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COMMENTARY

BRAIN ANGIOTENSIN: ON THE WAY TO BECOMING A WELL-STUDIED NEUROPEPTIDE SYSTEM

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More than thirty different peptides in the central nervous system have been described in recent years. Some of these peptides are well defined with respect to their biosynthetic pathways, their distribution in the body, and their biological functions; other peptides have been merely identified by immunohistochemical methods (for review see Refs. 1–3).

Angiotensin II (ANG II) is one such newly discovered neuropeptide. Its biosynthetic pathway and functions in the periphery as a plasma hormone are well recognized and characterized (see Fig. 1). However, when components of the renin–angiotensin system (RAS) were first described in the brain [4, 5], the reports went largely unnoticed and were then met with scepticism [6]. This lack of attention and the scepticism were perhaps related to the overwhelming attention given to the peripheral renin–angiotensin system, as well as to the little notice given to neuropeptides at the time.

Currently, the brain RAS is on its way to becoming one of the best studied enzyme–neuropeptide systems. This subject has been reviewed recently [7] and only new advances will be discussed here.

The high molecular weight precursor angiotensinogen

Definite evidence for an endogenous brain renin–angiotensin system required the demonstration that the brain synthesizes the angiotensin precursor angiotensinogen. The first evidence for the presence of angiotensinogen in the brain was obtained by Ganten *et al.* [8] when brain extracts were incubated with excess renin and tested for ANG I generating capacity. Significant amounts of brain angiotensinogen were found. In the cerebrospinal fluid, the specific activity even exceeded that observed in plasma. This observation has been confirmed by several authors using essentially similar methods. Subsequently, a specific regional regulation of brain angiotensinogen was demonstrated (see Ref. 9).

The complete sequence of angiotensinogen was determined recently from a clone selected from a rat liver cDNA bank and subjected to nucleotide sequence analysis [10]. The deduced amino acid sequence indicated that the precursor molecule consists of a mature angiotensinogen of 453 amino acid residues and a putative signal peptide of 24 amino acids. The predicted molecular weight and amino acid composition of angiotensinogen agreed well with those obtained by amino acid analysis of the purified protein [11]. The ANG I moiety is located at the amino-terminal part of the molecule, followed by a large carboxy-terminal sequence. This carboxy-terminal sequence contains two small internally homologous sequences and three potential glycosylation sites. The possibility that the carboxyl-terminal region of angiotensinogen has some biological role after the release of ANG I still awaits investigation.

Using the technique of cell free translation of mRNA, Campbell *et al.* [12] recently provided evidence that the same angiotensinogen molecule is synthesized in the liver and locally in the brain. In their studies, [^{35}S]methionine-labeled angiotensinogen precursors were synthesized by cell-free translation of either rat brain or rat liver mRNA and compared by immunoprecipitation, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and autoradiography. Rat liver mRNA synthesized two angiotensinogen precursors: a major precursor of molecular weight 52.5 k and a minor precursor of molecular weight 55.7 k. Precursor forms, identical and similarly abundant to those observed in liver, were synthesized by cell-free translation of rat brain mRNA. Both brain angiotensinogen precursors were cleaved by renin, resulting in a single cleavage product with a molecular weight of 47.5 k, identical to that observed for liver. Bilateral nephrectomy and dexamethasone administration produced less than a 2-fold increase in translatable levels of brain angiotensinogen mRNA, in contrast to the several-fold

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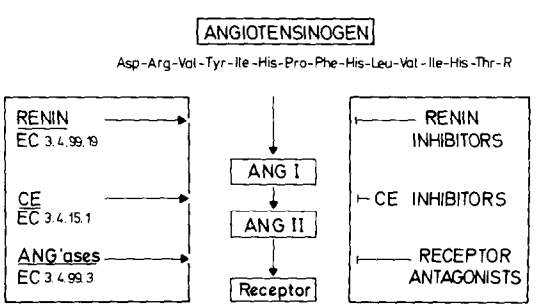

Fig. 1. General outline of the renin–angiotensin system with the enzymes on the left and possibilities for pharmacological interferences on the right. Abbreviations: CE: converting enzyme; ANG'ases: angiotensinases; EC: enzyme nomenclature classification; and ANG I, II: angiotensin I, II.

increase observed for liver. These results suggest that, although rat brain and liver angiotensinogen mRNAs appear to be products of the same gene(s), the regulation of their transcription is tissue specific [12].

Angiotensin

The missing link in the evidence for an active endogenous RAS in the brain has been the demonstration of local angiotensin synthesis in the central nervous system *in vivo*.

Recently, we reported that ANG I and ANG II could be extracted from brain of nephrectomized rats, rabbits and monkeys [13, *]. The peptides were characterized with high performance liquid chromatography (HPLC) capable of separating all angiotensins and their fragments including (Val^5)-ANG II from (Ile^5)-ANG II. The peptides extracted from brain corresponded to synthetic (Ile^5)-ANG II and (Ile^5)-ANG I, with small amounts (approximately 10%) of (Ile^5)-ANG(2-8) (ANG III) being present in the brain. Brain angiotensin thus appears to have the same amino acid sequence as plasma angiotensin. Identical peptides were cleaved from brain and plasma angiotensinogen upon incubation with renin *in vitro* and *in vivo* [13]. The accumulation of ANG I in brain tissue was increased in nephrectomized hypertensive rats when the conversion to ANG II was blocked *in vivo* by central application of a converting enzyme (CE) inhibitor. Concomitantly, the brain levels of ANG II were lower in hypertensive as compared to the CE inhibitor treated normotensive rats. This observation was interpreted to indicate that: (1) ANG I generation must occur in the brain with plasma components being eliminated after nephrectomy, and (2) turnover of brain angiotensin peptides was more rapid in the hypertensive animals.

Angiotensin pathways

The distribution of ANG II in the central nervous system has been investigated biochemically [13] as well as by immunohistochemical techniques [14–19]. The main locations of ANG II are the hypothalamus, the limbic system, the medulla oblongata and the spinal cord. High densities of ANG II-positive nerve terminals exist within the median eminence, in the nucleus paraventricularis, the supraoptic nucleus and the subfornical organ. Further, ANG II-positive brain areas are the substantia gelatinosa of the spinal cord, nucleus tractus spinalis nervi trigemini, nucleus amygdaloideus centralis, sympathetic lateral column, nucleus dorsomedialis hypothalami, and locus coeruleus.

The presence of ANG II in the paraventricular nucleus of the hypothalamus (PVN) is of particular interest in view of the capacity of ANG II to release adrenocorticotrophic hormone (ACTH) and in view of the projections from the PVN through the external layer of the median eminence to the portal blood circuit.

Lind *et al.* [19] recently studied the PVN/ANG II system in detail. It was confirmed that antiserum to ANG II stains neurosecretory neurons that synthesize vasopressin in magnocellular parts of the PVN, but it was also shown that ANG III-immunoreactive neurons were scattered throughout the parvocellular division. A subpopulation of ANG II-immunoreactive parvocellular neurons in the PVN projects to the neurohemal zone via the external layer of the median eminence. These ANG II-stained projections were eliminated after bilateral destruction of the PVN. In contrast, the ANG II-stained magnocellular neurons in the PVN appear to project through the internal lamina of the median eminence to the posterior pituitary. Bilateral lesions of the PVN reduce, but do not eliminate, ANG II staining in the internal lamina, the remaining fibers probably arising from ANG II-stained magnocellular neurons in the supraoptic nucleus.

These studies suggest that ANG II-stained fibers in the external and internal laminae of the median eminence arise from separate groups of neurons, a conclusion also supported by observations that these areas can be regulated independently. For instance, adrenalectomy leads to a selective enhancement of ANG II-stained fibers in the neurohemal zone as has also been described for corticotropin-releasing factor (CRF), oxytocin, and vasopressin. In contrast, the selective increase of ANG II staining in the internal lamina of the median eminence following water deprivation supports a functional relationship of this ANG II pathway to the posterior pituitary in vasopressin and oxytocin release.

Another area of the brain which is of particular interest for the regulation of fluid balance is the subfornical organ (SFO). The SFO is a small glomus-like convexity of the midline third ventricular ependyma near the interventricular foramen. It is densely vascularized, has relatively porous blood–brain barrier, and is strategically located to monitor plasma, cerebrospinal fluid (CSF) and neuronal inputs. ANG II receptors have been demonstrated on the SFO [20, 21], and stimulation of the receptors in water-satiated animals results in copious drinking.

Recently, Lind *et al.* [18] examined the ANG II pathways to and from the SFO by immunohistochemical methods. ANG II immunoreactive cell bodies and fibers were clearly identified in the SFO of the rat. Cells were distributed in an annulus around the periphery of the SFO. Fibers were observed in a plexus, located centrally within the ring of cells. Knife-cuts through the ventral stalk of the SFO diminished, but did not eliminate, fiber staining in the SFO. Ventral to the cut and, to a lesser degree, also dorsal to the cut, bright varicose ANG II immunoreactive fibers were described. The combination of immunohistochemistry with retrograde transport identified the perifornical zone of the lateral hypothalamus, the rostral zona incerta and the nucleus reuniens of the thalamus as the source of ANG II-stained inputs to the SFO, and the region of the median preoptic nucleus as a recipient of ANG II-immunoreactive SFO efferents. It was concluded that ANG II-stained pathways from the lateral hypothalamus and adjacent regions project to the SFO and that ANG II-stained neurons within the SFO project, in turn, to the preoptic region of the hypothalamus. Interestingly, the perifornical region of the lateral hypothalamic area, and rostral parts of

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the zona incerta that project to the SFO, are known to be involved in the regulation of thirst. Furthermore, the ANG II-stained projection from the SFO to the preoptic region is also thought to play a critical role in the initiation of drinking behavior [21].

The staining was not altered by water deprivation or by nephrectomy. Since the former procedure drastically increases and the latter drastically decreases circulating levels of ANG II, tissue bound peptide from the blood does not appear to have been responsible for the observed immunoreactivity. Nevertheless, circulating ANG II may also affect ANG II receptors in the SFO through the relatively permeable blood–brain barrier. Thus, the SFO could function as an integrating center where hormonal and neuronal angiotensin interact to control fluid balance.

Together with previously described data on the localization of ANG II in the brain, a picture begins to emerge which gives credence to a role for angiotensin pathways in blood pressure and volume control. This notion is corroborated by experiments in which inhibition of the brain angiotensin system was tested (see below).

Renin

Renin is a highly specific endoprotease which generates ANG I by cleaving the amino-terminal decapeptide from angiotensinogen. Interestingly, the first tissue from which renin was completely purified was not the kidney but the submaxillary gland of mice (see Ref. 22). Concomitantly, the primary structures of renin were determined by classical amino acid sequence analysis [22] and by determining the nucleotide sequences of cloned cDNAs complementary to their mRNA [23, 24]. The mature renin molecule consists of two chains. The heavy chain contains 288, and the light chain 48, amino acid residues.

Mouse submaxillary gland renin exhibited a 43% sequence homology with porcine pepsin, 34% indentity with bovine chymosin, and 22% identity with penicillopepsin [22]. The overall dimension and shape of the renin molecule appear to be similar to other acid proteases. Renins isolated from various sources including hog, rat and human kidney renin, bovine pituitary renin, and mouse submaxillary renin are all similar in such general molecular properties as amino acid composition, chain length, molecular weight, and isoelectric points (see Refs. 7, 22 and 25–27).

The fact that a number of acid proteases, including cathepsin D, can generate ANG I from angiotensinogen, had led to a controversy as to whether the brain contains "true" renin. This controversy was terminated when it was demonstrated that brain renin is active at neutral pH, can be separated from cathepsin D and other acid proteases, is inhibited by specific renin antibodies and peptide inhibitors, and is active *in vivo* [7, 25–28].

The ultimate proof for local synthesis of renin in the central nervous system will stem from recombinant DNA techniques as has been shown for brain angiotensinogen. We have used a renin cDNA probe for hybridization studies with mRNA isolated from various mouse tissues. The probe, plasmid pMSR 49 [24], contains a 700 bp insert of mouse submaxillary cDNA cloned into pBR 322. This approach was used for hybridization studies with RNA from brain and other organs of male mice* (Fig. 2).

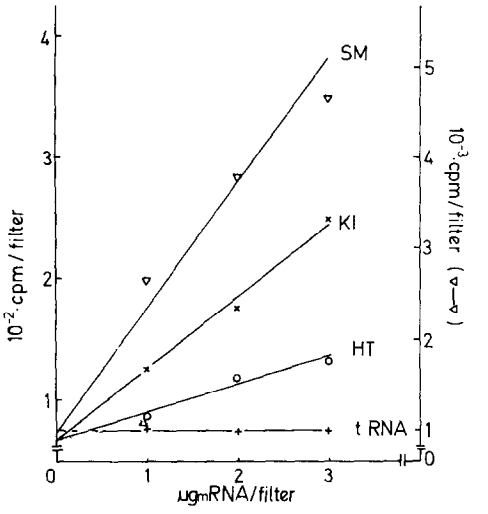

Fig. 2. Relative amounts of renin mRNA in mouse organs as determined by dotting analysis. Messenger RNA (mRNA) was spotted and fixed on nitrocellulose filters. Prehybridization, hybridization, washing and drying of filters were done under the same conditions as for Northern blotting. The dried filters were counted directly in liquid scintillation fluid. One, two and three micrograms each of mouse mRNA from submaxillary gland (SM) (▽——▽), kidney (KI) (×——×), and hypothalamus/thalamus (HT) (○——○) were applied per filter. Transfer RNA (tRNA) served as a control (see text).

Briefly, messenger RNA (mRNA) was isolated from total RNA by chromatography on oligo-(dT) cellulose, denatured and then fractionated electrophoretically by size on formaldehyde-agarose gels. The mRNA was then blotted from the gel onto nitrocellulose. Hybridization on these filters was performed with nicktranslated heat denatured (alpha-^{32}P)-labeled plasmid pMSR 49 containing submaxillary gland renin cDNA (Northern blot). After washing, hybrid bands were visualized by autoradiography using Kodak X-ray screen. Preliminary results show that the hybridization bands in brain corresponded to those found in submaxillary gland and in kidney. Relative amounts of renin mRNA in mouse organs were determined by dotting analysis. For this purpose mRNA was spotted and fixed on nitrocellulose filters. Hybridization was done under the same conditions as for Northern blotting, and the dried filters were counted directly in liquid scintillation fluid or exposed to X-ray film. Direct counting and densitometric analysis of the dot blots revealed the following rank order of hybridization: submaxillary gland > kidney > brain (Fig. 2).

These data are in harmony with the idea that the renin gene is expressed and that the protein is synthesized locally in brain. However, the final proof

* G. Ludwig, E. Lehmann, K. Murakami, R. E. Lang, Th. Unger and D. Ganten, manuscript submitted for publication.

will require one or more of various approaches including cell-free translation of brain mRNA and identification of the newly synthesized protein as renin, further characterization of the specificity of hybridization by digestion with nuclease S1 ("S1 mapping"), or sequencing of cDNA transcripts synthesized by reverse transcriptase. Such work is currently underway in several laboratories.

Converting enzyme (CE)

The biochemical properties of CE in the brain closely resemble the peptidyldipeptide carboxyhydrolases which have been purified from lung and kidney. However, the brain enzyme has not been completely purified, and it may not be identical with CE in peripheral tissue. The possibility also remains that different enzymes that are capable of activating ANG I exist in the brain.

CE is widely distributed throughout the brain. The localizations obtained in microdissection studies measuring CE catalytic activity and those described using immunohistochemical techniques are in reasonably good agreement with more recent data obtained by autoradiographic visualization of CE with [^{3}H]captopril [29–32].

The highest concentrations occur in the choroid plexus, subfornical organ, caudate-putamen, zona reticulata, substantia nigra, globus pallidus and median eminence. In certain areas (e.g. entopeduncular nucleus, medial habenula, and median preoptic area), however, there is disagreement between the autoradiographic and biochemical or immunological data [30]. Brain blood vessels also contain CE, but the enzyme is clearly also located neuronally as evidenced in cell culture and by ultracentrifugation studies. The latter techniques produced evidence that CE is present in synaptosomes [28, 31].

The striato-nigral localization of CE is of particular interest. Ibotenic acid selectivity destroys neuronal cell bodies intrinsic to the site of injection without damaging glial elements, extrinsic nerve terminals and axons of passage [29]. Injections of ibotenic acid into the caudate-putamen produced a decrease of CE at the site of injection and later on a depletion in the substantia nigra. On the other hand, the same injections into the substantia nigra were without effect on CE activity [29, 30]. The findings show that CE has a neuronal localization within the corpus striatum and that the CE-producing neurons (cell bodies) project to the ipsilateral substantia nigra. Glia seem to be devoid of CE [29, 30]. The decrease of CE in the caudate-putamen was associated with an increase in renin activity which could represent a compensatory effect [29]. The finding that the typical destruction of the corpus striatum in Huntington's disease is paralleled by a depletion of CE activity in the substantia nigra is noteworthy in this respect [33]. Surprisingly, it has not been possible to demonstrate ANG II receptors and ANG II immunoreactivity in the striato-nigral structures. CE may, nevertheless, play an important role in these structures, since the enzyme has a broad specificity and may hydrolyze other peptides as well.

Brain areas where ANG II has been shown to occur with no CE include parts of the spinal cord, the bed nucleus of the stria terminalis, and the central nucleus of the amygdala. The significance of this remains to be investigated. The different ratios of ANG I/ANG II in various brain areas [13] would be consistent with the interpretation that CE in different parts of the brain determines the activity of the RAS in the brain.

The presence of CE in specific brain regions has become of particular interest since inhibitors of the enzyme have been introduced as antihypertensive drugs [34]. These agents provide a new tool to study brain peptide metabolism, and there is increasing evidence that the antihypertensive effects of CE inhibitors are, at least in part, mediated by an action on brain CE [34–37].

Site of synthesis and site of action of ANG II

For the site of synthesis and biological action, several levels have to be considered. All components of the plasma RAS can, in principle, be taken up by peripheral tissue and by the brain, though the existence of a blood–brain barrier makes this latter possibility unlikely [38]. The locally synthetized or the plasma derived tissue components can interact to generate ANG II. The local synthesis can occur intracellularly and/or extracellularly. The effector peptide can be active intracellularly, in the interstitium, or be released into the circulation.

There is general agreement that ANG II can be generated in the blood by an interaction of the circulating components with each other. Though plasma CE is active and partly responsible for the conversion of ANG I to ANG II, the high concentration of CE in the endothelial lining of the vascular bed (especially, but not exclusively, the lung) makes it probable that activation of ANG I to ANG II occurs to a large degree by the endothelium.

Local ANG II generation has been studied best in the brain. In favor of local neuronal synthesis of ANG II are the intracellular localization of renin in synaptosomes [28], and the neuronal localization of CE [28, 30], probably angiotensinogen [9], and ANG II as demonstrated by electronmicroscopic studies [28]. Tissue culture studies also support the possibility of intracellular neuronal ANG II synthesis [39].

There appears to be an interaction between the circulating and the local RAS components which is not yet fully understood. Plasma ANG II, without any doubt, reaches several angiotensin sensitive sites such as the SFO, the OVLT, ME, anterior and posterior pituitary and the area postrema. Binding of plasma ANG II to these receptors can be diminished by an intraventricular (i.c.v.) administered ANG II analogue, indicating that peripheral and central ANG II could interact functionally at these sites. Other brain areas such as the paraventricular nucleus, the supraoptic nucleus or the cortex do not show binding of peripheral ANG II since they are located inside the blood–brain barrier [40]. At these sites, brain ANG II may be the exclusive receptor stimulus.

The SFO has been discussed above as an example for possible interactions between neuronal brain ANG II pathways and hormonal ANG II. Functionally, circulating ANG II could explain several of the peptide effects on thirst (SFO, OVLT), blood

pressure (OVLT, AP) and pituitary hormone release (ME, anterior and posterior pituitary). However, all these areas, also contain endogenous brain ANG II. The circulating peptides may act in concert with brain ANG II. Alternatively, they may also be antagonistic. Prolactin release, for example, is stimulated by a direct action of ANG II on the pituitary cells [41], while central ANG II is inhibitory, possibly via its influence on central catecholamines [42]. These relationships need to be clarified in the future.

Function of the brain RAS

ANG II has a large number of pharmacological effects which point to a possible broad spectrum of biological significance of the RAS. These include actions on membrane function, protein synthesis, cell growth, hormone synthesis, and memory (see Refs. 43 and 44). Of all these effects, the most reliable and best studied are those related to salt and volume homeostasis and blood pressure control. To the best of our knowledge there is no other peptide or other biological compound with similarly concerted effects on various regulatory systems in the body (arterial smooth muscle, adrenal gland, sympathetic nervous system, behavior) as is the case with ANG II.

In stroke-prone spontaneously hypertensive rats (SHRSP), elevated levels of renin were found in catecholaminergic nuclei of the brain, in the pineal organ and in the adeno- and neurohypophysis [45]. Angiotensin synthesis and degradation also appear to be higher in young SHRSP [13], and CSF angiotensinogen is found to be elevated in hypertensive patients, suggesting a stimulated brain RAS in hypertensive subjects [46].

Pharmacological interference with the brain RAS at various levels of the enzyme–peptide cascade has been used to investigate whether locally generated endogenous brain angiotensin was involved in blood pressure regulation [1, 34, 35, 47].

Acute blockade of brain angiotensin receptors by i.c.v. administration of competitive ANG II antagonists in SHR led to a consistent, dose-dependent decrease of blood pressure [47, 48]. When kidney renin was eliminated in SHRSP by nephrectomy, the blood pressure decrease was still found after i.c.v. ANG II receptor blockade.

Inhibition of converting enzyme in the brain with captopril equally lowered blood pressure in SHR. Low doses of i.c.v. captopril (5 μg) caused converting enzyme inhibition in the CSF and in the brain but not in the peripheral blood. Thus, the fall of blood pressure was centrally mediated [35, 36]. This observation has been confirmed in studies by Berecek *et al.* [37] who administered low doses of CE inhibitors chronically to SHR and found that the drug lowered blood pressure when administered centrally but not when given by the intravenous route at the same dose. The results indicate that an increase of baroreceptor reflex activity and a blunting of vascular reactivity might be involved in the depressor effect of central CE inhibitors. These data further support a contribution of brain angiotensin in the maintenance of high blood pressure. As reviewed elsewhere [34], there is increasing evidence that orally given CE inhibitors do have central effects and it is thus possible that part of the blood pressure lowering action of CE inhibitors is mediated by an effect of the orally administered drug on the brain.

In conclusion, since its discovery in 1971 [4, 5, 8], the RAS in the brain has come of age. "Existence or nonexistence" was the leitmotiv of the research efforts in the past. With improved techniques, most of the early results have been confirmed. The existence of all components of the RAS in the brain can now be considered established. The site of synthesis of the individual components of this complex enzyme–peptide systems, mapping of the pathways, functional aspects, more precise assessment of activity states and interactions with circulating hormones and chemical messengers in the brain, including enzymatic generation of new peptides in the nervous system, will be of great interest in the future.

Since the key genes for the RAS proteins are now known, studies at the cellular and molecular level have become possible. Moreover, the availability of pharmacological interferences at the sites of peptide generation and peptide action (Fig. 1), to an extent not equalled for most other enzyme–peptide systems, make the RAS one of the best studied peptide systems in the brain.

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