Chronic Production of Angiotensin IV in the Brain Leads to Hypertension That Is Reversible With an Angiotensin II AT1 Receptor Antagonist

Nadheige Lochard, Gaétan Thibault, David W. Silversides, Rhian M. Touyz, Timothy L. Reudelhuber

Abstract—Angiotensin IV (Ang IV) is a metabolite of the potent vasoconstrictor angiotensin II (Ang II). Because specific binding sites for this peptide have been reported in numerous tissues including the brain, it has been suggested that a specific Ang IV receptor (AT4) might exist. Bolus injection of Ang IV in brain ventricles has been implicated in learning, memory, and localized vasodilatation. However, the functions of Ang IV in a physiological context are still unknown. In this study, we generated a transgenic (TG) mouse model that chronically releases Ang IV peptide specifically in the brain. TG mice were found to be hypertensive by the tail-cuff method as compared with control littermates. Treatment with the angiotensin-converting enzyme inhibitor captopril had no effect on blood pressure, but surprisingly treatment with the Ang II AT1 receptor antagonist candesartan normalized the blood pressure despite the fact that the levels of Ang IV in the brains of TG mice were only 4-fold elevated over the normal endogenous level of Ang peptides. Calcium mobilization assays performed on cultured CHO cells chronically transfected with the AT1 receptor confirm that low-dose Ang IV can mobilize calcium via the AT1 receptor only in the presence of Ang II, consistent with an allosteric mechanism. These results suggest that chronic elevation of Ang IV in the brain can induce hypertension that can be treated with angiotensin II AT1 receptor antagonists. (Circ Res. 2004;94:1451-1457.)

Key Words: angiotensin IV ■ transgenic model ■ hypertension ■ brain renin–angiotensin system

The octapeptide hormone angiotensin II (Ang 1 to 8; Ang II) is considered to be the main effector of renin–angiotensin system (RAS) in the control and regulation of blood pressure.1 Ang II interacts with 2 types of receptors called AT1 and AT2.2,3 Most of the physiological actions of Ang II in the cardiovascular system are thought to be mediated by the activation of AT1 receptors. All the components of the RAS, including angiotensinogen, prorennin, renin, angiotensin-converting enzyme (ACE), and the angiotensin receptors are present not only in the circulation but also in certain tissues including the heart, kidney, and brain (reviewed in ref4). Whether these so-called local RAS have an activity independent of the circulating RAS is a matter of much debate. However, there is little doubt that the brain can synthesize angiotensin peptides locally because Ang II is not readily permeable to the blood-brain barrier and because it has been detected in brain of nephrectomized rats unable to generate circulating Ang II.5 Overactivation of the brain RAS is implicated in the maintenance of the hypertension in certain laboratory animal models including spontaneously hypertensive rats (SHR)6 and several transgenic mouse models.7

In the brain, as well as in the circulation, Ang II is rapidly metabolized (Figure 1). Aminopeptidases A and N act successively to remove 1 amino acid from the N-terminus of Ang II to produce Ang III (Ang 2 to 8) and Ang IV (Ang 3 to 8).8 Injection of Ang III peptide in cerebral ventricles has been found to have dose-dependent pressor responses similar to those of Ang II.9 In fact, the finding that inhibition of aminopeptidase A could prevent an increase in blood pressure after Ang II injection in cerebral ventricles has led some investigators to suggest that Ang III is the principal effector of the RAS in the brain.10 However, it is still not certain whether it is Ang III that performs these biological functions, or its further metabolite, Ang IV.

Because Ang IV binds with high affinity to sites that are both histologically and pharmacologically distinct from the AT1 and AT2 receptors, the possibility has been raised that a distinct receptor (the AT4 receptor) exists for this angiotensin peptide.11 In the brain, AT4 binding sites are found in regions associated with cognitive and motor function including neocortex, hippocampus, and cerebellum.12 Recent evidence has suggested that the AT4 receptor may be the insulin-regulated aminopeptidase (IRAP).13 The substrates of IRAP in vivo are not known, but it is possible that interaction of Ang IV with IRAP could inhibit degradation of endogenous neuropeptides, thereby increasing their half-life. In fact, Ang IV has been reported to increase memory recall and learning in passive
produce the peptide Ala and thereby increase the efficiency of peptide release. Second, the natural amino acid sequence of Ang IV. First, alanine (Ala) was described elsewhere.18,19 Two modifications were made to the fusion protein in brain, whole brain acid/alcohol extracts from 5 mice. To determine the form of angiotensin IV peptide released by the brain membranes were incubated with 6104 cpm of [I125]Ang IV in the presence of a mix of AT1 and AT2 antagonists, 10. Although these results suggest a number of physiological functions for Ang IV in the brain, they are largely based on experiments involving acute interventions with pharmacologic doses of the peptide delivered by nonphysiological routes. In the current study, we generated a transgenic mouse model designed to test for the cardiovascular effect of chronically elevated Ang IV levels in the brain. Our results suggest that a modest increase of Ang IV, specifically in the brain, contributes to hypertension that can be corrected with an angiotensin II AT1 receptor antagonist.

Materials and Methods

Generation of Transgenic Mice

The expression vector used for releasing Ang IV (m-Ang IV) is shown in Figure 2 and the details of its construction have been described elsewhere.18,19 Two modifications were made to the natural amino acid sequence of Ang IV. First, alanine (Ala) was added to the N-terminus to provide a good P1’ site for furin cleavage and thereby increase the efficiency of peptide release. Second, the proline at the penultimate position was substituted by glycine to produce the peptide Ala1-Gly1-Ang IV. This fusion protein was placed under the control of a 2.2-Kb fragment of the human glial fibrillary acidic protein promoter (hGFAP), which is specific for astrocytes.20 The transgene was excised from the plasmid vector and co-injected with a tyrosinase gene in the pronucleus of fertilized eggs of FVB/N females mice, as previously described,21 to produce GFAP-Ang IV transgenic mice. We obtained 3 founder lines for GFAP-Ang IV named GFAP10.1, 10.2, and 10.3.

All mice were used at 8 to 12 weeks unless otherwise specified. The animal protection and ethics committee of the Clinical Research Institute of Montreal approved all animal protocols.

Tissue Pattern of Transgene Expression

RNase protection assays (from total RNA) were performed as described previously.19,21 The pattern of transgene expression in brain was assessed by in situ hybridization as described previously.19

Preparation of Antibodies

A rabbit antiserum was raised against Ala1-Gly1-Ang IV. For immunization, the peptide was coupled to the chicken ovalbumin (Sigma A5503, Sigma-Aldrich, Oakville, Ontario, Canada) in molar ratio 20:1 using glutaraldehyde. Three rabbits were immunized at 2 subcutaneous and one intramuscular site. Primary immunization contained 1 mg of conjugated peptide mixed 1:2 with Freund’s complete adjuvant (Sigma F-5881). Three booster immunizations separated by 3 weeks were performed in the same way but using Freund’s incomplete adjuvant. Test bleeds (8 mL) were collected between the injections and evaluated for positive immune response by radioimmunoassay (RIA). At the end of the protocol, animals were exsanguinated and serum was collected and stored at −20°C. This antiserum is specific for Ala1-Gly1-Ang IV and shows 100% cross-reactivity with Gly2-Ang IV and no detectable reactivity with either Ang I, Ang II, or Ang IV.

Characterization of Transgenic Peptide Expression in GFAP-Ang IV Mice

To determine the form of angiotensin IV peptide released by the fusion protein in brain, whole brain acid/alcohol extracts from 5 mice from line GFAP10.2, or control were pooled and fractionated by reverse-phase HPLC as described previously.19,23 The elution fractions were lyophilized and subjected to RIA using the rabbit antibody raised against Ala1-Gly1-Ang IV. Synthetic peptides were used as migration standards. The levels of Ang II, Ang III, and Ang IV peptides in brain acid/alcohol extracts were determined by RIA using a polyclonal antibody that reacts equally well with all 3 metabolites and using [3H]-Ang II as tracer.19,24

Receptor-Binding Assays

Receptor binding assays were performed to determine the affinity of Ang IV, Ala1-Gly2-Ang IV, and Gly2-Ang IV for the AT1 and AT4 receptors using membrane preparations from whole brain of control mice or a stable CHO cell line expressing the rat AT1B receptor (CHO-AT1B). Brain membranes were prepared as described previously.25 Membrane fractions were resuspended in binding buffer (phosphate-buffered saline containing 2 tablets of complete protease inhibitor [Roche 1697498, Roche Diagnostics, Laval, Quebec, Canada] per 100 mL, 0.5% heat-treated bovine serum albumin (Sigma A-9018), 5 mmol/L MgCl2, 1 mmol/L PMSF, and 5 mmol/L DTT). For the competition assay, 100 μg of protein of a fresh preparation of brain membranes were incubated with 6x104 cpm of [125I]-Ang IV in the presence of a mix of AT1 and AT2 antagonists, 10−6 mol/L each of candesartan (gift from Astra-Zeneca), and PD 123 319 (gift from Parke-Davis, Canada), in a final volume of 0.25 mL. Increasing concentrations of Ang IV, Ala1-Gly2-Ang IV, Gly2-Ang IV, and Sar2-lys2-Ang II were added to the tubes. After an incubation period of 2 hours at 37°C with shaking, the bound ligand was separated from free ligand by filtration through glass fiber filters followed by 3 4-mL washes with 0.08 mol/L Tris/HCl pH 7.4 containing 0.9% NaCl. Radioactivity in the membranes was measured with a gamma counter. Nonspecific binding was estimated in presence of 10−6 mol/L Ang IV.

One day before the experiment, the CHO-AT1B were plated at 4x105 cells/well in 24-wells plate. The cells were washed twice with

![Diagram of the transgene used to target overexpression of Ang IV in brain.](https://example.com/diagram)

![Diagram of enzymatic reactions of the renin–angiotensin system (RAS) leading to the formation of Ang IV.](https://example.com/diagram)
DMEM free of serum and incubated with $5 \times 10^7$ cpm of $^{[125]I}$-Sar$^1$-Ile$^2$-Ang II for 90 minutes at room temperature in a final volume of 0.5 mL. For competition assays, increasing concentrations of Sar$^1$-Ile$^2$-Ang II, Ang IV, Ala$^1$-Gly$^5$-Ang IV, Gly$^5$-Ang IV, Sar$^1$-Ile$^2$-Ang II, candesartan, or PD 123 319 were included in the incubation medium. After the incubation period, the cells were washed twice with DMEM free of serum and solubilized with 0.5 mL of 1 N NaOH and the radioactivity in the lysate was quantified with a gamma counter. Non-specific binding was determined in the presence of $10^{-6}$ mol/L of Sar$^1$-Ile$^2$-Ang II.

Reverse Transcription Polymerase Chain Reaction Amplification and Quantification of AT1 Receptors in Brain of GFAP-Ang IV Mice

AT1 receptors expression in the brain was determined by reverse-transcription polymerase chain reaction (RT-PCR) and Southern blot. The oligonucleotides used detected AT1A and B receptors and are described in the online data supplement available at http://circres.ahajournals.org.

Intracellular Calcium Measurements

Intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}]_{i}$) in CHO-AT1B cells was determined as previously described. Briefly, cells were cultured on round glass coverslips. The cells were loaded with fura-2AM ($4 \times 10^{-6}$ mol/L) and 0.2% Pluronic acid in Hank's buffer and incubated at room temperature in the dark for 30 minutes. The cells were exposed either directly to Ang II ($10^{-9}$ mol/L) or Ang IV ($10^{-7}$ mol/L) or after pretreatment for 20 minutes with candesartan ($10^{-4}$ mol/L). Fluorescence was measured by photomicrographic digital imaging (Attofluor Ratiovision; Zeiss) using excitation wavelengths of 343/380 nm and an emission wavelength of 520 nm. [Ca$^{2+}]_{i}$ was determined from the Gryniewicz formula.

Physiological Measurements

Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (BP-2000 system; Visitec Systems, Apex, NC). Mice were trained for 7 days and systolic blood pressure (SBP) was recorded for an additional 3 days. After the initial 10 days of SBP recording, mice were treated with an ACE inhibitor (Captopril, Sigma) at 100 mg/kg per day intraperitoneal in 0.9% NaCl or the AT1 antagonist candesartan (15 mg/kg per day by gavage in 5% gum arabic) for a further 3 or 5 days while recording SBP.

Statistical Analysis

Results are expressed as mean±SEM. One-way ANOVA with Dunnett posttest or unpaired t test was performed using GraphPad Prism version 3.00 (GraphPad Software, San Diego, Calif).

Results

Affinity of Ang IV and m-Ang IV Peptides for AT4 and AT1 Receptors

Because overexpressing the entire RAS in a given tissue would result in the formation of a variety of angiotensin peptides including Ang II, III, and IV (Figure 1), we generated a fusion protein that allows the targeted production of specific angiotensin metabolites in whole animals. In the design of this fusion protein, a furin cleavage site is placed just before the sequence of the angiotensin peptide (Figure 2). Because of the furin cleavage preference, an alanine was added to N-terminus of the native Ang IV sequence (Figure 2) with the expectation that this extra amino acid would be removed by the abundant aminopeptidases present in brain. A second modification to the Ang IV sequence, substitution of a glycine residue for the proline normally found at position 5 (compare Figure 1 and Figure 2), has 2 main advantages.

First, this modification has been reported to increase the affinity of the peptide for AT4 receptors by ~10-fold. Second, using an antibody specific for this modified Ang IV, it is possible to discriminate between the exogenous and endogenous Ang IV peptides in the brains of transgenic animals. The transgenic peptide and its metabolic derivatives are hereafter referred to as modified Ang IV (m-Ang IV) to distinguish them from the endogenous angiotensin peptides.

Radioligand binding studies were performed to evaluate the affinity of these peptides for the AT4 and AT1 receptors. Figure 3B illustrates the comparative binding of bona fide Ang IV and the m-Ang IV peptides with fresh brain membrane preparations from control mice. To unequivocally demonstrate that these peptides interact with the AT4 receptors, the binding assay was performed in the presence of a mix of potent and specific AT1 and AT2 receptor antagonists (10$^{-6}$ mol/L each, candesartan, PD123 319) and the various peptides indicated. C, AT1 receptor affinity was determined by incubating CHO-AT1B with $^{[125]I}$-Sar$^1$-Ile$^2$-Ang II and the various peptides indicated. All experiments are performed in triplicate and the values are represented as mean±SEM. The analyses were performed with SigmaPlot 2002 with the ligand module.

While the substitution of glycine for proline does not significantly affect the affinity for AT1 receptors (Kd: Ang IV, 26 Briefly, cells were cultured on round glass coverslips. The cells were loaded with fura-2AM ($4 \times 10^{-6}$ mol/L) and 0.2% Pluronic acid in Hank’s buffer and incubated at room temperature in the dark for 30 minutes. The cells were exposed either directly to Ang II ($10^{-9}$ mol/L) or Ang IV ($10^{-7}$ mol/L) or after pretreatment for 20 minutes with candesartan ($10^{-4}$ mol/L). Fluorescence was measured by photomicrographic digital imaging (Attofluor Ratiovision; Zeiss) using excitation wavelengths of 343/380 nm and an emission wavelength of 520 nm. [Ca$^{2+}]_{i}$ was determined from the Gryniewicz formula.27

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Forms and Levels of Ang IV Present in the Brain of GFAP-10.X Mice

Because we were obliged to produce Ala\textsuperscript{-1}-Gly\textsuperscript{5}-Ang IV with the fusion protein and because the full-length peptide has a dramatically reduced affinity for the AT4 receptor (see previous), we sought to determine the efficiency with which the N-terminal alanine was removed (to produce the Gly\textsuperscript{5}-Ang IV form) in the brains of transgenic mice. Brain acid/alcohol extracts from line GFAP10.2 and control were fractionated by HPLC and eluted peptides were detected by radioimmunoassay. We found that while both modified Ang IV peptides are detectable in the brains of transgenic mice, approximately half of the peptide is in the form of Gly\textsuperscript{5}-Ang IV (Figure 5A), confirming that the N-terminal alanine is efficiently removed in vivo. Total modified Ang IV peptide synthesis in the brains of transgenic mice (Figure 5B) showed a direct correlation with the levels of transgene mRNA expression for the lines GFAP 10.2 and GFAP-10.3. Values are represented as mean±SEM minus the background from control mice; nd indicates undetectable; n=8 to 12.
Blood Pressure Measurement

The major purpose of this study was to evaluate the effect of a chronic elevation of brain Ang IV on SBP. We found a significant increase in the SBP of transgenic GFAP-10.X mice compared with control mice (Figure 6A). To rule out the possibility that this increase in SBP was caused by activation of the endogenous RAS, we treated mice from line GFAP10.2 with an ACE inhibitor (captopril 100 mg/kg per day intraperitoneally), which should only block the production of Ang II by the endogenous RAS (note that the transgene releases Ang IV directly). ACE inhibition did not decrease SBP in GFAP 10.2 mice to any greater extent than that seen in nontransgenic littermates (Figure 6B). These results support the conclusion that the increase in SBP of GFAP-10.2 mice is caused by the direct action of the m-Ang IV peptide in brain. To rule out the role of the AT1 receptor in this hypertension, animals were treated with a potent and specific AT1 receptor antagonist, which has been shown to cross the blood-brain barrier (candesartan 15 mg/kg per day orally). Surprisingly, this treatment normalizes the SBP in mice from line GFAP10.2 (Figure 6C). The levels and tissue distribution of AT1 receptors were not different in the brains of transgenic and nontransgenic littermates as demonstrated by the RT-PCR (Figure 7A and 7B) and the in situ hybridization (data not shown). The levels of endogenous brain angiotensin peptides (Ang II, III, and IV) were not increased in transgenic mice (control: 10.7±2.2; GFAP10.2: 6.8±0.2; GFAP10.3: 5.4±1.2 pg Ang peptide/g wet weight; n=12 to 18). Taken together, these results suggest that a chronic but modest increase in brain Ang IV can lead to an increase in SBP that can be reversed by an AT1 receptor antagonist.

To better-understand this phenomenon in light of the inability of the m-Ang IV peptides to displace Ang II from the AT1 receptor in binding studies (Figure 3C), we tested the possibility that Ang IV might potentiate the signaling capacity of the AT1 receptor in the presence of other angiotensin peptides. CHO-AT1B cells were tested for intracellular calcium mobilization after stimulation with Ang IV and Ang II, either alone or in combination, using fluorescence microscopy (Figure 8). Pretreatment of the cells with AT1 antagonist candesartan prevented both responses (Figure 8D). These results suggest that Ang IV can synergize with Ang II to promote signaling through the AT1 receptor.
Discussion

Previous studies of the physiological functions of Ang IV in the brain have required the direct injection of this peptide in the brain ventricles.12,14,31 In addition to being invasive, this strategy precludes the evaluation of long-term effects of this peptide. To explore the role of chronic elevations of Ang IV in brain, we used a fusion protein capable of targeting the direct release of an Ang IV peptide in the brain. The major finding of the present study is that moderate overproduction of Ang IV peptide in the brain of transgenic mice induced hypertension that could be reversed by an AT1 receptor antagonist. We have ruled out several of the more trivial explanations for this finding: First, the m-Ang IV peptides could display an increased affinity for the AT1 receptor or be produced in such large quantities that they are able to bind and activate the AT1 receptor. This is clearly not the case, however, because the m-Ang IV peptides show no capacity to displace Ang II from the AT1B receptor (Figure 3C) and thus the modest increases of m-Ang IV peptide content in the brain of the transgenic mice (as compared with endogenous peptides) cannot be replacing Ang II to activate the AT1 receptor. A second possible explanation would be that Gly5-Ang IV stimulates hypertension through its interaction with the AT4 receptor and that the doses of the AT1 antagonist used in this study were sufficient to block this interaction. This also seems like an unlikely explanation because micromolar concentrations of the AT1 antagonist did not have any significant effect on binding of Gly5-Ang IV to the AT4 receptor (Figure 3B).

Another possibility is that the levels of endogenous angiotensin peptides have been increased by the expression of Gly5-Ang IV. It has recently been reported that the insulin-regulated aminopeptidase (IRAP; the putative AT4 receptor) has the ability to cleave many peptides in vitro, including Ang III.13 If Gly5-Ang IV binding to IRAP decreased the metabolism of endogenous Ang III, the latter might accumulate to levels that would stimulate the AT1 receptor and result in an AT1 receptor antagonist-sensitive hypertension. However, the level of endogenous angiotensin peptides in the brain GFAP-10.X mice tends to be lower than in control mice, making this an unlikely explanation. It has been reported that Ang IV could act as an endogenous ACE inhibitor and thereby block the conversion of Ang I to Ang II in vitro.32 Perhaps such an effect explains the lower angiotensin peptide content in the brains of transgenic mice. Finally, no difference in the levels or distribution of AT1 receptors has been observed in the brain of GFAP-10.X mice as compared with the nontransgenic control mice.

A more compelling explanation for our findings is that angiotensin metabolites (including Ang III and Ang IV) exhibit a higher affinity for the AT1 receptor in vivo than is reflected in vitro.33,34 Likewise, injection of an aminopeptidase-resistant variant of Ang IV, N-hydroxymethylamine-Ang IV, into the brain ventricles has been shown to increase blood pressure.35 Pretreatment with an AT1 receptor antagonist but not with an AT4 antagonist, divalinal,35,36 prevented this increase in blood pressure. In addition, it has been reported that AT1 antagonists inhibit the pressure response after injection of Ang II, III, and IV in the systemic and hindquarter vascular beds in rat.37 How, then, can the apparent differences in the in vitro and in vivo results be reconciled? Kinetic, mutagenesis, and modeling studies support the notion that the AT1 receptor can adopt different conformations on binding of Gly5-Ang IV to the AT4 receptor (Figure 3B). Another possibility is that the AT1 receptor can adopt different conformations on binding of Gly5-Ang IV to the AT4 receptor (Figure 3B). This also seems like an unlikely explanation because micromolar concentrations of the AT1 antagonist did not have any significant effect on binding of Gly5-Ang IV to the AT4 receptor (Figure 3B). How, then, can the apparent differences in the in vitro and in vivo results be reconciled? Kinetic, mutagenesis, and modeling studies support the notion that the AT1 receptor can adopt different conformations on binding of Gly5-Ang IV to the AT4 receptor (Figure 3B). A more compelling explanation for our findings is that angiotensin metabolites (including Ang III and Ang IV) exhibit a higher affinity for the AT1 receptor in vivo than is reflected in the in vitro displacement assays. Several lines of evidence support this possibility: Injected Ang III peptide in brain ventricles has previously been reported to elicit a similar dose-dependent pressor response to Ang II.33,34 Likewise, injection of an aminopeptidase-resistant variant of Ang IV, N-hydroxymethylamine-Ang IV, into the brain ventricles has been shown to increase blood pressure.35 Pretreatment with an AT1 receptor antagonist but not with an AT4 antagonist, divalinal,35,36 prevented this increase in blood pressure. In addition, it has been reported that AT1 antagonists inhibit the pressure response after injection of Ang II, III, and IV in the systemic and hindquarter vascular beds in rat.37 How, then, can the apparent differences in the in vitro and in vivo results be reconciled? Kinetic, mutagenesis, and modeling studies support the notion that the AT1 receptor can adopt different conformations on binding of Gly5-Ang IV to the AT4 receptor (Figure 3B).

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Figure 8. Effects of Ang II (10⁻⁸ mol/L) and Ang IV (10⁻⁷ mol/L) on intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) in AT1-transfected CHO cells. Cells were loaded with fura-2AM (4 μmol/L) and fluorescence measured using alternating excitation wavelengths of 340 and 380 nm. Arrows indicate time of agonist addition. A and B, [Ca²⁺]ᵢ responses to Ang II and Ang IV, respectively. C, Effects of Ang IV in Ang II-prestimulated cells. D, Effects of Ang II and Ang IV in cells pretreated with candesartan (10⁻⁵ mol/L, 20 minutes). Each data point is the mean±SEM of 3 to 4 experiments, with each experimental field comprising 35 to 42 cells.
Alternatively, Ang IV might act as a potentiator of Ang II binding or signaling through the AT1 receptor. Consistent with this model, our calcium mobilization assays show that Ang IV can effectively activate the AT1 receptor only if Ang II is present (Figure 8C), although it does not compete for Ang II binding to this receptor (Figure 8C). This effect is specific to the activation of AT1, because it is prevented in cells pretreated with AT1 antagonist candesartan (Figure 8D). While such a model could explain the effect of Ang IV on blood pressure, it remains to be determined whether such an interaction could also explain the reported cognitive effects of Ang IV.

Acknowledgments
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References
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**RT-PCR amplification and quantification of AT1 receptors in brain of GFAP-Ang IV mice**

Total RNA from brain was prepared by the Trizol method (Invitrogen 15596-026). The first strand cDNA was synthesized with random primers and 5 μg of total RNA treated with the RQ1 RNase-free DNase (Promega, M6106) by using the Superscript™ II Reverse Transcriptase kit (Invitrogen 18064-022). Three primers were designed for AT1 receptors and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), two primers for the PCR amplification and one as the probe. The primers for AT1 were, forward primer: 5’-ATATTTGTCATCCCTAC-3; reverse primer: 5’-AATACACAATTCCGATGGATG-3’ and the probe: 5’-AGCGTCAGTTTCAACCTCTA-3’. The primers for GAPDH were, forward primer: 5’-TCGCCCCCTTCCGCTGATG-3’; reverse primer: 5’-CAGGAAGGCCATGCGCATGA-3’ and the probe: 5’CCA CAG TCC ATG CCA TCA CT-3’. PCR for AT1 was performed with 2 μl of cDNA at 52°C (Tm) for 30 cycles while the GAPDH PCR was performed at 61.6°C (Tm) for 20 cycles. The number of cycles and the quantity of cDNA used were optimized to be in the linear range of the PCR. The PCR products was electrophoresed on 1% agarose gel and transferred to the GeneScreen Plus membrane (NEN Life Science, NEF988). The southern was done as described in reference 1. Briefly, after the transfer the membrane was washed in 2X SSC. The membranes were placed for 2 hours in the prehybridization buffer (6X SSC; 20mM NaH₂PO₄; 0.4% SDS; 5X Denhardt’s and 500 μg/ml of denatured salmon-sperm) followed by an overnight incubation at 42°C in the hybridation buffer (6X SSC; 20mM NaH₂PO₄; 0.4% SDS; 500 μg/ml of denatured salmon-sperm and 1.5 X 10⁶ cpm of [γ-
32P]-ATP labeled probe). The membranes were subsequently washed in 2X SSC at 42°C and exposed for 2 hours in a phoshoimager cassette. The bands were quantified with the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). The results shown were reproduced in two independent experiments.

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