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# Adenovirus-Mediated Small-Interference RNA for In Vivo Silencing of Angiotensin AT<sub>1a</sub> Receptors in Mouse Brain

Yanfang Chen, Hao Chen, Andrea Hoffmann, David R. Cool, Debra I. Diz, Mark C. Chappell, Alex Chen, Mariana Morris

**Abstract**—Because of the lack of pharmacological approaches, molecular genetic methods have been required to differentiate between angiotensin type 1 (AT<sub>1</sub>) receptor subtypes AT<sub>1a</sub> and AT<sub>1b</sub>. RNA interference is a new tool for the study of gene function, producing specific downregulation of protein expression. In this study, we used the small hairpin RNA (shRNA) cassette method to screen target sites for selectively silencing AT<sub>1a</sub> or AT<sub>1b</sub> receptor subtypes in cultured Neuro-2a cells using real-time RT-PCR. For in vivo functional studies, we used C57BL mice with arterial telemetric probes and computerized licking monitors to test the effect of adenovirus carrying the DNA sequence coding AT<sub>1a</sub> shRNA (Ad-AT<sub>1a</sub>-shRNA). Ad-AT<sub>1a</sub>-shRNA was injected into the lateral ventricle (intracerebroventricular) or the brain stem nucleus tractus solitarius/dorsal vagal nucleus (NTS/DVN) with measurement of water intake, blood pressure (BP), and heart rate (HR) for up to 20 days after injection. Tissue culture studies verified the specificity and the efficiency of the constructs. In animal studies,  $\beta$ -galactosidase staining and Ang receptor binding assays showed expression of shRNA and downregulation of Ang AT<sub>1</sub> receptors in the subfornical organ and NTS/DVN by >70%. Intracerebroventricular injection of Ad-AT<sub>1a</sub>-shRNA increased water intake with no effect on BP or HR. In contrast, microinjection of Ad-AT<sub>1a</sub>-shRNA into NTS/DVN caused a decrease in BP with no effect on HR or water intake. Results demonstrate the use of the RNA interference method in site-directed silencing of gene expression and provide a method for the in vivo study of Ang AT<sub>1</sub> receptor function. (*Hypertension*. 2006;47:230-237.)

**Key Words:** blood pressure ■ brain ■ gene regulation ■ renin-angiotensin system

Brain angiotensin (Ang) has been implicated in the control of blood pressure (BP), water balance, and hormone secretion.<sup>1-3</sup> Ang type 1 (AT<sub>1</sub>) receptors are richly expressed in several brain regions, including the subfornical organ (SFO), paraventricular nucleus (PVN), dorsal nucleus vagus (DVN), nucleus tractus solitarius (NTS), and locus coeruleus.<sup>4,5</sup> In rodents,<sup>6</sup> and perhaps in humans,<sup>7,8</sup> there are 2 related AT<sub>1</sub> receptor subtypes, AT<sub>1a</sub> and AT<sub>1b</sub>. Recent studies by us<sup>4,5</sup> and others<sup>9</sup> demonstrate that AT<sub>1</sub> receptor subtypes AT<sub>1a</sub> and AT<sub>1b</sub> have similar distribution patterns in the mouse brain. In addition, several lines of reports showed that AT<sub>1a</sub> and AT<sub>1b</sub> receptor subtypes exhibit individual regulatory functions.<sup>10-12</sup> Our previous results demonstrated that AT<sub>1a</sub> and AT<sub>1b</sub> receptors are differentially expressed in response to increased dietary salt and fluid deprivation within mouse brain areas, such as the hypothalamus, anterior pituitary, and brain stem.<sup>4,5,13</sup> However, the precise functional roles of brain AT<sub>1a</sub> and AT<sub>1b</sub> receptors in specific brain regions remain to be determined.

AT<sub>1a</sub> and AT<sub>1b</sub> receptors share 95% homology in mRNA and amino acid sequences and have similar Ang II binding

characteristics.<sup>14</sup> It is difficult to differentiate the subtypes, because there are no specific agonists or antagonists. Thus, functional studies have relied on the use of gene knockout (KO) models selectively knocking out AT<sub>1a</sub> and AT<sub>1b</sub> receptors.<sup>15,16</sup> Davisson et al<sup>17</sup> showed that brain AT<sub>1a</sub> receptors were responsible for control of BP, whereas brain AT<sub>1b</sub> receptors were responsible for drinking activity. We showed that AT<sub>1a</sub> receptors are important in osmotic responsiveness and sympathetic balance.<sup>18,19</sup> However, KO models are not useful for determining whether there is regional specificity, because the receptors are absent from all tissues and at all times (from in utero onward). There are also questions as to upregulation of compensatory receptors in the KO. For example, we reported that brain stem Ang AT<sub>1b</sub> receptors were increased in Ang AT<sub>1a</sub> KO mice.<sup>20,21</sup>

It is for this reason that we set out to develop the RNAi system for inhibiting Ang AT<sub>1a</sub> expression in specific brain regions. The method represents a new and powerful tool, which provides for sequence-specific downregulation for an unlimited variety of targets.<sup>22</sup> For in vivo studies, it is coupled

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with tissue culture testing to evaluate the DNA sequences. For the Ang system, we relied on the Neuro-2a cell line, which expresses AT<sub>1a</sub>, AT<sub>1b</sub>, and AT<sub>2</sub> receptors.<sup>23</sup> A recent *ex vivo* study by Vazquez et al<sup>24</sup> showed the feasibility of silencing AT<sub>1a</sub> receptors using RNAi.

The objective of the present study was to develop the RNAi method for silencing the Ang AT<sub>1a</sub> receptors and to determine the cardiovascular and drinking effects of brain site-specific downregulation of receptor expression. We chose brain sites that show concentrations of Ang receptors and involvement in the control of cardiovascular and fluid balance, the SFO in the rostral forebrain, and NTS/DVN in the brain stem. The hypothesis is that brain Ang AT<sub>1a</sub> receptors may express different functions that are dependent on the regional tissue site.

## Methods

### RNAi Targets for Ang AT<sub>1a</sub> and AT<sub>1b</sub>

RNAi Target Finder software (GenScript Corporation) was used to select the RNAi candidate targets for murine AT<sub>1a</sub> and AT<sub>1b</sub> genes. Selection was performed according to guidelines established by Elbashir et al.<sup>25</sup> The DNA sequences coding shRNAs for AT<sub>1a</sub> (gene ID: 249945, target sequence 210 to 230) and AT<sub>1b</sub> (gene ID: 249947, target sequence 252 to 272) were cloned into expression cassettes (GenScript Corporation). The shRNA expression cassettes were linear double-stranded DNA consisting of the human U6 promoter followed by a DNA insert encoding an AT<sub>1a</sub>- or AT<sub>1b</sub>-specific shRNA flanked by a terminator sequence. Neuro-2a cells were grown in DMEM containing 10% FBS and 1× Pen-Strep, Fungizone. At 50% to 60% confluence, cells were transfected with lipofectamine using 1 μg/mL AT<sub>1a</sub> or AT<sub>1b</sub> shRNA expression cassettes. After 48 hours, RNA was extracted for evaluating the efficacy of the constructs using real-time RT-PCR.<sup>13</sup>

### Quantitative Real-Time RT-PCR

Details for the real-time PCR method for measuring AT<sub>1a</sub> and AT<sub>1b</sub> mRNAs was reported previously.<sup>13</sup> Briefly, total cellular mRNA was extracted using the RNeasy Protect mini kit and the RNase-Free DNase kit (Qiagen Inc). RNA was reverse transcribed using the TaqMan reverse-transcription kit (Applied Biosystems). Eukaryotic 18S rRNA was used as an endogenous reference control (Applied Biosystems). Real-time PCR (TaqMan 7700, Applied Biosystems) was performed using the TaqMan Universal PCR Master Mix. After PCR amplification, the threshold cycle values (CT) for AT<sub>1a</sub> and 18S were calculated using TaqMAN analysis software. The relative expression levels of AT<sub>1a</sub> and AT<sub>1b</sub> mRNA were obtained using the 2<sup>-ΔΔCT</sup> method.<sup>26</sup>

### Adenovirus Preparation for AT<sub>1a</sub> RNAi

The effective target sequence for AT<sub>1a</sub> RNAi was identified in the tissue culture study. The DNA coding AT<sub>1a</sub> shRNA was synthesized and cloned into an adenoviral shelter plasmid (Imagenex Corporation). The shelter plasmid and a backbone plasmid (Imagenex Corporation) were recombined in cell line 293 to generate adenovirus.<sup>27</sup> The virus concentrations were determined in the cultured cells. A standard Ad-LacZ virus was used as a control.

### Animal Surgery

C57BL mice (24 to 30 g, male) were housed singly in microisolator cages and fed standard mouse chow and water *ad libitum*. Mice were instrumented with intracerebroventricular (ICV) cannula for virus microinjection. A 22-gauge guide cannula was implanted in the lateral cerebral ventricle (model C313 GS; Plastics 1 Inc) using stereotaxic procedures. The cannula was implanted 1.0-mm lateral and 0.6-mm caudal to bregma. The cannula was fixed onto the skull with screws and dental adhesive. A telemetric catheter was inserted

into the carotid artery (model TA11PA-C20, Data Science International) for chronic BP measurement. The details of the surgeries have been described in previous studies.<sup>28,29</sup> Animals were allowed to recover for 5 to 7 days before experiments were conducted. Mouse husbandry followed the standard of animal biosafety level 2 set by the Centers for Disease Control and Prevention and the National Institutes of Health in their guideline for biosafety in microbiological and biomedical laboratories. All of the procedures were approved by the Committees of Animal Care and Use and Institutional Biosafety Committee at Wright State University.

### Experimental Protocols

Experiment 1 tested the effect of ICV injection of Ad-AT<sub>1a</sub>-siRNA virus (2.3×10<sup>16</sup> pfu/mL) with measurement of water intake (volume and licking activity), BP, and HR. Injections were delivered in 1 μL over a 1-minute period. Parameters were measure over the day and night periods under basal conditions and 5, 10, and 20 days after ICV injection. Ad-LacZ virus (1 μL, 1.9×10<sup>16</sup> pfu/mL) was used as a control.

Experiment 2 tested the effect of Ad-AT<sub>1a</sub>-shRNA virus (2.3×10<sup>16</sup> pfu/mL) injection into the NTS/DVN on water intake (volume and licking activity), BP, and HR. The Ad-AT<sub>1a</sub>-shRNA virus (200 nL) was injected bilaterally into the NTS/DVN (0.1- to 0.2-mm anterior and 0.3-mm lateral to the obex, 0.5-mm below the brain stem surface) in mice with arterial telemetric catheters. Cardiovascular and drinking parameters were continuously recorded for 24 hours on day 0 (before injection) and 10 and 20 days after injection. Ad-LacZ virus was used as a control. Microinjection was performed through a glass micropipette (50 μm) with a microinjection pump over 30 s (WPI).

### β-Galactosidase Staining

The marker gene (LacZ) exists in both Ad-AT<sub>1a</sub>-shRNA and Ad-LacZ vectors. The β-galactosidase (β-gal) staining was used as a marker for the site of the viral effect. After the completion of recording, mice were decapitated at each time point. Brain and brain stem were collected in dry ice. The tissues were sectioned into 14 μm, mounted on the slides, and fixed with 4% formaldehyde for 30 minutes. After washing with PBS, the slides were incubated with stain solution (ALPCO Diagnostics) at 37°C for 1 hour. The infected areas turned in blue. Slides were counterstained with cresyl violet to verify the injection site and infected areas.

### Ang AT<sub>1</sub> Receptor-Binding Assay

For determining the efficacy and time window of the Ad-AT<sub>1a</sub>-shRNA effect, we used Ang receptor autoradiography.<sup>30–32</sup> Brains were removed and frozen on dry ice before transferring to –80°C for storage. Series of adjacent coronal sections (14 μm) were cut and mounted on Superfrost/plus slides (Fisher Scientific). Tissues were incubated with 0.4 nmol/L of [<sup>125</sup>I-Sar<sup>8</sup>-Thr<sup>8</sup>]Ang II (2200 Ci/mmol), with and without 3 μmol/L AT<sub>1</sub> antagonist losartan, 3 μmol/L AT<sub>2</sub> antagonist PD123319, or Ang II to determine the proportion of AT<sub>1</sub> or AT<sub>2</sub> receptors and the nonspecific binding. Quantification was performed with the Fuji phosphor imaging system (Fujifilm Medical System) after exposure. The method used computerized densitometry of specific brain regions (documented with standard histology). Activity in tissue was compared with <sup>14</sup>C standards. A conversion factor relates <sup>14</sup>C to [<sup>125</sup>I-Ang II in μCi/mg protein. Slides were counterstained with cresyl violet to verify anatomic localization in SFO and NTS/DVN. Data are presented as specific binding and were competed by losartan, suggesting the majority of sites are AT<sub>1</sub> as reported previously in these brain regions.<sup>3,30,33–35</sup>

### Water Intake and Licking Activity

The volume of water consumed was measured daily. Licking activity was measured using a drinkometer system (Columbus Instruments) interfaced with a computerized Dataquest A.R.T. system (Data Science International). The cages were directly wired to the drinkometers with the positive input connected to metal tips on the water bottles and negative input to the metal grid flooring of the cage.

When the mice made simultaneous contact with the floor and water bottle, a lick was recorded via the drinkometer. Licking activity was used as the primary indicator of fluid consumption, because the precision of the method provided detailed information about the level of activity, as well as timing.

### BP and Heart Rate

The radiotelemetry system (model TA11PA-C20, Data Science International) was used for recording arterial pressure in conscious, freely moving mice, as reported previously.<sup>19</sup> Arterial pressure was recorded continuously (500 Hz) for 24 hours under basal conditions and on days 5, 10, and 20 after virus injection. HR and mean arterial pressure were calculated at each time point.

### Plasma Ang II Levels

A modified radioimmunoassay was used to measure plasma Ang II because of the small blood samples. Plasma Ang II was measured using a commercial radioimmunoassay kit (ALPCO Diagnostics).<sup>36</sup> Mouse plasma (100  $\mu$ L) pretreated with EDTA and Bestatin solution (ALPCO Diagnostics) was extracted through phenylsilylsilica column with methanol (ALPCO Diagnostics). The sample was reconstituted and incubated with Ang II antiserum and <sup>125</sup>I-Ang II at 4°C. After incubation, the antibody-bound fraction was precipitated using a secondary antibody. The samples were centrifuged (1000g) for 5 minutes. The supernatants were discarded, and pellets were counted with a gamma counter. The assay was modified to use a smaller volume (20  $\mu$ L) of antisera to increase the assay sensitivity.

The ED<sub>50</sub> was 46.1 pg/mL in our assay as compared with 54.5 pg/mL, which was reported in the kit description. We were able to measure Ang II levels in 20 to 50  $\mu$ L of plasma.

### Statistics and Data Analysis

BP is recorded using the telemetric system and transformed for measurement of systolic, diastolic, and mean pressure and HR. The digitized drinking data are converted to numerical form. All of the data are expressed as mean  $\pm$  SEM. Multiple and nested ANOVA were used for time course data, day/night data, and treatment analysis followed by Tukey's test. The statistical difference between 2 means was determined by an unpaired Student *t* test.  $P < 0.05$  was used as the criterion for significance.

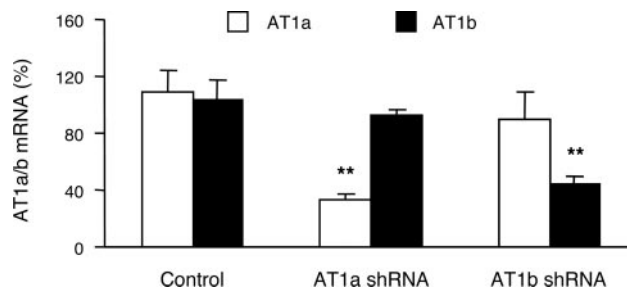
## Results

### Specific and Effective shRNA Targets for AT<sub>1a</sub> and AT<sub>1b</sub> Receptors

Cultured neuro-2a cells, known to express both AT<sub>1a</sub> and AT<sub>1b</sub> receptors,<sup>23</sup> were transiently transfected with AT<sub>1a</sub> or AT<sub>1b</sub> shRNA expression cassettes. Results showed a reduction of 60% for AT<sub>1a</sub> and 56% for AT<sub>1b</sub> in mRNA expression using real-time RT-PCR (AT<sub>1a</sub>: 40  $\pm$  8%,  $n = 7$ ,  $P < 0.05$  and AT<sub>1b</sub>: 44  $\pm$  18%,  $n = 7$ ,  $P < 0.05$ ). The effect was specific, because AT<sub>1a</sub> and AT<sub>1b</sub> mRNA levels were not significantly affected in presence of the opposite shRNA cassette (Figure 1). The results demonstrate that the chosen small-interference RNA expression cassettes can be used for selective knockdown of AT<sub>1a</sub> and AT<sub>1b</sub> receptor mRNAs.

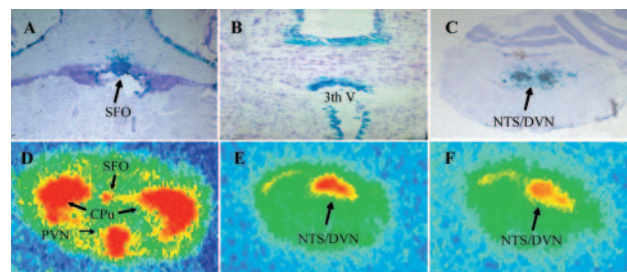
### Ad-AT<sub>1a</sub>-shRNA Mediates shRNA Expression and Downregulates AT<sub>1</sub> Receptors

Both Ad-AT<sub>1a</sub>-shRNA and Ad-LacZ adenoviral vectors encode the LacZ marker gene. This offers a useful tool for histological monitoring of the expression and location of adenoviral vector-mediated gene expression by  $\beta$ -gal staining. We used 2 modes of administration of Ad-AT<sub>1a</sub>-shRNA, ICV (1  $\mu$ L) or into the NTS/DVN (200 nL).  $\beta$ -gal staining showed, first, that the Ad-AT<sub>1a</sub>-shRNA virus ICV injection

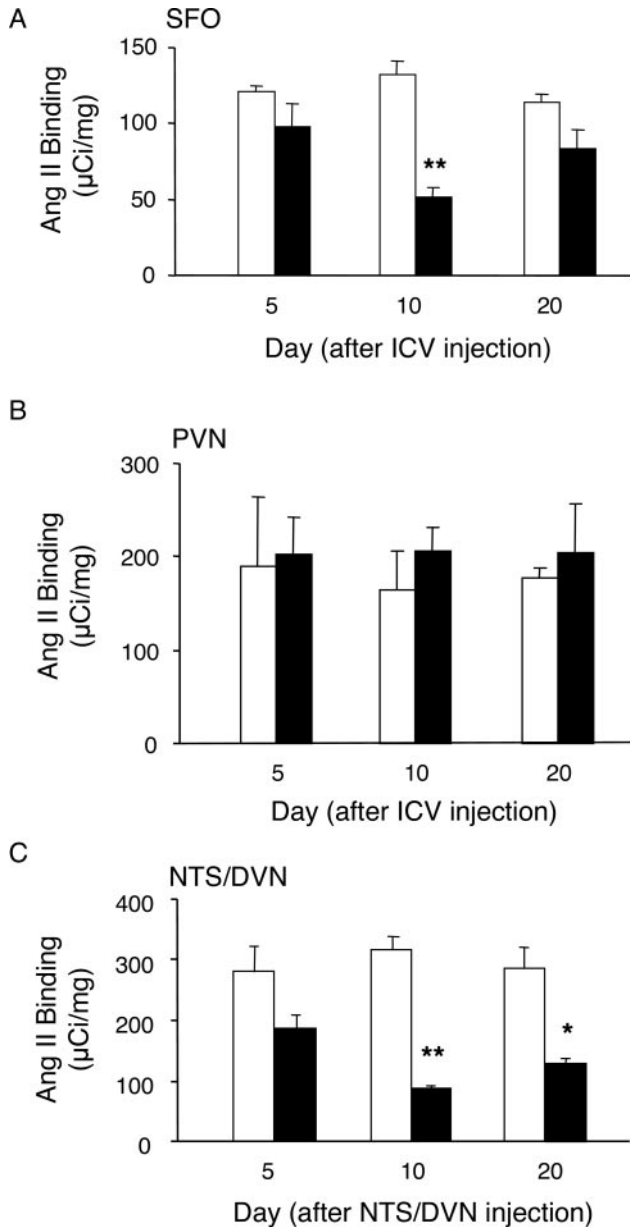


**Figure 1.** Real-time RT-PCR analysis of AT<sub>1a</sub> and AT<sub>1b</sub> receptor mRNA levels in Neuro-2a cells treated with shRNA cassette. The mRNA levels are expressed as a percentile of respective controls. The data are presented as mean  $\pm$  SEM. One-way ANOVA was used for analysis followed by Tukey's multiple comparison test. \*\* $P < 0.01$  vs control.

induced shRNA expression as seen by the blue staining in the SFO and around the cerebral ventricle (Figure 2A and 2B). There was no noticeable staining in other regions.  $\beta$ -gal staining also showed that when Ad-AT<sub>1a</sub>-shRNA virus was microinjected directly into the NTS/DVN, the adenoviral vector-mediated gene expression was confined to this brain region (Figure 2C). There was no  $\beta$ -gal staining in other brain regions. Third,  $\beta$ -gal staining showed that the LacZ gene expression appeared as early as 24 hours after injection and lasted for up to 2 weeks (data not shown). Figure 2D, 2E, and 2F show representative examples of <sup>125</sup>I-Sar<sup>1</sup>Thr<sup>8</sup>-Ang II binding in SFO, PVN, and NTS/DVN, respectively. After ICV injection ( $n = 8$ ) of Ad-AT<sub>1a</sub>-shRNA, there was a significant reduction in Ang binding in SFO with no change in PVN (Figure 3A and 3B). After adenovirus injection into the NTS/DVN ( $n = 8$ ), there was a selective decrease in binding in this region at day 10 and 20 (Figure 3C). The time course revealed that the effect was shorter after ICV injection (reduction on day 10 but not day 20). In contrast, after brain stem injection, receptors were reduced up to 20 days.



**Figure 2.**  $\beta$ -Gal staining and <sup>125</sup>I-Sar<sup>1</sup>Thr<sup>8</sup>-Ang II AT<sub>1</sub> receptor binding analysis of brain tissues after injection of Ad-AT<sub>1a</sub>-shRNA into ICV and bilateral NTS/DVN. ICV injection of Ad-AT<sub>1a</sub>-shRNA selectively delivered AT<sub>1a</sub> shRNA into SFO (A) and surrounding 3rd ventricle (B) as indicated by marker gene (LacZ) expression ( $\beta$ -gal staining showed blue color). Microinjection of the virus into bilateral NTS/DVN induced site-specific expression of AT<sub>1a</sub> shRNA (C) as indicated by marker gene (LacZ) expression ( $\beta$ -gal staining showed blue color). <sup>125</sup>I-Sar<sup>1</sup>Thr<sup>8</sup>-Ang II binding showed the receptors in SFO and PVN (D) and NTS/DVN (E and F). shRNA silenced down the AT<sub>1</sub> receptor-binding signal in NTS/DVN (F) compared with LacZ control (E).



**Figure 3.** Effects of ICV injection and bilateral NTS/DVN injection of Ad-AT<sub>1a</sub>-shRNA on AT<sub>1</sub> receptor expression. <sup>125</sup>I-Sar<sup>1</sup>Thr<sup>8</sup>-Ang II binding analysis in mice brain on days 5, 10, and 20 after injection of Ad-AT<sub>1a</sub>-shRNA. \**P*<0.05, \*\**P*<0.01, LacZ vs shRNA. (A) SFO, *F*(1, 22)=46.3, *P*=0.000; (B) PVN, *F*(1, 22)=1.14, *P*=0.30; (C) NTS/DVN, *F*(1, 26)=93.3, *P*=0.00; 2-way ANOVA.

**Cardiovascular and Water Intake Effects of Brain AT<sub>1a</sub> Gene Silencing**

One of the advantages of the Ad-AT<sub>1a</sub>-shRNA is that it can be used in specific brain sites to downregulate receptors, providing a useful tool for functional genetic studies. ICV injection of Ad-AT<sub>1a</sub>-shRNA produced an increase in water intake on day 10 (Table 1). This was associated with an increase in drinking activity during the dark (active) period on the same day (Figure 4A). There were no changes in BP or HR at any time point (Table 2). Water intake and drinking activity had returned to normal by day 20 (Table 1; Figure 4B), the same time course as Ang receptor expression in SFO.

In the brain stem NTS/DVN region, injection of Ad-AT<sub>1a</sub>-shRNA had no effect on water intake (Table 3; Figure 4B), but reduced BP on day 10 (Figure 5A). The BP effect was only evident during the dark period, a time when the mice are active and show higher BP levels.<sup>37</sup> There were no changes in HR (Figure 5B). BP had returned to basal levels on day 20 (Figure 5A), although AT<sub>1</sub> receptors were still at a low level (Figure 4).

**Effect of ICV Injection of Ad-AT<sub>1a</sub>-shRNA on Plasma Ang II**

To evaluate the interactions between central and peripheral Ang systems, we measured plasma Ang II after ICV injection of Ad-AT<sub>1a</sub>-shRNA. ICV injection of Ad-AT<sub>1a</sub>-shRNA had no effect on plasma Ang II, although there was a trend of increase at day 10 (Table 1).

**Discussion**

AT<sub>1</sub> receptor blockade is used routinely in the clinical treatment of hypertension and chronic heart failure.<sup>38,39</sup> Brain AT<sub>1</sub> receptors are involved in the control of BP, autonomic neural balance, volume homeostasis, and neuroendocrine secretion.<sup>1-3</sup> AT<sub>1</sub> receptors exist as 2 subtypes, AT<sub>1a</sub> and AT<sub>1b</sub> in rodents<sup>6</sup> and as splice variants in humans.<sup>7,8</sup> There is information on the brain distribution of receptor subtypes,<sup>4,5,11,40</sup> but there are questions as to whether there is regional functional specificity.

Ang AT<sub>1a</sub> and AT<sub>1b</sub> receptors are difficult to differentiate functionally because of the 95% homology in amino acid sequence and similar binding characteristics, as well as the lack of AT<sub>1</sub> subtype-selective agonists or antagonists.<sup>41</sup> For this reason, studies have relied on gene KO or overexpression mouse models.<sup>15,16,42,43</sup> Neuron-targeted overexpression of AT<sub>1a</sub> receptors resulted in enhanced cardiovascular respon-

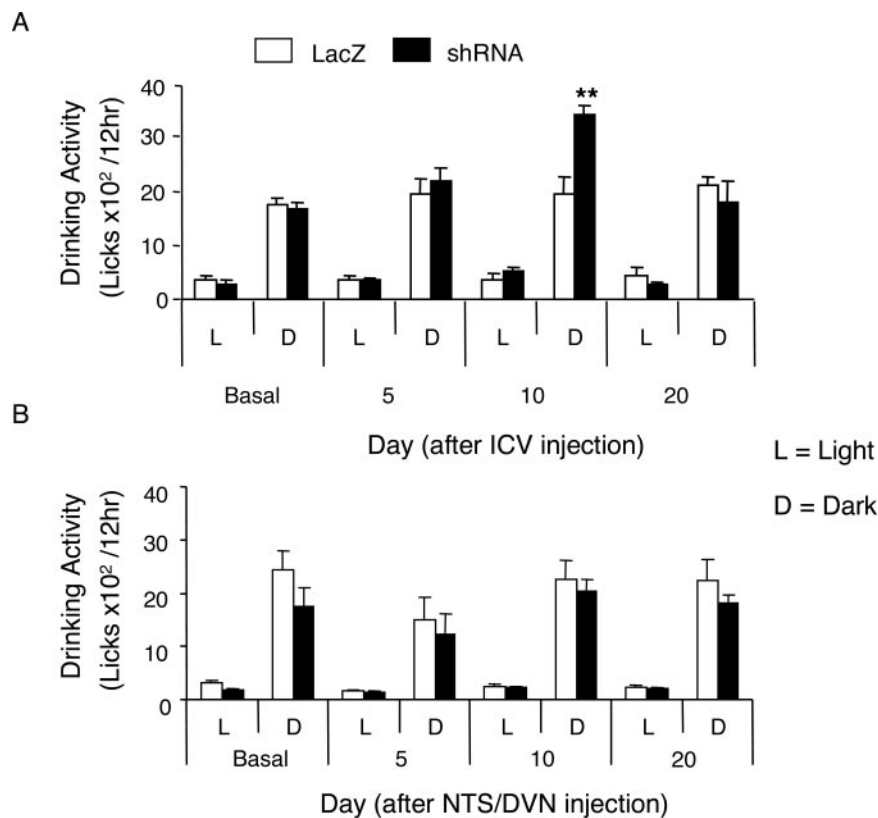
**TABLE 1. Effect of ICV Injection of Ad-AT1a-shRNA on Water Intake, Body Weight, and Plasma Ang II**

Variable	Group	Day 0	Day 5	Day 10	Day 20
Water intake (mL/24 h)	LacZ	4.6±0.2	4.7±0.7	4.3±0.4	3.9±0.9
	shRNA	4.3±0.2	5.6±0.7	8.3±0.5*	3.2±0.1
Body weight (g)	LacZ	33.0±2.4	32.8±2.1	31.9±2.3	30.2±1.3
	shRNA	28.7±1.1	28.9±0.9	28.0±1.2	29.5±1.3
Plasma Ang II (pg/mL)	shRNA	83.6±22.9	90.1±32.9	129.1±28.4	86.0±24.6

Values are mean±SEM.

\**P*<0.05, lacZ vs shRNA; water intake: *F*(1,6)=41.67, *P*=0.02; body weight: *F*(1, 27)=0.03, *P*=0.88; plasma Ang II: *F*(3, 21), *P*=0.45; 2-way ANOVA.

**Figure 4.** Effect of ICV (A) and bilateral NTS/DVN (B) injection of Ad-AT<sub>1a</sub>-shRNA on drinking activity. Drinking activity was recorded during light and dark periods on day 0 (basal), 5, 10, and 20 days after virus injection. There is a day/night rhythm for drinking activity [ICV: F(4, 13)=236.04, *P*=0.000; NTS/DVN: F(4, 11)=86.62, *P*=0.000; nested ANOVA]. There is a difference in ICV but not in NTS/DVN between LacZ and shRNA treatment [ICV: F(4, 13)=43.7, *P*=0.000; NTS/DVN: F(4, 11)=0.24, *P*=0.99]. \*\**P*<0.01, LacZ vs shRNA.



siveness to ICV injection of Ang II but not to other central pressor agents. Interestingly, baseline BP was not elevated.<sup>42</sup> Complete deletion of the AT<sub>1a</sub> receptor resulted in an animal with enhanced osmotic and sympathetic responses, as well as altered renal function.<sup>18,19,44</sup> However, there are limits to the use of these genetic models. First, because AT<sub>1</sub> receptors are widely expressed in brain and periphery, it is difficult to determine the regional functionality in models in which the receptor is globally deficient. Second, there is evidence for compensation in response to gene deletion and the fact that changes occur during development. For example, NTS AT<sub>1b</sub> receptors are upregulated in AT<sub>1a</sub> gene deletion mice.<sup>20</sup>

The novel RNAi method was demonstrated to efficiently and selectively silence mRNAs for a wide range of proteins.<sup>25</sup> McCaffrey et al<sup>45</sup> showed that gene expression can be suppressed in adult mice by synthetic small-interference RNAs and by small-hairpin RNAs transcribed in vivo from DNA templates. Makimura et al<sup>46</sup> demonstrated that RNAi

can be used to assess the physiological function of neuronal genes in vivo. This novel method has also been applied recently in the study of AT<sub>1a</sub> receptors. Vazquez et al<sup>24</sup> reported that transfection of AT<sub>1a</sub>-expressing Chinese hamster ovary cells with a synthetic AT<sub>1a</sub> shRNA resulted in an 80% decrease in the AT<sub>1a</sub> expression, which was maximal at day 3.

In this study, we identified the most effective targets for specific silencing and tested them using an ex vivo system (neuronal 2a cell line). The results showed a decrease of >60% for AT<sub>1a</sub> and AT<sub>1b</sub> receptor mRNAs 48 hours after shRNA cassette treatment, respectively. The ex vivo transfection efficiencies of the shRNA cassettes were ≈80% (data not shown). There was specificity of the treatment, because the AT<sub>1a</sub> shRNA did not affect AT<sub>1b</sub>, and vice versa for AT<sub>1b</sub>. We tested the silencing efficiency at 48 hours after shRNA cassette treatment, perhaps not the time point for maximum effect. On the basis of these results, we proceeded to in vivo studies using the same DNA sequences. Adenovirus vectors

**TABLE 2. Effect of ICV Injection of Ad-AT<sub>1a</sub>-shRNA on BP and HR**

Variable	Groups	Day 0		Day 5		Day 10		Day 20	
		Light	Dark	Light	Dark	Light	Dark	Light	Dark
MAP (mm Hg)	LacZ	104±5	117±6*	106±5	119±6*	100±5	106±4	101±3	113±5*
	shRNA	108±4	118±5*	106±3	117±4*	103±2	113±3†	100±3	118±4*
HR (bpm)	LacZ	576±22	630±25*	500±28	570±16*	563±30	600±42	580±20	618±16*
	shRNA	568±20	608±17*	560±13	600±16*	550±12	588±10*	512±16	592±19*

Values are mean±SEM. There is a day/night rhythm for both mean arterial pressure (MAP) and HR [MAP: F(4, 13)=21.39, *P*=0.000; HR: F(4, 13)=14.13, *P*=0.000. There is no significant treatment difference [MAP: F(8, 62)=1.00, *P*=0.44; HR: F(8, 62)=1.68, *P*=0.12; *n*=5 to 6, nested ANOVA].

\**P*<0.05, †*P*<0.01, light vs dark.

**TABLE 3. Effect of NTS/DVN Injection of Ad-AT1a-shRNA on Water Intake and Body Weight**

Variable	Group	Day 0	Day 5	Day 10	Day 20
Water intake (mL/24 h)	LacZ	4.4±0.4	2.9±0.5	4.1±0.3	4.0±0.4
	shRNA	3.9±0.2	30.0±0.6	3.7±0.7	4.0±0.2
Body weight (g)	LacZ	31.2±1.9	30.8±1.1	30.4±1.0	29.5±1.2
	shRNA	30.3±1.5	30.6±1.9	30.5±1.8	29.8±1.9

Values are mean±SEM. Repeated ANOVA shows no significance between lacZ and shRNA groups in water intake [F(1, 24)=0.28, *P*=0.61] and body weight [F(1, 36)=0.16, *P*=0.7].

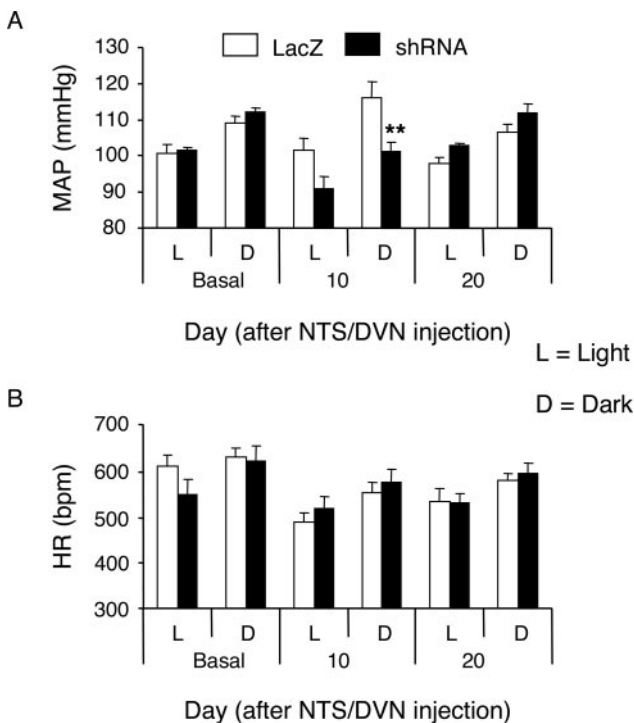
have achieved much success in gene delivery in in vivo studies.<sup>27,47,48</sup> When Ad-shRNA-AT<sub>1a</sub> was injected in the lateral ventricle, there was a decrease in AT<sub>1</sub> receptor binding in SFO. The decrease was noticeable at day 10 after injection, similar to the time course seen in a previous in vivo study.<sup>47</sup> There were no downstream effects in the hypothalamus or other regions. After adenovirus injection into the NTS/DVN region, there was downregulation in this region only. The effect was more pronounced than that seen in SFO with levels reduced up to 20 days after injection. The difference in time course is likely related to the mode of injection, the cerebral ventricle, as opposed to the brain parenchyma.

The physiological results revealed that the silencing of AT<sub>1a</sub> receptors in SFO and NTS/DVN produced different functional outcomes, suggesting regional specificity. Silenc-

ing of AT<sub>1a</sub> in SFO produced an increase in the volume of water intake, as well as an increase in licking activity. The increased drinking activity occurred during the night when mice are behaviorally active. There were no changes in BP or HR after ICV administration, showing that SFO AT<sub>1a</sub> receptors were specific for fluid balance. Indeed, there are much data that show that the SFO region is critical in the regulation of water intake and that these effects are mediated by Ang II. Early studies showed that Ang injection into the SFO induced activation of the hypothalamic neurons and increases in water intake.<sup>49,50</sup> Thus, our result showing that AT<sub>1a</sub> inhibition increased water intake is the opposite of what would be predicted based on the effects of Ang stimulation. However, one must consider that peptide injections stimulate all of the Ang receptors, whereas the shRNA approach is receptor specific and produces chronic, rather than acute, changes. Indeed, studies in mice lacking AT<sub>1a</sub> receptors show that water intake is also increased, similar to that seen in mice exposed to Ad-AT<sub>1a</sub>-shRNA.<sup>44</sup> This may be related to changes in renal function or to changes in the brain renin-angiotensin system.<sup>44</sup> Davisson et al<sup>17</sup> showed that brain AT<sub>1b</sub> receptors are important in the mediation of the Ang II-induced drinking responses. The current observation that inhibition of AT<sub>1a</sub> receptors in the SFO produced an increase in water intake could result from an enhancement in Ang AT<sub>1b</sub> signaling. It is possible that Ang II from a local or systemic source is available for stimulation of SFO AT<sub>1b</sub> receptors. We measured plasma Ang II levels and found no difference between Ad-AT<sub>1a</sub>-shRNA-treated and Ad-LacZ-treated mice. However, there was a trend for higher plasma Ang II at day 10 after injection, the time point at which we observed an increase in drinking. It is also possible that there are changes in central Ang peptides, resulting in stimulation of both AT<sub>1b</sub> and AT<sub>2</sub> receptors. Previous studies have suggested that brain AT<sub>2</sub> receptors are important in the regulation of water intake.<sup>51,52</sup>

With regard to the cardiovascular effects of ICV Ad-AT<sub>1a</sub>-shRNA treatment, there was no evidence of any change in BP or HR. This is also an unpredicted finding based on studies of SFO physiology. For example, the SFO is necessary for the rise in BP produced by Ang II infusion.<sup>53,54</sup> There is also evidence for neural connections among the SFO, PVN, and brain stem cardiovascular centers and evidence that Ang II serves as a central neurotransmitter in this pathway.<sup>55,56</sup> However, as mentioned above for water intake, there is little consideration of the separation of the receptor subtypes.

Another interesting finding is that silencing of AT<sub>1a</sub> receptors in the NTS/DVN reduced BP but produced no change in



**Figure 5.** Effect of bilateral NTS/DVN injection of Ad-AT<sub>1a</sub>-shRNA on BP (A) and HR (B). Blood pressure and heart rate were recorded on days 0 (basal), 10, and 20 after virus injection. There is a day/night rhythm for both mean arterial pressure and HR [mean arterial pressure: F(3, 10)=10.6, *P*=0.002; HR: F(3, 10)=5.15, *P*=0.021; nested ANOVA]. There is a difference in mean arterial pressure but not in HR between LacZ and shRNA treatment [mean arterial pressure: F(6, 20)=4.28, *P*=0.0062; HR: F(6, 20)=0.11, *P*=0.99; nested ANOVA]. \*\**P*<0.01 LacZ vs shRNA.

HR or drinking activity. The time course of the BP response was transient, seen only at day 10, whereas receptor changes were seen up to day 20. As with the drinking effect, the change was noticed only during the dark period when the mice are active. The data suggest that AT<sub>1a</sub> receptors in the brain stem NTS/DVN have a different functional role from that in the SFO. In fact, there are reports that the NTS is involved in setting the level of BP.<sup>57–59</sup> This is logical if you consider the structural neurochemistry of the region with input from baroreceptors and chemoreceptors in the heart and great vessels. Because BP was measured only at selected times after injection, we cannot make any statements related to the maximal changes or to the initiation of the effect. The fact that there were no changes in HR may signify that there were alterations in baroreflex function, although additional studies are required.

In terms of the limitations of the study, these are mainly related to a lack of detailed information on the complementary AT<sub>1b</sub> system. It would be useful to know if the Ad-shAT<sub>1a</sub> increased AT<sub>1b</sub> expression as observed in the Ang AT<sub>1a</sub> gene deletion model.<sup>20,21</sup> Likewise, it would be interesting to silence the AT<sub>1b</sub> receptors both singly and in combination with AT<sub>1a</sub> receptors. If the AT<sub>1b</sub> receptors are involved in control of water intake, one would predict that the drinking response would be abolished.

Results document that regional specific changes in brain Ang AT<sub>1a</sub> receptors produced different functional effects as related to water intake and BP. The results are different from those achieved using peptide administration and pharmacological blockade and raise the point that genetic modulation may be a more specific means to study function.

## Perspectives

This study introduces shRNA as a specific and powerful in vivo tool to study the role of neuropeptide receptors in the regulation of cardiovascular and fluid balance, with the advantage of offering regional selection. The results document that AT<sub>1a</sub> signaling is important in modulating drinking activity via inputs from the rostral forebrain, SFO, and is important in modulating BP via inputs from the brain stem NTS/DVN. These types of studies will prove useful in understanding how structurally specific receptors are involved in cardiovascular and fluid balance.

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## Correction

In the article by Chen et al in the February 2006 issue of *Hypertension* (Chen Y, Chen H, Hoffmann A, Cool DR, Diz DI, Chappell MC, Chen A, Morris M. Adenovirus-mediated small-interference RNA for in vivo silencing of angiotensin AT<sub>1a</sub> receptors in mouse brain. *Hypertension* 2006;47:230–237) the following errors occur. In the author list, Dr Chen's name should appear as Alex F. Chen. In the Experimental Protocols section, the virus concentration for Ad-LacZ virus should be  $1.9 \times 10^{12}$  pfu/mL, and the virus concentration for Ad-AT<sub>1a</sub>-siRNA virus should be  $2.3 \times 10^{10}$  pfu/mL. In Table 2, the last entry under the Light column of Day 0 should be  $567 \pm 20$ . The authors regret the errors.