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Amyloid precursor protein in aged nonhuman primates

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ABSTRACT **In individuals with Alzheimer disease and in aged nonhuman primates, deposits of amyloid occur in senile plaques in brain parenchyma and in the walls of some meningeal and cortical vessels. Amyloid is primarily composed of β/A4, a 4-kDa peptide derived from the transmembrane form of an amyloid precursor protein (APP). We examined the distribution of β/A4 and APP (outside the β/A4 domain) in cerebral cortices of monkeys ranging in age from 4 to 41 years. In all animals, APP immunoreactivity was present in cell bodies, proximal dendrites, and axons of cortical neurons. In aged animals, all of which showed senile plaques, large APP-positive axons were conspicuous, and APP immunoreactivity was present in neurites around β/A4-immunoreactive plaques. In some plaques, APP-immunoreactive elements were located in proximity to deposits of β/A4. The presence of APP immunoreactivity in neuronal perikarya, dendrites, axons, and in neurites within β/A4-containing plaques supports the hypothesis that neurons can serve as one source of amyloid deposited in brain parenchyma.**

Senile plaques, consisting of neurites (enlarged distal axons/nerve terminals/dendrites) surrounding deposits of extracellular amyloid fibrils, occur in the brains of subjects with Alzheimer disease and aged monkeys (1–3). The principal component of amyloid is β/A4, a 4-kDa peptide (4, 5) derived from the ecto- and transmembrane domains of the amyloid precursor protein (APP) (6, 7). Alternative splicing of the APP gene gives rise to several mRNAs, including those that encode integral membrane APP-695 (8), APP-751 (protease nexin II) (9, 10), and APP-770 (11–13). APP mRNAs are transcribed by neurons, but cell populations in various brain regions may express different levels of transcripts (14–23). Although APP is a membrane-associated glycoprotein and *in vitro* studies indicate that it is cleaved within the ectodomain of β/A4 (24, 25), the functions of APP are not known. APP immunoreactivity is present in neurons (21, 26, 27), but its distribution within cells and the relationships between APP-immunoreactive elements and deposits of β/A4 in the nonhuman primate brain have not been examined. The occurrence of senile plaques in the brains of aged monkeys (28, 29) provides an opportunity to study the spatial relationships between neurites and deposits of parenchymal β/A4. This investigation demonstrates the distribution of APP and β/A4 immunoreactivities in cerebral cortices of young and old nonhuman primates. Our observations suggest that neuronal APP may serve as one source of the β/A4 deposited in the brain parenchyma of older primates.

MATERIALS AND METHODS

Immunoblotting. To confirm the authenticity of APP-specific immunoreactivity (by immunoblotting), cingulate, temporal, orbitofrontal, and prefrontal cortices and corpus callosum were sampled (≈0.1 g) from a freshly frozen brain of a 37-year-old, lion-tailed macaque (*Macaca silenus*) with a 3-hr postmortem delay. Tissues were homogenized in buffer (0.5% SDS/50 mM Tris, pH 6.8/5 mM EDTA/pepstatin at 50 μg/ml/leupeptin at 50 μg/ml/0.25 mm phenylmethylsulfonyl fluoride/aprotinin at 10 μg/ml), boiled for 5 min, and centrifuged at 1200 × g for 10 min. Aliquots of total protein (50 μg) from the soluble fraction were boiled in 2% SDS/5% 2-mercaptoethanol/10% glycerol, fractionated by discontinuous SDS/PAGE in a 3–20% polyacrylamide gradient gel, and transferred to nitrocellulose membranes. Protein concentrations in soluble fraction were assayed using the bicinchoninic acid assay method (Pierce). APP immunoreactivity was visualized using conditions similar to those used for immunocytochemistry. Membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline, incubated (1:2000) overnight with monoclonal antibody (22C11) directed against the amino-terminal region common to all three APP isoforms (7), and developed by using a peroxidase-antiperoxidase method (30).

Immunocytochemistry. For immunocytochemistry, brain samples were obtained from the lion-tailed macaque and from rhesus monkeys (*Macaca mulatta*) ($n = 11$) ranging in age from 4 to 41 years. Monkeys were anesthetized and perfused with normal saline. Sections of unfixed frozen brain were cut (10 μm) serially, and adjacent sections were stained with cresyl violet, the Bielschowsky silver technique, or with monoclonal antibody (22C11), recognizing an epitope located between amino acids 60 to 100 in the amino-terminal part of the ectodomain of human APP (7); polyclonal antiserum (anti-Fd-preA4), directed against APP (7); polyclonal antibody recognizing amino acids 1–28 of the β/A4 region; monoclonal antibody (03-44), raised against a phosphorylated epitope of the 200-kDa neurofilament protein (31); and monoclonal antibody recognizing synaptophysin (Boehringer Mannheim), a membrane glycoprotein associated with presynaptic elements (32, 33). Sections for localization of APP and β/A4 were pretreated (30 min) with 15% formic acid (34). Primary antibodies were diluted as follows: monoclonal anti-APP (1:500); polyclonal anti-APP (1:250); anti-β/A4 (1:500); anti-03-44 (1:1000); and antisynaptophysin (1:25). Negative controls for monoclonal and polyclonal antibodies were mouse IgG and normal rabbit serum, respectively. Specificity of APP staining was assessed also by absorption of both monoclonal and polyclonal antibodies against denatured APP-695 synthesized in APP-695-transfected COS-1 cells (25). A diaminobenzidine reaction was used to disclose immunoreactivity.

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Abbreviation: APP, amyloid precursor protein.

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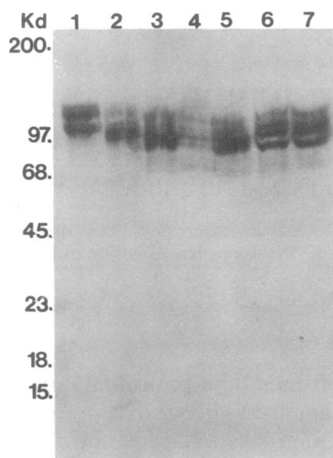

FIG. 1. Immunoblot of APP immunoreactivity in monkey brain. Lanes 1 and 2 demonstrate that the anti-APP antibody (22C11) recognizes APP synthesized in COS-1 cells transfected with full-length APP-770 and -695 cDNA, respectively. Samples of brain were obtained from frontal cortex (lane 3), corpus callosum (lane 4), and orbitofrontal (lane 5), temporal (lane 6), and cingulate (lane 7) cortices. Lightly stained bands below the 97-kDa marker probably reflect degradation products of APP. Immunoblots were performed on the soluble fraction. Molecular mass markers in kDa at left are prestained standards that migrate anomalously in SDS/PAGE.

RESULTS

Immunoblotting. Immunoblotting verified the presence of APP isoforms (≈100–135 kDa) in monkey brain (Fig. 1). The migration of immunoreactive proteins in denaturing acrylamide gels coincides with immunoreactive-protein species synthesized in COS-1 cells transfected with genes that code for APP-695 and APP-770 (35). The molecular mass heterogeneity of APP detected *in vivo* is consistent with its posttranslational modification (7, 36).

Immunocytochemistry. Distributions of APP immunoreactivity were similar in all monkeys, but some structures were more apparent in the brains of older animals. Controls, including APP antibodies preabsorbed against APP-695 synthesized in COS-1 cells, showed no immunostaining (Fig. 2).

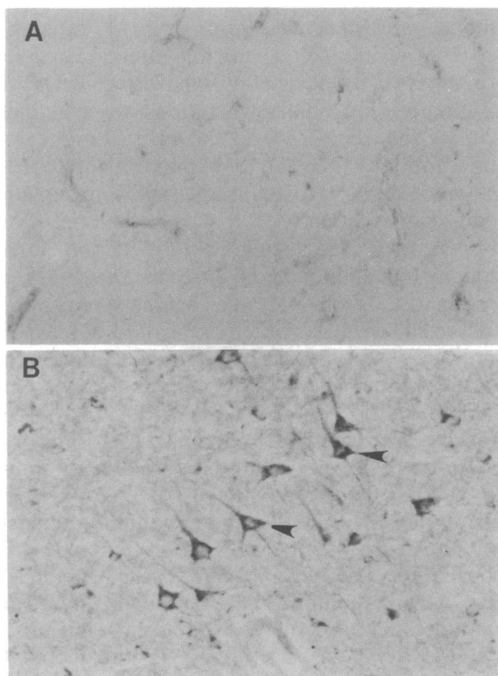

FIG. 2. Prefrontal cortex of a young monkey (9 years of age), illustrating the specificity of APP immunoreactivity. (×240.) (*A*) The section was incubated with polyclonal anti-APP antiserum after absorption overnight with APP synthesized in COS-1 cells. Immunoreactivity is absent. (*B*) Section incubated with polyclonal anti-APP antiserum shows specific immunoreactivity associated with neuronal cell bodies (arrowheads).

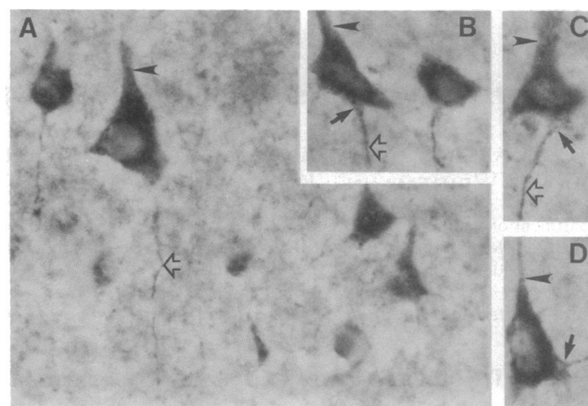

FIG. 3. Large and small cortical neurons (*A–D*) show APP immunoreactivity in perikarya, proximal dendrites (arrowheads), axon hillocks (short arrows), and axons (open arrows). Sections were stained with the polyclonal anti-APP antiserum. (×600.)

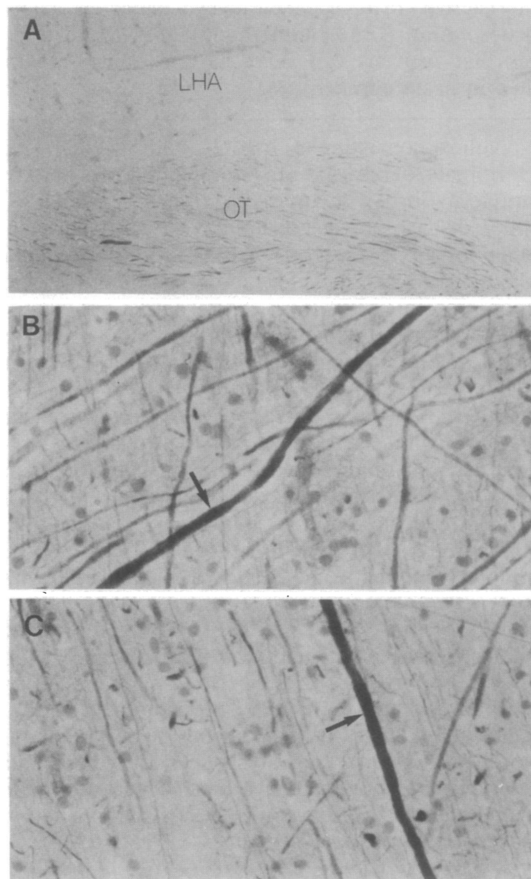

FIG. 4. APP immunoreactivity shown in axons of white matter of a 37-year-old macaque. Sections were stained with the monoclonal anti-APP antibody (22C11). (*A*) The optic tract (OT) shows APP immunoreactivity in axons. LHA, lateral hypothalamic area. (×60.) (*B* and *C*) Immunostained sections counterstained with cresyl violet show small and large APP-immunoreactive axons in the corona radiata in longitudinal profile. Large intensely stained axons (arrows) were found only in aged monkeys. (×240.)

In the cerebral cortex, APP was present in neuronal perikarya, proximal dendrites, axon hillocks, and initial segments (Fig. 3). Cortical neurons exhibited a range of APP immunoreactivities; some were highly immunoreactive, whereas others showed little or no APP (Fig. 3). The immunostaining was most intense in large pyramidal neurons (layers III and V), but some smaller neurons showed APP immunoreactivity (Fig. 3). Immunoreactivity was associated with intracytoplasmic aggregates that appeared, by fluorescence microscopy, to be distinct from accumulations of lipofuscin. In older monkeys, APP immunoreactivity was conspicuous in large axons (Fig. 4), particularly in the cortex, corona radiata, callosum, and optic tract. Throughout the cortical neuropil, fine puncta of APP immunoreactivity were present; these puncta may represent synaptic terminals because synaptophysin immunoreactivity showed similar terminal-like labeling in the cortex. APP immunoreactivity was not apparent in glial cells or blood vessels.

Cortices of older monkeys showed argentophilic fiber abnormalities, neurites, and senile plaques (most abundant in layers V and VI). Abnormal fibers and neurites contained APP, phosphorylated neurofilament, or synaptophysin epitopes. Some axons exhibited bulbous swellings (Fig. 5). Many neurites in plaques were in proximity to β/A4-positive deposits, and APP- and synaptophysin-immunoreactive elements were also present in plaques (Fig. 6). Only a subset of the total number of β/A4-containing plaques contained APP- and synaptophysin-immunoreactive neurites (Fig. 6). In individual plaques containing β/A4, APP, and synaptophysin immunoreactivities, the area decorated with β/A4 immunoreactivity was larger than the area containing APP- or synaptophysin-immunoreactive elements (Figs. 6 and 7). In these plaques, APP- and synaptophysin-immunoreactive structures were often surrounded by a halo of distorted neuropil that, in adjacent sections, contained β/A4 immunoreactivity (Fig. 7). Deposits of β/A4 immunoreactivity capped some neurites that showed APP, synaptophysin, or phosphorylated neurofilament immunoreactivities (Fig. 7). β/A4 was not apparent within neurites. In mature plaques, APP- and synaptophysin-immunoreactive globular neurites tended to occupy regions devoid of β/A4 immunoreactivity (Fig. 8). Most β/A4 immunoreactivity was visualized in the brain parenchyma, but some immunoreactivity was occasionally present in walls of meningeal and cortical vessels.

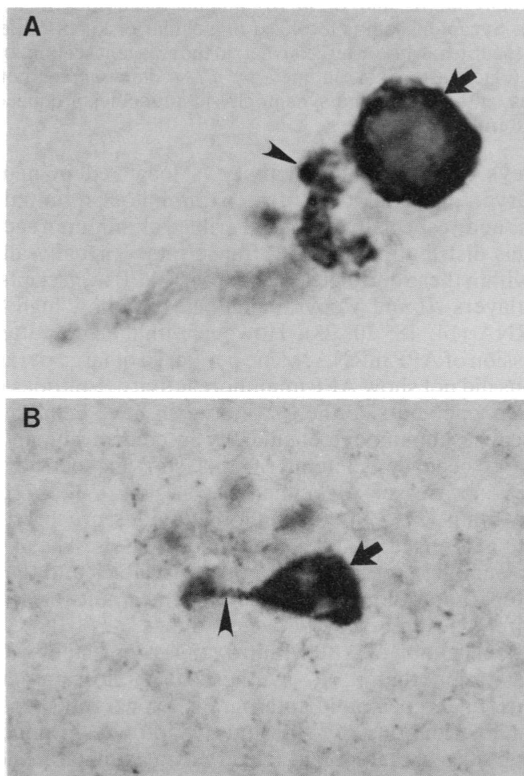

FIG. 5. Neurites in the temporal cortex of a 37-year-old monkey show immunoreactivity for APP (*A*) and synaptophysin (*B*). Note enlarged axons (arrowhead) and bulbous or knob-shaped swellings (arrow). In *A*, the neurite was visualized with the amino-terminal anti-APP antiserum. Monoclonal and polyclonal anti-APP antibodies generated similar patterns. (×600.)

DISCUSSION

Older macaques develop senile plaques and deposits of β/A4 that closely resemble plaques that occur in older humans,

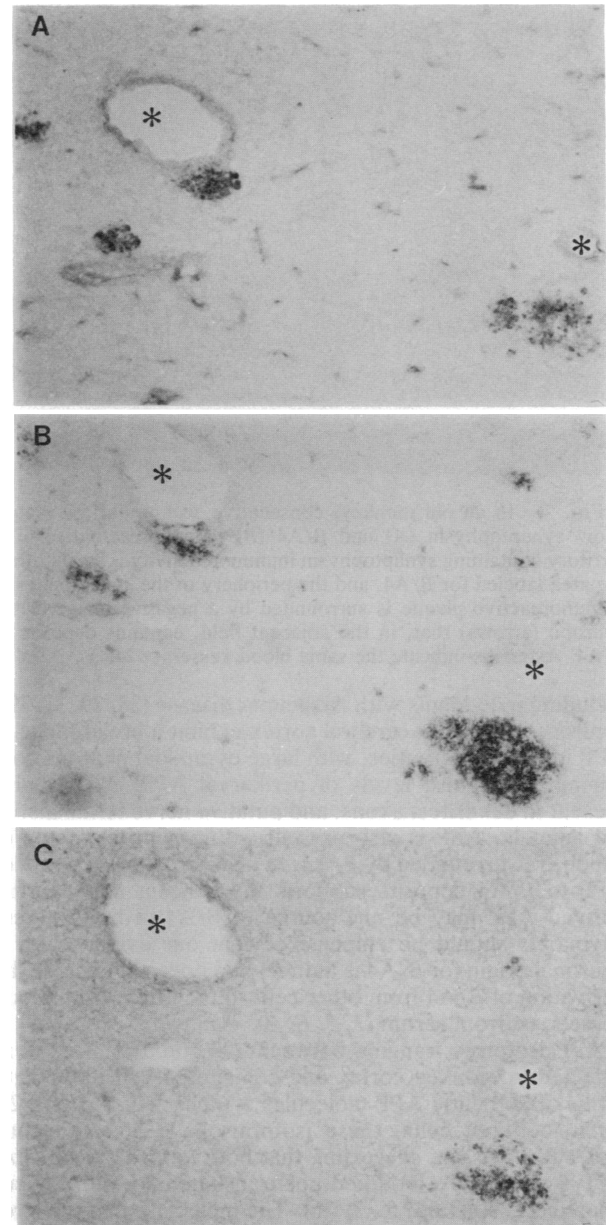

FIG. 6. APP (*A*), β/A4 (*B*), and synaptophysin (*C*) immunoreactivities are coextensive in some plaques in the temporal cortex of a 29-year-old monkey. Asterisks indicate the same blood vessel. In *A*, APP immunoreactivity was visualized with the polyclonal anti-APP antiserum; the monoclonal anti-APP antibody (22C11) produced a virtually identical pattern. (×120.)

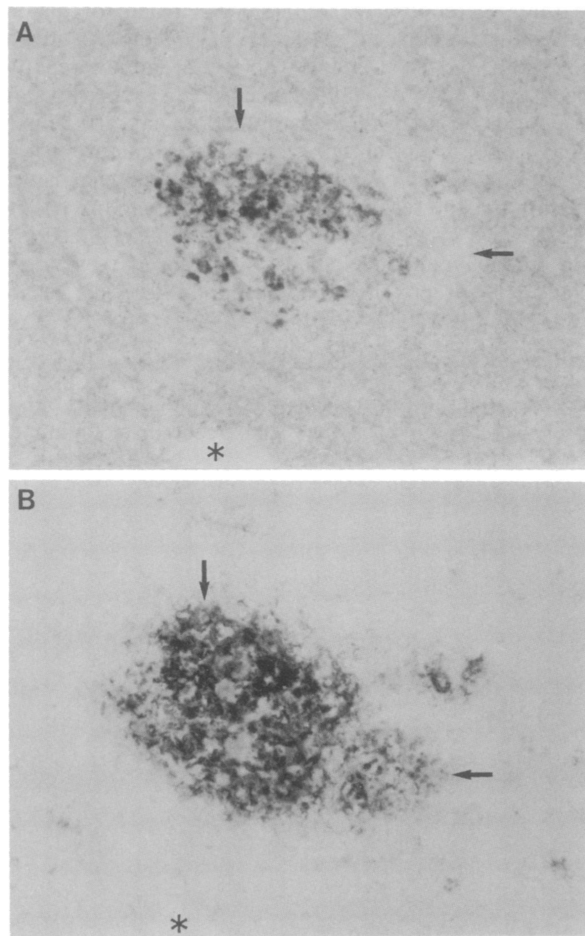

FIG. 7. In an old monkey, consecutive sections of the plaque show synaptophysin (*A*) and β/A4 (*B*) immunoreactivities. The territory containing synaptophysin immunoreactivity is smaller than the area labeled for β/A4, and the periphery of the synaptophysin-immunoreactive plaque is surrounded by a negative image in the neuropil (arrows) that, in the adjacent field, contains deposits of β/A4. Asterisks indicate the same blood vessel. (×240.)

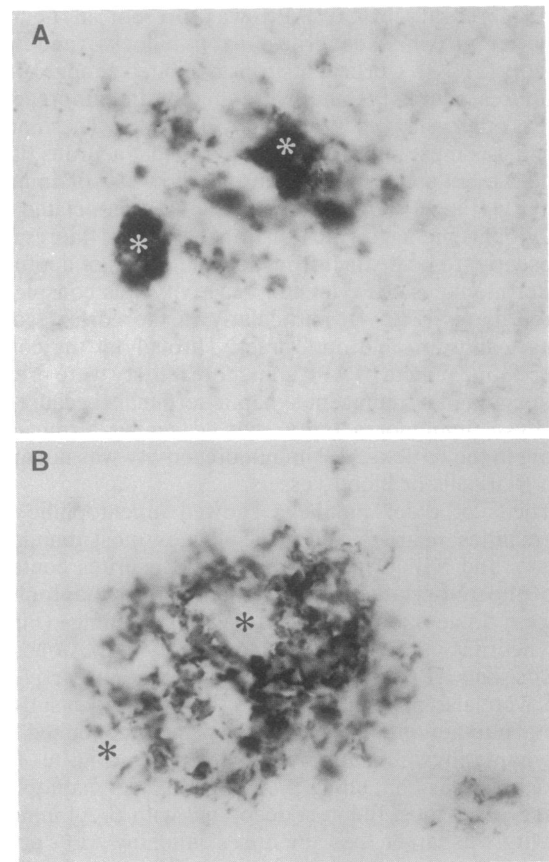

FIG. 8. In a 29-year-old monkey, adjacent sections demonstrate synaptophysin (*A*) and β/A4 (*B*) immunoreactivities in a senile plaque. Synaptophysin is localized in globular or knob-shaped elements (neurites, white asterisks) that, in the adjacent section, occupy areas (black asterisks) outlined by β/A4 immunoreactivity. In plaques, anti-APP and antisynaptophysin antibodies produced similar patterns. (×600.)

including individuals with Alzheimer disease (28, 29, 37–39). Neurons in monkey cerebral cortex exhibit a broad range of APP immunoreactivities, with large pyramidal neurons containing the highest levels of perikaryal APP. APP is also present in dendrites, axons, and putative nerve terminals. In old animals, APP is also present within neurites, some of which are surrounded by β/A4. In neurites, the proximity of APP to β/A4 deposits supports the concept that neurite-derived APP may be one source of β/A4 in brain parenchyma. It should be emphasized that our evidence for a neuronal origin for β/A4 is indirect and does not exclude the derivation of β/A4 from other cells in the brain, from blood vessels, or from serum (3, 5, 6, 40, 41).

APP isoforms, ranging between 100 and 135 kDa, were detected in monkey cortex and comigrated with full-length forms of authentic APP molecules synthesized *in vitro* (42). As in cultured cells, these isoforms have heterogeneous molecular masses, suggesting that both *in vivo* and *in vitro* APP isoforms are modified posttranslationally (i.e., glycosylated and sulfated) (6, 7, 36). The molecular mass heterogeneity is not restricted to APP-695 or -770 isoforms but, rather, is a combination of both these isoforms and, presumably, APP-751. These findings are consistent with results showing at least three major APP transcripts in adult monkey brain (43).

In cell bodies of neurons and perikarya in the rat, a variety of brain regions show APP immunoreactivities (21, 26). In monkeys, APP immunoreactivity is localized in neuronal perikarya, proximal dendrites, axon hillocks, initial segments, and, especially, axons. Perikaryal immunoreactivity parallels distributions of APP transcripts. Virtually all neurons within these cortical layers express APP transcripts, and large (layers III and V) pyramidal neurons show high levels of mRNA (14, 18, 20, 23). However, unlike the pattern of expression of APP mRNA, some perikarya of large pyramidal neurons did not show APP immunoreactivity. Epitopes of the APP sequence outside the β/A4 domain have been difficult to visualize immunocytochemically in control human brain but have been detected in regions showing neurites and plaques in cases of Alzheimer disease (44–47). In control human brain (27), APP immunoreactivities are present in cortical neurons, astrocytes, and blood vessels. The absence of confounding pre- and postmortem variables and the optimal preparation of monkey tissue may account for some of the differences in results between studies.

The localization of APP within axons is consistent with our demonstration that APP, synthesized in lumbar sensory neurons, undergoes rapid anterograde axonal transport (48). Similar distributions of APP and synaptophysin puncta in neuropil suggest that APP is delivered via axonal transport to nerve terminals. Ultrastructural immunocytochemical studies are needed to demonstrate that APP-immunoreactive puncta correspond to presynaptic elements. *In vitro* studies of transfected nonneuronal cells demonstrate that the large amino-terminal portion of APP is secreted into the extracellular compartment (7, 42) and that APP is cleaved at a site within the β/A4 region (24, 25). Similar processing may occur

in vivo because secreted APP is detected in the cerebrospinal fluid (7). However, the fate of APP, including the carboxyl-terminal region, membrane-spanning domain, and β/A4 ectodomain is not certain.

In conclusion, in aged primates, neurites containing APP epitopes (outside the β/A4 region) are capped by deposits of β/A4—an observation supporting the hypothesis that some parenchymal deposits of amyloid may be derived from neuronal APP. These neurites may not process APP properly on the membrane surface—i.e., the precursor may not be cleaved with normal efficiency within the β/A4 domain, and aberrant processing steps may occur, leading to the formation of β/A4. *In vitro* studies demonstrate that membrane-associated APP are substrates for a cleavage reaction, which results in secretion of the extracellular domain (24, 25). APP fragments and β/A4 associated with the membrane do not appear to self-assemble into amyloid fibrils, suggesting that the amyloidogenic fragment must be liberated from the membrane for self-assembly to occur. APP isoforms in neurites with damaged membranes that interact with other constituents, including proteases (or their inhibitors), appear to play a key role in the formation of β/A4 localized in brain parenchyma.

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