

Conditional Deletion of *Notch1* and *Notch2* Genes in Excitatory Neurons of Postnatal Forebrain Does Not Cause Neurodegeneration or Reduction of Notch mRNAs and Proteins^{*[5]}

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Background: Presenilin is essential for neuronal survival in adult brains. Notch is a key mediator of presenilin function in developing brains.

Results: Deletion of *Notch1/Notch2* in excitatory neurons of the adult cortex does not cause neurodegeneration or reduction of Notch expression.

Conclusion: Notch does not mediate presenilin-dependent survival of adult cortical neurons.

Significance: Notch expression is undetectable in excitatory neurons of the adult cortex.

Activation of Notch signaling requires intramembranous cleavage by γ -secretase to release the intracellular domain. We previously demonstrated that presenilin and nicastrin, components of the γ -secretase complex, are required for neuronal survival in the adult cerebral cortex. Here we investigate whether Notch1 and/or Notch2 are functional targets of presenilin/ γ -secretase in promoting survival of excitatory neurons in the adult cerebral cortex by generating *Notch1*, *Notch2*, and *Notch1/Notch2* conditional knock-out (cKO) mice. Unexpectedly, we did not detect any neuronal degeneration in the adult cerebral cortex of these *Notch* cKO mice up to ~2 years of age, whereas conditional inactivation of presenilin or nicastrin using the same α CaMKII-Cre transgenic mouse caused progressive, striking neuronal loss beginning at 4 months of age. More surprisingly, we failed to detect any reduction of Notch1 and Notch2 mRNAs and proteins in the cerebral cortex of *Notch1* and *Notch2* cKO mice, respectively, even though Cre-mediated genomic deletion of the floxed *Notch1* and *Notch2* exons clearly took place in the cerebral cortex of these cKO mice. Furthermore, introduction of Cre recombinase into primary cortical cultures prepared from postnatal floxed *Notch1/Notch2* pups, where Notch1 and Notch2 are highly expressed, completely eliminated their expression, indicating that the floxed *Notch1* and *Notch2* alleles can be efficiently inactivated in the presence of Cre. Together, these results demonstrate that Notch1 and

Notch2 are not involved in the age-related neurodegeneration caused by loss of presenilin or γ -secretase and suggest that there is no detectable expression of Notch1 and Notch2 in pyramidal neurons of the adult cerebral cortex.

Notch receptors are type I transmembrane proteins and are involved in a variety of cell-fate decisions during development (1). Upon ligand binding, Notch undergoes a proteolytic cleavage at the extracellular juxtamembrane region (site 2) by tumor necrosis factor- α -converting enzyme, a member of a disintegrin and metalloprotease (ADAM) family (2–5). The resulting C-terminal fragment is further cleaved at transmembrane domain site 3 by γ -secretase (6–9), releasing the Notch intracellular domain, which translocates to the nucleus where it binds to CSL³ (CBF1/RBP-J κ /Su(H)/Lag-1) and relieves the transcriptional suppression imposed by CSL. Presenilin (PS) encoded by the *PSEN* genes, two major genes linked with familial Alzheimer disease (AD), is an essential component of the γ -secretase complex and is required for the Notch intracellular domain production (7, 9). Our previous genetic analysis in mice showed that Notch is a key mediator of PS/ γ -secretase function in the developing brain (10–13). In addition, genetic ablation of either *Notch1* or *Notch2* results in phenotypes that resemble *PS1*^{-/-} or *PS2*^{-/-} mice (14–19). In contrast, *Notch3*^{-/-} or *Notch4*^{-/-} mice do not show embryonic lethality (20, 21), suggesting their unessential function in embryonic development.

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[5] This article contains supplemental Fig. 1.

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³ The abbreviations used are: CSL, CBF1/RBP-J κ /Su(H)/Lag-1; PS, presenilin; AD, Alzheimer disease; cKO, conditional knockout; α CaMKII, α -calcium calmodulin-dependent kinase II; DIV, days *in vitro*; GFAP, glial fibrillary acidic protein; CBP, CREB binding protein; CREB, cAMP response element-binding protein; nt, nucleotide(s); APP, amyloid precursor protein; VCP, vasolin containing protein.

In the central nervous system, the Notch signaling pathway is important for neural stem cell maintenance and proper neurogenesis in the embryonic brain (11–13, 22–25) as well as the adult brain (26–29). Interestingly, besides expression in the germinal zone where neural stem cells reside, Notch has been reported to express in terminally differentiated neurons in the adult cerebral cortex (30–32). Furthermore, altered expression of Notch receptors, its ligand Dll1, and effector Hes1 have been implicated in several brain disorders including AD, Down syndrome, and prion disease (33–35). Thus, despite its low expression in mature neurons, Notch may play an important role in the adult brain.

We previously reported that conditional inactivation of PS in excitatory neurons of the cerebral cortex causes progressive memory impairment and age-related neuronal degeneration (36, 37), raising the possibility that loss of PS function may underlie dementia and neurodegeneration in AD (38). Furthermore, similar inactivation of nicastrin, another key component of the γ -secretase complex, also leads to memory impairment and neurodegeneration (39), suggesting that γ -secretase-dependent activity of PS is important for neuronal survival. However, the molecular basis by which PS promotes neuronal survival in the adult brain remains to be elucidated. Although many γ -secretase substrates have been reported, most of them were identified using overexpression systems and cell lines (40, 41). Beyond the well established physiological substrates of γ -secretase, Notch and amyloid precursor protein (APP) (9, 42), the significance of γ -secretase-mediated cleavage of other substrates is often unclear. Based on prior genetic studies which demonstrated that Notch is an important downstream mediator of presenilin function in the developing brain (6, 7, 10, 12, 13, 43, 44), it was widely assumed that Notch is still a functional target of presenilin in the adult brain. In this study, we address this important question directly through the generation of *Notch* conditional knock-out (cKO) mice using the same α CaMKII-Cre transgenic mouse line that was used previously to inactivate presenilin and nicastrin selectively in mature excitatory neurons of the cerebral cortex beginning at the third to fourth postnatal week (36, 39, 45). Because Notch1 and Notch2 are reportedly the only two Notch receptors expressed in neurons of the adult rodent brain (46–48), we generated *Notch1*, *Notch2*, and *Notch1/2* cKO mice to determine whether Notch1 and Notch2 are similarly required for neuronal survival as presenilin and nicastrin. Surprisingly, these three lines of mutant mice do not exhibit any sign of neurodegeneration. In addition, these mutant mice do not show any reduction in the levels of Notch mRNAs and proteins in their cerebral cortices, where Cre-mediated genomic deletion of the floxed *Notch* exons occurred correctly. In contrast to these results, there was no detectable Notch1 and Notch2 proteins in primary cortical cultures derived from homozygous floxed *Notch1/2* neonatal pups infected with a Cre-expressing lentivirus, which we previously showed to infect all primary cultured neurons (49, 50), indicating that the floxed *Notch1* and *Notch2* alleles can be efficiently inactivated in the presence of Cre. Collectively, these results demonstrate that Notch1 and Notch2 are not involved in presenilin-dependent neuronal survival in the adult cerebral cortex and that there is no detectable Notch1 and Notch2

expression in excitatory pyramidal neurons of the hippocampus and the neocortex of the adult brain.

EXPERIMENTAL PROCEDURES

Generation of *Notch* cKO Mice—The generation of floxed *Notch1* (*fNotch1*) and floxed *Notch2* (*fNotch2*) mice (11, 51) and α CaMKII-Cre transgenic mice (45) was described previously. Exon1 and exon3 were floxed for the *Notch1* and *Notch2* genes, respectively. Forebrain-specific *Notch1* cKO mice and littermate control were obtained by crossing *fNotch1/fNotch1* mice with *fNotch1/fNotch1*; α CaMKII-Cre (*Notch1* cKO) mice. Likewise, *Notch2* cKO mice were obtained by crossing *fNotch2/fNotch2* mice to *fNotch2/fNotch2*; α CaMKII-Cre (*Notch2* cKO) mice. *Notch1/Notch2* cDKO mice were obtained by crossing *fNotch1/fNotch1*; *fNotch2/fNotch2* mice with *fNotch1/fNotch1*; *fNotch2/fNotch2*; α CaMKII-Cre (*Notch1/Notch2* cDKO) mice. We used female mice carrying the α CaMKII-Cre transgene for breeding to reduce the number of offspring bearing germ line deletions. All experimental mice were in the C57BL6/J 129 hybrid background, and littermates were used as controls. All procedures relating to animal care and treatment conformed to the Institutional and NIH guidelines.

PCR Genotype—Genomic PCR was performed using the primers for the deleted, the floxed, and the wild-type *Notch* alleles. For the *Notch1* gene, the following primers were used: 5'-ATTGAAAGCACATATGGAGAT-3' (forward primer at ~2600 nt upstream of exon1), 5'-GTATAAGCATGAAGTG-GTCCA-3' (reverse primer at ~2200 nt upstream of exon1), and 5'-CTCAGTTCAAACAAGATACGA-3' (reverse primer at ~1200 nt downstream of exon1). The first and the second primers amplify the wild-type and the floxed *Notch1* alleles, giving rise to PCR products of 400 and 500 bp, respectively. The first and the last primers amplify the deleted *Notch1* allele, yielding a 600-bp PCR product. For the *Notch2* gene, the following primers were used: 5'-AGCACTCAGTTGTGAAG-GAGC-3' (forward primer at ~500nt upstream of exon3), 5'-TGTTAGATACCAGCCTGGGAG-3' (forward primer at ~350 nt downstream of exon3), and 5'-TCCCTTCAAACCTC-TCCAAAGG-3' (reverse primer at ~700 nt downstream of exon3). The first and the last primers amplify the deleted *Notch2* allele and give rise to a 489-bp PCR product, whereas the second and the last primers amplify the wild-type and the floxed *Notch2* alleles, resulting in PCR products of 343 and 383 bp, respectively. PCR products were separated and analyzed in 2% agarose gel.

Primary Cortical Neuronal Cultures—Dissociated cortical neuronal cultures were prepared from newborn *fNotch1/fNotch1*; *fNotch2/fNotch2* pups as described previously (52). Briefly, cerebral cortex was dissected from the brain of neonates. Neurons were dissociated by trypsin treatment (Sigma; 2.5 mg/ml for 10 min at 37 °C), triturated with a siliconized Pasteur pipette, and then plated at 1.5×10^5 cells/cm² onto 12-well plates coated with Matrigel (BD Biosciences). Cultures were maintained in minimal essential medium, 5 g/liter glucose, 0.1 g/liter transferrin (Calbiochem), 0.25 g/liter insulin (Sigma), 0.3 g/liter glutamine, 5% fetal bovine serum (HyClone), 2% B-27 supplement (Invitrogen), and 2–4 μ M cytosine arabinoside (Sigma) at 37 °C in a humidified incubator

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gassed with 95% air and 5% CO₂ until 2–10 days *in vitro* (DIV) for biochemical analyses.

Lentivirus Production and Infection—Production of recombinant lentiviruses is achieved by transfecting HEK293T cells with three plasmids by FuGENE6 (Roche Applied Science). Vesicular Stomatitis Virus glycoprotein (VSVg) and Δ 8.9 are plasmids encoding the envelope and the gag/pol/tat proteins of lentivirus, respectively. pFUGW-EGFP-NLS-Cre and pFUGW-EGFP-NLS were previously described (49, 53). Viruses were harvested 48 h after transfection by collecting the medium from transfected cells and filtrated. Titer of the lentivirus was estimated by measuring the GFP-positive cells with flow cytometry after the infection of diluted lentivirus to HEK293 cells. Cortical neurons at DIV1 were infected with each lentivirus at a 3–4 multiplicity of infection.

Western Blot—The cortices were dissected from brains and homogenized in 1 ml of radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitors and phosphatase inhibitors mixture from Sigma). The protein concentration was measured by BCA assay (Pierce), and the same amount of protein per lane (30 μ g for cortex lysate, 10 μ g for cortical neuronal culture) was separated in NuPAGE Novex 3–8% Tris acetate gel (Invitrogen). Proteins were transferred to nitrocellulose or PDVF membrane, and the membranes were blocked in 5% nonfat milk, Tris-buffered saline (TBS) for 1 h. After that, the membranes were incubated at 4 °C overnight with the primary antibody against Notch1 rabbit polyclonal (used for primary neuronal cultures in Fig. 5 and mouse brains in supplemental Fig. S1; a kind gift of A. Israel), Notch1 rabbit monoclonal (used for mouse brains in Fig. 7; clone D1E11, Cell Signal Technology, #3608), Notch2 rat monoclonal (used for primary neuronal cultures in Fig. 5 and mouse brains in supplemental Fig. S1; clone C651.6DbHN, Developmental Studies Hybridoma Bank University of Iowa), Notch2 rabbit monoclonal (used for mouse brains in Fig. 7; clone D76A6, Cell Signal Technology #5732), Vasolin containing protein (VCP) rabbit polyclonal (Santa Cruz), and α -tubulin mouse monoclonal (Sigma). The membrane was then incubated with IRDye 800CW or IRDye 680-labeled secondary antibodies (LI-COR Bioscience) at room temperature for 1 h. Specific signals were developed by Odyssey Infrared Imaging System (LI-COR Biosciences).

Northern Blot—Total RNAs were isolated with TRI reagent (Sigma) according to manufacture's instruction. For Northern blot, ~20 μ g of total RNA were separated in formaldehyde agarose gels and transferred into nylon membrane (Amersham Biosciences). Hybridization was performed using [α -³²P]dCTP-labeled probes specific for *Notch1* (408-bp coding sequence from exons 6–8), *Notch2* (260-bp coding sequence from exon3), and *GAPDH* (452-bp coding sequence from exons 5–7). Specific signals were detected by autoradiography with Hyperfilm (Amersham Biosciences). Signal intensities were quantified by ImageJ software (NIH). Statistic significance was calculated using Student's *t* test.

Nissl Staining and Stereology—Mice were anesthetized with CO₂ and perfused with phosphate-buffered saline including heparin and procaine. We took out the brain, and hemibrains

were further immersed in 4% paraformaldehyde at 4 °C for 3 h and then processed for paraffin embedding. Serial sagittal sections were collected by microtome at 10 μ m in thickness. Sections from every 40 slides were deparaffinized, dehydrated, and stained with 0.5% Cresyl Violet (Sigma). Brain volumes were measured by BioQuant image analysis software.

Immunohistochemistry—Paraffin-embedded brain sections were deparaffinized, alcohol-dehydrated, and blocked in 5% normal horse serum, TBS for 1 h. Then sections were reacted with primary antibodies against NeuN (1:400, Chemicon) or GFAP (1:500, Sigma) at 4 °C overnight. These slides were then incubated with biotinylated secondary antibody (Vector Laboratories Inc.) at room temperature for 1 h. Specific signals were developed by Vectastain Elite ABC kit and DAB peroxidase substrate (Vector Laboratories, Inc.) and analyzed by BX50 microscope system (Olympus).

Counting Number of Cortical Neurons—The NeuN-stained sections (total number is 6 sections per animal, which spaced 0.4 mm apart each other) were analyzed by an unbiased neuronal counting by the fractionators and optical dissector method and showed the live image on the BioQuant image analysis software, which connected to the Leica DMRB microscope with a CCD camera. Forty optical dissectors were used to count the entire cortex area. Each optical dissector was a 50 \times 50- μ m sampling box. Then the number of neurons can be counted with an indicator of NeuN-positive cells through the 100 \times oil-immersion lens. The total number of neurons should be sum of the bilateral cortex neurons from all the picked slides. The genotype of each section was also blind to the experimenter. The coefficient of error from the counting technique was <0.10. Finally, the average number of neurons was calculated per genotype ($n = 4$ per genotype). Values are reported as the means \pm S.E.

RESULTS

Notch1, Notch2, and Notch1/2 cKO Mice Do Not Exhibit Age-related Neurodegeneration—Prior genetic studies have shown that activation of Notch receptors are presenilin- or γ -secretase-dependent and that Notch receptors are key mediators of presenilin function in cortical development (10–12, 14–19, 54–56). It was, therefore, widely assumed that Notch may still be a target of presenilin in mediating adult brain function and that γ -secretase inhibitors may have unwanted side effects due to its inhibition of Notch function. To address this question, we generated *Notch1*, *Notch2*, and *Notch1/2* cKO mice using the α CaMKII-Cre transgenic mouse, which we used previously to generate *presenilin* and *nicastrin* cKO mice (39, 45). In the presence of the α CaMKII-Cre transgene, Cre recombinase is expressed under the control of the α -calcium calmodulin-dependent kinase II promoter in excitatory neurons of the cerebral cortex beginning at approximately postnatal day 18 (45). Using the same Cre line, we will be able to compare directly the consequence of conditional deletion of *Notch1/2* with the phenotypes of *presenilin* and *nicastrin* cKO mice to determine whether presenilin and γ -secretase promote neuronal survival through the Notch signaling pathway. *Presenilin* and *nicastrin* cKO mice develop age-related neurodegeneration, and by 6–9 months the cerebral cortex shows severe atrophy and dramatic

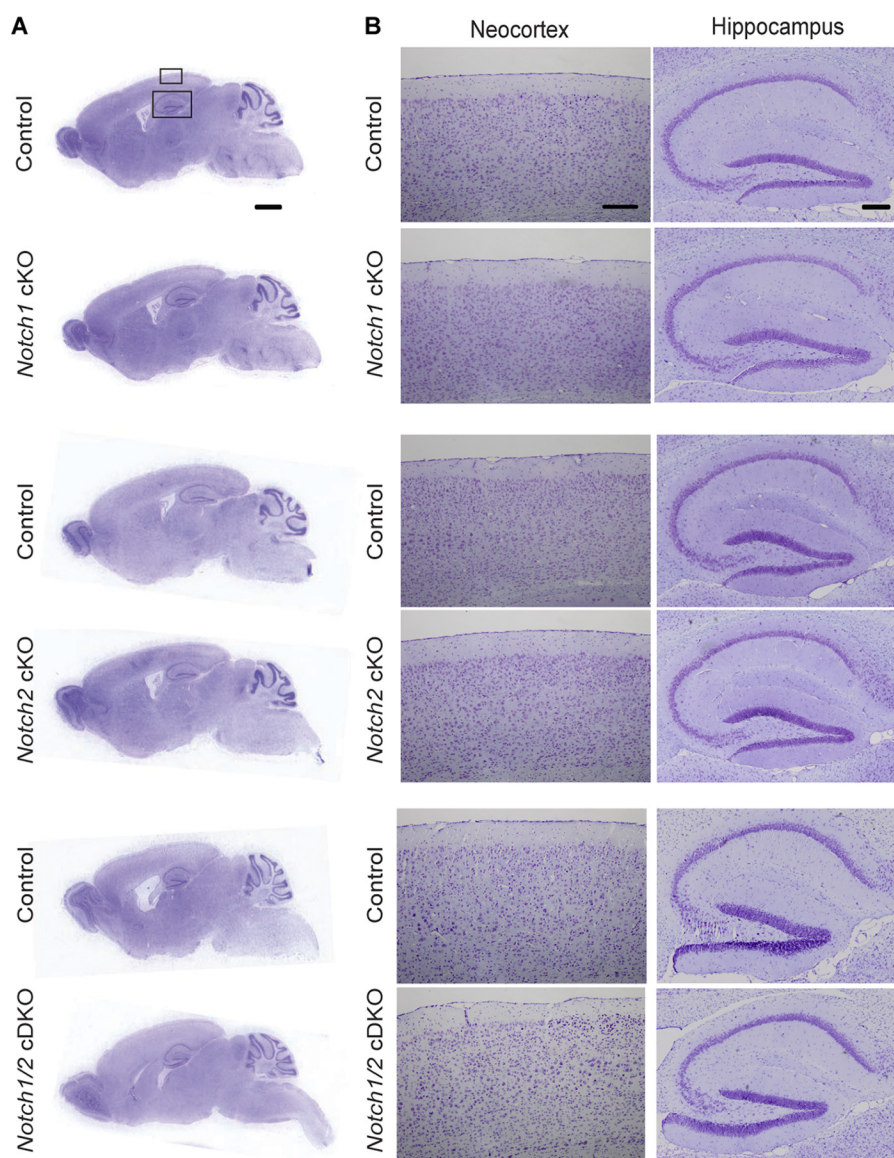


FIGURE 1. **Normal gross morphology of the cerebral cortex of *Notch* cKO mice.** *A* and *B*, shown is Nissl staining of comparable sagittal brain sections (10 μ m, paraffin) of aged *Notch* cKO and littermate control mice (24 months for *Notch1* cKO, 24 months for *Notch2* cKO, 12 months for *Notch1/2* cDKO). Representative images of sagittal brain sections at low magnification (*A*) and the boxed areas of the neocortex and hippocampus at a higher magnification (*B*) are shown. Scale bar, 1 mm (*A*) and 200 μ m (*B*).

loss of cortical volume and neurons (36, 37, 39). Our histological analysis of *Notch1*, *Notch2*, and *Notch1/2* cKO mice, however, did not yield any cortical atrophy (Fig. 1). Nissl staining of paraffin-embedded series sagittal sections showed no gross histological change in brain morphology in aged *Notch1* cKO (24 months), *Notch2* cKO (24 months), and *Notch1/2* cKO (12 months) mice (Fig. 1, *A* and *B*). We then measured cortical volume using stereological methods. Unlike *PS* cDKO mice, which showed progressive loss of cortical volume and neuron number (35% loss of cortical volume and 18% loss of cortical neurons at 6 months of age), the volume of the cerebral cortex was not significantly different ($n = 4$ per genotype) in each of the three *Notch* cKO mice relative to their respective littermate controls (Fig. 2*A*). Furthermore, we performed immunohistochemical analysis using an antibody specific for NeuN, which specifically stains the nucleus of neurons, followed by quantification of the number of NeuN-positive cells using unbiased

stereological methods. Again we found similar numbers of neurons in the neocortex in each of the three *Notch* cKO mice compared with their respective littermate control mice (Fig. 2*B*). These results indicate that conditional deletion of *Notch1* and/or *Notch2* in excitatory pyramidal neurons of the cerebral cortex does not cause loss of cortical neurons or volume during the life span of mice.

Because astrogliosis is often associated with ongoing neurodegeneration (36, 39, 57–64), we further looked for the presence of astrogliosis in these *Notch* cKO mice. We performed immunohistochemical analysis on glial fibrillary acidic protein (GFAP), a marker of astrogliosis, using brain sections of aged *Notch* cKO mice and littermate controls. We did not detect GFAP-immunoreactive astrogliosis in the hippocampus (Fig. 3*A*) and the neocortex (data not shown) in each of the three *Notch* cKO mice even at \sim 24 months of age, whereas we could easily see significant increases of GFAP immunoreactivity in

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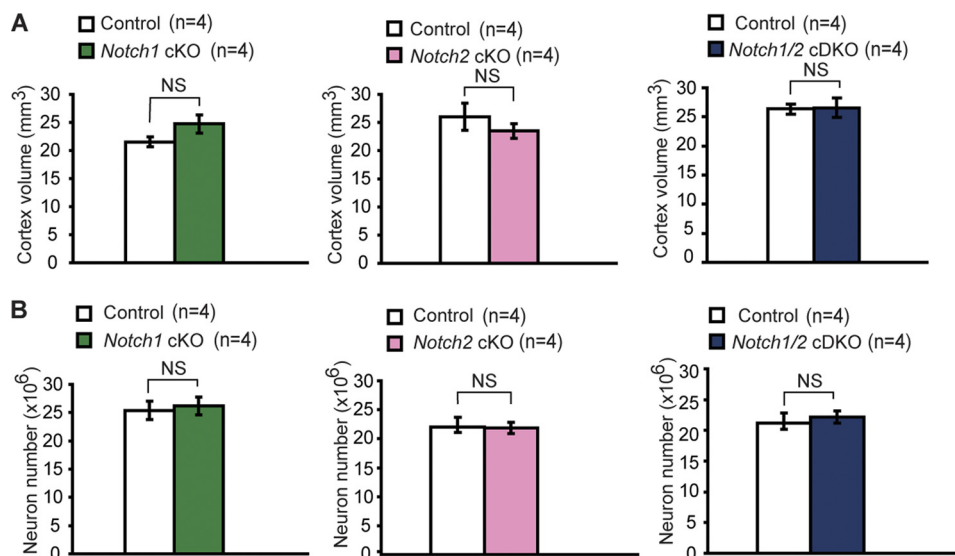


FIGURE 2. **No cerebral atrophy and neuronal loss in the neocortex of aged *Notch* cKO mice.** *A*, shown is stereological measurement of the cortical volume from *Notch* cKO and littermate control brains (16–24 months for *Notch1* cKO, 22–26 months for *Notch2* cKO, 6–12 months for *Notch1/2* cDKO mice). Values are presented per hemisphere. There is no significant (NS) difference in the neocortical volume between *Notch* cKO mice and their respective littermate controls ($n = 4$ per genotype, $p > 0.05$). *B*, shown is stereological quantification of neuronal number in the neocortex using NeuN staining. Similar numbers of cortical neuron are present between *Notch* cKO mice and the littermate control mice ($n = 4$ per genotype, $p > 0.05$). NS, not significant.

the hippocampus of *nicastrin* cKO mice at 9 months of age (Fig. 3A), when prominent neurodegeneration had taken place (39). Western analysis also showed no up-regulation of GFAP in the cerebral cortex of *Notch1* and *Notch2* cKO mice compared with littermate controls at 14–23 months of age (Fig. 3B), whereas 10-fold up-regulation of GFAP was detected in the cortex of *PS* cDKO mice at 6 months of age (58). Together, these results demonstrate the lack of neurodegeneration in *Notch1* and *Notch2* single and double cKO mice.

Floxed *Notch1* and *Notch2* Alleles Are Excised Correctly in Cerebral Cortex of *Notch1* and *Notch2* cKO Mice—The lack of neurodegeneration and other phenotypes in *Notch1* and *Notch2* cKO mice prompted us to examine whether the floxed *Notch1* and *Notch2* alleles (exon1 for *Notch1*, exon3 for *Notch2*) are appropriately excised in the cerebral cortex of these cKO mice. We, therefore, performed PCR using genomic DNA samples purified from the neocortex, the hippocampus, and the tail of *Notch* cKO mice and littermate controls at 2–3 months of age. PCR was performed using 3 primers that can amplify and distinguish all three alleles (floxed, deleted, and wild-type) for *Notch1* and *Notch2*. In the neocortex and the hippocampus, PCR products derived from both the correctly deleted allele (600 bp for *Notch1* and 489 bp for *Notch2*) and the floxed allele (500 bp for *Notch1* and 383 bp for *Notch2*) are present in *Notch1* and *Notch2* cKO mice, respectively, whereas only the floxed allele-derived single product was amplified in their littermate controls (Fig. 4, *A* and *B*). In contrast to genomic DNAs obtained from the cortical samples, single PCR products corresponding to the appropriate floxed allele were detected in the tail DNA of *Notch1* and *Notch2* cKO as well as their respective littermate controls (Fig. 4, *A* and *B*). The presence of the PCR products corresponding to the floxed *Notch1* and *Notch2* alleles in the hippocampus and the neocortex of the *Notch1* and *Notch2* cKO mice, respectively, is due to the selective expression of α CaMKII-*Cre* in excitatory pyramidal neurons (45, 50,

65). Thus, the floxed *Notch* alleles remain intact in cells other than excitatory neurons, such as interneurons, glia, and neural stem cells in the cerebral cortex where genomic DNA was isolated. Indeed we observed a similar proportion of PCR products derived from the deleted and the floxed alleles in the cerebral cortex of *PS* cDKO mice (data not shown), in which we used the same α CaMKII-*Cre* transgenic mice. These results confirm that excision of the floxed *Notch1* and *Notch2* exons occurred correctly in the cerebral cortex of *Notch1* and *Notch2* cKO mice, respectively.

Introduction of *Cre* Recombinase Efficiently Eliminated *Notch1* and *Notch2* Expression in Cultured Cortical Neurons Carrying Floxed *Notch1* and *Notch2* Alleles—Notch proteins are expressed highly in primary neuronal cultures compared with adult brains (30, 31). To determine whether introduction of *Cre* recombinase can effectively eliminate expression of *Notch1* and *Notch2* proteins in cultured cortical neurons bearing the floxed *Notch* alleles, we used a *Cre*-expressing lentivirus, which can efficiently infect all cultured neurons based on our earlier studies (49, 50). We, therefore, developed primary cortical neuronal cultures from neonates carrying homozygous floxed *Notch1* and *Notch2* alleles (*fNotch1/fNotch1;fNotch2/fNotch2*) in which both *Notch1* and *Notch2* can be inactivated upon introduction of the *Cre* recombinase. After one day *in vitro* (DIV1), we introduced a lentivirus carrying the cDNA encoding a functional *Cre* recombinase into the neuronal cultures and continued to culture primary neurons until the designated days. Western analysis was performed on the collected total cell lysates using antibodies specific for the C-terminal region of *Notch1* or *Notch2*. Levels of both full-length *Notch* (~270 kDa) and furin (S1)-cleaved products (~95 kDa) were unchanged over time in cultures infected with the control lentivirus, which carries the cDNA encoding a defective *Cre* recombinase (*Cre*⁻, Fig. 5, *A* and *B*). In contrast, *Notch1* and *Notch2* proteins are decreased in cortical cultures infected with

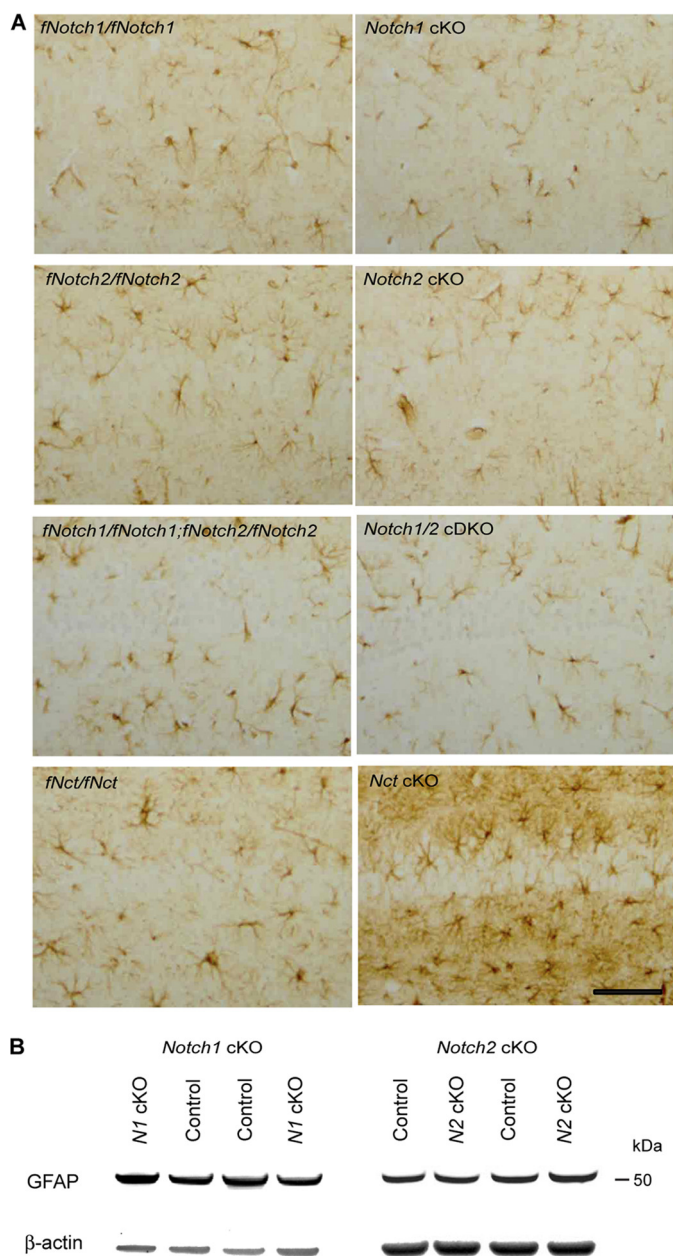


FIGURE 3. No astrogliosis or up-regulation of GFAP in the cerebral cortex of *Notch* cKO mice. *A*, shown is GFAP immunohistochemical analysis of *Notch1* cKO, *Notch2* cKO, and *Notch1/2* cDKO mice using paraffin sections of *Notch* cKO and littermate control mice at 12–26 months old of age. Representative views of hippocampal area CA1 at comparable levels are shown. There is no increase in GFAP immunoreactivity in any of the *Notch* cKO mice compared with the respective controls (*fNotch1/fNotch1* for *Notch1* cKO, *fNotch2/fNotch2* for *Notch2* cKO, *fNotch1/fNotch1;fNotch2/fNotch2* for *Notch1/2* cDKO). GFAP immunostaining of *Nicastrin* (*Nct*) cKO and littermate control (*fNct/fNct*) brains at 9 months of age is also included as the positive control for reactive astrogliosis. Scale bar, 100 μ m. *B*, shown is Western analysis of GFAP in the cerebral cortex of *Notch1* or *Notch2* cKO mice. The level of GFAP expression (50 kDa) is similar in cortical lysates of *Notch1* cKO and littermate control mice at 23 months of age or *Notch2* cKO and littermate control mice at 14 months of age. β -Actin is used as loading control.

a functional Cre lentivirus beginning at DIV2 and are completely absent by DIV4 (*Cre*⁺, Fig. 5, *A* and *B*). The reduction of Notch1 and Notch2 proteins after *Cre* transduction is more rapid than that of PS1 protein in cortical cultures derived from floxed *PS1* neonates, in which PS1 protein is completely eliminated by DIV7 (49). This difference could be due to the easier

accessibility of Cre recombinase to the *Notch* loci (66) or shorter half-life of Notch mRNAs and proteins (67–70).

Levels of Notch1 and Notch2 mRNAs and Proteins Are Unchanged in Cerebral Cortex of Notch1 and Notch2 cKO Mice—The genomic PCR and primary neuronal culture experiments confirmed that the floxed *Notch* alleles are deleted in the cerebral cortex of cKO mice and that inactivation of Notch is complete in cultured cortical neurons where Cre recombinase is efficiently introduced by lentivirus. We examined further whether Notch mRNA and protein expression are reduced in the cerebral cortex of *Notch* cKO mice similarly as in *PS1* cKO and *nicastrin* cKO mice carrying the same α CaMKII-*Cre* mice (39, 45). Although excitatory neurons are only a subset of cell types in the cerebral cortex, our previously generated *presenilin*, *nicastrin* and *CBP* cKO mice using this α CaMKII-*Cre* mouse all showed ~50% reduction of mRNAs and proteins in the cerebral cortex (39, 45, 71). Surprisingly, Northern analysis using total RNA isolated from the hippocampus and the neocortex of *Notch* cKO mice and littermate controls at 2 months or later ages showed similar levels of full-length *Notch1* mRNAs (~9.5 kb) in the neocortex and the hippocampus between *Notch1* cKO mice and littermate controls (Fig. 6*A*). Likewise, levels of *Notch2* mRNAs (~10.5 kb) were unchanged between *Notch2* cKO mice and controls (Fig. 6*B*). Western analysis using antibodies specific for Notch1 showed that levels of Notch1 proteins are much lower in hippocampal and neocortical lysates from 2–3-month-old mice compared with E14.5 embryonic brains when the same amount of total proteins (30 μ g/lane) was analyzed using rabbit monoclonal antibody specific for C-terminal region of Notch1 (Fig. 7*A*). Because Notch proteins are subjected to a furin-mediated cleavage at the S1 site, releasing a C-terminal fragment (~95 kDa), which is further cleaved by ADAM10 upon ligand binding (2–5, 72), we quantified the band of ~95 kDa in the hippocampal and neocortical samples from *Notch1* and *Notch2* cKO mice as well as their respective littermate controls. Consistent with the unchanged *Notch1* mRNA levels in cKO mice, levels of Notch1 proteins are also unchanged in the neocortex and hippocampus between *Notch1* cKO mice and littermate controls, whereas expression of Notch1 was almost completely inactivated in embryonic brains of *Notch1* cKO mice driven by *Nestin-Cre* transgenic mice (Fig. 7*A*), as shown previously (11). The higher molecular weight band (*) appears to be nonspecific, as this band is not present in samples from the embryonic brain or cultured neurons (Figs. 5*A* and 7*A*). In addition, the predicted molecular mass of the S1-cleaved Notch weight of the C-terminal fragment is ~95 kDa (based on 877 amino acid residues in mouse Notch1). Western analysis using rabbit monoclonal antibodies specific for the C-terminal region of Notch2 also showed that levels of Notch2 proteins are lower in the adult brain (30 μ g/lane) compared with cortical neuronal cultures (10 μ g per lane) (Fig. 7*B*). Similar to Notch1, the predominant species detected by Western is the furin-cleaved C-terminal fragment (~95 kDa). Consistent with the unchanged *Notch2* mRNA levels in cKO mice, levels of Notch2 proteins are also unaltered in the neocortex and the hippocampus of *Notch2* cKO mice relative to littermate controls, whereas expression of

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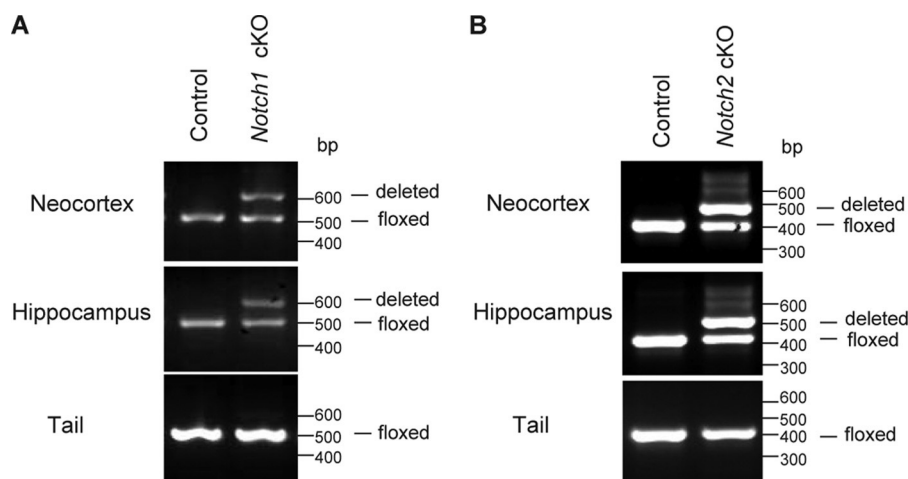


FIGURE 4. Deletion of the floxed *Notch* sequences in the cerebral cortex of *Notch1* and *Notch2* cKO mice. A and B, PCR using genomic DNA isolated from the neocortex and the hippocampus as well as the tail of *Notch1* cKO (A) and *Notch2* cKO (B) mice is shown. PCR was performed using three primers that can amplify and distinguish the floxed, deleted, and wild-type alleles. Only one PCR product (500 bp for the floxed *Notch1* allele, 383 bp for the floxed *Notch2* allele) is produced in the DNA samples from the neocortex, the hippocampus, and the tail of control (*fNotch1/fNotch1* for *Notch1* cKO, *fNotch2/fNotch2* for *Notch2* cKO) mice. In cKO mice, one PCR product (500 bp for the floxed *Notch1* allele, 383 bp for the floxed *Notch2* allele) is produced in the tail DNA sample, whereas two PCR products representing the floxed and the deleted (600 bp for *Notch1*, 489 bp for *Notch2*) alleles are produced in DNA samples from the neocortex and hippocampus, suggesting mosaic cell populations (some carrying the floxed allele, some carrying the deleted allele) in the cortex.

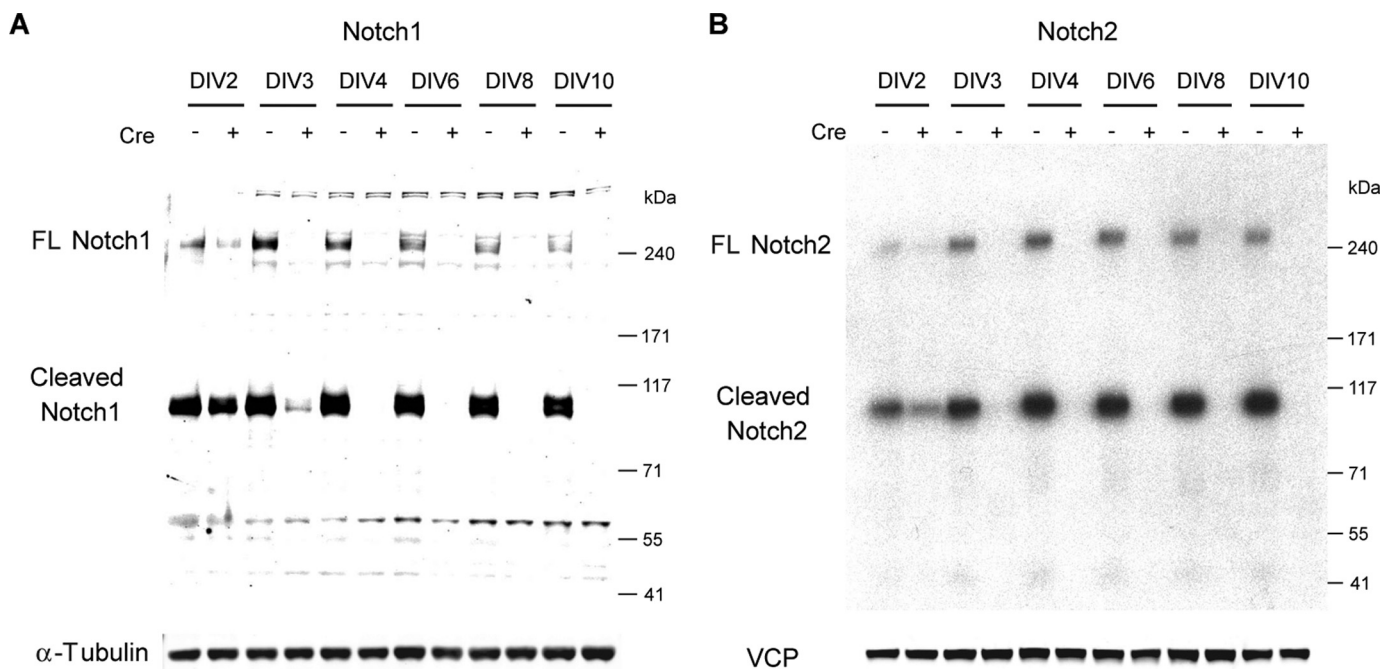


FIGURE 5. Elimination of Notch1 and Notch2 proteins in primary cortical neuronal cultures after Cre transduction. Shown is the time course of Notch1 (A) or Notch2 (B) inactivation in *fNotch1/fNotch1;fNotch2/fNotch2* cortical neuronal cultures after Cre transduction. The lentivirus carrying the cDNA encoding either a GFP/defective Cre (*Cre*⁻) or a GFP/functional Cre fusion protein (*Cre*⁺) was introduced to infect cortical neuronal cultures derived from *fNotch1/fNotch1;fNotch2/fNotch2* neonate pups at DIV1. Total cell lysates were collected at the designated days (DIV2–10) and were analyzed (10 μ g of lysates per lane) by Western blotting using specific antibodies for the Notch1 (rabbit polyclonal, from A. Israel) or the Notch2 (rat monoclonal, clone C651.6DbHN from the Hybridoma Bank, University of Iowa) C-terminal domain. α -Tubulin or VCP (Vasolin containing protein) was used as a loading control. Although most Notch proteins were subjected to S1 cleavage (Cleaved, \sim 95 kDa), full-length (FL) Notch (\sim 270 kDa) could be easily detected in this culture system. In the presence of Cre, inactivation of Notch1 and Notch2 occurs rapidly, and loss of Notch1 and Notch2 proteins is complete by DIV4.

Notch2 was completely absent in *fNotch1/fNotch1;fNotch2/fNotch2* cortical cultures 7 days after Cre transduction (Figs. 5B and 7B). We also used another set of antibodies (a rabbit polyclonal for Notch1, a rat monoclonal for Notch2) to confirm further our results. Again, similar levels of Notch1 or Notch2 proteins were found in *Notch* cKO mice and littermate controls (supplemental Fig. S1). Thus, postnatal deletion of the *Notch* alleles in excitatory neurons of the cerebral cortex does not

affect the amount of Notch proteins detected in the cortex, in contrast to our earlier studies using the same α CaMKII-Cre transgenic mouse to generate *PS1*, *nicastrin*, *CBP* cKO mice, all of which exhibit \sim 50% reduction of mRNAs and proteins in the cerebral cortex of cKO mice (39, 45, 71). Together, these results indicate that Notch1 and Notch2 are either normally not expressed or expressed at undetectable levels in the excitatory neurons of the hippocampus and the neocortex.

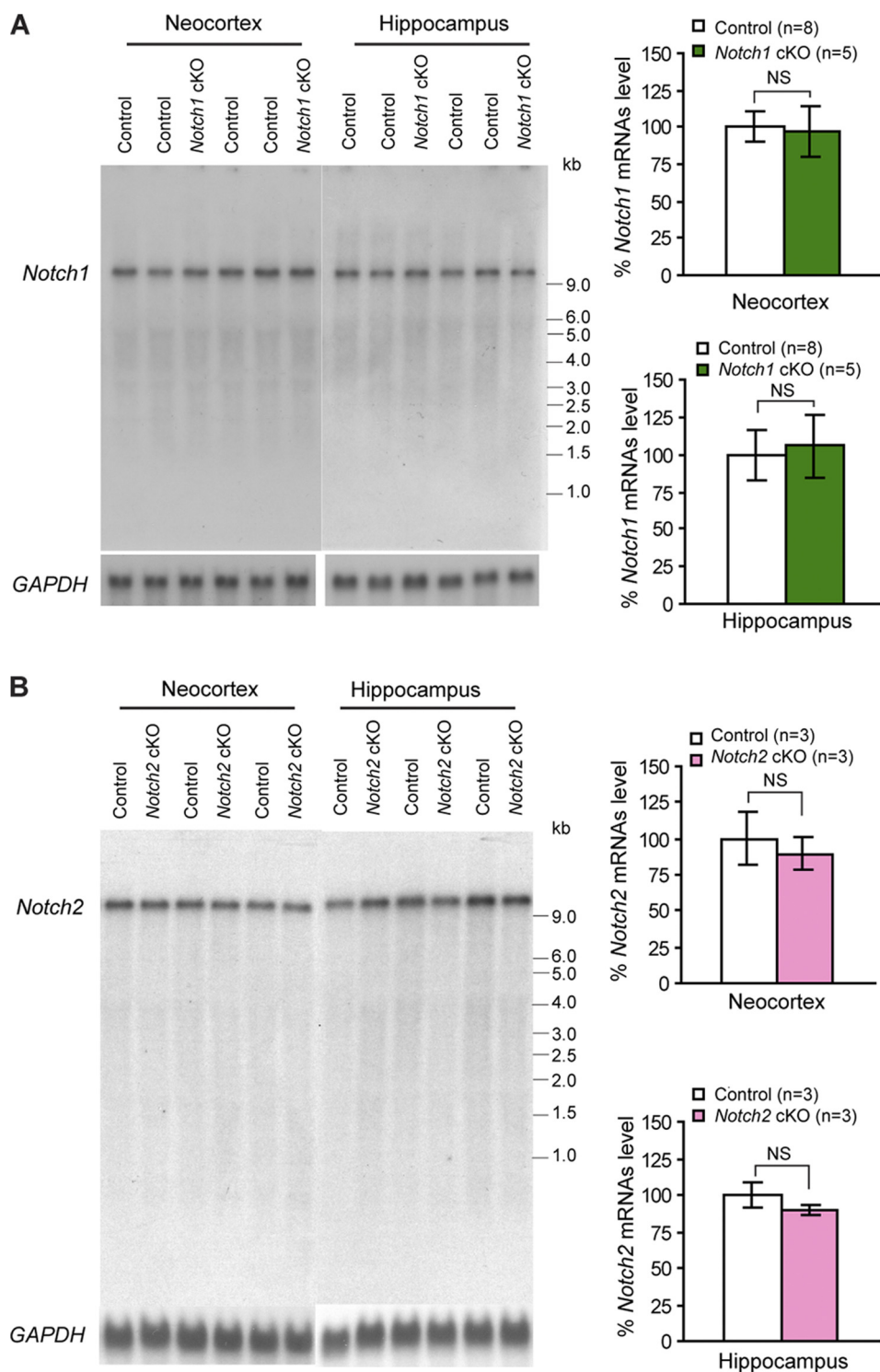


FIGURE 6. Unchanged levels of *Notch1* and *Notch2* mRNAs in the cerebral cortex of *Notch* cKO mice. *A*, Northern blots (left) and quantification (right) of *Notch1* mRNAs in *Notch1* cKO ($n = 5$) and littermate control ($n = 8$) mice show similar levels of *Notch1* mRNAs in total RNA samples isolated from the cortex of cKO and littermate controls. *B*, Northern blots (left) and quantification (right) of *Notch2* mRNAs in *Notch2* cKO ($n = 3$) and littermate control ($n = 3$) mice show similar levels of *Notch2* mRNAs in control and *Notch2* cKO mice. The level of *Notch* mRNAs is normalized to *GAPDH* mRNAs, and the value of littermate controls is set as 100%. All data are expressed as the mean \pm S.E. Statistical analysis was performed using two-tailed unpaired Student's *t* test. NS, not significant.

DISCUSSION

In this study we generated three lines of conditional knock-out mice in which the *Notch1* and/or *Notch2* genes are selectively deleted in excitatory neurons of the postnatal forebrain to investigate whether Notch receptors are crucial for neuronal

survival in the adult brain. Unexpectedly, we did not detect any neuronal degeneration in the adult cerebral cortex of *Notch1*, *Notch2* cKO and *Notch1/Notch2* cDKO mice up to ~ 2 years of age (Figs. 1–3), whereas conditional inactivation of presenilin or nicastrin, each of which is essential for γ -secretase-

mediated Notch activation, using the same α CaMKII-Cre transgenic mouse caused progressive neuronal loss beginning at 4 months of age (36, 37, 39). More surprisingly, we failed to detect any reduction of Notch1 and Notch2 mRNAs (Fig. 6) and proteins (Fig. 7 and supplemental Fig. S1) in the cerebral cortex of *Notch1* and *Notch2* cKO mice, respectively, even though Cre-mediated genomic deletion of the floxed *Notch1* and *Notch2* exons clearly took place in the cerebral cortex of these cKO mice (Fig. 4). Furthermore, introduction of Cre recombinase into primary cortical cultures prepared from the floxed *Notch1/Notch2* mice, where Notch1 and Notch2 are highly expressed, completely eliminated their expression (Fig. 5), indicating that Cre can efficiently delete the floxed *Notch1* and *Notch2* alleles. Based on these findings, we conclude that in the adult cerebral cortex, Notch is not a key mediator of presenilin-dependent neuronal survival and that there is no detectable expression of Notch1 and Notch2 in excitatory neurons of the adult cerebral cortex, where presenilin is highly expressed. Our current study argues against possible functional association between presenilins and Notch in mature pyramidal neurons of the adult cerebral cortex, although before our current study it was widely assumed that Notch1/2 are expressed in these excitatory neurons and could mediate presenilin function during aging.

The failure to identify neurodegenerative phenotypes in our *Notch* cKO models was somewhat unexpected given the striking neurodegenerative phenotypes observed in *presenilin* and *nicastrin* cKO mice (36, 37, 39), the essential requirement of presenilin/ γ -secretase in Notch activation (6–9), and the similarity of developmental phenotypes shared between *presenilin* and *Notch* germ line mutant mice (14–19). These findings highlight the difference in molecular targets regulated by presenilin in the mediation of its function in the developing and the adult brain. Although Notch is an important mediator of presenilin function in the developing brain, where they control the size of neural progenitor and neuronal population through the regulation of cell fate decision and apoptotic cell death (11–13, 22–25), the molecular target through which presenilin promotes neuronal survival in the adult cerebral cortex is entirely unknown. Although many γ -secretase substrates have been reported (40), most of them were identified in overexpression systems and cell lines, so it remains to be determined how many of them are physiological substrates of γ -secretase and which one(s) might be involved in mediating presenilin-dependent neuronal survival. Furthermore, the fact that *presenilin* and *nicastrin* cKO mice share similar phenotypes in memory impairment and neurodegeneration supports a γ -secretase-dependent involvement, but the reduction of presenilin levels in *nicastrin* cKO mice suggested that a γ -secretase-independent mechanism may still be at play (36, 37, 39).

We previously proposed that CBP (CREB-binding protein encoded by the *Crebbp* gene) is a putative downstream target of presenilin-mediated neuronal survival in the adult brain via Notch signaling, based on the reduced mRNA expression of the *Crebbp* gene and the CREB target genes and on the identification of the putative consensus sequences for CSL binding in the *Crebbp* promoter, which suggests that Notch activation should enhance CBP expression (36). However, conditional inactivation of CBP in excitatory neurons of the postnatal forebrain did

not cause neuronal loss in the cerebral cortex of *CBP* cKO mice although these mutant mice did exhibit cognitive deficits (71). In addition, using *CRE-luc* reporter assays, we found that *CRE-luc* activity is not reduced in primary *PS*-null cortical cultures, arguing against CRE-dependent gene expression being a direct target of PS-mediated Notch signaling pathway (49). Therefore, the modest reduction of CREB-CBP activity may be the consequence of other molecular changes caused by loss of presenilin, as suggested by a recent report (73). Collectively, Notch is unlikely an essential target of presenilin in mediating neuronal survival in the adult cerebral cortex. Consistent with this interpretation, *RBP-J κ* cKO mice, which were generated using independent α CaMKII-Cre lines, also failed to exhibit any phenotypes in excitatory neurons of the adult cerebral cortex,^{4,5} although adult neurogenesis and glial cell fate specification in the subventricular zone was affected (74). This is consistent with data from our *in situ* hybridization analysis that showed *Notch2* signals in the subventricular zone, the rostral migratory stream, and the olfactory bulb of the adult brain.⁶ Although we cannot exclude the possibility of compensatory effects provided by other Notch family members, Notch3 or Notch4, in our *Notch* cKO mice, their reported expression patterns, Notch3 being expressed exclusively in endothelial cells of the adult brain (75) and Notch4 expression being undetectable in the adult brain (76), make this possibility unlikely.

The most surprising finding of our current study is perhaps the absence of the reduced expression of Notch1 and Notch2 mRNAs and proteins, which we anticipated to find in the cerebral cortex of cKO mice. Using the same α CaMKII-Cre transgenic line, which expresses Cre recombinase in most if not all excitatory pyramidal neurons in the cerebral cortex (45, 50), we previously generated *PS*, *nicastrin*, and *CBP* cKO mice, all of which showed ~50% reduction of protein expression in the cerebral cortex (39, 45, 71). The remaining ~50% protein is likely due to expression of these genes in other cell types, such as glia and interneurons, where Cre expression is not targeted. In *Notch1* and *Notch2* cKO mice, we were able to confirm that deletion of the floxed DNA sequences indeed took place selectively in the cerebral cortex (Fig. 4). Furthermore, in primary cortical cultures where Notch1 and Notch2 are relatively highly expressed compared with the adult brain, introduction of Cre recombinase efficiently eliminated all of the Notch proteins (Fig. 5). Thus, the only reasonable interpretation of these results is that there is no Notch1 and Notch2 expression in excitatory neurons of the cerebral cortex. Consistent with this interpretation, the deletion of the floxed exons in excitatory neurons of the cerebral cortex in *Notch1* and *Notch2* cKO mice has no detectable effect on Notch mRNA and protein levels. These results appear to be at odds with earlier studies showing high levels of Notch immunoreactivity in the nucleus of the pyramidal neurons in the adult cerebral cortex (30–32). However, our Northern blot, which showed a single clean band of the correct size on the entire blot, does not rely upon specificity of available Notch antibodies. We were able to find two Notch2

⁴ K. Tanigaki, Research Institute, Shiga Medical Center, personal communication.

⁵ R. Kopan, unpublished results.

⁶ M. Wines-Samuels and J. Shen, unpublished data.

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antibodies that are rather specific and recognize Notch2-specific bands on Western blots using lysates from adult cortical samples as well as primary cortical cultures (Figs. 5 and 7 and supplemental Fig. S1). Notch1 antibodies, however, recognize nonspecific bands on Western blots, especially when adult cortical lysates were used (Fig. 7 and supplemental Fig. S1), making them less reliable indicators of Notch expression in the adult brain.

In summary, despite of the prevailing concerns of unwanted side effects on Notch while using γ -secretase inhibitors for treatment of Alzheimer disease, our current genetic study shows that Notch is not required for age-dependent survival of cortical pyramidal neurons, which are particularly vulnerable in AD. Thus, Notch is not a target of presenilin in promoting survival of excitatory neurons in the adult cerebral cortex. Although presenilin is highly expressed in pyramidal neurons in the cortex, Notch expression is undetectable in the same neurons, making it even less likely that presenilin and Notch families, which are tightly linked in the same signaling pathway during development, functionally act together in the adult brain. Future investigation is needed to identify molecular targets, through which presenilin exerts its neuronal protection in the aging cerebral cortex. The identification of such targets may provide novel targets to combat neurodegeneration in AD.

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