Cerebral Aβ deposition in an Aβ-precursor protein-transgenic rhesus monkey

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Abbreviations: APP, Aβ-precursor protein; APPSWE/IND, Gene coding for the Aβ-precursor protein with the Swedish (K670N/M671L) and Indiana (V717F) mutations; CAA, cerebral amyloid angiopathy; LCOs, luminescent conjugated oligothiophenes; pTau, Tau phosphorylated at threonine 181.

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A B S T R A C T

With the ultimate goal of developing a more representative animal model of Alzheimer’s disease (AD), two female amyloid-β-(Aβ) precursor protein-transgenic (APPtr) rhesus monkeys were generated by lentiviral transduction of the APP gene into rhesus oocytes, followed by in vitro fertilization and embryo transfer. The APP-transgene included the AD-associated Swedish K670N/M671L and Indiana V717F mutations (APPSWE/IND) regulated by the human polyubiquitin-C promoter. Overexpression of APP was confirmed in lymphocytes and brain tissue. Upon sacrifice at 10 years of age, one of the monkeys had developed Aβ plaques and cerebral Aβ-amyloid angiopathy in the occipital, parietal, and caudal temporal neocortices. The induction of Aβ deposition more than a decade prior to its usual emergence in the rhesus monkey supports the feasibility of creating a transgenic nonhuman primate model for mechanistic analyses and preclinical testing of treatments for Alzheimer’s disease and cerebrovascular amyloidosis.

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1. Introduction

The defining pathologic signature of Alzheimer’s disease (AD) is the accumulation of multimeric assemblies of amyloid-β (Aβ) in Aβ- ('senile') plaques along with hyperphosphorylated Tau in neurofibrillary tangles [1–4]. Genetic, pathologic and biomarker findings implicate the misfolding and seeded aggregation of Aβ as an essential early step in this pathogenic process, followed by tauopathy and numerous nonspecific neurodegenerative changes [2,5–7]. Genetically modified rodent models have yielded important insights into cellular and molecular mechanisms driving the pathogenesis of AD, but the development of effective treatments has been hampered by the lack of an animal model that fully manifests all features of the disorder [8–15]. Owing to their relative longevity and their phylogenetic proximity to humans, nonhuman primates have the potential to more faithfully replicate the pathobiology of AD [9,14–21].

Aβ is enzymatically released from the Aβ-precursor protein (APP) [22,23], a 695–770 amino acid protein that is highly homologous among primates [19,24]. All primate species analyzed to date express human-sequence Aβ [19,25]. As they age, nonhuman primates naturally exhibit cerebral Aβ-amyloidosis (see [13–15,19,20,25,26] for reviews). Tauopathy, while occasionally present, is generally mild or absent, and a dementia-like behavioral state involving profound impairments in multiple cognitive domains has not been demonstrated in an aged nonhuman primate [14].

One possible reason for the resistance of nonhuman primates to AD is chronological age; in most humans, clinical dementia does not set in until after the age of 65 years [27], and the accumulation of Aβ begins 20 years or more prior to the first clear signs of dementia [28,29]. Rhesus monkeys (Macaca mulatta) have a known maximum lifespan of over 40 years [30]. Assuming a maximum lifespan in humans of just over 120 years [31], rhesus monkeys thus can be considered to age at approximately three times the rate of humans. The monkeys begin to deposit Aβ in the brain (as Aβ plaques and cerebral Aβ-amyloid angiopathy [Aβ-CAA]) in their mid-20s [19]. In old age, some rhesus monkeys (and the closely related cynomolgus monkey [Macaca fascicularis]) show signs of incipient tauopathy [26,32–35], but this does not resemble in quantity or anatomical distribution the tauopathy of advanced AD [14,16,35]. It is possible that two or more decades are required for cerebral Aβ-proteopathy to incite AD-like tauopathy (profuse and widespread neurofibrillary tangles) and other neurodegenerative sequelae [14]. If so, it may be necessary to induce Aβ aggregation earlier in life to fully model AD-like pathology in a nonhuman primate. As a first step toward this goal, we generated transgenic rhesus monkeys expressing human APP with the AD-associated ‘Swedish’ and ‘Indiana’ mutations. By 10 years of age, one of the two transgenic monkeys had developed neocortical Aβ plaques and cerebral Aβ-amyloid angiopathy.

2. Materials and methods

2.1. Subjects

Two female rhesus monkeys (Macaca mulatta) were generated via lentiviral gene delivery into mature oocytes, followed by in vitro fertilization and transfer of embryos into surrogate female monkeys, as previously described [36]. Using established assisted reproductive techniques in rhesus monkeys [36–38], oocytes were recovered from adult, hormone-stimulated females for in vitro fertilization and culture. The lentiviral solution was injected into the perivitelline space of metaphase-II-arrested oocytes. The oocytes then were fertilized by the intracytoplasmic injection of sperm, followed by in vitro culture until the embryos were transferred to the surrogate females at the 4–8-cell stage [36–38]. The surrogates were chosen based on hormonal evidence that their reproductive stage was compatible with the embryos. (For details of our lentiviral transgenesis methodology for nonhuman primates, see [36–39]).

After delivery, the monkeys were reared in the primate nursery of the Emory National Primate Research Center according to procedures developed by Sackett and colleagues [40] to allow normal growth and the development of species-specific social skills. These procedures included daily social interactions with peers and intensive human contact (for specifics of the rearing conditions, see [41]). The animals were exposed to a 12 h/12 h light/dark cycle from birth. They were housed in quads (4 animals per large enclosure) starting at 6–7 months of age, and in pairs after approximately 12 months of age.

The primary control monkeys for the study were two age-matched, non-transgenic (wild-type) females that were nursery-reared in the same way as the APPtg monkeys, and who also served as controls for a parallel study of huntingtin-transgenic monkeys [42–44]. These animals (RCK12 and RFK12) were the controls for all imaging analyses and behavioral tests through 5 years of age, for longitudinal CSF measurement of Aβ and Tau through 9 years, for analysis of transgene expression in lymphocytes and neocortex, and for histological analysis at the end of the study. After 5 years of age, no further behavioral testing was undertaken with these monkeys, so three 6-year-old, nontransgenic nursery-reared females (designated Neo-C1, Neo-C3 and Neo-C5), which were controls for an investigation of early hippocampal lesions [45], served as controls on the Emotional Reactivity and Visual Paired Comparison tasks.

At the end of the study, the APPtg animals were humanely sacrificed (below) at the ages of 10 years, 45 weeks (APP1) and 10 years, 2 weeks (APP2). Control monkeys for histopathology (RCK12 and RFK12) both were sacrificed at 10 years, 4 weeks of age. All procedures were approved by the Emory University Institutional Animal Care and Use Committee and were performed in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services “Guide for the Care and Use of Labo-
The APP gene coding for human-sequence APP with the Swedish K670N/M671L + Indiana V717F (APPSWE/IND) mutations, regulated by the human polyubiquitin-C promoter, was inserted into a lentiviral vector. High titer lentiviruses were generated by co-transfection of the lentiviral vector coding for APPSWE/IND (pLV-APPSWE/IND), pΔ8.9, and pVSV-G encoding the vesicular stomatitis virus envelope protein (Invitrogen, now part of ThermoFisher Scientific, Waltham, Massachusetts) into a 293FT packaging cell (Invitrogen). Viruses were then concentrated by ultracentrifugation using a previously described method [36].

2.3. Quantitative (real time) reverse transcription-polymerase chain reaction (qRT-PCR) in lymphocytes and brain tissue

For analysis of APP expression in lymphocytes, peripheral blood was collected from the two APPtg monkeys and two age- and sex-matched control monkeys (RCk12 and RFk12) at 9–10 years of age. APP expression also was analyzed in samples of necortex collected and frozen at necropsy (see Supplementary Material for methodological details).

2.4. Magnetic resonance imaging (MRI)

MRI data were acquired with a Siemens 3-Tesla (3 T) Trio clinical scanner (Siemens Medical Solutions USA, Malvern, PA) using the Siemens CP extremity volume coil at two years of age and with the Siemens 8-channel knee array coil thereafter (when the scanner was upgraded to the Siemens Total Imaging Matrix [TIM] Trio system). Scans were performed when the transgenic animals were approximately 2, 5, and 8 years of age. The wild-type female rhesus monkeys RCK12 and RFK12 served as controls for the MRI analysis at approximately 2 years and 5 years of age. We also measured the volumes of the hippocampus (a structure affected early in AD [46–47]) and the cerebellum (a structure affected later in AD) in the APPtg monkeys and control monkeys at 5 years of age, at which point the monkeys had reached full adulthood. At the 8-year timepoint only the transgenic monkeys were scanned in order to seek potential transgene-related structural abnormalities in the brain, of which none were found.

The anesthetized monkeys were immobilized with a custom-made head-holder and placed in the supine position. Anesthesia was maintained with 1–1.5% isoflurane mixed with O₂. End-tidal CO₂ (Et-CO₂), inhaled CO₂, O₂ saturation, blood pressure, heart rate, respiration rate, and body temperature were monitored continuously and maintained within normal ranges. 3D T1-weighted images with magnetization-prepared rapid acquisition with gradient echo (MP-RAGE) sequence with the following parameters: repetition time (TR) = 2500 ms, echo time (TE) = 3.48 ms, inversion time (TI) = 950 ms, field-of-view (FOV) = 96 mm × 96 mm, data matrix = 192 × 192, flip-angle = 8°, slice thickness = 0.5 mm, 208 slices, 6 averages.

2.4.1. Brain volume calculation

3D T1-weighted images were used for calculation of the brain volumes. Bias-field correction and brain-extraction (plus manual adjustment to exclude the non-brain tissues on T1-weighted images) were carried out using FMRIB Software Library (FSL) software (Oxford University). The volumes of the whole brain (excluding the cerebrospinal fluid), the hippocampus (bilateral) and cerebellum were calculated by multiplication of the voxel number by the voxel size (0.5 mm X 0.5 mm X 0.5 mm).

2.5. Behavioral testing

Behavioral testing was undertaken from birth to 5 years of age to assess the early developmental trajectory of the monkeys relative to wild-type controls. At 6 years of age, the monkeys were assessed for emotional reactivity, and at 9 years they were tested for recognition memory. The tests included the Infant Neurobehavioral Assessment Scale (INAS), a series of four discrimination tasks (Simple Object Discrimination (SOD), Pattern Discrimination (PD), Concurrent Discrimination (CD), and Object Discrimination Reversal (ODR)), a Barrier Detour task, Emotional Reactivity task, and Visual Paired Comparison (VPC) tasks (object recognition memory after increasing delays (VPC-Delays) and memory for spatial locations (VPC-Spatial) (Table 1). For details of the behavioral tests, see Supplementary Material.

2.6. Biomarkers: Aβ and Tau in the cerebrospinal fluid

Cerebrospinal fluid (CSF) samples were collected from the two APPtg monkeys and two wild-type female control monkeys at the ages of 2, 4, 6, 8, and 9 years. CSF was withdrawn from the cisterna magna under general anesthesia between the hours of 10 AM and noon. The levels of Aβ42, total Tau, and Tau phosphorylated at threonine 181 (pTau) were measured in a Luminex 200 platform using Alzbio3 (Fujirebio Diagnostics, Malvern, PA), a standard diagnostic instrument for the assessment of AD biomarkers in humans [48].

2.7. Sacrifice and tissue preparation

The animals were humanely sacrificed after an overdose of sodium pentobarbital (100 mg/kg, i.v.). The brains were rapidly removed, and the two hemispheres separated along the midline. One hemisphere was immersion-fixed in a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) for 2–3 weeks at 4°C. The other hemisphere was sub-dissected into regions of interest that were placed in Eppendorf tubes, quickly frozen on dry ice, and stored at −80°C until analysis.
The paraformaldehyde-fixed hemisphere was used for histological investigation. In brief, the hemisphere was cut into 2–3 cm-thick coronal slabs that were then cryo-protected in a solution of 30% sucrose (in 0.1 M phosphate buffer, pH7.4) for at least 1 week before being frozen and serially sectioned at a thickness of 50 μm on an HM450 freezing-sliding microtome (Thermo Fisher). The sections were placed in an antifreeze solution consisting of 30% glycerol and 30% ethylene glycol in 0.1 M phosphate buffer, and stored at −20 °C until further processing.

### 2.8. Histopathology

#### 2.8.1. Antibodies

The following antibodies were used for immunohistochemistry: 6E10 (1:5000) mouse IgG1 monoclonal antibody (Covance, Princeton, NJ; Antibody Registry # AB_662798) to an epitope at residues 3–8 of Aβ [49], 4G8 (1:5000) mouse IgG2b monoclonal antibody (Covance; Antibody Registry AB_662812) to an epitope at residues 18–22 of Aβ [49]; rabbit polyclonal antibodies R361 and R398 (both at 1:2,000), provided by Dr. Pankaj Mehta (Institute for Basic Research on Developmental Disabilities, Staten Island, NY; Antibody Registry AB_2315240 and AB_2315241, respectively), raised against synthetic Aβ32-40 and Aβ33-42, respectively; CP13 (1:5,000) mouse IgG1 monoclonal antibody, a gift from Dr. Peter Davies (The Feinstein Institute for Medical Research, Manhasset, NY; Antibody Registry AB_2314223), raised against a synthetic peptide representing the region around phosphoserine residue 202 of the Tau protein [50], PHF1 (1:5,000) mouse IgG1 monoclonal antibody, also from Dr. Davies (Antibody Registry AB_2315150), raised against detergent-extracted PHF preparations, with an epitope around phosphoserine 396/404 [51], anti-glia fibrillary acidic protein (GFAP) (1:5000) purified immunoglobulin fraction of rabbit anti-serum from Dako (Carpinteria, CA; Antibody Registry AB_10013382), raised against glial fibrillary acidic protein isolated from cow spinal cord; anti-Iba1 (1:1000) rabbit polyclonal antibody (Wako, Osaka, Japan; Antibody Registry # AB_839504), raised against the C-terminal region of the ionized calcium-binding adaptor molecule 1 (Iba1), a protein that is expressed by macrophages/microglia.

#### 2.8.2. Immunohistochemistry

Endogenous peroxidase in tissues for immunostaining was inactivated with 3–10% H2O2, and nonspecific reagent-binding was blocked with 1–2% normal serum in 0.2% Tween, each for one hour at room temperature. For Aβ-immunodetection, sections were pretreated for 3–10 min in concentrated formic acid to expose antigenic sites. Tissues stained for comparison of antibodies R398 and R361 were incubated in 1% sodium borohydride in buffer following the H2O2 step to optimize antigen retrieval. Sections were incubated in primary antibody (diluted in buffer with blocking serum) overnight at 4 °C. Vectastain kits (Vector Laboratories, Burlingame, CA) were used for immunodetection of antigen–antibody complexes via the avidin–biotin complex (ABC) method. After rinsing, sections were incubated for one hour at room temperature in biotinylated secondary antibody, rinsed, immersed for 30 min in avidin–biotin complex, and then developed with diaminobenzidine (DAB) (Vector Laboratories). Tissue from human Alzheimer’s disease cases was used as positive control material, and non-immune mouse IgG or rabbit sera were used in place of the primary antibodies as negative controls. In some instances, a light hematoxylin counterstain was applied after immunostaining.

#### 2.8.3. Other stains and non-nervous tissues

Some sections were stained with Congo Red, Thioflavin-S, luminescent conjugated oligothiophenes (LCOs) [52] (kindly provided by Dr. K.P.R. Nilsson, Linköping University), Prussian Blue (Perls stain), Perls-DAB (an enhanced iron stain that detects microglia [53]), or hematoxylin and eosin (H&E).

Tissue samples also were taken from the heart, liver, spleen, and kidney for routine pathologic analysis. These samples were fixed in 4% paraformaldehyde in PBS, embedded in paraffin wax, sectioned at 8–10 um thickness, and stained with H&E and with each of the anti-Aβ antibodies 6E10, 4G8 and R398.

Photomicrographs were taken with a SPOT FLEX digital camera (Diagnostic Instruments, Sterling Heights, MI) on a Leica DMLB microscope (Wetzlar, Germany), or with a Moticam 5+ digital camera (Motic, Hong Kong) on a Leica DMLS microscope.
2.9. **Statistical analyses**

Simple group comparisons were undertaken statistically with either t-tests or analysis of variance (ANOVA) tests. For cognitive tasks administered at only one age, group differences were analyzed with t-tests, but for cognitive tasks administered at different time points, multivariate analysis of variance (MANOVA) (Group X Age) with repeated measures for the last factor were used. For the Human Intruder task, data were analyzed using a MANOVA (Group X Condition) with repeated measures for the last factor, followed by post-hoc t-tests when appropriate. Further details regarding analyses of behavioral data are in the Supplementary Material. In all analyses, significance levels are based on two-tailed distributions. Given the small group sizes, the statistical results overall should be interpreted cautiously.

### 3. Results

#### 3.1. Genotyping

The genotype of the APPtg monkeys was examined in tissues derived from the ectoderm (frontal cortex), mesoderm (peripheral blood cells), and endoderm (lung) using PCR analysis followed by sequencing to confirm the presence of the Swedish (GA→TC) and Indiana (G→T) mutations. The mutant APP transgene was detected in tissues of the three germ layers of both transgenic monkeys (Fig. 1).

#### 3.2. Transgene expression

Using qRT-PCR, the estimated normalized expression of APP mRNA in lymphocytes of the transgenic monkeys was 7.3 X control levels in APP1 and 5.6 X control levels in APP2. The estimated normalized neocortical expression of APP mRNA was 2.1 X control levels in APP1 and 2.3 X control levels in APP2.

#### 3.3. Magnetic resonance imaging

The brains of the APPtg monkeys were comparable in size to those of age- and sex-matched control monkeys at 2 years and 5 years of age, with the possible exception of the somewhat smaller brain of APP1 (Fig. 2). There were no significant differences between the transgenic and control groups at 2 years (t = 0.25, p = 0.41) or 5 years of age (t = 0.64, p = 0.64). The volumes of the hippocampus and cerebellum generally corresponded to the volume of the whole brain in each animal, and neither structure showed a statistically significant difference between the transgenic and control monkeys (Supplementary Fig. 1). In the APPtg monkeys at 8 years of age, there was no evidence of hemorrhage, white matter lesions or overt structural abnormalities in the brain (Fig. 3 and Supplementary Fig. 2), nor were anomalies present in any monkey at younger ages (Supplementary Fig. 3).

#### 3.4. CSF biomarkers

The CSF biomarker data are summarized in Table 2. The small n relative to the number of measures per subject precluded the use of ANOVA for repeated measures; therefore we computed t-tests (two-tailed) for each time point to highlight potentially meaningful differences between the APPtg and control monkeys.

The highest peak CSF Aβ42 levels were seen in APP1 and APP2 at the 2- and 4-year timepoints, whereas thereafter

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**Fig. 1.** Genotyping confirmed the presence of the mutant APPSWE/IND transgene in tissues derived from the three germ layers of monkeys APP1 and APP2.
the levels were similar to those in the two control monkeys (Fig. 4). Of these timepoints, the only statistically significant difference was at 4 years of age ($t = 8.74$, $p = 0.01$). At the two youngest ages (2 and 4 years), when Aβ levels are most likely to reflect Aβ expression levels prior to the onset of deposition in the brain, the CSF Aβ42 was approximately 2 times higher in the transgenic monkeys than in the non-transgenic controls, similar to the estimated APP mRNA expression levels in the brain (see the transgene expression levels, Section 3.2).
The CSF total Tau levels were significantly higher in the APPtg monkeys than in control monkeys at 2 years ($t = 10.13$, $p = 0.01$) and 4 years ($t = 6.07$, $p = 0.03$) of age (Fig. 5). This difference was not significant at later ages, although there was a trend for the total Tau levels to increase in the APPtg monkeys at 9 years of age ($t = 3.05$, $p = 0.09$).

Neither the total Tau/Aβ42 ratio nor the pTau levels differed significantly between the transgenic and nontransgenic monkeys at any age. The pTau/Aβ42 ratio differed

Table 2
The levels of Aβ42, total Tau and pTau (phosphorylated at threonine 181), and the ratios of total Tau and pTau to Aβ42 in the CSF of APPtg monkeys and nontransgenic control monkeys at 2, 4, 6, 8 and 9 years of age. Significant differences as determined by $t$-tests are indicated in bold.

| CSF Biomarkers | Group | Monkey | 2 years | 4 years | 6 years | 8 years | 9 years |
|----------------|-------|--------|---------|---------|---------|---------|---------|
|                | CSF Aβ42 (pg/ml) | {APPtg} APP1 | 608.61 | 938.60 | 408.67 | 370.12 | 422.34 |
|                |       | APP2  | 1039.37 | 948.34 | 566.30 | 251.29 | 360.13 |
|                |       | Control Rck12 | 535.72 | 450.89 | 370.57 | 451.99 | 189.00 |
|                |       | RFk12 | 483.45 | 324.09 | 456.15 | 407.41 | 353.49 |
|                | $t$ (probability) | 1.45 (0.28) | 8.74 (0.01) | 0.83 (0.50) | 1.88 (0.20) | 1.36 (0.31) |
|                | CSF Total Tau (pg/ml) | {APPtg} APP1 | 27.15 | 26.18 | 23.71 | 17.81 | 26.07 |
|                |       | APP2  | 28.88 | 26.48 | 22.6 | 20.74 | 28.8 |
|                |       | Control Rck12 | 19.18 | 16.13 | 22.24 | 13.27 | 15.26 |
|                |       | RFk12 | 18.72 | 19 | 26.56 | 19.82 | 20.79 |
|                | $t$ (probability) | 0.12 (0.91) | 1.69 (0.23) | 1.12 (0.38) | 1.33 (0.31) | 0.08 (0.95) |
|                | CSF Total Tau/Aβ42 | {APPtg} APP1 | 0.044 | 0.028 | 0.058 | 0.048 | 0.062 |
|                |       | APP2  | 0.028 | 0.028 | 0.040 | 0.083 | 0.080 |
|                |       | Control Rck12 | 0.036 | 0.036 | 0.060 | 0.029 | 0.081 |
|                |       | RFk12 | 0.039 | 0.059 | 0.058 | 0.049 | 0.059 |
|                | $t$ (probability) | 0.012 (0.91) | 1.69 (0.23) | 1.12 (0.38) | 1.33 (0.31) | 0.08 (0.95) |
|                | CSF pTau (pg/ml) | {APPtg} APP1 | 44.64 | 48.16 | 37.27 | 38.95 | 36.46 |
|                |       | APP2  | 31.29 | 42.68 | 29.78 | 30.25 | 45.99 |
|                |       | Control Rck12 | 92.31 | 36.53 | 31.5 | 42.88 | 35.81 |
|                |       | RFk12 | 25.13 | 23.82 | 26 | 26.08 | 29.49 |
|                | $t$ (probability) | 0.012 (0.91) | 1.69 (0.23) | 1.12 (0.38) | 1.33 (0.31) | 0.08 (0.95) |
|                | CSF pTau/Aβ42 | {APPtg} APP1 | 0.073 | 0.051 | 0.091 | 0.105 | 0.086 |
|                |       | APP2  | 0.030 | 0.045 | 0.053 | 0.120 | 0.128 |
|                |       | Control Rck12 | 0.172 | 0.081 | 0.085 | 0.095 | 0.189 |
|                |       | RFk12 | 0.052 | 0.073 | 0.057 | 0.064 | 0.083 |
|                | $t$ (probability) | 0.095 (0.44) | 5.93 (0.03) | 0.04 (0.97) | 1.94 (0.19) | 0.52 (0.66) |
significantly only at 4 years of age ($t = 5.93$, $p = 0.03$) (Table 2).

3.5. Behavior

On most behavioral tasks conducted from infancy through 16 months of age - INAS (Supplementary Table 1), VPC-Delays, VPC-Location, SOD, PD, CD, and ODR - the APPtg monkeys as a group did not differ significantly from the control monkeys. The APPtg monkeys tended to perform more poorly on several components of the Barrier Detour task at 16 months of age (Supplementary Table 2), but the only statistically significant difference was in the percent retrieval of rewards ($t = 7.22$, $p = 0.02$). At 5 years of age, this difference was no longer evident, and the only significant group difference was in perseveration following the reversal (RP Reaches) ($t = 6.55$, $p = 0.02$). APP1 in particular had a stronger tendency to perseverate (TP Reaches and RP Reaches) than did the control monkeys at 16 months and at 5 years, i.e., repeatedly attempting to reach through the transparent barrier rather than reaching around it (Supplementary Table 2).

When tested in the Emotional Reactivity task at 6 years of age, the wild-type controls exhibited the species-typical response to the human intruder, in that they primarily froze during the Profile condition and primarily expressed hostile and anxious behaviors during the Stare condition. The APPtg monkeys presented an atypical pattern of behavior, with more freezing during the Stare condition than in the Profile condition (Group X Condition: $F(2,6) = 15.47$, $p = 0.025$; Fig. 6a.). Unlike wild-type controls, the APPtg monkeys displayed more hostility during the Alone condition (Group X Condition: $F(2,6) = 21.68$, $p = 0.003$; Fig. 6b) and more displacement behaviors during the Alone and Profile conditions (Group X Condition: $F(2,6) = 8.72$, $p = 0.017$; Fig. 6c). In addition, the APPtg monkeys demonstrated significantly elevated motor stereotypies across all three conditions compared to the controls (Group: $F(1,3) = 24.19$, $p = 0.016$; Fig. 6d).

At 9 years of age, the novelty preference of the APPtg group did not differ significantly from that of the wild-type control group on the VPC-Location and VPC-Replace tasks ($t = 0.71$, $p = 0.53$, and $t = 2.27$, $p = 0.11$, respectively). However, the APPtg monkeys showed significantly less preference for the novel image than did control monkeys on the Object-in-Place task ($t = 3.61$, $p = 0.04$) (Supplementary Table 4).

3.6. Histopathology

3.6.1. Brain

In transgenic monkey APP1, Aβ deposition was present primarily in association with cerebral blood vessels (Aβ-CAA) and as mostly diffuse or small, clustered parenchymal deposits (Fig. 7 and Supplementary Fig. 7). The Aβ deposits were most abundant in caudal (parietal, occipital, and caudal temporal) neocortical regions, whereas more rostral brain regions were devoid of lesions (Fig. 8). No Aβ deposition was present in non-neocortical structures, including the diencephalon, basal ganglia, hippocampal formation, amygdala, brainstem, and cerebellum. No Aβ-plaques or Aβ-CAA were present in the second APP-transgenic monkey (APP2) (Supplementary Fig. 4) or in the non-transgenic control monkeys (Supplementary Fig. 5).

The Aβ-CAA affected both large vessels and capillaries, and it was immunoreactive with antibodies 6E10 (Fig. 7), 4G8, R361, and R398 (Supplementary Figs. 6 and 7). CAA and dense parenchymal deposits often stained with both antibodies R398 and R361, whereas R398 was more sensitive to diffuse deposits (Supplementary Fig. 7). Some vessels showed birefringence after staining with Congo red (Fig. 9A) and some were strongly fluorescent upon staining with Thioflavin-S (Fig. 9C) and luminescent conjugated oligothiophenes (Supplementary Fig. 8). Cored plaques were very rare, but a few parenchymal deposits were congophilic (Fig. 9B) and Thioflavin-S-positive (Fig. 9D). Antibody CP13 to Tau protein did not reveal tauopathy in any brain region (Supplementary Fig. 9). Consistent with the MRI findings (Section 3.3, above), there was no histologic evidence of overt hemorrhage, siderosis, or white matter abnormalities. There was little histological evidence of overt inflammation such as reactive microgliosis in association with the Aβ deposits (Supplementary Figure 10).
3.6.2. Other organs

Histological examination of the APPtg monkeys revealed no abnormalities in heart muscle, liver, spleen or kidney, and no Aβ deposition was identified in these organs when immunostained with antibodies 6E10, 4G8 or R398.

4. Discussion

The presence of Aβ-plaques and Aβ-CAA more than a decade before their usual appearance in rhesus monkeys indicates that the intracerebral deposition of Aβ can be accelerated in a nonhuman primate by the overexpression of transgenic APP bearing AD-associated mutations. The resulting lesions were located only in more posterior regions of the neocortex (occipital, parietal, and caudal temporal neocortices), whereas no Aβ deposition was present in the frontal lobe, medial temporal lobe, or in non-neocortical structures. This exclusively neocortical pattern of Aβ deposition approximately corresponds to Thal Phase 1 of AD [54–55], and to Stage 1 Aβ-CAA [56] in humans.

No Aβ deposition was detected in a second APPtg rhesus monkey (APP2) in which the estimated APP expression was comparable to that in APP1. Although the unaffected monkey was slightly younger at the time of death (10 years, 2 weeks) than was monkey APP1 (10 years, 45 weeks), it is likely that other, still unknown factors, interact with APP expression to incite the aggregation of Aβ. Studies of APP duplication [57] and triplication [58] in humans have noted variability in the age of disease onset and clinical features in patients with the same genetic anomaly, so the absence of obvious lesions in one of the monkeys is not unexpected at this young stage of life. As in humans, phenotypic variability is likely to be a characteristic of outbred animal models such as monkeys.

Analysis of biomarkers in the CSF found high levels of Aβ42 in the APPtg monkeys early in life, followed by a relatively steep decline from 2 to 6 years of age (Fig. 4). This temporal pattern is reminiscent of that in humans at preclinical stages of dominantly inherited AD (i.e., high levels of Aβ initially, followed by a decline as the disease progresses [59]). However, in a cross-sectional study of wild-type cynomolgus monkeys, CSF Aβ levels were shown to decrease with age [60]. Thus, the longitudinal decline of

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**Fig. 6.** Altered emotional responses in the 6-year-old APPtg monkeys (grey bars) compared to three non-transgenic, 6-year-old female controls (white bars). Bars represent the means for (a) freezing, (b) hostility, (c) displacement behaviors, and (d) motor stereotypies during the Alone, Profile, and Stare conditions for APPtg monkeys (the yellow square represents APP1, and the red square represents APP2) and wild-type controls (blue circles represent the three individual control monkeys). *Indicates significant group differences (p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Aβ in the present study may reflect normal, age-associated changes in CSF levels of the protein. The amount of total Tau in CSF was greater in the transgenic monkeys at 2 years, 4 years, and (non-significantly) at 9 years, but the levels of phospho-Tau (which is thought to be a relatively specific biomarker of AD-type pathology [61]) were similar in the two groups. The ratios of Tau and pTau to Aβ42 (except for a [possibly anomalous] spike in pTau/Aβ42 at...
4 years of age) were largely similar in APPtg and control monkeys, consistent with the absence of tauopathy histologically.

Behavioral tests found that the APPtg monkeys were hyperactive and irritable as young adults compared to control animals that were similarly reared in the nursery. Neuropsychiatric symptoms have been reported to predict progression to mild cognitive impairment (MCI) among cognitively normal patients [62], to predict progression to AD among prodromal patients [63], and to be prognostic of earlier progression to severe AD dementia [64–66]. When tested at 9 years of age, the overall performance of the transgenic monkeys on visual paired comparison tasks was generally similar to that of wild-type control monkeys. A possible exception was the diminished preference of the APPtg monkeys for the novel images in the relatively difficult Object-in-Place task. In humans, the VPC task has been shown to predict the progression from an unimpaired state to mild cognitive impairment (MCI) and from MCI to AD [67].

Despite somewhat deficient performance on some behavioral tasks, particularly for monkey APP1, there was no evidence of a dementia-like cognitive state, i.e., extreme impairments in multiple cognitive domains [14] in either transgenic monkey. This is not unexpected, given the limited degree of Aβ deposition and the absence of tauopathy, along with the paucity of inflammation and other degenerative changes that characterize the later stages of AD. Monkey APP2, which lacked histologically detectable Aβ deposition, also displayed some behavioral differences from the control animals. However, owing to the small number of subjects and the somewhat younger age of the control monkeys (6 years) for the VPC assessment, the behavioral findings should be interpreted guardedly. Furthermore, while the behavioral anomalies of the APPtg monkeys may indicate the initial effects of increased APP and/or Aβ (possibly in oligomeric form), we cannot rule out potential non-specific effects such as those that might result from the unpredictable genomic integration sites of the transgene.

Transgene-induced premature Aβ-amyloidogenesis, combined with a potential lifespan of 40+ years [30], could favor the eventual emergence of profuse, human-like neurofibrillary tangles in transgenic rhesus monkeys. Studies of very old wild-type monkeys of several species have found evidence of incipient tauopathy [9,26,32–35,68], indicating that the complete pathologic phenotype of AD might be achievable in nonhuman primates if the disease process is initiated early in life. Since tauopathy follows Aβ deposition in human AD [2,5–7], and in light of the early stage of Aβ deposition in the affected monkey, the absence of tauopathy is not unexpected. We should note that non-fibrillar tau is rapidly de-phosphorylated postmortem, and that by quickly inactivating phosphatases, perfusion-fixation can enable the detection of non-fibrillar phosphorylated tau [26]. Because our histologic analysis used immersion-fixed tissue, the immunohistochemical methods may not have detected a very early stage of aberrant tau phosphorylation. Future studies are needed to determine if fully human-like AD tauopathy can be modeled in transgenic monkeys.

There was little histopathological evidence of reactive gliosis in the monkeys, even in areas with evident Aβ deposition. However, histology may fail to detect early bio-

Fig. 9. Congo Red- and Thioflavin-S-stained blood vessels (A,C) and parenchymal plaques (B,D) in the caudal neocortex of monkey APP1. The amyloid was birefringent under crossed polarizing filters when stained with Congo Red (A,B), and yielded a fluorescent signal when stained with Thioflavin-S (C,D). Hematoxylin counterstain in A and B. Bar in D = 50 µm in A, B and D, and 100 µm in C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
chemical changes associated with inflammation. In humans with AD, reactive astrocytes [69] and microglia [70–72] are most strongly linked to the later stages of AD pathogenesis, and they appear to emerge subsequent to Aβ deposition [69]. The microenvironment of Aβ plaques in nonhuman primates is similar to that in humans [21]. Thus, a similar glial response might be expected in APP-transgenic monkeys as the pathogenic process progresses, a possibility that must be addressed in longer-term analyses.

Even if transgenic monkeys fail to display human-like AD, they might serve as more representative models of Aβ-CAA. The buildup of Aβ in the vascular wall is a feature of nearly all AD brains [73–75], although the degree of Aβ-CAA is variable, being severe in only about a quarter of the cases [76]. Cerebrovascular amyloidosis has been linked to lobar intracerebral hemorrhage, white matter lesions, cerebral ischemia, and cognitive impairment, and thus is itself a significant source of morbidity and mortality [77]. None of these sequelae were apparent in the affected APPtg monkey, although the Aβ-CAA was probably at an early stage of development. CAA-related anomalies should be possible in transgenic nonhuman primates, inasmuch as naturally occurring Aβ-CAA has been associated with white matter lesions and neuroinflammation in an aged, wild-type squirrel monkey (Saimiri sciureus) [78]. It is noteworthy that cases of APP duplication and triplication in humans are characterized by early-onset AD with prominent Aβ-CAA [58,79–81]. Many cases of trisomy 21 in which an extra copy of the APP gene is present also manifest early-onset AD with Aβ-CAA [79,82]. The pathologic phenotype in the APPtg rhesus monkeys thus has parallels with cases of human APP-gene multiplication, albeit at an early stage in the monkeys. As in the case of Aβ plaques, further aging will be required to evaluate the full phenotype resulting from transgene-induced Aβ-CAA in monkeys.

Rhesus monkeys are advantageous primates for modeling AD due to their extensive use in aging research and their relatively close evolutionary relationship to humans [17,21,83,84]. Although the long timecourse of pathogenesis is a shortcoming of the model, it is conceivable that an extended period of pathogenesis is necessary to completely model the pathobiology of AD, including neurofibrillary tangles, neuroinflammation, and the progressive loss of neurons and their connections. Several research groups are developing primate models of AD-like pathology using germline genetic modification (e.g., [85,86]). One such paradigm uses the marmoset (Callithrix jaccus) [85], a small, New World primate with a known maximum lifespan of around 22 years [87]. Although they are more evolutionarily distant from humans, if the accumulation of misfolded Aβ can be accelerated in these animals, which can manifest AD as it occurs in humans.

Conflict of interest statement

W.T.H. has patents on CSF-based diagnosis of frontal-temporal lobar degeneration (FTLD) with TDP-43 inclusions (FTLD-TDP), and diagnosis of MCI due to AD and spinal muscular atrophy; has received research support from Fujirebio Diagnostics; and has consulted for Apelis Pharmaceuticals, Fujirebio Diagnostics, and Hoffman-LaRoche. The other authors declare no competing interests. This work was prepared while Anthony W.S. Chan and Rebecca F. Rosen were employed at Emory University. The opinions expressed in this article are the authors’ own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

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Author contributions

A.W.S.C and L.C.W. designed the study with input from J.B., J.R., S.P.M., X.Z., J.G.H., R.F.R., W.T.H., J.J.L., A.I.L. and Y.S. A.W.S.C and S.-H.Y. created the transgenic monkeys. C.X.L. and J.B. designed and interpreted the cognitive assessments (FTLD-TDP), and prognosis of MCI due to AD and spinal muscular atrophy; has received research support from Fujirebio Diagnostics; and has consulted for Apelis Pharmaceuticals, Fujirebio Diagnostics, and Hoffman-LaRoche. The other authors declare no competing interests. This work was prepared while Anthony W.S. Chan and Rebecca F. Rosen were employed at Emory University. The opinions expressed in this article are the authors’ own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbas.2022.100044.
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