and N-cadherin. Here again, we found that lack of S-palmitoylation on nicastrin and APH-1 had no effect on intramembrane proteolysis of Notch and N-cadherin. Taken together, it is evident that S-palmitoylation of nicastrin and APH-1 is not essential for γ- and e-site cleavage of substrates.

How do we reconcile the marked effect of S-palmitoylation on nascent nicastrin and APH-1 polypeptide stability with the observation that neither PS1 complex formation nor γ-secretase activity is impaired by the expression of mutant subunits? We suggest that in the culture systems employed in this investigation, overexpression of nicastrin and APH-1 is sufficient to overcome protein instability associated with lack of S-palmitoylation. Thus, despite nascent polypeptide instability protein overexpression drives the formation of PS1 complexes, allowing us to determine that S-palmitoylation does not directly contribute to substrate recognition or proteolysis. Nevertheless, to fully understand the physiological role of γ-secretase S-palmitoylation, it may be necessary to generate mouse models where the palmitoylation site mutations are introduced into NCSTN and APH1 alleles by knock-in strategy. The roles of S-palmitoylation in γ-secretase trafficking and intramembranous cleavage of APP and other substrates in neurons and transgenic mice remain to be investigated.

Acknowledgments—We thank Dr. Guojun Bu for suggesting the use of BFA. We thank Drs. Yueming Li and Todd Golde for providing the γ-secretase inhibitors L685,458 and CompE, respectively.

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S-Palmitoylation of γ-Secretase