Enhanced Amyloidogenic Processing of the β-Amyloid Precursor Protein in Gene-targeted Mice Bearing the Swedish Familial Alzheimer’s Disease Mutations and a “Humanized” Aβ Sequence*

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The processing of the β-amyloid precursor protein (APP) in vivo has been characterized in a novel animal model that recapitulates, in part, the APP genotype of a familial form of Alzheimer’s disease (AD). A gene-targeting strategy was used to introduce the Swedish familial AD mutations and convert mouse Aβ to the human sequence. The mutant APP is expressed at normal levels in brain, and cleavage at the mutant β-secretase site is both accurate and enhanced. Furthermore, human Aβ production is significantly increased to levels 9-fold greater than those in normal human brain while nonamyloidogenic processing is depressed. The results on Aβ production extend similar findings obtained in cell culture to the brain of an animal and substantiate Aβ as a etiological factor in Swedish familial AD. These animals provide several distinguishing features over others created by conventional transgenic methodologies. The spatial and temporal expression patterns of others created by conventional transgenic methodologies are expected to be faithfully reproduced because the gene encoding the mutant APP remains in its normal chromosomal context. Thus, the neuropathological consequences of human Aβ overproduction can be evaluated longitudinally in the absence of potential mitigating effects of APP overexpression or presence of the mouse Aβ peptide.

Alzheimer’s disease (AD) is a complex multi-genic neurodegenerative disorder characterized by progressive impairments in memory, behavior, language, and visuo-spatial skills, ending ultimately in death. Hallmark pathologies within vulnerable brain regions include extracellular β-amyloid deposits, intracellular neurofibrillary tangles, synaptic loss, and neuronal cell loss. Considerable human genetic evidence has implicated the major proteinaceous component of the β-amyloid deposit (7, 8).

The Aβ peptide is produced and secreted normally by many cell types in one of at least two intracellular processing pathways of APP. Under normal conditions, a majority of APP is processed through the nonamyloidogenic pathway, where cleavage occurs within the Aβ domain at the α-secretase site, resulting in secretion of the large N-terminal ectodomain of APP (preA), production of a cell-associated 9-kDa C-terminal fragment, and destruction of the Aβ domain (9, 10). Processing in the amyloidogenic pathway produces APP fragments bearing the Aβ peptide or the Aβ peptide itself. In this pathway, APP is cleaved at the β-secretase site located at the N terminus of the Aβ domain, leading to secretion of an N-terminal APP/β fragment and appearance of a cell-associated 12-kDa C-terminal fragment (11). Paired cleavage at the γ-secretase site within the transmembrane domain leads to secretion of the Aβ peptide, ranging in size from 39 to 43 amino acids, and production of a cell-associated 6-kDa fragment (12, 13). The proteases responsible for these cleavages have not been isolated and are defined by their cleavage site specificities.

A correlation between APP overexpression and development of β-amyloid deposits is found in Down’s syndrome in which trisomy of chromosome 21 results in an extra copy of the APP gene. These individuals show a 50% overexpression of APP mRNA (14) and develop manifestations of AD by their fourth decade of life (15, 16). However, because many other genes are trisomic in Down’s syndrome, the AD-like phenotype cannot be attributed solely to APP overexpression. More compelling evidence for a causative role of altered APP processing in AD neuropathology comes from a subset of families predisposed to AD, where mutations linked to the diseases map to the APP gene (17–20). The familial AD (FAD) mutations immediately surround or lie within the Aβ domain and have been shown to increase either total Aβ production (21–23) or secretion of the more fibrillogenic Aβ1–42 peptide relative to Aβ1–40 (24, 25). One class of FAD mutations has been mapped to two amino acids immediately upstream of the Aβ N terminus (K670N/M671L) in a large Swedish kindred (20). These mutations enhance total Aβ secretion up to 6-fold in DNA transfection assays (21, 22) and are correlated with enhanced Aβ secretion in cultured fibroblasts isolated from affected individuals of the Swedish FAD family (26).

Numerous reports have described attempts to recapitulate the hallmark pathologies of AD in the rodent brain by overexpression of human APP or APP fragments in transgenic models (27–37). To date, only one of these models exhibits robust AD-like neuropathology. Five- to 10-fold overexpression relative to endogenous APP of APP751 and APP770 from an APP minigene bearing the FAD V717F mutation under control of the human PDGFβ promoter results in age-dependent depo-
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Cloning and Characterization of the Mouse APP Exon 16 Region—Mouse APP genomic DNA was cloned from a phage library created from 129/Sv mouse DNA (41) using a 300-bp probe homologous to APP cDNA sequences found in exons 15 through 17. From a screen of approximately 12,000 recombinant bacteriophages, five independent clones covering a total of 20.2 kb were identified. Exons 15, 16, and 17 were then positioned on a restriction map of the clones using exon-specific probes generated by PCR. Exons 15 and 17 were only localized to within the limits of a 4.4-kb fragment and a 1.9-kb fragment, respectively (Fig. 1).

APP Gene-targeting Vector Construction—The universal targeting vector pPTNloxP was created by modification of pPTN (42). loxP recombinase sites and multiple cloning sites were inserted on either side of the neocassette by subcloning with overlapping oligonucleotides. To create an APP targeting vector, a 4.5-kb HindIII fragment (position 6.5–11.0, Fig. 1) was used as the 5' arm of homology, and a 5.6-kb HindIII fragment (positions 11.0–17.0, Fig. 1) was used as the 3' arm of homology. Both fragments were subcloned initially into pBlueScriptSK− (Stratagene cloning systems). A total of six base pair changes were introduced into exon 16 found in the 3' arm of homology using a two-step PCR mutagenesis strategy. The primary structure of the mutated portion of exon 16 was confirmed by nucleotide sequencing. To assemble the final targeting vector, the mutated 3' arm of homology, isolated as an EcoRIV/KpnI fragment, was inserted into pPTNloxP between the EcoRI (blunt-ended with Klenow polymerase) and KpnI sites. The 5' arm of homology isolated as an XbaI (blunt-ended with Klenow polymerase)/NofI fragment was then inserted between the NofI and HpaI sites forming pAPP-TV.

Homologous Recombination in ES Cells and Mouse Chimeric Production—The R1 line of ES cells (43) kindly provided by Dr. Janet Rossant, Mt. Sinai Hospital, Toronto, Ontario, Canada) were grown as described previously (44), except that gelatinized plates were used as a substrate rather than feeder cells, and the growth medium contained 1000 units/ml leukemia inhibitory factor. NofI-linearized pAPP-TV DNA was introduced into ES cells by electroporation. Putative homologous recombinants were selected by growth in the presence of G418 and ganciclovir. DNA samples were prepared as described previously (44). APP mutant ES cells were used to make chimeric mice by aggregating the mutant ES cells to E2.5 embryos and transferring the aggregated embryos to pseudopregnant females (45, 46).

Excision of the neo Cassette—pBS185 plasmid DNA (47) encoding the Cre recombinase was introduced into the selected ES cells by electroporation (10 μg circular DNA/700 ES cells/cuvette). The electroporated cells were plated onto a gelatinized plate and grown in tissue culture medium in the absence of drug selection. Medium was changed every 48 h. After 8 days, individual colonies were picked into 24-well plates and expanded. DNA was prepared from the expanded cell lines as described (44).

Antibodies—Monoclonal antibody 6E10 (48), specific for human APP1–17, and monoclonal antibody 4G8 (48), directed against APP72–74 was directed against mouse APP (AP741–770). Both of these C-terminal domains are specifically internalized by astrocytes and microglia in the brain (49). Rabbit polyclonal antibody 97 is a functional equivalent of previously described antibody 10 (49) directed against APP72–74 (APPPT770 numbering). Rabbit polyclonal antibody 97 is a functional equivalent of previously described antibody 12 (49) directed against the last 30 amino acids of APP (AP741–770). Both of these C-terminal domains are specifically internalized by astrocytes and microglia in the brain (49).

Detection of Full-length APP, APPα, and APPβ—To prepare extracts for measuring full-length APP and APPα, one-half (0.25 g) mouse brain cut longitudinally (40–138-day-old animals) and 0.25 g normal human frontal cortex (70-year-old male) were sonicated in 4 volumes of TBS (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, and 200 mM phenylmethylsulfonyl fluoride, pH 8.0) and centrifuged at 13,000 × g for 15 min. To prepare extracts for measuring APPβ, one-half mouse brain and 0.25 g normal human frontal cortex were homogenized in 3 ml of B buffer (20 mM Tris-HCl, 2 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 1 mM diethiothreitol, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and centrifuged at 100,000 × g for 15 min. Pellets from this detergent-free extraction were saved for detection of 12- and 9-kDa C-terminal derivatives of APP (see below). Twenty-five μg of total protein from each supernatant, as measured by Bio-Rad protein assay (51), were resuspended in 1× Laemmli sample buffer (52) and electrophoresed on 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose by electrotransfer as described previously (53). Filters containing proteins extracted with B buffer were incubated in 1:500 dilution was used as primary blotting reagent to enabled detection of a rabbit immunized with human APP1–8 (DAEFHRDS) linked through a C-terminal GC to bovine serum albumin. The antibody was affinity-purified by binding and elution from a column of CNBr-activated Sepharose 4B to which a peptide-bovine thyroglobulin had been linked. To test for specificity of 149ST for the free NH2 terminus of the APP domain, competition ELISA was performed with peptides corresponding to human APP1–8 (GC), APP2–8 (AC), and APP1–9 (AC). A 1:30,000 dilution of Ab149ST was incubated for 1 h with various concentrations of competing peptides in Tris-buffered saline (1 μM containing 3% nonfat dry milk and then added to a microtiter plate previously coated with APP1–8 (GC).

Detection of 12- and 9-kDa C-Terminal APP Derivatives—Pellets obtained from the 100,000 × g spin of samples extracted in B buffer were washed in an additional 3 ml B buffer. Following centrifugation at 100,000 × g, pellets were sonicated in 1× RIPA (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, 1% benzamidine, 0.05 mM leupeptin, and 0.02 mM pepstain A), followed by centrifugation at 100,000 × g for 1 h. Supernatant proteins were immunoprecipitated as described previously (30) with antibody 97 and Pansorbin (Calbiochem, La Jolla, CA), resolved by electrophoresis on 16% Tris-Tricine polyacrylamide gels (53) (Novex, San Diego, CA), and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories) by electroblotting. Antibody 6E10 (1:2000) was used as the primary blotting reagent to enable detection specifically of human and humanized 12-kDa C-terminal derivatives. Antibody 9 at a 1:500 dilution was used as primary blotting reagent to enable detection of mouse, human, and humanized 12-kDa C-terminal derivatives and the 9-kDa C-terminal derivatives. After washing with TBST, blots were incubated in either goat anti-mouse IgG- or goat anti-rabbit IgG-conjugated to horseradish peroxidase, and immunoactive species were
visualized using ECL.

For detection of 12-kDa immunoreactive polypeptides with an amino terminus at Asp672 (β1) of the Aβ peptide, 1 × RIPA supernatants were immunoprecipitated with antibody 1948T. Immunoprecipitates were electrophoresed and blotted as described above. Filters were incubated in either antibody 6E10 (1:2000) or antibody 12 (1:500), followed by goat anti-mouse IgG- or goat anti-rabbit IgG-conjugated to horseradish peroxidase, respectively, and ECL was used for detection.

**RESULTS**

**APP Gene Targeting**—The mutagenesis strategy to humanize the mouse Aβ gene sequence and simultaneously introduce the FADK670N/M671L mutations is outlined in Fig. 1A. The Aβ coding sequences are split between exons 16 and 17 of the mouse APP gene on chromosome 16. The three amino acid differences between mouse and human Aβ and the K and M residues that are mutated to N and L, respectively, in the Swedish FAD kindreds are located in exon 16 (54, 55). This makes possible the introduction of all coding sequence mutations (Fig. 1B) using a single targeting vector in a gene replacement strategy (56). The mutagenesis in ES cells was done in two steps. Using a positive-negative selection scheme (57), the exon 16 mutations were introduced along with a neo-selectable marker in intron 15 approximately 1.7 kb upstream of exon 16. In the second step, the PGKneo cassette was excised by Cre-mediated recombination at the flanking loxP sites.

Following transfection of the DNA targeting vector into R1 ES cells, DNA samples from individual G418R and GANCR...
clones were screened by Southern hybridization using an APP probe flanking the 5’ arm of homology in the targeting vector and another probe flanking the 3’ arm of homology. Of 242 ES clones screened with the 5’ probe, 22 clones were identified as having the 6-kb XbaI fragment predicted from a homologous recombination event. DNA samples from these 22 clones were screened with the 3’ probe, and 9 clones were identified as proper homologous integrants, as judged by the appearance of an 8-kb ApaI fragment. Depending on where crossover took place during recombination in the 3’ arm, the exon 16 mutations may or may not have been incorporated into the genomic DNA of the nine bona fide homologous recombinants. Therefore, a third Southern hybridization screen was done using an internal probe to detect the predicted 1.8-kb fragment diagnostic for the novel XbaI restriction site created by the K670N/M671L mutation. Recombination events occurring prior to the exon 16 mutations would result in the appearance of a 3.7-kb XbaI fragment. Four of the nine bona fide homologous recombinant cell lines incorporated the novel XbaI. Following PCR amplification of exon 16 DNA and nucleotide sequence analysis, each of the four cell lines that carried the exon 16 XbaI site were found to contain all of the nucleotide substitutions underlying the FADK670N/M671L and Aβ-humanizing mutations. The mutant APP allele containing the neomycin-selectable marker in intron 15 (neo+) and the exon 16 mutations was designated as APPneo..

Chimeric founder mice were obtained with three of the neo+ ES cell clones, and one clone (APP139) was shown to transmit the ES cell phenotype through the germ line. The presence of the mutant APP allele in the F1 and subsequent generation progeny was determined by the Southern hybridization assay described above, using the internal probe on XbaI-restricted tail DNA. Mice heterozygous for APPneo+APP+ have 8.8- and 2.0-kb XbaI fragments that hybridize with this probe, and mice homozygous for the mutant APP allele (APPneoAPPneo) have only the 2.0-kb XbaI DNA fragment.

Due to the uncertainty of the effect of the neo-selectable marker on APP expression in vivo, the PGK-neo gene was excised by recombination at the flankingloxP sites after transient expression of the Cre recombinase. APP139 ES cells were electroporated with a cytomegalovirus/cre expression vector (pBS185), and individual cells from the transfected population were cloned in the absence of G418 selection. Loss of the PGK-neo gene was scored by Southern hybridization. A total of 149 clones was screened, and five presumptive excision events were scored. The integrity of the APP locus in four of the presumptive neo− clones was confirmed by comprehensive restriction enzyme mapping (data not shown). A chimeric founder mouse produced by embryo aggregation with one of these neo− clones (APP139−34) exhibited germ line transmission of the mutant APP allele (termed APPneo). From this founder, heterozygous (APPneoAPP+) and homozygous (APPneoAPPneo) lines for the neo− APP mutant allele were established.

Measurement of Humanized APP in Brain—To determine if the APP gene-targeted mice produced APP with a humanized Aβ domain, immunoblot assays were performed on brain samples prepared from wild-type APP+APP+, APPneoAPPneo, APPneoAPP+, APPneoAPPneo, and APPneoAPPneo mice. The protein samples were treated with the human APP-specific 6E10 antibody raised against the first 17 amino acids of human Aβ, the domain that contains all three amino acid differences between human and mouse Aβ (Fig. 2A). Immunoreactive species that co-migrate with the APP signal from human cortex were detected in brain extracts from the APP gene-targeted mice (APPneoAPP+, APPneoAPP+, APPneoAPPneo, and APPneoAPPneo) but not in the wild-type APP+APP+ brain sample. As expected, the increase in gene dosage between the APPneoAPP+ and APPneoAPPneo mice was directly related, with a 2-fold increase in expression of the humanized APP. Immunoblot analysis of the same gene-targeted samples was also done with antibody 4G8 raised against Aβ17−24 (Fig. 2B). This antibody, which recognizes both mouse and humanized APP equally, showed similar levels of APP immunoreactivity between the wild-type and APP gene-targeted mouse brains.

These results indicate that APP with a humanized Aβ domain is being produced in the APP gene-targeted mice. Comparisons of the signal intensities between the APPneoAPP+ and APPneoAPP+ mice from multiple experiments using the human APP-specific 6E10 antibody indicate that presence of the neo selectable marker in intron 15 resulted in only a 30−50% decrease in APP accumulation. There was no evidence with any of the antibodies reported here or with N-terminal-specific APP antibodies (data not shown) that the neo-selectable marker caused the appearance of aberrant or truncated forms of APP.

Enhanced Amyloidogenic Processing in Vivo in APP Gene-targeted Mice—The central hypothesis to the APP gene-targeting strategy is that amyloidogenic processing at the β-secretase site would be enhanced due to the introduction of the FADK670N/M671L mutations. To determine if this was indeed occurring in vivo, the relative levels of the 9- and 12-kDa fragments, two C-terminal APP processing derivatives produced in the nonamyloidogenic and amyloidogenic pathways, respectively, were measured in brains of wild-type and APP gene-targeted mice. The APP fragments and the antibodies used to identify them are schematically represented in Fig. 3. Brain homogenates were initially immunoprecipitated with antibody 97, specific for the last 30 amino acids of APP, to concentrate all APP fragments bearing C-terminal epitopes. The immunoprecipitates were then analyzed on immunoblots using antibody 9 that recognizes both the 12-kDa β-secretase and 9-kDa α-secretase products from wild-type mouse and APP gene-targeted mouse and human brains (Fig. 4A). In wild-type mice, a weak immunoreactive species that co-migrated with the recombinant 12-kDa size standard (C100) was detected, while a doublet in the 9-kDa size range predominated. These results were consistent with a majority of native APP being processed through the nonamyloidogenic pathway. In the APP gene-targeted mouse brains, Twenty-five µg of total brain protein from each APP gene-targeted mouse brain (nNLh+/+, nNLh/nNLh, and NLh+/), a wild-type mouse brain (+/+) and normal human frontal cortex (Hu) were immunoblotted and polypeptides visualized by ECL. A, humanized full-length APP and APPa were detected in both heterozygous (nNLh+/+ and NLh+/+) and homozygous (nNLh/nNLh) APP gene-targeted mouse brains using human Aβ1−17-specific 6E10 antibody. Immunoreactive bands in the gene-targeted brain extracts co-migrated with those seen from human cortex (Hu). B, full-length APP (humanized and/or mouse) from wild-type and APP-targeted mouse brains was detected using antibody 4G8.
Fig. 3. Schematic of relevant APP processing fragments and pertinent antibodies. Antibodies and their respective epitopes used to detect full-length APP, APPα, APPβ, and C-terminal derivatives are shown. Details for each antibody are presented under “Experimental Procedures.” Cleavage sites for the α-, β- and γ-secretases are indicted by the arrows. APP processing by the β-secretase results in production of the secreted APPβ fragments ending in either the amino acid sequence SEVKM (wild-type mouse and human) or SEVNL (APP-targeted mouse). Also shown are C-terminal derivatives of APP processing including the 12- and 9-kDa fragments produced after cleavage by the β- and α-secretases, respectively, and the Aβ peptide.

The identification of the immunoreactive species as the 9- and 12-kDa processing fragments was further supported by analysis of immunoprecipitated samples on immunoblots with the human-specific 6E10 antibody (Fig. 4B), which recognizes the 12-kDa but not the 9-kDa C-terminal APP derivatives. As expected, only the 12-kDa immunoreactive species were detected in brain homogenates from the APP gene-targeted mice and human brain, whereas the 9-kDa species that lacks the 6E10 epitope (β1–17) was not visualized. The 12-kDa species were absent in the wild-type samples due to the human specificity of the antibody. Consistent with enhanced expression at the neo versus neo− mutant APP locus, there is an approximate 30% increase in levels of the 12-kDa species in brain homogenates from APPNLh/APP+ over APPNLh/APP− mice. Quantitation of the 9- and 12-kDa species in Fig. 4A by densitometric scanning showed that in the APPNLh/APP− and APPNLh/APP− brains, the 12-kDa species increased approximately 3.5- and 7-fold, respectively, relative to wild type (Fig. 4C), together with a corresponding 2-fold decrease in the 9-kDa species in the homozygous gene-targeted brains. The calculated 12 kDa:9 kDa ratio, therefore, increased approximately 12-fold in homozygous APPNLh/APP− mouse brains relative to wild type. This dramatic increase reflected the overall effect of the humanization of Aβ and simultaneous introduction of the FADK670N/M671L mutations on amyloidogenic APP processing in mouse brain. A virtually identical 12 kDa:9 kDa ratio was obtained with the APPNLh/APPNLh mouse (data not shown). This is consistent with the neo-selectable marker affecting overall expression levels from the mutant APP allele but not differentially influencing accumulation of specific processing fragments of the mature protein. A 2-fold increase in the 12 kDa to 9 kDa ratio was measured from wild-type mouse to normal human brain, suggesting that the overall enhancement in amyloidogenic processing in the APP gene-targeted mouse brain is due primarily, but not exclusively, to the FADK670N/M671L mutations.

Enhanced Cleavage at the β-Secretase Site Occurs at β1.—To verify that the mutant APP is being cleaved at the authentic β-secretase site and to confirm the identity of the 12-kDa species, brain homogenates from APP gene-targeted mice were immunoprecipitated with antibody 1948T raised against Aβ1–8. Based on competition ELISA, antibody 1948T was found to be highly selective (>100-fold) for C-terminal derivatives of APP that begin at Asp-672 or β1 of the Aβ peptide, as compared with Aβ peptides extended or truncated by one residue (Fig. 5A, lower panel). As expected, a 12-kDa species was immunoprecipitated by Ab1948T from human brain (Fig. 5A). In the APP gene-targeted mice, an immunoreactive species that co-migrated with the human 12-kDa species was also detected. These results indicate that the 12-kDa product in gene-targeted mouse brains begins at β1 and, therefore, is the C-terminal cleavage product of mouse brain β-secretase.

We have noted in this and previous experiments that the 12-kDa species migrates as a doublet in brain, whereas only a single band that co-migrates with C100 is observed in extracts from cells in culture. Specific detection of the 12-kDa doublet by 1948T in brain indicates that both these fragments begin at β1. Therefore, differences in migration could result from either C-terminal truncation of the 12-kDa product or posttranslational modification. The recombinant 12-kDa (C100) fragment co-migrates with the faster migrating fragment from brain. These results indicate that truncation is not occurring, and the slower migrating immunoreactive fragment most likely arises from posttranslational modification. In support of this conclu-

8 M. Savage, unpublished results.
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and homozygous \((APP^{NLh}/APP^{NLh})\) gene-targeted brains, Swedish FAD mutant APP\(\beta\) was detected and increased in abundance with gene dosage. As expected, no immunoreactivity with Ab54 was detected in wild-type mouse brain or in normal human brain. This is unequivocal evidence that the APP gene-targeted mice express APP containing the FADK670/N671L mutations and supports observations made on the 12-kDa C-terminal derivative that cleavage by the mouse \(\beta\)-secretase occurs at \(\beta1\).

Production of Human \(\alpha\beta\) in the APP Gene-targeted Mouse Brain—We next examined whether enhanced cleavage at the \(\beta\)-secretase site was associated with an increase in human \(\alpha\beta\) accumulation in the APP gene-targeted mouse brain. An immunoprecipitation and immunoblotting method using two distinct \(\alpha\beta\)-specific antibodies identified a 4-kDa polypeptide that co-migrated with synthetic human \(\alpha\beta\) in the brains of \(APP^{NLh}/APP^{NLh}\), \(APP^{NLh}/APP^{NLh}\), \(APP^{NLh}/APP^{NLh}\) and \(APP^{NLh}/APP^{NLh}\) mice (Fig. 7. A and B). Due to the selectivity of Ab 6E10 for human \(\alpha\beta\), no immunoreactivity was found in the wild-type \(APP^{NLh}/APP^{NLh}\) mouse brains. Levels of immunoreactivity in the APP gene-targeted brains also corresponded directly with gene dosage, providing further evidence on the identity of the immunoreactive species as human \(\alpha\beta\). Comparative levels of human \(\alpha\beta\) in brains from the various genotypes of the APP gene-targeted mice appear in Fig. 7C. An increase of approximately 50% in human \(\alpha\beta\) is correlated with excision of the neo-selectable marker in mice that are heterozygous \((APP^{NLh}/APP^{NLh})\) or homozygous \((APP^{NLh}/APP^{NLh})\) or homozygous \((APP^{NLh}/APP^{NLh})\) and \((APP^{NLh}/APP^{NLh})\) for the mutant APP allele. In the \(APP^{NLh}/APP^{NLh}\) mouse brain, human \(\alpha\beta\) levels were approximately 9-fold greater than those found in normal aged human brain.

**DISCUSSION**

To accurately model familial AD in an animal, we have used a gene-targeting strategy to enhance the production of human \(\alpha\beta\) in the mouse brain without changing the levels of APP synthesis. Gene-targeted mouse lines have been established in which site-specific mutations have been introduced into exon 16 of the mouse APP gene to humanize the \(\alpha\beta\) gene sequence and introduce the FADK670/N671L mutations. Two aspects of this animal model that distinguish it from models established by conventional cDNA and yeast artificial chromosome-based transgenic methodologies are that human \(\alpha\beta\) is produced in the absence of mouse \(\beta\), and the developmental and tissue-specific expression patterns of human \(\alpha\beta\) are expected to be accurately reproduced because the gene encoding the mutant precursor protein remains in its normal chromosomal location. Accordingly, these animals can be considered a faithful genotypic emulation of FAD in the Swedish kindred.

In the brains of the APP gene-targeted mice we have shown that mouse APP with a humanized \(\alpha\beta\) domain is expressed, and amyloidogenic processing at the \(\beta\)-secretase site is both accurate and elevated. This in turn is correlated with significant accumulation of human \(\alpha\beta\) to levels that far exceed those in normal human brain. Unfortunately, the lack of a detection method for mouse \(\alpha\beta\) precludes a direct measurement on the fold increase of human \(\alpha\beta\) over endogenous mouse \(\alpha\beta\). Although processing effects due specifically to either the FADK670/N671L or \(\alpha\beta\)-humanizing mutations cannot be distinguished in the current animals, we have found a 2-fold increase in the 12 kDa:9 kDa ratios in the normal human versus wild-type mouse brains. It has been reported previously that humanization of the mouse \(\alpha\beta\) gene sequence results in an approximate 3-fold increase in amyloidogenic processing of recombinant APP in rat hippocampal neurons infected with Semliki Forest Virus expression constructs (59). Together, these results argue that humanization results in a subtle but distinct
increase in amyloidogenic processing of APP in rodents, and the FADK670N/M671L mutations cause a further and more substantial increase. These in vivo results are critical in supporting the hypothesis that the Swedish mutations cause AD by enhancing Aβ production; a proposal based previously on APP processing results in cell culture (21, 22, 26, 59). Here, we have demonstrated measurable increases in Aβ production caused by the Swedish FAD mutations when placed in context of the normal gene and expressed in the organ uniquely susceptible in the disease.

An alternate explanation to account for the apparent increase in levels of the 12-kDa fragment in the APP gene-targeted mice is enhanced stability of the humanized fragment. Although this possibility cannot be ruled out completely, it is made unlikely by the corresponding decrease in levels of the 9-kDa fragment that is identical in sequence between the wildtype and APP gene-targeted mice. Also, levels of the humanized 12-kDa fragment and human Aβ were substantially higher in the APP gene-targeted brain (APPNLh/APPNLh) than in normal aged human brain. If stability was the sole factor, one would expect these levels to be more similar based on the near identical amounts of APP (per gram of brain) between the two species.

In addition to increased levels of the 12-kDa C-terminal β-secretase product, levels of the 9-kDa C-terminal α-secretase product were diminished, indicating that elevated amyloidogenic processing was accompanied by a reduction in nonamyloidogenic processing. This suggests that either β-secretase cleavage of APP bearing the FADK670N/M671L mutations occurs in a subcellular compartment upstream of α-secretase and the 12-kDa fragment is not a preferred α-secretase sub-
strate, or much of the mutant APP is diverted to a pathway where it encounters only the β-secretase. The first possibility is consistent with proposed models describing α-secretase cleavage at or near the plasma membrane (60, 61) and more proximal β-secretase cleavage in an acidic compartment, such as the Golgi (62, 63). Coupling of the amyloidogenic and nonamyloidogenic pathways, where alterations in one pathway affect levels processed in the other, has been reported in cell culture (21, 22, 64–66). However, evidence to the contrary from cell culture experiments has also been reported (67, 68). We show, for the first time, that the two pathways are indeed associated in vivo.

Excision of the neo-selectable marker from intron 15 of the APP-targeted allele resulted in an average 50% increase of APP expression, as judged by the relative accumulation of various full-length and processing derivatives in the neo+ and neo− lines. This is likely to be caused by more efficient transcriptional readthrough at the targeted locus in the neo− lines due to removal of the neo gene cassette with its RNA processing signals. In fact, truncated APP species were detected in the neo−, but not neo+, APP-targeted ES cells lines used to create the data (not shown). However, the appearance of truncated APP species was not observed in vivo. Alternatively, there may be inherent differences in stabilities of the primary transcripts from the neo+ and neo− APP-targeted alleles.

The lack of APP overexpression and the absence of mouse Aβ in the APP gene-targeted mice are important features of this animal model. Potent neuroprotective activities of the secreted forms of APP (APPα) have been reported in primary cultures of rat hippocampal and septal neurons as well as human cortical neurons (39). The administration of APPα intraventricularly protects against ischemic brain damage (40), and moderate overexpression of APP695 or APP751 in the brains of transgenic mice has been reported to cause marked synaptotrophic responses (29). These results suggest that APP overexpression may modify any neuropathological effects caused by Aβ overproduction. Also, several findings in vitro argue for a reduced amyloidogenic potential of mouse versus human Aβ, including aggregation studies done in membrane-mimicking solvents (69), or in the presence of zinc (70) or free radical-generating agents (71). The question remains whether, in vivo, the presence of mouse Aβ can interfere with the deposition of human Aβ or any other Aβ-associated neuropathological events. The elimination of mouse Aβ in the APP gene-targeted mice precludes this possibility.

In conclusion, we have shown by site-specific mutagenesis of

**FIG. 6.** Comparison of wild-type and Swedish mutant APP β expression in human, wild-type mouse, and gene-targeted mouse brains. Brains were extracted in Tris-based buffer containing no detergents, as described under “Experimental Procedures.” Twenty-five µg of Tris-soluble protein fraction from wild-type (+/+), gene-targeted mouse brains (nNLh/+), nNLh/nNLh, and NLh/+), and normal human frontal cortex (Hu) were electrophoresed on 8% Tris-glycine SDS-polyacrylamide gels and subsequently immunoblotted with either antibody 53, specific for wild-type APP β (A), or antibody 54, specific for Swedish mutant APP β (B).

**FIG. 7.** Human Aβ expressed in gene-targeted mouse brains. A, one-half (0.25 g) of each heterozygous gene-targeted mouse brain (nNLh/+ and NLh/+) as well as a wild-type control (+/+ +) were extracted in 6 M guanidine as described under “Experimental Procedures” and immunoprecipitated with antibody 1153. A separate wild-type control brain (+/+ + spike) received a spike of 50 ng of synthetic human Aβ1–40 (Bachem Bioscience Inc., King of Prussia, PA) immediately following brain homogenization. Synthetic human Aβ1–40 alone was included on the immunoblot for size comparison. To detect specifically human Aβ, immunoblots shown in A and B were incubated with antibody 6E10, followed by goat anti-mouse IgG conjugated to horseradish peroxidase and ECL. B, quantitative comparison of the Aβ signal from heterozygous (nNLh/+ and NLh/+) and homozygous (nNLh/nNLh and NLh/NLh) gene-targeted mouse brains (0.25 g) and normal human cortex (0.25 g). Wild-type brains alone (+/+ +) and wild-type brains spiked with 25 ng synthetic human Aβ1–40 (+/+ + spike) were included as controls. C, quantification of the relative amounts of Aβ present in each of the gene-targeted brains and normal human frontal cortex. The data presented are the means of two experiments (n = 2) and were compiled using the Docugel V Scanalytic system (Mitsubishi); bars, S.D.
the mouse genome that introduction of the FADK6707/NM761L and Aβ-humanizing mutations promoted a re-routing of APP intracellular trafficking in vivo from the nonamyloidogenic to amyloidogenic processing pathway. Furthermore, production of human Aβ was approximately one order of magnitude greater than that found in normal human brain. The neuropathological and behavioral consequences of the alterations in APP processing and human Aβ accumulation were assessed using the APP^NLh^/APP^NLh^ line.

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Enhanced Amyloidogenic Processing of the β-Amyloid Precursor Protein in Gene-targeted Mice Bearing the Swedish Familial Alzheimer's Disease Mutations and a "Humanized" Aβ Sequence

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