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Widespread Expression of Amyloid Beta-Protein Precursor Gene in Rat Brain

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The neuritic plaque is a characteristic finding in Alzheimer's disease. A major component of the plaque core is a 4.2 kd polypeptide, amyloid beta-protein (ABP), which is derived from the C-terminus of a larger precursor protein (ABPP). The authors have studied the transcription of ABPP mRNA in the adult rat brain by Northern analysis and* in situ *hybridization, and report that the ABPP gene gives rise to essentially the same multitranscript family of mRNAs as in the human, and that differential transcription patterns exist between brain and kidney. Morphologically, ABPP mRNA is expressed ubiquitously in neurons of the fore and hindbrain. ABPP transcripts also are present less frequently in occasional glial cells and at moderate to low frequency in nonneural cell types, namely, the choroid plexus epithelium, ependymal cells, and leptomeningeal membranes. Neuronal transcripts are most abundant in cerebral cortical layers II and V, the pyramidal cell layer of the hippocampus, the olfactory cortex, nucleus basis pontis, cranial nerve nuclei, and, significantly, in Purkinje cells and cerebellar granule cells. Because the cerebellum is relatively uninvolved in Alzheimer's disease, these findings suggest that high intraneuronal expression of ABPP may be a necessary but not sufficient requirement for plaque formation. (Am J Pathol 1989, 134:1253–1261)

A 4.2 kd polypeptide, referred to as amyloid beta-protein (ABP) or A4 protein, is the major component of the amyloid cores of neuritic plaques (NP) and of cerebrovascular amyloid in Alzheimer's disease (AD), Down's syndrome, and the aging brain.[1-7] ABP is a 40 to 42 amino acid polypeptide that arises from the C-terminus of a larger precursor protein (ABPP) consisting of 695 to 770 amino acids.[8-11] Although the function of ABPP has not yet been determined, it has the characteristics of a transmembrane protein[8] and may play a role in cell contact,[12] or may undergo processing to release a peptide ligand.[13] The gene for ABPP is located on the long arm of chromosome 21,[8-11] is highly conserved among species,[9,11] and is expressed in a wide variety of tissues in human and other species.[9,10] The ABPP gene is not linked to the locus for familial AD[14,15] and, contrary to initial reports,[16,17] is not duplicated in either sporadic or familial AD.[18-20]

The factors responsible for the excessive accumulation of ABP in AD and its relationship to the larger precursor species have not yet been determined. The human ABPP gene generates a family of mRNA transcripts by differential splicing.[21-23] Some forms of the ABPP transcript contain an additional exon that encodes a protease inhibitor domain.[21-23] Another serum protease inhibitor, alpha-1 antichymotrypsin, is a component of NP and cerebrovascular amyloid.[24] Although it has been suggested that antiprotease activity may be relevant to the mechanism of ABP fibril deposition in AD,[21-24] at present this is speculative.

An understanding of the molecular basis for the excessive accumulation of ABP in AD requires precise knowledge of the cell populations expressing the ABPP gene. In humans, selective transcription of the ABPP gene in subsets of neurons has been reported;[25-28] however, these studies have been restricted to specific brain regions such as the hippocampus or neocortex. Therefore, to define the extent of ABPP expression in the mammalian brain, we performed a survey of the entire adult rat brain by the technique of *in situ* hybridization. We report a widespread distribution of ABPP mRNA in both neural and non-neural structures, including neuronal expression at high levels in regions that, in AD, are not major sites of NP formation.

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Materials and Methods

Reagents

Restriction enzymes were from New England Biolabs (Beverly, MA); placental RNAse inhibitor (RNAsin) and T_7 polymerase were from Promega Biotec (Madison, WI; Bluescribe M13− plasmid was from Stratagene (La Jolla, CA); (alpha-[^{35}S]thio)-UTP and (alpha-^{32}p)-dATP were from New England Nuclear (Boston, MA); OTC embedding compound was from Miles Inc.; NTB_2 nuclear track emulsion, D19 developer, fixer and XAR x-ray film were from Kodak (Rochester, NY); ultrapure DNAse was from Cooper Biomedical (Westchester, PA); ultrapure sucrose, random primed DNA labeling kit, and unlabeled NTPs were from Boehringer Mannheim (Indianapolis, IA); guanidine isothiocyanate was from BRL (Gaithersberg, MD); all other reagents were from Sigma Chemical Co (St. Louis, MO). The human fetal muscle cDNA library was a gift of F. S. Walsh (The National Hospital, Queen Square, London).

Preparation of Probes

We have reported previously the isolation of a cDNA clone encoding human ABPP.[29] For Northern blotting, ^{32}p-labeled probes were prepared by the random-primer method[30] to a specific activity of approx 1×10^9 cpm/μg DNA, using as template the 1.0 kb *Eco*RI-*Eco*RI fragment (corresponding to nucleotides 1796 to 2851 of the ABPP sequence of Kang et al)[8] or a 212 by *Taq*I-*Ava*I fragment (corresponding to nucleotides 862 to 1073 of the longer ABPP sequence reported by Kitaguchi et al).[23] For *in situ* hybridization, the 1.0 kb *Eco*RI-*Eco*RI fragment was subcloned into the polylinker *Eco*RI site of Bluescribe M13− vector in the sense and anti-sense orientations. One microgram of plasmid DNA was linearized with *Bam*HI, and single-stranded cRNA probes were prepared according to the manufacturer's specifications in a 20 μl reaction containing 150 pmol ^{35}S-UTP, 500 pmol each of GTP, CTP and ATP, 20 units of RNAsin, and 20 units of T_7 polymerase, to a specific activity of about 1 to 2×10^8 cpm per μg DNA template. After transcription, template DNA was removed by treatment with 20 μg/ml ultrapure DNAse. ^{35}S-labeled transcripts were extracted with phenol/chloroform and precipitated with ethanol.

Northern Analysis

Immediately after the rats were killed, rat organs were removed and frozen rapidly in liquid nitrogen. Total RNA was extracted by the guanidine-cesium chloride procedure.[31] Ten micrograms of total RNA from each organ was electrophoresed through a 1% agarose-formaldehyde gel[32] and transferred to nitrocellulose filters in 20× SSC (20× SSC = 3M NaCl + 0.3M Na citrate) overnight.[33] The filter was prehybridized at 65 C for 3 hours in 50% formamide, 5× SSC, 10× Denhardt's solution (1× Denhardt's = 0.02% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 0.1% SDS, 20 mM Na phosphate, and 250 μg/ml sonicated denatured salmon sperm DNA, and hybridized overnight at 42 C in the same solution containing 5×10^6 cpm/ml denatured probe. The following day the filter was washed three times for 10 minutes each at room temperature in 2× SSC and 0.1% SDS and once for 30 minutes at 50 C in 0.5× SSC and 0.1% SDS.

Tissue Preparation

Adult male Sprague-Dawley rats were killed by intraperitoneal injection of a lethal dose of pentobarbital, rapidly thoracotamized, and the systemic circulation perfused by intra-aortic administration of 4% paraformaldehyde in sterile phosphate-buffered saline (PBS), pH 7.4, at 4 C for 20 minutes, with exit through the right atrium. Whole brains, livers, kidneys, and spleens were removed and immersed in the same fixative at 4 C overnight, after which they were immersed in 15% sucrose solution in sterile PBS for 24 hours. Tissue blocks were sectioned at 8 to 10 μ on a cryostat at −20 C and sections taken up onto polylysine-coated glass slides. Slides were stored at −70 C in the presence of a dessicant until use.

In Situ Hybridization

We used published methods[34,35] with minor modifications. Before hybridization, slides underwent further fixation by exposure to formaldehyde vapors and were then treated for 10 minutes with 5 μg/ml proteinase K at room temperature. After washing in 2× SSC, sections were then prehybridized with a solution containing 50% formamide, 600 mM NaCl, 10 mM TRIS (pH 7.5), 1 mM EDTA, 0.12% polyvinylpyrrolidone, 0.12% Ficoll, 0.6% bovine serum albumin, 0.5% denatured salmon sperm DNA, 0.5 mg/ml total yeast RNA, and 50 μg/ml yeast tRNA at 50 C for 2 hours. Sections were then hybridized overnight at 50 C with the same solution containing 10 mM DTT, 10% dextran sulfate, and the ^{35}S-labeled cRNA probe (final activity, 5×10^6 cpm per ml). The following day sections were washed in 2× SSC, treated with 60 μg/ml RNAse A at 37 C for 45 minutes, and washed in 2× SSC for 60 minutes at 50 C, followed by 0.5× SSC for 3 hours at 50 C. Sections were then dehydrated, exposed to x-ray film for 10 to 14 days, dipped in NTB_2 emulsion,

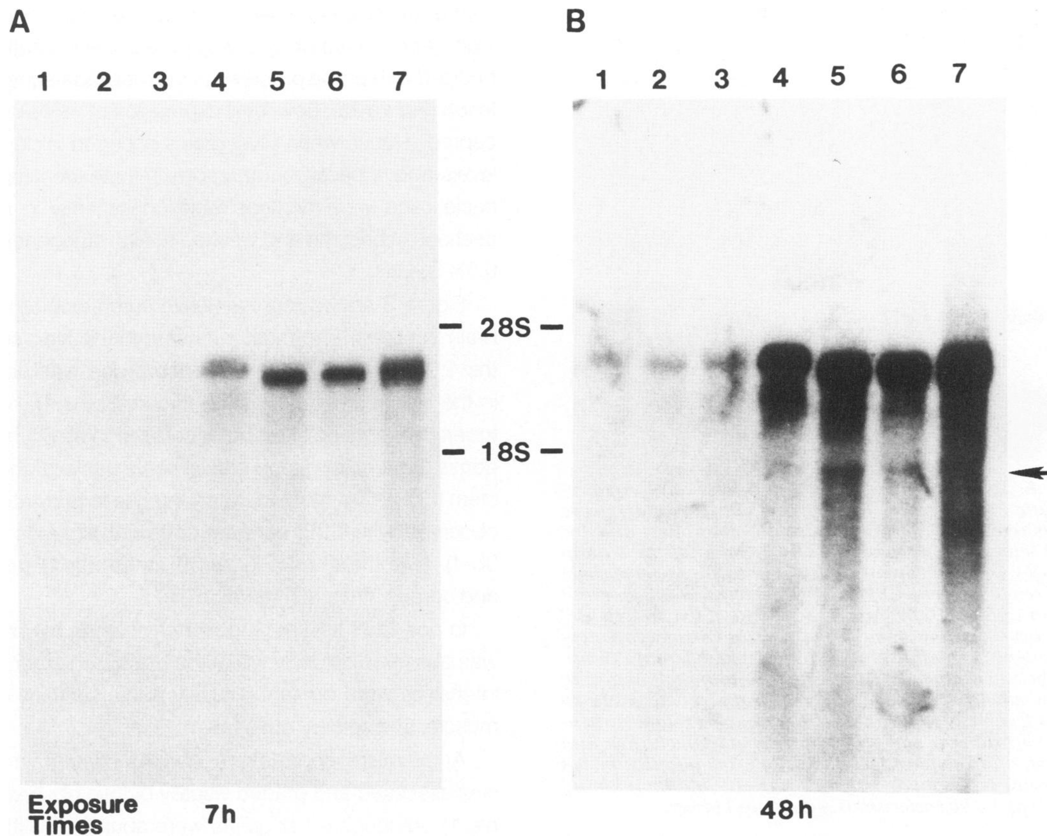

Figure 1. *Ten micrograms of total RNA from rat spleen (lane 1), skeletal muscle (lane 2), liver (lane 3), kidney (lane 4), hindbrain (cerebellum + pons-medulla, lane 5) and forebrain (lane 6), and 2.5 μg of poly(A)$^+$ RNA from adult human skeletal muscle (lane 7), were separated on a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a 1.0-kb* EcoRI *fragment derived from the 3'-portion of the human ABPP cDNA. A: after 7-hours exposure at −70 C. Differences in sizes and levels of ABPP mRNA among tissues are evident. Note the relative thickness of the hybridizing bands. B: The same filter after 48-hours exposure at −70 C. Faint hybridizing bands are now detected in lanes 1 to 3, and an additional 1.8 kb hybridizing band is detected in lanes 4 to 7 (arrow).*

and stored in the dark for 4 to 6 weeks. After development in D19 developer, slides were counterstained with hematoxylin and eosin (H & E), mounted, and viewed under combined brightfield/epipolarized light illumination on a Leitz Orthoplan microscope.

Results

Northern Analysis

Under stringent Northern hybridization conditions, the human ABPP 1.0 kb *Eco*RI fragment recognized mRNA species in all of the rat tissues studied (Figures 1, 2A). For equal amounts of total RNA loaded, the most intense hybridization was present in the fore and hindbrain (Figure 1A, lanes 5 and 6); moderately intense hybridization was present in the kidney (Figure 1A, lane 4) and, after longer exposures, faint hybridization was detected in spleen, muscle, and liver homogenates (Figure 1B, lanes 1, 2 and 3, respectively).

Notably, the size of the detected mRNA species varied slightly among tissues (Figures 1A, 2A). Rat spleen, muscle, liver, and kidney appeared to contain a larger species, predominantly, migrating at about 3.7 kb, whereas the fore and hindbrain homogenates appeared to contain slightly smaller mRNA species (3.5 to 3.6 kb). A similar heterogeneity has been described in human tissues.[21–23]

To clarify further whether the rat ABPP gene generates a multitranscript family similar to that found in the human,[21–23] we performed Northern analysis using as probe the 212 bp *Taq*I- *Ava*I restriction fragment of the human ABPP cDNA, containing the two "additional" exon sequences specifying the Kunitz-type protease inhibitor domain (Figure 2). Our results indicate that rat cortex, cerebellum, and kidney contain a cross-hybridizing mRNA species migrating at 3.7 kb (Figure 2B), suggesting

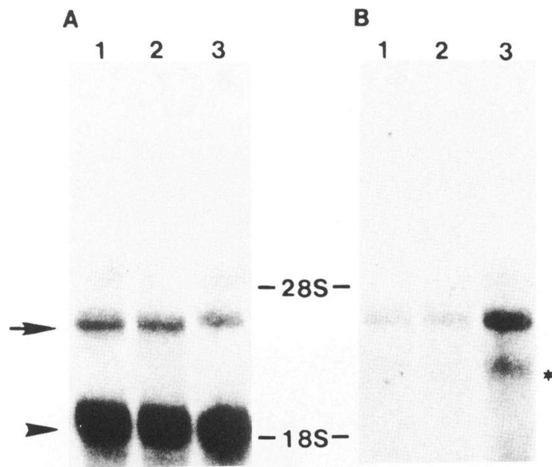

Figure 2. A. *Ten micrograms of total RNA from adult rat forebrain (lane 1), hindbrain (lane 2), and kidney (lane 3) were electrophoresed, transferred to nitrocellulose filter, and hybridized with a 1.0-kb* EcoRI-EcoRI *fragment of the human ABPP cDNA and with human beta-actin cDNA. Note the slight size difference between ABPP mRNA detected in the fore and hindbrain (3.5 to 3.6 kb, arrow) and that of the kidney (3.7 kb). The hybridization signal for beta-actin (arrowhead) is of similar intensity in all tissues and shows no difference in migration between lanes.* B: *After exposure to x-ray film for 18 hours, the filter was stripped and rehybridized with a 212-bp* TaqI-AvaI *fragment of the ABPP cDNA. Note the relative intensity of the hybridizing band in kidney compared with fore and hindbrain, and compared with panel A. Note also that mRNA in all tissues is of similar size (3.7 kb). An additional band migrating at 2.9 kb (asterisk) is seen in the kidney.*

strongly that, in these tissues, the rat ABPP primary transcript is processed as it is in the human. The relative proportion of the mRNA recognized by the 212 bp probe (containing the Kunitz-type domain) to mRNA recognized by the 1.0 kb probe (common to all transcripts) was considerably greater in the kidney than in the brain, suggesting that there is a regional variation in the pattern of transcript splicing.

After longer exposure of the Northern blots shown in Figure 1 (48 hours), an additional 1.8 kb RNA species was detected with the 1.0 kb probe in homogenates of human muscle, rat fore and hindbrain, and rat kidney (Figure 1B). A similar finding has been reported by others.[26] Furthermore, an additional band, migrating at 2.9 kb, was detected with the 212 bp probe in kidney homogenates but not in brain (Figure 2B). The significance of these additional mRNA species is presently unclear.

In Situ Hybridization

Sections prepared at 50-μ intervals from the entire adult rat brain were examined. Immediately adjacent sections were hybridized with probes in the message and anti-message-sense orientations, respectively. Specificity of hybridization was evident from the fact that virtually no signal above background was detectable in sections hybridized with probe prepared in the message-sense orientation (Figure 3a). Low-level expression of mRNA was accepted as such when silver grains occurred in clusters, at levels above background, in proximity to identifiable cell nuclei, and were demonstrable consistently in multiple sections hybridized and washed at high stringency (50 C, 0.5× SSC).

Figure 3 shows representative autoradiograms of rat brain sections. The most intense hybridization signal to the 1.0 kb *Eco*RI ABPP antisense probe was observed in the hippocampal formation (Figure 3b, e, f), olfactory tubercle (Figure 3c), cerebellar cortex (Figure 3g, h), basis pontis (Figure 3b, g) and cranial nerve nuclei of the brainstem (Figure 3g, h). Moderately intense hybridization was observed within the cerebral cortex of all lobes (Figure 3b–f), hypothalamus (Figure 3b), thalamus (Figure 3b), and corpus striatum (Figure 3b, d).

In non-CNS tissues, moderately intense hybridization was observed diffusely within the kidney, and much lower intensities were present diffusely within sections of liver, muscle, and spleen (not shown).

At the microscopic level (Figure 4), silver grain density was assessed and graded visually by two observers (Table 1). Although silver grains were abundant within neurons throughout the brain, the greatest concentrations were present within large neurons of the cerebral cortex (layers II and V) (Figure 4a), hippocampal pyramidal neurons (sectors CA1 through CA4) (Figure 4b), neurons of the olfactory cortex (not shown), Purkinje cells (Figure 4c), neurons of the basis pontis, and neurons of cranial nerve nuclei (Figure 4e). Grain density was assessed as moderately intense in the following structures: neurons of cortical layers III, IV, and VI (Figure 4a); granule cells of the hippocampal dentate fascia (Figure 4b); cerebellar granule cells (Figure 4c), neurons of the amygdaloid and caudate nuclei, thalamus, hypothalamus (Figure 4d), parahippocampal gyrus and raphe nuclei, and choroid plexus epithelial cells (Figure 4g).

In addition, silver grains were present at low density within selected oligodendrocytes (Figure 4f), astrocytes, leptomeninges, and ependymal cells, but not in endothelial cells or smooth muscle of the subarachnoid vasculature (Figure 4h). Oligodendrocytes were identified in the cerebral subcortical white matter as cells with round nuclei and scant cytoplasm aligned in tandem arrays along white matter tracts. Hybridization-positive oligodendrocytes were situated preferentially in the hippocampal white matter, and were not detected in the hindbrain. The basal leptomeninges were more heavily labeled than the meninges over the hemispheres, and labeling of the cho-

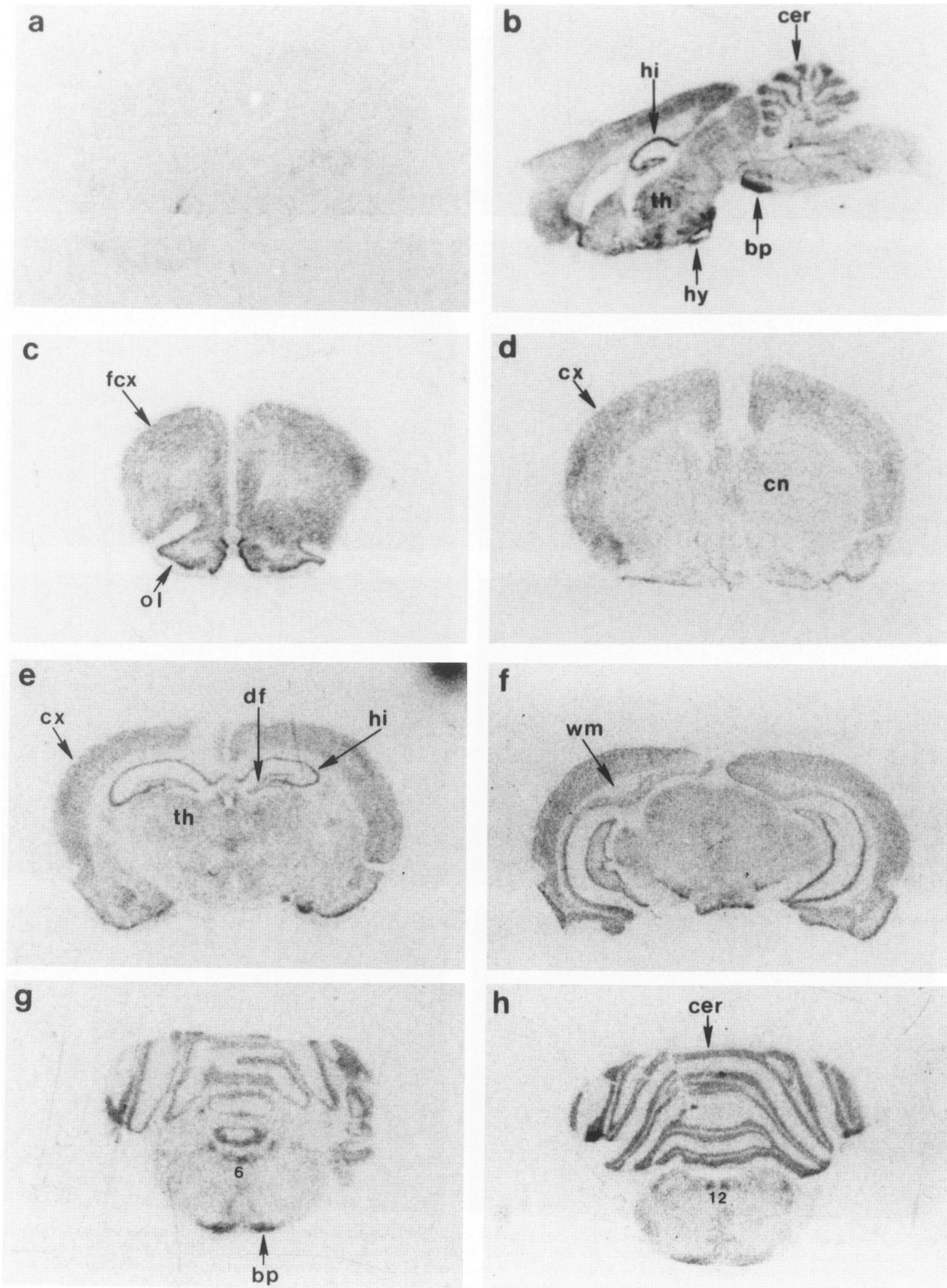

Figure 3. *Autoradiograms of coronal* (a, c to h) *and parasagittal* (b) *rat brain sections hybridized with* ^{35}S*-labeled cRNA probes of the ABPP cDNA 1.0 kb* EcoRI *fragment in the sense (a) and antisense (b to h) orientations. Exposure time, 10 to 14 days. Level of sections: a and e: thalamus; c: olfactory tubercle; d: caudate nucleus; f: lateral geniculate body; g: midpons; h: pontomedullary junction. bp, basis pontis; cx, parietal cortex; cer, cerebellum; cn, caudate nucleus; df, dentate fascia; fcx, frontal cortex; hi, pyramidal cell layer of hippocampal formation; hy, hypothalamus; ol, olfactory cortex; th, thalamus; wm, subcortcial white matter. Numerals 6 and 12 refer to the corresponding cranial nerve nuclei.*

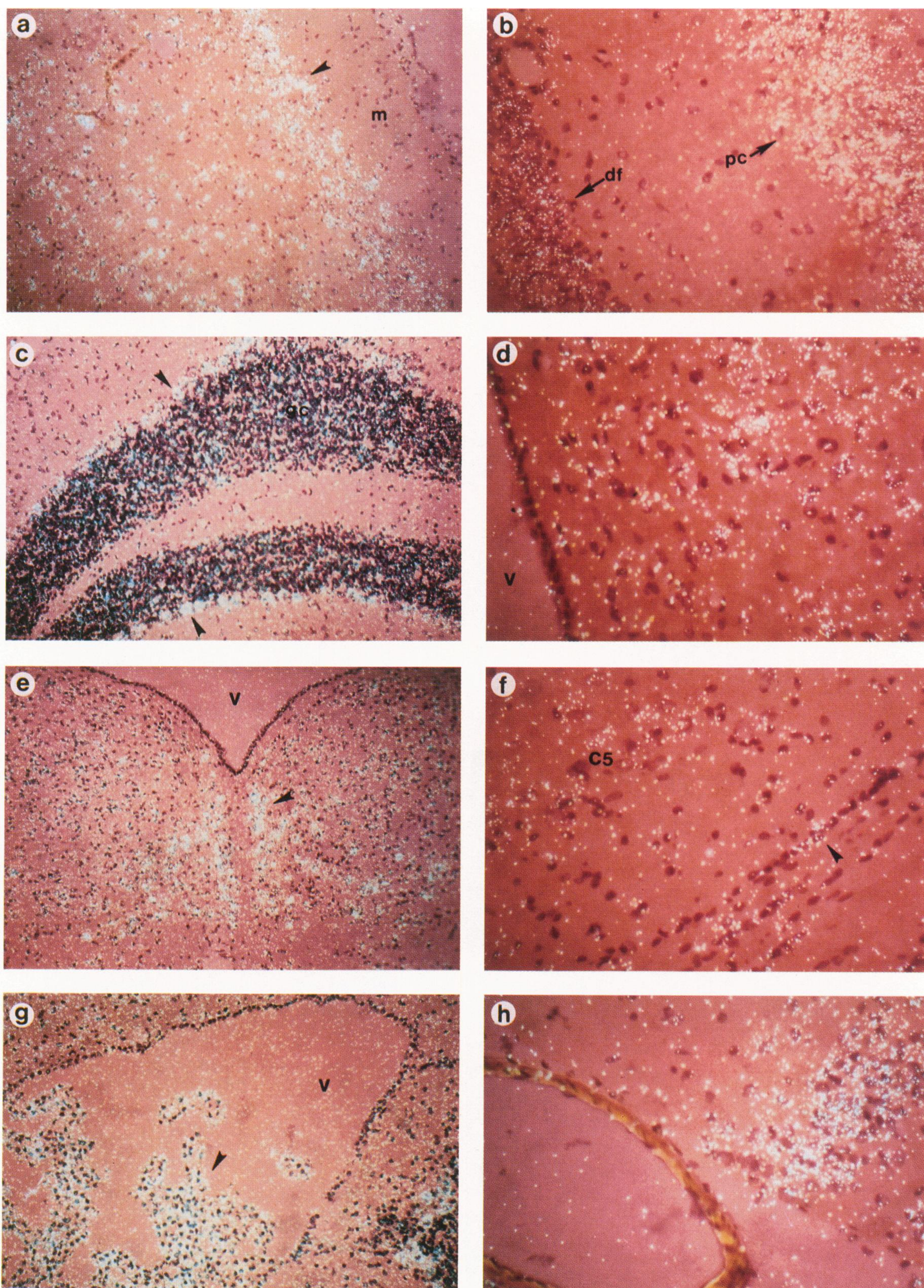

Figure 4. *Photomicrographs of tissue sections after hybridization with ^{35}S-RNA anti-sense probe of the ABPP cDNA 1.0 kb* EcoRI *fragment and exposure to nuclear track emulsion for 4 to 6 weeks. Hybridization to ABPP mRNA is visualized as silver-blue grains by combined brightfield/epipolarized light microscopy (Leitz) (counterstain, H & E).* **a**: *Parietal cortex. Arrowhead, cortical later II; m, molecular layer.* **b**: *Hippocampal formation. pc, pyramidal cell layer; df, dentate fascia.* **c**: *Cerebellum. Arrowheads, Purkinje cell layer; gc, granule cell layer.* **d**: *Periventricular and paraventricular hypothalamic nuclei. v, third ventricular space.* **e**: *Upper medulla. Arrowhead, hypoglossal nucleus; v, fourth ventricular space.* **f**: *Junction of cerebral cortex and subcortical white matter. Arrowhead, clusters of silver grains surrounding oligodendrocyte nuclei; C5, fifth cortical lamina.* **g**: *Fourth ventricle. Arrowhead, choroid plexus epithelium; v, ventricular space.* **h**: *Subarachnoid blood vessel within hippocampal flexure.*

roid plexus epithelium of the fourth ventricle was more intense than that of the lateral or third ventricles.

Discussion

This study revealed three important findings. First, it appears that, as in humans, rat ABPP precursor mRNA is processed to yield a family of transcripts, the composition of which varies among tissues. Second, the transcription of the ABPP gene, although most abundant in neurons, is not restricted to that cell type but also includes non-neural elements, such as choroid plexus epithelium, and occasional glial cells. Finally, although neuronal transcription appears highest in those areas that, in AD, are most susceptible to neuritic plaque formation, ie, the hippocampus and cerebral cortex, equally high levels of mRNA expression also are present in regions that are relatively unaffected, most notably the cerebellum.

In humans, the ABPP gene gives rise to at least three mRNA transcripts, presumably by alternative splicing of a large ABPP precursor RNA.[21-23] The two larger species contain a 168 bp "additional" exon that encodes a Kunitz-type protease inhibitor domain.[21-23] Our Northern analysis suggests that, in the rat also ABPP mRNA is transcribed in a wide variety of systemic organs (Figures 1, 2)[9,10] and is processed to yield a multitranscript family, the composition of which may vary among tissues. Notably, the adult kidney expresses preferentially the larger mRNA species containing the protease-inhibitor domain, whereas in the brain the smaller mRNA species is relatively abundant (Figure 2). This finding is similar to that described in humans.[22]

The *in situ* hybridization experiments reported here were performed with a 1.0 kb *Eco*RI fragment derived from the 3′ region of the cDNA, which is common to all three known (human) mRNA species. Our results demonstrate widespread transcription of the ABPP gene in neurons throughout the rat brain, most abundantly in cerebral cortical layers II and V, olfactory neurons, hippocampal pyramidal cells, dentate fascia, Purkinje cells, cerebellar granule cells, nucleus basis pontis, and the cranial nerve nuclei. This is consistent with other reports on the specific neuronal localization of ABPP transcripts in rodent[12,37] and human[25-28] brain. The human studies were limited to the neocortex, however, and did not include other regions such as the cerebellum, choroid plexus, brainstem, and deep gray structures.

In addition to abundant ABPP transcription in neurons, we also detected low-level expression of ABPP mRNA in occasional oligodendrocytes and astrocytes, and in non-neuronal structures such as the choroid plexus, leptomeninges, and ependymal lining of the ventricles, particularly in the hippocampus. Using a probe to the homologous region in the rat ABPP cDNA, Shivers et al found little or no hybridization to oligodendrocytes or choroid plexus epithelium.[12] Although we agree that the intensity of hybridization over these structures was considerably less than over neurons, nevertheless we consistently observed accumulations of silver grains above background. We attribute our detection to the longer exposure times employed in our studies.

Our interpretation of ABPP expression in the choroid plexus is consistent with the report that, in humans, age-related accumulations of intracellular fibrils occurring within choroid plexus epithelial cells (Biondi rings)[37] are apparently derived from ABP.[38] Our finding of ABPP expression in a subset of glial cells is also consistent with the reported detection of ABPP mRNA in oligodendrocytes in human brain[25] and in glioblastoma cell lines.[23]

It is noteworthy that we were unable to detect ABPP mRNA in endothelial cells or smooth muscle of the cerebral vasculature. Microvascular deposits of amyloid are a consistent pathologic feature of the AD brain. Some authors have favored a vascular origin for the amyloid fibril protein,[3] whereas others have favored a parenchymal source.[6] Our inability to demonstrate ABPP mRNA in vascular endothelium or smooth muscle in the rat argues against these cells as the origin of the amyloid found in blood vessel walls in AD.

Although neuronal transcription of the ABPP gene was widespread, ABPP mRNA was most abundant in those regions that most commonly develop neuritic plaques in human AD, such as the hippocampal pyramidal cell layer, amygdala, parahippocampal gyrus, and the cerebral cor-

Table 1. *Cellular Distribution of ABPP mRNA*

Cell type	Grain density
Hippocampal pyramidal neurons (CA1 to CA4)	4+
Purkinje cells	4+
Cerebral cortical neurons (layers II, V)	4+
Neurons of basis pontis	4+
Neurons of olfactory cortex	4+
Neurons of cranial nerve nuclei	3-4+
Other cortical neurons (layers III, IV, VI)	3+
Granule cells of dentate fascia, cerebellum	3+
Neurons of basal ganglia, thalamus, hypothalamus, parahippocampal gyrus, median raphe nuclei	3+
Choroid plexus epithelial cells	2+
Oligodendrocytes	1-2+
Leptomeninges	1-2+
Ependymal cells	1+
Astrocytes	0-1+
Smooth muscle of subarachnoid blood vessels	0
Endothelial cells	0

Estimated density of silver grains, representing ABPP mRNA, over cellular populations of the adult rat brain. Density was estimated visually by two observers and graded on a scale from 0 to 4+.

tex. Paradoxically, however, high levels of ABPP transcription also were detected in regions that, in AD, are not major sites of amyloid plaque formation, such as the cerebellum. ABPP transcription also has been reported in the human cerebellum.[22]

The basis for regional susceptibility to pathologic change in AD is not yet understood. It has been suggested that the pattern of amyloid plaque formation in AD may be related to differential expression of the various ABPP mRNA transcripts in various brain regions.[23] Initial reports suggest that, in humans, different patterns of ABPP transcription[22,23] and post-translational modification[39] may exist among different organs and among brain regions. To address this question in rat brain, we are currently performing *in situ* hybridization studies using probes specific to each of the additional exons or to the variety of possible exon junctions.[23]

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