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Effect of repetitive icv injections of ANG II on c-Fos and AT_1-receptor expression in the rat brain

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[1]*Institute of Pharmacology, University of Kiel, 24105 Kiel;* [2]*German Institute for High Blood Pressure Research, 69120 Heidelberg; and* [3]*Gesselschaft Für Strahlenforschling-National Research Center for Environment and Health, Institute of Mammalian Genetics, 85764 Neuherberg, Germany*

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Moellenhoff, Eva, Annegret Blume, Juraj Culman, Bimal Chatterjee, Thomas Herdegen, Christine J. Lebrun, and Thomas Unger. Effect of repetitive icv injections of ANG II on c-Fos and AT_1-receptor expression in the rat brain. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R1095–R1104, 2001.—ANG II has been implicated in neuroplastic processes via stimulation of inducible transcription factors (ITF) in the brain. In the present study, we investigated the effects of acute vs. repetitive once daily intracerebroventricular injections of ANG II for 7 days on the expression of ITF and constitutive transcription factor (CTF) and the AT_1 receptor in the median preoptic area (MnPO), the subfornical organ (SFO), and the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON). After repetitive injections, the expression of c-Fos declined by ~50% in MnPO, SFO, PVN, and SON compared with controls injected once. The desensitization of c-Fos occurred on the transcriptional level as shown in the SON by RT-PCR. Apart from a novel expression of c-Jun in the SON, the ITF c-Jun, JunB, JunD, and Krox-24 did not change after repetitive stimulation. Neither were the CTF, calcium response element binding protein, activating transcription factor 2, and serum response factor altered after repetitive vs. single injections of ANG II. The AT_1 receptor was coexpressed with c-Fos/c-Jun. Immunohistochemical stainings suggest an increase in AT_1-receptor number in MnPO, SFO, PVN, and SON on chronic stimulation compared with once-injected controls. These findings demonstrate that repetitive periventricular stimulation with ANG II essentially alters the expression of transcription factors compared with acute stimulation and suggest c-Fos and c-Jun as major intermediates of the AT_1-receptor transcription.

chronic stimulation; desensitization; inducible transcription factor; constitutive transcription factor; angiotensin AT_1 receptor; intracerebroventricular

IN THE CENTRAL NERVOUS SYSTEM, ANG II induces neuroendocrine and behavioral responses, including drinking, vasopressin and oxytocin release, natriuresis, and pressor response (41). Whereas the role of the AT_2 receptor in these effects is still controversially discussed, the involvement of the AT_1 receptor in the maintenance of volume, electrolyte, and cardiovascular homeostasis is generally accepted knowledge. Forebrain areas, such as the median preoptic area (MnPO), the subfornical organ (SFO), and the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON), known to be involved in osmoregulation, predominately contain AT_1 receptors (16, 34, 37). The AT_1 receptor also mediates the finely tuned expression of inducible transcription factors (ITF) of the Fos, Jun, and Krox families in these brain regions after a single intracerebroventricular injection of ANG II, suggesting a profound effect of ANG II on neuronal gene expression (2, 26). The induction of genes encoding for ITF depends on preexisting constitutively expressed transcription factors (CTF), such as calcium/cAMP response element binding protein (CREB), serum response factor (SRF), or activating transcription factor 2 (ATF-2). In addition to their well-known activation on the posttranslational level, a regulation of the CTF on the transcriptional level has been demonstrated in recent studies (4, 9, 13).

Apart from the CTF, the expression of the ITF showed substantial changes after chronic or repetitive administration of various stimuli (10, 18–21, 25, 43). Therefore, we addressed the question as to what extent repetitive intracerebroventricular injections of ANG II would affect the expression of ITF and CTF compared with a single stimulation of periventricular ANG receptors. Furthermore, we examined the expression of AT_1 receptors in the brain, which is the origin of the ANG II-activated signal-transcription coupling and which is regarded as putative target gene of the AP-1- and CRE-binding transcription factors, such as Fos, Jun, CREB, and ATF-2 proteins (2, 26).

MATERIALS AND METHODS

Animals

Male Wistar rats (250–300 g) were acquired from Dr. K. Thomae, Germany, and housed under standard conditions with respect to temperature, humidity, and light periodicity

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(12:12-h light-dark cycle). They were allowed free access to food (Altromin 1320 standard diet) and water.

Surgical Methods

Chronic intracerebroventricular cannulas (PP20, Portex) were implanted into the right lateral ventricle under chloralhydrate anesthesia (400 mg/kg ip) using the coordinates 0.6 mm caudal, 1.3 mm lateral to bregma, and 5.0 mm below the skull surface. The cannulas were fixed in position by dental cement anchored to the bone by three stainless steel screws. After surgery, the rats were housed individually for a 1-wk postoperative period and handled daily to familiarize them with the injection procedure and to reduce unspecific stress-dependent expression of ITF on the day of experiments.

Preparations of the ANG II Solution

ANG II (Bachem, Bubendorf, Switzerland) was stored in stock solutions (1 mg/ml) at −20°C until use and was diluted to a final concentration of 100 pmol/μl on the day of the experiment.

Single and Repetitive Application of ANG II

All injections were given intracerebroventricularly in a volume of 1 μl flushed with 4 μl isotonic sodium chloride between 9:00 and 9:30 AM. The animals were randomly divided into 12 groups.

Experimental protocol 1. Group 1 ($n = 9$) received intracerebroventricular injections of 100 pmol ANG II. *Group 2* ($n = 8$) received isotonic saline in 24-h intervals for a 1-wk period (7× ANG II, 7× NaCl). *Group 3* ($n = 5$) was injected intracerebroventricularly with isotonic saline in 24-h intervals for 6 days followed by a single injection of 100 pmol ANG II on *day 7* (6× NaCl + 1× ANG II). *Groups 4* ($n = 7$) and *5* ($n = 7$) were given single intracerebroventricular injections of either 100 pmol ANG II or 0.9% NaCl, respectively (1× ANG II, 1× NaCl). Ninety minutes after the last injection, rats were deeply anesthetized with chloralhydrate and transcardially perfused with 200 ml PBS (10 mM) followed by 200 ml 4% paraformaldehyde (4°C) for fixation of brain tissue. Brains were removed and postfixed in the same fixative overnight at 4°C. The tissues were subsequently cryoprotected with 30% sucrose at 4°C for 72 h for immunocytochemistry.

Experimental protocol 2. Rats of *groups 6-9* (*group 6*: $n = 10$, *group 7*: $n = 9$, *group 8*: $n = 10$, *group 9*: $n = 10$) underwent the same protocol, with the exception that brains were removed 60 instead of 90 min after the last injection and immediately frozen on dry ice. Tissues were stored at −70°C until RNA preparation.

Experimental protocol 3. Rats of *groups 10-12* (*group 10*: $n = 8$, *group 11*: $n = 9$, *group 12*: $n = 6$) received intracerebroventricular injections of 10 pmol ANG II, 100 pmol ANG II, or 0.9% NaCl, respectively, once daily for a 1-wk period. The animals' water intake was monitored for a period of 20 min after each intracerebroventricular injection.

Immunocytochemistry

Coronal cryostat sections (40 μm) were processed free floating for immunocytochemistry. They were incubated with antisera against c-Fos, c-Jun, JunB, JunD, Krox-24, CREB, SRF, ATF-2, and AT_1 receptor for 72 h (4°C). Immunoreactivity was visualized by the biotin-avidin-peroxidase reaction (Vectastain, Vector Laboratories) using diaminobenzidine as chromogen (14). For double staining, c-Fos and c-Jun immunocytochemistry preceded the AT_1-receptor immunocytochemistry using VIP Vector or Vector SG (Vector Laboratories), respectively, as second chromogen.

The dilutions of the antisera were as follows: anti-c-Fos 1:10,000, anti-c-Jun 1:30,000, anti-JunB 1:4,000, anti-JunD 1:8,000, anti-Krox-24 1:6,000 (generous gifts of Dr. R. Bravo, Princeton, NJ), anti-CREB 1:25,000 (generous gift of Dr. W. Schmid, Deutsches Krebsforschungszentrum, Heidelberg, Germany), anti-SRF 1:2,000 (generous gift of Dr. D. Ginty, Boston, MA), anti-ATF-2 1:2,500 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-AT_1 receptor 1:3,500 (Chemicon International, Temecula, CA). Apart from the monoclonal ATF-2 antiserum, all antisera used in this study were polyclonal. Generation and specificity of the primary antibodies have been described previously (14, 23, 24, 30, 34).

Quantification and Statistics

For quantification of the ITF and CTF, the number of stained neurons of MnPO, SFO, PVN, and SON was counted by two investigators in a blinded manner for each treatment condition on at least two sections from each rat brain. Sections were taken from the same level of each brain region identified according to the atlas of rat brain of Paxinos and Watson (33). No attempt was made to quantify the intensity of the staining. The mean number of labeled neurons ± SD was calculated for each region and group. Statistical analysis was performed using ANOVA followed by Student-Newman-Keuls test (SPSS). Results were considered to be significantly different when $P < 0.05$.

To estimate changes in the AT_1-receptor expression, stainings were compared on photographs taken from each brain region of at least two sections from each rat brain.

Competitive RT-PCR

Preparation of RNA. SON tissue was micropunched from frozen rat brain slices (250 μm) with a 0.5-mm diameter stainless needle. Tissues of five animals were pooled, and RNA was isolated according to the method of Chomczynski and Sacchi (6). The isolated precipitated RNA was dissolved in diethyl pyrocarbonate-treated water.

As internal standard, the plasmid containing mutant c-Fos cDNA (deletion of 48 bp) was linearized by digestion with *Hind*III. The sense strand of the cDNA was reverse transcribed by SP6 RNA polymerase according to the protocol of the manufacturer (Biozym, Hess. Oldendorf, Germany). As control, the plasmid containing native c-*fos* cDNA was submitted the same procedure.

The intactness of the RNAs was checked by gel electrophoresis according to the method of Gruendemann and Koepsell (12). Spectralphotometric measurement at 260 nm wavelength was used to calculate the concentration of the RNAs. Only intact RNA with a ratio of at least 1.7 was used for determination of gene expression.

Oligonucleotides. The sequence of oligonucleotides refers to the sequence of the rat c-fos DNA (8).

For the reverse transcription procedure, antisense-primer 5′-TGA CAA CGG GAG TGC ACA-3′ (18 mer) for PCR, the sense-primer 5′-cag aag ggg caa agt aga gca g-3′ (22 mer), and the antisense primer 5′-att gag aag agg cag ggt gaa g-3′ (22 mer) were synthesized. The predicted size of the PCR products was for the native c-*fos* cDNA 364 bp, and, for the mutant c-*fos* cDNA (internal standard), 316 bp. Because the primer spans two introns (PCR sense primer was located on the 2nd exon, the antisense primer on the 4th exon), amplified c-*fos* DNA was easily distinguishable from genomic DNA, which could possibly be coamplified.

Competitive RT-PCR. Four hundred nanograms of the RNA template was coreverse transcribed with an equimolar amount of competitive cRNA template (internal standard) using 18-mer antisense primer in a concentration of 5 pmol and SuperScript II RNase H^- Reverse Transcriptase from Life Technologies (Eggenstein, Germany). The reaction mixture was heated to 50°C for 60 min followed by 2 min at 94°C. One and one-half microliters of the resulting cDNAs were subsequently amplified with Expand High Fidelty Polymerase (Boehringer Mannheim, Germany) using the 22-mer sense and antisense primer for PCR (each 50 pmol) in 100-µl reaction volume. The PCR was carried out in the thermocycler GeneAmp System 9600 (Perkin Elmer, Weiterstadt, Germany) for 32 cycles for 45 s at 95°C for denaturation, 30 s at 57.8°C for annealing, and 45 s at 72°C for elongation. For extension, a 7-min lasting post-PCR incubation at 72°C followed the last cycle. The successful amplification was verified on 1.5% agarose-gels in 1× TBE.

Quantification and Statistics

Two to twenty microliters of the PCR products were directly separated and quantified by HPLC-ultraviolet using the TSK-DEAE-NPR ion exchange column (TosoHaas, Stuttgart, Germany) and HRLC-800 (Biorad, München, Germany) as described previously (5). The starting copy number of the native c-*fos* cDNA was determined by calculating the ratio of the peak integrals of the native and mutant c-*fos* product yields of at least three PCR reactions. The mean ± SD was calculated for each group. Statistical analysis was performed using ANOVA followed by Student-Newman-Keuls test (SPSS). Results were considered to be significantly different when $P < 0.05$.

Determination of the Water Consumption

Animals received ad libitum access to bowls of tap water. The drinking response was determined by weighing the bowls before and 20 min after each intracerebroventricular injection. The mean water intake of each group ± SE was calculated, and statistical analysis was performed using ANOVA followed by Student-Newman-Keuls test (SPSS). Results were considered to be significantly different when $P < 0.05$.

RESULTS

Immunohistochemistry

ITF expression after injection of ANG II into the lateral brain ventricle was restricted to four forebrain areas, namely the MnPO, SFO, PVN, and SON (2, 26). In the present study, investigations of alterations in ITF and CTF expression were therefore restricted to these brain regions as well as estimation of AT_1-receptor expression.

ITF Expression

In the MnPO, SFO, PVN, and SON, no basal expression of the ITF, c-Fos, c-Jun, JunB, and Krox-24, was

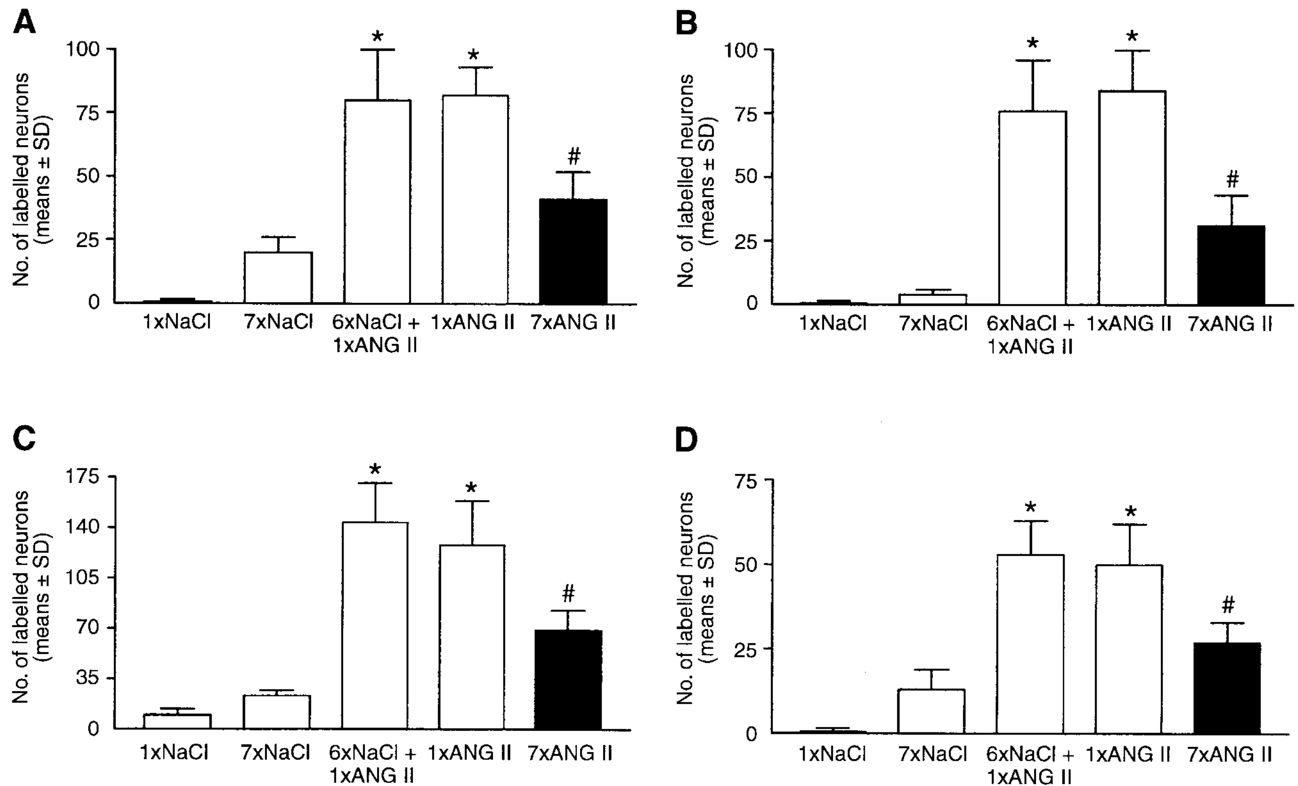

Fig. 1. Bar graph showing the decrease in c-Fos expression in median preoptic area (MnPO; *A*), subfornical organ (SFO; *B*), paraventricular nucleus (PVN; *C*), and supraoptic nucleus (SON; *D*) after repetitive injections of ANG II. Animals received repetitive intracerebroventricular injections of either 100 pmol ANG II or 0.9% NaCl (7× ANG II; 7× NaCl). Controls were given single intracerebroventricular injections of either 100 pmol ANG II or 0.9% NaCl (1× ANG II; 1× NaCl) or repetitive intracerebroventricular injections of 0.9% NaCl followed by a single injection of 100 pmol ANG II (6× NaCl + 1× ANG II). *$P < 0.05$ vs. a single intracerebroventricular injection of 0.9% NaCl; #$P < 0.05$ vs. a single intracerebroventricular injection of ANG II with or without pretreatment with 0.9% NaCl and vs. repetitive intracerebroventricular injections of 0.9% NaCl.

detected. However, JunD showed a substantial basal staining in these brain areas.

Single intracerebroventricular injections of ANG II, even subsequent to repeated NaCl injections, caused a marked expression of c-Fos in the MnPO, SFO, PVN, and SON. After repeated injections of ANG II, c-Fos expression declined in all brain regions compared with a single injection. However, c-Fos expression was still higher than in controls treated with isotonic saline. Repetitive treatment with isotonic saline induced a slight increase in c-Fos expression in all investigated brain regions, whereas a single NaCl injection did not induce any c-Fos expression (Figs. 1 and 2, Table 1). Single ANG II injections induced the expression of c-Jun in the MnPO, SFO, and PVN. Whereas the c-Jun expression was unchanged in these brain regions after repeated injections, c-Jun appeared additionally in the SON. After single or repetitive injections of isotonic NaCl, there was no expression of c-Jun (Fig. 3, Table 1). The expression of JunB was induced in the MnPO and SFO after a single intracerebroventricular injection of ANG II, but did not change after repeated treatment with ANG II. Single or repetitive injections of isotonic saline did not induce JunB expression in the investigated brain regions (Table 1). The expression of JunD did not change in all investigated brain areas after either a single or repetitive ANG II injections compared with its basal levels (Table 1). The expression of Krox-24 in MnPO, SFO, PVN, and SON after repetitive injections of ANG II was similar to that induced after a single injection. Single or repetitive injections of isotonic NaCl did not induce Krox-24 expression (Table 1).

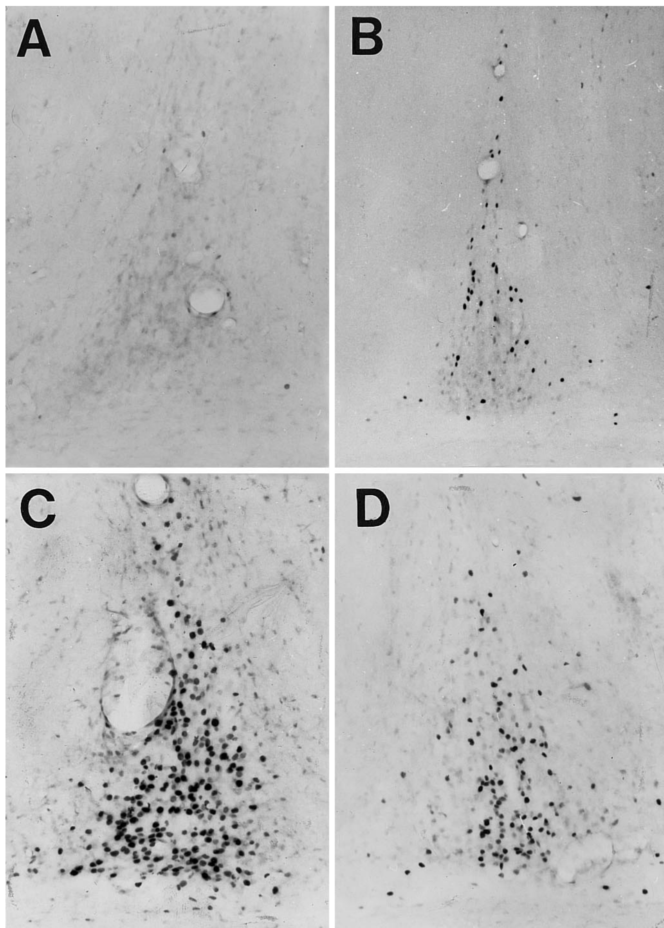

Fig. 2. Photographs of coronal sections of the rat brain showing the habituation of c-Fos expression in the MnPO after repetitive injections of ANG II. *A*: 1× NaCl; *B*: 7× NaCl; *C*: 1× ANG II; *D*: 7× ANG II.

Table 1. *Expression of Fos, Jun, and Krox proteins after single or repetitive intraventricular injections of either 0.9% NaCl and/or 100 pmol ANG II*

	1×NaCl	7×NaCl	6×NaCl+ 1×ANG II	1×ANG II	7×ANG II
c-Fos					
MnPO	0±0	20±6*	80±18*†	82±10*†	41±11*†‡
SFO	0±0	4±2*	76±24*†	84±16*†	31±12*†‡
PVN	0±0	23±4*	144±27*†	128±31*†	69±14*†‡
SON	0±0	13±6*	54±9*†	50±11*†	27±6*†‡
c-Jun					
MnPO	0±0	0±0	—	42±8*†	36±13*†
SFO	0±0	0±0	—	44±10*†	34±11*†
PVN	0±0	0±0	—	122±11*†	101±15*†
SON	0±0	0±0	—	0±0	50±9*†
Jun B					
MnPO	0±0	0±0	—	36±7*†	30±4*†
SFO	0±0	0±0	—	33±8*†	31±12*†
PVN	0±0	0±0	—	0±0	0±0
SON	0±0	0±0	—	0±0	0±0
Jun D					
MnPO	98±24	112±22	—	106±16	96±16
SFO	96±11	105±27	—	115±23	130±15
PVN	201±33	214±42	—	233±46	217±37
SON	76±9	85±10	—	81±14	93±16
Krox-24					
MnPO	0.0±0	0.0±0	—	76±10*†	69±11*†
SFO	0±0	0±0	—	50±9*†	47±9*†
PVN	0±0	0±0	—	156±29*†	139±31*†
SON	0±0	0±0	—	64±15*†	52±10*†

Data show the number of neurons (means ± SD) stained for the inducible transcription factors, c-Fos, c-Jun, Jun B, Jun D, and Krox-24 in the median preoptic area (MnPO), subfornical area (SFO), paraventricular nucleus (PVN), and the supraoptic nucleus (SON) after single or repetitive intracerebroventricular injections of either 0.9% NaCl and/or 100 pmol ANG II. *$P < 0.05$ vs. 1× NaCl, †$P < 0.05$ vs. 7× NaCl, ‡$P < 0.05$ vs. 1× ANG II and vs. 6× NaCl plus 1× ANG II.

CTF Expression

Similar to the ITF, CTF are involved in the regulation of transcriptional activity. In the present study, we investigated the expression of the CTF, CREB, SRF, and ATF-2.

A high expression of CREB was found in neurons and glial cells of untreated animals. Single or repetitive injections of ANG II or isotonic NaCl did not change the expression pattern of CREB (Table 2). SRF was intensively expressed in cortex, hippocampus, and striatum but was absent in the areas of interest. SRF expression was not altered by single or repetitive intracerebroventricular injections of ANG II or 0.9% NaCl in all areas screened (Table 2). A high basal expression of ATF-2 was observed throughout the

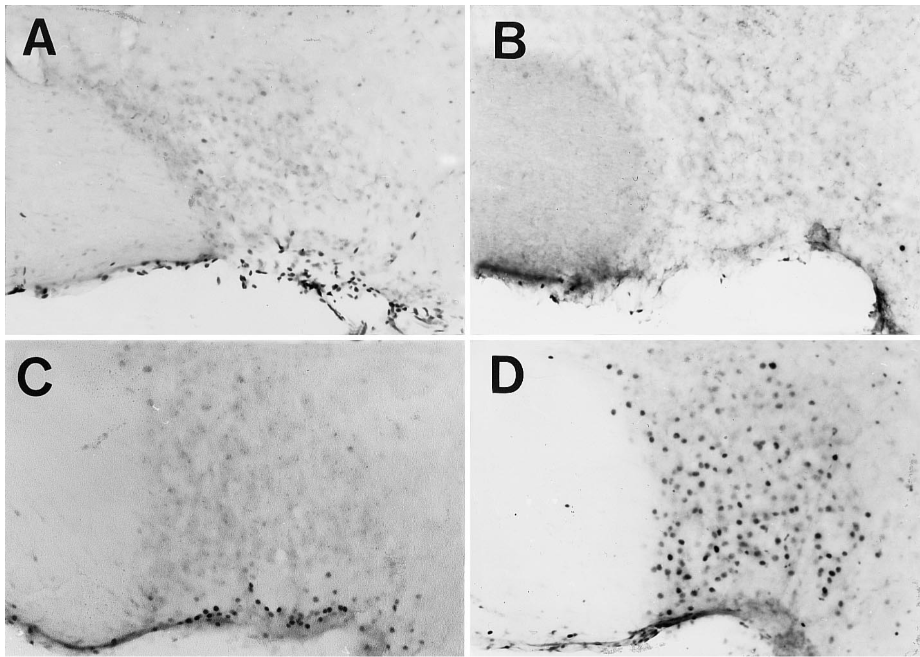

Fig. 3. Photographs of coronal sections of the rat brain showing the novel expression of c-Jun in the SON after repetitive injections of ANG II. *A*: 1× NaCl; *B*: 7× NaCl; *C*: 1× ANG II; *D*: 7× ANG II.

brain. It was exclusively neuronal. Expression of ATF-2 did not change after single or repetitive intracerebroventricular injections of ANG II or isotonic NaCl (Fig. 4, Table 2).

AT_1-Receptor Expression

Immunohistochemical stainings revealed the AT_1 receptor in structures of the medulla, the brain stem, and the forebrain. In the areas of interest, the MnPO, SFO, PVN, and SON, staining was most prominent in the magnocellular neurons of the PVN and SON. Although AT_1-receptor number was not exactly quantified, a single injection of ANG II seems not to alter the expression of the AT_1 receptor compared with a single injection of isotonic saline. However, the expression of the AT_1 receptor seems to be upregulated in all four brain areas investigated after repetitive injections of ANG II compared with once-injected controls (Fig. 5).

Table 2. *Expression of the CREB, SRF and ATF-2 after single or repetitive intraventricular injections of either 0.9% NaCl or 100 pmol ANG II*

	1× NaCl	7× NaCl	1× ANG II	7× ANG II
CREB				
MnPO	252 ± 64	208 ± 34	269 ± 59	248 ± 62
SFO	209 ± 32	225 ± 35	239 ± 75	208 ± 24
PVN	833 ± 135	887 ± 133	986 ± 148	862 ± 187
SON	302 ± 61	326 ± 69	341 ± 78	360 ± 91
ATF-2				
MnPO	78 ± 9	79 ± 15	99 ± 14	93 ± 28
SFO	84 ± 9	81 ± 8	101 ± 38	92 ± 18
PVN	167 ± 34	208 ± 41	180 ± 46	192 ± 30
SON	73 ± 8	80 ± 16	83 ± 9	76 ± 8
SRF				
MnPO	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SFO	0 ± 0	0 ± 0	0 ± 0	0 ± 0
PVN	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SON	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Data show the number of neurons (means ± SD) stained for the constitutive transcription factors, calcium responsive element binding (CREB), serum response factor (SRF) and activating transcription factor 2 (ATF-2), in the MnPO, SFO, PVN, and the SON after single or repetitive intracerebroventricular injections of either 0.9% NaCl or 100 pmol ANG II. There are no significant differences between groups.

Coexpression of c-Fos and c-Jun with the AT_1 Receptor

The expression of c-Fos and c-Jun was demonstrated by a brown nuclear staining, the AT_1 receptor by a red or blue staining on or within the cell bodies. After periventricular angiotensin receptor stimulation, immunocytochemical double staining revealed colocalization of many c-Fos- and c-Jun-labeled neurons with the AT_1 receptor and of many AT_1-labeled neurons with c-Fos and c-Jun in all four forebrain regions studied. The magnocellular neurons of PVN and SON showed the highest portion of colabeled cells (Fig. 6).

The number of colabeled neurons was not precisely quantified because of the limited number of rats and sections that were double stained.

Competitive PCR

To determine whether the changes in ITF expression after single vs. repetitive periventricular angiotensin receptor stimulation as observed on the protein levels were due to changes in ITF gene transcription, c-fos mRNA expression was measured in one of the areas studied, the SON. The gene expression of c-fos in the SON was dramatically increased after a single injection of ANG II, but declined after repetitive treatment nearly to control levels. Repetitive injections of isotonic

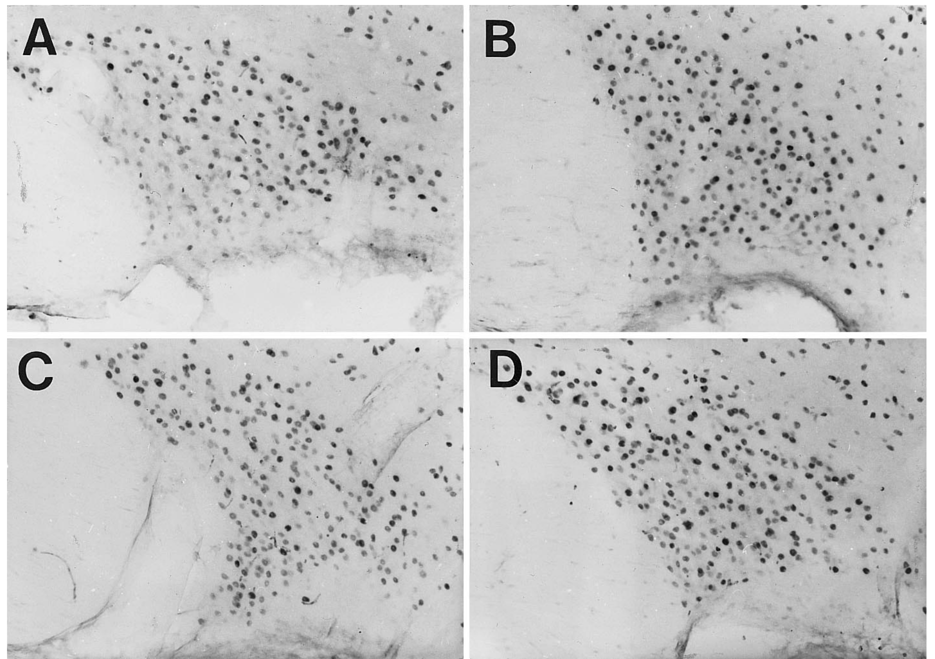

Fig. 4. Photographs of coronal sections of the rat brain showing the constitutive expression of activating transcription factor 2 in the SON after single or repetitive injections of ANG II. *A*: 1× NaCl; *B*: 7× NaCl; *C*: 1× ANG II; *D*: 7× ANG II.

saline resulted in a slight increase of c-*fos* gene expression (Fig. 7).

Water Consumption

Single intracerebroventricular injections of ANG II elicited water drinking in a dose-dependent manner. The dipsogenic response was potentiated after repetitive intracerebroventricular injections of ANG II. The maximum water intake was reached on *days 5* and *6* of the experiment, respectively (Table 3).

DISCUSSION

Previous studies have shown that a single intracerebroventricular injection of ANG II induces a complex and finely tuned expression pattern of transcription factors exclusively in four areas of the forebrain, the MnPO, SFO, and the hypothalamic PVN and SON (2, 3, 26). The present study provides evidence that repetitive ANG II stimulation induces an expression pattern of transcription factors that substantially differs from the one after acute stimulation. Compared with single

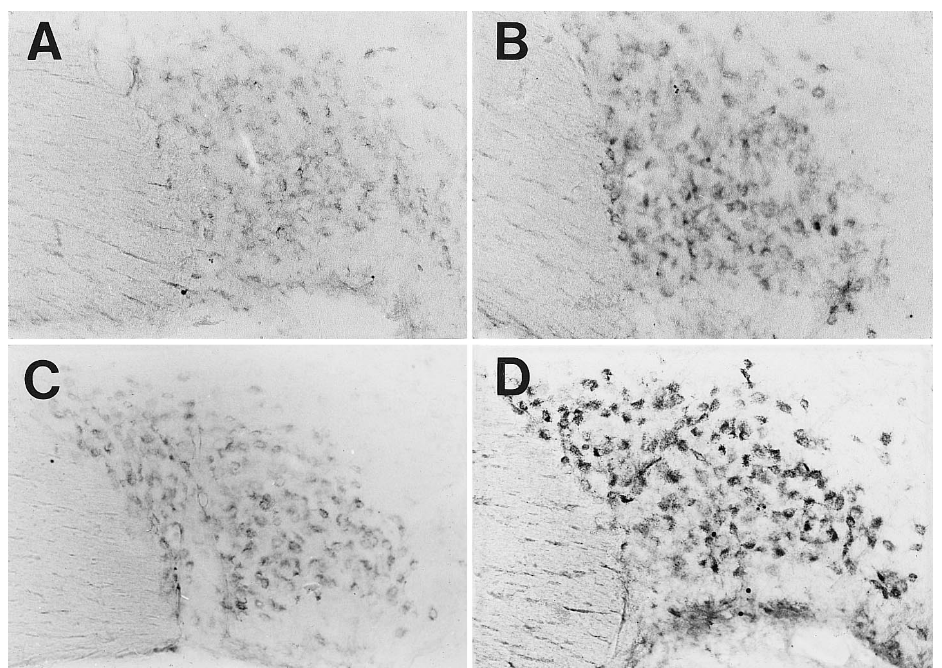

Fig. 5. Photographs of coronal sections of the rat brain showing the expression of AT_1 receptor in the SON after single or repetitive injections of ANG II. *A*: 1× NaCl; *B*: 7× NaCl; *C*: 1× ANG II; *D*: 7× ANG II.

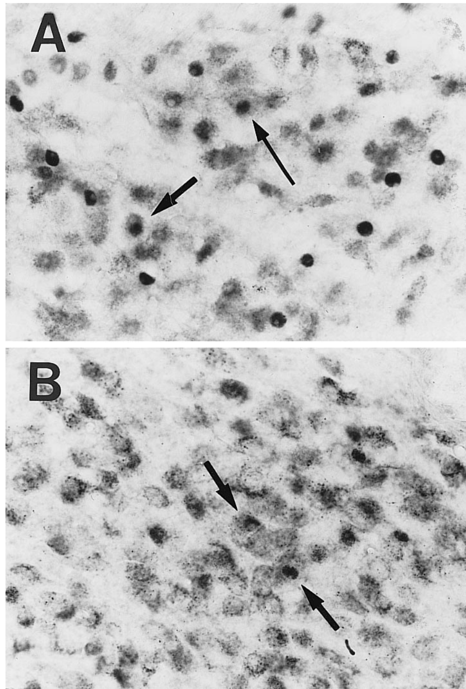

Fig. 6. Photographs of coronal sections of the rat brain showing colocalization of c-Fos and c-Jun with the AT_1 receptor after intracerebroventricular injections of ANG II (100 pmol ANG II). *A*: colocalization of c-Fos with the AT_1 receptor in the SON. *B*: colocalization of c-Jun with the AT_1 receptor in the PVN.

ANG II injections, repetitive ANG II injections induced c-Jun in the SON and reduced c-Fos immunoreactivity in the MnPO, SFO, PVN, and SON. The dramatic decrease of c-*fos* mRNA expression in the SON after repeated intracerebroventricular ANG II injections suggests that the observed changes in transcription factor proteins occurred on the transcriptional level. Interestingly, repetitive treatment with ANG II seems to increase AT_1 receptors in the four brain areas investigated that mediate most of the central effects of the peptide.

Expression of Immediate Early Gene/ITF

A single injection of ANG II (with or without pretreatment with 0.9% NaCl) resulted in a marked increase, whereas repetitive treatment for 1 wk resulted in a marked decrease of c-Fos expression compared with once-injected controls. A similar regulation of c-Fos protein expression has also been shown after acute and repeated/chronic administration of diverse stimuli, such as cocaine or ethanol, inflammation, electroconvulsive seizures, or ultraviolet irradiation (10, 18–21, 25, 43). The change in c-Fos protein levels were mirrored by those in c-*fos* mRNA expression in the SON. Thus it can be assumed that the regulatory changes occurred at the transcriptional level.

Expression of c-*fos*/c-Fos induced by a single injection of ANG II is transient (3, 26, 27). This may be due to destabilizing features of the mRNA as well as autodepression, in which newly synthesized c-Fos protein represses transcription through its own promoter (9, 38). Furthermore, the c-Fos protein is unstable with a half-life of only ~2 h (8).

The mechanisms by which repetitive stimulation downregulates c-*fos*/c-Fos expression are not known. As shown in the present study, the expression of CREB and SRF, major regulators of the c-*fos* promoter (9) did not change. Furthermore, a reduced sensitivity of the AT_1 receptor subsequent to the first ANG II injection seems to be unlikely, because the Jun and Krox proteins remained inducible. Thus we postulate the activation or induction of mechanisms that suppress the c-*fos* promoter, for example, the induction of the transcription factor inducible cAMP early repressor, which has been demonstrated after noxious or osmotic stimulation (28, 32). Moreover, repression of c-Fos may be due to the induction of FosB and Fos-related proteins (Fra) that are able to repress the transcription of the c-fos gene (11, 31). FosB and Fra proteins, which were not investigated in the present study, have much longer half-lives than c-Fos. We previously showed a lasting expression of FosB after a single intracerebroventricular injection of ANG II that surpasses the presence of c-Fos (3). FosB and Fra can persist for days after an acute neuronal stimulation, and their levels may rise gradually after repetitive treatment (19, 20, 39).

Repetitive intracerebroventricular injections of isotonic saline induced a slight expression of c-Fos in all investigated brain areas. Bacterial infections, inflammations, etc., processes that may influence the transcription factor expression, can be excluded because expression of transcription factors was limited to c-Fos. The Jun and Krox proteins were not induced. Furthermore, animals never appeared sick, and the daily water intake of each group was not affected (data not shown). The different course of c-Fos expression after repetitive injections of NaCl and ANG II and the specific immunohistochemical pattern of ITF expression

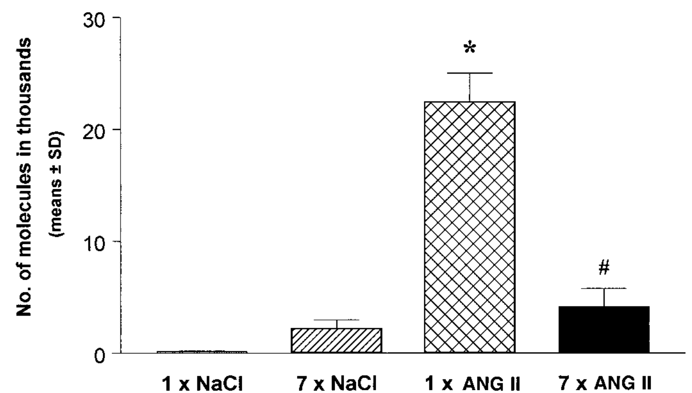

Fig. 7. Illustration of the diminution of c-*fos* gene expression in the SON after repetitive stimulation with ANG II. Animals received repetitive intracerebroventricular injections of either 100 pmol ANG II or 0.9% NaCl (7× ANG II; 7× NaCl). Controls were given single intracerebroventricular injections of either 100 pmol ANG II or 0.9% NaCl (1× ANG II; 1× NaCl). $^*P < 0.05$ vs. a single intracerebroventricular injection of 0.9% NaCl; $\#P < 0.05$ vs. a single intracerebroventricular injection of ANG II and vs. repetitive intracerebroventricular injections of 0.9% NaCl.

Table 3. *Twenty-minute water intake (ml) after intracerebroventricular injection of either 0.9% NaCl, 10 pmol ANG II, or 100 pmol ANG II*

	Day 1	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*
NaCl 0.9%	0.13 ± 0.03	0.16 ± 0.02	0.11 ± 0.01	0.25 ± 0.10	0.18 ± 0.02	0.23 ± 0.14	0.11 ± 0.01
ANG II 10 pmol	4.09 ± 0.68*	4.60 ± 0.92*	5.49 ± 0.62*	6.52 ± 0.29*	7.03 ± 0.56*‡	7.58 ± 0.83*‡	7.11 ± 0.42*‡
ANG II 100 pmol	8.05 ± 0.79*†	8.62 ± 0.74*†	9.75 ± 0.74*†	10.09 ± 0.79*†	11.12 ± 0.74*†‡	10.81 ± 1.31*†‡	10.73 ± 0.99*†‡

The water intake (means ± SE) was determined in a period of 20-min after each intracerebroventricular injection. Rats received intracerebroventricular injections of either 0.9% NaCl, 10 pmol ANG II, or 100 pmol ANG II in 24-h intervals for a 1-wk period. Statistical analysis was performed using ANOVA followed by Student-Newman-Keuls test. *$P < 0.05$ vs. 0.9% NaCl on the respective day, †$P < 0.05$ vs. 10 pmol ANG II on the respective day, ‡$P < 0.05$ vs. once intracerebroventricular injected animals (*day 1*).

after repeated saline injections suggest that ITF expression on repeated NaCl injections was mediated via pathways different from those activated by ANG II.

In addition to c-Fos, repetitive stimulation with ANG II influences the expression of c-Jun that appears in the SON. The novel expression of c-Jun in the SON after repeated ANG II injections has to be distinguished from the delayed c-Jun expression after a single ANG II injection (3). By down- and upregulation of c-Fos and c-Jun expression, respectively, repetitive ANG II evokes the formation of different AP-1 complexes, compared with acute stimulation, that substantially differ in their transcriptional properties (19, 20, 31, 36). Such stimulus-dependent variability of transcriptional complexes could contribute to the stimulus-dependent induction of the AT_1 receptor and other target genes.

In contrast to c-Fos, expression of c-Jun, JunB, and Krox-24 remained fully inducible, and JunD did not differ from its basal level. Thus the habituation of c-Fos, the inducibility of JunB, and the persistence of JunD reflect general properties of the expression of these transcription factors as observed under different conditions (9, 21, 25).

Expression of CTF

In this study, CREB and ATF-2 were constitutively expressed in the MnPO, SFO, PVN, and SON and showed no alteration after single or repetitive injections of ANG II. SRF, which was absent in these brain areas after a single injection of ANG II, remained undetectable even after repetitive administration of ANG II.

Although repetitive intracerebroventricular injections of ANG II do not alter the expression of CREB and ATF-2, the involvement of these CTF in ANG II-induced expression of ITF or genes activated by these ITF cannot be excluded. In contrast, the lack of SRF immunoreactivity in the ANG II-associated brain regions suggests that the signal transduction pathways by which ANG II induces gene expression does not involve SRF. SRF may be substituted by other transcription factors (3). However, because the CTF expression shows no regulation, the ITF seem to play the decisive role in the transcriptional control of AP-1 and CRE-triggered target genes.

Expression of AT_1 Receptors

Immunohistochemical stainings revealed the AT_1 receptor in structures of the medulla, brain stem, and the forebrain. In the areas of interest, the MnPO, the SFO, and the hypothalamic PVN and SON, the expression of the AT_1 receptor seems to be increased after repetitive, but not on acute periventricular, stimulation with ANG II.

The presence of AT_1 receptors in these brain regions is in agreement with previous immunohistochemical, autoradiographic, and binding studies (16, 34, 37). In vivo and in vitro studies have shown that binding of ANG II to the AT_1 receptor leads to internalization of the ligand receptor complex-mediating desensitization and reduction of the AT_1 receptors at the cell surface (35). Internalized receptors are partly recycled, partly degraded. Degraded receptors are substituted by newly synthesized binding sites. In this study, immunohistochemical stainings of the AT_1 receptor did not show a reduction of the receptor number after single ANG II injections. Ninety minutes after exposure to ANG II, when brains were removed, the AT_1 receptor may have been recycled in parts, but resynthesis will not be finished. Therefore, it can be assumed that the receptors were generated from a pool of presynthesized/preexisting receptors (40). On the other hand, immunohistochemical staining does not only detect receptors at the cell surface, but also those internalized.

In contrast to the lack of AT_1-receptor regulation after a single intracerebroventricular injection, immunohistochemical stainings showed an increase in AT_1-receptor number after repetitive stimulation. ANG II stimulates neurons in the MnPO, SFO, PVN, and SON, suggesting that the increase of the AT_1-receptor number may be partly due to an increase in neuronal activity. An upregulation of AT_1 receptors in vivo was also observed subsequent to water deprivation in the SFO on (repeated) stress in the PVN and SFO and after treatment with mineralocorticoids in the MnPO, the SFO, and the PVN (1, 37). An increase in AT_1-receptor number in the MnPO and the SFO has also been seen after salt loading (37). Under all these conditions, the concentrations of vasopressin, gluco- and mineralocorticoids, and/or ANG II are elevated in the brain. In primary cell cultures, mineralocorticoids and catecholamines have already been shown to upregulate the number of AT_1 receptors (37).

The enhanced expression of AT_1 receptors in distinct brain areas after varying conditions suggests that the regulation of the AT_1 receptor depends on several factors. Expression of AT_1 receptors seems to be positively regulated by gluco- and mineralocorticoids, and its transcriptional control seems to be modified by ANG II itself (1, 37).

Colocalization of c-Fos and c-Jun with the AT_1 Receptor

Immunohistochemical double staining revealed the coexpression of nuclear c-Fos- and c-Jun-labeled neurons with cytoplasmic AT_1 receptors in MnPO, SFO, PVN, and SON and vice versa after intracerebroventricular injections of ANG II.

The promoter region of the AT_1-receptor gene comprises an AP-1 and a CRE site in addition to three GRE consensus sequences. Thus initiation of mRNA synthesis could be influenced by the leucine zipper proteins. Fos and, in particular, Jun proteins can bind to AP-1 and CRE consensus sequences as AP-1 homo- or heterodimers or as heterodimers with CRE binding protein or activating factor proteins and activate gene expression (9). These findings support the hypothesis that the AT_1-receptor gene may constitute a target gene of c-Fos and c-Jun in response to centrally applied ANG II.

After repetitive stimulation, the proposed enhanced expression of the AT_1 receptor coincides with a downregulation of c-Fos. As described above, stimulation with ANG II induces a persisting expression of FosB and probably Fra proteins (3). Therefore, AP-1 complexes formed after repetitive ANG II exert longer half-lives compared with AP-1 complexes generated after acute stimulation. The binding properties of these AP-1 complexes will differ, resulting in a persisting gene transcription. Thus an increased expression of the AT_1 receptor probably reflects the fact that the expression of the AT_1-receptor gene is under positive control by AP-1 activity. Because mineralo- and glucocorticoids have been shown to upregulate the transcription of the AT_1 receptor, synergistic effects with the GRE element seem to be likely.

On the other hand, AP-1 proteins have been shown to antagonize the GRE-mediated gene expression (15). Thus the downregulation of c-Fos could result in disinhibition of GRE-mediated gene transcription. Therefore, it cannot be excluded that inhibition of repressive mechanisms in the transcriptional control of the AT_1-receptor gene activity may also account for the suggested increase in the number of AT_1 receptors.

Angiotensin AT_1-Receptor Expression and Drinking

A single intracerebroventricular injection of ANG II elicited drinking behavior in a dose-dependent manner. After repetitive ANG II injections, water intake was even potentiated.

Drinking behavior is mediated via the AT_1 receptor and stimulation of periventricular AT_1 receptors by ANG II-induced dipsogenic action as has been shown in numerous studies (16, 17, 41, 42). The water intake is not only dose dependent but seems to be dependent on the number of receptors, too. The increased water consumption measured after repeated ANG II injections is probably a result of the enhanced expression of the AT_1 receptor. However, the water intake did not exceed a maximum. The regulation of the receptor number seems to be dependent on the strength of the stimulus. On the other hand, second messenger systems may limit the effects resulting from “overstimulation” to protect cells from damage. Moreover, AT_2 receptor-mediated mechanisms might counterbalance the AT_1 receptor-mediated actions (16, 17, 42).

Perspectives and Pathophysiology

The study analyzed the expression of ITF and CTF and AT_1 receptors after repetitive stimulation of periventricular angiotensin receptors in the rat.

Desensitization of c-Fos will probably change the composition of the AP-1 complex. This could prevent rapid responses to certain stimuli, indicating a loss of neuronal plasticity at the genetic level. The altered expression after repetitive stimulation does not mean a loss of plasticity per se, because c-*fos*/c-Fos becomes reinducible after a period of latency (21, 29, 43). Thus habituation of ITF expression may be due to a loss of novelty. Mechanisms leading to habituation and reinduction of ITF expression are still unidentified.

The presented data suggest the AT_1-receptor gene as target gene of the leucine zipper proteins Fos and Jun and the CTF, CREB, and ATF-2. Further investigations are necessary to elucidate the complex mechanisms by which CTF and ITF control target gene activity, and further efforts have to be made to enlighten the interplay of AT_1 and AT_2 receptors, because influences of the AT_2 receptor on AT_1 receptor-mediated effects via interference in the mitogen activated protein kinase pathway seem to be possible (17, 22).

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REFERENCES

1. **Aguilera G, Kiss A, and Luo X.** Increased expression of type 1 angiotensin II receptors in the hypothalamic paraventricular nucleus following stress and glucocorticoid administration. *J Neuroendocrinol* 7: 775–783, 1995.
2. **Blume A, Lebrun CJ, Herdegen T, Bravo R, Linz W, Möllenhoff E, and Unger T.** Increased brain transcription factor expression by angiotensin in genetic hypertension. *Hypertension* 29: 592–598, 1997.
3. **Blume A, Seifert K, Lebrun CJ, Moellenhoff E, Gass P, Bravo R, Unger T, and Herdegen T.** Differential time course of angiotensin-induced AP-1 and Krox proteins in the rat lamina terminalis and hypothalamus. *Neurosci Lett* 241: 87–90, 1998.
4. **Brecht S, Gass P, Anton F, Bravo R, Zimmermann M, and Herdegen T.** Induction of c-Jun and suppression of CREB transcription factor proteins in axotomized neurons of substantia nigra and covariation with tyrosine hydroxylase. *Mol Cell Neurosci* 5: 431–441, 1994.
5. **Chatterjee B, Gimbel W, Goerblich T, and Werner T.** HPLC purification of PCR products for direct sequencing (Abstract). *Trends Genet* 9: 406, 1993.

6. **Chomczynski P and Sacchi N.** Single-Step method of RNA Isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
8. **Curran T, Miller AD, Zokas L, and Verma IM.** Viral and cellular fos proteins: a comparative analysis. *Cell* 36: 259–268, 1984.
9. **Gass P and Herdegen T.** Neuronal expression of AP-1 proteins in excitotoxic-neurodegenerative disorders and following nerve fiber lesions. *Prog Neurobiol* 47: 257–290, 1995.
10. **Gillardon F, Eschenfelder C, Uhlmann E, Hartschuh W, and Zimmermann M.** Differential regulation of c-fos, fosB, c-jun, junB, bcl-2 and bax expression in rat skin following single or chronic ultraviolet irradiation and in vivo modulation by antisense oligodeoxynucleotide superfusion. *Oncogene* 9: 3219–3225, 1994.
11. **Gius D, Cao X, Rauscher FJ, III, Cohen DR, Curran T, and Sukhatme VP.** Transcriptional activation and repression by Fos are independent functions: the C terminus represses immediate-early gene expression via CArG element. *Mol Cell Biol* 10: 4243–4255, 1990.
12. **Gruendemann D and Koepsell H.** Ethidium bromide staining during denaturation with glyoxal for sensitive detection of RNA in agarose gel electrophoresis. *Anal Biochem* 216: 459–461, 1994.
13. **Guitart X, Thompson MA, Mirante CK, Greenberg ME, and Nestler EJ.** Regulation of cyclic AMP response element-binding protein (CREB) phosphorylation by acute and chronic morphine in the rat locus coeruleus. *J Neurochem* 58: 1168–1171, 1992.
14. **Herdegen T, Kovary K, Leah J, and Bravo R.** Specific temporal and spatial distribution of Jun, Fos, and Krox-24 proteins in spinal neurons following noxious transsynaptic stimulation. *J Comp Neurol* 313: 178–191, 1991.
15. **Herdegen T and Leah JD.** Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res Rev* 28: 370–490, 1998.
16. **Hoehle S, Blume A, Lebrun C, Culman J, and Unger T.** Angiotensin receptors in the brain. *Pharmacol Toxicol* 77: 306–315, 1995.
17. **Hoehle S, Spitznagel H, Rascher W, Culman J, and Unger T.** Angiotensin AT1 receptor-mediated vasopressin release and drinking are potentiated by an AT2 receptor antagonist. *Eur J Pharmacol* 275: 277–282, 1995.
18. **Hope B, Kosofsky B, Hyman SE, and Nestler EJ.** Regulation of immediate early gene expression and AP-1 binding in the rat nucleus accumbens by chronic cocaine. *Proc Natl Acad Sci USA* 89: 5764–5768, 1992.
19. **Hope BT, Kelz MB, Duman RS, and Nestler EJ.** Chronic electroconvulsive seizure (ECS) treatment results in expression of a long-lasting AP-1 complex in brain with altered composition and characteristics. *J Neurosci* 14: 4318–4328, 1994.
20. **Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS, and Nestler EJ.** Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatment. *Neuron* 13: 1235–1244, 1994.
21. **Hsieh T-F, Simler S, Vergnes M, Gass P, Marescaux C, Wiegand SJ, Zimmermann M, and Herdegen T.** BDNF restores the expression of Jun and Fos inducible transcription factors in the rat brain following repetitive electroconvulsive seizures. *Exp Neurol* 149: 161–174, 1998.
22. **Huang X-C, Richards EM, and Sumners C.** Mitogen-activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors. *J Biol Chem* 271: 15635–15641, 1996.
23. **Hummler E, Cole TJ, Blendy JA, Ganss R, Aguzzi A, Schmid W, Bermann F, and Schuetz G.** Target mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. *Proc Natl Acad Sci USA* 91: 5647–5651, 1994.
24. **Kovary K and Bravo R.** Expression of different Jun and Fos protein during Go-to-G1 transition in mouse fibroblasts: in vitro and in vivo associations. *Mol Cell Biol* 11: 2451–2459, 1991.
25. **Lanteri-Minet M, de Pommery J, Herdegen T, Weil-Fugazza J, Bravo R, and Menetrey D.** Differential time course and spatial expression of Fos, Jun, and Krox 24 proteins in spinal cord of rats undergoing subacute or chronic somatic inflammation. *J Comp Neurol* 333: 223–235, 1993.
26. **Lebrun CJ, Blume A, Herdegen T, Seifert K, Bravo R, and Unger T.** Angiotensin II induces a complex activation of transcription factors in the rat brain: expression of Fos, Jun and Krox proteins. *Neuroscience* 65: 93–99, 1995.
27. **Lebrun CJ, Moellenhoff E, Bao G, Culman J, and Unger T.** Angiotensin II induces the expression of c-fos mRNA in the central nervous system of the rat. *Clin Exp Hypertens* 17: 877–893, 1995.
28. **Luckman SM and Cox HJ.** Expression of inducible cAMP early repressor (ICER) in hypothalamic magnocellular neurons. *Mol Brain Res* 34: 231–239, 1995.
29. **Melia KR, Ryabinin AE, Schroeder R, Bloom FE, and Wilson MC.** Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. *J Neurosci* 14: 5929–5938, 1994.
30. **Misra RP, Rivera VM, Wang JM, Fan PD, and Greenberg ME.** The serum response factor is extensively modified by phosphorylation following its synthesis in serum-stimulated fibroblasts. *Mol Cell Biol* 11: 4545–4554, 1991.
31. **Nakabeppu Y and Nathans D.** A naturally occurring truncated form of fosB that inhibits fos/jun transcriptional activity. *Cell* 64: 751–759, 1991.
32. **Naranjo RN, Mellstroem B, Carrion AM, Lucas JL, Foulkes NS, and Sassone-Corsi P.** Peripheral noxious stimulation induces CREM expression in dorsal horn: involvement of glutamate. *Eur J Neurosci* 9: 2778–2783, 1997.
33. **Paxinos G and Watson C.** *The Rat Brain in Stereotaxic Coordinates.* New York: Academic, 1982.
34. **Phillips MI, Shen L, Richards EM, and Raizada MK.** Immunohistochemical mapping of angiotensin AT 1 receptors in the brain. *Regul Pept* 44: 95–107, 1993.
35. **Reagan LP, Ye X, Maretzski CH, and Fluharty SJ.** Down-regulation of angiotensin II receptor subtypes and desensitization of cyclic GMP production in neuroblastoma N1E-115 cells. *J Neurochem* 60: 24–31, 1993.
36. **Ryseck RP and Bravo R.** c-Jun, Jun B, and Jun D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. *Oncogene* 8: 333–342, 1991.
37. **Saavedra JM.** Brain and pituitary angiotensin. *Endocr Rev* 13: 329–380, 1992.
38. **Schoenthal A, Buescher M, Angel P, Rahmsdorf HJ, Ponta H, Hattori K, Chiu R, Karin M, and Herrlich P.** The fos and jun/AP-1 proteins are involved in the downregulation of fos transcription. *Oncogene* 4: 629–636, 1989.
39. **Sonnenberg JL, Macgregor-Leon PF, Curran T, and Morgan JI.** Dynamic alterations occur in the levels and composition of transcription factor AP-1 complexes after seizure. *Neuron* 3: 359–365, 1989.
40. **Ullian ME and Linas SL.** Role of receptor cycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. *J Clin Invest* 84: 840–846, 1989.
41. **Unger T, Badoer M, Ganten D, Lang RE, and Rettig R.** Brain angiotensin: pathways and pharmacology. *Circulation* 77: 1–40, 1988.
42. **Unger T, Chung O, Csikos T, Culman J, Gallinat S, Gohlke P, Hoehle S, Meffert S, Stoll M, Stroth U, and Zhu Y-Z.** Angiotensin receptors. *J Hypertens* 14: 95–103, 1996.
43. **Winston SM, Hayward MD, Nestler EJ, and Duman RS.** Chronic electroconvulsive seizures down-regulate expression of immediate-early genes c-fos and c-jun in rat cerebral cortex. *J Neurochem* 54: 1920–1925, 1990.