Protection Against HIV-1 gp120–induced Brain Damage by Neuronal Expression of Human Amyloid Precursor Protein

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Summary

Expression of the HIV-1 envelope protein gp120 in brains of transgenic (tg) mice induces extensive neurodegeneration (Toggas, S. M., E. Masliah, E. M. Rockenstein, G. F. Rall, C. R. Abraham, and L. Mucke. 1994. Nature [Lond.]. 367:188–193.). To further analyze the pathogenesis of gp120-induced neurotoxicity and to assess the neuroprotective potential of human amyloid precursor proteins (hAPPs) in vivo, different hAPP isoforms were expressed in neurons of gp120/hAPP-bigenic mice: hAPP751, which contains a Kunitz-type protease inhibitor domain, or hAPP695, which lacks this domain. Bigenic mice overexpressing hAPP751 at moderate levels showed significantly less neuronal loss, synapto-dendritic degeneration, and gliosis than singly tg mice expressing gp120 alone. In contrast, higher levels of hAPP695 expression in bigenic mice failed to prevent gp120-induced brain damage. These data indicate that hAPP can exert important neuroprotective functions in vivo and that the efficiency of this protection may depend on the hAPP isoform expressed and/or on the level of neuronal hAPP expression. Hence, molecules that mimic beneficial APP activities may be useful in the prevention/treatment of HIV-1–associated nervous system damage and, perhaps, also of other types of neural injury.

A significant number of people infected with HIV-1 develop central nervous system (CNS) damage that can culminate in dementia and paralysis (1). The effective prevention and treatment of AIDS dementia complex is still impossible at the present time and will likely require a better understanding of the molecular and cellular processes that underlie HIV-1–associated brain damage. Data obtained in diverse experimental models suggest that the HIV-1 envelope glycoprotein gp120 can induce significant neurotoxicity (for review see reference 2), most likely via macrophage/microglia–derived mediators (3, 4). In contrast, one group of investigators recently reported that they were unable to detect neurodegeneration after intracerebral/ventricular gp120 injections in rats (5). However, these negative results are difficult to interpret because no evidence was provided that significant amounts of gp120 were deposited into the brain parenchyma or that gp120 maintained for prolonged periods in minipump reservoirs remained bioactive. Furthermore, the only histopathological method applied in this study (counting of Cresyl fast violet–stained cells by conventional light microscopy) provides no quantitative information on the integrity of dendrites, the neuronal structures affected most prominently by gp120 (2, 6–8), and was used to evaluate only subpopulations of hippocampal neurons that are less susceptible to gp120-induced neurotoxicity than neurons in the neocortex (6–8).

As discussed in detail elsewhere (9), fusion gene constructs expressed in transgenic (tg) animals allow the prolonged, reproducible delivery of selected proteins (devoid of any contaminations) to specific areas/cells within the intact CNS and circumvent many of the problems associated with direct CNS injections (e.g., secondary responses to mechanical trauma and gross variations in the amount of protein delivered). Recently, we demonstrated that expression of a fusion gene encoding secretable HIV-1 gp120 in brains of tg mice induced a spectrum of neuronal and glial alterations that resembled neuropathological abnormalities found in patients with AIDS (6). Because a variety of studies indicate that derivatives of the human amyloid protein precursor (hAPP) may fulfill neurotrophic/neuroprotective functions (10–17), we wanted to assess whether hAPP can prevent or ameliorate gp120-induced brain damage in vivo. For this purpose, different hAPP iso-

forms were expressed in neurons of gp120/hAPP-bigenic mice. Our results demonstrate that moderate levels of hAPP751 expression effectively protect the CNS against gp120-induced neuronal injury.

Materials and Methods

Animals and DNA Analysis. Male and female B6xSJL mice (4–14 mo old) were used. Animal care was in accordance with institutional guidelines. Transgenes were detected by slot blot analysis of genomic DNA extracted from tail biopsies using ^{32}P-labeled probes that recognize either gp120 (6) or an SV40 sequence at the 3' end of hAPP-encoding constructs (17).

Expression of Transgene Products. Transgene-derived mRNAs were detected by solution hybridization and RNase protection assay, carried out essentially as described (18), using 10 μg of RNA per sample in combination with the following ^{32}P-labeled antisense riboprobes (protected sequences indicated in parentheses): APP (nucleotides 2468–2657 of APP mRNA [GenBank accession number X06989]), gp120 (nucleotides 2532–2656 of SV40 [GenBank accession number M24914] at the 3' end of glial fibrillary acidic protein (GFAP)-gp120–derived transcripts), and β-actin (nucleotides 480–559 of mouse β-actin mRNA [GenBank accession number X03672]). hAPP protein expression was detected by Western blot analysis as described (17).

Quantitative Immunohistopathological Analysis. Mice were 4–14 mo of age and there were no significant differences in the average age of mice across the different groups compared by one-factor analysis of variance (ANOVA). Brains were fixed, sectioned, (immuno) stained, and analyzed as described previously (6, 17). Hemibrains were assigned code numbers (by E. M. Rockenstein) to ensure objective assessment. Codes were not broken until the analysis was complete. For each mouse and immunostain, three serial sections of corresponding brain regions were analyzed. For the assessment of neuronal changes, sections were examined using a laser scanning confocal microscope (MRC-600; Bio-Rad Labs., Richmond, CA) (19, 20) mounted on an Axiovert Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY). Digitized images (4/section/case), 0.5 μm in thickness, were transferred to a Macintosh IIci, running the public domain program of Wayne Rasband (Image 1.23) (20). The area of the neuropil occupied by immunolabeled neuronal dendrites and presynaptic terminals was expressed as a percentage of the total image area, as described previously (19, 20). Neuronal counts were determined in brain sections stained with cresyl violet using the Quantimet 570C as described (21). Corrected optical density values for the relative levels of GFAP and F4/80 immunoreactivity were obtained with the Quantimet 570C as described (20). Statistical comparisons between individual groups of mice were done by unpaired two-tailed Student's *t* test.

Results and Discussion

The tg mice used in this study were from previously characterized tg lines, namely GFAP-gp120 line 2 (6), neuron-specific enolase (NSE)-hAPP695m line 19, and NSE-hAPP751m line 57 (17). The astroglial expression of a truncated HIV-1 env gene encoding soluble, secretable gp120 in GFAP-gp120 tg (gp120 tg) mice was directed by a modified murine GFAP gene and the neuronal expression of full-length hAPP cDNAs in NSE-hAPP tg (hAPP tg) mice by the NSE promoter (Fig. 1). Gp120 line 2 was selected because it showed the most severe neurodegenerative changes of all gp120 lines previously

analyzed (6). The hAPP lines were chosen for this study primarily because they showed the highest levels of hAPP expression of all hAPP expressor lines that could be maintained (17). Several different APP isoforms are normally derived from the endogenous APP gene by alternative splicing (22). The APP cDNAs expressed here encoded either hAPP751 which contains a Kunitz-type serine protease inhibitor (KPI) domain or hAPP695 which lacks this domain (Fig. 1, *A* and *C*). Both hAPPs expressed contained a conservative Val to Ile substitution (23); this change did not significantly affect the synaptotrophic potential of hAPPs when mutated and nonmutated hAPP isoforms were compared in tg lines with similar levels of cerebral hAPP expression (17). hAPP695 and hAPP751 tg mice displayed a similar widespread neuronal expression of hAPP, with highest levels found in the neocortex (17). Notably, the neocortex is also the brain region that showed the greatest amount of damage in the gp120 tg model (6). This presumably fortuitous topographic overlap indicated that gp120/hAPP bigenic mice should be suitable for the assessment of neuroprotective hAPP effects in vivo.

Compared with age-matched non-tg mice, adult hAPP singly tg mice displayed no evidence for neurodegeneration, whereas adult gp120 singly tg mice showed statistically significant losses of neuronal dendrites, presynaptic terminals and large pyramidal neurons, abundant vacuolizations of apical dendrites, as well as a prominent activation of astrocytes and microglia (Figs. 2 and 3). Whereas brains of gp120 singly tg neonates were indistinguishable at the structural level from brains of non-tg littermate controls, neuronal damage (distortion of apical dendrites and decrease in the area of neuropil occupied by MAP-2–positive dendrites) was evident in 7-d-old gp120 tg mice but not in non-tg littermate controls (data not shown). This early development of brain damage in gp120 tg mice is consistent with the postnatal increase in expression directed by the GFAP promoter (24). Notably, it also correlates well with the developmental expression of NMDA receptors (25), which appear to play an important role in gp120-induced neurotoxicity (2). The early development of neuropathology in gp120 tg mice implies that, to be effective, preventative therapeutic interventions may have to be initiated before or shortly after birth. Previous studies (26) have shown that the NSE promoter is active before birth making it suitable for the expression of potentially neuroprotective factors in gp120 tg mice.

To generate gp120/hAPP695 and gp120/hAPP751 bigenic mice, gp120 singly tg-heterozygous mice were crossed with either hAPP695 or hAPP751 singly tg-heterozygous mice. Following Mendelian genetics, the offspring from such crosses were singly tg for either hAPP or gp120, bigenic for gp120/hAPP or non-tg, each group comprising ~25% of any given litter. Brains of bigenic mice were compared quantitatively with brains of non-tg or singly tg littermates. Moderate levels of neuronal expression of hAPP751 in gp120/hAPP bigenic mice significantly decreased the neuronal loss and synapto-dendritic damage found in singly tg mice expressing gp120 alone (Fig. 2, *A* and *B*). This protection was so effective that the structural integrity of neurons
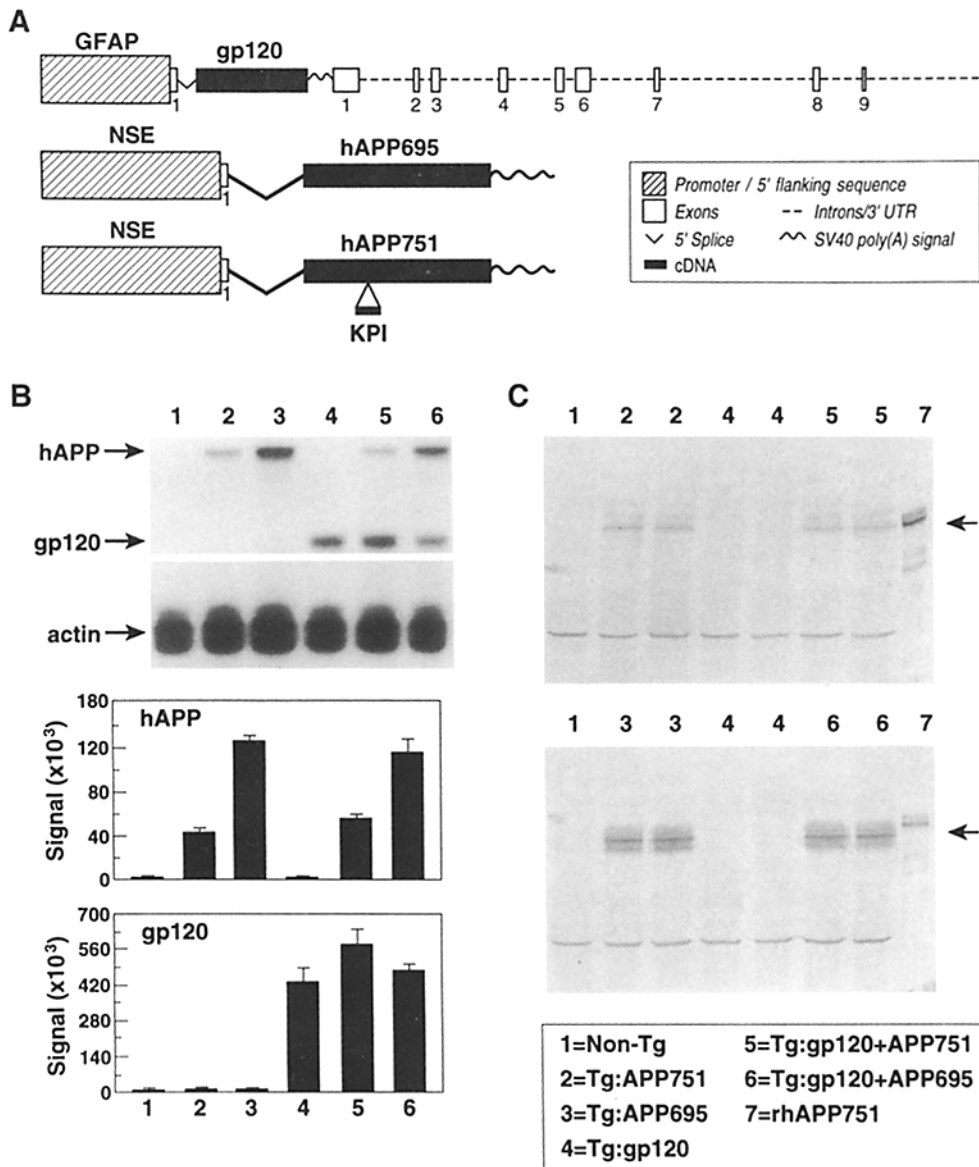
Figure 1. Structure of GFAP-gp120, NSE-hAPP695 and NSE-hAPP751 transgenes (not drawn to scale) (*A*). The construction of these fusion genes and their expression in singly tg mice have been described previously (6, 17). Gp120/hAPP bigenic mice were generated as described in the text. Expression of transgene-derived mRNAs in brains of singly tg and bigenic mice (*B*). Numbers 1–6 identify samples obtained from different groups of mice as indicated in the key (see *C*). RNA was isolated from hemibrains of 4–8-mo-old mice ($n = 3$/group) and levels of specific RNAs analyzed by solution hybridization and RNase protection assays. Radioactive signals were quantitated by integrating pixel intensities over defined volumes using a Phosphorimager SF (Molecular Dynamics, Sunnyvale, CA) and the ImageQuant software. Actin signals were used to correct for differences in RNA content/loading as described (18). Columns and error bars represent means and SEM, respectively. The differences in gp120 mRNA levels between gp120 singly tg and bigenic mice were not statistically significant. hAPP detection by Western blot analysis (*C*). Immunoblotting of brain homogenates, carried out as described (17), revealed similar hAPP levels in hAPP singly tg and bigenic mice expressing the same hAPP isoform, whereas control brains from mice lacking NSE-hAPP transgenes showed no or only minimal background staining. Two mice were analyzed per group; the same two gp120 singly tg samples were included in both Western blots. Arrows indicate hAPP bands. rhAPP751, recombinant full-length human APP751 (50 ng).

in gp120/hAPP751 bigenic mice was essentially indistinguishable from that in non-tg controls (Fig. 3). In contrast, neuronal expression of hAPP695 at higher levels had no neuroprotective effects (Figs. 2 and 3).

Although gp120/hAPP751 bigenic mice also showed significantly less gliosis (astrocytosis and microgliosis) than gp120 singly tg mice ($p < 0.002$), the inhibition of the gliosis was clearly incomplete (Fig. 2 *C*). It is possible that the gliosis in gp120/hAPP751 bigenic mice represents a residual glial response to chemical distress signals from neurons that appear structurally intact but are functionally impaired, or results from more direct effects of gp120 on astrocytes and/or microglia.

In vitro, gp120 or gp120-induced mediators appear to induce excitotoxicity by synergizing with glutamate to elevate neuronal intracellular-free calcium levels ($[Ca^{2+}]_i$) (27), whereas secretable forms of hAPP diminish the glutamate-induced rise in $[Ca^{2+}]_i$ (28). Although there is currently no reliable method to directly measure neuronal $[Ca^{2+}]_i$ in vivo, our demonstration of neuroprotective hAPP effects in gp120/hAPP bigenic mice indicates that these molecules may have similar effects in vivo. It is interesting in this context that $[Ca^{2+}]_i$-imaging of cultured neurons revealed the APP-mediated reduction in neuronal $[Ca^{2+}]_i$ to be particularly marked in dendrites (28), since these neuronal structures show prominent damage in gp120 tg mice (Figs. 2, *A* and *B* and 3). Because in aggregated form the APP derivative Aβ has calcium-destabilizing and neurodegenerative effects in vitro (29), it is important to note that none of our NSE-hAPP tg lines (age range of mice analyzed: 2–24 mo) showed evidence for

A

B

C
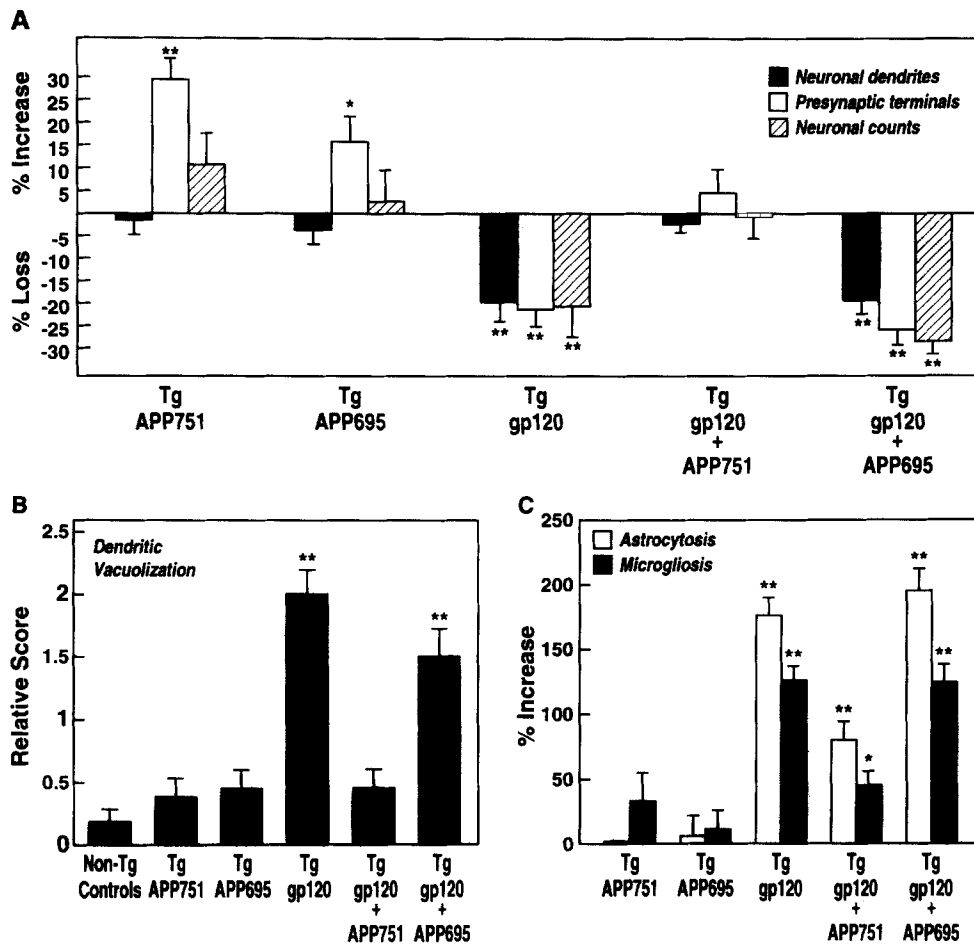
Figure 2. Computer-aided quantitation of neuroprotective hAPP effects. Hemibrains of singly tg (hAPP751 vs. hAPP695 vs. gp120), bigenic (gp120 + hAPP751 vs. gp120 + hAPP695), and non-tg mice (6–11 mice analyzed per group) were fixed, sectioned, and either immunolabeled with antibodies against MAP-2 (neuronal dendrites), synaptophysin (presynaptic terminals), GFAP (astrocytes), or F4/80 (macrophages/microglia) to analyze structural features, or stained with Cresyl violet to determine counts of large pyramidal neurons. Quantitative assessments were carried out as described in Materials and Methods. To facilitate comparisons across different groups of mice and different neuronal/glial parameters, for each parameter, the median of measurements obtained in 9–11 non-tg controls was used as the normal baseline value and arbitrarily defined as 100%. The data shown in A and C represent deviations (mean increase or loss [± SEM]) from this non-tg baseline. The extent of vacuolization of neocortical neuronal dendrites (B) was graded semiquantitatively (0, none; 1, mild; 2, moderate; 3, intense) based on the inspection of confocal images from three MAP-2–immunostained sections per case. Only the columns marked by asterisk(s) showed statistically significant differences from results obtained in normal non-tg controls: ** $p < 0.01$, * $p < 0.05$.

Aβ/amyloid deposits or neurodegeneration when examined with a variety of antibodies that readily detect such alterations in brains of patients with Alzheimer's disease (17).

Although it is reasonable to postulate that gp120-induced neurotoxicity was prevented in gp120/hAPP bigenic mice by hAPP-mediated stabilization of the intraneuronal calcium homeostasis, alternative mechanisms also deserve consideration. Coexpression of hAPPs in bigenic mice did not significantly alter the cerebral levels of gp120 mRNA compared with gp120 singly tg mice (Fig. 1 B). However, it has so far been difficult to unequivocally identify soluble gp120 in brains of gp120 tg mice or in brains of patients with HIV-1 encephalitis (6) and the current study was not designed to evaluate whether cerebral hAPP expression affects gp120 protein levels or alters the concentration of gp120-induced neurotoxic mediators. Both hAPP695 and hAPP751 contain a metalloprotease inhibitor domain (30) and protease inhibitors could mediate a variety of important biological effects in the CNS (31, 32). Because hAPP751 expression protected against gp120-induced neurotoxicity, whereas hAPP695 ex-

pression did not, one might be tempted to speculate that this difference relates to an activity of the KPI domain that is present in hAPP751 and absent from hAPP695. However, the two hAPP lines evaluated in the current study also differed with respect to their level of hAPP expression, line NSE-hAPP695m-19 showing significantly higher levels of hAPP expression than line NSE-hAPP751m-57 (Fig. 2, B and C). Notably, a more extensive investigation of synaptotrophic hAPP effects in multiple lines of hAPP tg mice suggested that the dose–response curve for potentially beneficial hAPP effects might be bell-shaped with progressively less neurotrophism/protection seen at higher levels of expression (17). Experiments are currently in progress (a) to confirm neuroprotective hAPP effects in additional hAPP tg lines, (b) to differentiate KPI domain-related from hAPP dosage effects, and (c) to compare the neuroprotective capacity of wild-type versus mutated hAPPs.

In conclusion, our in vivo results are consistent with the postulate that gp120-induced neurotoxicity involves derangements of the neuronal calcium homeostasis. The prominent
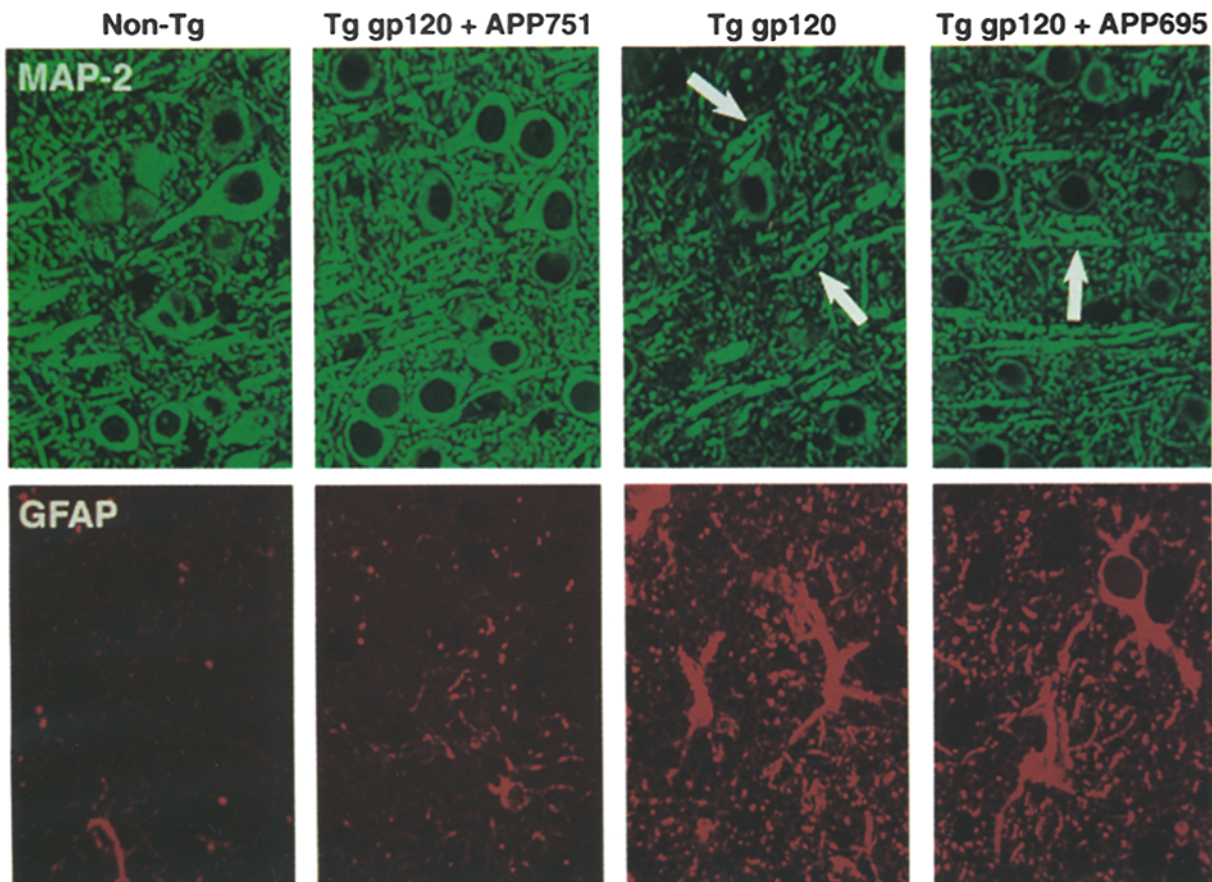
Figure 3. Neuroprotective hAPP effects revealed by confocal microscopy of brain sections (frontal cortex) immunolabeled with antibodies against the neuronal dendritic marker MAP-2 (*green*) or the astroglial marker GFAP (*red*). Hemibrains of non-tg, singly tg, and bigenic mice were paraformalde-hyde fixed, sectioned, immunostained, and analyzed by laser scanning confocal microscopy as described (6). Compare the normal appearance of neurons and paucity of astroglial activation in non-tg controls and gp120/hAPP751 bigenic mice with the rarefaction and vacuolization (*arrows*) of neuronal dendrites and the reactive astrocytosis seen in gp120 singly tg and gp120/hAPP695 bigenic mice.

neuroprotective effect of hAPP demonstrated here indicates that drugs that mimic beneficial APP activities might be useful in the treatment or prevention of HIV-1–associated nervous system damage and, perhaps, also of other types of neural injury.

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