Effect of repetitive icv injections of ANG II on c-Fos and AT$_1$-receptor expression in the rat brain

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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 subformical organ (SFO), and the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON). After repetitive injections, the expression of c-Fos declined by $\sim$50% in MnPO, SFO, PVN, and SON compared with controls injected once. The desensitization of c-Fos occurred on the transcriptional level as shown in the SON by RT-PCR. Apart from a novel expression of c-Jun in the SON, the ITF c-Jun, JunB, JunD, and Krox-24 did not change after repetitive stimulation. Neither were the CTF, calcium response element binding protein, activating transcription factor 2, and serum response factor altered after repetitive vs. single injections of ANG II. The AT$_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.

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 subformical organ (SFO), and the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON), known to be involved in osmoregulation, predominantly 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.
(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 of 4% paraformaldehyde (4°C) for fixation of brain tissue. Brains were removed and postfixed in the same fixative (4% paraformaldehyde, 4°C) for 72 h. Immunoreactivity preceded the AT1-receptor immunocytochemistry using VIP Vector or Vector SG (Vector Laboratories, respectively, as second chromogen).

**Quantification and Statistics**

For quantification of the ITF and CTF, the number of stained neurons of MnPO, SFO, PVN, and SON was counted on photographs taken from each brain region. 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 AT1-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 HindIII. 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 to the same procedure.

The intactness of the RNAs was checked by gel electrophoresis according to the method of Gruendemann and Koeppel (12). Spectrophotometric 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 5’-TGA CAA CCG 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 cag ggt gaa 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 amplified.
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 Fidelity 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 AT1-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.](http://ajpregu.physiology.org/)
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).

**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

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**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**

<table>
<thead>
<tr>
<th></th>
<th>1× NaCl</th>
<th>6× NaCl</th>
<th>1× ANG II</th>
<th>1× ANG II + 100 pmol ANG II</th>
<th>1× ANG II + 7× NaCl</th>
<th>7× NaCl</th>
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<tbody>
<tr>
<td>c-Fos</td>
<td>MnPO 0±0 20±6 80±18 82±10 41±11</td>
<td>SFO 0±0 4±2 76±24 84±16 31±12</td>
<td>PVN 0±0 23±4 144±27 128±31 69±14</td>
<td>SON 0±0 13±6 54±9 50±11 27±6</td>
<td></td>
<td></td>
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<tr>
<td>c-Jun</td>
<td>MnPO 0±0 0±0 42±8 36±13</td>
<td>SFO 0±0 0±0 44±10 34±11</td>
<td>PVN 0±0 0±0 122±11 101±15</td>
<td>SON 0±0 0±0 0±0 50±9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jun B</td>
<td>MnPO 0±0 0±0 36±7 30±4</td>
<td>SFO 0±0 0±0 33±8 31±12</td>
<td>PVN 0±0 0±0 0±0 0±0</td>
<td>SON 0±0 0±0 0±0 0±0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jun D</td>
<td>MnPO 95±24 112±22 106±16 96±16</td>
<td>SFO 96±11 105±27 115±23 130±15</td>
<td>PVN 201±33 214±42 233±46 217±37</td>
<td>SON 76±9 85±10 81±14 93±16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krox-24</td>
<td>MnPO 0±0 0±0 76±10 69±11</td>
<td>SFO 0±0 0±0 50±9 47±9</td>
<td>PVN 0±0 0±0 156±29 139±31</td>
<td>SON 0±0 0±0 64±15 52±10</td>
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</tbody>
</table>

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.

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**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.**
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).

**AT1-Receptor Expression**

Immunohistochemical stainings revealed the AT1 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 AT1-receptor number was not exactly quantified, a single injection of ANG II seems not to alter the expression of the AT1 receptor compared with a single injection of isotonic saline. However, the expression of the AT1 receptor seems to be upregulated in all four brain areas investigated after repetitive injections of ANG II compared with once-injected controls (Fig. 5).

**Coexpression of c-Fos and c-Jun with the AT1 Receptor**

The expression of c-Fos and c-Jun was demonstrated by a brown nuclear staining, the AT1 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 AT1 receptor and of many AT1-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

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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**

<table>
<thead>
<tr>
<th></th>
<th>1× NaCl</th>
<th>7× NaCl</th>
<th>1× ANG II</th>
<th>7× ANG II</th>
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<tbody>
<tr>
<td>CREB</td>
<td>MnPO</td>
<td>252 ± 64</td>
<td>208 ± 34</td>
<td>269 ± 59</td>
</tr>
<tr>
<td></td>
<td>SFO</td>
<td>209 ± 32</td>
<td>225 ± 35</td>
<td>239 ± 75</td>
</tr>
<tr>
<td></td>
<td>PVN</td>
<td>833 ± 135</td>
<td>887 ± 133</td>
<td>986 ± 148</td>
</tr>
<tr>
<td></td>
<td>SON</td>
<td>302 ± 61</td>
<td>326 ± 69</td>
<td>341 ± 78</td>
</tr>
<tr>
<td>SRF</td>
<td>MnPO</td>
<td>78 ± 9</td>
<td>79 ± 15</td>
<td>99 ± 14</td>
</tr>
<tr>
<td></td>
<td>SFO</td>
<td>84 ± 9</td>
<td>81 ± 8</td>
<td>101 ± 38</td>
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<tr>
<td></td>
<td>PVN</td>
<td>167 ± 34</td>
<td>208 ± 41</td>
<td>180 ± 46</td>
</tr>
<tr>
<td></td>
<td>SON</td>
<td>73 ± 8</td>
<td>80 ± 16</td>
<td>83 ± 9</td>
</tr>
</tbody>
</table>

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.
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).

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**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
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 AT1 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 AT1 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...
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 AT1 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.
The enhanced expression of AT₁ receptors in distinct brain areas after varying conditions suggests that the regulation of the AT₁ receptor depends on several factors. Expression of AT₁ receptors seems to be positively regulated by glucocorticoids and, its transcriptional control seems to be modified by ANG II itself (1, 37).

**Colocalization of c-Fos and c-Jun with the AT₁ Receptor**

Immunohistochemical double staining revealed the coexpression of nuclear c-Fos- and c-Jun-labeled neurons with cytoplasmic AT₁ receptors in MnPO, SFO, PVN, and SON and vice versa after intracerebroventricular injections of ANG II.

The promoter region of the AT₁-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 AT₁-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 AT₁ 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 AT₁ receptor probably reflects the fact that the expression of the AT₁-receptor gene is under positive control by AP-1 activity. Because mineralocorticoids have been shown to upregulate the transcription of the AT₁ 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 AT₁-receptor gene activity may also account for the suggested increase in the number of AT₁ receptors.

**Angiotensin AT₁-Receptor Expression and Drinking**

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

Drinking behavior is mediated via the AT₁ receptor and stimulation of periventricular AT₁ 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 AT₁ 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, AT₂ receptor-mediated mechanisms might counterbalance the AT₁ receptor-mediated actions (16, 17, 42).

**Perspectives and Pathophysiology**

The study analyzed the expression of ITF and CTF and AT₁ 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 inducible 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 induction of ITF expression are still unidentified.

The presented data suggest the AT₁-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 AT₁ and AT₂ receptors, because influences of the AT₂ receptor on AT₁ receptor-mediated effects via interference in the mitogen activated protein kinase pathway seem to be possible (17, 22).

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