

## Cerebral $\beta$ -amyloid angiopathy in aged squirrel monkeys

H.A. Elfenbein<sup>1</sup>, R.F. Rosen<sup>1</sup>, S.L. Stephens<sup>1</sup>, R.C. Switzer<sup>3</sup>,  
Y. Smith<sup>1,2</sup>, J. Pare<sup>1</sup>, P.D. Mehta<sup>4</sup>, R. Warzok<sup>5</sup> and L.C. Walker<sup>1,2</sup>

<sup>1</sup>Yerkes National Primate Research Center and <sup>2</sup>Department of Neurology, Emory University, Atlanta, USA,

<sup>3</sup>Neuroscience Associates, Knoxville, USA, <sup>4</sup>New York State Institute for Basic Research, Staten Island, NY, USA and

<sup>5</sup>Department of Pathology, University of Greifswald, Greifswald, Germany

**Summary.** Cerebral  $\beta$ -amyloid angiopathy (CAA) is an age-related disorder of the brain vasculature that is involved in up to 20% of non-traumatic cerebral hemorrhage in humans. CAA is a risk factor for cognitive decline, and may exacerbate the dementia of Alzheimer's disease. Progress in discovering the cause and potential therapies for this disorder has been hindered by the paucity of animal models, particularly models of idiopathic CAA. The squirrel monkey (*Saimiri spp*) develops significant CAA in the natural course of aging. To evaluate the suitability of *Saimiri* as a model of human CAA, we studied the distribution and composition of A $\beta$  subtypes in CAA and parenchymal (senile plaque) deposits in the brains of aged squirrel monkeys, as well as the relationship between vascular  $\beta$ -amyloid deposition and comorbid vasculopathies that occur in aged humans. Our findings show that: 1) CAA consists ultrastructurally of classical amyloid fibrils and is the principal type of cerebral  $\beta$ -amyloidosis in squirrel monkeys; 2) The two primary isoforms of A $\beta$  (A $\beta$ 40 and A $\beta$ 42) coexist in most microvascular and parenchymal lesions of *Saimiri*, although A $\beta$ 40 tends to predominate in larger arterioles; 3) CAA and parenchymal plaques overlap to a considerable degree in most affected brain areas, and are distributed symmetrically in the two hemispheres; 4) Both CAA and plaques are particularly abundant in rostral regions and comparatively sparse in the occipital lobe; 5) Capillaries are especially vulnerable to CAA in squirrel monkeys; and 6) When CAA is severe, it is associated with a small, but significant, increase in other vasculopathies, including microhemorrhage, fibrinoid extravasation and focal gliosis. These findings, in the context of genetic, vascular and immunologic similarities between squirrel monkeys and humans, support the squirrel monkey as a biologically advantageous model for studying the basic biology of idiopathic, age-related CAA, and for testing

emerging therapies for human  $\beta$ -amyloidoses such as Alzheimer's disease.

**Key words:** Alzheimer, Apolipoprotein E, Inflammation, Hemorrhage, Vascular dementia

### Introduction

Cerebral amyloid angiopathy (cerebrovascular amyloidosis) is a vascular disorder that results from the accumulation of amyloidogenic proteins in the walls of cerebral blood vessels (Vinters, 1992; Walker and Durham, 1999; Revesz et al., 2003). The protein most commonly involved in cerebrovascular amyloidosis is the  $\beta$ -amyloid peptide (A $\beta$ ) (Vinters, 2001), a proteolytic fragment that also is a primary component of senile plaques in aging and Alzheimer's disease (AD). Cerebral A $\beta$ -amyloid angiopathy (CAA) occurs in ~30% of individuals over 60 years of age (Rensink et al., 2003). Autopsy studies have shown that nearly all end-stage AD patients have some degree of vascular amyloidosis, and in approximately 25% of those cases, CAA is moderate to severe (Ellis et al., 1996; Kalaria and Ballard, 1999; Vinters, 2001; Jellinger, 2002).

Leptomeningeal and cortical arteries and arterioles are particularly vulnerable to  $\beta$ -amyloidosis in humans, whereas veins and capillaries are less frequently affected (Vinters, 1987; Preston et al., 2003). All cortical regions can develop CAA, although there is substantial variation among brain areas and among individuals; the cerebellum is sometimes afflicted, but CAA is seldom seen in the diencephalon, basal ganglia or lower brainstem (Vinters, 2001). While the etiology of the disorder remains obscure, failure of active (Shibata et al., 2000; Vogelgesang et al., 2004) and/or passive (Preston et al., 2003) elimination of A $\beta$  from the aging brain may be an important factor.

CAA increases the risk of intracerebral bleeding, and is associated with up to 20% of non-traumatic hemorrhagic strokes in the elderly (Vinters, 1992; Kase,

1994; Jellinger, 2002). In some instances, vascular/perivascular inflammation (Yamada et al., 1996; Eng et al., 2004; Scolding et al., 2005), fibrinoid necrosis (Vonsattel et al., 1991; Yamada et al., 1996; Greenberg and Vonsattel, 1997; Eng et al., 2004; Scolding et al., 2005) and leukoencephalopathy (Oh et al., 2004) are linked to vascular A $\beta$  deposition. CAA exacerbates cognitive decline in AD (Jellinger, 2002; Thal et al., 2003), and is a risk factor for dementia in its own right (Rensink et al., 2003; Xu et al., 2003; Greenberg et al., 2004; van Horssen et al., 2005). Moreover, the likelihood of hemorrhage in patients with CAA is increased by anticoagulants/thrombolytics (Melo et al., 1993; Sloan et al., 1995; Rosand et al., 2000; Winkler et al., 2002; McCarron and Nicoll, 2004) and possibly by A $\beta$ -immunization therapy for AD (Ferrer et al., 2004; Gandy and Walker, 2004). Additionally, CAA may be involved in the encephalitic reaction of some patients to A $\beta$ -immunotherapy (Nicoll et al., 2003), an adverse event that was not anticipated by numerous studies of transgenic mouse models (Gandy and Walker, 2004). Currently there are no practical diagnostic tests or treatments for CAA.

Some genetically modified mouse models develop CAA, but there is a need for valid and biologically proximate models of *sporadic* CAA – by far the most common form of the disorder - to evaluate the normal physiological and biochemical mechanisms underlying vascular amyloidogenesis. Squirrel monkeys (*Saimiri* spp) are small, New World primates that naturally develop A $\beta$  deposits in the form of CAA and senile plaques beginning around 13 years of age (Walker et al., 1987, 1990). These animals have a maximum lifespan in captivity of up to 30 years, and their cerebrovascular and immune systems are similar to those in humans (Mackic et al., 1998, 2002; Bading et al., 2002; Fukuda and Zoppo, 2003; Maeda et al., 2005). The  $\beta$ -amyloid precursor protein ( $\beta$ APP<sub>751</sub>) in squirrel monkeys is 99.5% homologous to human  $\beta$ APP<sub>751</sub>, and the amino acid sequence of A $\beta$  is identical in the two species (Levy et al., 1995). An important feature of cerebral A $\beta$  deposition in squirrel monkeys is their species-specific predilection to CAA rather than senile plaques (Walker et al., 1990). A preliminary study found that A $\beta$ 40 predominates histologically in CAA of an aged squirrel monkey (Sawamura et al., 1997), but it is important to undertake a more comprehensive assessment of A $\beta$  isoforms comprising CAA in this species, as well as the ultrastructural characteristics of cerebrovascular amyloid, and the relationship of CAA to coincident lesions such as gliosis and leukoaraiosis.

## Materials and methods

### Subjects

Five squirrel monkeys (*Saimiri* spp), four aged (15-23 years old) and one young (7 years old), formed the core of this study (Table 1). In all of these cases, the

entire rostro-caudal extent of the brain was systematically sampled as described below. In addition, we analyzed brain samples from ten supplemental aged squirrel monkeys (17-24 years old) from which tissue was available only from specific coronal levels. All animals had been in captivity for many years; the birthdates of seven subjects were known, and the ages of the other eight were estimated at the time of acquisition. All studies were conducted in accordance with federal and local guidelines for the humane care and use of animals.

Core animals were restrained with ketamine (15 mg/kg, i.m.) and deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.v.). They were then perfused transcardially with phosphate-buffered saline (PBS, RT) followed by 10% neutral buffered formalin (NBF). One animal (89U) died of natural causes at 15 years of age. The brain was removed and immersed in NBF within 2 hours of death. Following fixation, brains were prepared for immunohistochemical, neuro-pathologic and ultrastructural evaluation. In all animals, the brains were grossly normal.

### Histochemistry

For light-microscopic analysis, tissues were coronally sectioned at 10-25  $\mu$ m thickness and then immunostained with one or more antibodies to A $\beta$ : Monoclonal 4G8 [Signet Labs, Dedham, MA] to amino acids 17-24 of A $\beta$ ; monoclonal 6E10 to amino acids 1-17 of A $\beta$ ; monoclonal 10D5 to amino acids 1-16 of A $\beta$  (Dale Schenk, Elan Pharmaceuticals, South San Francisco, CA); rabbit polyclonal to A $\beta$  (Colin Masters, University of Melbourne, Melbourne, Australia); rabbit polyclonal antibodies R163 to C-terminal A $\beta$ 40, and R165 to C-terminal A $\beta$ 42 [Pankaj Mehta, Staten Island, NY]; or polyclonal antibodies to A $\beta$ 40 and A $\beta$ 42 from Biosource [Camarillo, CA]). The antibodies to A $\beta$  all were highly sensitive to both CAA and senile plaques, and, except for a slight preference of A $\beta$ 40 antibodies for large amyloidotic vessels (below), all antibodies yielded quantitatively similar lesion profiles in the aged animals. Microglia were stained with a polyclonal antibody to a macrophage/microglia-specific antigen (ionized calcium-binding adapter molecule 1, Iba-1; Wako Chemicals USA, Richmond, VA). Astrocytes were immunolabeled for glial fibrillary acidic protein (GFAP, Boehringer Mannheim, Indianapolis, IN), the microtubule-associated tau protein was immunolabeled with antibody AT8 (Endogen, Woburn, MA) or Alz50 (Benjamin Wolozin, Boston University, Boston, MA).

Primary antibodies were detected using the ABC avidin-biotin-peroxidase system (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). In brief, sections were preincubated for 30 minutes in 1% H<sub>2</sub>O<sub>2</sub> followed by 0.3% Triton X-100 in Tris-buffered saline for 10 minutes and 5% blocking serum for 30 minutes. For A $\beta$ -immunodetection, antigenic sites were exposed by pre-treatment with formic acid. Tissue was then

reacted overnight at 4°C with primary antibodies in Tris-buffered saline. After washing, the antigen-antibody complex was visualized with the avidin-biotin method and 3,3'-diaminobenzidine (DAB) as the chromogen. Tissue sections from AD cases with CAA were routinely included as positive controls, and non-immune sera/mouse IgG replaced the primary antibodies as negative controls, followed by the full ABC protocol (above).

The DAB-enhanced Perls' method, a sensitive marker of ferric iron, was used to identify hemosiderin deposits indicative of prior hemorrhage. Additional sections were stained with the Campbell-Switzer modification of the Gallyas silver method for AD pathology, with Congo Red for amyloid, and with hematoxylin and eosin for assessment of general pathologic changes.

#### *Histological analysis*

Deposits of immunoreactive A $\beta$  in the vasculature and parenchyma of the brain, as previously defined (Walker et al., 1990), were mapped and enumerated in representative coronal sections throughout the forebrain of the five core subjects using the NeuroLucida image analysis system (MicroBrightField Inc., Williston, VT). Every A $\beta$ -deposit was indicated in all brain regions of each coronal section; however, because CAA and senile plaques were most abundant in the neocortex (Fig. 1; see below), the neocortex was selected for comparative quantitation of these lesions. The total planar area of neocortex was quantitated in each coronal section by stereological point counting methods (Mouton et al., 1997). Briefly, a symmetrical grid of (+) symbols was superimposed on a low magnification image of the tissue section, with each symbol representing a micrometer-calibrated square of known area. All (+) symbols in which the interior corner of the upper right quadrant overlay the region of interest were counted, and the total cortical area established. Parenchymal and vascular amyloidotic profiles were quantitated in the region of interest, and any instances of microhemorrhage, fibrinoid accumulation or other pathologic changes were noted. The relative abundance of A $\beta$ 40- and A $\beta$ 42-immunoreactivity was determined in specific vessel types (capillaries - characterized by a uniformly small (5-10  $\mu$ m) diameter - vs. larger vessels) and parenchymal lesions, and total numbers and densities of A $\beta$  deposits were calculated. The cortical ratios of CAA to senile plaques were determined in whole brain sections at multiple rostrocaudal levels in the five core subjects (bold in Table 1), and at specific coronal levels from the ten subjects from which only limited tissue was available. In these latter ten cases, the coronal levels sampled (according to Emmers and Akert, 1963) are indicated in Table 1. Light-micrographs were taken with a Leica DMLB microscope (Wetzlar, Germany) and SPOT XPlorer and FLEX digital cameras (Diagnostic Instruments, Sterling Heights, MI).

#### *Ultrastructural analysis*

Tissue samples from the superior temporal cortex (level A8.0 – A9.0, Emmers and Akert, 1963) and superior parietal cortex (level P5.0 - P6.0) of one aged animal (23 years old) were postfixed in 4% paraformaldehyde and 0.5% glutaraldehyde, washed in phosphate buffer (PB; 0.1M, pH 7.4) and immersed in osmium tetroxide (1% in PB) for 20 min. They were then rinsed in PB and dehydrated in a graded series of ethanol and propylene oxide. Uranyl acetate (1%) was added to the 70% ethanol (35 minute immersion) to improve contrast in the electron microscope. The sections were then embedded in resin (Durcupan ACM; Fluka, Ft. Washington, PA, USA) on microscope slides and placed in the oven for 48hrs at 60°C. Areas of interest were selected, excised from the slide and glued onto resin blocks. Ultrathin sections were then cut with an ultramicrotome (Leica Ultracut T2, Wetzlar, Germany), collected onto Pioloform-coated single-slot copper grids, stained with lead citrate and examined with a Zeiss EM10-C electron microscope (Oberkochen, Germany).

#### *Statistical analysis*

The non-parametric chi-square ( $X^2$ ) test was used to evaluate relative lesion frequencies.

## **Results**

### *CAA and parenchymal A $\beta$ -plaques in squirrel monkeys*

Vascular and parenchymal  $\beta$ -amyloid deposits were present in all squirrel monkeys aged 15 years or older. The young (7 year-old) squirrel monkey showed no evidence of A $\beta$  deposition in any part of the brain. Overall, A $\beta$ -immunoreactive vascular profiles were consistently more abundant than were A $\beta$  plaques in neocortex of aged squirrel monkeys (Figs. 1, 2; Table 1), although some regions (such as the neostriatum) tended to show a relatively higher proportion of plaque-like deposits. There was variability among aged animals in the numerical density of lesions and, to some extent, in the presence and distribution of specific types of A $\beta$  deposits (Table 1). The parenchymal deposits consisted of both dense-cored and diffuse A $\beta$ , as described by Walker et al. (1990).

A $\beta$  plaques are usually discrete, spherical or ovoid structures, whereas the vasculature forms a continuous network in brain. Amyloidotic vessels can show extended stretches of mural A $\beta$ , or only patchy deposits. In general, the number of A $\beta$ -containing vascular profiles in a tissue section is a reliable index of the overall severity of CAA (which is a function of the number of vessels affected and the degree to which A $\beta$  invades individual vessels), but in comparing the incidence of vascular and parenchymal deposits, it is important to bear in mind the morphological

idiosyncrasies of the two types of lesion.

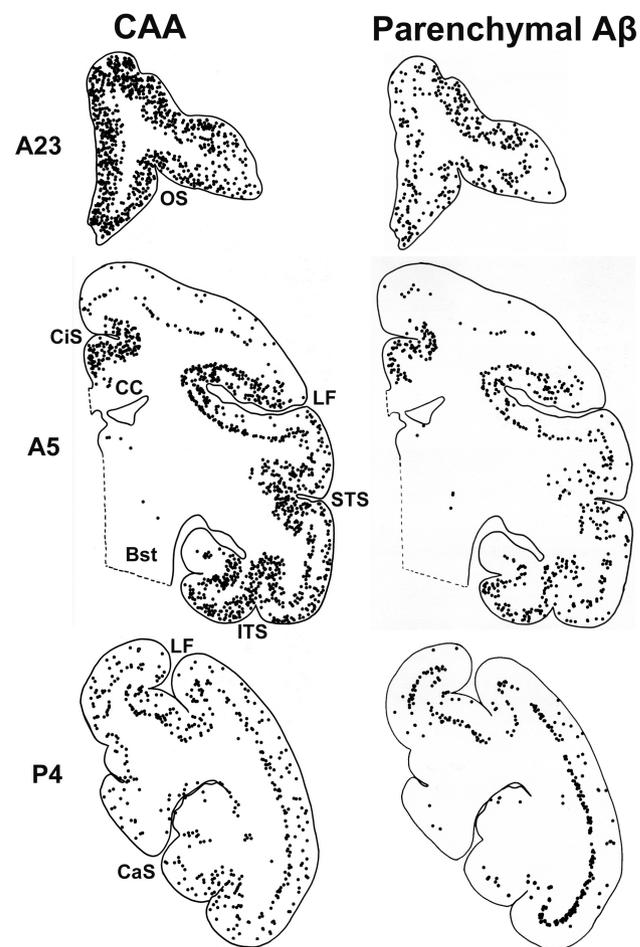
#### Brain distribution of vascular and parenchymal A $\beta$ -deposits

CAA in squirrel monkeys generally resembles that in human AD cases with abundant CAA, except that large plaques are relatively infrequent and capillary CAA is relatively profuse in squirrel monkeys (Fig. 2). There is extensive overlap in the distribution of CAA and parenchymal A $\beta$  deposits in the squirrel monkey brain, although most of these lesions are spatially discrete in tissue sections, i.e. there is a mixture of distinct vascular and parenchymal profiles, as well as a subpopulation of adjoining lesions (Figs. 3, 4). Both types of A $\beta$ -deposit occur predominantly in the neocortex, and the areal density of the lesions is greatest in the rostral cortex and diminishes caudally (Fig. 1). The distribution and quantity of parenchymal and vascular A $\beta$  deposits are highly symmetrical in the left and right hemispheres (Fig. 3). In certain neocortical regions there is a conspicuous laminar organization of CAA and plaques, with numerous lesions at the border between neocortical layer 6 and the white matter (Figs. 1, 4C), and a separate band of deposits in more superficial layers (Fig. 1). This pattern is particularly evident in the dorsal and lateral neocortex. Other areas that develop A $\beta$ -amyloidosis, to a lesser degree than in neocortex, include the amygdala, claustrum, hippocampus, septum verum, neostriatum (particularly the nucleus accumbens) and, very rarely, the diencephalon, globus pallidus, and lower brainstem. A $\beta$  accumulations in the white matter are sparse, and when found they typically are diffuse and occur near the

interface between white and grey substance. The cerebellum, spinal cord and pituitary gland are generally devoid of vascular and parenchymal  $\beta$ -amyloid lesions in aged squirrel monkeys.

#### Histomorphology of CAA in squirrel monkeys

In squirrel monkeys, aggregated A $\beta$  occupies meningeal and parenchymal arteries/arterioles as well as numerous parenchymal capillaries (Figs. 2A, 4B). When stained with Congo Red, many arterioles show



**Fig. 1.** Distribution of A $\beta$ 42-immunoreactive CAA and parenchymal (plaque-like) deposits at 3 coronal levels in a 21 year-old squirrel monkey (84L). Each point represents a single vascular (left) or parenchymal (right) A $\beta$ -positive profile. The density of total neocortical A $\beta$ -positive profiles in this animal was 32.1 lesions/mm<sup>2</sup> in prefrontal cortex and 2.1 lesions/mm<sup>2</sup> in occipital cortex. Note the laminar segregation of neocortical deposits, particularly dorsally and laterally. A23, A5 and P4 indicate the approximate coronal levels (Emmers and Akert, 1963); the lower brainstem and cerebellum were devoid of A $\beta$  deposits and are not depicted at the caudalmost level [P 4] (Antibody Anti-A $\beta$ 42). Abbreviations: Bst, brainstem; CaS, calcarine sulcus; CC, corpus callosum; CiS, cingulate sulcus; ITS, inferior temporal sulcus; LF, lateral fissure; OS, orbital sulcus; STS, superior temporal sulcus.

**Table 1.** Age, sex and overall ratio of CAA: senile plaque profiles.

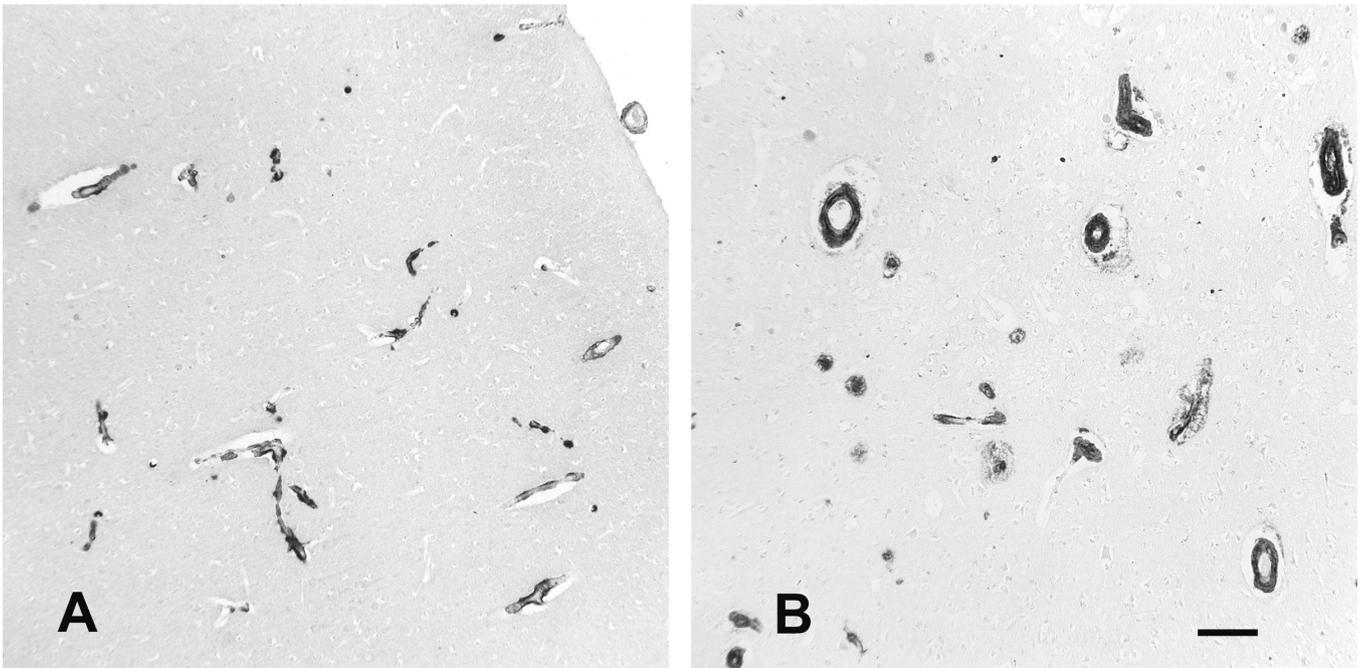
Subject	Age	Sex	CAA:SP [Level]
84L	~21	M	2.4 [Multiple]
83GO	20	M	6.2 [Multiple]
81EK	23	M	2.0 [Multiple]
89U	15	M	2.4 [Multiple]
2539	7	M	No lesions [Multiple]
92-11	24	M	2.0 [A15.5]
88-7	~23	M	3.0 [A8.5]
92-13	~17	F	2.9 [A18.0]
91-7	18	F	1.6 [A22.0; A9.0]
93-1	~16	M	1.9 [A8.5]
93-7	17	F	3.4 [A14.0]
6613	~19	M	2.9 [A5.0]
8601	~20	M	2.7 [A13.5]
7842	~19	M	2.9 [A14.0]
7140	~18	M	4.2 [A7.5]

Fifteen squirrel monkeys were used in this study; the first five form the core of the analysis. The anterior (A) coronal levels according to Emmers and Akert (1963) at which CAA and plaques were quantitated are indicated in parentheses in the right-hand column. The number of CAA profiles was consistently greater than that of senile plaques ( $\chi^2[1] = 3.46, p < 0.0001$ ), although the ratio of CAA to plaques varied among the animals. ~ indicates estimated ages; all other ages were known.

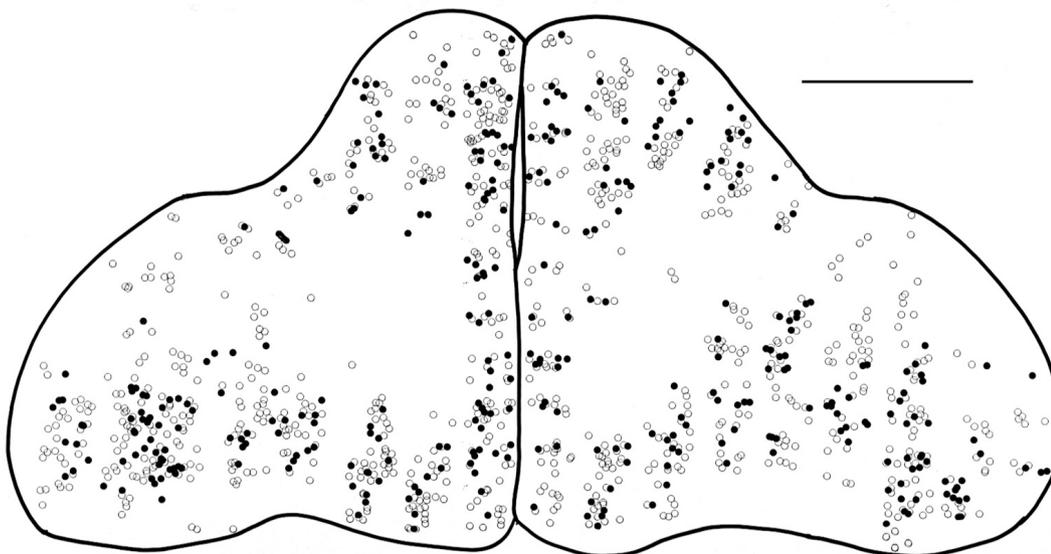
*Cerebral amyloid angiopathy in Saimiri*

characteristic birefringence using crossed polarizing filters (Fig. 4D), but few capillaries are strongly congophilic. As in humans, veins are seldom amyloidotic. The involvement of superficial (Fig. 5A) and parenchymal (Figs. 2A, 4D, 5B) arterioles is most apparent in animals with profuse capillary CAA. Within the arteriolar wall, the basal lamina of the tunica media

is a principal site of A $\beta$  accumulation, and the tunica adventitia also is frequently involved. When severe, CAA is associated with effacement/hypocellularity of the vascular wall, particularly within the tunica media (Fig. 5B). Ultrastructurally, the basal lamina of amyloidotic vessels is enlarged and distended by masses of classical amyloid fibrils (Fig. 6). In capillaries (which



**Fig. 2.** Cortical CAA in a 17 year-old squirrel monkey (92-13)(A) and a human AD patient with abundant CAA (B). Capillaries are frequently amyloidotic in Saimiri, whereas large vessels are more often involved in humans (Antibody 10D5). Bar: 100  $\mu$ m.



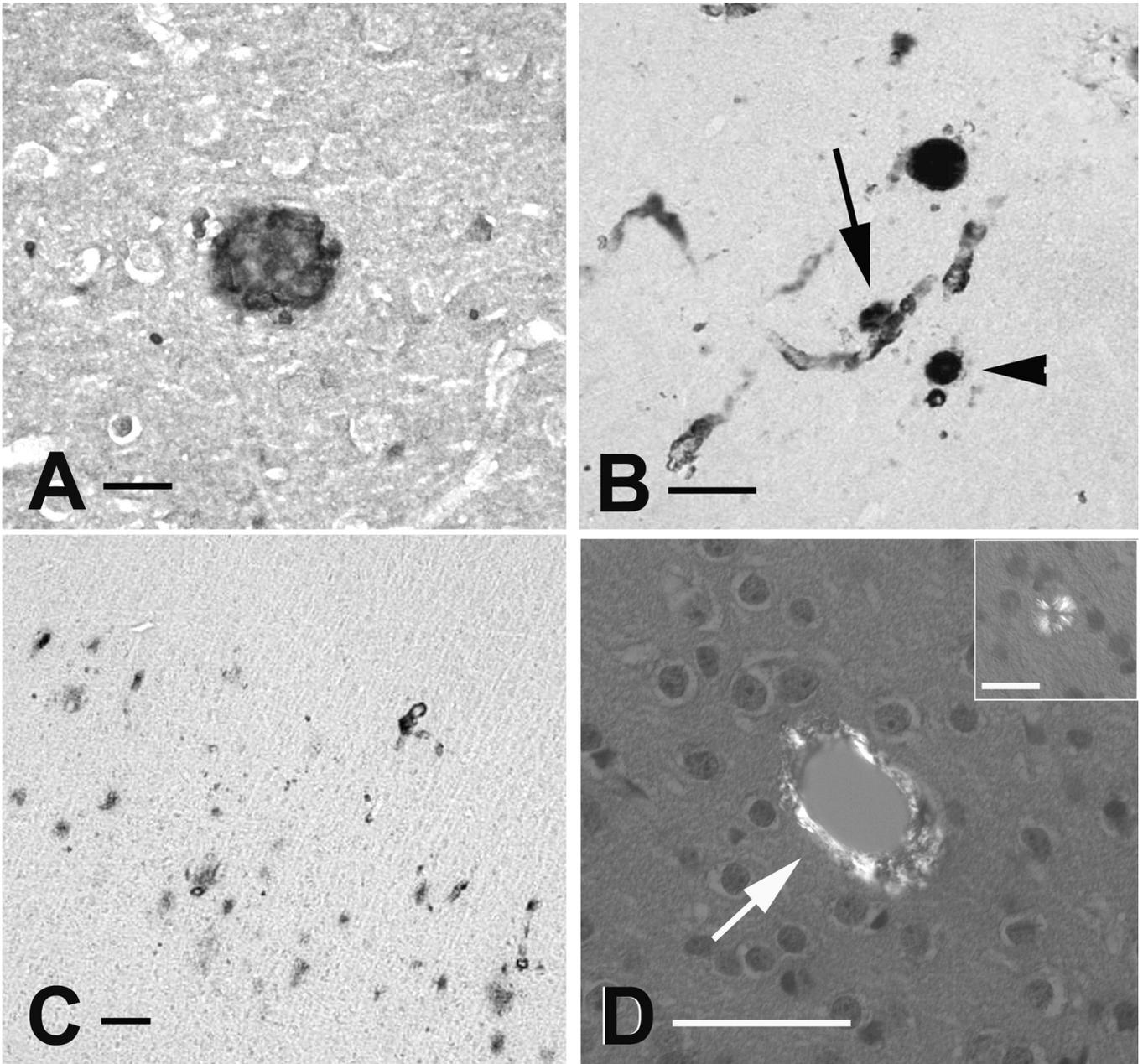
**Fig. 3.** The bilateral symmetry of A $\beta$ -immunoreactive parenchymal deposits (filled circles) and CAA profiles (empty circles) is illustrated in this plot of an A $\beta$ -immunostained section through the rostral frontal lobes of an 18 year-old squirrel monkey (91-7) at approximately anterior level +22 according to Emmers and Akert (1963). In this section there were 417 vascular deposits and 177 parenchymal deposits in the hemisphere on the left, and 400 vascular deposits and 174 parenchymal deposits on the right (Antibody 10D5). Bar: 5  $\mu$ m.

lack a tunica media proper), A $\beta$  condenses in the basal lamina and tunica adventitia. In some instances, the A $\beta$  in parenchymal and vascular lesions is coextensive (Fig. 4B, arrow).

*Histomorphology of parenchymal A $\beta$  and cellular tau*

*deposits*

Large, parenchymal senile plaques (Fig. 4A) are relatively infrequent in aged squirrel monkeys. More often, A $\beta$  deposits in the neuropil of the brain are small



**Fig. 4.**  $\beta$ -amyloid pathology in aged squirrel monkeys. **A.** Senile plaque in neocortex of a 23 year-old squirrel monkey (81EK); Antibody R163. Bar: 25  $\mu$ m. **B, C.** CAA and parenchymal A $\beta$  deposits in neocortex of a 21 year-old squirrel monkey (84L); Antibody Anti-A $\beta$ 40. **B.** The arrow denotes an amyloid "bloom" adjacent to an amyloidotic capillary, and the arrowhead marks a small parenchymal deposit. Bar: 50  $\mu$ m. **C.** Low magnification view of a thin layer of mostly diffuse, parenchymal A $\beta$ -deposits at the border between neocortical grey matter (upper right) and white matter (lower left). A few immunoreactive capillaries also are present. Bar: 50  $\mu$ m. **D.** Congo Red-stained arteriole (arrow) and senile plaque (inset), viewed with crossed polarizing filters, in neocortex of a 23 year-old squirrel monkey (81EK). Bars: 50  $\mu$ m, and 25  $\mu$ m (inset).

## Cerebral amyloid angiopathy in Saimiri

(mostly 10-25  $\mu\text{m}$  in diameter), spherical (Fig. 4B) and generally devoid of a prominent neuritic corona in silver-stained or tau-immunostained material. Some of these plaque-like deposits are similar to the amyloid tufts adjoining capillaries (Fig. 4B, arrow). A subset of parenchymal plaques are congophilic (Fig. 4D, inset), but the number of parenchymal lesions stained by Congo Red was much less than that detected by immunohistochemistry (in general, Congo Red was much less sensitive to the presence of A $\beta$  than was immunohistochemistry or the Campbell-Switzer silver stain). There also are numerous diffuse, parenchymal A $\beta$  deposits in some regions, such as those forming a thin band at the border between gray and white matter in the dorsolateral neocortex (Fig. 4C).

Occasional cortical neurons as well as abnormal neurites around plaques are immunoreactive with anti-tau antibodies Alz50 and AT8 (not shown). As in other nonhuman primates, neither tau immunostaining nor silver- (Campbell-Switzer Gallyas) histostaining revealed fully developed neurofibrillary tangles in any of the squirrel monkeys that we examined.

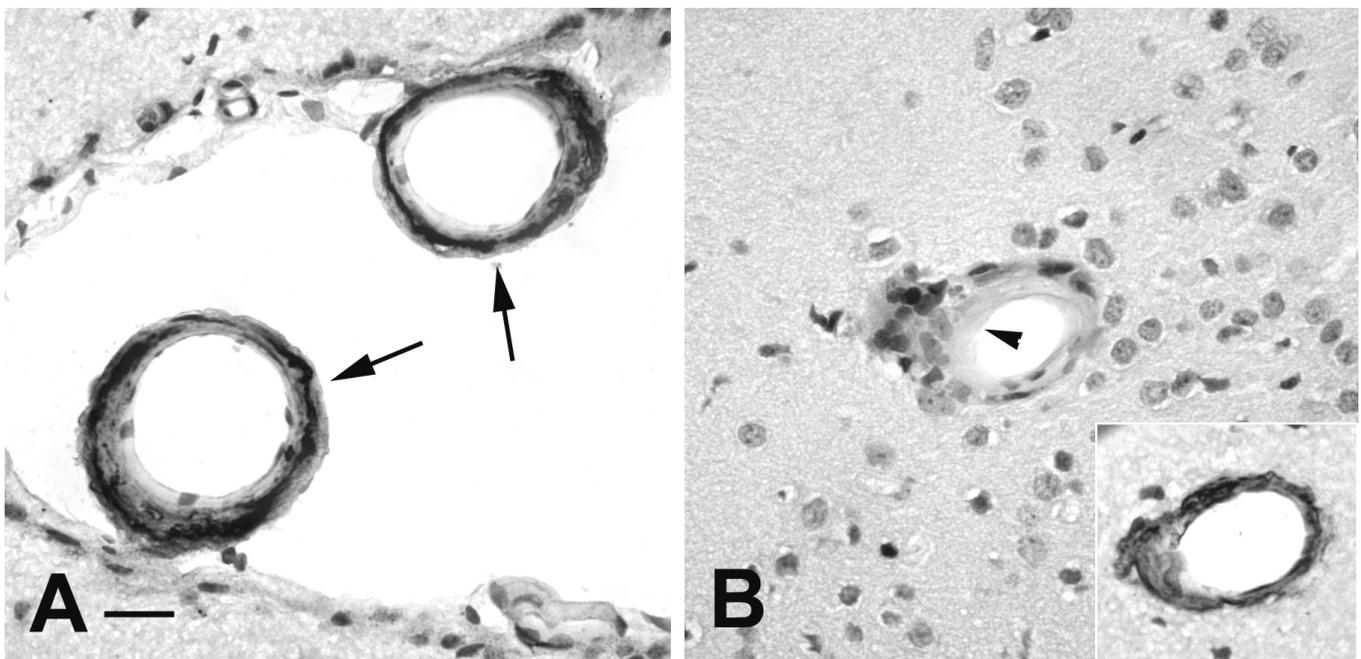
### Deposition of A $\beta$ 40 and A $\beta$ 42 in aged squirrel monkeys

A $\beta$ 40 and A $\beta$ 42 each were detected using antibodies from two sources (R163 [Mehta] and polyclonal anti- $\beta$ -amyloid 1-40 [Biosource] for A $\beta$ 40, and R165 [Mehta]

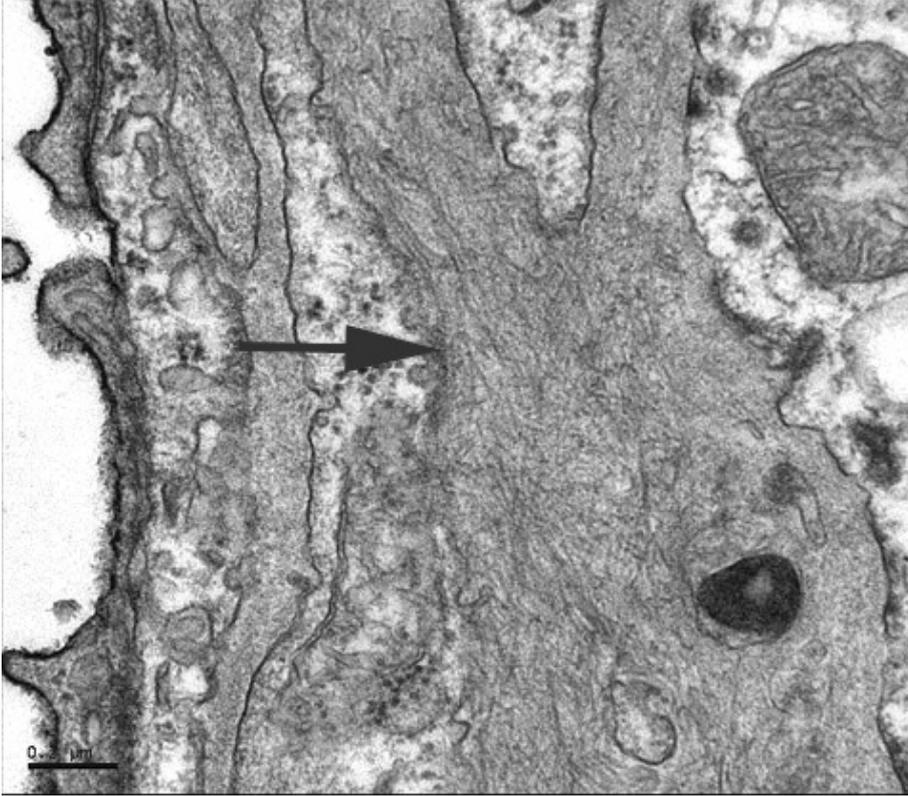
and polyclonal anti- $\beta$ -amyloid 1-42 [Biosource] for A $\beta$ 42). Antibodies from both sources yielded similar immunolabeling patterns. Parenchymal A $\beta$ -deposits and capillary CAA (which together constitute the great majority of lesions) generally are immunopositive both for A $\beta$ 40 and A $\beta$ 42, whereas somewhat more numerous large vessels are immunoreactive for A $\beta$ 40 than for A $\beta$ 42. As in humans, individual aged monkeys differ in the relative abundance of CAA and senile plaques (the CAA:Plaque numerical ratio varied from 1.6:1 to 6.2:1 in our sample; Table 1), and in the tendency of these lesions to contain different isoforms of A $\beta$ .

### CAA and coincident vasculopathies

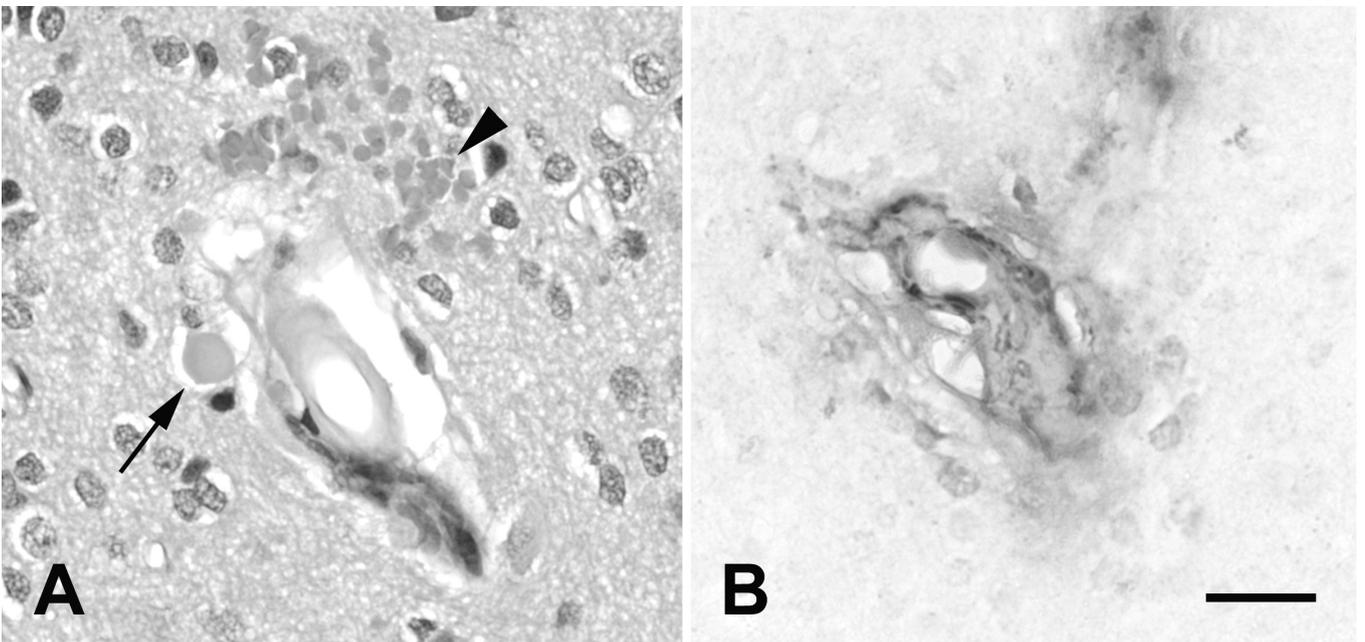
Coincident CAA-related vasculopathies arise in aged squirrel monkeys, but such lesions are uncommon. In severely affected animals there is evidence of microhemorrhage and fibrinoid exudation in some amyloidotic vessels (Fig. 7), but these changes are very rare, even in animals with pronounced cerebrovascular amyloid. Overt inflammation/encephalitis was not present in any aged animal. Although amyloidotic vessels occasionally were surrounded by reactive microglia, most often peri-CAA microgliosis was negligible (Fig. 8A,B). Much more often, CAA was associated with reactive astrocytes (Fig. 8C,D).



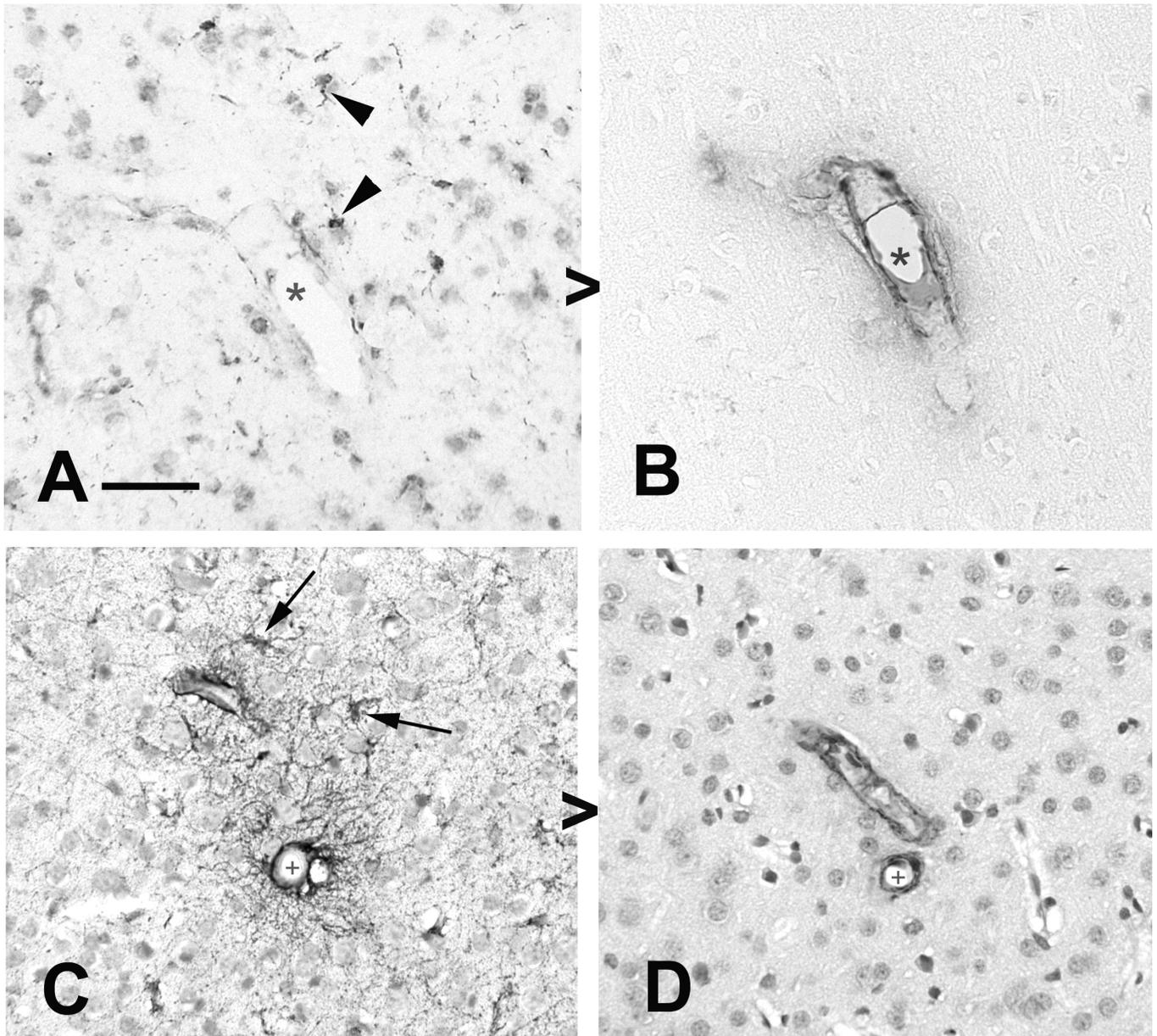
**Fig. 5.** CAA in a 23 year-old squirrel monkey (81EK). **A.** Large superficial arterioles are immunopositive for A $\beta$  (arrows); Antibody 4G8, hematoxylin counterstain. **B.** Intracortical amyloidotic arteriole with effacement of the vascular wall (arrowhead) and a focal clustering of cells, including a multinucleated giant cell, in the adventitia and adjacent parenchyma to the left (H+E stain). Inset: the same vessel in an adjacent tissue section is positively immunostained for A $\beta$  (antibody 4G8, light hematoxylin counterstain). Bar: 25  $\mu\text{m}$ .



**Fig. 6.** Ultrastructure of the wall of an amyloidotic arteriole in a 23 year-old squirrel monkey (81EK). The lumen is to the left; the basal lamina (arrow) contains copious  $\beta$ -amyloid fibrils and is greatly increased in width and tortuosity. Bar:  $0.2 \mu\text{m}$ .



**Fig. 7.** CAA-associated vasculopathy in a 23 year-old squirrel monkey (81EK). **A.** Neocortical arteriole stained with hematoxylin and eosin showing effacement of the vessel wall, eosinophilic fibrinoid exudate (arrow), and red blood cells in the brain parenchyma (arrowhead). **B.** The same vessel as in A, in an adjacent section, immunostained with antibody 4G8 to A $\beta$ . Bar:  $25 \mu\text{m}$ .

Cerebral amyloid angiopathy in *Saimiri*

**Fig. 8.** Glial cells and CAA in a 23 year-old squirrel monkey (81EK). **A.** Microglia immunostained with an antibody to Iba1 (arrowheads) near a blood vessel (\*) that is immunopositive for A $\beta$  in an adjacent section (**B**; antibody 4G8). Reactive microglia are uncommon near CAA in normal aged squirrel monkeys. **C.** Vascular profiles (one is denoted by +) that are surrounded by reactive astrocytes (two are marked by arrows) and strongly immunoreactive GFAP-positive astrocytic processes. Hematoxylin counterstain. **D.** The same region as in C, in an adjacent section, immunostained with antibody 4G8. Note the immunopositivity of the vascular wall for A $\beta$ . Reactive astrocytosis frequently accompanies CAA in squirrel monkeys. Hematoxylin counterstain. Bar: 50  $\mu$ m.

## Discussion

CAA increases the risk of hemorrhagic stroke in the elderly (Vinters, 1992; Kase, 1994; Jellinger, 2002), and appears to contribute to cognitive decline even in the absence of stroke (Rensink et al., 2003; Xu et al., 2003; Greenberg et al., 2004; Nicoll et al., 2004). Progress in understanding cerebrovascular amyloidosis has been hindered by a dearth of appropriate animal models

(Revesz et al., 2003). Whereas age-associated CAA has been noted in a variety of vertebrate species (Walker, 1997, 2000; Brellou et al., 2005), presently there are few well-established, natural models of idiopathic CAA. Aged dogs and nonhuman primates have been most thoroughly investigated (Walker, 2000; Walker and Cork, 1999; Walker and Durham, 1999). Squirrel monkeys are particularly suitable for modeling human

idiopathic CAA, since they share a number of biological similarities with humans, and because CAA is the primary form of cerebral  $\beta$ -amyloidosis in this species.

In the present study, we confirm the predilection of squirrel monkeys to age-related CAA, and show that: 1) Some amyloidotic blood vessels in squirrel monkeys are congophilic, and are afflicted by classical amyloid fibrils at the ultrastructural level; 2) A $\beta$ 40 and A $\beta$ 42 coexist in most microvascular and parenchymal lesions, although A $\beta$ 40 tends to predominate in larger arterioles; 3) The density and anatomical distribution of CAA and parenchymal plaques overlap and show bilateral symmetry; 4) In neocortex, which is heavily affected, parenchymal and vascular A $\beta$  deposits are most numerous rostrally and relatively sparse in the occipital lobe; 5) Capillaries are particularly vulnerable to CAA in squirrel monkeys; and 6) Severe CAA is linked to a low but unambiguous increase in other vasculopathic changes such as microhemorrhage, fibrinoid extravasation and focal gliosis. In light of the cerebrovascular and immunologic similarities between squirrel monkeys and humans (Mackic et al., 1998; Bading et al., 2002; Fukuda and Zoppo, 2003; Maeda et al., 2005), these findings support the squirrel monkey as a biologically optimal model for modeling idiopathic CAA, and for testing the safety and efficacy of A $\beta$ -reduction strategies such as A $\beta$ -immunization therapy for AD.

The two most common isoforms of A $\beta$  in the brain are 40 and 42 amino acids in length (A $\beta$ 40 and A $\beta$ 42). Whereas cells generally produce much more A $\beta$ 40, A $\beta$ 42 has a greater tendency to polymerize, and is the first isoform to appear as parenchymal, plaque-like deposits in aging humans (Walker et al., 2000). For reasons that remain unclear, A $\beta$ 40 is the main component of CAA in humans (Castano et al., 1996; Attems et al., 2004), although there is evidence for the early deposition of A $\beta$ 42 in the vessel wall (Roher et al., 1993; Revesz et al., 2003). In squirrel monkeys, our studies show that capillaries are particularly disposed to CAA, and most of these small vessels contain both A $\beta$ 40 and A $\beta$ 42. However, as in humans, A $\beta$ 40 immunoreactivity is more frequent than that of A $\beta$ 42 in arterioles. While capillary CAA is comparatively uncommon in humans, when present in AD patients, it is significantly correlated with the degree of neuritic pathology (Attems and Jellinger, 2004; Attems et al., 2004). Our observation of substantial overlap of CAA and small, parenchymal, plaque-like deposits in squirrel monkeys suggests that these plaques may be pathogenically linked to capillary CAA. Future studies can profitably evaluate the temporal emergence of parenchymal and vascular lesions, establish the relationship between CAA and behavioral performance, and determine whether the efflux of A $\beta$  via perivascular drainage pathways (Weller and Nicoll, 2003) in *Saimiri* differs from that of primate species manifesting relatively greater proportions of parenchymal lesions, for instance rhesus monkeys (*Macaca mulatta*). The relative

sparing of brain regions such as the occipital lobe (an area frequently involved in human CAA) offers additional opportunities to identify endogenous risk factors for CAA, including physiological and biochemical influences, regional variation of vascular factors, or heterogeneity of gene expression profiles.

The immunoreactivity of most senile plaques and amyloidotic vessels in *Saimiri* for both A $\beta$ 40 and A $\beta$ 42 supports data from rhesus monkeys and chimpanzees that A $\beta$ 40 is particularly abundant in nonhuman primates compared to humans (Gearing et al., 1996). Whether nonhuman primate cells produce comparatively higher levels of A $\beta$ 40 than A $\beta$ 42, whether they differentially transport or metabolize the two forms, or whether the longer peptide is enzymatically trimmed to the 40th residue in simian A $\beta$ -deposits, remains unknown. Despite the universal expression of A $\beta$  pathology in aged primates, the full behavioral and pathologic phenotype of Alzheimer's disease is manifested only in humans, suggesting that elucidation of human-specific genetic, physiologic and/or biochemical factors could yield useful clues to the origins of this disorder.

Genetic modifications that are associated with A $\beta$ -CAA in humans include mutations in  $\beta$ APP and in presenilins 1 and 2, as well as polymorphisms in apolipoprotein E (ApoE) (Rensink et al., 2003; Revesz et al., 2003). No comparable mutations are present in squirrel monkeys that would account for their predisposition to cerebrovascular amyloidosis.  $\beta$ APP<sub>751</sub> is 99.5% homologous in humans and squirrel monkeys, and there are no mutations in or near A $\beta$  that would be expected to favor vascular A $\beta$  deposition, as occurs in human familial forms of CAA (Levy et al., 1995). The apolipoprotein E $\epsilon$ 4 allele is a significant risk factor for CAA in humans with AD (Love, 2004), but, among primates, only humans have the variations at amino acid positions 112 and 158 that define the human apoE types 2, 3 and 4. All nonhuman primates so far analyzed, including squirrel monkeys, are homozygous for apoE $\epsilon$ 4 according to the human nomenclature (Morelli et al., 1996). However, nonhuman primates have an additional substitution (threonine for arginine at position 61) that causes simian apoE to interact with lipoproteins similarly to human apoE3, that is, squirrel monkey apoE is functionally equivalent to human apoE3 (Morelli et al., 1996). It is worth noting, however, that capillary CAA is more frequent in apoE $\epsilon$ 4-positive humans than in those lacking apoE $\epsilon$ 4 (Thal et al., 2002), and this type of amyloid angiopathy (called Type 1 CAA) more closely resembles the phenotype seen in squirrel monkeys than does Type 2 CAA, which afflicts mainly arterioles.

Intriguingly, squirrel monkeys have a polymorphism in the gene for the cysteine protease inhibitor *cystatin C* at the locus that causes a rare human disorder known as hereditary cerebral hemorrhage with amyloidosis-Icelandic type (HCHWA-I) (Wei et al., 1996). In HCHWA-I, the vascular amyloid deposits consist of a truncated form of cystatin C rather than A $\beta$ . However, a

## Cerebral amyloid angiopathy in Saimiri

Croatian man with a sporadic 'Icelandic'-like mutation in cystatin C and recurrent intracranial hemorrhage was found at autopsy to have severely amyloidotic brain vessels in which cystatin C co-deposited with A $\beta$  (Graffagnino et al., 1995). A $\beta$  and cystatin C also colocalize in cerebral vessels of Saimiri (Wei et al., 1996), but whether the Icelandic-like polymorphism in cystatin C influences the predisposition of squirrel monkeys to A $\beta$ -CAA remains uncertain. A similar polymorphism in cystatin C is not present in rhesus monkeys (Wei et al., 1996).

A small percentage of amyloidotic vessels in normal aged squirrel monkeys exhibit microhemorrhage and/or perivascular fibrinoid material. A local cellular gliotic reaction sometimes accompanies these changes, but there was no sign of widespread vasculitis, encephalitis or leukoariosis in any animal. Hence, as in humans, CAA is a risk factor for other vasculopathies in squirrel monkeys; the relatively low incidence of these changes in Saimiri, however, even in the context of profuse CAA, provides an optimal baseline from which to assess the adverse effects of therapeutic manipulations such as A $\beta$ -immunotherapy or secretase inhibition on CAA-associated vasculopathy, hemorrhage, and meningo-encephalitis.

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*Paradoxes in nuclear structure and function*

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