Angiotensin II–Independent Angiotensin-(1–7) Formation in Rat Hippocampus
Involvement of Thimet Oligopeptidase

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Abstract—The involvement and relevance of the renin–angiotensin system have been established clearly in cardiovascular diseases, and renin–angiotensin system involvement has also been investigated extensively in the central nervous system. Angiotensin II acts classically by binding to the AT₁ and AT₂ receptors. However, other pathways within the renin–angiotensin system have been described more recently, such as one in which angiotensin-(1–7) (Ang-(1–7)) binds to the receptor Mas. In the central nervous system specifically, it has been reported that this heptapeptide is involved in learning and memory processes that occur in central limbic regions, such as the hippocampus. Therefore, this prompted us to investigate the possible role of the Ang-(1–7)–receptor Mas pathway in epileptic seizures, which are also known to recruit limbic areas. In the present study, we show that Ang-(1–7) is the main metabolite of angiotensin I in rat hippocampi, and, strikingly, that thimet oligopeptidase is the major enzyme involved in the generation of Ang-(1–7). Furthermore, elevations in the levels of thimet oligopeptidase, Ang-(1–7), and of receptor Mas transcripts are observed in chronically stimulated epileptic rats, which suggest that the thimet oligopeptidase–Ang-(1–7)–receptor Mas axis may have a functional relevance in the pathophysiology of these animals. In summary, our data, which describe a new preferential biochemical pathway for the generation of Ang-(1–7) in the central nervous system and an increase in the levels of various elements of the related thimet oligopeptidase–Ang-(1–7)–receptor Mas pathway, unveil potential new roles of the renin–angiotensin system in central nervous system pathophysiology. (Hypertension. 2013;62:879-885.) ● Online Data Supplement

Key Words: angiotensins • angiotensin-(1–7) • angiotensin-(1–7) receptor Mas • central nervous system • renin-angiotensin system • thimet oligopeptidase

The increase in knowledge about the functional diversity of the renin–angiotensin system (RAS) during the past several years is demonstrative of the remarkable complexity of this system. The classical linear signaling pathway ultimately results in the angiotensin II (AngII)–induced activation of the AT₁ and AT₂ receptors. AngII is the most widely studied effector in the RAS and is mainly produced by angiotensin-converting enzyme (ACE) after AngI degradation. More recently, our understanding of the RAS has been enriched by descriptions of other receptors, functionally active angiotensin metabolites and enzymes. Previous data have proposed that AngIII, AngIV, and Ang-(1–7) are new angiotensin fragments that have important biological activities. Ang-(1–7) has been studied extensively in the past years, and it was described as an endogenous ligand for receptor Mas by Santos et al. Several studies have shown that Ang-(1–7) and the receptor Mas are important components of the RAS and are involved in diverse pathophysiological conditions, such as heart failure, nephropathies, and diabetes mellitus.

Ang-(1–7) can be generated directly from the cleavage of AngI or AngII by endopeptidases or carboxypeptidases, respectively. The major enzymes involved in the production of Ang-(1–7) from AngI are thimet oligopeptidase (TOP), neutral endopeptidase, and prolyl oligopeptidase (POP). Both TOP³ and POP⁴ have been reported to be directly responsible for the formation of Ang-(1–7) from AngI in vascular smooth muscle cells. The involvement of neutral endopeptidase in the Ang-(1–7) generation using AngI as substrate was studied in spontaneously hypertensive rats. In addition, ACE2, a newly discovered homolog of ACE, and carboxypeptidase A can generate Ang-(1–7) from AngII. The general understanding of the functionality of the RAS has become more complex
as a result of not only the description of new ligands, receptors, and enzymes that are involved in it but also because of its broad presence in several different tissues and organs, such as heart, kidney, skin, testes, ovaries, and brain.\textsuperscript{13,14}

Both the existence and the functional involvement of many components of the RAS in the brain have been widely demonstrated since 1971 when Ganten et al\textsuperscript{15} first reported renin activity in the central nervous system (CNS). For the most part, RAS activity in the CNS has been associated with the regulation of various homeostatic processes, including body temperature control, thirst, and cardiovascular events. Most findings of RAS involvement in cardiovascular events are related to the interaction between AngII and the AT\(_1\) receptor, but several reports also demonstrated the role of Ang-(1–7).\textsuperscript{16}

One previous report anticipated a possible functional role for Ang-(1–7) originated from AngI by an ACE-independent pathway in the brain,\textsuperscript{17} and more recently it has been reported that Ang-(1–7) is involved in the control of heart rate and the baroreflex via the nucleus of the tractus solitarii.\textsuperscript{18,19} Importantly, Ang-(1–7) has also been associated with the enhancement of long-term potentiation in the limbic regions of the brain (eg, the hippocampus),\textsuperscript{20} and it has been shown to be related to the memory processes that are associated with object recognition.\textsuperscript{21} Epilepsies are a pathogenic condition that affect ≈1% to 3% of the world’s population, and the limbic regions of the brain (eg, the hippocampus and the amygdala) are known to be recruited during temporal lobe epilepsy (TLE), which is the most common type of epilepsy in humans.\textsuperscript{22} Thus, we were prompted to investigate the possible role of Ang-(1–7) and its receptor, Mas, in TLE on the basis of the aforementioned observations. To conduct this investigation, we used chronically stimulated Wistar audiogenic rats (WARs), an experimental model of epilepsy associated with behavioral, electroencephalography, and cellular features of human TLE.\textsuperscript{23–27}

In previous studies, we have demonstrated that several classical components of the RAS were upregulated in the hippocampi of kindled WARs (which were used as animal models of TLE). Moreover, treatment with losartan (AT\(_1\) receptor antagonist) was capable of decreasing the severity of seizures of WARs with TLE.\textsuperscript{28} On the basis of those studies, we aimed to investigate the metabolism of AngI in the hippocampus and related enzymatic pathways. We first show that Ang-(1–7) is the AngI metabolite that is preferentially generated by hippocampal enzymes; second, we show that TOP is one of the major enzymes involved in the AngI metabolism, and it is responsible for ≈50% of the Ang-(1–7) formation in the hippocampus. Furthermore, the conversion of AngI to Ang-(1–7), the expression of TOP and receptor Mas transcripts, and protein levels were significantly increased in kindled WARs as compared with the control group.

Taken together, these findings demonstrated a possible involvement of the TOP–Ang-(1–7)–receptor Mas pathway in a model of TLE and highlighted a new potential role for RAS in complex pathophysiological conditions that affect the CNS.

**Materials and Methods**

All experiments were performed using 200- to 250-g adult female Wistar rats and WARs. The local Animal Care and Use Committee approved previously the use of animals before the start of the study (protocol FMRP-USP 200/2005). The detailed Material and Methods section, which includes experimental model of TLE,
Western blotting, evaluation of proteolytic activities, inhibition assays, and other methods, can be found in the online-only Data Supplement.

**Results**

**Evaluation of AngI-Metabolite Profile From Rat Hippocampi**

Initially, we determined the angiotensin fragments generated after performing reactions with hippocampi extracts from Wistar rats using AngI as a substrate. The profile was obtained by high-performance liquid chromatography using the following standard peptides: Ang-(1–4), Ang-(1–7), Ang-(5–8), Ang-(1–9), Ang-(3–8), Ang-(4–8), AngII, and AngI. Samples for which there was no standard peptide were analyzed by amino acid analysis. Figure 1 shows that Ang-(1–7) is the main metabolite resulting from the AngI metabolism in Wistar rats hippocampi extracts. In addition to Ang-(1–7), other angiotensin metabolites were detected to a lower extent, including Ang-(4–10), Ang-(5–10), and Ang-(8–10), identified by amino acid analysis (Figure 1). Interestingly, AngII was not detected as a metabolite, resulting from AngI degradation. Likewise, Ang-(1–9), AngIII, and AngIV were also not detected. To further investigate this observation, we repeated the reaction using AngII as the substrate, as the octapeptide AngII may also be a substrate-producing Ang-(1–7). Nevertheless, no Ang-(1–7) was detected after this reaction (Figure 2A), reinforcing that Ang-(1–7) production from AngI incubation was indeed AngII independent. We also investigated the potential involvement of ACE and serine proteases in Ang-(1–7) formation. Assays using specific inhibitors, such as chymostatin, lisinopril, and captopril, revealed that these enzymes do not contribute to AngI metabolism (Figure 2B and 2D). We have not performed assays with ACE2 inhibitors activity because we did not observe formation of either Ang-(1–9) or Ang-(1–7) when using AngI or AngII as substrates, respectively (Figure 1B and 1C), and therefore concluded that in the hippocampus there is no significant ACE2 activity for these 2 substrates. Taken together, these data suggest that Ang-(1–7) formation may result from an endopeptidase activity.

**TOP Is the Main Enzyme Responsible for the Generation of Ang-(1–7) in Rat Hippocampi**

To determine which endopeptidases could participate in the generation of Ang-(1–7) from AngI, we performed specific assays in the presence of neutral endopeptidase and TOP inhibitors (phosphoramidon and JA-2, respectively). Our results show that incubation with 10 μmol/L phosphoramidon did not affect the AngI metabolism profile, whereas incubation with 10 μmol/L JA-2 inhibited ≈60% of Ang-(1–7) production relative to the level of Ang-(1–7) production in a control reaction (Figure 3A). It is interesting to note that the inhibition assay performed with 0.3 μmol/L JA-2 already resulted in ≈50% of inhibition (Figure 3B). The highest concentration of JA-2 tested was 30 μmol/L resulting in ≈75% inhibition.

**Figure 2.** Representative profile of the inhibition assay of rat hippocampal extracts with angiotensin I (AngI). Hippocampal extracts were preincubated without inhibitor (A), with chymostatin 100 μmol/L (B), with lisinopril 10 μmol/L (C), and with captopril 10 μmol/L (D) before AngI incubation. Angiotensin fragments were identified by reverse-phase high-performance liquid chromatography using peptide standards.
TOP activity in the hippocampus was also evaluated using a pull-down assay with a specific anti-TOP antibody. The formation of Ang-(1–7) was decreased by ≈50% after pull-down with the anti-TOP antibody compared with Ang-(1–7) formation after the control reaction (Figure 3C); this finding corroborates the data obtained with the inhibition assay. On the basis of these results, we concluded that the endopeptidase TOP is the main enzyme involved in the production of Ang-(1–7) in rat hippocampi.

**Evaluation of TOP and Receptor Mas Expression Levels and Ang-(1–7) Formation From Hippocampi of Kindled W ARs (An Animal Model of TLE)**

Multiple inductions of audiogenic seizures in the W AR strain lead to recruitment of limbic areas, such as the hippocampus, mimicking the human TLE.24,25 This involvement prompted us to investigate the AngI metabolism in the hippocampi of W ARs with TLE, following the same conditions of assay as performed with hippocampi of Wistar rats. Our results show that in this model of epilepsy, Ang-(1–7) is also the most prevalent metabolite of AngI in the hippocampus (Figure 4A). Moreover, it is worth noting that we found evidence of an ≈2-fold increase in Ang-(1–7) formation in W ARs with TLE (14 days of stimuli, W AR-14 dS) as compared with normal Wistar rats and W ARs that did not receive acoustic stimulation (Figure 4B).

We also analyzed the levels of the receptor Mas and TOP in the hippocampi of W ARs with TLE. We observed a coordinated upregulation of the receptor Mas at both the transcript and the protein levels (3.8- and 5.4-fold, respectively; Figure 5). We also observed a significant 2.6-fold increase of TOP protein level in W ARs with TLE, as compared with Wistar rats and W ARs without acoustic stimulation (Figure 6). Unexpectedly, TOP transcript levels were not upregulated, suggesting that the cellular content of this peptidase might be preferentially regulated by protein trafficking and secretion rather than by gene expression.30

**Discussion**

The central role of the peptide Ang-(1–7) has remained unclear for many years since its presence in the brain was described.31
The first report on the biological activity of Ang-(1–7) was published by Schiavone et al. when it was described that this peptide was responsible for releasing vasopressin from the rat hypothalamo-neurohypophyseal system. Chappell et al. also described the endogenous presence of Ang-(1–7) in the CNS and showed that this peptide was the most common metabolite of AngI degradation, where AngII was an intermediate substrate, in both the amygdala and the hypothalamus. One year before, Santos et al. have shown that Ang(1–7) was the major metabolite generated from AngI in the dog brain stem, and that it was not affected by ACE inhibition, suggesting that other enzymes could be involved. Although TOP participation in the generation of Ang-(1–7) was first reported in vascular smooth muscle cells, it had been described previously to act on other neuropeptides, and some studies have found that this enzyme is widely distributed among different brain areas, such as cerebellum, hippocampus, cortex, and nucleus tractus solitarii.

In the current study, we show that Ang-(1–7) is the preferential peptide generated from AngI metabolism in hippocampal extracts from rats. Importantly, we describe that TOP plays a major enzymatic contribution to Ang-(1–7) formation, and that AngII formation is not observed. Previous reports have described the presence of AngII-generating enzymes, such as ACE or serine proteases, in the rat amygdala and hypothalamus, and indeed our own group has reported previously ACE activity in the hippocampus of rats when assessed using the ACE-specific substrate Hip-His-Leu. Here, it is important to highlight that in the present study, enzymatic activities were assessed by metabolites formation using the promiscuous substrate AngI, which allowed evaluating the action of other enzymes than only ACE. Therefore, taking our previous and the present results together, the lack of AngII formation from AngI suggests that, despite the likely presence and activity of local ACE, it is probably not the favored enzyme to act on AngI metabolism. We think that this differential regional processing of angiotensin peptides in the brain may be of functional relevance.

Since 1998, the role of the Ang-(1–7)–receptor Mas axis has been investigated in learning and memory. The target disruption of receptor Mas leads to anxious behavior and increased duration of long-term potentiation (LTP) in the dentate gyrus in the 129/C57BL/6 genetic background mice. Hellner et al. showed that in the CA1 hippocampal region high-frequency stimulation-induced LTP was significantly more pronounced in Mas knockout (Mas−/−) mice than in controls. However, in Mas−/− mice, Ang-(1–7) caused a significant decrease in CA1-LTP, probably via AT1 receptor because losartan recovers the decrease in CA1-LTP. Furthermore, in the wild-type mice, Ang-(1–7) acting via receptor Mas was able to increase LTP. The Mas ablation in the FVB/N genetic background mice and the blockade of receptor Mas in CA1 in...
control animals were able to impair object recognition memory. Moreover, the blockade of AT₁, but not of AT₂, recovers the object recognition memory impairment in the FVB/N-Mas knockout. Although the authors of those studies associate their data with memory and learning processes specifically, limbic regions, such as the amygdala and the hippocampus, are also known to participate in certain neurological disorders, such as the epilepsies. Therefore, we have extended our investigation of the functional role(s) of the Ang-(1–7)–receptor Mas axis in the CNS including the evaluation of hippocampi from epileptic animals from the WAR strain. It is pertinent to note that the limbic seizures in WARs are considered a representative model of TLE,26,27 which is the most common type of epilepsy in humans.22 Those kind of seizures are associated with behavioral, structural, and electroencephalographic alterations, which are coupled with the recruitment of forebrain regions, such as the hippocampus, during epileptic events.24,26,37 There are some reports on the role of angiotensins in epileptic events. For example, when applied via intrahippocampal microinjections in rats, AngII has an excitatory effect.38 Additional studies have demonstrated that AngIV (which is produced via AngII degradation) has both anticonvulsant and antiepileptic effects.39 In a previous study, we found evidence of upregulation of ACE and of the AT₁ receptor in the hippocampi of WARs with TLE, and we showed that blockade of the AT₁ receptor by systemic administration of the antagonist losartan significantly decreased the severities of seizures, therefore suggesting a key role for AngII. Nevertheless, as described above, we surprisingly found Ang-(1–7) to be the main metabolite generated after AngI degradation, whereas AngII formation was not detected. At a first glance this might seem contradictory, but in our previous work,28 despite the observation of ACE upregulation, it was not possible to assure that AngII would be eventually generated from AngI metabolism because ACE activity was evaluated using the synthetic substrate Hip-His-Leu that is a selective substrate cleavable only by ACE. Similarly, high ACE activity has been reported before in dog brain stem extracts using Hip-His-Leu as substrate, but Ang-(1–7) formation showed to be independent of ACE activity.17 Besides that, AngII can also be generated in other regions that are close or connected to the hippocampus. Our previous work28 also showed that treatment with enalapril was able to decrease the seizures in the WAR strain, therefore suggesting a relevant contribution of ACE. Taking the previous and the current data together, we think that those apparently contradictory results must be discussed considering that both enalapril and losartan treatments were performed systemically, and therefore ACE and AT₁ receptor activities were impaired in other CNS regions that could also contribute in seizure triggering. Concerning the hippocampus, our previous and current data suggest that although there is ACE activity, it must be disfavored by the presence of other angiotensin-generating enzymes, mainly by TOP activity as suggested by our current study.

In this work, we presented evidence of a novel enzymatic pathway for Ang-(1–7) production from AngI in hippocampi from Wistar rats, consisting of TOP as the main enzyme responsible for this conversion. Besides the higher Ang-(1–7) generation from AngI in hippocampi extracts from epileptic animals, we also found an upregulation of TOP and receptor Mas expression levels in this tissue from epileptic animals, suggesting a possible correlation between this pathway and its potential functional role in epileptogenesis or the establishment of TLE. Furthermore, we can suggest that TOP–Ang-(1–7)–receptor Mas axis may have a possible role in the pathogenesis of other neurological disorders that are associated with the hippocampus; however, further studies are needed to investigate this hypothesis. Taken together, the novelty of our findings is that we revealed a new enzymatic pathway for AngI metabolism in hippocampus, leading to AngII-independent Ang-(1–7) production.

**Perspectives**

Our study sheds light on the TOP–Ang-(1–7)–receptor Mas axis as possibly having a major functional role in RAS activity in the hippocampus. Investigation of this axis and of its relevance in neurological disorders and their associated pathologies certainly represent a rich avenue of research.

**Acknowledgments**

We thank Professor Emer Suavinho Ferro from ICB at University of São Paulo for the kind gift of thimet oligopeptidase inhibitor (JA-2) and antibodies.

**Sources of Funding**

This study was supported by the São Paulo State Research Foundation (FAPESP, Grants 02/09406-5, 06/61810-6, 07/50261-4, and FAPESP-Cinapce 05/56447-7) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). C.M. Costa-Neto, M.D. Gomes, E.B. Oliveira, N. Garcia-Cairasco, and M.C.O. Salgado hold CNPq Research Fellowships.

**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is New?**
- Angiotensin-(1–7) (Ang-(1–7)) is an active heptapeptide of the renin–angiotensin system, classically generated by angiotensin-converting enzyme 2 action over AngII. In this article, we identified that Ang-(1–7) is the main peptide in the rat hippocampus and is generated by an AngII-independent and angiotensin-converting enzyme 2–independent fashion, involving the enzymatic action of the endopeptidase thimet oligopeptidase.

**What Is Relevant?**
- Our data describe the Ang-II–thimet oligopeptidase–Ang-(1–7)–receptor Mas axis as a pivotal pathway in the hippocampus and strongly suggest that it may play a role in central diseases, which in turn are relevant issues to hypertension and other vascular disorders. This new axis also sheds light on possible new targets to functional blockade or modulation of the renin–angiotensin system.

**Summary**
In this study, we describe an AngII-independent biochemical pathway for generation of Ang-(1–7) in the central nervous system. Various elements of this pathway, including the enzyme thimet oligopeptidase and the receptor Mas, show an increased expression level in animals after epileptic seizures, unveiling potential new roles for the renin–angiotensin system in central nervous system pathophysiology.
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Hypertension. 2013;62:879-885; originally published online September 16, 2013; doi: 10.1161/HYPERTENSIONAHA.113.01613

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Expanded Materials and Methods

Animals

Experiments were performed using 200-250 g adult female Wistar rats and WARs; 2-day-old newborn animals were also used in some experiments. Food and water were provided ad libitum to the animals, and all of the animals were housed in a controlled environment with a constant 12:12-hour light-dark cycle. Experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee prior to the start of the study (protocol FMRP-USP 200/2005).

Audiogenic kindling as an experimental model of TLE and seizure severity evaluation

In an acoustically isolated chamber, individual animals were exposed to a high-intensity sound (120 dB SPL) until either the onset of a tonic seizure or a maximum of 1 min had elapsed. Behavior was recorded for an additional 1 min. Acoustic stimulation was either performed once (single stimulus group), or it was performed twice a day at fixed times over a period of 2 weeks (14-day stimuli). The severities of any tonic-clonic seizures that occurred were evaluated on the basis of the categorized mesencephalic severity index,\(^1\) and TLE seizures were evaluated on the basis of Racine’s scale (a limbic index).\(^2\)
Tissue collection, RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

All of the tissues that were analyzed were collected in an RNAse-free environment and were immediately frozen in liquid nitrogen. Unless otherwise specified, all of the reagents that we used were obtained from Invitrogen (Carlsbad, CA, USA). Frozen tissues were pulverized and homogenized in Trizol reagent, and the total RNA from each sample was isolated according to the instructions from the manufacturer. RNA samples were then treated with DNase (Amersham Pharmacia; Piscataway, NJ, USA) for 15 min at room temperature to remove any potential genomic DNA contaminants. The RNA concentration in each sample was measured spectrophotometrically using a wavelength of 260 nm. Oligo-dTs were used to generate cDNA from the total RNA samples (1 µg of total RNA from each sample was used in the generation of cDNAs). Specific primers for the PCR amplification of transcript fragments corresponding to receptor MAS and β-actin were designed on the basis of the available GenBank sequences for these genes and were used to perform PCR using Platinum Taq polymerase (Invitrogen Life Technologies, Grand Island, NY, USA). The PCR products were densitometrically quantified using ImageJ software (http://rsb.info.nih.gov/ij) after being separated in agarose gels.

Measurement of TOP and receptor Mas protein levels by Western blotting

After normalizing the protein concentrations of the samples (50 µg of protein per sample), the protein extracts were subjected to 12% SDS-PAGE and were subsequently
transferred to nitrocellulose membranes (GE Healthcare, Pittsburgh, PA, USA). The membranes were blocked in a 1% solution of BSA, and they were then incubated overnight with a 1:500 solution of rabbit anti-receptor Mas (Cell Signaling Technology, Inc., Boston, MA, U.S.A.) and a 1:3000 solution of rabbit anti-TOP (provided by Prof. Emer Suavinho Ferro) at a temperature of 4°C as described before. Following incubation with the primary antibodies, the membranes were incubated with a secondary horseradish peroxidase-conjugated antibody (1:2000 dilution, KPL, Maryland, VA, USA) for 1 h at room temperature and were subsequently developed using the ECL system (Santa Cruz, Santa Cruz, CA, USA) for chemiluminescence. In addition, the membranes were stripped and were subsequently incubated with a monoclonal anti-β-actin antibody (1:10000 dilution, Millipore, Billerica, MA, USA) for 1 h at room temperature. The Image J software program (http://rsbweb.nih.gov/ij/) was used to densitometrically quantify band intensities blottings.

**Tissue preparation and determination of proteolytic activities in the hippocampus**

Each hippocampus was removed and was subsequently frozen in liquid nitrogen until processing. The HPLC profiles of angiotensin fragments that were generated via AngI metabolism were investigated to establish the proteolytic activities of the hippocampus over AngI.

The frozen hippocampal tissues were homogenized in 2 ml of 0.02 M potassium phosphate buffer (pH 8.3) and were centrifuged at 40,000 g for 20 min at 4°C. From this preparation, 50 μL of supernatant was incubated with 50 μL of a reaction mixture with a pH of 8.3 for 2 h at 37°C. The reaction mixture contained 0.3 M NaCl, 0.01% Triton X-
100, $10^{-4}$ M ZnCl$_2$ and 25 nmol of AngI (as the substrate) in a 0.1 M phosphate buffer solution.

Reactions were terminated by the addition of 4 $\mu$L of a 5% trifluoroacetic acid (TFA) solution and 96 $\mu$L of water, and the resultant Ang I fragments were separated via reversed-phase HPLC using Shimadzu 6B equipment that had been fitted with a Shim-Pack CLC-ODS column (4.6 x 150 mm) and an ultraviolet detector at a wavelength of 215 nm. Separations were performed over a 25–47% linear gradient of acetonitrile concentration in a 0.1% TFA solution at a flow rate of 1.0 ml/min. Each of the generated angiotensin fragments was identified by comparison of its retention time with the retention times of standard peptides.

**Inhibition assays**

Drugs used: captopril, lisinopril, chymostatin, phosphoramidon (Sigma Chemical, St. Louis, MO, USA); JA-2 (provided by Prof. Emer Suavinho Ferro).

To evaluate the activities of possible enzymes involved in the formation of Ang-(1-7), we used specific inhibitors for ACE (10 $\mu$M captopril or 10 $\mu$M lisinopril), serine proteases (100 $\mu$M chymostatin), NEP (10 $\mu$M phosphoramidon) and TOP (0.3, 1, 3, 10, 20 and 30 $\mu$M JA-2). The hippocampal extracts were pre-incubated with these inhibitors 30 min before the reaction with AngI; these reactions were performed in the same manner as the reactions described in the previous section.

Supernatants from the centrifuged hippocampal extracts were incubated with 1 $\mu$L of anti-TOP, 1 $\mu$L of anti-cyclophilin and 1 $\mu$L of Protein A Sepharose for 24 h at 4°C with gentle rocking. Following incubation, the treated extract preparations were subjected
to 1 h of pull-down with 10 µl of Protein A Sepharose beads (Invitrogen, Grand Island, NY, USA) at 4°C with gentle rocking and were then centrifuged at 3,000 rpm for 15 min at 4 °C. The supernatants were incubated with AngI as described in the previous section.

**Statistical Analyses**

Data are given as means±s.e.m. of the indicated number of independent experiments. Statistical analyses were performed using one- or two-way analyses of variance (ANOVAs) followed by either Newman-Keuls multiple comparison tests or unpaired Student's t-tests, as appropriate. Statistical analysis was performed using the GraphPad Prism software program (San Diego, CA, USA), and a p-value of $P<0.05$ was used to establish statistical significance.

**References cited in the Expanded Materials and Methods**

