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An *in vivo* model for the neurodegenerative effects of β amyloid and protection by substance P

(Alzheimer disease/neurotoxin/cytoskeleton/tachykinin)

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ABSTRACT **Deposition of the β-amyloid protein in senile plaques is a pathologic hallmark of Alzheimer disease (AD). Focal deposition of β amyloid in the adult rat cerebral cortex caused profound neurodegenerative changes, including neuronal loss and degenerating neurons and neurites. Chronic induction of the Alz-50 antigen appeared in neurons around focal cortical deposits of β amyloid. Immunoblot analysis showed that β amyloid induced Alz-50-immunoreactive proteins in rat cerebral cortex that were very similar to the proteins induced in human cerebral cortex from patients with AD. The neuropeptide substance P prevented β-amyloid-induced neuronal loss and expression of Alz-50 proteins when coadministered into the cerebral cortex. Systemic administration of substance P also provided protection against the effects of intracerebral β amyloid. Thus, β amyloid is a potent neurotoxin in the adult brain *in vivo*, and its effects can be blocked by substance P.**

Excessive deposition of the β-amyloid protein in the brain is characteristic of patients with Alzheimer disease (AD) and aging individuals with Down syndrome (1–5). Deposition of β amyloid is one of the earliest pathological changes observed in the brains of individuals with Down syndrome, preceding the appearance of neurofibrillary tangles and the development of clinical dementia (6–8). The appearance of β-amyloid deposits can also be observed in normal elderly individuals, but it is usually considerably less extensive than in AD or Down syndrome and reflects a very gradual accumulation (7, 9, 10). The accumulation of low levels of β amyloid has been observed in tissues other than the brain (11), raising the possibility of a systemic defect in the processing of the amyloid precursor protein (APP) in AD.

A central issue in the pathophysiology of AD is the role of β amyloid in the neurodegenerative process. Exposure of primary hippocampal neurons to β amyloid causes neuronal degeneration, giving rise to the hypothesis that β amyloid may also cause neuronal degeneration when it accumulates abnormally in AD (12, 13). In this report, we examine the effects of β amyloid *in vivo* in the adult rat brain. We find that intracerebral deposition of β amyloid causes neuronal degeneration that is accompanied by induction of the Alz-50 antigen. The neurodegenerative effects of β amyloid could be prevented by intracerebral or systemic administration of substance P.

MATERIALS AND METHODS

Intracerebral Injection of Peptides and Histopathological Analysis of Tissue. Adult male Sprague–Dawley rats (175–200 g) were used in all experiments. A peptide corresponding to the first 40 amino acids of β amyloid [β-(1–40)] was synthesized, purified, and dissolved in 35% acetonitrile/0.1% trifluoroacetic acid as described (12). This vehicle was used in all control injections. Substance P was obtained from Bachem Fine Chemicals (Torrance, CA) and was dissolved in water. In 69 rats, microinjections of 1 μl of the designated peptides or vehicle alone were made into the deep frontal cortex or hippocampus with a Kopf stereotaxic apparatus. Injections were made with a 10-μl Hamilton syringe fitted with a 30-gauge blunt-tipped needle over 1 min, and the needle was left in place for an additional 2 min before being slowly withdrawn. Seven days after injections, rats were anesthetized and killed by transaortic perfusion–fixation with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.3). Serial 35-μm frozen sections were collected and processed by the Gallyas silver degeneration method (14) or for immunocytochemistry as described (15). A rabbit polyclonal antibody (antibody 1280) made against the synthetic β-(1–40) peptide was used at a 1:1000 dilution. The Alz-50 and tau-2 mouse monoclonal antibodies were used at dilutions of 1:200. The area of maximal lesion size was chosen based on examination of serial sections through the lesion. Lesion areas were calculated in triplicate on digitalized images with a Macintosh SE computer.

Neuronal Counts. Neuronal counts were performed on cresyl violet-stained coronal sections from animals sacrificed 3–7 days after intracerebral injection. Determinations were made at ×100 magnification with a calibrated eyepiece graticule. Three contiguous 0.64-mm^2 areas were counted at the maximal lesion site identified from serial sections. The region counted spanned layers II–V of cerebral cortex.

Immunoblot Analysis of Cortical Tissue. Cortical brain tissue was removed as an ≈8-mm^3 block containing the injection site and surrounding tissue. Brain tissue was homogenized in 4% SDS/10% 2-mercaptoethanol/20% (vol/vol) glycerol/3 mM EGTA/0.5 mM $MgSO_4$/0.0625 M Tris·HCl, pH 6.8, with protease inhibitors (leupeptin at 10 μg/ml, aprotinin at 100 μg/ml, pepstatin at 5 μg/ml, and 0.3 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (50 mM imidazole, 50 mM potassium fluoride, 50 mM β-glycerophosphate, 50 mM sodium pyrophosphate, 100 μM sodium orthovanadate, and 0.1 mM zinc chloride). Homogenates were boiled for 1 min and then 15 μg of protein per lane (Bio-Rad protein assay) was loaded onto an SDS/4–20% polyacrylamide gel. After electrophoresis, the separated proteins were electrotransferred to nitrocellulose. Nitrocellulose membranes were blocked with 5% nonfat dry milk/0.1% Tween 20 and then incubated with the mouse monoclonal antibody Alz-50 (1:85 dilution) for 12 hr at 4°C. The reaction product was visualized with an alkaline phosphatase-

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Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein.
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conjugated goat anti-mouse IgM (1:1000 dilution, Boehringer Mannheim). Postmortem human cortex was obtained from Brodmann area 20/21.

RESULTS

Neurodegenerative Effects of β-(1–40). β-(1–40) (12) was microinjected unilaterally into the adult rat cerebral cortex under stereotaxic guidance. The contralateral cerebral cortex was infused with equimolar concentrations of control peptides or vehicle alone. Rats were sacrificed 7 days after treatment, and the brains were fixed and serially sectioned for histochemical and immunocytochemical analysis. A focal β-amyloid deposit was evident at the site of microinjection in the deep frontal cortex or in the hippocampus (Fig. 1*a*). The β-(1–40) deposit caused significant neuronal degeneration in the CA1 layer of the hippocampus located 0.5–1.0 mm from the injection site (Fig. 1*b*). Degenerating neurons and neurites were evident as discrete argyrophilic perikarya and neuropil threads adjacent to the β-(1–40) deposit (Fig. 1 *b* and *c*). Intracerebral injection of a non-β-amyloid peptide (A37), derived from the N-terminal region of the APP (16), or vehicle alone produced a minor local gliotic reaction but did not cause significant neuronal or neuritic degeneration outside of the immediate injection site (Fig. 1 *d* and *e*). Cortical injection of β-(1–40) produced a neurodegenerative lesion with an area of 1.09 ± 0.13 mm^2 in the coronal plane of section (mean ± SEM; $n = 8$ animals).

We performed neuronal counts on β-(1–40) treated and control brains from cresyl violet-stained cortical sections adjacent to the injection sites (Table 1). Injection of the A37 control peptide resulted in 10% ± 13% neuronal loss, which was not statistically significant. Focal intracerebral deposition of 3 nmol and 20 fmol of β-(1–40) resulted in 73% ± 3% and 56% ± 4% neuronal loss, respectively ($P < 0.001$; Table 1). A β-(1–40)-derived peptide (CA4) synthesized with several amino acid substitutions showed diminished neurotoxic potency. A peptide with the same amino acid composition as β-(1–40) but with the exact reverse sequence, designated β-(40–1), did not show any significant neurotoxic activity relative to controls. Coadministration of β-(1–40) with an antibody to native β amyloid significantly diminished the extent of neuronal loss (data not shown).

Table 1. Effects of β-(1–40) and other peptides on cortical neuronal number

Treatment	Neurons per mm^2
Untreated	1167 ± 43
A37 (3 nmol)	1051 ± 146
β-(40–1) (3 nmol)	987 ± 86
CA4 (3 nmol)	675 ± 55
β-(1–40) (20 fmol)	509 ± 49
β-(1–40) (3 nmol)	313 ± 29

Peptides were microinjected into the cerebral cortex and neurons were counted as described in *Matherials and Methods*. The A37 control peptide represents APP residues 261–280 (16). The β-(40–1) peptide is identical in amino acid composition to the β-(1–40) peptide (native β amyloid), but the N- to C-terminal sequence is reversed. The β-(1–40)-derived peptide CA4 contains the substitutions Arg^3, Glu^5, Val^7, Lys^{13}, His^{16}, Asp^{18}, Ser^{19}, Tyr^{20}, Pro^{26}, Val^{30}, Ala^{31}, $norLeu^{35}$, $Ileu^{38}$, Ala^{39}, Gly^{40}. The treatments A37 (3 nmol), CA4 (3 nmol), β-(1–40) (3 nmol), and β-(1–40) (20 fmol) were statistically different ($P < 0.001$ by analysis of variance); β-(1–40) (3 nmol) and β-(1–40) (20 fmol) were each significantly different from β-(40–1) ($P < 0.01$ by Scheffe post hoc analysis). Values are given as neurons per mm^2 of cortex and represent the mean ± SEM [$n = 3$–5 animals except for β-(1–40) (3 nmol) where $n = 16$].

Induction of Alz-50 Immunoreactive Proteins by β-(1–40). To determine whether neuronal degeneration in response to β-(1–40) deposition showed features of the neurodegenerative process in AD, we assayed for induction of the AD-associated Alz-50 antigen (17–21). Significant induction of Alz-50 immunoreactivity appeared in cortex surrounding a focal deposit of β-(1–40) (Fig. 2 *A* and *C*). Abnormal Alz-50 immunoreactivity appeared in neuronal perikarya and in neurites (Fig. 3*B*). A similar distribution of abnormal immunoreactivity was observed with antibodies to the tau protein (Fig. 3*A*). Immunoblot analysis of cortical tissue showed that the Alz-50 antibody reacts with three or four major species of molecular mass 55–68 kDa in human and rat cerebral cortex

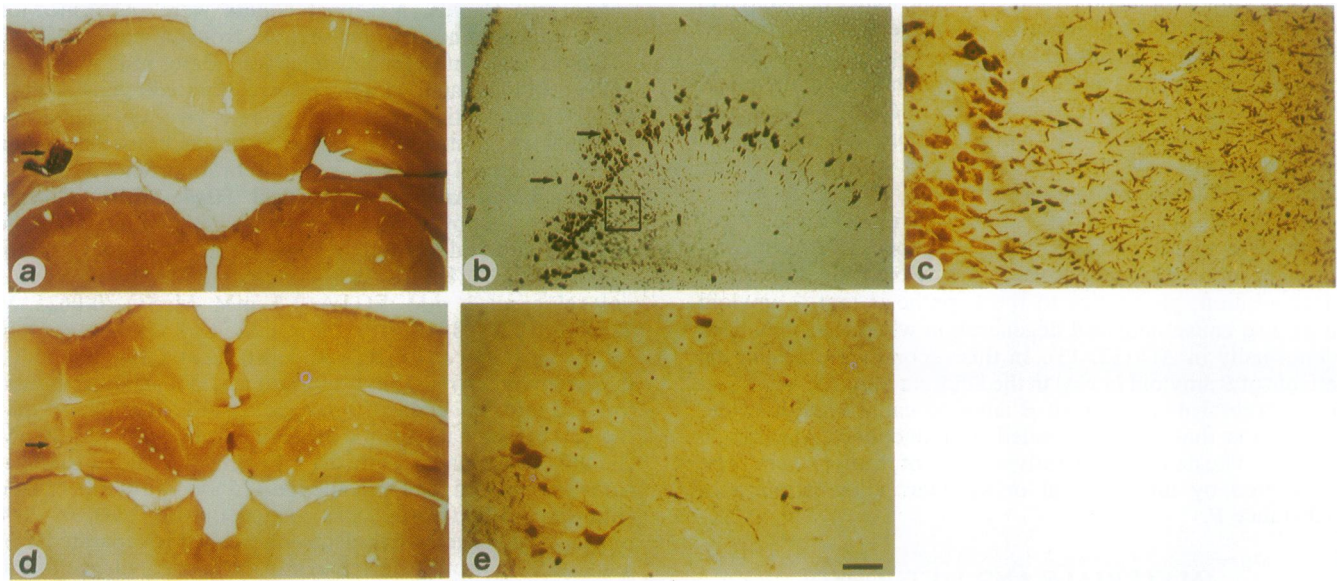

FIG. 1. Intracerebral β-(1–40) causes neuronal and neuritic degeneration. (*a* and *d*) Injection sites (arrows) of β-(1–40) (*a*) and A37 control peptide (*d*) in the rat hippocampus after reaction with the β-(1–40) antibody 1280. Note the deposit that is immunoreactive with β-(1–40) antibody in the hippocampal formation in *a*. (*b*) Silver stain of a hippocampal section adjacent to a β-(1–40) deposit shows argyrophilic degenerating pyramidal neurons (arrows) in the CA1 layer. (*c*) Higher-power view of the boxed region in *b* shows abundant argyrophilic degenerating neurites, which appear as neuropil threads (arrowheads). (*e*) Silver stain of a hippocampal section (CA1) adjacent to the A37 control injection does not show significant neuronal or neuritic degeneration. Animals were sacrificed 7 days after intracerebral injection of 3 nmol of peptide. (*a* and *d*, bar = 400 μm; *b*, bar = 80 μm; *c* and *e*, bar = 20 μm.)

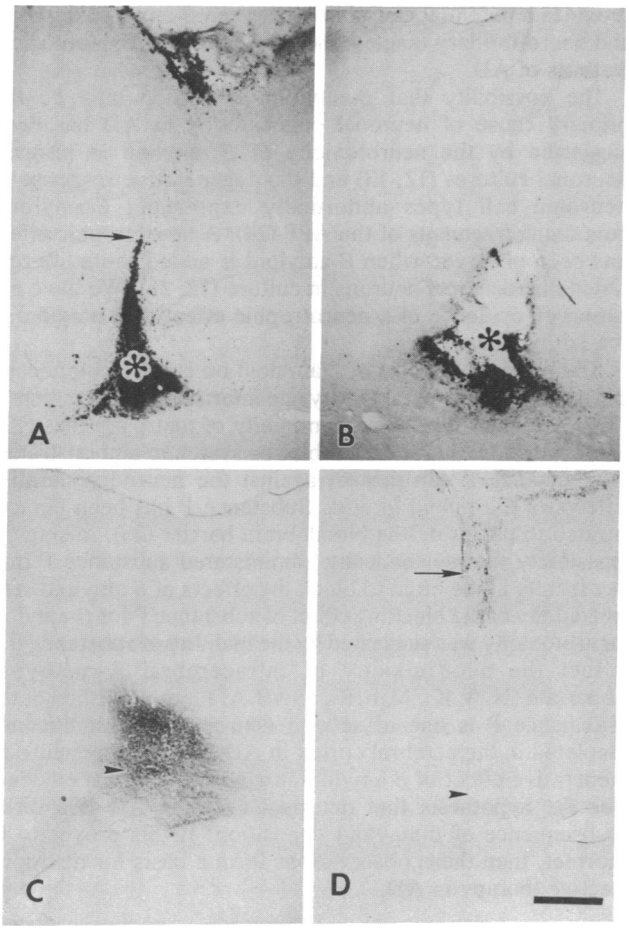

FIG. 2. Induction of the Alz-50 antigen by β-(1–40). (*A* and *C*) Intracerebral injection of β-(1–40) (3 nmol) induces Alz-50 immunoreactivity in the surrounding cortex 7 days later. (*B* and *D*) Coinjection of substance P (200 pmol) with β-(1–40) prevents induction of Alz-50 immunoreactivity. *A* and *B* are stained with β-amyloid antibody; *C* and *D* are stained with Alz-50 antibody. Staining with β-amyloid and Alz-50 antibodies was done on adjacent sections from the same region. Control injections of A37 peptide, the reverse β-(40–1) peptide, or vehicle alone did not produce significant Alz-50 immunoreactivity (data not shown). Arrowheads and asterisks, injection sites; arrows, needle track. (Bar = 200 μm.)

(Fig. 4). Intracerebral injection of β-(1–40) resulted in a significant induction of the Alz-50 immunoreactive species in cortical tissue dissected from the lesion site, which did not occur after injection of the A37 control peptide or in untreated cortex (Fig. 4*A*). Immunoblot analysis of human cerebral cortex showed that the Alz-50 immunoreactive species that were induced in AD were very similar in electrophoretic mobility to those induced by intracerebral β-(1–40) in the rat (Fig. 4*B*). Intracerebral injection of β-(1–40) in the rat caused chronic induction of neuronal Alz-50 immunoreactivity, which was observed after 14 days.

Substance P Protects Against the Neurodegenerative Effects of β-(1–40). The finding that substance P can inhibit the effects of β amyloid on cultured hippocampal neurons (12) prompted an examination of the effects of this neuropeptide *in vivo*. Substance P was coinjected with β-(1–40) into the rat cortex and compared to β-(1–40) injected alone at the same level in the contralateral hemisphere. Substance P prevented β-(1–40)-induced neuronal loss in a dose-dependent manner with intracerebral administration of 2–200 pmol (Fig. 5*A*). Induction of Alz-50 immunoreactivity by β-(1–40) was prevented by intracerebral substance P, as determined by immunocytochemistry and immunoblot analysis (Figs. 2 *B* and *D* and 4*A*).

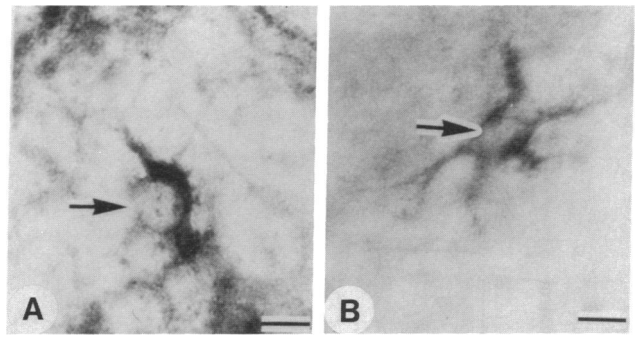

FIG. 3. Abnormal tau and Alz-50 immunoreactivity in cortical neurons surrounding a β-(1–40) deposit. (*A*) Neuron stained with the tau-2 monoclonal antibody. (*B*) Neuron stained with the Alz-50 antibody. (Bars = 10 μm.)

We also determined that there was a protective effect of substance P administered systemically. The neuropeptide was administered by intraperitoneal injection 30 min before intracerebral injection of β-(1–40). Neuronal loss induced by β-(1–40) was effectively inhibited by systemic administration of substance P in a dose range of 2–200 nmol/kg (Fig. 5*B*). Induction of Alz-50 immunoreactivity by β-(1–40) was also inhibited by systemic administration of substance P (Fig. 4*A*). This protective effect of substance P was provided by a single dose before intracerebral injection of β-(1–40) and was evident 7 days later (Fig. 5). The chronic induction of Alz-50 immunoreactivity could also be reversed by systemic administration of substance P up to 24 hr after intracerebral deposition of β-(1–40), but efficacy diminished considerably when substance P was administered 72 hr after β-(1–40) deposition (data not shown).

DISCUSSION

We have demonstrated that the β-amyloid protein is a potent neurotoxin in the adult brain *in vivo*. Intracerebral β amyloid caused neuronal and neuritic degeneration accompanied by induction of the Alz-50 immunoreactive proteins. These effects were observed after intracerebral deposition of 20 fmol of β-(1–40), the approximate quantity of β-amyloid protein contained in a single senile plaque in AD (22). The subacute loss of neurons observed after intracerebral deposition of β amyloid in the rat appears to occur more rapidly than the chronic neurodegenerative process in human AD. In

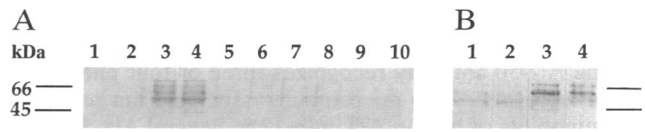

FIG. 4. (*A*) Immunoblot analysis of Alz-50 immunoreactive proteins in rat cerebral cortex. Lanes: 1 and 2, untreated cortex; 3 and 4, intracerebral β-(1–40) (3 nmol); 5 and 6, intracerebral β-(1–40) and substance P (200 pmol); 7 and 8, intracerebral β-(1–40) and systemic substance P (200 nmol/kg); 9 and 10, intracerebral A37 control peptide (3 nmol). Each lane shows a separate sample representative of five or six animals for each treatment. Animals were sacrificed 7 days after intracerebral injection and a cortical block containing the injection site and surrounding tissue was dissected and homogenized. (*B*) Alz-50 immunoreactive proteins in normal and AD postmortem human cerebral cortex. Lanes: 1, normal 86 year old; 2, normal 89 year old; 3, AD 81 year old; 4, AD 74 year old. Alz-50 immunoreactivity was increased by 4- to 10-fold in β-(1–40)-treated rat cortex and AD human cortex relative to controls by densitometric analysis. Molecular size markers were from Sigma.

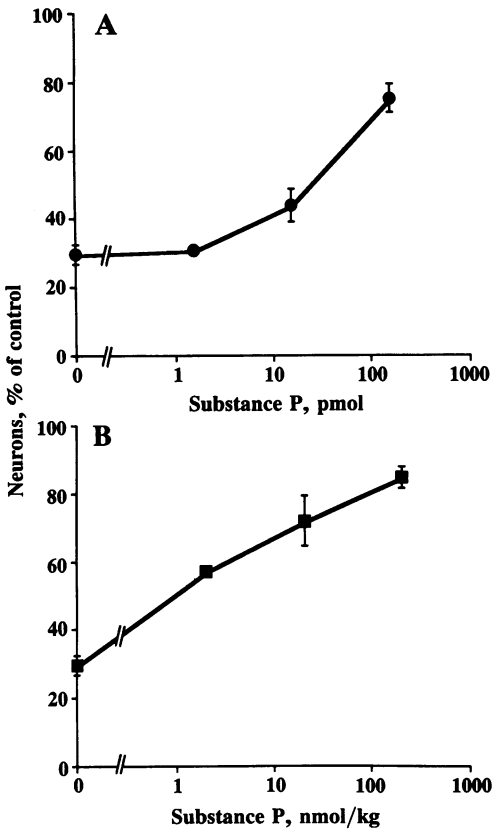

FIG. 5. β-(1–40)-induced neuronal loss is inhibited by intracerebral or systemic substance P. (*A*) β-(1–40) and intracerebral substance P. (*B*) β-(1–40) and systemic substance P. Intracerebral injections of 3 nmol of β-(1–40) were made either alone (0 point) or in the presence of the designated quantity of substance P. Substance P was administered systemically by intraperitoneal injection 30 min before intracerebral injection of β-(1–40). Intraperitoneal injections of vehicle alone were used as controls in *B*. Animals were sacrificed 7 days after intracerebral injection and neurons were counted. Values are expressed as % of neuron number in control injections of A37 peptide and represent the mean ± SEM (n = 3–5 animals).

this experimental paradigm, the neurotoxicity of β amyloid may be accelerated by the rapid presentation of relatively high local concentrations of solubilized β amyloid. Alternatively, the degenerative response to β amyloid may occur more acutely in rat neurons than in primate neurons. In addition to subacute neuronal loss, intracerebral β amyloid also caused chronic changes in the rat cortex that bear some resemblance to the pathology of human AD, including persistent induction of the Alz-50 antigen in neurons and neurites surrounding β-amyloid deposits.

The Alz-50 antibody recognizes three or four phosphorylated isoforms of the microtubule-associated tau protein in immunoblots of human AD brain (21). Several Alz-50 immunoreactive species of similar electrophoretic mobility were induced in rat cortex after deposition of β amyloid. In addition, the rat cortical Alz-50 immunoreactivity could be abolished by phosphatase pretreatment (J.B. and B.A.Y., unpublished data), suggesting that these proteins are phosphorylated isoforms as is the case in human AD (23). The Alz-50 immunoreactive proteins are normally expressed in the brain during fetal development and then decline in the adult (18). They are reexpressed in the brain in AD and appear in neurons undergoing neurofibrillary degeneration (17–20). Purified Alz-50 immunoreactive proteins polymerize *in vitro* into paired helical filaments, which are indistinguishable from those observed *in situ* in the AD brain (21). The induction of Alz-50 immunoreactive proteins by β amyloid provides a potential causal link between β-amyloid deposits and neurofibrillary tangles, the two major neuropathological features of AD.

The possibility that β-amyloid deposition may be the primary cause of neuronal degeneration in AD has been suggested by the neurotoxicity of β amyloid in primary neuronal cultures (12, 13) and the degenerative response of neuronal cell types abnormally expressing β-amyloid-containing fragments of the APP (24). A neurotrophic effect has been observed when β amyloid is added to undifferentiated hippocampal neurons in culture (12, 25). We have not observed evidence of a neurotrophic effect of β amyloid in the adult brain *in vivo*.

The biological effects of β amyloid on cultured hippocampal neurons are mediated by an internal sequence that is homologous to the tachykinin family of neuropeptides (12). Here we show that intracerebral or systemic administration of substance P can protect against the neurodegenerative effects of β amyloid *in vivo*. Substance P has been demonstrated to penetrate the blood–brain barrier (26), raising the possibility that systemically administered substance P may act locally in the brain to block the effects of β amyloid. The specificity of the blocking effect of substance P for β-amyloid neurotoxicity was suggested by the inability of substance P to affect the neurotoxicity of intracerebral *N*-methyl-D-aspartate (N.W.K., M.F.B., and B.A.Y., unpublished data). Substance P is one of several neuropeptides significantly depleted in the cerebral cortex in AD (27–30). The neurodegenerative effect of β amyloid *in vivo* is additional evidence for the hypothesis that neuronal death in AD is a direct consequence of β-amyloid deposition. If this proves to be correct, then these observations form a basis for neuroprotective therapy in AD.

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