Differentiation of Brain Angiotensin Type 1a and 1b Receptor mRNAs
A Specific Effect of Dehydration

Yanfang Chen, Mariana Morris

Abstract—The objective was to examine the effect of dehydration on the expression of the angiotensin type 1 (AT\textsubscript{1}) receptor subtype mRNAs in mice by using an in situ hybridization method. The method used free-floating brain sections with \textsuperscript{35}S-labeled probes specific for the untranslated 5' (AT\textsubscript{1a}) and 3' (AT\textsubscript{1b}) regions. AT\textsubscript{1a} and AT\textsubscript{1b} mRNA levels in the subfornical organ (SFO) and anterior third ventricle (AV3V) were quantified by using a phosphor-imaging system. Emulsion autoradiography with cresyl violet counterstaining was used to show cellular expression. Adult male C57BL mice (25 to 30 g) were given water ad libitum or were deprived of water for 48 hours. Dehydration produced increases in plasma osmolality (349±6 versus 314±4 mOsm/kg) and hematocrit (58±2% versus 47±1%). In situ hybridization showed that there was expression of AT\textsubscript{1a} and of AT\textsubscript{1b} mRNA in SFO and AV3V. Dehydration produced an increase in AT\textsubscript{1a} mRNA in both regions, with no changes noted for AT\textsubscript{1b}. AT\textsubscript{1a} mRNA was increased in the AV3V region from 0.3±0.2 to 0.7±0.2 mCi/g and in the SFO from 0.6±0.3 to 1.0±0.2 mCi/g. These results provide information regarding the localization and physiological importance of a subset of angiotensin receptors that are important in volume and blood pressure regulation. AT\textsubscript{1a} and AT\textsubscript{1b} mRNAs showed a similar pattern of expression in rostral forebrain osmosensitive regions. However, osmotic/volume stimulation with dehydration produced specific activation of AT\textsubscript{1a} receptors. This verifies the role of AT\textsubscript{1a} receptors in volume regulation but raises a question concerning the physiological role of the AT\textsubscript{1b} subtype. (Hypertension. 2001;37[part 2]:692-697.)

Key Words: angiotensin II ■ brain ■ hybridization ■ receptors, angiotensin ■ renin-angiotensin system

Angiotensin II (Ang II) is the key effector of the renin-angiotensin system, producing a variety of effects in the cardiovascular, renal, neural, and endocrine systems. The peptide acts through its peripheral receptors to increase vascular resistance, cardiac output, sodium reabsorption, and blood volume. In addition, all components of the renin-angiotensin system are present in the brain, including the angiotensin peptides and their synthetic and degradative enzymes.\textsuperscript{1} There is much evidence to suggest that the central Ang II system is critical in the regulation of blood pressure and volume.

Ang II receptors can be discriminated into subtypes 1a, 1b, and 2 (AT\textsubscript{1a}, AT\textsubscript{1b}, and AT\textsubscript{2}, respectively).\textsuperscript{2} AT\textsubscript{1} and AT\textsubscript{2} receptors can be distinguished on the basis of their affinities for different ligands, but it has not been possible to pharmacologically discriminate between AT\textsubscript{1a} and AT\textsubscript{1b} subtypes. The genes and the receptor proteins are almost identical, sharing >94% sequence homology.\textsuperscript{3} Initial studies using Northern blot analysis showed low levels of AT\textsubscript{1} receptor mRNA in brain and an inability to distinguish between the subtypes.\textsuperscript{4} Differential tissue-specific expression of the mouse AT\textsubscript{1a} and AT\textsubscript{1b} genes was achieved by using reverse transcriptase (RT)–polymerase chain reaction (PCR) restriction fragment length polymorphism. AT\textsubscript{1a} and AT\textsubscript{1b} mRNAs were detected in the brain, although it was not possible to show cellular distribution. Using RT-PCR in specific brain regions, Kakar et al\textsuperscript{5} demonstrated central nervous system (CNS) localization. They found that AT\textsubscript{1b} mRNA was predominant in the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), regions that mediate Ang II–induced drinking behavior. For detailed anatomically specific expression, in situ hybridization has proved to be a useful technique. By use of cRNA probes that differentiate AT\textsubscript{1a} from AT\textsubscript{1b}, there is evidence for AT\textsubscript{1a} mRNA expression in rostral forebrain regions, such as the OVLT, SFO, and the median preoptic and periventricular hypothalamus.\textsuperscript{6,7} AT\textsubscript{1b} mRNA was not detected in any of the brain regions. Barth and Gerstberger\textsuperscript{8} used an oligonucleotide probe specific for rat AT\textsubscript{1a} mRNA and reported upregulation in the SFO after dehydration. This is consistent with reports on Ang II receptor regulation that show increased AT\textsubscript{1} receptors in the paraventricular nucleus (PVN) and SFO after dehydration and salt loading.\textsuperscript{9–11}
There is little information regarding the localization and regulation of the brain angiotensin system in the mouse. Johren et al. performed a detailed analytical localization pattern for angiotensin peptides and receptors and ACE. They showed low levels of ACE throughout the hypothalamus. Ang II–immunoreactive neurons and AT<sub>1</sub> receptors were detected in the PVN and median eminence. AT<sub>1</sub> receptors were also detected in the rostral forebrain region, SFO, OVLT, and PVN areas. However, there is some disagreement as to whether there is precise overlap of angiotensin receptors and receptor mRNA.

Another method for the study of angiotensin receptor function is the use of gene-deletion models. We examined angiotensin receptor regulation in mice lacking the AT<sub>1a</sub> gene and found an increase in receptor expression after dehydration in control but not in AT<sub>1a</sub> knockout animals. Further examination of CNS responses to dehydration (c-Fos and vasopressin mRNA) showed greater activation in the AT<sub>1a</sub> knockout mice. Studies by Davison et al. suggested that central AT<sub>1b</sub> receptors may be important in volume control, particularly in the absence of AT<sub>1a</sub> receptors.

It is clear that there are significant gaps in our knowledge of the CNS angiotensin system that are specifically related to the differentiation of the AT<sub>1a</sub> and AT<sub>1b</sub> receptors, anatomically and functionally. The objective of the present study was to use in situ hybridization methods to distinguish between the receptor subtype mRNAs and to test the effect of osmotic/volume stimulation. The method developed is based on the use of oligonucleotide probes for the noncoding 5′ regions, which are structurally different between the subtypes, and the use of free-floating brain sections.

**Methods**

**Animals and Tissue Preparation**

Male C57BL mice (36 ± 2.7 g) were randomly divided into 2 groups and housed in single cages at 22°C and under a 12-hour light/12-hour dark cycle. The control group had free access to standard chow and drinking water. For the dehydration test, drinking water was withdrawn for 48 hours with chow available ad libitum. Body weights were determined before and after dehydration. At the end of the experiment, the animals were decapitated, and trunk blood was collected for the determination of hematocrit and plasma osmolality. Brains were rapidly removed and put into precooled 4% paraformaldehyde in PBS. After overnight fixation at 4°C, the brains were trimmed and further fixed in 4% paraformaldehyde/20% sucrose for 4 days (4°C). Brains were stored at −80°C until analysis.

**Preparation of Oligodeoxynucleotide Probes**

The sequences of the AT<sub>1a</sub> receptor–specific (35-bp) and AT<sub>1b</sub> receptor–specific (33-bp) antisense oligonucleotides were derived from mouse cDNAs. For AT<sub>1a</sub>, the sequence was derived from the 5′ untranslated region: 5′-CTTTTCTGGGTAGTGGCTGAGGCACTGTTC-3′, nucleotides 35 to 69. For AT<sub>1b</sub>, the sequence was derived from the 3′ untranslated region: 5′-GTGAGCTGAACTGCTGACAAATGCTGTCTGGG-3′, nucleotides 1146 to 1178. Another oligonucleotide probe (34 bp) that recognizes both AT<sub>1a</sub> and AT<sub>1b</sub> was derived from the following coding region: 5′-CAGGTAGCGATCGATGTTGA-3′, nucleotides 423 to 456 for AT<sub>1a</sub> cDNA and nucleotides 934 to 967 for AT<sub>1b</sub> cDNA, 100% homology to both AT<sub>1a</sub> and AT<sub>1b</sub> mRNAs and receptor mRNA.

Another method for the study of angiotensin receptor regulation in mice lacking the AT<sub>1a</sub> gene is the use of gene-deletion models. We examined angiotensin receptor regulation in mice lacking the AT<sub>1a</sub> gene and found an increase in receptor expression after dehydration in control but not in AT<sub>1a</sub> knockout animals. Further examination of CNS responses to dehydration (c-Fos and vasopressin mRNA) showed greater activation in the AT<sub>1a</sub> knockout mice. Studies by Davison et al. suggested that central AT<sub>1b</sub> receptors may be important in volume control, particularly in the absence of AT<sub>1a</sub> receptors.

It is clear that there are significant gaps in our knowledge of the CNS angiotensin system that are specifically related to the differentiation of the AT<sub>1a</sub> and AT<sub>1b</sub> receptors, anatomically and functionally. The objective of the present study was to use in situ hybridization methods to distinguish between the receptor subtype mRNAs and to test the effect of osmotic/volume stimulation. The method developed is based on the use of oligonucleotide probes for the noncoding 5′ and 3′ regions, which are structurally different between the subtypes, and the use of free-floating brain sections.

**In Situ Hybridization**

Consecutive coronal brain sections were cut at 20 μm at −20°C with a cryostat. The sections were collected in PBS (0.01 mol/L) in tissue culture wells. For the in situ hybridization reaction, the sections were transferred into autoclaved 1.5-mL tubes and washed in 2× SSC for 30 minutes. The hybridization buffer containing labeled probe (0.1 to 0.3×10<sup>6</sup> cpm/100 μL) was added, and the tissues were incubated overnight (18 hours) at 40°C. The hybridization buffer consisted of 50% deionized formamide, 4× SSC, 10% dextran sulfate, 1× Denhardt’s solution, 0.5 mmol/L dithiothreitol, 250 μg/mL sperm DNA, and 250 μg/mL yeast tRNA. After hybridization, tissue sections were washed sequentially in 1× SSC (50°C for 30 minutes), 2× SSC/50% formamide (40°C, 3 times for 30 minutes each), and 0.5× SSC (50°C for 30 minutes).

**Autoradiography and Quantification**

Sections were mounted on slides, dried, and exposed to Fuji film for 5 days. The film cassette contained a 14C standard slide, which was used for quantification. The film was read by using a phosphor-imaging system (Fuji FLA-2000). Quantification was performed by using a computerized densitometry system (Image reader V1.7E and Image gauge V3.3). For measurement, the areas of interest were selected and outlined. A background area was selected with the same dimensions as the sample. The signal intensity was calculated with background subtraction: net signal = total signal − background [as signal − background/mm<sup>2</sup>]. The results were then compared with the 14C standard curve, yielding data expressed as μCi/g. For cellular localization, slides were dipped in Kodak NTB2 liquid emulsion and exposed for 1 to 2 weeks. After development, sections were counterstained with cresyl violet. The areas selected for density measurements (SFO and AV3V) were verified by microscopic evaluation.

**Statistical Analysis**

The data are presented as mean ± SEM. The Student’s t test was used for determining significance.

**Results**

In situ hybridization studies were conducted by using brain sections from the optic chiasm through the rostral forebrain. There was specific labeling for both AT<sub>1a</sub> and AT<sub>1b</sub> mRNAs in AV3V and SFO (Figures 1 and 2). Figure 1 shows an example of a film autoradiograph including the 14C standards. The pattern of labeling was similar for AT<sub>1a</sub> (Figure 1A and 1C) and AT<sub>1b</sub> (Figure 1B and 1D). A series of experiments was conducted to verify the specificity of the reaction by using 35S-labeled sense probes (Figure 1F) and by using a probe directed toward the coding region of the AT<sub>1</sub> receptor mRNA (Figure 1E). Emulsion and radiography showed the detailed pattern for cellular expression (Figure 2). There was clear neuronal labeling along the ventricular surface in the AV3V region.

To examine the functional role of AT<sub>1</sub> receptors, we tested the effect of dehydration on volume/electrolyte status and AT<sub>1a</sub> and AT<sub>1b</sub> expression. Dehydration produced a significant loss of body weight and increase in hematocrit and plasma osmolality (Table). Hematocrit was increased from 46.9% to 57.7%; osmolality was increased by 35 mOsm. These changes indicate that the dehydration model produces both extracellular volume depletion and extracellular hyperosmotic. Quantitative evaluation of the hybridization signal was accomplished by using the phosphor-imaging system. This allows for the measurement of radioactivity in specific brain regions. Dehydration produced a 2- to 3-fold increase in...
Figure 1. Example of phosphor image (Fuji FLA 2000) of the in situ hybridization for AT$_1$ receptor mRNA subtypes in mouse brain. Images show radioactive signal in specific brain regions. $^{14}C$ standard is shown at the bottom. This is used for the quantification of the radioactive signal (conversion to $\mu$Ci/g). A through D, Labeling for AT$_{1a}$ mRNA in the AV3V region (A) and SFO region (B) and for AT$_{1b}$ mRNA in the AV3V region (C) and SFO region (D) with use of 5' and 3' subtype-specific probes. E, Labeling with a probe for the common coding region, which interacts with both AT$_{1a}$ and AT$_{1b}$. F, Examples of labeling with the sense control probe.
AT₁a mRNA in both the SFO and AV3V (Figure 3). In contrast, there were no changes in AT₁b mRNA expression.

**Discussion**
A newly developed hybridization technique was used to differentiate between AT₁a and AT₁b mRNAs in the mouse brain. The method uses oligonucleotide probes that are designed to interact with the nontranslated 5' and 3' regions, the only regions showing sequence difference between the receptor subtypes.8 Our results show that there is robust labeling for both AT₁a and AT₁b receptor mRNAs in the rostral forebrain. A test of the effect of dehydration showed

### Table of Body Weight, Hematocrit, and Plasma Osmolality in Mice

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<th>Dehydration</th>
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<td>36.4±2.7</td>
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<td>35.8±2.9</td>
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<tr>
<td>Hematocrit, %</td>
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<tr>
<td>Osmolality, mOsm/kg</td>
<td>314.3±4.4</td>
<td>349.6±6.1*</td>
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Values are mean±SEM.

*P<0.01 control vs dehydration.

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**Figure 2.** Example of an emulsion autoradiograph of the in situ hybridization reaction for AT₁ receptor subtypes. A and B, AT₁a (A) and AT₁b (B) mRNA expression in AV3V region. C and D, Higher power photomicrograph for AT₁a (C) and AT₁b (D) signal in periventricular cells.

**Figure 3.** Effect of dehydration on AT₁ receptor subtype mRNA expression in the AV3V and SFO regions of mouse brain. AV3V SFO

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that there were increases in AT\textsubscript{1a}, but not AT\textsubscript{1b} expression in the SFO and AV3V.

The renin-angiotensin system plays an important role in the regulation of blood pressure, body fluid, and electrolyte homeostasis through its main effector peptide, Ang II. The vascular and CNS effects of Ang II occur mainly through AT\textsubscript{1} receptors. AT\textsubscript{1} receptors exist in 2 gene variants, AT\textsubscript{1a} and AT\textsubscript{1b}, which are very similar in structure. Johren et al\textsuperscript{a} and Lenkei et al\textsuperscript{7} mapped the distribution of AT\textsubscript{1a} and AT\textsubscript{1b} receptor mRNA in rat brain by using riboprobes for in situ hybridization. They reported that the AT\textsubscript{1a} subtype was the predominant form. However, other studies using RT-PCR techniques showed evidence for the expression of both AT\textsubscript{1a} and AT\textsubscript{1b} in mouse and rat brain.\textsuperscript{4,5}

Because there was little information on the cellular distribution of the receptor subtypes in mice and their functional regulation, the goal was to establish a method for visualization and quantification of AT\textsubscript{1a} and AT\textsubscript{1b} mRNA. An in situ hybridization method was set up by using oligonucleotide probes specific for the 5' and 3' untranslated regions, which show only 35% homology between the subtypes. A recent study by Barth and Gerstberger\textsuperscript{8} demonstrated that oligonucleotides were effective in labeling AT\textsubscript{1a} mRNA in rat brain. The specificity of the method and the probes can be verified as follows: (1) There was no in situ hybridization signal when the respective control sense probes were used. (2) The in situ hybridization experiments were performed under strict incubation and washing conditions. (3) There was competitive suppression of the hybridization signal when an excess of unlabeled probe was used (data not shown). (4) The same expression patterns were observed with an AT\textsubscript{1b} probe deduced from a different region in the 3' untranslated region or a probe to the common coding sequence (highest homology between receptors). (5) There was a specific enhancement in AT\textsubscript{1a} expression in response to physiological stimulation, showing that there were differences in the receptors.

This is the first study to show anatomically specific labeling for both AT\textsubscript{1a} and AT\textsubscript{1b} in the mouse brain. The pattern for the 2 subtypes was essentially identical with cellular labeling in the rostral forebrain, specifically in the SFO and AV3V. These regions are known to be osmosensitive and to contain angiotensin receptors and AT\textsubscript{1a} mRNA. Although there is general consensus regarding the expression pattern for AT\textsubscript{1} receptors, questions remain as to the differentiation between AT\textsubscript{1a} and AT\textsubscript{1b}. Previous studies in rats documented the presence of AT\textsubscript{1b} receptors in the SFO and pituitary.\textsuperscript{5} These results were not confirmed in subsequent studies.\textsuperscript{6,7} The reason that we were able to distinguish between the subtypes is likely related to the animal species and to the use of a very sensitive method. The use of free-floating brain sections is an important consideration. This method allows better access of the probe to the tissue and more effective washing. With the immersion technique, lower concentrations of radioactive probe can be used, resulting in lower background. The net result is an increase in the signal-to-noise ratio. Other important points are the specificity of the oligonucleotide probes, the labeling efficiency with [\textsuperscript{35}S]dATP, and the probe stability.

In the present study, we used a phosphor-imaging system to detect and measure the hybridization signal. This method offers advantages compared with standard film densitometry. There is improved sensitivity so that the reaction can be visualized within 1 to 2 days of exposure. With regard to quantification, there is a wider range for signal detection, which is directly related to the amount of radioactivity. The levels can be measured and compared with \textsuperscript{14}C standards, providing information on the amount in mCi/g. The specific area of interest is always verified by histological examination of the cresyl violet–stained tissue. In addition, all of the tissues were measured at the same time and under the same conditions. As shown in Figure 1, this system provides a simple, quick, and sensitive method for the detection and quantification of any in situ hybridization signal.

For the study of functionality, we tested the effect of osmotic/volume stimulation with dehydration. Previous studies in mice showed that dehydration produced increases in brain angiotensin receptors, c-Fos immunoreactivity, and vasopressin mRNA levels.\textsuperscript{11,15} Forty-eight hours of water deprivation resulted in a loss in body weight and increases in plasma osmolality and hematocrit. In situ hybridization for AT\textsubscript{1a} and AT\textsubscript{1b} receptor mRNA revealed differences in the responses. There was a specific increase in AT\textsubscript{1a} mRNA and almost a 2-fold change in the SFO, AV3V, and parvocellular PVN region (data not shown). These results are consistent with a report showing that dehydration in rats produced an increase in AT\textsubscript{1a} mRNA in the SFO.\textsuperscript{8} For AT\textsubscript{1b} mRNA, the signal was present in the same regions, but there was no effect of stimulation. This indicates that there are functional differences between the receptor subtypes.

Most effects of Ang II in the CNS have been reported to be mediated through the AT\textsubscript{1a} subtype. This is based on the supposed predominance of AT\textsubscript{1a} receptors in the CNS and the results of studies using receptor knockout mice. For example, mice lacking the AT\textsubscript{1a} receptor show hypotension and an inability to respond to the blood pressure effects of Ang II.\textsuperscript{17} In addition, the removal of AT\textsubscript{1b} receptors results in few phenotypic changes.\textsuperscript{18} However, there is some evidence of the role of AT\textsubscript{1b} receptors in the control of drinking, with results obtained by comparing the dipsogenic effect of Ang II in animals lacking AT\textsubscript{1a} and AT\textsubscript{1b} receptor subtypes.\textsuperscript{16} This new finding is consistent with the previous RT-PCR results\textsuperscript{5} and our present in situ hybridization results, which show AT\textsubscript{1b} mRNA expression in the osmosensitive SFO region. However, it appears that changes in volume and osmotic status are not sufficient to activate the AT\textsubscript{1b} system, at least in control animals.

In conclusion, in situ hybridization methods coupled with quantitative autoradiography proved to be a useful means for the study of the central expression of AT\textsubscript{1} receptor subtypes. Results showed that there was cellular expression of both AT\textsubscript{1a} mRNA and AT\textsubscript{1b} mRNA in areas of the brain that are important in fluid and pressure regulation and changes in mRNA expression in response to dehydration. The specificity of the response for the AT\textsubscript{1a} subtype provides support for its physiological role. Further studies are required to determine the function of AT\textsubscript{1b} receptors.
Acknowledgments
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