Activation of the Brain Angiotensin System by In Vivo Human Angiotensin-Converting Enzyme Gene Transfer in Rats

Shigefumi Nakamura, Atsushi Moriguchi, Ryuichi Morishita, Kazuo Yamada, Tadahiko Nishii, Naruya Tomita, Mitsuhiro Ohishi, Yasufumi Kaneda, Jitsuo Higaki, Toshio Ogihara

Abstract—The possibility of the brain-specific expression of a component of the renin-angiotensin system was evaluated in the present study. We used the hemagglutinating virus of Japan-liposome complex to transfect human angiotensin-converting enzyme (ACE) cDNA, driven by the cytomegalovirus enhancer and β-actin promoter, into the lateral cerebroventricle of male Sprague-Dawley rats. We evaluated the time course of hemodynamics, the tissue levels of angiotensin (Ang) II and vasopressin, and ACE activity. Intracerebroventricular transfection of the human ACE gene increased both blood pressure and heart rate. Transfected rats exhibited higher concentrations of brain Ang II and increased brain ACE activity. This activation of the brain angiotensin system was accompanied by increased vasopressin production. The increases in blood pressure and heart rate were abolished by intracerebroventricular administration of an ACE inhibitor or Ang II type 1 receptor antagonist. The expression of the transgene was widely distributed in the periventricular cell layer, the cortex, the hypothalamic nuclei, and the brain stem. Expression in the neuronal cells persisted for up to 14 days. Thus, this hemagglutinating virus of Japan-liposome method is a highly efficient system for gene delivery and is extremely useful for functional gene transfection. This novel hypertensive model may enable characterization of the functions of the renin-angiotensin system in the brain and determination of its role in the pathogenesis of hypertension. (Hypertension. 1999;34:302-308.)

Key Words: renin-angiotensin system ▪ central nervous system ▪ hypertension, essential ▪ parainfluenza virus type 1 ▪ gene transfer

Recent studies have broadened our understanding of the role of the renin-angiotensin system (RAS) in the pathogenesis of essential hypertension. The overactivity of the RAS may explain the pathogenesis of malignant hypertension and many of the renin-dependent forms of renovascular disease.1 The tissue RAS appears to be a neuroendocrine modulator of the vasopressor system in the brain. The injection or infusion of renin, angiotensin (Ang) I, or Ang II into the ventricular spaces of the brain stimulates drinking, the release of arginine vasopressin (AVP) and corticotropin, natriuresis, and a rise in blood pressure (BP).2,3 The intracerebroventricular (ICV) administration of angiotensin-converting enzyme (ACE) inhibitors or Ang II antagonists blocks these actions, which suggests that these Ang peptides are produced in the brain. Neurons and neuroglia can bring about complex changes in nervous system function by acting on various neural, hormonal, and immune mechanisms. This raises the possibility that the brain is involved in BP control by a mechanism that, in part, requires RAS. Indeed, recent observations by Phillips and colleagues4,5 have demonstrated that antisense oligodeoxynucleotides injected directly into the brain and targeted to the Ang II type 1 receptor or the angiotensinogen gene produce a decrease in BP. The establishment of an animal model to depict brain-specific overactivation of the RAS is required to examine the detailed brain mechanisms of RAS in BP regulation.

We attempted to establish a hypertension model in which the brain RAS is activated by transfection of the human ACE (hACE) gene. This approach involved an efficient gene transfer technique that used the hemagglutinating virus of Japan (HVJ) complexed with liposomes to transfer the hACE cDNA. We used this technique to test our hypothesis by transfecting the hACE gene into intact rat brains in vivo. We then studied the biochemical and physiological effects of the overexpression of ACE within the central nervous system.

Methods

Plasmid Construction
An EcoRI fragment that contained the truncated hACE gene released from the RB 35 to 15 vector by EcoRI digestion and 2 putative active
sites was ligated into the EcoRI site of the pUC-CAGGS vector (kindly provided by Junichi Miyazaki, Osaka University, Suita, Japan) with T4 DNA ligase. The hACE cDNA was a generous gift of F. Soubrier (INSERM, Paris, France). The pUC-CAGGS contains an open reading frame that codes for the entire envelope protein. This open reading frame consists of 3 translation initiation sites that correspond to the N termini of the large, middle, and major surface polypeptides. These sequences are downstream of the cytomegalovirus enhancer and the chicken β-actin promoter. The control vector was pUC-CAGGS without the hACE cDNA.

Preparation of HVJ-Liposomes

The preparation of HVJ-liposomes has been previously described. In brief, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4:8:2. This lipid mixture (10 mg) was then deposited on the sides of a flask by removing the tetrahydrofuran solvent in a rotary evaporator. The dried lipid was hydrated in 200 μL of balanced salt solution (137 mM NaCl, 5.4 mM KCl, 13 mM Tris-HCl, pH 7.6) containing DNA-HMG (high mobility group)-1 complex (300 μg:96 μg), which had previously been incubated at 20°C for 1 hour. Liposomes were formed by shaking and sonicating this mixture. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg mm² s⁻¹) for 3 minutes just before use. The liposome suspension (0.5 mL, containing 10 mg of lipids) was mixed with HVJ (20 000 hemaglutinating units) in a total volume of 4 mL of the balanced salt solution. The mixture was incubated at 4°C for 10 minutes and then at 37°C for 30 minutes with gentle shaking. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient that contained the HVJ-liposomes was collected for use.

General Surgical Procedures

Male Sprague-Dawley rats (400 to 500 g, Charles River Breeding Laboratories, Osaka, Japan) were anesthetized with sodium pentobarbital (50 mg/kg IP). Anesthetized rats were then placed in a stereotaxic frame (Narishige Scientific Instrument Laboratory), and the skull was exposed. A stainless cannula (30 gauge, Becton Dickinson) with a specially designed Teflon connector (FEP tube, Bioanalytical Systems) was introduced into the left lateral cerebroventricle, as previously described. The stereotaxic coordinates were the lateral brain ventricle 1.3 mm posterior to the bregma, 2.1 mm lateral to the midline, and 3.6 mm below the skull surface. This procedure was completed by administration of a prophylactic antibiotic (penicillin G, 30 000 U IM).

In Vivo hACE Gene Transfer

The HVJ-liposome complex (20 μL) containing either 10 μg/mL of hACE cDNA (n=38) or the same amount of control vector (n=20) was infused into the cannula in the left lateral cerebroventricle at a infusion rate of 2 μL/min with a micro-infusion pump (CMA-100, Carnegie Medicine). After infusion of the HVJ-liposome complexes, the infusion cannula was removed.

We then measured the time course of BP and heart rate (HR) changes in these rats with the tail-cuff method (BP-98A, Softron) until 14 days after transfection. Rats in a separate group were decapitated at day 5 (hACE, n=18; control, n=18) and at day 14 (hACE, n=5; control, n=4) after transfection. Brain tissue was carefully removed and, with the collected blood, assayed for Ang II concentration, ACE activity, and AVP concentration.

To evaluate the localization of the transfected gene, we performed immunohistochemical analysis with anti–ACE antibody, as previously described. In brief, rats were euthanized 1 day after transfection. Tissue extracts were lyophilized and resuspended in radioimmunoassay buffer with additional dilution of the tissue extracts when necessary. The plasma samples were then extracted in acetone/petroleum ether. The detection limit for this radioimmunoassay was ~0.1 pg at 95% binding: the cross-reactivity with oxytocin and other structurally related peptides was <0.01%.

For the measurement of brain and plasma ACE activity, tissue and blood samples were prepared. Blood samples were placed in tubes containing heparin sulfate. After centrifugation at 4°C, all samples of plasma were quickly frozen on dry ice and stored at −20°C until assay. Brain tissue was promptly removed and immediately frozen in liquid nitrogen. All brain tissue was stored at −70°C before use. On the day of assay, the brain tissue was thawed at 4°C, weighed, and then homogenized in 0.1 N hydrochloric acid with a polytron. Each sample was then centrifuged at 20 000g for 30 minutes at 4°C. To extract Ang II, the supernatant was applied to an Amprer C8 mini-column (Amersham), as previously described, and quantified as described below.

Biochemical Determinations

Rats were euthanized 5 days after transfection for the measurement of brain and plasma Ang II concentrations. Blood samples were collected by decapitation, without use of anesthesia, in preheched tubes containing EDTA-2Na (1 mg/mL whole blood) and 2.5 mmol/L PMSF, and centrifuged at 4°C. To measure Ang II, plasma was stored at −70°C before assay. A 1-mL sample of each freshly separated plasma specimen was promptly concentrated in an Amprer C8 mini-column (Amersham), as previously described, and quantified as described below.

Brain tissues were promptly removed and immediately frozen in liquid nitrogen. All brain tissue was stored at −70°C before use. On the day of assay, the brain tissue was thawed at 4°C, weighed, and then homogenized in 0.1 N hydrochloric acid with a polytron. Each sample was then centrifuged at 20 000g for 30 minutes at 4°C. To extract Ang II, the supernatant was applied to an Amprer C8 mini-column that was prewashed with 4 mL of methanol and 4 mL of 0.1% trifluoroacetic acid (TFA). After washing the column with an additional 10 mL of 0.1% TFA, Ang II was eluted with 2 mL of ethanol/water/TFA (80:19:9.0.1%, v/v/v). The eluate was dried with the use of a centrifugal vacuum concentrator (CC-181, Tomy). The recovery of Ang II with this procedure was 98±2% (n=5), which was verified by 125I–Ang II detection. The data were not corrected for recovery because the variation of recovery was negligible. The resultant Ang II–containing residue was suspended in 100 μL of 0.1% TFA. High-performance liquid chromatography characterization was then performed as previously described. In the appropriate peak fractions, samples were collected and dried in a vacuum centrifuge and redissolved in 0.1 mol/L Tris acetate, pH 7.4, containing 2.6 mmol/L EDTA-2Na, 1 mmol/L PMSF, and 0.1% bovine serum albumin. The elution times for Ang II, Ang III, and Ang I were 19.0, 20.7, and 23.7 minutes, respectively. The immunoactive Ang II was measured by radioimmunoassay with a specific antibody donated by Kazuaki Shimamoto, MD (Sapporo, Japan). The sensitivity of this immunoassay was 0.1 pg/tube. The recovery of Ang II after high-performance liquid chromatography was 85±5%. The cross-reactivity of the Ang II antibody was 100% for Ang II and <0.1% for either Ang I, Ang III, or Ang-(1–7).

Plasma and tissue levels of AVP were measured with a specific radioimmunoassay described elsewhere. Briefly, tissue extracts were lyophilized and resuspended in radioimmunoassay buffer with additional dilution of the tissue extracts when necessary. The plasma samples were then extracted in acetone/petroleum ether. The detection limit for this radioimmunoassay was ~0.1 pg at 95% binding: the cross-reactivity with oxytocin and other structurally related peptides was <0.01%.

The amount of His-Leu generated during ACE incubation with Hippuryl-His-Leu, generated against human lung ACE. The specificity of 9B9 has been previously reported.
ICV Administration of ACE Inhibitor or Ang II Type 1 Receptor Antagonist to hACE-Transfected Rats

To investigate whether the elevation in BP observed in the hACE-transfected rats was mediated by the activation of the brain RAS, we administered the ACE inhibitor delapril (100 μg/10 μL; Takeda Chemical Industries, Ltd) into either the cerebroventricle (10 μL, n=8) or femoral vein (100 μL, n=5) in both hACE gene-transfected rats and control rats 5 days after transfection. Administration of the specific AT1 antagonist CV-11974 (300 μg/10 μL; Takeda Chemical Industries, Ltd) was also performed according to a similar protocol (into the cerebroventricle, 10 μL, n=5; into the femoral vein, 100 μL, n=5, respectively). Rats were fitted with arterial and venous catheters 24 hours before the procedure under anesthesia with urethane (1.5 g/kg IP). A polyethylene catheter (pericardial effusion [PE]-50, Clay Adams) was inserted into the abdominal aorta for continuous measurement of MAP and HR through the arterial catheter, by use of a pressure transducer (TP-400T, Nihon Koden Electronics), amplifier (AP-641G, Nihon Koden Electronics), and cardiometer (AT-601G, Nihon Koden Electronics). A venous catheter was also advanced into the inferior vena cava by way of the femoral vein for the injection of drugs. The free end of both catheters was tunneled to the back of the neck, as described elsewhere.14 We evaluated BP and HR responses to either ACE inhibitor or AT1 antagonist in conscious, freely moving animals.

Reverse Transcription-Polymerase Chain Reaction for hACE mRNA Measurement

RNA was extracted from the brain tissue of hACE gene-transfected rats and control plasmid-transfected rats 5 days after transfection with the use of RNazol B (Tel-Test Inc). The levels of hACE and GAPDH mRNA were measured by reverse transcription-polymerase chain reaction (RT-PCR).9 The primers for ACE were 5' TTGGAGGACCTGGTGGTGGCCAC-3' (forward) and 5' AAAGTGTATGTCATGTGCTGCCTGCT-3' (reverse), which selectively amplify a 216-bp fragment of hACE (nucleotides 2960 to 3175 of cDNA), which begins in exon 21 and terminates in exon 22 of the human, but not hACE, gene. Primers for GAPDH were 5'-CCCATACCATCTCAGGAG-3' (forward) and 5'-GTGTCTGATGGATGGCC-3' (reverse), which amplify a 284-bp fragment of human GAPDH (nucleotides 211 to 495 of cDNA). The amplification profiles consisted of RT at 65°C for 40 minutes and PCR with 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. First cycle denaturation was for 4 minutes; last cycle extension was for 8 minutes. After the completion of RT-PCR, the products were separated by electrophoresis through a 2% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis

Data are expressed as mean±SEM. One-way ANOVA was used for multiple comparisons of the effects of the hACE gene or control vector transfection. Two-way ANOVA was used to assess differences between hACE gene-transfected rats and control rats. When appropriate, the repeated-measures option was applied to the ANOVA. The Duncan multiple range test was applied to assess differences whenever statistical significance was found. The Student t test was also used for paired and unpaired observations. For statistical purposes, values below the detection levels of an assay were recorded as the detectable level. Statistical significance was established at P<0.05.

Results

Effects of hACE Gene Transfection on Hemodynamics and Brain RAS

Figure 1 shows the changes in systolic BP (top) and HR (bottom) in response to ICV transfection of either the hACE gene or control plasmid with the use of the HVJ-liposome method. Transfection of the hACE gene into the brain resulted in a significant increase in both the systolic BP and HR. The BP of hACE-transfected rats increased from 127±5 mm Hg (day 0) to 150±4 mm Hg (day 5), and this elevation continued through day 14 (137±2 mm Hg). A concomitant increase in HR was also observed (Figure 1). Conversely, no changes in systolic BP and HR were observed in rats transfected with control vector. No behavioral changes such as convulsions or abnormal movements of the extremities were observed in any animal undergoing ICV injections.

Characteristics of the Brain and Plasma Angiotensin Systems and AVP Content in hACE Gene-Transfected Rats

We measured the ACE activity and the Ang II and AVP concentrations in the brain tissue and blood 5 days after transfection. The brain ACE activity was significantly higher in hACE gene-transfected rats than control rats (60±12 U versus 18±6 U, P<0.05) (Figure 2, top left). Brain Ang II concentrations were also significantly higher in hACE-transfected rats than in control-transfected rats (11.1±0.4 versus 7.5±0.3 pg/g, P<0.05) (Figure 2, top right) and had returned to comparable levels by day 14 (7.2±0.5 versus 6.9±0.4 pg/g, NS). However, both the serum ACE activities and plasma Ang II concentrations were comparable between hACE gene-transfected and control-transfected rats (Figure 2, bottom). Higher AVP concentrations were observed both in brain tissue and plasma in the hACE gene-transfected rats than the control rats (brain, 308±18 versus 134±21 pg/g; plasma, 18.6±2.8 versus 11.8±2.2 pg/mL, P<0.05, respectively).

Effect of ACE Inhibitor or AT1 Antagonist on BP and HR

To confirm that these hypertensive events were caused by acceleration of the brain RAS, we administered either the
ACE inhibitor delapril or the AT1 antagonist CV-11974 into the cerebroventricle or femoral vein of both hACE gene-transfected and control-transfected rats. ICV administration of 100 μg of delapril caused a significant reduction in mean arterial pressure (MAP) (−27±5 mm Hg at 15 minutes after injection, P<0.05) and HR (−25±8 bpm at 15 minutes after injection, P<0.05) in conscious, unrestrained hACE gene-transfected rats. No changes were observed in MAP (−25±6 mm Hg at 15 minutes after injection, NS) or HR (−11±6 bpm at 15 minutes after injection, NS) in response to delapril ICV in control rats. Figure 3 shows typical examples of these hemodynamic responses to this ACE inhibitor in hACE gene-transfected rats (top) and control rats (bottom). The same dose (100 μg) of delapril administered through the femoral vein did not cause any changes in arterial BP or HR in either group of rats (data not shown). ICV administration of 300 μg of CV-11974 also caused a significant reduction in MAP (−32±6 mm Hg at 15 minutes after injection, P<0.05) and HR (−48±10 bpm at 15 minutes after injection, P<0.05) in conscious, unrestrained hACE gene-transfected rats (Figure 4). No changes were observed in MAP (−4±5 mm Hg at 15 minutes after injection, NS) or HR (−21±15 bpm at 15 minutes after injection, NS) in response to CV-11974 in control rats (Figure 6).

**Distribution of the Transgene and mRNA Expression in the Brain**

To verify successful gene transfer and to localize the transgene, we performed immunocytochemical analysis with the use of a monoclonal anti-ACE antibody antiserum. Positive staining was then evaluated in brain sections 1 day, 5 days, and 14 days after transfection. Positive cellular staining for hACE was observed throughout a broad area of brain tissue 5 days after transfection. Staining was evident in the brain cortex, the subfornical organ, the supra optic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus, and in the ventral area of the brain stem, including the rostral ventrolateral medulla (RVLM). Figure 5 shows the positive staining of hACE in the PVN, the SON, and the RVLM 5 days after gene transfection. In the control gene-transfected rats, positive staining was not apparent throughout the 14 days after transfection. Moreover, at day 14, the positive staining was weak but still persisted in the brain regions containing the transfected gene with HVJ-liposome complex, which demonstrates a prolonged effect of gene transfection by this HVJ-liposome method.

Consistent with the increase in hACE activity in the brain, stronger hACE mRNA expression was observed in hACE gene-transfected rats than control rats, whereas no apparent change in GAPDH mRNA was observed between gene-transfected and control rats at 5 days after hACE gene transfection (Figure 6).

**Figure 2.** Brain (top) and serum (bottom) activities of ACE (left) and concentrations of Ang II (right) measured at 5 days after transfection of the hACE gene or control vector. *P<0.01 vs control.

**Discussion**

The present study provides the first direct evidence that hACE gene transfection into the rat brain mediates the elevation of systemic BP and HR by activation of the brain RAS independent from the circulating RAS. This study also indicates that the HVJ-liposome vector system is an efficient tool for targeted gene delivery even in the central nervous system. Furthermore, the use of this novel hypertensive rat
model can address the as yet unknown pathophysiology of BP regulation as governed by brain RAS mechanisms. In several species and brain regions, the presence of Ang II in the brain has been demonstrated by the detection of Ang II immunoreactive cells and fibers, and by the presence of renin and the mRNA encoding for renin and angiotensinogen. Some earlier studies have suggested the functional importance of RAS in the brain, because injections of renin, Ang I, or Ang II into the ventricular spaces stimulated drinking response, the release of AVP, natriuresis, and a rise in BP. The central administration of an ACE inhibitor or an Ang II receptor antagonist blocked these actions. These findings suggest that the angiotensin peptide is produced within the brain. The precise mechanism by which this system regulates BP and its role in the development of hypertension, the identity of the regions of the nervous system that mediate the action of Ang II, and the functions that are specific to the brain RAS remain to be clarified.

We used an in vivo gene-transfer technique that permits the target gene, such as renin, ACE, and chymase, to be transfected directly into the brain, thereby avoiding any systemic effects. In addition, the consequences of local over-expression of the transfected gene within its physiological/pathophysiological concentration range may be studied. The problems surrounding gene transfer techniques include safety, efficiency, and continuance. HVJ complexed with liposomes has been reported as an effective and harmless vector for local gene delivery to vascular tissue, kidney, and liver. Recent studies in our laboratory indicate that the HVJ-liposome method prolongs the half-life of oligodeoxynucleotides and concentrates them in the nuclei of cultured neuronal cells in vitro and in rat brains in vivo. Moreover, this study reports the broad transfection of oligodeoxynucleotides into the brain by the HVJ-liposome method. We further confirmed the safety of this method by histological examination and behavioral observation. The present study applied this novel vector system to the local delivery of a gene into the central nervous system without induction of any behavioral changes, such as convulsions or abnormal movement of the extremities, during the experiment. We were able to study the long-term expression of the transfected gene (hACE gene) and the function of its gene product (Ang II) in the brain. As shown in Figure 5, bottom right, hACE expression was weak, but still present, at day 14 after gene transfection. However, the increases in BP and HR had diminished by day 14. The Ang II concentration in the brain of hACE gene-transfected rats at day 14 was slightly, but not significantly, higher than that of control rats. Therefore, we speculate that at day 14, ACE activity was not high enough to convert Ang I to Ang II. Further work is required to clarify the precise cellular fate or degradation process of the transgene and the mechanisms of the diminished physiological effect of hACE in the brain.

The transgene was widely distributed at the brain cortex, the PVN and SON of the hypothalamus, and the brain stem.
including the nucleus tractus solitarii and the ventrolateral medulla. The transfected hACE gene produced ACE and was mainly localized in the neuronal cells, but also, in part, in the glial cells (Figure 5b). Expression in these neuronal cells persisted for up to 14 days. The activation of brain RAS by hACE gene transfection in the cardiovascular center may explain the mechanism of BP elevation. The PVN and SON of the hypothalamus are considered key areas of cardiovascular regulation. These nuclear regions contain a high concentration of neurosecretory neurons, which possess a high metabolic rate and a dense capillary network. These neurons innervate the median eminence, the brain stem and spinal cord, and the posterior pituitary. Colocalization of Ang II with AVP neurons in the PVN and SON has been described, and endogenous brain angiotensin peptides are thought to regulate AVP release. In the present study, we observed the augmented release of both central and peripheral AVP in hACE-transfected rats. Thus, the activation of neurons in the PVN and SON may cause high BP in this hypertensive rat model.

The vasomotor neurons of the medulla oblongata (nucleus tractus solitarii and ventrolateral medulla) that control respiration and determine vascular tone are also important in BP regulation. The bulbar vasomotor neurons receive important angiotensinergic input from the neuronal circuits in the vagal-solitarii region. Indeed, the topical application of Ang II to the vasomotor structures of the RVLM causes increases in BP that are mediated by enhanced peripheral sympathetic drive. The neuronal circuits of the medulla oblongata that subserve cardiovascular function are endowed with angiotensin receptors. Ang II appears to act within the central nervous system pathways at various sites that determine the excitatory neural and endocrine mechanisms governing BP, vascular resistance, and renal function. In the present study, strong expression of the transgene was observed in the ventral area of the brain stem. Thus, increased Ang II production by the transfected hACE gene may enhance the actions of Ang II that augment peripheral sympathetic drive. Moreover, this sympathetic activation also may relate to a specific mechanism involved in the pathogenesis of arterial hypertension in these hACE gene-transfected rats.

ACE hydrolyzes a variety of peptide hormones such as bradykinin, enkephalin, or substance P, in addition to Ang I. Some of these peptides play a role in the regulation of BP in the brain. For instance, it is well known that administration of bradykinin into the lateral ventricle of the brain causes an increase in BP. Although we did not evaluate changes in these peptides in the brain, alterations in the level of neuropeptides might modify the changes in BP and HR in hACE gene-transfected animals. Further study will be necessary to know the detailed evaluation of the kinetics of neuropeptides and their contribution to the BP regulation mechanisms. Nevertheless, the present study showed that the hACE gene, when successfully transfected into neuronal cells, could cleave Ang I to produce Ang II. The brain angiotensin pathway in BP regulation may be more important than other peptidergic pathways because ICV administration of both ACE inhibitor and AT1 antagonist reversed the physiological effect of increased ACE in gene-transfected animals.

In conclusion, the present findings provide evidence that the brain angiotensin system regulates BP independently from the circulating RAS. The successful transfer of the hACE gene into the brain achieved in the present study serves as a paradigm for the elucidation of the role and interaction of other neuro-endocrine mediators in the pathogenesis of diseases such as hypertension, cardiac disease, and stroke. Our data demonstrate that localized in vivo gene transfer is a
useful experimental tool for studying complex pathophysiology logic autocrine/paracrine mechanisms in vivo.

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