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Neuronal localization of amyloid beta protein precursor mRNA in normal human brain and in Alzheimer's disease

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Clones for the amyloid beta protein precursor gene were isolated from a cDNA library prepared from the frontal cortex of a patient who had died with a histologically confirmed diagnosis of Alzheimer's disease; they were used to investigate the tissue and cellular distribution of amyloid beta protein precursor mRNA in brain tissues from control patients and from Alzheimer's disease patients. Amyloid beta protein precursor mRNA was expressed in similar amounts in all control human brain regions examined, but a reduction of the mRNA level was observed in the frontal cortex from patients with Alzheimer's disease. By *in situ* hybridization amyloid beta protein precursor mRNA was present in granule and pyramidal cell bodies in the hippocampal formation and in pyramidal cell bodies in the cerebral cortex. No specific labelling of glial cells or endothelial cells was found. The same qualitative distribution was observed in tissues from control patients and from patients with Alzheimer's disease. Senile plaque amyloid thus probably derives from neurones. The tissue distribution of amyloid beta protein precursor mRNA and its cellular localization demonstrate that its expression is not confined to the brain regions and cells that exhibit the selective neuronal death characteristic of Alzheimer's disease.

Key words: Alzheimer's disease/amyloid beta protein precursor gene/*in situ* hybridization

Introduction

Senile plaques and neurofibrillary tangles constitute the neuropathological characteristics of Alzheimer's disease (Alzheimer, 1907). Whereas the molecular nature of the paired helical filament, the major tangle constituent, remains unknown, the 43 amino acid beta protein (also called A4 protein) forms the main protein component of cerebrovascular and plaque amyloid (Glenner and Wong, 1984; Masters *et al.*, 1985; Kang *et al.*, 1987). Recently the gene for the amyloid beta protein precursor was cloned and sequenced from normal human brain (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987; Tanzi *et al.*, 1987). It encodes a 695 amino acid protein of unknown function with the characteristics of a transmembrane protein (Kang *et al.*, 1987). The beta protein is located towards the carboxy-terminal end of the precursor and terminates within the putative transmembrane region. The C-terminal cleavage of the precursor during amyloid formation thus might occur within the membrane. The amyloid beta protein precursor gene is located in the proximal region of the long arm of chromosome 21 (Goldgaber *et al.*, 1987; Tanzi *et al.*, 1987), a region to which the genetic defect responsible for the familial form of Alzheimer's disease has also been mapped (St George-Hyslop *et al.*, 1987). At present it is unclear why the amyloid beta protein is deposited in large amounts in the disease and whether this represents a primary or a secondary event in the disease process. As the amyloid deposits are extracellular it is not known which cells manufacture the beta protein precursor and whether there exists a correlation between the neuronal cell loss characteristic of Alzheimer's disease and the expression of the beta protein precursor.

I have isolated cDNA clones for the amyloid beta protein precursor gene from a cDNA library prepared from the frontal cortex of a patient who had died with a histologically confirmed diagnosis of Alzheimer's disease, and used them to investigate the levels and cellular localization of amyloid beta protein precursor gene mRNA in the brain by Northern blotting and *in situ* hybridization.

Results

Five clones (called Am1–Am5) for the amyloid beta protein precursor gene were isolated from a cDNA library prepared from the frontal cortex of a patient who had died with a histologically confirmed diagnosis of Alzheimer's disease. Clones Am1–Am3 consist of a hybridization-positive 1.1 kb insert comprising 291 nucleotides of open reading frame and 772 nucleotides of 3′ untranslated sequence. It encodes the carboxy-terminal 41 amino acids of the amyloid beta protein. Clone Am4 contains an additional 424 nucleotides of open reading frame, whereas clone Am5 is 3.2 kb in length and encodes the whole open reading frame

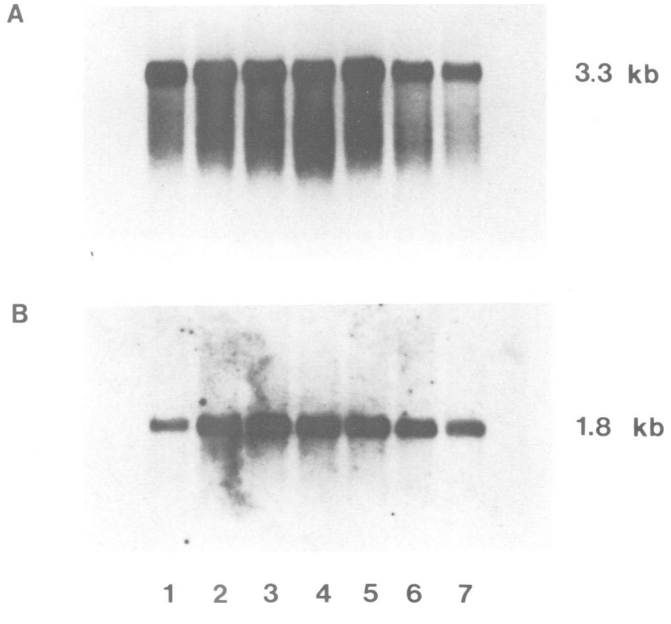

Fig. 1. Northern blot analysis of poly(A)$^+$ RNA from control human brain using ^{32}P-labelled human amyloid beta protein precursor DNA (**A**) or mouse beta actin DNA (**B**) as a probe. Each lane contained 2 μg poly(A)$^+$ RNA. **Lanes: 1**, frontal cortex; **2**, temporal cortex; **3**, striatum; **4**, hippocampus; **5**, cerebellum; **6**, brainstem; **7**, thalamus. The blot was hybridized with the actin probe following dehybridization of the amyloid probe. Rat rRNAs served as size markers. The autoradiographic exposure times were 5 h for A and 3 h for B.

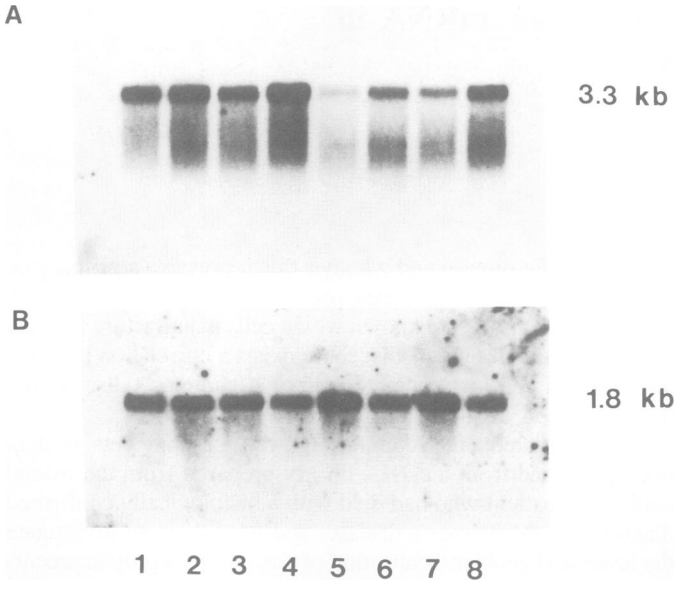

Fig. 2. Northern blot analysis of poly(A)$^+$ RNA from frontal cortex of control patients and of patients who had died with Alzheimer's disease using ^{32}P-labelled human amyloid beta protein precursor DNA (**A**) or mouse beta actin DNA (**B**) as a probe. Each lane contained 2 μg poly(A)$^+$ RNA as a probe. **Lanes: 1–4**, frontal cortex from control patients; **5–8**, frontal cortex from Alzheimer's disease patients. The blot was hybridized with the actin probe following dehybridization of the amyloid probe. Rat rRNAs served as size markers. The autoradiographic exposure times were 5 h for A and 3 h for B.

of the amyloid beta protein precursor gene. In view of the fact that the carboxy-terminal cleavage of the amyloid beta protein has to occur within the putative transmembrane region of the precursor the open reading frame of the 1.1 kb fragment was sequenced from all five clones. In every case the nucleotide sequence was found to be identical with that determined from normal human brain (Kang *et al.*, 1987).

The tissue distribution of amyloid beta protein precursor mRNA was investigated in frontal cortex, temporal cortex, hippocampus, striatum, thalamus, cerebellum and brainstem from a patient who had died with no neurological or psychiatric disorders (Figure 1). One major transcript of 3.3 kb was observed by Northern blotting, and substantial levels of amyloid beta protein precursor mRNA were present in all regions (Figure 1A), indicating that it represents quite an abundant mRNA species. Only very small regional differences were observed when the results were normalized using hybridization with a mouse beta actin cDNA clone (Figure 1B). Similar results were obtained by using the same tissues from a different control brain.

The levels of amyloid beta protein precursor mRNA were investigated in the frontal cortex from four control patients and from four patients who had died with a histologically confirmed diagnosis of severe Alzheimer's disease (Figure 2). The results were normalized using the mouse beta actin cDNA clone (Figure 2B). The same major 3.3 kb band was observed, and an overall reduction in its level was found in Alzheimer's disease (Figure 2A). The latter result has been confirmed using frontal cortex tissue from a further four cases (data not shown). In addition

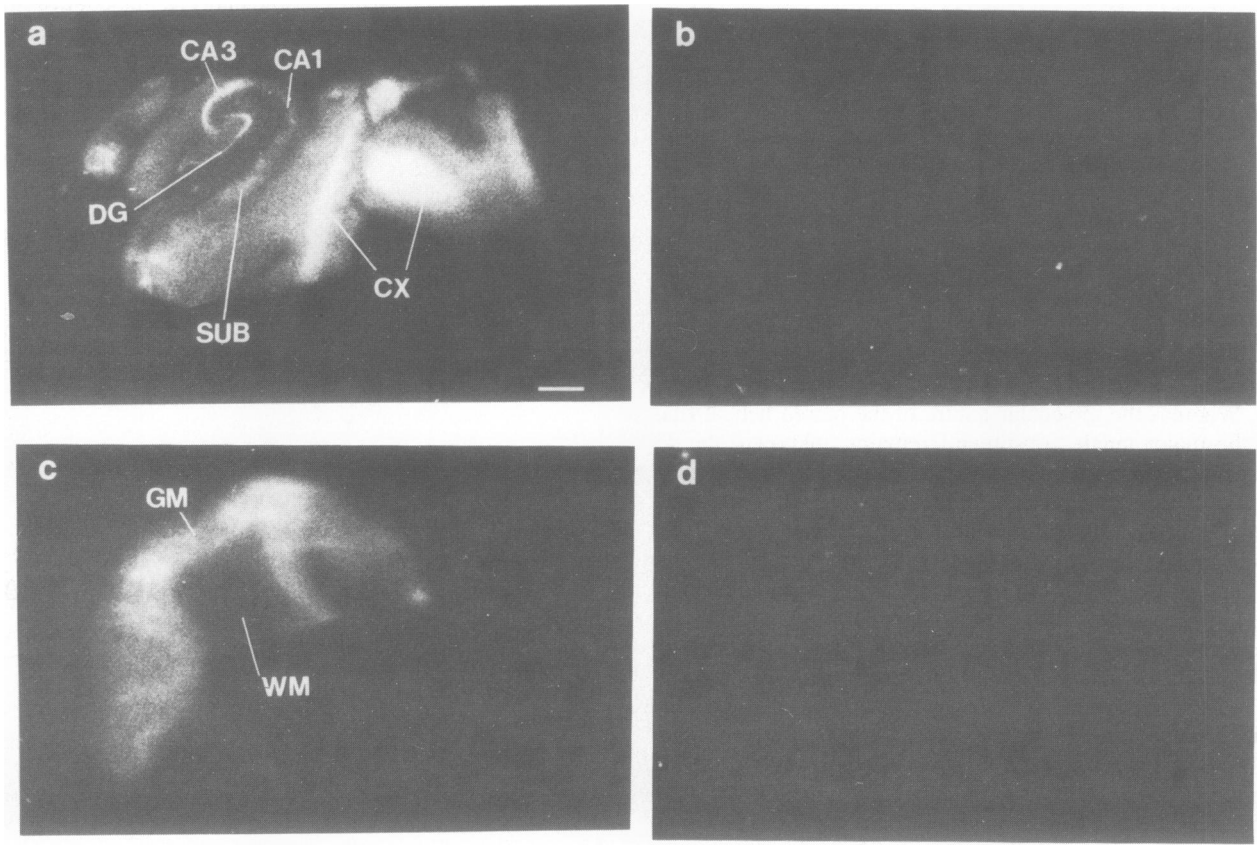

Fig. 3. *In situ* hybridization of control human hippocampal formation (**a**) and frontal cortex (**c**) brain sections with the amyloid beta protein precursor probe Am3 in the anti-mRNA sense orientation; (**b**) and (**d**) show *in situ* hybridization of hippocampal formation (**b**) and frontal cortex (**d**) sections with the Am3 probe in the mRNA sense orientation. The probes were labelled with [^{35}S]dATP and the sections photographed under dark-field. The hybridization-positive region in (**a**) between DG and CA3 corresponds to the medial geniculate body. Abbreviations: **DG**, dentate gyrus; **SUB**, subiculum; **CA3**, cornu ammonis region 3; **CA1**, cornu ammonis region 1; **CX**, middle lobe of the infratemporal cerebral cortex; **GM**, grey matter; **WM**, white matter. Scale bar, 2 mm.

the amyloid beta protein precursor probe gave a broad smear of hybridization that centred around 1.7 kb in all samples, with some faint bands within it in some samples from Alzheimer's disease cases. This was not due to general degradation, as no such smear was seen with the actin probe. But the extra bands were too faint for any definite difference between samples from control and Alzheimer's disease patients to be established. Single-stranded DNA probes in the anti-mRNA sense orientation were used to investigate the cellular localization of amyloid beta protein precursor mRNA by *in situ* hybridization, in the hippocampus and cerebral cortex from three control patients and from four Alzheimer's disease patients. The same qualitative distribution of hybridization-positive cells was observed in both groups, and the results were highly reproducible between the tissues from different patients within each group. In both the cerebral cortex and hippocampus the specific silver grains were confined to neuronal cell bodies, and these perikarya were predominantly those of projection neurones (Figures 3–7). No specific labelling of glial cells or endothelial cells was observed. The hybridization was specific, as only the background labelling was seen when a probe in the mRNA sense orientation was used as a control for non-specific labelling (Figures 3B, 3D, 4B, 7B).

In the hippocampal formation there was strong labelling of granule cells in the dentate gyrus, pyramidal cells throughout Ammon's horn and pyramidal cells in the subiculum (Figures 3A, 4A, 4C, 6A, 6B, 7D). All granule cells and all pyramidal cells appeared to be hybridization-positive; the pyramidal cell labelling was stronger in the CA3 than in the CA1 region. In the cerebral cortex hybridization-positive cells were only seen in the grey matter (Figure 3C), where they were present in all layers (Figure 5); relatively few labelled cells were seen in the superficial layers, with most positive cells in the deeper layers (Figures 5, 6C, 6D, 7A). The distribution of these cells and their size (Feldman, 1984) indicate that pyramidal cells constitute the cortical cell type that predominantly if not exclusively expresses amyloid beta protein precursor mRNA. No specific labelling of blood vessel walls was observed (Figure 7C). In all these cases

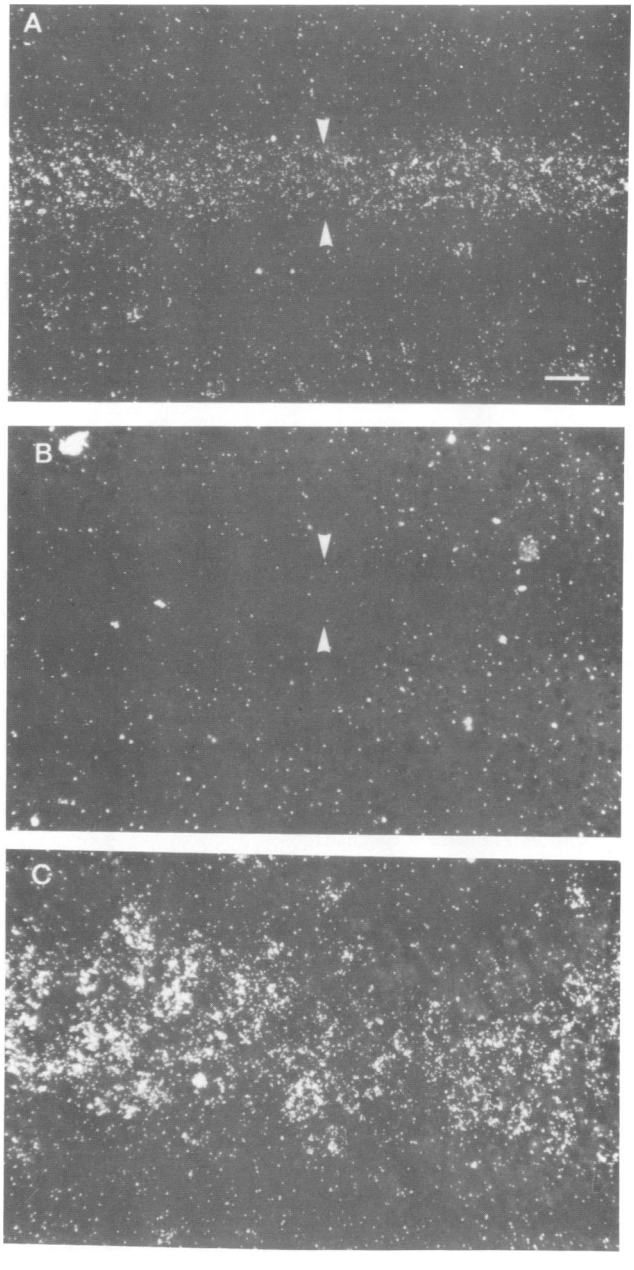

Fig. 4. Cellular localization of amyloid beta protein precursor mRNA in the hippocampus from a control patient. **A**, Dark-field photomicrograph of dentate gyrus after hybridization with a probe in the anti-mRNA sense orientation. **B**, Dentate gyrus following hybridization with a probe in the mRNA sense orientation. **C**, Dark-field photomicrograph of the CA3 region following hybridization with a probe in the anti-mRNA sense orientation. The probes were labelled with [^{35}S]dATP; the arrows point to the dentate gyrus. Scale bar, 30 μm.

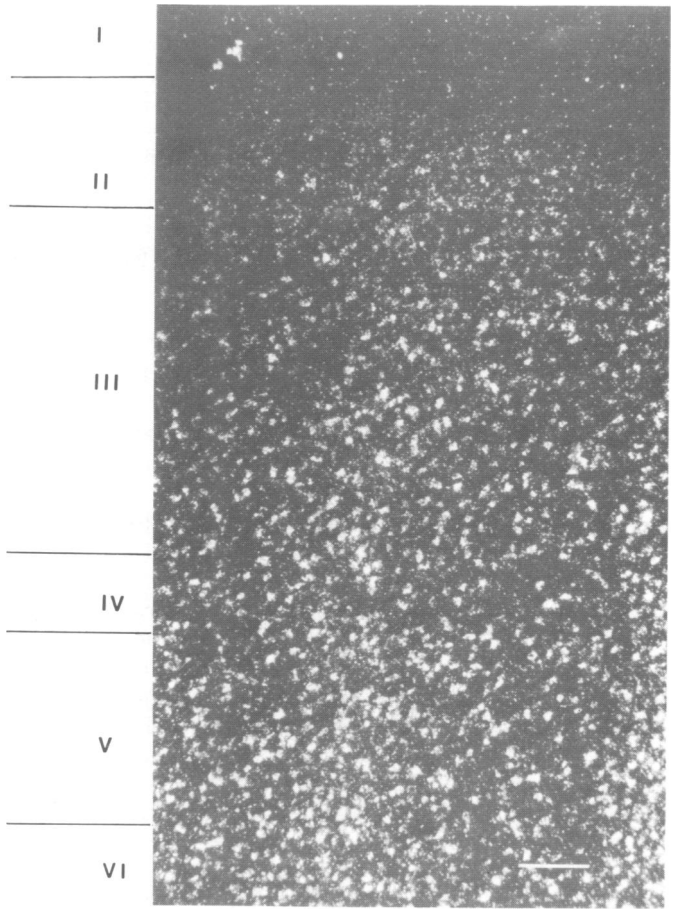

Fig. 5. Dark-field photomicrography of the cellular localization of amyloid beta protein precursor mRNA in the frontal cortex from a control patient. The probe was labelled with [^{35}S]dATP. The roman numerals indicate the cellular layering of the cerebral cortex (only part of layer VI is shown). Scale bar, 150 μm.

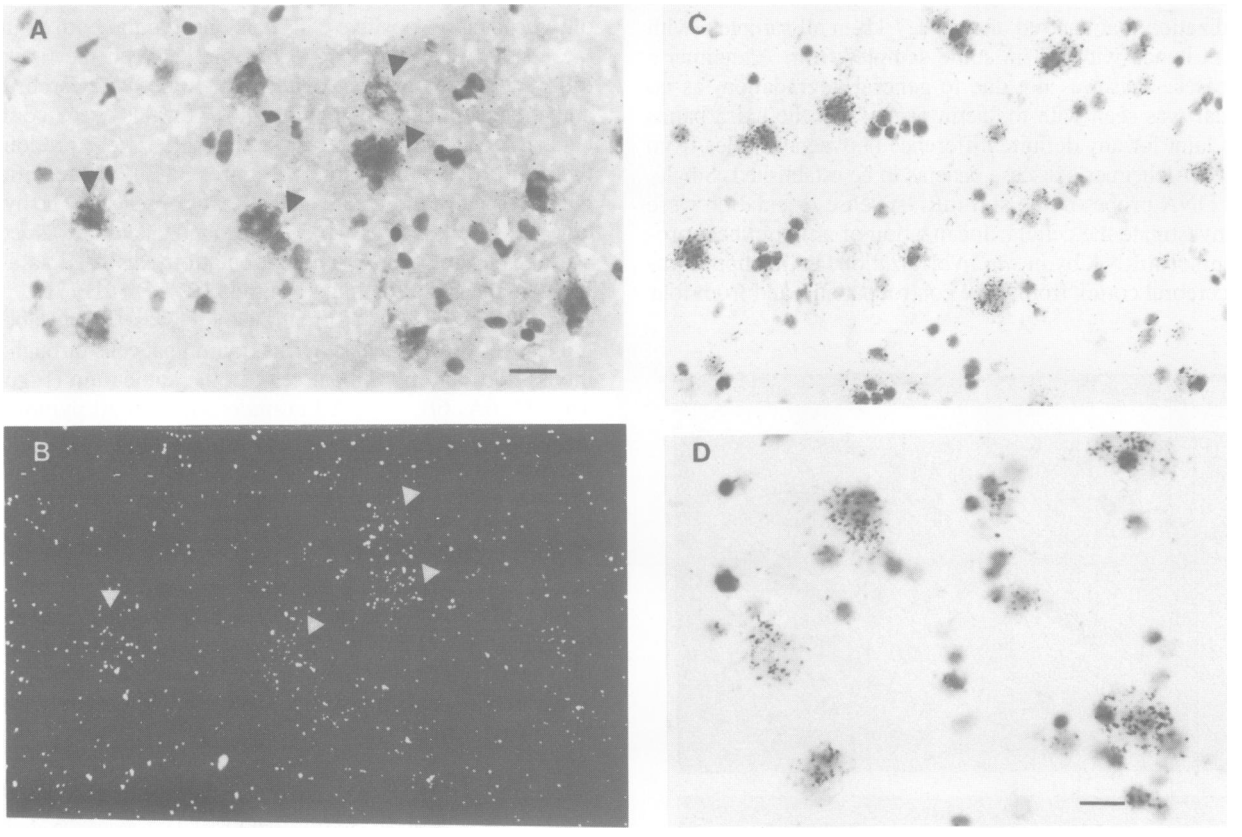

Fig. 6. Cellular localization of amyloid beta protein precursor mRNA in subiculum (**A,B**) and frontal cortex (**C,D**) from a control patient. **A** and **B** represent light- and dark-field photomicrographs of the same tissue section; the arrows point to the hybridization-positive cells. **C** and **D** show light-field photomicrographs of hybridization-positive cells in the frontal cortex. The probe was labelled with [^{35}S]dATP. Scale bar in A (for A–C), 25 μm; scale bar in D, 17 μm.

no distinction in labelling could be seen between tissues from control brains and from brains of patients who had died with Alzheimer's disease.

Discussion

The results of the Northern blotting experiments indicate that amyloid beta protein precursor mRNA is widely distributed in the normal human brain and that in the frontal cortex, the only region examined, its mRNA levels are reduced in Alzheimer's disease. The latter result probably reflects the loss of large neurones that is observed in the course of the disease (Price, 1986). The findings in normal human brain confirm and extend previous results (Tanzi *et al.*, 1987); amyloid beta protein precursor mRNA showed very little variation between the different brain regions examined, and there existed no correlation between the regions affected in the disease and the presence of amyloid beta protein precursor mRNA; thus, the levels seen in the frontal and temporal cortex and in the hippocampus, all of which are regions that are severly affected in Alzheimer's disease, were not significantly different from those found in the striatum and thalamus, two regions that show only very few neuropathological changes. All regions showed the presence of one major 3.3 kb transcript; a broad lower molecular weight band was also observed. Similar results were obtained in the rat brain (data not shown). It is at present unclear whether this reflects a great susceptibility of amyloid beta protein precursor mRNA to degradation or whether it indicates the presence of multiple transcripts.

By *in situ* hybridization amyloid beta protein precursor mRNA was present in granule and pyramidal cell bodies in the hippocampal formation and in pyramidal cell bodies in the cerebral cortex. No specific labelling of glial cells or blood vessel walls was observed. The same was true for the medial geniculate body, where magnocellular neurones constituted the hybridization-positive cells (data not shown). The overall results were identical in tissues from control patients and from patients who had died with Alzheimer's disease.

At present the cellular origin of the extracellular amyloid beta protein deposits of Alzheimer's disease is unknown. It has been proposed that amyloid beta protein deposition represents the consequence of a breakdown of the blood–brain barrier, implying a peripheral origin for the amyloid beta protein (Glenner, 1979). This appears improbable, as it would imply a selective breakdown of the blood–brain barrier, and no amyloid beta protein plaques are found in peripheral tissues in Alzheimer's disease; moreover, a radioimmunoassay study has failed to detect amyloid beta protein in human serum (Pardridge *et al.*, 1987). The presence of microglial cells at very early stages of neuritic plaque formation has led to the suggestion that non-neuronal cells may constitute the source of plaque amyloid (Terry and Wisniewski, 1972; Wisniewski *et al.*, 1981). The present results make that seem unlikely, as the hybridization-positive cells were invariably neuronal in nature. It therefore appears that the senile plaque amyloid beta protein derives from nerve cells. It remains to be determined how this neuronal origin can lead to the cerebrovasular amyloidosis that is observed in a large proportion of Alzheimer's disease cases (Mandybur, 1975; Morimatsu *et al.*, 1975; Wong *et al.*, 1985).

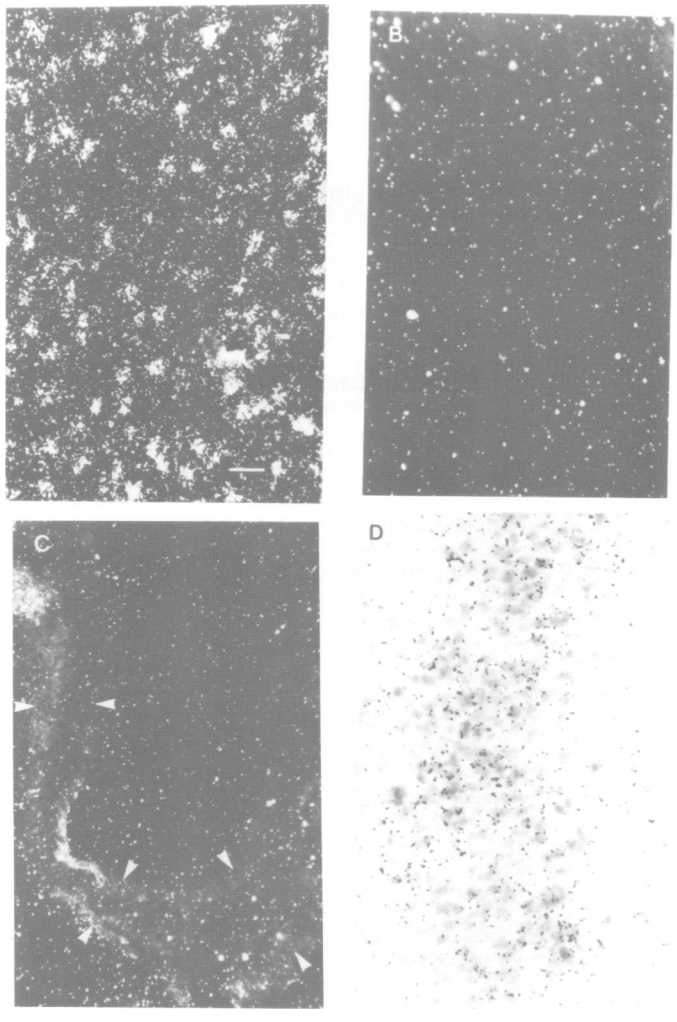

Fig. 7. Cellular localization of amyloid beta protein precursor mRNA in cerebral cortex and hippocampus from a patient who had died with a histologically confirmed diagnosis of Alzheimer's disease. **A**, Dark-field photomicrograph of frontal cortex layer 5 (identified under light-field) after hybridization with a probe in the anti-mRNA sense orientation. **B**, Adjacent section hybridized with a probe in the mRNA sense orientation. **C**, Dark-field photomicrograph of a longitudinal section through an arteriole following hybridization with a probe in the anti-mRNA sense orientation. Arrows mark edges of the arteriole. Note the absence of specific labelling. **D**, Light-field photomicrograph of the dentate gyrus after hybridization with a probe in the anti-mRNA sense orientation. The probes were labelled with [^{32}P]dATP. Scale bar, 25 μm.

It has been proposed that amyloid beta protein deposition represents the primary event in Alzheimer's disease and that neurofibrillary tangle formation and neuronal cell loss represent the direct consequence of an abnormal or an abnormally regulated amyloid beta protein precursor gene (Delabar *et al.*, 1987). The findings reported here are difficult to reconcile with such a view, assuming that the expression of the mRNA reflects the presence of the amyloid beta protein precursor. Although pyramidal cells in the cerebral cortex, hippocampus and subiculum are lost in the course of the disease, this is not the case for hippocampal granule cells (Ball, 1977; Terry *et al.*, 1981; Hyman *et al.*, 1984), and these, as shown in this study, also express substantial levels of amyloid beta protein precursor mRNA; moreover, both the hippocampal CA3 and CA1 regions express high levels of amyloid beta protein precursor mRNA, although it is mostly the CA1 region which shows the neuropathological changes (Hyman *et al.*, 1984). Amyloid beta protein precursor mRNA is thus found in brain regions that are not affected in Alzheimer's disease, and it is conversely expressed in a region which is affected in the disease (the hippocampus), where it is found in a class of neurones (granule cells) that do not degenerate. The present results are in keeping with neuropathological observations which have indicated that enlarged abnormal neurites represent the earliest ultrastructural manifestations of the neuritic plaque. The first amyloid fibrils were only observed after a cluster of four or five abnormal neurites had formed (Kidd, 1964; Terry and Wisniewski, 1972), suggesting that amyloid beta protein deposition is not the cause of abnormal neurite formation.

In conclusion, the present results indicate that the selective distribution of the neuronal cell death in Alzheimer's disease cannot be explained through the expression of an abnormal or an abnormally regulated amyloid beta protein precursor gene. It is conceivable that the selective vulnerability requires the presence of additional factors. Alternatively amyloid fibril formation may represent a secondary event in the development of Alzheimer's disease.

Materials and methods

cDNA library construction and screening

RNA was isolated from the frontal cortex of a 65 year old patient who had died with a histologically confirmed diagnosis of Alzheimer's disease (Chirgwin *et al.*, 1979); the tissue was obtained 3 h after death. Poly(A)$^+$ RNAs were enriched for by oligo(dT) cellulose affinity chromatography (Aviv and Leder, 1972). First strand cDNA synthesis was carried out using murine reverse transcriptase in the presence of actinomycin D (40 μg/ml), using oligo(dT) as a primer.

Double-stranded cDNA was generated by a modification of the procedure described by Gubler and Hoffman (1983), using RNase H, DNA polymerase I and *Escherichia coli* DNA ligase. After treatment with S1 nuclease and *Eco*RI methylase, the size-selected double-stranded cDNA was cloned into the imm^{434} *Eco*RI insertion vector λgt10 (Huynh *et al.*, 1985) using *Eco*RI linkers. Ten micrograms of poly(A)$^+$ RNA yielded a library of 6.2 × 10^6 clones. Replica filters of a portion of this library (50 000 plaques) were screened in duplicate with two mixed synthetic oligonucleotides [5′TT(^T_C)TG(^A_G)TG(^A_G)TG(^A_G)AC(^T_C)TG(^A_G)TA3′ and 5′TT(^T_C)TG(^A_G)TG(^A_G)TG(^T_G)AC(^T_C)TG(^A_G)TA3′] derived from the amino acid sequence of cerebrovascular amyloid (Glenner and Wong, 1984) and labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Five positive clones were obtained; they were subcloned into M13mp18 and partially sequenced using a modification (Biggin *et al.*, 1983) of the dideoxy chain termination method (Sanger *et al.*, 1977).

Single-stranded DNA probes

The 1.1 kb *Eco*RI fragment of the beta amyloid precursor cDNA clone Am3 was subcloned into the *Eco*RI site of M13mp19. Messenger RNA sense and anti-mRNA sense orientations were distinguished by dideoxy sequencing. To prepare ^{32}P-labelled probe 1 pmol of phage DNA and 0.1 pmol of the M13 sequencing primer (5′GTAAAACGACGGCCAGT3′) were annealed for 1 h at 60°C in 10 μl 10 mM Tris, pH 8.5, 50 mM $MgCl_2$. One nmol dTTP, dCTP and dGTP and 25 pmol [α-^{32}P]dATP (800 Ci/mmol) were added and synthesis initiated by the addition of 5 units Klenow fragment of DNA polymerase I. After 15 min at room temperature 3 nmol dATP, dGTP, dTTP and dCTP were added for another 15 min, followed by digestion with *Pst*I for 15 min at 37°C. The reaction was stopped with EDTA, and incorporated and non-incorporated nucleotides were separated using Sephadex G-50 gel chromatography. The samples were denatured in 50% formamide at 95°C and electrophoresed on a 1% agarose gel. The labelled fragment band was localized by autoradiography and electrophoresed onto a glass fibre filter backed by dialysis tubing. The labelled DNA was removed from the glass fibre filter by centrifugation, phenol/chloroform extracted twice, and ethanol-precipitated in the presence of carrier tRNA. The specific activity of the probe was 1.3 × 10^9 c.p.m./μg. ^{35}S-labelled probes were prepared as above, except that 5 pmol [α-^{35}S]dATP (4000 Ci/mmol) was used and the DNA was restricted with *Sma*I for 15 min at 25°C. The specific activity of the probe was 6.6 × 10^9 c.p.m./μg.

In situ hybridization

Brain tissues from three control patients (ages 68, 73 and 79 years) and from four patients with a histologically confirmed diagnosis of Alzheimer's disease (ages 74, 76, 78 and 82 years) were removed less than 4 h after death and processed using a modification of a previously described method (Goedert and Hunt, 1986). Frontal cortex and hippocampus were dissected, frozen and cut on a cryostat

at 20–30 μm. The sections were thawed onto gelatine/L-polylysine-coated microscope slides and kept at −70°C until use. They were then fixed for 10 min at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by three washes in PBS. The sections were treated with 250 μg/ml pronase in 50 mM Tris, pH 7.5, containing 5 mM EDTA for 10 min at room temperature. They were then treated for 1 min with 2 mg/ml glycine, refixed for 10 min in 4% paraformaldehyde, washed twice in PBS, dehydrated with 70% ethanol and air dried. The sections were prehybridized for 2 h at room temperature in 50% formamide, 5× SSC (1× SSC = 150 mM sodium chloride, 15 mM sodium citrate), 50 mM sodium phosphate buffer, pH 7, 250 μg/ml acid/base cleaved salmon sperm DNA, 5× Denhardt's solution (1× Denhardt's = 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin), 200 μg/ml heparin and 0.1% sodium dodecylsulphate. Hybridization was performed at 42°C in the above solution containing 10% Dextran sulphate and denatured, radioactively labelled probe in the anti-mRNA sense orientation (10^6 c.p.m./section). In control experiments probes in the mRNA sense orientation (10^6 c.p.m./section) were used. The sections were washed in three changes of 2× SSC, followed by an overnight wash at room temperature in 50% formamide, 0.6 M sodium chloride, 10 mM Tris, pH 7.4 and 1 mM EDTA. When ^{35}S was the isotope, 20 mM dithiothreitol was included in all hybridization and washing solutions. The slides were dehydrated in 70% ethanol, air dried and exposed to X-ray film (Kodak XAR-5) for 48 h at room temperature. They were then dipped in Ilford K-5 emulsion and kept at −20°C for 10 days. They were developed in safe-light conditions using Kodak D19 developer, post-fixed in Carnoy's solution, stained with haematoxylin, dehydrated, cleared in Histoclear and mounted using Depex.

Northern blot analysis

Human tissues were obtained less than 6 h after death and stored at −70°C until use. Seven different brain regions were dissected from the brains of a 68 and a 74 year old patient who had both died with no neurological or psychiatric disorders. The frontal cortex was used from the same two patients and from two others who had also died without neurological or psychiatric diseases (ages 79 and 83 years) and from four patients who had died with a histologically confirmed diagnosis of Alzheimer's disease (ages 65, 78, 80 and 83 years). Total cellular RNA was extracted (Chirgwin *et al.*, 1979) and enriched for poly(A)$^+$ RNA using oligo(dT) cellulose (Aviv and Leder, 1972). The poly(A)$^+$ RNA was electrophoresed on a 2.2 M formaldehyde-containing 0.9% agarose gel (Lehrach *et al.*, 1977) and directly transferred to a Nylon membrane (Hybond-N) with 20× SSC (Thomas, 1980). After fixation by UV irradiation the blots were prehybridized for at least 3 h at 42°C. Hybridization was carried out for 16 h at 42°C in prehybridization solution containing 10% Dextran sulphate and 10^6 c.p.m. denatured labelled probe in the anti-mRNA sense orientation/ml. The filters were washed twice at room temperature in 2× SSC/0.1% sodium dodecylsulphate, followed by two washes in 0.1× SSC/0.1% sodium dodecylsulphate at 60°C. The filters were then exposed to Fuji X-ray film with a Du Pont Cronex intensifying screen at −70°C. Following autoradiography the blots were washed for 1 h at 95°C in 0.5× Denhardt's solution, 50 mM Tris, pH 7.4, 0.1% sodium dodecylsulphate, followed by a 15 min wash in water at 65°C. The blots were rehybridized at 42°C with a mouse beta actin cDNA clone (K.Willison and J.Rogers, personal communication) labelled by nick-translation (Rigby *et al.*, 1977). The filters were washed twice in 2× SSC/0.1% sodium dodecylsulphate at room temperature, followed by two washes in 0.1 × SSC/0.1% sodium dodecylsulphate at 50°C and exposure to Fuji X-ray film at −70°C using a Du Pont Cronex intensifying screen.

Note Added

During the preparation of this manuscript a report was published showing the presence of amyloid beta protein precursor mRNA in pyramidal cells in human hippocampus and cerebral cortex (Bahmanyar *et al.*, *Science*, **237**, 77–80, 1987).

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