

Early-onset and Robust Cerebral Microvascular Accumulation of Amyloid β -Protein in Transgenic Mice Expressing Low Levels of a Vasculotropic Dutch/Iowa Mutant Form of Amyloid β -Protein Precursor*

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Cerebrovascular deposition of amyloid β -protein ($A\beta$) is a common pathological feature of Alzheimer's disease and related disorders. In particular, the Dutch E22Q and Iowa D23N mutations in $A\beta$ cause familial cerebrovascular amyloidosis with abundant diffuse amyloid plaque deposits. Both of these charge-altering mutations enhance the fibrillogenic and pathogenic properties of $A\beta$ *in vitro*. Here, we describe the generation of several transgenic mouse lines (Tg-SwDI) expressing human neuronal $A\beta$ precursor protein ($A\beta$ PP) harboring the Swedish K670N/M671L and vasculotropic Dutch/Iowa E693Q/D694N mutations under the control of the mouse Thy1.2 promoter. Tg-SwDI mice expressed transgenic human $A\beta$ PP only in the brain, but at levels below those of endogenous mouse $A\beta$ PP. Despite the paucity of human $A\beta$ PP expression, quantitative enzyme-linked immunosorbent assay measurements revealed that Tg-SwDI mice developed early-onset and robust accumulation of $A\beta$ in the brain with high association with isolated cerebral microvessels. Tg-SwDI mice exhibited striking perivascular/vascular $A\beta$ deposits that markedly increased with age. The vascular $A\beta$ accumulations were fibrillar, exhibiting strong thioflavin S staining, and occasionally presented signs of microhemorrhage. In addition, numerous largely diffuse, plaque-like structures were observed starting at 3 months of age. *In vivo* transport studies demonstrated that Dutch/Iowa mutant $A\beta$ was more readily retained in the brain compared with wild-type $A\beta$. These results with Tg-SwDI mice demonstrate that overexpression of human $A\beta$ PP is not required for early-onset and robust accumulation of both vascular and parenchymal $A\beta$ in mouse brain.

The progressive accumulation of amyloid β -protein ($A\beta$)¹ in senile plaques and the cerebral vasculature is a prominent feature of Alzheimer's disease and several related disorders (1,

2). The $A\beta$ peptide is derived from the $A\beta$ precursor protein ($A\beta$ PP), a type I integral membrane protein, through sequential proteolytic processing mediated by β - and γ -secretase activities (1, 3). Several mutations that are linked to familial forms of early-onset Alzheimer's disease have been identified in the $A\beta$ PP gene. These mutations tend to cluster around the β - and γ -secretase cleavage sites within $A\beta$ PP and lead to increased production of total $A\beta$ or a preferential increase in the levels of the longer, more pathogenic $A\beta$ 42 peptide (1, 3, 4). On the other hand, several mutations in the $A\beta$ PP gene that reside within residues 21–23 of $A\beta$ and that give rise to familial forms of cerebral amyloid angiopathy (CAA) have been found. The first recognized of these was the Dutch E22Q mutation, which is associated with diffuse $A\beta$ deposition in the neuropil and severe CAA, leading to recurrent and often fatal hemorrhagic episodes at mid-life (5–7). More recently, the Iowa D23N mutation in $A\beta$ was identified in a cohort presenting with late-onset dementia accompanied by severe CAA with numerous small cortical hemorrhages, cortical and subcortical infarcts, and neurofibrillary tangles (8). The reason as to why mutations in this region within $A\beta$ lead preferentially to a strong accumulation of cerebrovascular amyloid remains unclear.

It is noteworthy that both the Dutch E22Q and Iowa D23N mutations result in loss of a negative charge in the $A\beta$ peptide. In previous studies, we (9, 11) and others (10) showed, that compared with wild-type $A\beta$, the Dutch E22Q and Iowa D23N mutant $A\beta$ peptides exhibit enhanced fibrillogenic and pathogenic properties in cultured cerebrovascular cells used as *in vitro* models for CAA. Moreover, an experimental $A\beta$ peptide containing the Dutch and Iowa mutations together (E22Q/D23N) possesses even more robust fibrillogenic and pathogenic properties *in vitro* compared with either single mutation alone (11). These combined findings suggest that the loss of negative charges and gain of pathogenicity in $A\beta$ associated with the Dutch and Iowa mutations may directly correlate with the accumulation of $A\beta$ in the brain, particularly around the cerebral vasculature.

To further investigate this *in vivo*, we generated transgenic mice expressing human Swedish, Dutch, and Iowa triple-mutant $A\beta$ PP (Tg-SwDI) in brain that produce Dutch/Iowa E22Q/D23N double-mutant $A\beta$. Although these transgenic mice were

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¹ The abbreviations used are: $A\beta$, amyloid β -protein; $A\beta$ PP, amyloid

β -protein precursor; CAA, cerebral amyloid angiopathy; Tg-SwDI, transgenic mouse expressing human Swedish, Dutch, and Iowa triple mutant $A\beta$ PP; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; Tricine, N-[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]glycine.

found to express human A β PP at levels below those of endogenous mouse A β PP, three independent Tg-SwDI mouse lines developed strikingly similar early-onset and robust accumulation of A β in the brain with high association with the cerebral microvasculature. Importantly, these results demonstrate that overexpression of human A β PP is not necessary for the development of A β pathology in mouse brain. Furthermore, functional studies revealed, that compared with wild-type A β , Dutch/Iowa mutant A β was poorly cleared from mouse brain into the circulation. These findings suggest that the observed clearance deficit of Dutch/Iowa mutant A β likely contributes to its strong accumulation in and around cerebral blood vessels and in the parenchyma of the Tg-SwDI mice and possibly in patients with either the Dutch or Iowa familial CAA mutations.

EXPERIMENTAL PROCEDURES

Vector Construction and Generation of Transgenic Mouse Lines—A pcDNA3 vector containing 2.1 kb of human A β PP (isoform 770) cDNA was used to introduce mutations Swedish K670N/M671L, Dutch E693Q, and Iowa D694N using the QuikChange kit (Stratagene, La Jolla, CA). The A β PP770-SwDI cDNA was amplified by PCR using primers containing the NheI 5'-linker and SacII 3'-linker. The PCR product was digested and subcloned between exons II and IV of a Thy1.2 expression cassette (a gift from Dr. F. LaFerla, University of California, Irvine, CA) using NheI and SacII restriction sites. The completed construct was entirely sequenced to confirm its integrity. The 9-kb transgene was liberated by NotI/PvuI digestion, purified, and microinjected into pronuclei of C57Bl/6 single cell embryos at the Stony Brook Transgenic Mouse Facility. Three founder transgenic mice were identified by Southern blot analysis of tail DNA. Transgenic offspring from each line were determined by PCR analysis of tail DNA using the following primers specific for human A β PP: 5'-CCTGATTGATAC-CAAGGAAGGCATCCTG-3' and 5'-GTCATCATCGGCTTCTTCTTCT-TCCACC-3' (generating a 500-bp product). All subsequent analyses were performed with heterozygous transgenic mice.

Immunoblot Quantitation of A β PP—Mouse forebrain, distinct mouse brain regions, or various peripheral tissues were homogenized in 10 volumes of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1% SDS, 0.5% Nonidet P-40, 5 mM EDTA, and proteinase inhibitor mixture (Roche Applied Science). The tissue homogenates were clarified by centrifugation at $14,000 \times g$ for 10 min. Protein concentrations of the resulting supernatants were determined using the BCA protein assay Kit (Pierce). The levels of A β PP in the tissue homogenate samples were determined by performing quantitative immunoblotting as described (11). Briefly, 35 μ g of total protein from each sample were electrophoresed on SDS-10% polyacrylamide gels, and the proteins were transferred onto Hybond nitrocellulose membranes (Amersham Biosciences). Unoccupied sites on the membranes were blocked overnight with 5% nonfat milk in phosphate-buffered saline with 0.05% Tween 20. The membranes were probed either with monoclonal antibody P2-1, which is specific for human A β PP (12), or with monoclonal antibody 22C11 (Chemicon International, Inc., Temecula, CA), which is specific for mouse and human A β PP, and then incubated with a secondary peroxidase-coupled sheep anti-mouse IgG antibody at a dilution of 1:1000. The peroxidase activity on the membranes was detected using Super-signal Dura West (Pierce). Bands corresponding to A β PP were measured using a VersaDoc 3000 imaging system (Bio-Rad) with the manufacturer's Quantity One software and compared with standard curves generated from known quantities of purified A β PP.

Enzyme-linked Immunosorbent Assay (ELISA) Quantitation of A β Peptides—Soluble pools of A β 40 and A β 42 were determined by specific ELISAs of carbonate-extracted mouse forebrain tissue; and subsequently, the insoluble A β 40 and A β 42 levels were determined by ELISA of guanidine lysates of the insoluble pellets resulting from the carbonate-extracted brain tissue (13, 14). Brain microvessels were isolated from mouse forebrains as described (15). Total vascular A β 40 and A β 42 levels were measured in guanidine lysates of the brain microvessels isolated from Tg-SwDI mice. In the sandwich ELISAs, A β 40 and A β 42 were captured using their respective carboxyl terminus-specific antibodies m2G3 and m21F12, and biotinylated antibody m3D6, specific for human A β , was used for detection (13). Because antibody m3D6 recognizes an epitope in the first five amino acids of A β , this ensured that the sandwich ELISA was measuring amino-terminally intact A β peptides.

Immunohistochemical Analysis—Mice were killed at specific ages, and the brains were removed and, in most cases, bisected in the mid-

sagittal plane. One hemisphere was snap-frozen and used for the protein analyses described above. The other hemisphere was placed in 70% ethanol overnight and subjected to increasing sequential dehydration in ethanol, followed by xylene treatment and embedding in paraffin. Sections were cut from mouse brain hemispheres in the sagittal plane at 5 μ m using a microtome, placed in a flotation water bath at 45 °C, and then picked on glass slides. Paraffin was removed from the sections by washing with xylene, and the tissue sections were rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by treating the tissue sections with proteinase K (0.2 mg/ml) for 10 min at 22 °C. Primary antibodies were detected with horseradish peroxidase-conjugated or alkaline phosphatase-conjugated secondary antibodies and visualized either with a stable diaminobenzidine solution (Invitrogen) or with the fast red substrate system (Spring Bioscience, Fremont, CA), respectively, as substrate. Sections were counterstained with hematoxylin. Thioflavin S staining for fibrillar amyloid was performed as described (16). Prussian blue iron staining was performed to detect hemosiderin to reveal signs of previous microhemorrhage as described (17, 18). The following antibodies were used for immunohistochemical analysis: monoclonal antibody 66.1, which recognizes residues 1–5 of human A β (19), and rabbit polyclonal antibody to collagen type IV (Research Diagnostics Inc., Flanders, NJ). The percent of A β -associated blood vessels in the frontotemporal cortex, thalamic, and subiculum regions was determined in four mice at each of the specified ages using stereological principles as described (19).

Radioiodination of Synthetic A β Peptides—Radioiodination was carried out using the lactoperoxidase method (20). After radiolabeling, the preparations were subjected to HPLC to separate the monoiodinated non-oxidized forms of A β (which is the tracer we used) from di-iodinated A β , unlabeled non-oxidized A β , and oxidized A β species. The content of material in the peaks eluted by HPLC was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to ensure the purity of the radiolabeled species. These analyses confirmed that oxidized species of 125 I-labeled wild-type A β 40 and Dutch/Iowa mutant A β 40 were not present in the preparations. At specific activities between 55 and 85 μ Ci/ μ g, the radiolabeled peptides were stabilized using ethanol as a quenching system and kept for up to 96 h. Prior to infusion into animals, we performed HPLC purification of the tracer to ensure use of monomeric A β species.

Brain Clearance Model—Central nervous system clearance of 125 I-labeled wild-type A β 40 or Dutch/Iowa mutant A β 40 was determined simultaneously with [14 C]inulin (a metabolically inert reference marker) in male C57Bl/6 wild-type mice (8–10 weeks old) as described (15). A stainless steel guide cannula was implanted stereotactically into the right caudate putamen of anesthetized mice (60 mg/kg sodium pentobarbital administered intraperitoneally), and 0.5 μ l of tracer fluid containing 125 I-labeled wild-type A β 40 or Dutch/Iowa mutant A β 40 (1–120 nM) were injected over 5 min along with [14 C]inulin using the Ultra Micropump (World Precision Instruments, Inc., Sarasota, FL). Radioactivity analysis was performed within 30 min.

Tissue Sampling and Radioactivity Analysis—Brains were sampled and prepared for radioactivity analysis. Degradation of 125 I-labeled A β 40 peptides was initially studied by trichloroacetic acid precipitation. Previous studies with 125 I-labeled A β 40 demonstrated an excellent correlation between the trichloroacetic acid and HPLC methods (15, 19, 21). Brain samples were mixed with trichloroacetic acid (10% final concentration) and centrifuged at $14,000 \times g$ for 8–10 min at 4 °C, and the radioactivity in the precipitate, water, and chloroform fractions was determined in a γ -counter. The integrity of 125 I-labeled wild-type A β or Dutch/Iowa mutant A β injected into the brain was $\geq 99\%$ as determined by trichloroacetic acid analysis. Degradation of 125 I-labeled A β peptides in the brain was further studied by HPLC and SDS-PAGE analyses. Following intracerebral injections of 125 I-labeled A β , brain tissue was homogenized in phosphate-buffered saline containing proteinase inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 mM *p*-aminobenzamide) and centrifuged at $100,000 \times g$ for 1 h at 4 °C. The supernatant was then lyophilized. The resulting material was dissolved in 0.005% trifluoroacetic acid (pH 2.0) in water before injection onto a Vydac C₄ column (Separations Group, Hesperia, CA). Separation was achieved with a 30-min linear gradient of 25–83% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min as described (15). Under these conditions, the A β standards eluted between 29.1 and 31.2 min for the wild-type A β 40 and Dutch/Iowa mutant A β 40 peptides. The eluted fractions were collected and counted. The integrity of 125 I-labeled A β peptides injected into the brain was $>98\%$ as determined by HPLC analysis, confirming the results of trichloroacetic acid analysis. For SDS-PAGE analysis, trichloroacetic acid-precipitated samples were resuspended in 1% SDS, vortexed, incubated at 55 °C for 5 min, neu-

tralized, boiled for 3 min, homogenized, and analyzed by electrophoresis on 10% Tris/Tricine gels, followed by fluorography. Lyophilized HPLC fractions were resuspended in sample buffer, neutralized, boiled, and electrophoresed as we reported previously (15, 19).

Calculations—The percentage radioactivity remaining in the brain after microinjection was determined from the following equation: % recovery in brain = $100 \times (N_b/N_i)$, where N_b is the radioactivity remaining in the brain at the end of the experiment, and N_i is the radioactivity injected into the brain. In all calculations, the dpm values for [^{14}C]inulin and the cpm values for trichloroacetic acid-precipitable [^{125}I] radioactivity reflecting the intact peptide were used. Inulin was studied as a reference marker that is neither transported across the blood-brain barrier nor retained by the brain and therefore reflects the rate of transport via passive diffusion of the interstitial fluid (interstitial fluid) bulk flow (15, 19). In the case of Aβ, there are two possible

physiological pathways of elimination: direct transport across the blood-brain barrier into the bloodstream and elimination via interstitial fluid bulk flow into the cerebrospinal fluid and cervical lymphatics (15). All calculations were based on 30-min experiments.

Statistical Analysis—Data were analyzed by multifactorial analysis of variance, Student's *t* test, and Dunnett's *t* test.

RESULTS

Generation of Tg-SwDI Mice Expressing Vasculotropic Mutant AβPP—The E22Q and D23N mutations within Aβ primarily manifest as strong cerebrovascular amyloid-depositing disorders (5–8). To assess the effects of these CAA-associated Aβ mutations *in vivo*, we generated transgenic mice expressing the human AβPP770 isoform harboring the Swedish, Dutch, and Iowa mutations in neurons of the central nervous system under the control of the mouse Thy1.2 promoter (Fig. 1). The Swedish K670N/M671L mutation was included in the AβPP transgene to enhance β-secretase processing and production of Aβ (4, 22). The adjacent Dutch E693Q and Iowa D694N mutations were included in the human AβPP transgene since we previously showed that the presence of both these mutations in Aβ markedly enhances the *in vitro* cerebrovascular pathogenic properties of Aβ compared with either single mutation (11). The transgenic mice were generated by microinjection of the AβPP770-SwDI construct into oocytes in a pure C57Bl/6 background. The presence of the transgene was confirmed by PCR analysis. All mice used in the subsequent characterization studies were heterozygous for the human AβPP transgene.

AβPP Expression and Aβ Production in Tg-SwDI/B Mice—The first transgenic mouse line generated (designated Tg-SwDI/B) showed expression of human AβPP in the brain (Fig. 2A). Although human AβPP expression was observed in the cortex, hippocampus, and brain stem, much lower levels were observed in the cerebellum, with no detectable expression in other non-neural tissues (Fig. 2B). However, analysis using a

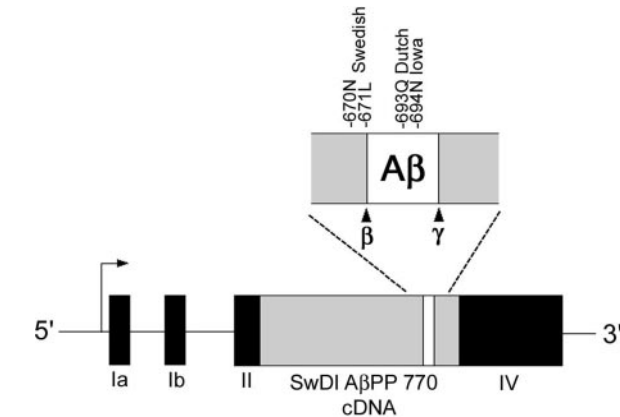
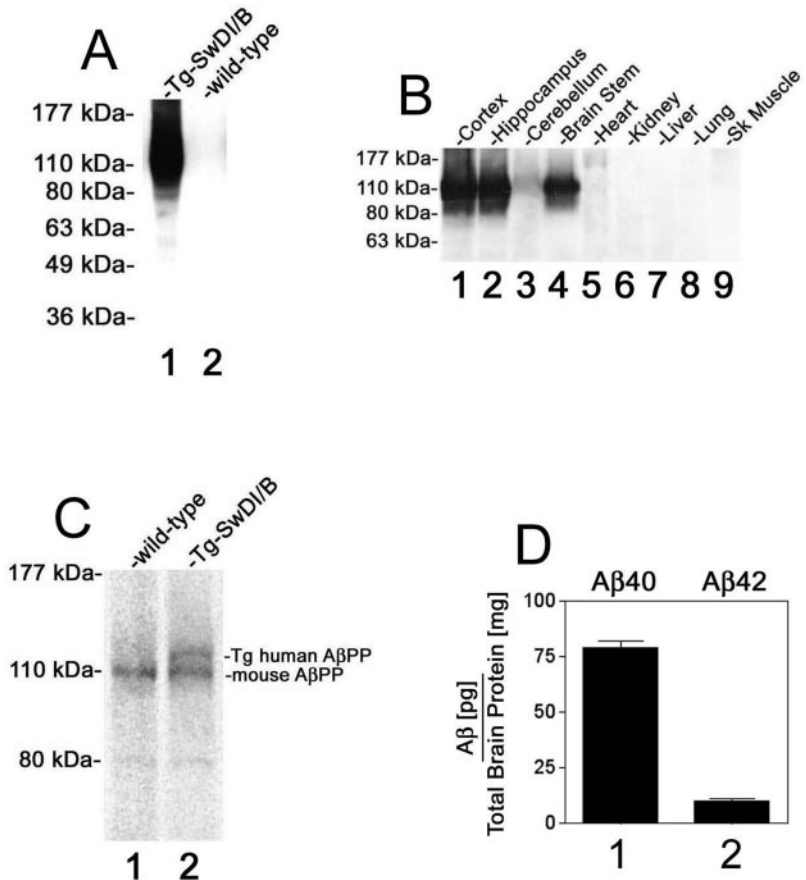


FIG. 1. Schematic of the mouse Thy1.2 promoter-transgenic human SwDI mutant AβPP construct. The 9-kb transgene construct used to produce the transgenic mice was composed of cDNA from human AβPP770, containing the Swedish K670N/M671L, Dutch E693Q, and Iowa D694N mutations, subcloned between exons II and IV of a Thy1.2 expression cassette.

FIG. 2. Analysis of transgenic human AβPP expression and Aβ levels in Tg-SwDI/B mice. Quantitative immunoblotting was performed as described under "Experimental Procedures." A, immunoblot analysis of human AβPP expression in total brain homogenates from Tg-SwDI/B (lane 1) and wild-type (lane 2) mice. B, immunoblot analysis of human AβPP expression in tissue homogenates prepared from different brain regions and peripheral tissues of Tg-SwDI/B mouse. Sk, skeletal. C, immunoblot analysis of endogenous mouse AβPP and transgenic (Tg) human AβPP in wild-type mouse brain (lane 1) and Tg-SwDI/B mouse brain (lane 2) homogenates. D, the levels of total Aβ40 (lane 1) and total Aβ42 (lane 2) determined in 2-month-old Tg-SwDI/B mouse forebrains by ELISA measurements as described under "Experimental Procedures." The data presented are the means ± S.D. of triplicate measurements in three mice.



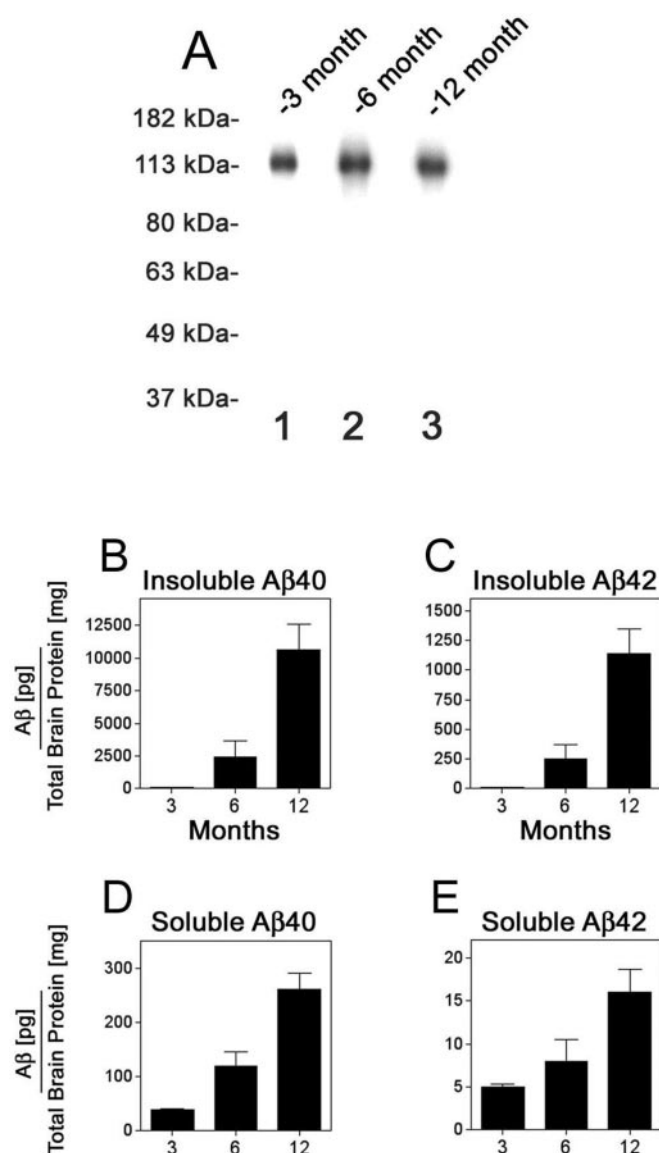


FIG. 3. Quantitation of temporal expression of A β PP and temporal accumulation of A β peptides in Tg-SwDI/B mouse brain. A, shown are the results from immunoblot analysis of the temporal expression of transgenic human A β PP in Tg-SwDI/B mouse forebrain tissue homogenates. B–E, the temporal accumulation of insoluble (B and C) and soluble (D and E) A β 40 and A β 42 was determined by sequential carbonate and guanidine extraction, respectively, from mouse forebrains as described under “Experimental Procedures.” The data presented are the means \pm S.D. from six to eight Tg-SwDI/B mice/time point.

monoclonal antibody that detects both endogenous mouse A β PP and transgenic human A β PP unexpectedly revealed that expression of transgenic human A β PP was modest, and it was estimated by quantitative image analysis to be only at $<50\%$ the level of endogenous mouse A β PP (Fig. 2C). Consistent with the low level expression of transgenic human A β PP, young Tg-SwDI/B mice (≈ 2 months old) exhibited very low levels of total A β 40 and A β 42 in the brain, with the predominant species being the shorter A β 40 peptide (Fig. 2D).

Over the course of 1 year, the expression of transgenic human A β PP protein remained consistently low and was estimated to be $\approx 33 \pm 4$ ng/mg of total brain protein based on comparative quantitative immunoblot measurements against known concentrations of purified human A β PP (Fig. 3A). Despite the continuous paucity of transgenic human A β PP expression, quantitative ELISA analysis revealed a progressive

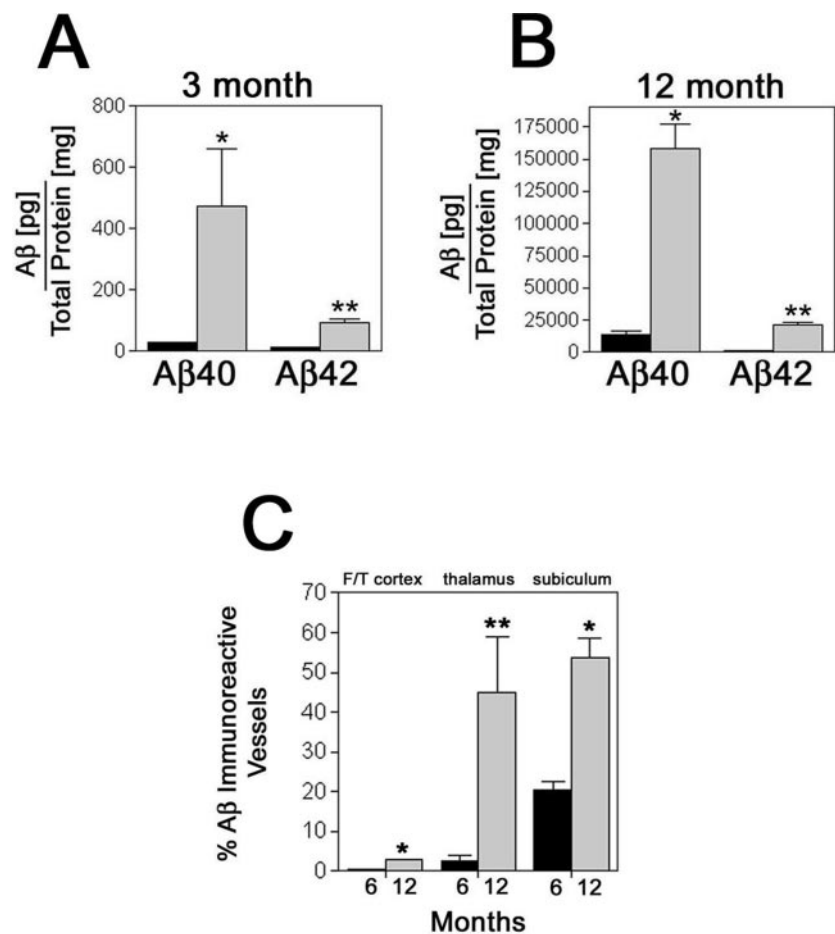
and robust accumulation of insoluble A β 40 and A β 42 in the Tg-SwDI/B mice (Fig. 3, B and C, respectively). The increase in insoluble A β peptides first appeared at 3 months and markedly increased by 12 months. The levels of soluble A β 40 and A β 42 increased several-fold through 12 months (Fig. 3, D and E, respectively), but composed a minor fraction compared with the amount of accumulating insoluble A β peptides (Fig. 3, B and C). In each case of insoluble and soluble A β peptides, the A β 40 levels were ≈ 10 -fold higher than the A β 42 levels. The antibodies used to detect A β in Tg-SwDI/B mice were human-specific, *i.e.* did not recognize mouse A β , and were directed to the first five amino acids of A β , indicating that they possess an intact amino terminus (13, 14). Furthermore, a monoclonal antibody directed against the mid-region of wild-type A β did not recognize either synthetic Dutch/Iowa A β 40 or A β in Tg-SwDI/B mouse brain (data not shown). These findings indicate that, despite low expression of transgenic human SwDI mutant A β PP, the resulting Dutch/Iowa mutant A β peptides exhibit a striking early and robust accumulation in mouse brain.

Early and Progressive Vascular A β Deposition in Tg-SwDI/B Mice—Since the Dutch and Iowa mutations are associated with familial disorders that develop prominent cerebrovascular amyloid deposition, we were particularly interested in the level of brain vascular accumulation of A β in the Tg-SwDI/B mice. Quantitative ELISA measurement of vascular A β showed that, at 12 months, the levels of A β 40 and A β 42 were 12- and 14-fold higher, respectively, in brain microvessels isolated from Tg-SwDI/B mice forebrains than in the whole forebrain tissue homogenates (Fig. 4B). At ~ 3 months of age, the levels of A β 40 and A β 42 were similarly significantly higher in brain microvessels than in the whole forebrains of Tg-SwDI/B mice (Fig. 4A), but the absolute levels were much lower compared with the 12-month-old mice, as expected. Quantitative regional analysis of Tg-SwDI/B mice forebrain tissue showed that vascular A β in the thalamus and subiculum was highly prominent and significantly increased with age; 45–55% of the vessels were affected in these regions at 12 months (Fig. 4C).

Moreover, immunohistochemical analysis revealed that, starting at ~ 6 months and increasing with age, numerous A β accumulations in and around microvessels, particularly in the thalamic and subiculum regions of the brain (Fig. 5, A and C, respectively), were observed in the Tg-SwDI/B mice. The microvascular A β accumulations were mainly fibrillar, displaying strong thioflavin S staining (Fig. 5B). Although much less common, some arterioles in these regions showed strong vascular and perivascular A β deposition (Fig. 5, D and E). Other blood vessel-rich regions, including the hippocampal fissure, consistently exhibited large accumulations of perivascular A β (Fig. 5F). Occasionally, evidence of microhemorrhage was observed as assessed by hemosiderin staining adjacent to microvessels with A β deposits (Fig. 5G).

In addition to vascular amyloid deposits, immunohistochemical analysis revealed the presence of A β plaque-like deposits in the brains of Tg-SwDI/B mice beginning at ~ 3 months of age (Fig. 6, A and D). These deposits initially appeared in the regions of the subiculum, hippocampus, and cortex. As the Tg-SwDI/B mice aged to 6 months, the A β plaque-like deposits became more numerous and appeared in the olfactory bulb and thalamic region as well (Fig. 6, B and E). By 12 months of age, the Tg-SwDI/B mice showed A β deposition throughout most of forebrain (Fig. 6, C and F). In contrast to the fibrillar vascular A β deposits, the overwhelming majority of the parenchymal A β accumulations presented as diffuse plaque-like deposits (Fig. 6G), consistent with the parenchymal A β deposits that are present in patients with the Dutch and Iowa disorders (5–8). Occasionally, A β plaque-like deposits that had a more compact

FIG. 4. Quantitation of the temporal and regional accumulation of vascular A β in Tg-SwDI/B mouse brain. The A β 40 and A β 42 concentrations were determined in total forebrain homogenates (black bars) and forebrain isolated microvessels (gray bars) from 3-month-old (A) and 12-month-old (B) Tg-SwDI/B mice. The data presented are the means \pm S.D. from four mice at each age. *, $p < 0.05$; **, $p < 0.001$. The vascular A β profiles in the frontotemporal (F/T) cortex, thalamus, and subiculum in 6-month-old (black bars) and 12-month-old (gray bars) Tg-SwDI/B mice were quantitated as described under "Experimental Procedures" (C). The data presented are the mean \pm S.D. from four mice at each age. *, $p < 0.001$; **, $p < 0.05$.



structure and that stained with thioflavin S were observed in the brain, suggesting that they were fibrillar (Fig. 6, *H* and *I*). However, it is notable that these more compact, thioflavin S-positive A β deposits were very rare and randomly found and did not appreciably increase with age in the Tg-SwDI/B mice.

Consistent Robust Accumulation of Dutch/Iowa Mutant A β in Independent Tg-SwDI Mouse Lines—We generated two additional Tg-SwDI mouse lines to confirm the unique properties of Dutch/Iowa mutant A β in the brain observed in the initial findings presented above with Tg-SwDI/B mice. Tg-SwDI/A and Tg-SwDI/B mice were found to express similar low levels of transgenic human A β PP protein, whereas Tg-SwDI/C mice expressed nearly twice the amount of human A β PP in the brain compared with the former two lines (Fig. 7, *A* and *B*). Nevertheless, the levels of human A β PP in Tg-SwDI/C mice were still no higher than those of endogenous mouse A β PP in the brain. ELISA measurement of A β 40 and A β 42 levels at 6 months of age showed that Tg-SwDI/A and Tg-SwDI/B mice, which expressed similar levels of human A β PP, accumulated similar levels of soluble and insoluble A β 40 and A β 42 (Fig. 7, *C* and *D*). However, Tg-SwDI/C mice, which expressed nearly twice the levels of human A β PP compared with the other two Tg-SwDI lines, accumulated nearly four times the amount of soluble and insoluble A β 40 and A β 42 in the brain (Fig. 7, *C* and *D*).

Immunohistochemical analysis for A β showed that, at 1 year of age, all three Tg-SwDI lines developed strong accumulation of A β in the brain microvasculature (Fig. 8, *A–C*) and largely diffuse, plaque-like deposits in the parenchyma (Fig. 8, *D–F*), with similar regional deposition in the brain (Fig. 8, *G–I*). Together, these findings demonstrate that, despite the low levels of transgenic human A β PP expression, the early-onset and robust accumulation of Dutch/Iowa mu-

tant A β in the cerebral microvasculature and brain parenchyma is a unique and consistent phenotype in independent Tg-SwDI lines.

Increased Brain Retention of Dutch/Iowa Mutant A β —We next determined whether the early and robust accumulation of Dutch/Iowa mutant A β at perivascular/vascular sites and in the parenchyma in Tg-SwDI mice could be attributed to reduced elimination of this mutant form of A β in the brain. To test this, we compared the retention of Dutch/Iowa mutant A β 40 and wild-type A β 40 peptides from mouse brain after intracerebral microinjections of radiolabeled peptides at different carrier concentrations (15). In these experiments, we focused on A β 40 peptides since this is largely the predominant form that accumulates in the Tg-SwDI mice (Figs. 3, 4, and 7). Retention was estimated within the first 30 min after intracerebral injections of peptides, as described previously for mice and squirrel monkeys (15, 23). At 1 nM peptide, which is somewhat higher than the physiological levels of A β in the cerebrospinal/brain interstitial fluid in mice (24, 25), the retention of Dutch/Iowa mutant A β 40 was \approx 10-fold greater than that of wild-type A β 40 (Fig. 9A). At the higher concentration of 12 nM, corresponding to the pathophysiological A β 40 cerebrospinal/brain interstitial fluid levels in transgenic mice (24, 25), wild-type A β 40 exhibited reduced elimination compared with that at the lower concentration of 1 nM, consistent with a saturable nature of the A β brain clearance mechanism *in vivo* (15). However, the retention of Dutch/Iowa mutant A β 40 at 12 nM was still significantly higher by \approx 2.5-fold compared with wild-type A β 40 at 12 nM (Fig. 9A). At 120 nM, Dutch/Iowa mutant A β 40 was almost completely retained in the brain, whereas wild-type A β 40 still exhibited measurable disappearance, but approached its elimination limit close to 120 nM

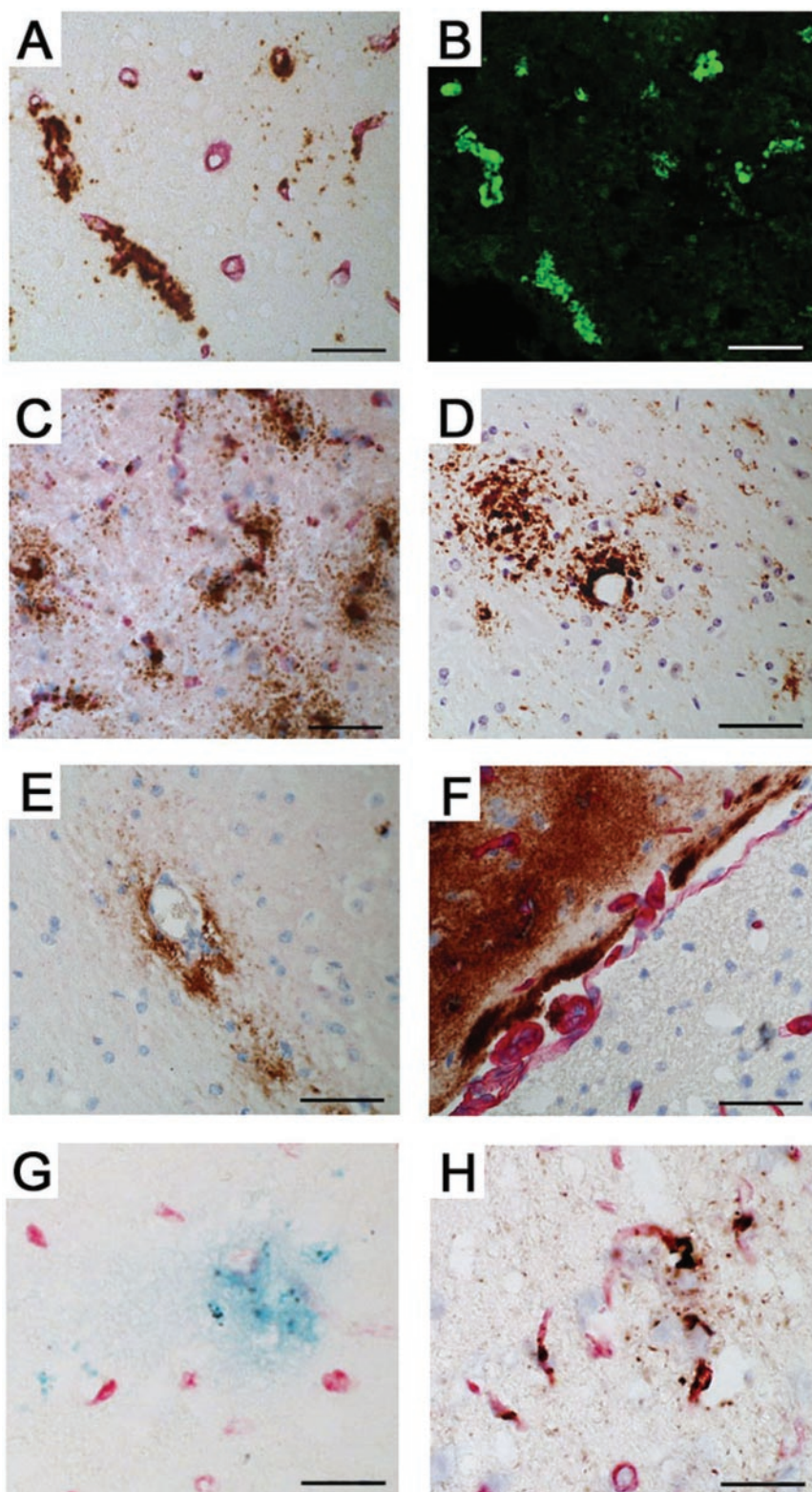


FIG. 5. Immunohistochemical analysis of cerebrovascular A β accumulation in Tg-SwDI/B mouse brain. Tg-SwDI/B mouse brain sections from 12-month-old animals were double-immunostained for A β (brown) and collagen type IV (red) or stained with thioflavin S to identify fibrillar deposits as described under "Experimental Procedures." A, double immunostaining showing prominent microvascular A β deposits in the thalamus; B, thioflavin S staining of vascular A β deposits in the thalamus; C, double immunostaining showing prominent microvascular A β deposits in the subiculum; D and E, double immunostaining showing vascular and perivascular A β deposits in larger cerebral vessels in the thalamus; F, double immunostaining of A β accumulations in the blood vessel-rich hippocampal fissure; G, Prussian blue staining revealing previous microhemorrhage in the thalamus; H, adjacent double-immunostained tissue section showing microvascular A β deposits at the site of previous microhemorrhage. Scale bars = 50 μ m.

(15). The interstitial fluid drainage assessed by elimination of the simultaneously infused reference marker inulin in the presence of either A β peptide was not significantly affected (Fig. 9B). These results suggest that the differences in retention between the two peptides are unlikely to reflect differences in removal through cerebrospinal/brain interstitial fluid bulk flow and may therefore reflect primarily significantly reduced clearance of Dutch/Iowa mutant A β from the brain into the bloodstream.

DISCUSSION

A β deposition in the cerebral vasculature and plaque-like structures are prominent pathological features of Alzheimer's disease and several rare familial CAA disorders (1–3, 7, 8). Investigation into the pathogenic effects of A β in Alzheimer's disease has been bolstered by the generation of several transgenic mouse models that express human forms of A β PP in the brain and that develop age-dependent A β deposits in the central nervous system (26–30). Generally, the successful mouse

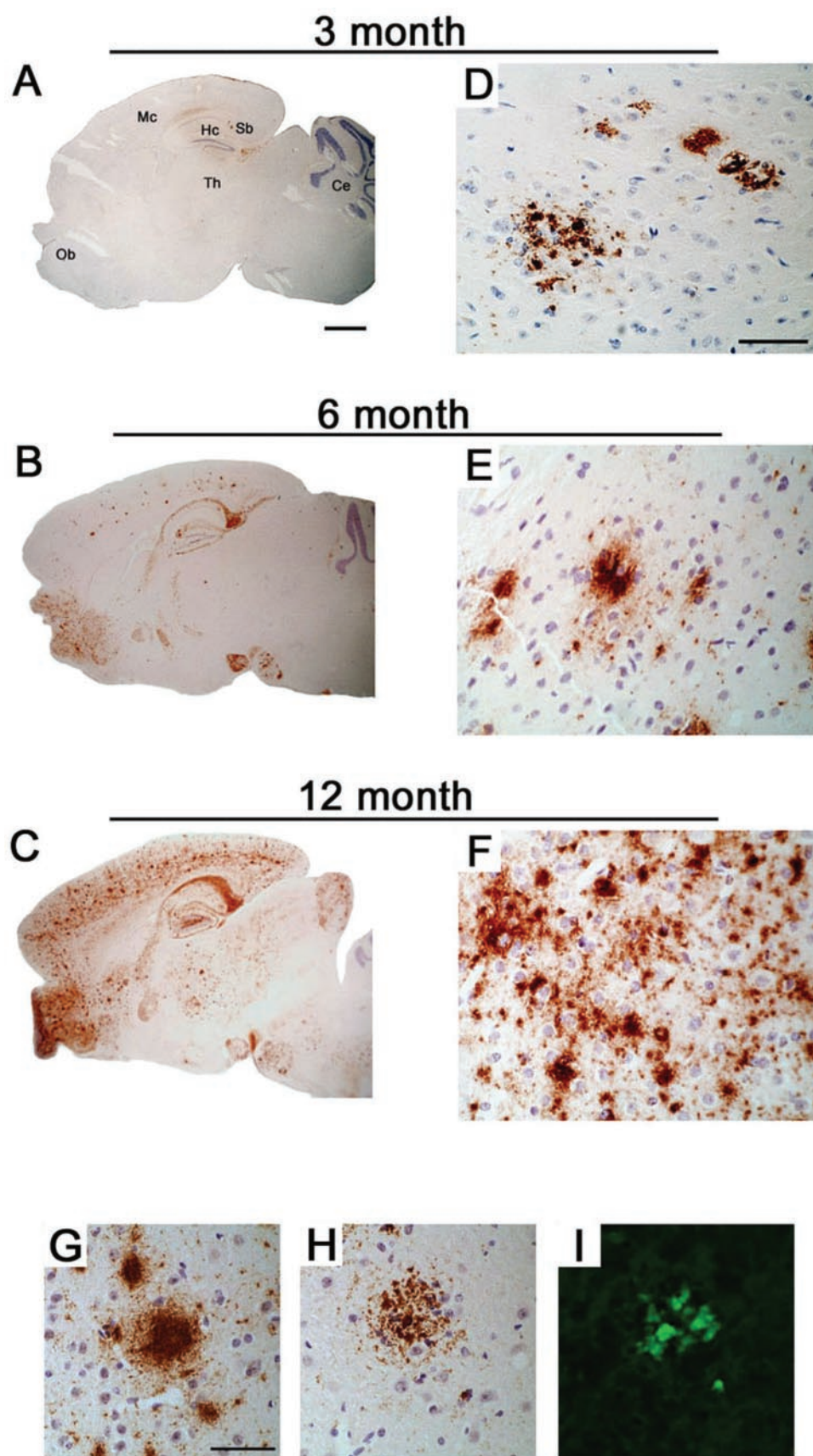


FIG. 6. **Immunohistochemical analysis of parenchymal A β accumulation in Tg-SwDI/B mouse brain.** A–C, Tg-SwDI/B mouse brain sections (3, 6, and 12 months old, respectively) immunostained for A β as described under “Experimental Procedures.” Ce, cerebellum; Hc, hippocampus; Mc, motor cortex; Ob, olfactory bulb; Sb, subiculum; Th, thalamus. Scale bar = 1 mm. D–F, higher magnifications of A β deposits in the motor cortex of Tg-SwDI/B mouse brain (3, 6, and 12 months old, respectively). Scale bar = 50 μ m. G and H, immunostaining of diffuse and compact A β plaque-like deposits, respectively. I, thioflavin S staining of compact fibrillar A β deposits. Scale bar = 50 μ m.

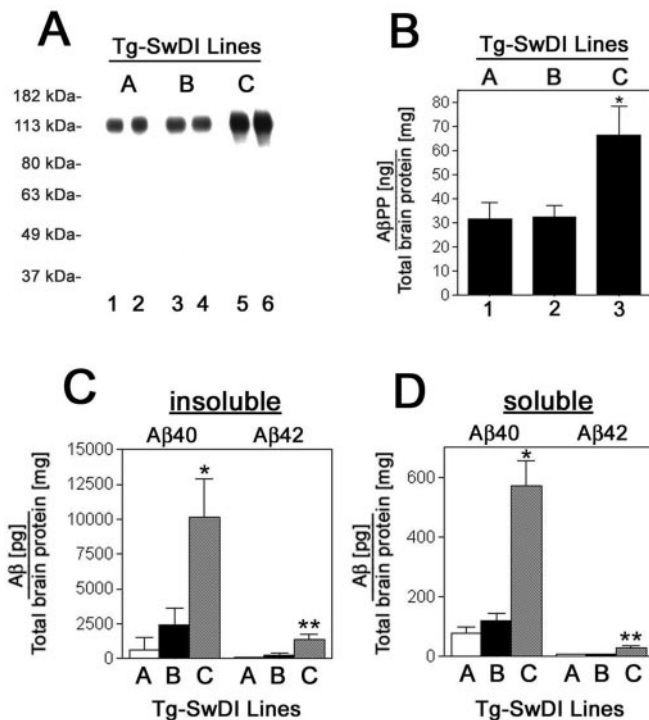


FIG. 7. Quantitation of A β PP expression and A β accumulation in different Tg-SwDI mouse lines. Shown are the results of immunoblot analysis of transgenic human A β PP expression in 3-month-old Tg-SwDI mouse forebrain tissue homogenates (A). Lanes 1 and 2, Tg-SwDI/A mice; lanes 3 and 4, Tg-SwDI/B mice; lanes 5 and 6, Tg-SwDI/C mice. The levels of transgenic human A β PP expression in Tg-SwDI mouse forebrain tissue homogenates were determined by quantitative immunoblotting as described under "Experimental Procedures" (B). Lane 1, Tg-SwDI/A mice; lane 2, Tg-SwDI/B mice; lane 3, Tg-SwDI/C mice. The data presented are the means \pm S.D. from four mice in each line. *, $p < 0.01$. The accumulation of insoluble (C) and soluble (D) A β 40 and A β 42 was determined by sequential carbonate and guanidine extraction, respectively, from 6-month-old mouse forebrains from the different Tg-SwDI lines as described under "Experimental Procedures." The data presented are the means \pm S.D. from four to six mice in each Tg-SwDI line. *, $p < 0.05$; **, $p < 0.01$.

models to date have relied on marked overexpression of human A β PP containing one or more mutations flanking the β - and γ -secretase processing sites associated with familial forms of Alzheimer's disease (26–30). Mutations at these key processing sites in the A β PP transgene were included to promote total production of A β peptides and/or to increase the production of the longer, more pathogenic A β 42 isoform. Most of these transgenic mouse lines primarily accumulate A β plaque-like deposits, although several lines have been found to start developing CAA after 12 months of age (18, 31–33). However, in each of these mouse models, A β PP overexpression is required to produce and deposit human wild-type A β peptides. Here, we have described the generation and initial characterization of transgenic mice that express, in the brain, very low levels of human A β PP containing Dutch/Iowa CAA double mutations that produce vasculotropic mutant A β model peptides.

To generate the Tg-SwDI mice, we used the mouse Thy1.2 promoter, which provides neuronal expression of the transgene in the central nervous system. Although the use of this promoter is somewhat limiting in that it provides for only a neuronal source of A β PP and A β , it was previously used to successfully generate two independent transgenic mouse lines overexpressing Swedish K670N/M671L mutant A β PP and producing wild-type A β peptides that developed extensive fibrillar plaque deposits in the brain and some cerebrovascular amyloid after 12 months (18, 31–33). In our transgenic A β PP cDNA

construct, we similarly included the tandem Swedish mutations to help promote A β production (Fig. 1). However, we also incorporated the tandem Dutch and Iowa mutations (E693Q/D694N) in A β PP, resulting in the production of Dutch/Iowa CAA double mutant A β peptides. Our earlier *in vitro* studies showed that Dutch/Iowa mutant A β , with the loss of two negative charges at positions 22 and 23, possesses more robust fibrillogenic and pathogenic properties (11). Therefore, the present Tg-SwDI/B mice provide a novel *in vivo* model to investigate the central nervous system activities of this experimental peptide with amplified fibrillogenic and pathogenic properties compared with either CAA single mutant form of A β .

Analysis of human A β PP expression in the Tg-SwDI/B mouse line showed restriction to the brain, consistent with neuronal expression of the Thy1.2 promoter. However, further comparative analysis indicated that transgenic human A β PP was modestly expressed at levels actually below those of endogenous mouse A β PP. Furthermore, the initial levels of A β peptides in very young 2-month-old Tg-SwDI/B mice were quite low, consistent with the low transgenic human A β PP expression. At first glance, this was disappointing since previous successes in generating A β pathology in earlier transgenic mouse models relied on marked overexpression (as high as 7-fold) of transgenic human A β PP (26–30). Therefore, it was surprising to find that, as the Tg-SwDI/B mice continued to age, they accumulated large amounts of human insoluble mutant A β 40 and A β 42 peptides. In fact, A β accumulation in Tg-SwDI/B mice was much earlier in onset and more robust than that reported for the commonly used Tg-2576 mouse line, which highly overexpresses human A β PP (27). The Tg-SwDI/B mice expressed much lower levels of human A β PP than reported for other A β PP-expressing transgenic mice, indicating that the presence of the tandem charge-altering Dutch/Iowa mutations within the A β peptides produced in the present model has a striking effect on the accumulation of A β in mouse brain.

Although the ELISA measurements of total forebrain homogenates clearly showed robust accumulation of A β in Tg-SwDI/B mouse brain, they did not reveal in which structures this accumulation was most pronounced. The subsequent compartmental quantitative ELISA analysis showed a 12–14-fold higher association of A β with isolated brain microvessels compared with whole forebrain tissue at 3 and 12 months, underscoring the vasculotropic nature of the Dutch/Iowa mutant A β peptides. Furthermore, immunohistochemical analysis confirmed that Tg-SwDI/B mice developed progressive and extensive A β deposits in cerebral blood vessels, most notably in the microvasculature. It is noteworthy that, in the regions of the subiculum and thalamus, with high numbers of microvessels with deposited A β (Fig. 4C), A β was found to congregate in and closely around the microvessels, with little deposition in the parenchymal tissues between the vessels (Fig. 5, A–C). This observation emphasizes the highly vasculotropic nature of Dutch/Iowa mutant A β peptides in these brain regions. Although other human A β PP-expressing transgenic mice develop cerebrovascular A β deposition, this has been found to generally occur in mice older than 12 months of age, with predominant accumulation in meningeal vessels (18, 31–33). On the other hand, the cerebrovascular A β deposits in Tg-SwDI/B mice developed much earlier (*i.e.* starting at 6 months) and were abundant in the microvasculature of the thalamic and subiculum regions. Although there was much less involvement of the meningeal and cortical vessels in Tg-SwDI mice, the extent of cortical vascular A β (*i.e.* $\approx 4\%$) was still appreciably higher than in other A β PP-expressing transgenic mice (33). The find-

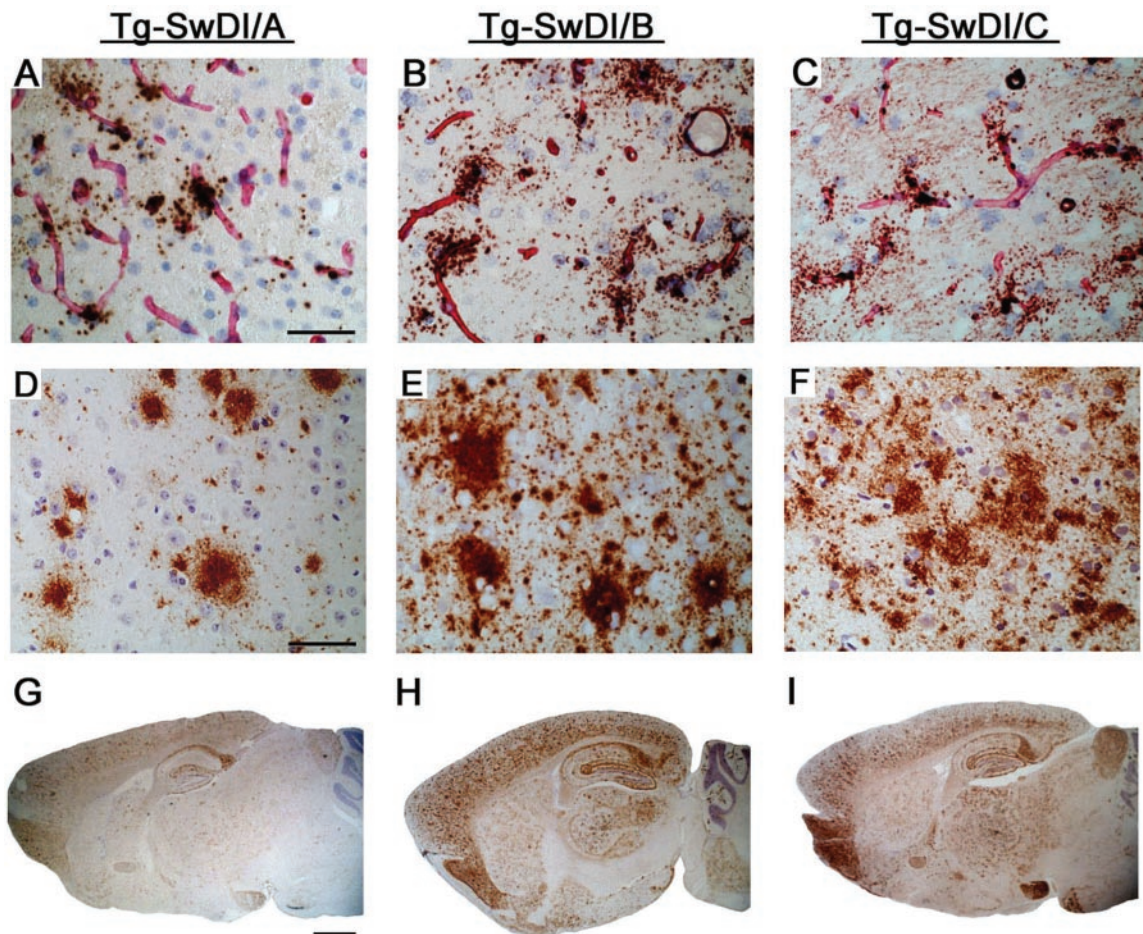
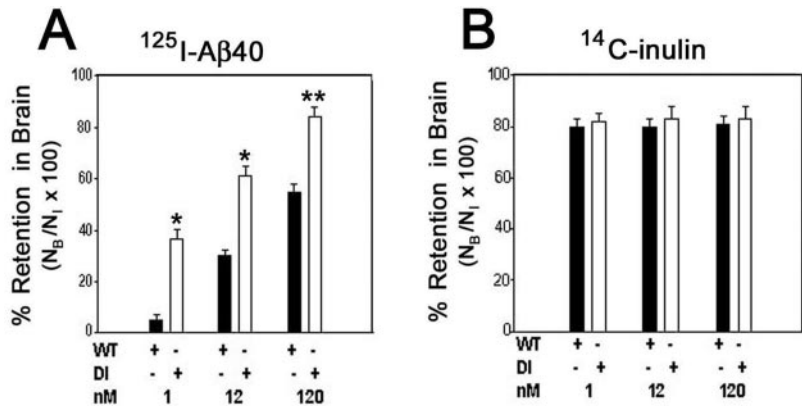


FIG. 8. **Immunohistochemical analysis of Aβ accumulation in Tg-SwDI mouse brains.** Mouse brain sections from 12-month-old animals of three independent Tg-SwDI lines were double-immunostained for Aβ (brown) and collagen type IV (red) to identify Aβ deposits as described under “Experimental Procedures.” A–C, double immunostaining showing prominent microvascular Aβ deposits in the thalamic region in each Tg-SwDI line. Scale bar = 50 μm. D–F, immunostaining showing diffuse Aβ deposits in the motor cortex in each Tg-SwDI line. Scale bar = 50 μm. G–I, immunostaining showing similar regional Aβ accumulation in each Tg-SwDI line. Scale bar = 1 mm.

FIG. 9. **Deficient clearance of Dutch/Iowa mutant Aβ40 peptides from mouse brain.** A, retention of ¹²⁵I-labeled wild-type Aβ40 (WT; black bars) and Dutch/Iowa mutant Aβ40 (DI; open bars) in the brain at different peptide concentrations. B, slow elimination of simultaneously infused [¹⁴C]inulin in the experiments shown in A indicating that inulin clearance was not affected by either of the Aβ peptides. The data presented are the means ± S.D. from three to eight separate measurements for each concentration. *, *p* < 0.001; **, *p* < 0.01.



ing that the vascular Aβ deposits were largely fibrillar (showing strong thioflavin S staining) is consistent with the nature of the cerebrovascular Aβ deposits found in patients with either the Dutch- or Iowa-type CAA disorder (7, 8, 34). Occasionally, evidence of prior microhemorrhage was observed in 12-month-old Tg-SwDI/B mice using Prussian blue iron staining to identify residual hemosiderin adjacent to amyloid-laden microvessels. Although these events were rare at 12 months, it is anticipated that they will become more common as the Tg-SwDI/B mice age.

Tg-SwDI/B mice showed the appearance of Aβ plaque-like deposits at an early 3 months of age, with a progressive and

widespread accumulation by 1 year. Similar to the early onset of microvascular Aβ deposits, the accumulation of Aβ plaque-like structures markedly precedes the development of deposits in other Aβ-depositing, human AβPP-expressing transgenic mice. Since Dutch/Iowa mutant Aβ strongly assembles into fibrils *in vitro* (11), it was interesting to find that the overwhelming majority of the Aβ plaque-like deposits were of the diffuse type, whereas fibrillar, thioflavin S-staining plaques were extremely rare. However, this is highly consistent with the pathological findings of largely diffuse Aβ plaques in patients with either the Dutch- or Iowa-type CAA disorder (7, 8, 34). This suggests that the enhanced fibrillogenic properties of

Dutch and Iowa mutant A β observed *in vitro* do not directly translate *in vivo* to the parenchymal A β deposits in the brain. Perhaps other A β -interacting molecules in the brain, which likely facilitate or impede parenchymal fibril formation, possess different specificity for wild-type and Dutch and Iowa mutant A β peptides.

Two additional Tg-SwDI mouse lines subsequently generated, again with levels of human A β PP expression at or below those of endogenous mouse A β PP, exhibited early-onset and robust accumulation of A β peptides in the brain similar to that found in Tg-SwDI/B mice. A dosage effect was observed where Tg-SwDI/C mice, which expressed approximately twice the amount of human A β PP compared with Tg-SwDI/A and Tg-SwDI/B mice, accumulated significantly more A β in the brain compared with the latter two transgenic lines. However, all three Tg-SwDI lines showed consistent A β accumulations as strong microvascular deposits and diffuse plaque-like structures. These findings, from three independent Tg-SwDI mouse lines, clearly demonstrate the unique behavior of Dutch/Iowa mutant A β peptides in mouse brain. Importantly, this novel and reproducible transgenic mouse paradigm demonstrates that vast overexpression of human A β PP is not required for the accumulation of A β in mouse brain. This suggests that Tg-SwDI mice provide a unique *in vivo* model to study A β accumulation in the brain without the confounding issue of abnormally high expression of human A β PP that is commonly found in other A β PP-expressing transgenic mice.

The mechanisms responsible for cerebrovascular accumulation of A β remain unclear, although recent studies suggest that they may involve ineffective transport of A β out of the central nervous system and into the circulation (15, 35–37). Therefore, it was interesting to find that, in addition to A β deposition in the microvessels, there was a pronounced accumulation of A β around the immediate vicinity of the vessels and in blood vessel-rich regions such as the hippocampal fissure (Fig. 4F). This suggests that the deficient clearance of Dutch/Iowa mutant A β from the central nervous system at the cerebral vasculature may account for this striking accumulation around cerebral blood vessels and also in the parenchyma. Accordingly, *in vivo* clearance studies of ¹²⁵I-labeled A β peptides at varying carrier concentrations suggested that, compared with wild-type A β 40, Dutch/Iowa mutant A β 40 was retained in the brain (Fig. 9). This is similar to the finding of Monro *et al.* (38) in guinea pigs that demonstrated decreased elimination of Dutch mutant A β from the cerebrospinal fluid. It has been suggested the blood-brain barrier removes A β 40 from the brain largely via low density lipoprotein receptor-related protein-1 (15). Thus, further studies are needed to clarify whether retention of Dutch/Iowa mutant A β reflects its reduced affinity for low density lipoprotein receptor-related protein-1-mediated clearance.

Recent work indicated that bidirectional transport of A β across the blood-brain barrier is the dominant pathway regulating the concentrations of A β in the central nervous system under physiological and pathophysiological conditions (19, 21, 23, 37–41). Peripheral A β -sequestering agents, including anti-A β antibody (40, 41), a soluble form of the receptor for advanced glycation end products (19), and gelsolin and ganglioside GM1 (42), may all promote clearance of central nervous system-derived A β into the bloodstream as shown in different types of transgenic mice expressing A β PP. Several physiological and pharmacological modifiers of A β transport clearance systems have been described, including insulin-like growth factor I (43) and apolipoprotein E, α_2 -macroglobulin, and apolipoprotein J (21, 39, 44). The present data suggest that the observed retention of Dutch/Iowa mutant A β in the brain likely contributes to its striking accumulation around cerebral blood

vessels and in the parenchyma of the Tg-SwDI mice. Although not a precise model of Dutch- or Iowa-type familial CAA, Tg-SwDI mice mimic many of the features of these disorders and provide a novel and useful experimental paradigm to investigate the effects of these vasculotropic mutations *in vivo* and potential therapies for CAA.

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Early-onset and Robust Cerebral Microvascular Accumulation of Amyloid β -Protein in Transgenic Mice Expressing Low Levels of a Vasculotropic Dutch/Iowa Mutant Form of Amyloid β -Protein Precursor

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