We further suggest that astrocyte expression of peptides occurs early and that the peptides produced may play a role in brain development. Exposure of animals or humans to opiates has been shown to affect development of the central nervous system (*20*); enkephalins might act endogenously in a similar capacity. Thus, the peptides may represent a new class of neurotrophic factors. Alternatively, some evidence exists that peptides can stimulate production of neurotrophic agents (*21*).

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Neurotoxicity of a Fragment of the Amyloid Precursor Associated with Alzheimer's Disease

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Amyloid deposition in senile plaques and the cerebral vasculature is a marker of Alzheimer's disease. Whether amyloid itself contributes to the neurodegenerative process or is simply a by-product of that process is unknown. Pheochromocytoma (PC12) and fibroblast (NIH 3T3) cell lines were transfected with portions of the gene for the human amyloid precursor protein. Stable PC12 cell transfectants expressing a specific amyloid-containing fragment of the precursor protein gradually degenerated when induced to differentiate into neuronal cells with nerve growth factor. Conditioned medium from these cells was toxic to neurons in primary hippocampal cultures, and the toxic agent could be removed by immunoabsorption with an antibody directed against the amyloid polypeptide. Thus, a peptide derived from the amyloid precursor may be neurotoxic.

AMYLOID IS A 4.2-KD POLYPEPTIDE (also called the β protein or the A4 peptide) that is deposited in neuritic plaques and along the walls of the cerebral vasculature in Alzheimer's disease, Down syndrome, and to a lesser extent in normal aging (*1*). Complementary DNA (cDNA) clones for the amyloid precursor protein (APP) encode a protein considerably larger than the amyloid polypeptide (*2*). One form of the precursor (APP-1), distinguished by the absence of a protease inhibitor domain (*3*), is selectively expressed in the nervous system (*4*). We designed three constructs containing different segments of APP-1 (Fig. 1) in the retroviral expression vector DO (*5*). The recombinant AS1 carries the entire APP-1 coding sequence, whereas the recombinants AB1 and AD1 both contain the internal amyloid sequence with different extents of the remaining COOH-terminal portion of APP-1. These constructs were transfected into PC12 and NIH 3T3 cells by a modified version of the calcium phosphate method (*6*). Stable transfectants were selected with the neomycin analog G418, and resistant colonies were subcloned and expanded.

Cells expressing the transfected constructs synthesize, under the control of the 5′ long terminal repeat of Moloney murine leukemia virus, a fusion mRNA that is transcribed from the cDNA insert and the neomycin resistance gene (*neo*). The fusion mRNA is then translated to give the peptide encoded by the cDNA insert. The *neo* gene product is predominantly synthesized from a second transcript originating from an internal simian virus 40 promoter (*5*). The RNA hybridization patterns for transfected PC12 cell clones expressing constructs AB1 and AS1 (designated PC12-AB1 and PC12-AS1, respectively) and transfected 3T3 cell clones expressing constructs AB1 and AD1 (designated 3T3-AB1 and 3T3-AD1, respectively) were examined (Fig. 2). The RNA blots were probed with ^{32}P-labeled APP cDNA. The probe hybridized to the 3.4- to 3.6-kb endogenous APP transcript present in normal PC12 and 3T3 cells and to the bands of higher molecular mass corresponding to the fusion RNA species transcribed from the transfected constructs (Fig. 2A). It was confirmed that the larger transcripts originated from the transfected vector by hybridization of identical RNA blots with ^{32}P-labeled cDNA for the *neo* gene (Fig. 2B). In PC12-AS1 cells, the predicted 7.1-kb RNA was identified (Fig. 2A). The blots were also hybridized with a ^{32}P-labeled Eco RI–Xho I fragment of the gene for APP, which represents the NH_2-terminal portion of APP and does not overlap with the AB1 or AD1 sequences. This NH_2-terminal probe hybridized with the transfected RNA only in PC12-AS1 cells and to the endogenous APP 3.4- to 3.6-kb band in all the clones (*7*). The PC12-DO clone was transfected with the DO vector alone and expressed the *neo* gene (Fig. 2B).

Immunoblot analysis of the transfected cells was performed with an affinity-purified

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S. Fisher and M. L. Oster-Granite, Developmental Genetics Laboratory, Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

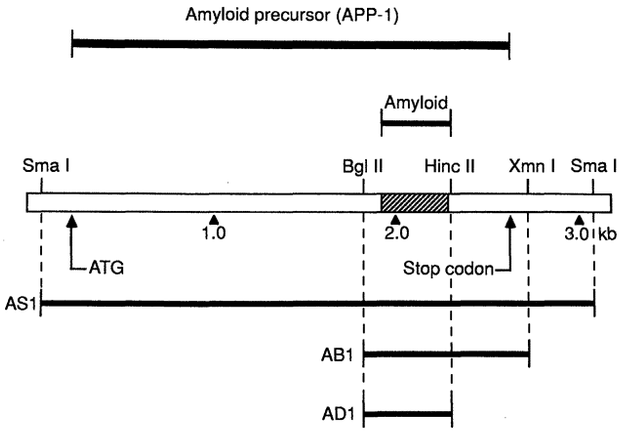

Fig. 1. Construction of AS1, AB1, and AD1. To make AS1 [nucleotides −95 to 2953 of APP-1, using the numbering system in Kang *et al.* (*2*)], we digested the full-length cDNA with Sma I and ligated it to the Sal I–cleaved DO vector, whose cohesive ends had been filled in with T4 DNA polymerase to create blunt ends. To make AB1 (nucleotides 1769 to 2373), we cleaved the APP-1 cDNA with Bgl II and Xmn I, ligated it to Xho I linkers to convert the blunt Xmn I end to an Xho I–cohesive end, and then inserted it into the DO vector, which had been digested with Bam HI–Sal I. To make AD1 (nucleotides 1769 to 1959), we cleaved the cDNA with Bgl II and Hinc II and ligated it to DO that had been cleaved with Sal I, filled in with T4 DNA polymerase, and then cleaved with Bam HI. Constructs were made in two variants of the DO vector, DOL in which the backbone of the vector contains the polyoma early region (*5*) and DOJ in which the polyoma backbone is replaced with pBR322 (*20*). The DOL constructs were transfected into 3T3 cells and the DOJ constructs into PC12 cells. AB1 and AD1 were designed with the goal of creating transfectants that constitutively synthesize the amyloid polypeptide without upstream sequences. An internal methionine immediately preceding the amyloid sequence was used as an initiation codon because it had been shown to efficiently initiate translation in vitro (*11*).

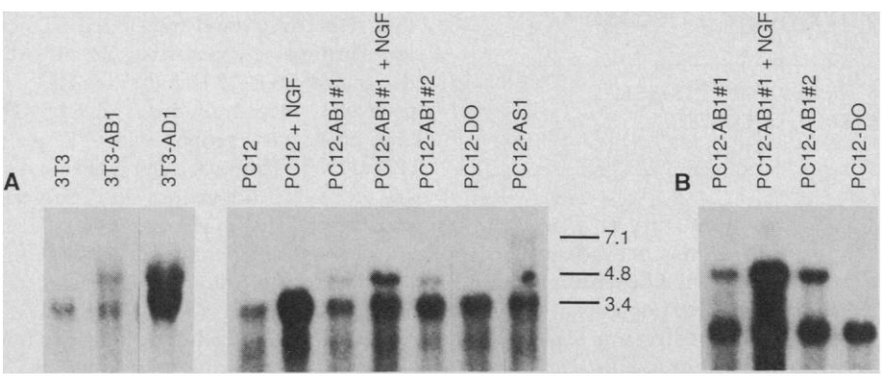

Fig. 2. RNA blot analysis of transfected clones. (**A**) Hybridization with FB68L, a cDNA clone carrying the 1.1-kb region of the gene for APP corresponding to the COOH-terminal portion of APP (*2*) that is common to all identified APP RNAs. Sizes of transcripts are expressed in kilobases. (**B**) Hybridization with the *neo* gene probe, which consisted of the Bgl II–Hind III fragment of the *neo* gene in DO. The RNA was isolated, fractionated on agarose-formaldehyde gels, transferred to a Biotrans membrane (ICN Biomedicals, Costa Mesa, California), and hybridized with ^{32}P-labeled probe, as described (*21*). Total RNA (10 μg) was electrophoresed in each lane. The blots were subsequently hybridized with an actin probe to confirm uniform loading of RNA in all of the lanes.

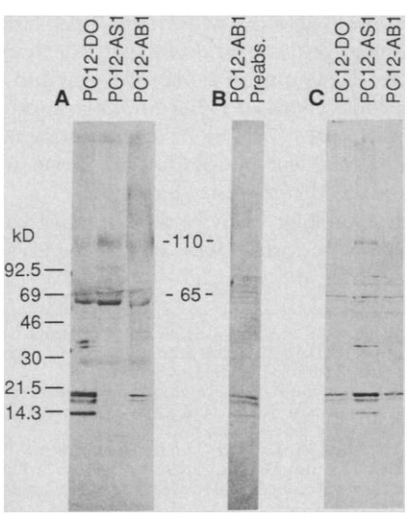

Fig. 3. Immunoblot analysis of amyloid peptides in transfected cell lines. (**A**) Conditioned medium. (**B**) Conditioned medium from PC12-AB1 cells analyzed with M4 antibody that had been preabsorbed with synthetic amyloid peptide. (**C**) Cell lysates. Serum-free conditioned medium from 4 × 10^6 cells of each cell line was concentrated by dialysis against 50 m*M* ammonium acetate, lyophilization, and resuspension in SDS sample buffer containing 5% β-mercaptoethanol. Cell lysates were obtained by direct suspension of cells in SDS sample buffer. The samples were boiled, electrophoresed on a 3 to 27% polyacrylamide gradient gel, and then transferred to nitrocellulose. The nitrocellulose was incubated with M4 antibody (200 ng/ml), and immunodetection was performed with an alkaline-phosphatase–coupled goat antibody to rabbit immunoglobulin G. Preabsorption of the antibody was performed with APP peptide (120 μg per microgram of M4 antibody).

rabbit antibody (M4) directed against the first 15 amino acids of the amyloid polypeptide; this antibody detects the APP in both human and murine brain (*8*). The immunoblot profile of conditioned medium from the transfected PC12 cell lines showed a prominent band at 60 to 65 kD and a fainter, diffuse band at 110 kD (Fig. 3A), similar to reported results for normal PC12 cells (*9*). These bands were more intense in the precursor-transfected PC12-AS1 cells. The profile of conditioned medium from PC12-AB1 cells showed an additional smear corresponding to aggregated immunoreactive material of high molecular mass, which was not present in the other PC12 cell lines (Fig. 3A) and was significantly diminished in intensity after peptide preabsorption of the antibody (Fig. 3B). Aggregated immunoreactive material was also detected with the M4 antibody in 3T3-AB1 cells but not in 3T3 cells (*10*). The immunoblot profile of cell lysates of the transfected PC12 cell lines showed the 110-kD band, which was most intense in PC12-AS1 cells (Fig. 3C). The 65-kD protein present in conditioned medium was less prominent in cell lysates. The high molecular mass, aggregated amyloid products in PC12-AB1 cells were also found predominantly in conditioned medium. The propensity of the AB1 fragment to aggregate has also been demonstrated by in vitro translation of the AB1 fragment (*11*).

The transfected PC12 clones showed similar morphology and viability to nontransfected PC12 cells. Differences appeared after the cells were treated with nerve growth factor (NGF), which causes PC12 cells to differentiate into a neuronal cell type (*12, 13*). Addition of NGF to PC12-AB1 cells resulted in the initial outgrowth of small neuritic processes. However, after 3 to 4 days the cells deviated from the normal course of differentiation, with a slowing of neurite outgrowth and the development of vacuolar inclusions. This was followed by swelling of the soma, which also became granular, and subsequent cell death (Fig. 4A). After 7 days of treatment with NGF, 50 to 75% of the cells had died (Table 1). In contrast, PC12-AS1 cells (Table 1) and PC12-DO cells (*14*) differentiated normally in response to NGF, without any evidence of degenerative changes or cell death. The degeneration of PC12-AB1 cells was NGF-dependent and was greatest 5 to 8 days after addition of NGF, the period in which maximal process outgrowth normally occurs. The AB1 and AD1 transfectants in the 3T3 cell line, which does not respond to NGF, showed no degenerative changes.

In an attempt to determine whether the 3T3-AB1 clone also produced this toxic activity, we added conditioned medium

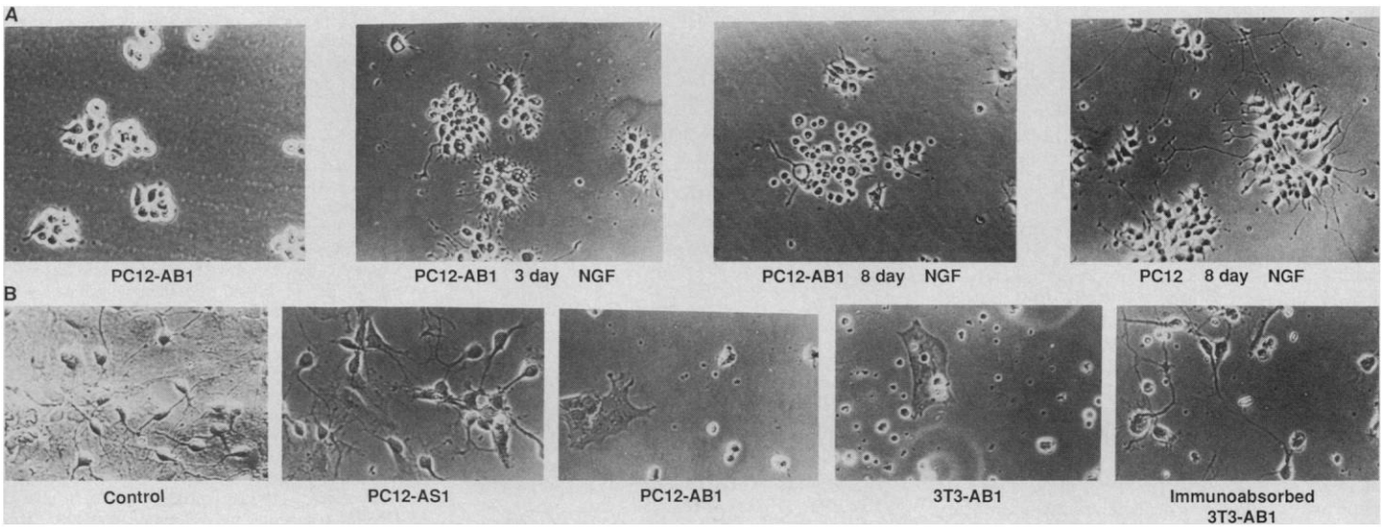

Fig. 4. (**A**) Degeneration of AB1-transfected PC12 cells after treatment with NGF. A clone of PC12-AB1 cells is shown before NGF addition, after 3 days in NGF when vacuolar inclusions were apparent, and after 8 days in NGF when considerable cellular degeneration had occurred. Also shown for comparison are normal untransfected PC12 cells after 8 days in NGF. (**B**) Effect of transfectant-conditioned media on primary rat hippocampal cultures. Cultures shown were either untreated (control) or treated for 48 hours with conditioned medium from PC12-AS1, PC12-AB1, or 3T3-AB1 (*13*). Control cultures and cultures treated with conditioned medium from PC12-AS1 cells showed greater than 90% neuronal viability, whereas cultures treated with conditioned media from PC12-AB1 or 3T3-AB1 cells showed virtually complete neuronal degeneration with necrotic cell profiles and cellular debris. A culture treated with conditioned medium from 3T3-AB1 cells that had been immunoabsorbed with the M4 antibody showed surviving neurons. Magnification, ×94, as shown.

from 3T3-AB1 cells to normal, differentiated PC12 cells that had been treated with NGF. During the next 4 to 7 days, the cells gradually retracted neuritic processes and degenerated. Conditioned medium from normal 3T3 cells had no effect on differentiated PC12 cells. Addition of conditioned medium from 3T3-AB1 cells to undifferentiated PC12 cells or 3T3 cells also had no effect.

As the degenerative effects of AB1 in PC12 cells were specific for the differentiated neuronal phenotype, we assayed the effect of AB1-conditioned medium on primary neuronal cultures derived from the hippocampus, a region of the brain severely affected in Alzheimer's disease. The morphological identification of neurons was verified by immunocytochemical analysis with an antibody to the neuron-specific 68-kD neurofilament protein (*15*). Conditioned medium from either of the two PC12-AB1 clones or the 3T3-AB1 clone, when added to cultures derived from embryonic day 18 (E18) rat hippocampi, caused neuronal degeneration within 24 hours, as characterized by loss of phase brightness, roughening of the soma, and disintegration of neuritic processes. After 48 to 72 hours, virtually all neurons in the cultures had died (Fig. 4B). Glial cells and a small population of bipolar neurons were the dominant surviving cell types. The same effects were observed in serum-containing or serum-free N2-supplemented medium (*16*). Conditioned medium from PC12-AS1 (Fig. 4B) and 3T3-AD1 cells had no apparent effect on the viability of hippocampal neurons. Control conditioned medium from nontransfected PC12 cells and from PC12-DO cells also had no effect on hippocampal neurons (*15*). The neurotoxic effect of AB1-conditioned medium was also observed in primary cultures of murine hippocampal and neocortical neurons.

We then used antibodies directed against two different domains of the AB1 amyloid polypeptide to immunoabsorb the AB1 polypeptide out of conditioned medium. Cultures treated with control 3T3-AB1–conditioned medium showed only 27% neuronal viability relative to untreated cultures (Table 2). Cultures treated with 3T3-AB1–conditioned medium that had been immunoabsorbed with the M4 antibody or antibody C1 [directed against the COOH-terminal 20 amino acids of AB1 (*17*)] showed 69 and 46% neuronal viability, respectively (Table 2 and Fig. 4B). Immunoabsorption of AB1-conditioned medium with an antibody to neural cell adhesion molecule (anti–N-CAM) or with rabbit preimmune serum did not affect the neurotoxicity.

Thus, our results suggest that the AB1 fragment of the amyloid precursor, which contains the COOH-terminal 105 amino acids, is neurotoxic. The PC12 transfectants expressing the entire human amyloid precursor (APP-1) gene do not degenerate when treated with NGF. Moreover, the conditioned medium from cells expressing the APP-1 amyloid precursor or from cells expressing the amyloid protein alone without the flanking COOH-terminal sequence is not neurotoxic. A peptide containing the NH_2-terminal 28 amino acids of amyloid increases the survival of hippocampal neurons in culture (*18*), but this peptide does not contain the COOH-terminal region of APP, which may explain its lack of toxicity.

Genetic linkage studies have demonstrat-

Table 1. Viability of transfected PC12 cell lines after treatment with NGF. Normal PC12 cells and PC12 cells transfected with AS1 or AB1 were plated on polylysine-coated wells at a density of 2 × 10^4 cells per well. Cells were incubated in the absence (−) or presence (+) of NGF for 8 days. The cells were then dissociated in trypsin, and viable cells that excluded trypan blue were counted in a hemocytometer. Each value represents the mean of eight determinations (±SEM) in a single experiment. Although there was a small difference in the growth rates of the different PC12 clones in the absence of NGF, there was no apparent evidence of cell death or degeneration. In the presence of NGF, the PC12-AB1 cultures, but not PC12 or PC12-AS1 cultures, showed clear evidence of cellular degeneration and death, accounting for their decreased cell numbers (Fig. 4A). After treatment with NGF, PC12 cells became post-mitotic (*12*), thus accounting for the decreased cell numbers relative to cells grown in the absence of NGF. The selective degeneration of NGF-treated PC12-AB1 cells was observed in ten experiments.

Cell line	10^3 viable cells per well	
	−NGF	+NGF
PC12	145 ± 7	58 ± 4
PC12-AS1	128 ± 7	65 ± 7
PC12-AB1#1	110 ± 7	30 ± 5
PC12-AB1#2	108 ± 9	15 ± 3

Table 2. Immunoabsorption of AB1 neurotoxicity with amyloid-specific antibodies. M4 is an affinity-purified antibody to an APP peptide (residues 597 to 611), and C1 is a rabbit antiserum to an APP peptide (residues 676 to 695) (*17*). Anti–N-CAM is a rabbit antiserum to the neural cell adhesion molecule. Polystyrene immunoassay plates were coated with the designated antibodies (M4 at 10 μg/ml; C1 and anti–N-CAM at 1:100 dilutions) for 2 hours at room temperature. After blockage of nonspecific sites with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 hours, the wells were washed with PBS, and conditioned medium was applied. After 3 hours, the medium was transferred to a second set of antibody-treated wells for an additional 3 hours. As a control, 3T3- or 3T3-AB1–conditioned medium was treated in a parallel fashion in wells blocked with BSA but not treated with antibody. The medium was finally filter-sterilized and applied to hippocampal cultures; neuronal viability was determined 36 hours later (*13*). Each value represents the mean of 9 to 17 determinations in two experiments. CM, conditioned medium.

Treatment	Neuronal viability ± SEM (%)
Untreated	100 ± 7
3T3 CM	101 ± 7
3T3-AB1 CM	27 ± 2
3T3-AB1 CM immunoabsorbed with M4 antibody	69 ± 4
3T3-AB1 CM immunoabsorbed with C1 antibody	46 ± 3
3T3-AB1 CM immunoabsorbed with anti–N-CAM	22 ± 3

ed that the genes for familial Alzheimer's disease (FAD) and the amyloid precursor both localize to chromosome 21 but represent distinct loci (*19*). Although this suggests that a mutation in the amyloid gene is not a cause of FAD, defective processing of the amyloid precursor may still play a role in the disease. A peptide that is equivalent to our AB1 fragment has been synthesized in vitro (*11*) and can be processed to a polypeptide the size of amyloid by treatment with proteinase K. Moreover, the COOH-terminal portion of the amyloid precursor, which is contained in the AB1 fragment, is found in senile plaques in Alzheimer's disease (*17*). Altered processing or overexpression of APP in Alzheimer's disease may result in the generation of a neurotoxic peptide.

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8. The M4 antibody was prepared by injecting 350 μg of an uncoupled peptide representing amino acids 597 to 611 of murine APP-1 (that is, the first 15 amino acids of the murine homolog of the amyloid polypeptide) into rabbits. Second and third injections of 200 μg each were given at 4 and 6 weeks. The antibody was affinity-purified on a peptide column. Immunoblot analysis with the antibody revealed a prominent, positively reacting band at 135 kD in membrane fractions of murine and human brain homogenates; preabsorption of the antibody with the peptide to which it was made abolished this immunoreactivity.
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13. The PC12 cells were cultured (*12*) in Dulbecco's modified essential medium (DMEM) containing 10% fetal calf serum (FCS) and 5% horse serum. The β-NGF was added at a concentration of 50 ng/ml; medium and β-NGF were changed every 2 days. Conditioned medium was prepared by incubating subconfluent undifferentiated cultures with DMEM supplemented with 2 m*M* glutamine and insulin (5 μg/ml) for 48 hours, and then withdrawing the medium and filtering it. Primary hippocampal cultures were established by dissecting hippocampi from E18 rat fetuses, incubating them for 30 min with 0.03% trypsin in Ca^{2+}- and Mg^{2+}-free Earle's salt solution, and then incubating for 20 min in Earle's salts. After trituration, cells were plated in 24 well plates on polylysine-coated glass cover slips in DMEM supplemented with 2 m*M* glutamine, 20 m*M* KCl, 1 m*M* pyruvate, 0.5% glucose, and 10% FCS. After 24 hours, media were replaced with DMEM containing the N2 supplements (*15*) with or without 50% of conditioned medium. In some experiments with conditioned medium, cells were maintained in the FCS-containing medium. Neuronal viability, as assessed by trypan blue exclusion, phase brightness, and intactness of neurites, was determined in ten fields of four sister cultures for each condition.
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