The EMBO Journal vol.7 no.5 pp.1365 - 1370, 1988

Alzheimer's disease amyloidogenic glycoprotein: expression pattern in rat brain suggests a role in cell contact

Brenda D.Shivers, Caroline Hilbich, Gerd Multhaup, Michael Salbaum, Konrad Beyreuther and Peter H.Seeburg

Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, FRG

Communicated by P.H.Seeburg

The cloned cDNA encoding the rat cognate of the human A4 amyloid precursor protein was isolated from a rat brain library. The predicted primary structure of the 695-amino acid-long protein displays 97% identity to its human homologue shown previously to resemble an integral membrane protein. The protein was detected in rodent brain and muscle by Western blot analysis. Using *in situ* hybridization and immunocytochemistry on rat brain sections, we discovered that rat amyloidogenic glycoprotein (rAG) and its mRNA are ubiquitously and abundantly expressed in neurons indicating a neuronal original for the amyloid deposits observed in humans with Alzheimer's disease (AD). The protein appears in patches on or near the plasma membranes of neurons suggesting a role for this protein in cell contact. Highest expression was seen in rat brain regions where amyloid is deposited in AD but also in areas which do not contain deposits in AD. Since amyloid deposits are rarely observed in rat brain, we conclude that high expression of AG is not the sole cause of amyloidosis.

Key words: Alzheimer's disease/amyloid/cell contact/receptor

Introduction

The human amyloidogenic glycoprotein (hAG), 695 residues in length (Kang *et al.*, 1987; Goldgaber *et al.*, 1987; Tanzi *et al.*, 1987b), is, in some as yet ill-defined manner, responsible for the deposits of amyloid associated with the neuropathology of Alzheimer's disease (AD) (for review see Selkoe, 1987). This responsibility stems from the fact that a 42–43-amino acid-long polypeptide sequence (A4) contained within hAG is the major constituent of the amyloid deposits (Glenner and Wong, 1984; Masters *et al.*, 1985). The A4 sequence both precedes, and forms part of, the unique putative membrane-spanning segment of the AG glycoprotein and possesses self-aggregating properties (Kang *et al.*, 1987). Aberrant catabolism of hAG or overexpression of this protein (Delabar *et al.*, 1987) have been advanced as causes of amyloidosis and AD.

The rat offers a unique model system to test these hypotheses, as it is among the mammals which show no, or exceptionally few, amyloid deposits in aged individuals (Vaughan and Peters, 1981). Thus, the primary structure of the rat homologue and the expression of this protein in rat brain may illuminate causes for the human neuropathology, and reveal how this membrane glycoprotein normally participates in brain function. Our preliminary results have appeared in abstract form (Shivers *et al.*, 1987).

Results

Structure of the predicted rAG protein

To determine the structure of the rat homologue of hAG, a cDNA libary was constructed in λ gt10 from rat brain mRNA and screened with a single oligodeoxynucleotide probe encoding the N-terminal 17 amino acids of the 42-residue-long human A4 amyloid polypeptide (Masters *et al.*, 1985). Such screening produced a large number of hybridizing clones, indicating high abundance of the cognate sequence in rat brain mRNA. The open reading frame of the cloned cDNAs (Figure 1) encodes a protein of 695 amino acid residues with 676 residues identical to the recently determined hAG and therefore identical domain features (Kang *et al.*, 1987). The 19 amino acid differences between both proteins are somewhat clustered with 13 occurring in the N-terminal half and five in the region preceding and within the amyloid A4 polypeptide sequence itself (Figure 1).

Size of rAG protein

Western blot analysis was performed on tissue extracts from rodent brain and muscle. A molecular form having an M_r of 92 kd was detected in both tissues but was in higher abundance in muscle (Figure 2). Since AG is a glycosylated 695 amino acid protein and its expected mol. wt should exceed 76 kd, the observed 92 kd form is reasonable. AG is expected to be expressed in many tissues (Tanzi *et al.*, 1987b), including muscle (Zimmermann *et al.*, 1988).

Location, relative abundance and cell-type containing rAG mRNA

Tissue distribution and cellular location of mRNA encoding rAG was determined by hybridizing rat brain sections with radioactively labelled complementary RNA (cRNA). Film autoradiograms from sections hybridized with [^{32}P]cRNA revealed that the mRNA is widely and abundantly distributed in grey matter with hybridization densities varying distinctly across brain regions (Figure 3). Regions displaying the highest densities included olfactory areas, cerebral cortex, hippocampus, dentate gyrus, amygdala and cerebellum. Thalamic nuclei had nearly as high levels of rAG mRNA.

Inspection of emulsion autoradiograms from rat brain sections hybridized with [^{32}P]- or [^{3}H]rAG cRNA (Figure 4A–F) confirmed the neuronal location of the majority of rAG mRNA, as suggested by our film autoradiographic results. Within each region, rAG mRNA content varied with neuronal-cell type. Most, if not all, neurons appeared to express the mRNA, and the largest neuronal cell bodies within a region were often the most impressively labelled. Little or no hybridization signal was seen in cells in the white matter (oligodendrocytes), illustrated here by the absence

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-15                                          .....CCACGCAGGATCACG

  1 ATGCTGCCCAGCCTGGCACTGCTCCTGCTGGCCGCCTGGACGGTTCGGGCTCTGGAGGTG
  1 M  L  P  S  L  A  L  L  L  L  A  A  W  T  V  R  A  L  E  V
               G                                        A
 61 CCCACTGATGGCAATGCCGGTCTGCTGGCAGAACCCCAGATCGCCATGTTCTGTGGTAAA
 21 P  T  D  G  N  A  G  L  L  A  E  P  Q  I  A  M  F  C  G  K
                                                        R
121 CTCAACATGCACATGAATGTGCAGAATGGAAAATGGGAGTCAGACCCATCAGGGACCAAA
 41 L  N  M  H  M  N  V  Q  N  G  K  W  E  S  D  P  S  G  T  K
                                          D
181 ACCTGCATTGGCACCAAGGAGGGAATCCTGCAGTACTGCCAAGAGGTCTACCCTGAACTG
 61 T  C  I  G  T  K  E  G  I  L  Q  Y  C  Q  E  V  Y  P  E  L
          D
241 CAGATCACAAACGTGGTGGAAGCCAACCAGCCAGTGACCATCCAGAACTGGTGCAAGCGG
 81 Q  I  T  N  V  V  E  A  N  Q  P  V  T  I  Q  N  W  C  K  R

301 GGCCGCAAGCAGTGCAAGACGCACACCCACATCGTGATTCCTTACCGGTGCCTAGTTGGT
101 G  R  K  Q  C  K  T  H  T  H  I  V  I  P  Y  R  C  L  V  G
                           P     F
361 GAGTTTGTAAGCGATGCCCTTCTCGTGCCCGACAAGTGCAAGTTTCTACACCAGGAGCGG
121 E  F  V  S  D  A  L  L  V  P  D  K  C  K  F  L  H  Q  E  R

421 ATGGACGTTTGTGAGACCCATCTTCACTGGCATACTGTTGCCAAAGAGACATGCAGTGAG
141 M  D  V  C  E  T  H  L  H  W  H  T  V  A  K  E  T  C  S  E

481 AAGAGCACTAACTTGCACGACTATGGCATGCTGCTGCCCTGTGGCATCGACAAGTTCCGA
161 K  S  T  N  L  H  D  Y  G  M  L  L  P  C  G  I  D  K  F  R

541 GGGGTCGAGTTCGTGTGCTGCCCGTTGGCGGAGGAGAGCGACAGCATCGATTCTGCGGAC
181 G  V  E  F  V  C  C  P  L  A  E  E  S  D  S  I  D  S  A  D
                                                 N  V
601 GCAGAGGAGGACGACTCCGATGTCTGGTGGGGTGGAGCGGACACAGACTATGCTGATGGC
201 A  E  E  D  D  S  D  V  W  W  G  G  A  D  T  D  Y  A  D  G

661 GGTGAAGACAAAGTCGTAGAAGTAGCCGAAGAGGAGGAAGTGGCCGATGTTGAGGAAGAA
221 G  E  D  K  V  V  E  V  A  E  E  E  E  V  A  D  V  E  E  E
     S                                             E
721 GAAGCTGAGGATGACGAGGATGTGGAGGATGGGGATGAGGTGGAGGAGGAGGCCGAGGAG
241 E  A  E  D  D  E  D  V  E  D  G  D  E  V  E  E  E  A  E  E
        D           D
781 CCCTACGAAGAGGCCACAGAGAGAACAACCAGCATTGCCACCACTACCACAACTACCACT
261 P  Y  E  E  A  T  E  R  T  T  S  I  A  T  T  T  T  T  T  T

841 GAGTCTGTGGAGGAGGTAGTCCGAGTTCCCACGACGGCAGCCAGCACCCCTGACGCAGTC
281 E  S  V  E  E  V  V  R  V  P  T  T  A  A  S  T  P  D  A  V

901 GACAAGTACCTGGAGACCCCCGGAGATGAGAACGAGCACGCCCATTTCCAGAAAGCCAAA
301 D  K  Y  L  E  T  P  G  D  E  N  E  H  A  H  F  Q  K  A  K

961 GAGAGGTTGGAAGCCAAGCACCGAGAGAGAATGTCCCAGGTCATGAGAGAATGGGAGGAG
321 E  R  L  E  A  K  H  R  E  R  M  S  Q  V  M  R  E  W  E  E

1021 GCAGAACGTCAAGCCAAGAATTTGCCCAAAGCTGACAAGAAGGCCGTTATCCAGCATTTC
341 A  E  R  Q  A  K  N  L  P  K  A  D  K  K  A  V  I  Q  H  F

1081 CAGGAGAAAGTGGAATCTCTGGAACAGGAAGCAGCCAACGAGAGGCAGCAGCTTGTAGAG
361 Q  E  K  V  E  S  L  E  Q  E  A  A  N  E  R  Q  Q  L  V  E

1141 ACACACATGGCCAGAGTTGAAGCCATGCTCAATGATCGCCGTCGCCTGGCCCTCGAGAAT
381 T  H  M  A  R  V  E  A  M  L  N  D  R  R  L  A  L  E  N

1201 TACATCACCGCACTGCAGGCGGTGCCTCCAAGGCCTCATCATGTGTTCAACATGCTGAAG
401 Y  I  T  A  L  Q  A  V  P  P  R  P  H  H  V  F  N  M  L  K
                                      R
1261 AAGTACGTCCGTGCAGAGCAGAAGGACAGACAGCACACCCTAAAGCATTTTGAACATGTG
421 K  Y  V  R  A  E  Q  K  D  R  Q  H  T  L  K  H  F  E  H  V

1321 CGCATGGTGGACCCCAAGAAAGCTGCTCAGATCCGGTCCCAGGTTATGACACACCTCCGT
441 R  M  V  D  P  K  K  A  A  Q  I  R  S  Q  V  M  T  H  L  R

1381 GTGATCTACGAGCGCATGAACCAGTCTCTGTCCCTGCTCTACAACGTCCCTGCCGTGGCT
461 V  I  Y  E  R  M  N  Q  S  L  S  L  L  Y  N  V  P  A  V  A

1441 GAGGAGATTCAAGATGAAGTTGACGAGCTGCTTCAGAAGGAGCAGAACTACTCCGACGAC
481 E  E  I  Q  D  E  V  D  E  L  L  Q  K  E  Q  N  Y  S  D  D

1501 GTCTTAGCCAACATGATCAGTGAACCCAGAATCAGTTACGGCAACGATGCTCTCATGCCT
501 V  L  A  N  M  I  S  E  P  R  I  S  Y  G  N  D  A  L  M  P

1561 TCTTTGACTGAAACGAAGACCACTGTGGAGCTCCTTCCCGTGAATGGCGAATTCAGCCTG
521 S  L  T  E  T  K  T  T  V  E  L  L  P  V  N  G  E  F  S  L

1621 GATGATCTCCAACCGTGGCATCCTTTTGGGGTGGACTCTGTGCCAGCCAATACAGAAAAT
541 D  D  L  Q  P  W  H  P  F  G  V  D  S  V  P  A  N  T  E  N
                        S     A
1681 GAAGTTGAGCCTGTCGACGCCCGCCCCGCTGCTGACCGAGGACTGACCACTCGACCAGGG
561 E  V  E  P  V  D  A  R  P  A  A  D  R  G  L  T  T  R  P  G

1741 TCTGGGTTGACAAACATCAAGACAGAAGAGATCTCAGAAGTGAAGATGGATGCGGAGTTC
581 S  G  L  T  N  I  K  T  E  E  I  S  E  V  K  M  D  A  E  F

1801 GGACATGATTCAGGCTTCGAAGTCCGCCATCAAAAACTGGTGTTCTTTGCAGAAGATGTG
601 G  H  D  S  G  F  E  V  R  H  Q  K  L  V  F  F  A  E  D  V
     R           Y     H
1861 GGTTCAAACAAAGGTGCCATCATTGGACTCATGGTGGGTGGCGTTGTCATAGCAACAGTG
621 G  S  N  K  [hatched]                                  V  I  A  T  V

1921 ATTGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATCCATCATGGCGTG
641 [hatched]                 K  K  K  Q  Y  T  S  I  H  H  G  V

1981 GTGGAGGTTGACGCTGCTGTGACCCCGGAGGAGCGCCACCTCTCCAAGATGCAGCAGAAT
661 V  E  V  D  A  A  V  T  P  E  E  R  H  L  S  K  M  Q  Q  N

2041 GGATATGAGAATCCAACATACAAGTTCTTTGAGCAGATGCAGAACTAAACCCCGCCCCCC
681 G  Y  E  N  P  T  Y  K  F  F  E  Q  M  Q  N  *

2101 GCCACAGCAGCGGCCTCTGAACTTGACAGCAAAACCATTGCTTCACTACCCATCG.....
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Fig. 1. Nucleotide and deduced protein sequence of cloned cDNA for rat amyloidogenic glycoprotein. The coding sequence is shown together with 15 nt of 5′-(negative numbers) and 65 nt of 3′-untranslated sequence. The protein sequence (695 amino acid residues) is presented in the standard one-letter code below the nucleotide sequence. At positions where the rat and human sequences differ the human amino acid residue is indicated below the rat sequence. The hatched residues denote the presumptive transmembrane region (Kang *et al.*, 1987). The rat equivalent of the human amyloid A4 polypeptide is located between amino acid residues 598–640.

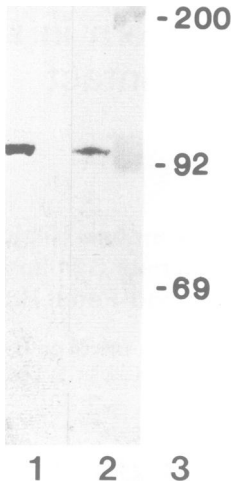

Fig. 2. Western blot mouse muscle (**lane 1**) and brain (**lane 2**) showing the existence of an immunoreactive 92 kd form of amyloidogenic glycoprotein. This staining pattern was not seen when the synthetic peptide, which had been conjugated to keyhole limpet hemocyanin and used to immunize a rabbit, was added to the primary antiserum. The protein standards are shown in **lane 3**.

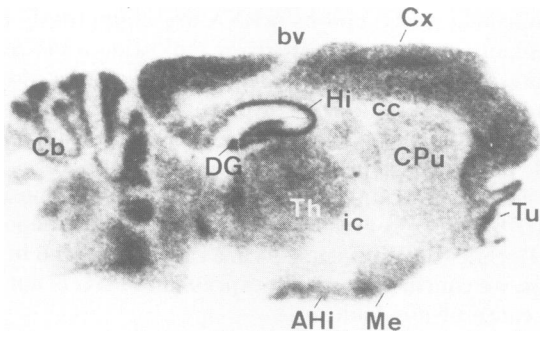

Fig. 3. Film autoradiogram of a parasagittal section of adult rat brain hybridized with ^{32}P-labelled rat amyloidogenic glycoprotein (rAG) cRNA and exposed to LKB ^{3}H-sensitive film for 9 days. Brain structures labelled on autoradiogram were identified following emulsion autoradiography and staining of this section with reference to a sagittal rat brain atlas (Paxinos and Watson, 1982). Abbreviations: AHi, amygdalohippocampal area; bv, blood vessel; CPu, caudate-putamen; Cb, cerebellum; Cx, cerebral cortex; cc, corpus callosum; DG, dentate gyrus; Hi, hippocampus; ic, internal capsule; Me, medial amygdaloid nucleus; Tu, olfactory tubercule; Th, thalamus.

of signal in the corpus callosum and internal capsule and cerebellar cortical white matter (Figure 3). Other non-neuronal brain cells showing little or no rAG mRNA content were the epithelial cells lining the ventricles and blood vessels and cells of the choroid plexus (not shown).

Recently in humans, an alternative form of amyloid mRNA containing a 168-bp insert has been discovered (Tanzi *et al.*, 1987a). If alternate forms exist in the rat, the cRNA probe used in this study would not distinguish between the two alternative forms described for hAG transcripts but hybridize to both mRNAs alike.

Brain distribution and cellular location of rAG immunoreactivity

Immunocytochemical localization of the rAG in rat brain was performed with a mouse polyclonal antiserum raised against a synthetic peptide of identical sequence in both rAG and

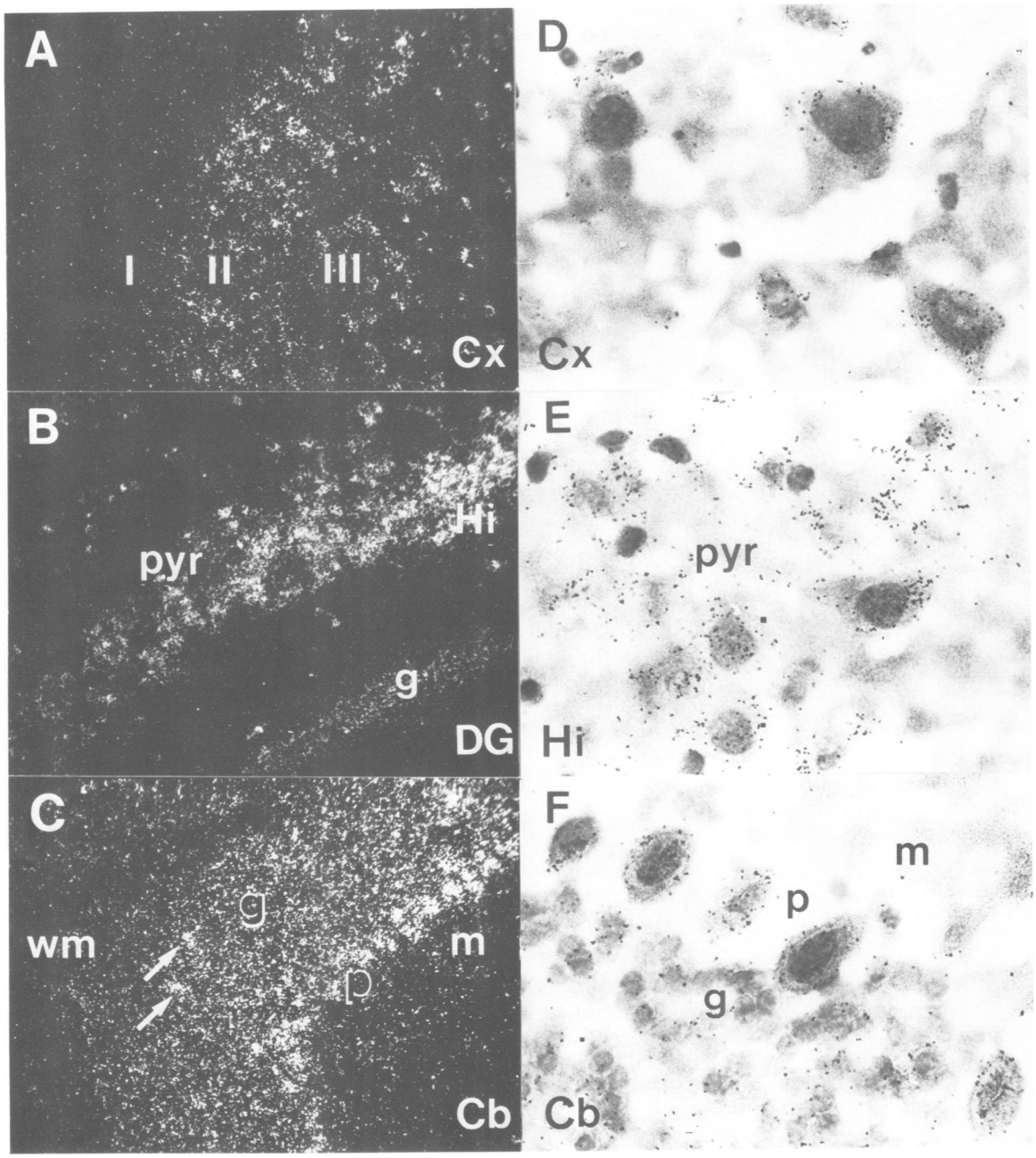

Fig. 4. Darkfield (**A**–**C**) and brightfield (**D**–**F**) photomicrographs of emulsion autoradiograms from three rat brain areas expressing high levels of rAG mRNA: cerebral cortex (**A** and **D**); hippocampal formation (**B** and **E**); and cerebellar cortex (**C** and **F**). Sections in (A–C) were hybridized with [^{32}P]cRNA and exposed for 8 days; sections in (D–F) were hybridized with [^{3}H]cRNA and exposed for 54 days. Cerebral cortex (Cx). Principal and interneurons in all cortical layers expressed rAG. In (A) only the first three layers are shown; no neurons, which are infrequent in layer I, appear in this example. Hippocampal formation. Pyramidal neurons (B,E) in all subdividions of the hippocampus (Hi); granule cells (B) of the dentate gyrus (DG); and the polymorphic cells (B) all contain rAG mRNA. Cerebellar cortex (Cx). The highest expression of rAG mRNA was found in Purkinje cells (C and F) and Golgi II neurons (C, arrows) followed by stellate cells, basket cells (not shown) and granule cells (C and F). The mRNA content per cell was, as in all brain areas, related to cell size. Magnification in (A–C) 215 × and in (D–F) 900 ×. Other abbreviations: g, granule cell layer; m, molecular cell layer; pyr, pyramidal cell layer; p, Purkinje cell layer; wm, white matter.

hAG (Materials and methods). The amount of immunoreactivity associated with cells in each brain region correlated with the mRNA content as predicted from the intensity of *in situ* hybridization signals and argues for its authenticity. Neuronal subtypes in cerebral cortex, hippocampus and cerebellar cortex were among the cells most highly decorated and were, therefore, chosen for illustration here (Figure 5).

The immunoreactivity had a patchy appearance and was frequently associated with cell body contours and sometimes proximal regions of dendrites, suggesting that the normal cellular location of this glycoprotein is on or near plasma membranes. These results do not exclude the possibility that rAG immunoreactivity also exists in another compartment, e.g. endosomes, lysosomes, Golgi or endoplasmic reticulum. However, the appearance of rAG immunoreactivity differed from that seen with antiserum recognizing lysosomes (data not shown).

In acetone-fixed tissue, the immunoreactive rAG deposits associated with neuronal cell bodies were punctate, more numerous and more evenly dispersed (Figure 6) than were

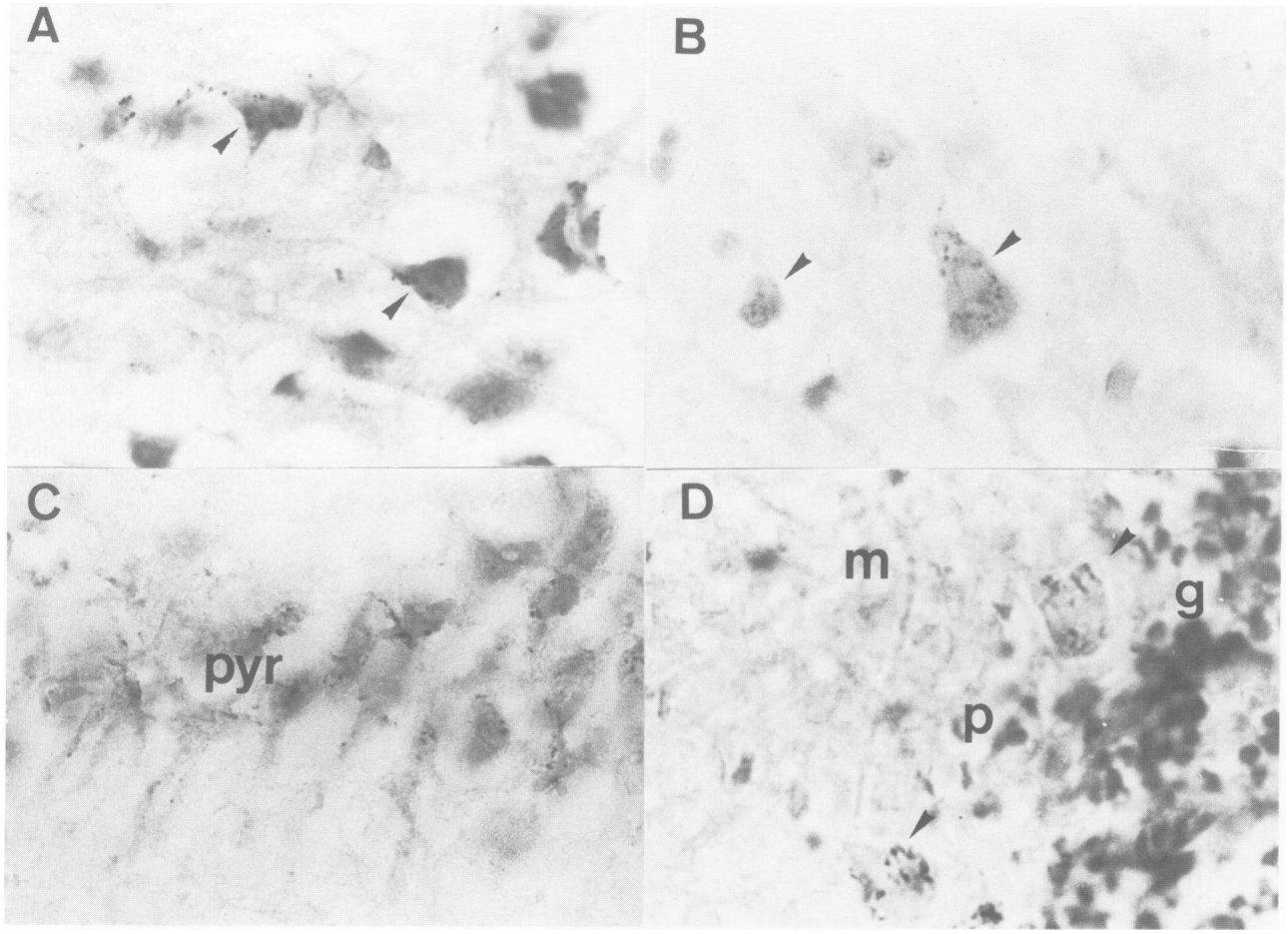

Fig. 5. In immunocytochemically stained sections of rat brain fixed with paraformaldehyde, the DAB reaction product appears as patches on or near the plasma membrane of neurons. Sections in (**A**, **B** and **D**) were counterstained with cresyl violet. Brain areas shown are cerebral cortex (**A** and **B**), hippocampus (**C**) and cerebellar cortex (**D**). Prominently labelled cells are indicated by arrowheads. A, C magnified 690×; B, D magnified 960×. Abbreviations as in Figure 4 legend.

the patches associated with paraformaldehyde-fixed cells (Figure 5). The reason for the different appearance of the immunoreactive rAG in acetone-fixed as compared with paraformaldehyde-fixed tissue is unknown but perhaps paraformaldehyde, a cross-linking fixative, aggregates the protein producing fewer but larger patches.

Discussion

The primary structure of rAG as predicted from the cloned cDNA displays an extraordinarily high degree of sequence identity to hAG (Figure 1), suggesting functional importance but also limited tolerance for structural changes. It may be that such changes increase the propensity for amyloid fibril formation by facilitating release of the A4 polypeptide from its precursor. It is not known if the few substitutions in rAG hinder the formation of the A4 polypeptide. However, a synthetic rat A4 sequence containing the three amino acid substitutions in which the rat peptide differs from the human forms fibrillary structures *in vitro* just as readily as the human sequence (C.Hilbich, P.Gibson and K.Beyreuther, unpublished).

In determining expression levels of rAG our results, in agreement with those of others (Bahmanyar *et al.*, 1987; Goedert, 1987), clearly show that rAG mRNA is abundant and ubiquitous in rat brain (Figures 3 and 4). The mRNA occurs, if not exclusively, certainly in the highest amounts in neurons. This indicates that the majority of the amyloid found extracellularly in neuritic plaques and associated with cerebral vasculature in AD, Down's Syndrome and aged mammals (Price *et al.*, 1986) is neuronal in origin. Importantly, there is differential expression of rAG mRNA in different brain regions. Highest expression levels were not confined to those areas which typically contain amyloid deposits in afflicted humans. This strongly suggests high expression by itself does not cause amyloidosis and corroborates similar conclusions recently reached from inspecting monkey and normal human and Alzheimer brains (Bahmanyar *et al.*, 1987; Goedert, 1987). This finding also indicates that factors common to amyloid-containing regions initiate fibril formation and represent the more proximal cause of amyloidosis. Such a concept is in accordance with the lack of evidence for a hAG gene duplication in sporadically occurring AD (Tanzi *et al.*, 1987a), and the lack of concordance concerning the chromosomal positions of the genes for familial AD and AG (Tanzi *et al.*, 1987c; Van Broeckhoven *et al.*, 1987).

The phylogenetic conservation as well as the abundance and distribution in brain presage an important role for the AG protein in neurons. Our immunocytochemical studies

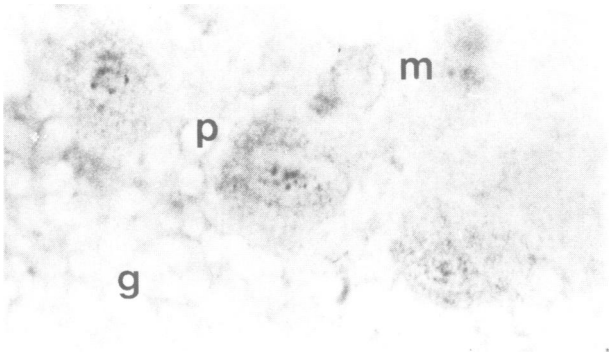

Fig. 6. Immunocytochemically stained rat cerebellar cortex fixed in acetone:alcohol (1:1) after cryosectioning. The section was not counterstained. The DAB reaction product was finer and more dispersed over cell bodies than was seen in paraformaldehyde-fixed tissue (Figure 5). It was not possible in the acetone:alcohol fixed tissue to determine the relation of the reaction product to the plasma membrane. Magnified 830×. Abbreviations as in Figure 4 legend.

revealed a patchy and punctate appearance of rAG suggesting that the protein occurs in aggregates, a state perhaps promoted by the unique properties of its transmembrane segment. Our provisional location of this protein on or near the neuronal plasma membrane is consistent with the notion that AG constitutes an integral membrane protein but ultrastructural studies are needed for confirmation. While many possible functions for integral membrane proteins exist, the patchy appearance of AG suggested to us a function for rAG in cell−cell or cell−matrix interactions, perhaps in the recognition, formation or stabilization of such contacts.

How then is this proposed function related to the pathological formation of amyloid deposits in AD and aged individuals? Amyloid deposition may be an indirect consequence of the location of AG in the cell membrane and the aggregatory properties of the A4 polypeptide—a product of AG breakdown. The location of AG in the plasma membrane renders the protein vulnerable to extracellular proteases whose attack could be eventuated by injury to neuronal membranes in certain neurodegenerative diseases. During the course of the present studies, we noted that the amount of the ageing pigment, lipofuscin, in neurons correlated with rAG mRNA content. Since the occurrence of lipofuscin is related to free-radical injury and lipid peroxidation (Contron, 1987), it is tempting to speculate that the metabolism of certain chemical messengers or the processes which form and maintain functional cell contacts generate free-oxygen radicals. Certainly, brain areas where plasticity is thought to underlie cognitive functions are primary sites for amyloid deposition. In longer-lived mammals, then, the mechanisms protecting against free-radical injury may fail, and account for amyloid deposits in some aged mammalian brains (humans, monkeys, dogs, polar bears) (Selkoe *et al.*, 1987).

An important neuropathology associated with AD is the paired helical filaments (PHF) found in neurofibrillary tangles (NFT). Although the association of A4 amyloid with PHF (Masters *et al.*, 1985) is controversial, there is agreement that the NFT contain ubiquitin (Mori *et al.*, 1987). The accrued evidence, though circumstantial, supports the concept that ubiquitinated NFT signal the location of injured cells whose proteolytic systems are overburdened, and the amyloid deposits are the legacy of dead neurons.

Materials and methods

rAG cDNA isolation

A rat forebrain cDNA library constructed in λ gt10 was screened with a ^{32}P-labelled oligodeoxynucleotide encoding the N-terminal 17 residues of the human amyloid polypeptide (5′ GATGCTGAGTTCCGTCATGAC-TCTGGCTATGAGGTTCATCATCAGAAACTG 3′). Approximately one in 5000 clones hybridized with the probe. Eight isolates were analysed and found to be derived from an identical mRNA sequence.

Western blot analysis

Mouse brain or muscle (20% w/v) was homogenized in 5 M urea, 5% β-mercaptoethanol, 2.5% SDS, 0.1 mM PMSF in a Dounce homogenizer, sonicated (2 × 10 s, Branson Sonifier, Microtip), incubated at 50°C for 15 min and centrifuged for 10 min at 10 000 *g*. The homogenates (5−20 μl) were subjected to electrophoresis (Laemmli, 1970). Proteins were separated on a 10% polyacrylamide gel and transferred to nitrocellulose (Towbin *et al.*, 1979). Blots were blocked in 100 mM Tris−HCl, pH 7.4, 150 mM EDTA, 0.25% gelatin, reacted with a rabbit antiserum diluted 1:3000, washed and developed with alkaline-phosphatase-conjugated second antibody (Promega). The antiserum was generated against a synthetic peptide conjugated to keyhole limpet hemocyanin. The peptide sequence was from the predicted cytoplasmic domain (CT II, amino acids 666−676). This antiserum was not useful for immunocytochemical studies.

In situ hybridization

An *Eco*RI-generated cDNA fragment of 1.1 kb was inserted in both orientations into the plasmid vector pSP65. Following plasmid linearization radiolabelled antisense RNA (cRNA) or sense RNA (mRNA) was synthesized using SP6 RNA polymerase in the presence of one radiolabelled nucleotide to yield RNA of sp. act. 6 × 10^8 c.p.m./μg (500 μM each of ATP, GTP and CTP; 12.5 μM [^{32}P]αUTP, Amersham, sp. act. 3000 Ci/mmol). Tritiated cRNA was also made as described above except that 20 μM tritiated UTP ([5,6-^{3}H]uridine 5′-triphosphate; 52 Ci/mmol, Amersham) was used instead of [^{32}P]αUTP. Sp. act. of the [^{3}H]cRNA was about 10^7 c.p.m./μg.

Tissue for *in situ* hybridizations was obtained from five adult male rats euthanized with gaseous CO_2 and then decapitated. Brains were frozen using powdered dry ice. Frozen sections (10 μm) were thaw-mounted onto slides coated with poly-L-lysine, fixed in 3% neutral-buffered paraformaldehyde containing 0.02% DEP, and stored frozen at −80°C. Details of the hybridization procedure were published by Shivers *et al.* (1986), except that more stringent washing conditions were employed here (two 15-min washes in 0.1 × SSC, 0.05% inorganic sodium pyrophosphate at 50°C). Each section received 30 μl containing either 100 000 c.p.m. of [^{32}P]-cRNA, 100 000 c.p.m. of [^{32}P]mRNA, 50 000 c.p.m. of [^{3}H]cRNA or probe diluent alone (as a positive chemography control). No hybridization signal was observed in sections pretreated with RNase A (20 μg/1 ml) for 30 min at 30°C, or hybridized with labelled mRNA. For emulsion autoradiography the sections were dipped in Kodak NTB2 emulsion and following autoradiogram development, the sections were stained with 1% fast green and 0.5% cresyl violet.

Immunocytochemistry

A synthetic peptide was conjugated to keyhole limpet hemocyanin and used to immunize a mouse. The peptide sequence was from the predicted extracellular domain (Alz 2, amino acids 361−371). Best results were obtained with a 1:2500 dilution of antiserum. Immunocytochemistry was performed on 25-μm cryostat sections (without further permeabilization) of rat brain obtained from adult male rat perfused with 3% neutral-buffered paraformaldehyde and cryoprotected with sucrose. An anti-mouse avidin-biotinylated horseradish peroxidase (ABC) kit was used according to supplier's recommendations (Vector Burlingame, CA) and the HRP visualized with 0.05% diaminobenzidine and 0.001% H_2O_2 (60 min). Substitution of primary antiserum with normal mouse serum produced no staining pattern; preabsorption of primary antisera with 10 μM peptide nearly eliminated the immunoreactive patches associated with cells. In addition, mouse Alz 2 antiserum recognized the gene product (M_r 92 kd) of the human AG gene transfected into mammalian cells (A.Weidemann and K.Beyreuther, unpublished observations). In one case, unfixed frozen rat brain was sectioned at 25 μm and the sections post-fixed for 2 min in acetone:ethanol (1:1) followed by dehydration and the standard immunocytochemical procedure for detection of immunoreactive rAG.

Acknowledgements

We thank Klaus Schenck and Heinz Horstmann for expert technical assistance, Matthias Cramer for preparing and characterizing the antiserum, and Jutta Rami and Ina Nonnenmacher for word processing. This work was supported by grants from the Bundesministerium für Forschung und Technologie, Thyssen Stiftung and the Fonds der Chemischen Industrie.

References

Bahmanyar,S., Higgins,G.A., Goldgaber,D., Lewis,D.A., Morrison,J.H., Wilson,M.C., Shankar,S.K. and Gajdusek,D.C. (1987) *Science*, **237**, 77–80.

Contron,R.S. (1987) In Robbins,S.L. and Kumar,V. (eds), *Basic Pathology*. W.B.Saunders Co., Philadelphia, p. 21.

Delabar,J.M., Goldgaber,D., Lamour,Y., Nicole,A., Huret,J., De Grouchy, J., Brown,P., Gajdusek,D.C. and Sinet,P.M. (1987) *Science*, **235**, 1390–1392.

Glenner,G.G. and Wong,C.W. (1984) *Biochem. Biophys. Res. Commun.*, **122**, 1131–1135.

Goedert,M. (1987) *EMBO J.*, **6**, 3627–3632.

Goldgaber,D., Lerman,M.I., McBridge,O.W., Saffiotti,U. and Gajdusek, D.C. (1987) *Science*, **235**, 877–880.

Kang,J., Lemaire,H.G., Unterbeck,A., Salbaum,J.M., Masters,C.L., Grzeschik,K.H., Multhaup,G., Beyreuther,K. and Müller-Hill,B. (1987) *Nature*, **325**, 733–736.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Masters,C.L., Multhaup,G., Simms,G., Pottgiesser,J., Martins,R.N. and Beyreuther,K. (1985) *EMBO J.*, **4**, 2757–2763.

Mori,H., Kondo,J. and Ihara,Y. (1987) *Science*, **235**, 1641–1644.

Paxinos,G. and Watson,C. (1982) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, New York.

Price,D.L., Whitehouse,P.J. and Strubles,R.G. (1986) *Trends Neurosci.*, **9**, 29–33.

Selkoe,D.J. (1987) *Trends Neurosci.*, **10**, 181–184.

Selkoe,D.J., Bells,D.S., Podlisny,M.B., Price,D.L. and Cork,L.C. (1987) *Science*, **235**, 873–877.

Shivers,B.D., Schachter,B.S. and Pfaff,D.W. (1986) *Methods Enzymol.*, **124**, 497–510.

Shivers,B., Hilbich,C., Beyreuther,K. and Seeburg,P.H. (1987) *Abstracts of the Society for Neuroscience*, **13**, 819.

Tanzi,R.E., Bird,E.D. and Reve,R.L. (1987a) *Abstracts of the Society for Neuroscience*, **13**, 818.

Tanzi,R.E., Gusella,J.F., Watkins,P.C., Burns,G.A.P., St George-Hyslop, P., Van Keuren,M.L., Patterson,D., Pagan,S., Kurnit,D.M. and Neve, R.L. (1987b) *Science*, **235**, 885–890.

Tanzi,R.E., St George-Hyslop,P.H., Haines,J.L., Polinsky,R.J., Nee,L., Foncin,J.-F., Neue,R.L., McClatchey,A.I., Coneally,P.M. and Gusella, J.F. (1987c) *Nature*, **329**, 156–157.

Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.

Van Broeckhoven,C., Genthe,A.M., Vandenberghe,A., Horsthemke,B., Bachhovens,H., Raeymackers,P., Van Hut,W., Wehnert,A., Gheuens,J., Cras,P., Bruyland,M., Martin,J.J., Salbaum,M., Multhaup,G., Masters, C.L., Beyreuther,K., Gurling,H.M.D., Mullan,M.J., Holland,A., Barton, A., Irving,N., Williamson,R., Richards,S.J. and Hardy,J.A. (1987) *Nature*, **329**, 153–155.

Vaughan,D.W. and Peters,A. (1981) *J. Neuropathol. Exp. Neurol.*, **40**, 472–487.

Zimmermann,K., Herget,T., Salbaum,J.M., Schubert,W., Hilbich,C., Cramer,M., Masters,C.L., Beyreuther,K. and Starzinski-Powitz,A. (1988) *EMBO J.*, **7**, 367–372.

Received on January 7, 1988; revised on February 11, 1988