Abstract—Intracerebroventricular administration of angiotensins causes pronounced pressor and dipsogenic responses. The suggestion that angiotensin III rather than angiotensin II is the active peptide in the brain spawned what we call The Angiotensin III Hypothesis. To test this hypothesis, 5 angiotensin II analogs containing zero or one position substitutions conferring resistance to aminopeptidases were administered intracerebroventricularly to determine their pressor and dipsogenic efficacies. Two aminopeptidase-resistant analogs caused significantly greater pressor responses than angiotensin II, whereas 3 analogs caused pressor responses similar to angiotensin II. Latency to cause a pressor response for 4 of the 5 aminopeptidase-resistant angiotensin II analogs was the same as for angiotensin II. There was no detectable formation of 125I-angiotensin III from 1 of the intracerebroventricularly administered analogs, 125I- N-Methyl-L-Asp1-angiotensin II, indicating its aminopeptidase resistance. Latency to drink also did not differ between the angiotensins. After the initial dipsogenic response, water was removed until 25 minutes after angiotensin administration to avoid interfering with the pressor response. The dipsogenic stimulus was sustained 25 minutes after intracerebroventricular injection of angiotensin II and its aminopeptidase-resistant analogs. Comparison of angiotensin III and angiotensin II showed equivalent pressor responses with similar latencies and durations. The latency to drink was similar for angiotensin III and angiotensin II. However, there was no dipsogenic response to angiotensin III 25 minutes after intracerebroventricular injection. These data do not support The Angiotensin III Hypothesis and suggest that conversion of exogenously applied angiotensin II to angiotensin III is not necessary to cause brain-mediated pressor or dipsogenic responses. (Hypertension. 2007;49:1328-1335.)

Key Words: brain angiotensin receptors ■ intracerebroventricular ■ dipsogenesis ■ metabolism

The brain rennin–angiotensin system (RAS) is distinct from the peripheral RAS and regulated independently.1–3 Hyperactivity of the brain RAS is known to cause hypertension.4 Angiotensin II (Ang II) receptors in circumventricular organs (which respond to both blood-borne and centrally produced Ang II), as well as in the hypothalamus and brain stem, cause cardiovascular, endocrine, and behavioral effects.5 Centrally administered angiotensins stimulate both angiotensin II type 1 (AT1) and type 2 (AT2) receptor subtypes in the brain, but the cardiovascular effects arise from AT1 receptor-mediated vasopressin, oxytocin and aldosterone release, activation of sympathetic neuronal activity, and enhanced thirst and salt appetite.6,7 Fitzsimons demonstrated that angiotensin III (Ang III) which is des Asp1 Ang II also acts in the brain, to cause thirst.8 Subsequent studies demonstrated that intracerebroventricular (ICV) Ang II and Ang III were equipotent at causing dipsogenic and pressor responses.9 Harding and Felix10 were able to increase the response to Ang III by iontophoretically applying the aminopeptidase inhibitor bestatin into the paraventricular nucleus of the hypothalamus (PVH). They also administered amastatin, which was thought to inhibit the metabolism of Ang II to Ang III, and blocked the ability of iontophoretically administered Ang II to activate neurons of the PVH.10 This was interpreted to mean that Ang II must be converted to Ang III in the brain to activate Ang II receptors in the PVH10 and can be viewed as the initial evidence on which The Angiotensin III Hypothesis was based. Additional studies using amastatin and bestatin as aminopeptidase inhibitors to examine the ability of ICV-administered Ang II and Ang III to cause dipsogenic and pressor responses were also interpreted as being consistent with a primary role of Ang III in mediating the activation of brain Ang II receptors.11 However, Zini et al12 challenged these observations, because
Ang III is necessary to activate brain AT₁ receptors mediating pressor and dipsogenic responses.\textsuperscript{10–12,14,15}

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Harlan, Indianapolis, Ind) 300 to 450 g were fed with Purina 5001 rodent chow and given water ad libitum. Artificial light (12 hour light/dark cycle) was maintained. All animal procedures were carried out using a protocol approved by the University of Mississippi IACUC and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Telemetry Probe and ICV Guide Cannula Implantation

Rats were anesthetized with isoflurane gas anesthesia (Halocarbon Laboratories) and the abdominal area was cleaned for a midline surgical incision along the abdomen. Sterile conditions were maintained throughout all surgical procedures. The lower portion of the abdominal aorta was exposed. A small nick was made at the site of aortic entry with Vetebond (3 mol/L, IACUC approved products). The transmitter was placed into the peritoneal cavity and the incision was sutured, wound clamped, and wiped with chlorhexidine.

Animals were next placed in a stereotaxic apparatus equipped with a nose cone to sustain anesthesia. A midline incision exposed the skull and the stereotaxic coordinates for guide cannula insertion were marked. A stainless steel 22-gauge guide cannula with an attachable dummy cannula stylette (Plastics One) was implanted at AP: −1.0 mm bregma, L: 1.5 mm DV: 2.5 mm. Dental cement was used to secure the guide cannula to 2 anchoring screws and the cranium. Postoperative ketoprofen, 5 mg/kg (Ft Dodge Animal Health) was administered intramuscularly. The animals were recovered 3 to 6 days before the onset of experimental procedures.

Angiotensin Analog Syntheses

The following analogs of Ang II: N-Methyl-L-Asp₁-Ang II, β-D-Asp₁-Ang II, Beta-L-Asp₁-Ang II, D-Ala₁ Ang II, Sar⁰ Ang II (Figure 1) were synthesized using Fmoc chemistry on an Applied Biosystems 431A peptide synthesizer, according to manufacturer’s instructions and purified by high pressure liquid chromatography (HPLC) using a C₁₈ column. Verification of the molecular weight of the purified peptide was made by mass spectroscopic analysis. Human/ rat Ang II was purchased from commercial suppliers. The analogs and Ang II were diluted to 50 μmol/L in artificial cerebrospinal fluid (aCSF), NaCl 128 mmol/L, KCl 2.5 mmol/L, CaCl₂ 1.3 mmol/L, MgCl₂ 0.93 mmol/L.

Testing Protocol

Blood pressure and heart rate data were collected continuously and averaged at 10 second intervals. Ang II and its analogs were administered after steady baseline blood pressures and heart rates were obtained.

The experimental design was: Day 1: Each rat was injected with Ang II, 100 pmol in 2 μL of aCSF, to determine the animal’s responsiveness during a 40-minute period. Approximately 5 minutes before dosing, the injector cannula, attached to a 10-μL Hamilton syringe via a long (~60 cm) piece of polyethylene tubing, was inserted into the guide cannula. The injector cannula extended 1 to 2 mm beyond the guide cannula. After the rat’s blood pressure returned to a stable baseline, Ang II was slowly administered, ~30 seconds. Immediately on initiation of a drinking response after administration of Ang II a time mark was recorded to indicate latency of the dipsogenic response and the water bottle was removed from the cage so the drinking response would not interfere with the pressor response. Approximately 5 minutes after administration of Ang II the injector cannula was unobtrusively removed from the

Figure 1. 0 or 1 position amino acid structures of Ang II and aminopeptidase-resistant Ang II analogs.

These aminopeptidase inhibitors do not selectively alter Ang II and Ang III metabolism.

However, development and use of more specific aminopeptidase inhibitors reinforced the suggestion that conversion of Ang II to Ang III was required for activation of brain AT₁ receptors. The specific aminopeptidase A (E.C.3.4.11.7, APA) inhibitor, ([R,S]-3-aminomercaptobutanesulfonate), named EC33,\textsuperscript{13} substantially reduced vasopressin release to ICV Ang II in mice\textsuperscript{12} and the pressor response to ICV Ang II in rats.\textsuperscript{14} Another aminopeptidase inhibitor, ([S]-2-aminopentan-1,5-dithiol) named EC27,\textsuperscript{13} selective for aminopeptidase N (E.C. 3.4.11.2, APN), which can protect Ang III from metabolic inactivation, increased vasopressin release to ICV Ang III in mice.\textsuperscript{15} Additional evidence in support of the Angiotensin III Hypothesis was the report that ICV administration of Ang II analogs given ICV to cause pressor and dipsogenic actions.12,15

Pursuant to the formulation of the Angiotensin Hypothesis, efforts have been directed to developing inhibitors of APA in the brain as potential antihypertensive agents.\textsuperscript{16,17} However, for such agents to be efficacious, there needs to be absolutely certain that brain Ang II is unable to cause pressor pressor actions of ICV Ang II in rats, without affecting responses to ICV Ang III.\textsuperscript{15}

In the present study, a series of 0 and 1-substituted analogs of Ang II were synthesized (Figure 1) which varied in their amino acid characteristics of aminopeptidase resistance.\textsuperscript{19} The ability of these Ang II analogs given ICV to cause pressor and dipsogenic responses in rats was compared with Ang II to test the Angiotensin III Hypothesis, that conversion of Ang II to
were prepared and administered ICV. Please see supplemental data, Figures S1–S4, for recordings of other analogs. All analogs caused short latency pressor responses, and a subsequent dipsogenic response. Both the pressor and dipsogenic responses to N-Methyl-L-Asp\(^{1}\)-Ang II were inhibited (76% and 96%, respectively) by the AT-1 antagonist losartan. Please see supplemental data, Figure S5, which shows this antagonism.

Ang II increased MAP by 18.6±0.7 mm Hg. N-methyl-L-Asp\(^{1}\)-Ang II and Beta-D-Asp\(^{1}\)-Ang II caused 26 and 8% greater increases in MAP (t=2.9, P=0.021; t=2.7, P=0.029, respectively) than Ang II (Figure 4). The pressor responses to the other 3 aminopeptidase-resistant Ang II analogs did not differ significantly from Ang II (Figure 4).
The peak pressor response to Ang II occurred 93.4 ± 6.8 sec after ICV administration. The latency to attain the peak pressor response to the aminopeptidase-resistant Ang II analogs did not differ from Ang II, except for Beta- L-Asp1-Ang II (paired t = 2.6, P = 0.034) which showed a longer (150 ± 31%) latency to attain an initial peak pressor response compared with Ang II (Figure 5). The duration of the pressor response to aminopeptidase-resistant Ang II analogs was not significantly different from Ang II (Table). The change in heart rate corresponding to the initial peak pressor response to the aminopeptidase-resistant Ang II analogs (Table) also was not significantly different from the response to Ang II.

There was no significant difference in the latency of the dipsogenic response to Ang II and its aminopeptidase-resistant analogs (Table). The dipsogenic response (Figures 2 and 3) was sustained 25 minutes after ICV administration of Ang II and its aminopeptidase-resistant analogs. As shown in Figures 2 and 3, rats quickly began to drink after placement of a calibrated drinking tube into the cage. The amount of water consumed (Figure 6) 25 minutes after the ICV injection of Ang II was 5.9 ± 0.5 mL. The dipsogenic response to D-Ala1 Ang II was 50% greater (paired t = 2.5, P = 0.035) than for Ang II, whereas the dipsogenic response to B-d-Asp1-Ang II was 50% less (paired t = 2.81, P = 0.026) than for Ang II (Figure 6). The dipsogenic responses of the other aminopeptidase-resistant Ang II analogs did not differ significantly from Ang II.

Because most studies of aminopeptidase resistant analogs of Ang II have used peripheral tissues, we investigated the aminopeptidase resistance of one of the novel analogs used for this study by radiolabeling it and comparing its metabolic fate with that of Ang II in the brain. Both 125I/127I-Ang II and 125I/127I-N-Methyl-L-Asp1/Ang II caused short-latency pressor and dipsogenic responses that were not qualitatively different from those of the uniodinated peptides (data not shown). In rats infused with 125I/127I-Ang II, little or no 125I-Ang II was detected in the brain extract of rats euthanized at the time of initiation of the dipsogenic response. However, 125I-Ang III, 125I-Ang IV, and 125I-Ang4–8 (Figure 7A) were detectable. In rats infused with 125I/127I-N-Methyl-L-Asp1/Ang II, a small fraction of 125I-N-Methyl-L-Asp1/Ang II was present in the brain extracts of the 125I/127I-N-Methyl-L-Asp1/Ang II-treated rats. However, there was no detectable 125I-Ang III or 125I-Ang IV in any of the extracts tested (Figure 7B).

To further address the question of conversion of Ang II to Ang III, 100 pmoles of Ang III and Ang II were administered ICV to a subset of 3 rats. There was no significant difference in latency to drink, although the response to Ang II (105 ± 5 s) tended to be faster than with Ang III (156 ± 42 s). The latency to attain the maximal pressor response to Ang II (71.5 ± 3.8 s) was moderately, but not significantly faster than Ang III (103 ± 29 s). There was an equivalent increase in blood pressure with Ang II and Ang III (Ang II 17.4 ± 2.5 mm Hg, Ang III 17.2 ± 1.0 mm Hg). There was a tendency for the duration of the pressor response to Ang II (14.2 ± 4.5 minutes) to be longer than for Ang III (7.9 ± 0.8 minutes). Finally, when the calibrated drinking tube was placed into the cage 25 minutes after ICV administration of Ang III, the rats did not drink. This contrasts with the copious, short-latency drinking response with Ang II (3.1 ± 0.5 mL).

**Discussion**

In this study, aminopeptidase-resistant analogs of Ang II with varying similarity to Ang II were shown to be equivalent or more active pressor and dipsogenic agents than Ang II in the brain. N-methyl-L-Asp1-Ang II and Beta-d-Asp1-Ang II caused pressor responses that were significantly greater than those to paired administration of Ang II, which argues strongly against requisite formation of Ang III. If aminopeptidases could metabolize these analogs, the product would be Ang III, and their effects would not differ from Ang II if formation of Ang III is required for activity.
All but one of the aminopeptidase-resistant analogs caused pressor and dipsogenic effects with equivalent or shorter latency than Ang II. Because these analogs should undergo slower metabolism to Ang III, this also suggests that metabolism to Ang III is not required for Ang II–induced dipsogenic or pressor actions.

The proposed lack of responsivity of brain AT₁ receptors to Ang II implies that brain AT₁ receptors differ from peripheral AT₁ receptors, which are considered to be fully responsive to Ang II. If this is the case, then Ang II either does not bind to AT₁ receptors in the brain, or Ang II binds to brain AT₁ receptors without causing a response. This latter explanation would characterize Ang II as an antagonist at brain AT₁ receptors. Consistent with this latter possibility, Harding and Felix reported that Sar⁴ Ang II blocked both Ang II– and Ang III–induced neuronal firing in septal neurons.

With respect to Ang II binding to AT₁ receptors in the brain, there continue to be many unanswered questions. Receptor autoradiographic studies of brain angiotensin receptors with Ang II suggest that it binds to brain regions known to be responsive to exogenously applied Ang II. However, to our knowledge, no one has verified that what is bound is Ang II and not Ang III. Abhold et al. identified both Ang II and Ang III, bound with high affinity and specificity, to rat brain membranes. But, there was an additional low affinity binding site exclusive to Ang II. Thus although Ang II appears to be able to bind to brain AT₁ receptors, it is still unclear how this might...
compare with the ability of Ang III to bind to and activate these receptors.

The duration of the pressor response after ICV-administered aminopeptidase-resistant Ang II analogs did not differ statistically from paired Ang II responses. This could indicate similar sustained activations of intracellular signaling mechanisms. But, more likely it indicates that the peptides are metabolically inactivated at a similar rate. Other metabolic routes for Ang II via diaminopeptidases, carboxypeptidases, or endopeptidases can occur24-27 (see also review in Reference 22).

It is noteworthy that the pressor effects of Ang II and its analogs preceded the dipsogenic response. Blood pressure is elevated during drinking behavior28 so this observation rules out the possibility that the increase in blood pressure is secondary to the drinking response. Camargo et al29 stated that ICV administration of Ang II into the subfornical organ (SFO) caused a pressor response that preceded the dipsogenic response. However, no data were presented to document that statement. The present results confirm this claim. The pressor response to Ang III also preceded the dipsogenic response.

Water access was prevented after onset of a dipsogenic response to preclude its interference with the pressor response.28 Surprisingly, avid dipsogenic responses were sustained 25 minutes after ICV administration of Ang II and its aminopeptidase-resistant analogs, but not Ang III. A transient pressor response accompanied this dipsogenic response. Ang II, as well as Ang III, are rapidly metabolized in the rat30 as well as the mouse12 brain, and the pressor response had largely dissipated. Dipsogenic responses to ICV Ang II rarely last more than 15 minutes.9,31 A similar phenomenon has been observed by Fregly and Rowland32 and Evered33 after subcutaneous and intravenous, respectively, administration of Ang II to rats. Rats denied access to water retained a subcutaneous and intravenous, respectively, administration of Ang II and 3H-Ang III in mouse brain suggest that only a small fraction (≤15%) of Ang II metabolism is inhibited by EC33.12 Moreover, less than 7% of 3H-Ang II is present as 3H-Ang III after 30 seconds, regardless of whether mice were pretreated with EC33 or EC27.12 This also suggests that other brain peptidases may be major determinants of Ang II metabolism. Because the reported specificity of EC33 and EC27 is based only on their selectivity for APA versus APN, it is not known whether these inhibitors affect other brain peptidases.

A third concern arises from the 3- to 10-fold greater amounts of EC27 administered into the mouse brain relative to EC33.12 In vitro studies of EC27 and EC33 indicated that EC27 was ≈8-fold more potent at inhibiting APN than EC33 was at inhibiting APA.13 This amount of EC27 would have been sufficient to inhibit APA as effectively as the 10-fold lower amount of EC33. Also unexplained is the need to use a higher amount of Ang III relative to Ang II in the studies of both Reaux et al14 and Song et al.15 If Ang II must be converted to Ang III to be active, then one might expect Ang II to be substantially less potent than Ang III, because only a fraction of 3H-Ang II is metabolized to 3H-Ang III.12

Lastly, is the question of whether EC33 affects the stimulation of vasopressin release by Ang III and whether EC27 affects the stimulation of vasopressin release by Ang II. These were not determined in the original mouse studies.12 Because EC27 increased the half-life of 3H-Ang III derived from ICV-administered 3H-Ang II,12 EC27 should be able to increase vasopressin release to ICV Ang II. However, the pressor response to ICV administered D-Asp1 Ang II in rats after administration of PC18 was not prolonged.46 Recently,
we reported that PC18 and EC33 inhibited the dipsgenic and saline intake responses of rats to an aminopeptidase-resistant Ang II given ICV, and that PC18 delayed the dipspogenic and saline intake responses of rats to an aminopeptidase-resistant Ang III given ICV.43 This again suggests possible adverse effects of these inhibitors on AT1 receptor function in the rat brain.

Perspectives
This study contradicts the hypothesis that Ang III is the only bioactive angiotensin in the brain. Ang II analogs with characteristics of aminopeptidase resistance, but with the pharmacophore of Ang II in the position one amino acid, retain or exceed the pressor and dipspogenic activity of Ang II when administered ICV. Also, Ang II, its aminopeptidase-resistant analogs, and Ang III showed no significant difference in latency to initial peak pressor response, which argues against a requisite formation of Ang III to effect a response.

In view of the long period of time that has elapsed between the first reports of The Angiotensin III Hypothesis, and this challenge to that hypothesis, additional testing of this hypothesis should be sought. It is possible that heretofore undefined mechanisms of receptor-agonist interactions could affect the responses we have observed in this study and alter the conclusions. Other aminopeptidase inhibitors such as glutamate phosphate66 should be used to determine whether the inhibition of the actions of Ang II in the brain by EC33 arise solely from its ability to inhibit aminopeptidase A, or if they derive from other effects, unrelated to aminopeptidase A inhibition. Ongoing studies in our laboratory are addressing the ability of radiolabeled and unlabeled Ang II, Ang III, and aminopeptidase-resistant analogs of Ang II to bind to brain Ang II receptors.

It is critical to resolve this question in view of the importance of the brain renin–angiotensin system as a regulator of blood pressure6 and the proposed development of antihypertensive agents whose mechanism of action is the blockade of conversion of Ang II to Ang III in the brain by inhibiting brain aminopeptidase A.16,17

Acknowledgments
Thanks to Drew Hendon for his technical support and Gerhard Munske for the synthesis of the Ang II analogs used in this study. We thank our colleagues Steven Cutler, Ziaeddin Shariat-Madar, and Kristine Willett for their thoughtful and critical review of this manuscript. Losartan was a gift of Dr Ron Smith, DuPont-Merck, Whitehouse, New Jersey.

Sources of Funding
This work was supported by funding from the American Heart Association, Grant-in-Aid 0350481Z, and the Peptide Radioidination Service Center, University of Mississippi.

Disclosures
None.

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Central Pressor Actions of Aminopeptidase-Resistant Angiotensin II Analogs: Challenging the Angiotensin III Hypothesis
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Hypertension. 2007;49:1328-1335; originally published online April 30, 2007; doi: 10.1161/HYPERTENSIONAHA.107.087130
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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Supplementary Material


Materials and Methods:
Analysis of in vivo metabolism of Ang II and N-Methyl-L-Asp\textsuperscript{1} Ang II: To assess the metabolism of the exogenously administered Ang II and aminopeptidase-resistant Ang II analogs, instrumented rats were administered \textsuperscript{125/127}I-Ang II or \textsuperscript{125/127}I-N-Methyl-L-Asp\textsuperscript{1}Ang II ICV. The ratio of \textsuperscript{125}I to \textsuperscript{127}I was 1:19 to allow for administration of 50 – 100pmole of the angiotensin to cause a response. All radioiodinated angiotensins were prepared and purified using procedures described elsewhere\textsuperscript{1,2} except that the mobile phase for HPLC purification of \textsuperscript{125/127}I-Ang II and \textsuperscript{125/127}I-N-Methyl-L-Asp\textsuperscript{1}Ang II was acetonitrile:acetic acid, pH 4.0. The fraction containing \textsuperscript{125/127}I-Ang II and \textsuperscript{125/127}I-N-Methyl-L-Asp\textsuperscript{1}Ang II was lyophilized and resuspended in aCSF at a concentration of 20–50\mu M. Twenty-five \mu l of the radioiodinated peptide was administered ICV. As soon as the rat showed a dipsogenic response it was sacrificed by decapitation and the head was microwaved for 12 sec in a conventional microwave unit (1 kilowatt of power, Model JES738BH01, General Electric), to inactivate brain peptidase activity. The brain was quickly removed from the skull and homogenized in HPLC mobile phase: ~23% acetonitrile and ~77% triethylamine phosphate (83 mM PO\textsubscript{4}) at pH 3.0. The homogenate was centrifuged at 48,000 x g for 20 min at 4\textdegree C. The supernatant was decanted and centrifuged in a Spin-X (Costar) microcentrifuge tube through a 0.2 micron filter to eliminate small particulates. The filtrate was analyzed by HPLC using a 250 x 4.6mm C\textsubscript{18} column (Varian, Microsorb-MV 100-5) and the HPLC mobile phase. \textsuperscript{125}I-Ang II, \textsuperscript{125}I-N-Methyl-L-Asp\textsuperscript{1} Ang II, \textsuperscript{125}I-Ang III, \textsuperscript{125}I-Ang IV, \textsuperscript{125}I-Ang 4-8 \textsuperscript{125}I-Ang 1-5 and \textsuperscript{125}I-Ang 2-5, also radioiodinated in this laboratory, were applied to the C\textsubscript{18} column under identical conditions prior to and following
administration of the brain extracts to characterize the peaks eluting from the brain extracts. The majority of the $^{125}$I-labeled material extracted from the rat brains eluted shortly after the void volume of the column and was not characterized because it was not possible to determine if this was $^{125}$I that was released from the tyrosine due to the heating of the brain, or small fragments of the peptide, including $^{125}$I-tyrosine.

Reference List


Figure Legends

FigureS1. Representative plot of MAP for 40 minutes duration after 100 pmol of β-D-Asp$^1$ Ang II given ICV. Markers on the tracing indicate:

1. Beginning of ICV administration the angiotensin.
2. Completion of ICV administration.
3. First dipsogenic response (water bottle immediately removed from the cage).
4. Injector cannula removed.
5. Placement of a calibrated drinking tube in the cage 25 minutes after beginning of ICV administration.
6. Dipsogenic responses after the placement of the calibrated tube in the cage.

After placement of calibrated drinking tubes in the cage, various activities e.g., cessation and initiation of drinking, eating, sleeping, and rearing of the animals were marked. These markers
appear after the marker number 6 and are not labeled. No consistent behavior patterns were observed between animals.

Figure S2. Plot of MAP for 40 minutes duration after ICV administration of 100 pmol D-Ala₁-Ang II. Markers are as noted for Figure I.

Figure S3. Plot of MAP for 40 minutes duration after ICV administration of 100 pmol Sar₀-Ang II. Markers are as noted for Figure I.

Figure S4. Plot of MAP for 40 minutes duration after ICV administration of 100 pmol Beta-L-Asp¹-Ang II. Markers are as noted for Figure I.

Figure S5. Inhibition of aminopeptidase-resistant angiotensin analog-induced pressor and dipsogenic responses by the AT-1 receptor antagonist losartan. The responses to ICV administration of 100 pmoles of N-Methyl-L-Asp¹ Ang II were inhibited by ICV administration of 10 nmoles of losartan 20 minutes beforehand. Panel A) Pressor response. Panel B) Dipsogenic response 25 minutes after administration of N-Methyl-L-Asp¹ Ang II. Error bars indicate SEM.