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Astrocytes Synthesize Angiotensinogen in Brain

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Cell types associated with angiotensinogen mRNA in rat brain were identified in individual brain sections by in situ hybridization with tritiated RNA probes or with a sulfur-35–labeled oligonucleotide combined with immunocytochemical detection of either glial fibrillary acidic protein (GFAP) for astrocytes or microtubule-associated protein (MAP-2) for neurons. Autoradiography revealed silver grains clustered primarily over GFAP-reactive soma and processes; most grain clusters were not associated with MAP-2–reactive cells. These results demonstrate that, in contrast to other known neuropeptide precursors, angiotensinogen is synthesized by glia.

ANGIOTENSINOGEN IS THE PRECURSOR of the angiotensin peptides. In the brain, these peptides elicit a dipsogenic response, an increased salt appetite, vasopressin release, and an increased sympathetic outflow. All the components of the angiotensin-generating system are endogenous to the brain, including angiotensinogen and its mRNA, isorenin activity, angiotensin converting enzyme, and angiotensin receptors (*1*). Our previous results (*2, 3*) demonstrated that angiotensinogen mRNA accumulates throughout the rat brain at a low level and, in addition, several dozen nuclei in the hypothalamus, midbrain, and brainstem contain high levels of this mRNA. Cell-associated immunoreactive angiotensinogen is associated predominantly with glia (*4–6*), although some angiotensinogen-immunoreactive neurons also have been described (*6*). However, immunocytochemistry does not necessarily reveal the site of synthesis, since most brain angiotensinogen is extracellular (*7*), and these antisera do not discriminate between the renin substrate (angiotensinogen) and product [des-(Ang I)-angiotensinogen]. To address directly the question of whether brain angiotensinogen is synthesized in neurons or glia (or both), we have used the combined techniques of immunocytochemistry and high-resolution in situ hybridization to assign angiotensinogen mRNA sequences to particular cell types.

To achieve the resolution necessary for cellular localization, we hybridized tritiated RNAs synthesized from an angiotensinogen cDNA (*2*) to coronal sections from six adult male Sprague-Dawley rat brains or a sulfur-35–labeled oligonucleotide (five rats) in combination with peroxidase antiperoxidase immunostaining for glial fibrillary acidic protein (GFAP) or microtubule-associated protein (MAP-2) (*8–10*). We previously determined (*3*), by the use of sense and antisense RNA probes and in situ thermal melt curves of oligonucleotide-mRNA hybrids, that our methods (*8*) accurately detect angiotensinogen mRNA sequences. Clusters of silver grains associated with GFAP-positive cells (Fig. 1) were consistently observed in hypothalamic and brainstem areas, regions previously (*2, 3*) shown to have high levels of angiotensinogen mRNA. Adjacent sections treated for MAP-2 immunoreactivity in combination with in situ hybridization for angiotensinogen mRNA sequences did not exhibit a noticeable coincidence of grain clusters and immunoreactivity (Fig. 1 and Table 1). The correlation between GFAP immunostaining and silver grain clusters was repeatedly observed in all brain areas

Table 1. Silver grain counts associated with neuronal (MAP-reactive) or glial (GFAP-reactive) cells after in situ hybridization with tritiated angiotensinogen RNA probes. Brain sections from three rats were processed with combined immunohistochemical and in situ hybridization procedures with the mRNA (sense probe) used as the nonhybridization control (*8*). Silver grains located within 5 μm of a cell soma or its processes were counted in the ION or ARC. All cells in the ION or ARC that were associated with one or more silver grains were counted on the right-hand side of randomly selected brain sections. Values are presented as the mean number of silver grains ± SEM per cell exhibiting one or more silver grains, where *n* represents the total number of these cells in the designated brain area. Analysis of variance performed for each separate animal revealed a highly significant *F* ratio, and subsequent comparisons between the cRNA + GFAP group and each of the three other groups within each animal were made with Bonferroni *t* test. Asterisks indicate $P < 0.001$ for *t* values. A statistically significant hybridization signal was thus identified in association with glia but not with neurons in the two brain structures we examined. There was no significant difference between any cRNA + MAP group and its respective mRNA + MAP group, indicating no discernible hybridization signal over background associated with neurons.

Histochemical procedure	Silver grain counts per GFAP- or MAP-reactive cell			
	ION			ARC
	Animal 1 (*n*)	Animal 2 (*n*)	Animal 3 (*n*)	Animal 3 (*n*)
cRNA + GFAP	16.2 ± 0.6* (92)	21.0 ± 1.1* (89)	12.2 ± 0.5* (87)	15.4 ± 1.5* (23)
mRNA + GFAP	4.0 ± 0.4 (88)	4.9 ± 0.2 (100)	3.1 ± 0.2 (93)	4.0 ± 0.4 (31)
cRNA + MAP	3.5 ± 0.2 (106)	6.1 ± 0.3 (105)	2.6 ± 0.1 (102)	4.5 ± 0.6 (26)
mRNA + MAP	2.4 ± 0.3 (74)	8.3 ± 0.4 (100)	3.5 ± 0.2 (72)	6.4 ± 0.9 (27)

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examined, including the supraoptic nucleus of the hypothalamus, paraventricular (PVN) and periventricular hypothalamic nuclei, arcuate nucleus of the hypothalamus (ARC), amygdala, parabrachial complex, vestibular nucleus, inferior olivary nucleus (ION), nucleus of the solitary tract, hypoglossal nucleus, dorsal motor nucleus of the vagus, and reticular formation. This correlation was quantified for the ION and ARC in Table 1. Those areas with higher angiotensinogen mRNA levels, such as ION and the nucleus of the solitary tract (*3*), contained far more astrocytes positive for angiotensinogen mRNA than did areas with less angiotensinogen mRNA, such as the reticular formation. Furthermore, in areas that are known to contain the lowest levels of angiotensinogen mRNA, such as the cortex (*2*), few astrocytes contain this mRNA although there are numerous GFAP-positive cortical astrocytes.

Earlier suggestions that angiotensinogen is synthesized in neurons were based on immunocytochemical localization (*6*) or low-resolution in situ hybridization without immunocytochemistry (*11*). We examined the two groups of neurons that had been suggested as sites of angiotensinogen synthesis, the supraoptic magnocellular neurons (*6*) and the paraventricular magnocellular neurons of the hypothalamus (*11*). Neither area showed dual labeling of these neurons with antibody to MAP-2 and angiotensinogen hybridization probes; conversely, both areas exhibited angiotensinogen mRNA-positive astrocytes. However, it is still possible that low levels of angiotensinogen mRNA in neurons were undetected in our experiments; furthermore, we have not yet conducted a detailed examination of the mesencephalon or telencephalon with the combined technique. Nevertheless, our finding that angiotensinogen mRNA accumulates in astrocytes in all brain areas examined strongly suggests that most, and possibly all, brain angiotensinogen is synthesized by glia.

Angiotensin II–like immunoreactive material is present in the brain, and the identity of the reactive peptide has been confirmed by high-performance liquid chromatography (HPLC) (*12*). Furthermore, localization of angiotensin-like immunoreactivity in specific groups of neurons is suggestive of a neurotransmitter role for angiotensin peptides (*13, 14*). Since angiotensinogen, the only known precursor to these neuropeptides, is synthesized in astrocytes, the source of the neuronal peptide invites speculation. One possibility is that neuronal angiotensinogen mRNA has escaped detection in our assays, either because of its low concentration, or, alternatively, because a distantly related (non–cross-hybridizing) angiotensinogen mRNA exists in neurons. Another possibility is that angiotensin peptides that are generated extracellularly are taken up and released by neurons, contrasting with the known synthesis pattern of other central nervous system neuropeptides, where all components of peptide synthesis are intraneuronal.

Finally, we wish to stress that not all astrocytes synthesize angiotensinogen. The reason for the highly uneven pattern of rat brain angiotensinogen mRNA accumulation (*2, 3*) is that a large proportion of astrocytes associated with certain hypothalamic, midbrain, and brainstem nuclei express the angiotensinogen gene, whereas relatively few astrocytes outside these nuclei do so. The available data indicate that astrocytes synthesize, and presumably secrete, unprocessed angiotensinogen. Thus, this angiotensinogen may function in the brain as in the periphery, as an extracellular reservoir of angiotensin peptides. The subset of astrocytes that synthesize angiotensinogen may fulfill the same role as hepatocytes in the periphery. It remains to be determined why angiotensinogen-synthesizing astrocytes are much more numerous in certain brain nuclei (*3*) and whether the angiotensin peptides (or angiotensinogen) can be taken up and released by neurons.

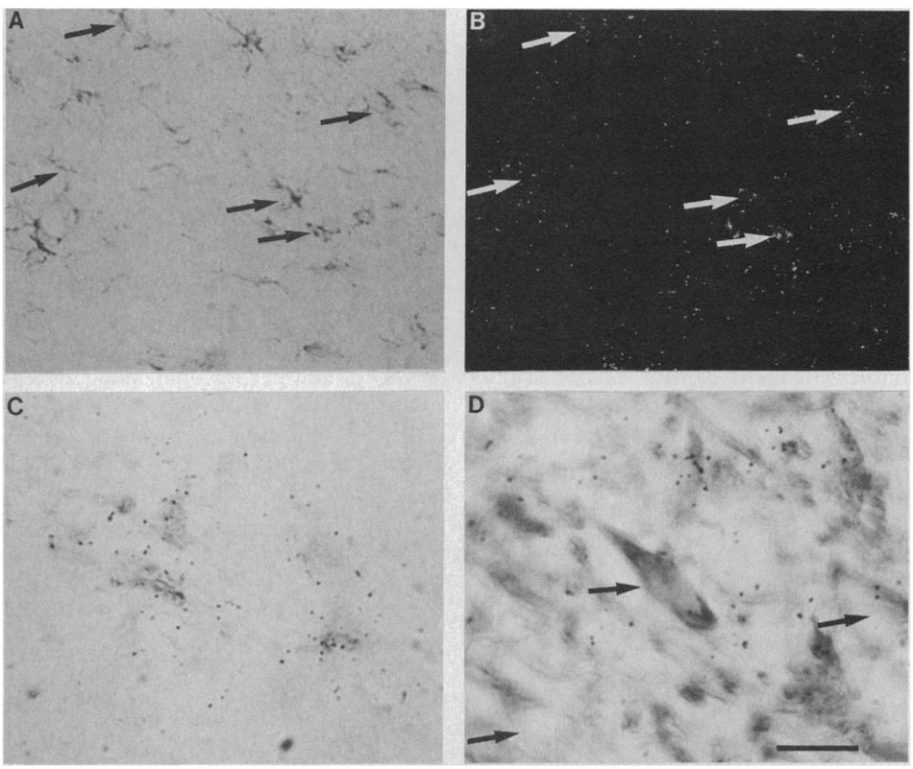

Fig. 1. Glial localization of angiotensinogen mRNA sequences in rat brain demonstrated by combined in situ hybridization and immunocytochemistry. (**A**) GFAP-labeled astrocytes in the PVN of the hypothalamus. Arrows show the location of some of these cells. (**B**) Darkfield photomicrograph of A, enabling visualization at this magnification of clusters of silver grains over same astrocytes. The coincidence of silver grain clusters with GFAP-labeled cells indicates the presence of sulfur-35–labeled angiotensinogen oligonucleotide in association with glia. (**C**) GFAP-labeled astrocytes in the PVN photographed at higher magnification. The silver grains are clustered over the glial cell bodies showing the association of angiotensinogen mRNA with astrocytes. (**D**) MAP-2–labeled neurons, indicated by arrows, in the PVN. The lack of association of silver grains with these cell bodies indicates the absence of angiotensinogen mRNA in neurons in this area. However, there are clusters of silver grains adjacent to the MAP-2–labeled neurons. Several neuronal cell bodies are out of focus because the silver grains in the emulsion layer are on a slightly higher plane than the tissue section. Scale bar: 30 μm, A and B; 75 μm, C and D.

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37°C for 4 to 12 hours. RNAs (sense and antisense) were synthesized in vitro in the presence of [^{3}H]uridine triphosphate on a full-length angiotensinogen cDNA template (*2*), to a specific activity of 1.3×10^8 dpm/pmol. After limited base hydrolysis (to a length of 50 to 100 nucleotides), RNA probes were added directly to the prehybridization solution at 10 to 15 p*M*; the sense RNA generates a background control. Alternatively, a 42-residue oligonucleotide complementary to the region of rat angiotensinogen mRNA encoding the amino-terminal tetradecapeptide Ang I-Leu-Tyr-Tyr-Ser (DRVY-IHPFHLLYYS; abbreviations are D, Asp; F, Phe; H, His; I, Ile; L, Leu; P, Pro; R, Arg; S, Ser; V, Val; and Y, Tyr) was end-labeled with [^{35}S]deoxyadenosine 5′-(α-thio)triphosphate to a specific activity of 5×10^6 dpm/pmol and added directly to the prehybridization solution at 1.0 to 1.5 n*M*; a 20- to 50-fold molar excess of unlabeled oligonucleotide was added to some sections as a background control (*3*). After addition of the probe, the sections were returned to 37°C for 18 to 56 hours. Sections were then rinsed through decreasing concentrations of salt solutions (*3*), with a final rinse at 45°C for 20 min. At this point, tissues hybridized with tritiated ribroprobe were incubated with ribonuclease A (20 μg/ml) for 45 min at 37°C, followed by a 45 min incubation at 37°C with the ribonuclease buffer alone. Then sections were postfixed in 4% paraformaldehyde for 30 min and rinsed before following standard immunocytochemistry procedures. Nonspecific antibody binding was blocked with a 1-hour incubation in 5% bovine serum albumin in 50 m*M* tris-saline (*p*H 7.4). Sections were rinsed and incubated overnight at 4°C in primary antibody [1:2000 dilution of a mouse monoclonal antibody to MAP-2 (*9*); or a 1:500 dilution of a mouse monoclonal antibody to GFAP (Boehringer Mannheim). The anti-GFAP is a commercially available antibody first developed and tested as the clone G-A-5 (*10*), which selectively stains astrocytes in rat brain sections]. Sections were rinsed and incubated in rabbit antibody to goat immunoglobulin G (1:100). Sections were then incubated in goat peroxidase-antiperoxidase (PAP) (1:200), rinsed, and reacted with 0.04% diaminobenzidine in 50 m*M* tris buffer and 0.0075% hydrogen peroxide and 10 m*M* imidazole. After further rinsing, sections were mounted onto gelatin-coated slides, dehydrated through a graded alcohol series, and dipped in Kodak NTB2 emulsion. Exposure time was 4 to 8 weeks.

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Metabolic Correction of Defects in the Lipid Anchoring of Thy-1 in Lymphoma Mutants

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Many plasma membrane proteins, including Thy-1, are anchored by a carboxyl terminal glycophospholipid. This unit is absent from the Thy-1 of several lymphoma mutants that synthesize the Thy-1 polypeptide but fail to express it at the cell surface. Recessive mutants of complementation groups A to C, E, and F contain Thy-1 mRNA of normal size, which suggests that their Thy-1 polypeptide is normal. To identify possible metabolic lesions, each mutant was grown with various supplements. The class F and B mutants exhibited a reversible induction of surface lipid anchored Thy-1 when grown with the aminoglycoside G418. Other aminoglycosides, sugars, and ethanolamine were inactive. These unexpected observations are discussed in the context of lipid anchor biosynthesis.

MEMBRANE PROTEINS SUCH AS Thy-1 are anchored to the plasma membrane by a complex moiety composed of ethanolamine phosphate, mannose, glucosamine, *N*-acetylgalactosamine, and phosphatidylinositol (PI) (*1*) that is sensitive to PI-specific phospholipase C (*2*). This unit is added to the COOH-terminus of Thy-1 after the completion of polypeptide synthesis and excision of the most COOH-terminal 31 amino acids, which includes a putative membrane-spanning sequence (*3*). A preassembled unit including most or all of the "anchor components" may be involved (*4*), and dolichol-phosphorylmannose appears to be a precursor of some of the mannose residues (*5*); however, none of the metabolic paths and enzymes involved in anchor assembly or addition have been definitively identified.

There is a family of Thy-1–negative murine T lymphoma mutants (*6*) that synthesizes immunoprecipitable Thy-1 of slightly abnormal gel mobility and expresses essentially none of the antigen on the plasma membrane. These mutants do, however, express normal amounts of transmembrane proteins. Cell fusion studies have assigned the recessive mutants that synthesize Thy-1 to six complementation groups.

Each of the recessive mutants does not add the lipid anchor to Thy-1, and two of them (classes B and E) secrete a hydrophilic form of Thy-1 (*5, 7*). Thus, these cells may have (i) metabolic defects that interrupt anchor biosynthesis or cause anchor hydrolysis, or (ii) mutations that interrupt the normal splicing and processing of the Thy-1 pre-mRNA, thereby producing a variant form of Thy-1 that cannot accept an anchor.

We extracted RNA from wild-type and mutant cell lines to determine the size of each Thy-1 mRNA species (Fig. 1). Each cell line produced an mRNA of 1.8 kb, the same size as in selected wild-type lymphoid and nonlymphoid cells (*8*). Thus, as previously suggested (*6*), the cell-associated Thy-1 of each of these cells may have the same primary structure. The amount of Thy-1 mRNA is, however, variable among the cells examined.

Since several mammalian cell mutations that affect post-translational glycosylation

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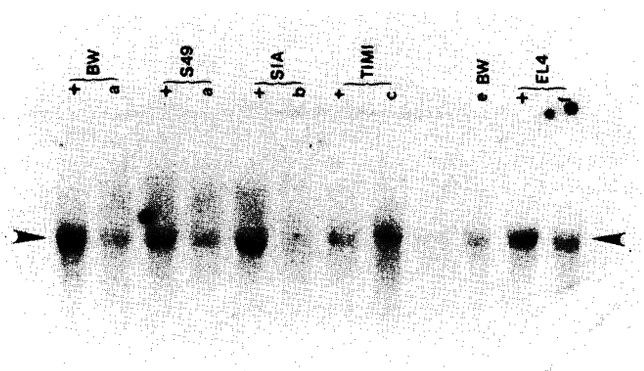

Fig. 1. Analysis of Thy-1 mRNA. RNA was extracted with guanidine isothiocyanate and purified by sedimentation through CsCl from wild-type (+) Thy-1 expressing cells (S49, BW5147, SIA, T1M1, and EL4) and mutant (−) cells (S49-a, BW5147-a, SIA-b, T1M1-c, BW5147-e, and EL4-f). Samples (20 μg) were fractionated on a 1.5% agarose formaldehyde gel, blotted to GeneScreen, and probed with a single-stranded ^{32}P-labeled DNA transcript of a Sac I fragment of the Thy-1 gene (*27*) that had been cloned into M13. The probe was purified and eluted from a low temperature–melting agarose gel before use.