Nitric Oxide Synthase mRNA Levels Correlate with Gene Expression of Angiotensin II Type-1 but not Type-2 Receptors, Renin or Angiotensin Converting Enzyme in Selected Brain Areas

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Summary
Recent data suggest that there is interaction between peripheral angiotensin II and nitric oxide. However, sparse information is available on the mutual interaction of these two compounds in the brain. The potential intercourse of nitric oxide with brain neuropeptides needs to be substantiated by assessing its local production and gene expression of the synthesizing enzymes involved. The aim of the present study was to evaluate whether the gene expression of brain nitric oxide synthase (bNOS) is related to the sites of gene expression of different components of the rat brain renin angiotensin system (renin, angiotensin converting enzyme (ACE) or angiotensin receptors of AT₁ and AT₂ subtypes). The levels of corresponding mRNAs were measured and correlated in nine structures of adult rat brain (hippocampus, amygdala, septum, thalamus, hypothalamus, cortex, pons, medulla and cerebellum). As was expected, positive correlation was observed between renin and angiotensin-converting enzyme mRNAs. Moreover, a significant correlation was found between brain NO synthase and AT₁ receptor mRNAs, but not with mRNA of the AT₂ receptor, ACE and renin. Parallel distribution of mRNAs coding for bNOS and AT₁ receptors in several rat brain structures suggests a possible interaction between brain angiotensin II and nitric oxide, which remains to be definitely demonstrated by other approaches.

Key words
Angiotensin II • Nitric oxide synthase • AT₁ receptors • AT₂ receptors • Rat brain • Gene expression

Introduction
The existence of the brain renin-angiotensin system (RAS) is well recognized and is known to be involved in cardiovascular control, fluid homeostasis and pituitary hormone release (Ganong 1984, Saavedra 1992, Höhle et al. 1995). Conclusive evidence for angiotensin production and action in the brain has been provided by the
demonstration of genetic message for individual components of the RAS. Thus, angiotensinogen mRNA was found to be present mainly in astrocytes and to be spread diffusely throughout the brain with intense areas of expression in the hypothalamus and brainstem (Stornetta et al. 1988, Bunnemann 1993). The expression of genes encoding enzymes participating in angiotensin II synthesis, namely renin and angiotensin-converting enzyme (ACE), is also well documented (Phillips et al. 1993). Extensive information is available on characteristics and gene expression of angiotensin II receptors of pharmacologically defined types, type-1 (AT1) and type-2 (AT2). In rodents, two AT1 receptor subtypes, AT1A and AT1B, have been isolated. Studies using in situ hybridization histochemistry with specific riboprobes resulted in a detailed mapping of AT1A, AT1B, and AT2 receptor mRNAs in adult rat brain (Lenkei et al. 1997, 1998). The observed distribution pattern supports the view that central effects of angiotensin on body fluid and cardiovascular homeostasis are mediated mainly via the AT1A receptor subtype. In most of the nuclei of the adult rat brain, a good correlation was observed between the distribution of angiotensin binding sites and angiotensin receptor mRNA expression (Allen et al. 1992).

Central angiotensin II interacts with other neurotransmitters, such as norepinephrine, natriuretic peptides, serotonin and tachykinins (Saavedra 1990, 1992). Recent data suggest that there is an interaction between angiotensin II and nitric oxide (NO). NO has been implicated particularly in the function of the peripheral RAS and studies using cell cultures have demonstrated that angiotensin II may influence NO production through mechanisms involving AT2 or other, yet unidentified, receptor subtypes (McLay et al. 1995, Schelman et al. 1997). Moreover, NO and NO-releasing compounds inhibit ACE activity in a concentration-dependent and competitive way in the endothelium (Ackermann et al. 1998). In the brain, some studies indicate that NO might participate at least in some angiotensin II-regulated processes including drinking behavior (Zhu and Herbert 1997) and development of stroke in spontaneously hypertensive rats (Ahmad 1997). Furthermore, centrally produced NO was reported to maintain resting arterial blood pressure through modulation of the brain angiotensin system and prostaglandins (Liu et al. 1998).

NO is produced by several NO-synthases and the mRNA levels of brain-type NO-synthase (bNOS) are present in many neurons of various brain regions as observed by in situ hybridization (Iwase et al. 1998). The aim of the present study was to evaluate whether the gene expression of bNOS is related to the sites of gene expression of renin, ACE or angiotensin receptors of AT1 or AT2 subtypes. Because this potential relationship might be region specific, levels of mRNA coding for individual components of RAS and for bNOS were measured in nine anatomically and functionally well-defined rat brain regions using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique.

**Methods**

**Tissue collection**

Adult male Wistar rats weighing 300-350 g, supplied by Dobra voda (Slovakia), were maintained under conditions of 12 h light-dark cycle (lights on from 06.00h), constant temperature (22-24 °C) and free access to water and pelleted food.

Rats were killed by decapitation and their brains were quickly removed. Nine brain regions were selected and isolated according to the atlas of Palkovits and Brownstein (1988). The hypothalamus and hippocampus were removed as complete areas by forceps. The septum, thalamus, corpus amygdaloideus and piriform cortex were excised of coronal sections using a razor blade. The pons and medulla were used as whole anatomical pieces separated from each other with a razor blade. One third of the right hemisphere of the cerebellum was separated by scissors. All tissues were rapidly frozen in liquid nitrogen.

**RNA preparation**

RNA was isolated according to the procedure of Chomczynski and Sacchi (1987) using guanidine isothiocyanate (Fisher Scientific, USA) and phenol-chloroform extraction. Briefly, the tissues were homogenized in guanosine-thiocyanate with 2-mercaptoethanol, phenol, sodium acetate and a mixture of chloroform: isoomyl alcohol (24:1 v/v) and mixed thoroughly. After 15 min of the incubation on ice, the homogenate was centrifuged for 20 min (12 000 rpm, 4 °C). An equal volume of isopropanol was added to the aqueous phase and kept at –20°C for 1 hour. After precipitation, the mixture was centrifuged for 20 min (12 000 rpm, 4 °C). The RNA pellet was dissolved in RNase free water and extracted twice by phenol : chloroform (1:1 v/v). The last aqueous RNA phase was mixed with 1/10 volume of 3 M sodium acetate and twofold volume of 96 % ethanol. RNA was precipitated overnight at –20 °C. Afterwards, the RNA pellet was washed with 75 % ethanol and dissolved in RNase free...
water. Concentration and purity of RNA was determined spectrophotometrically on Shimadzu UV-3000 (Kyoto, Japan).

**Northern blot analysis**

One series of samples was analyzed using a Northern blot technique and subsequent hybridization with radiolabeled bNOS and AT1 probe (Sambrook et al. 1989). The probe was found to bind to a single band, however, even after 3 days of film exposure, the signal was still very weak. Therefore, a more sensitive RT-PCR approach was chosen for further experiments.

**Relative quantification of mRNA levels by RT-PCR**

Reverse transcription was done using Ready-To-Go You-Prime First-Strand Beads (Pharmacia Biotech), with a pd(N)₆ primer. For each RT-PCR analysis, 1 µg of the total RNA was used. The number of cycles was determined for each kind of RT-PCR separately, testing 15, 20, 25, 30, 35, 37, and 40 cycles. We have verified that the amplification of each product was in the linear range at the used number of cycles. To determine whether RNA samples were contaminated with DNA, control reactions were performed without reverse transcription. Specific PCR for renin was done using RR1 (5'-TCT CAG CAA CAT GGA CTA TGT GC-3') and RR2 (5'-TTA GCG GGC CAA GGC GAA CC-3') primers designed according to Pieruzzi et al. (1995) giving the 190 bp fragment. The PCR program included 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and polymerization at 72 °C for 1 min. As a control for quantitative evaluation of PCR, GAPDH primers (GPH1: 5'-AGA TCC ACA ACG GAT ACA TT-3', GPH2: 5'-TCC CTC AAG ATT GTG AGC AA-3') were used to amplify the 309 bp fragment (Terada et al. 1993).
from each first strand sample. After denaturation at 94 °C for 5 min, 30 cycles of PCR at 94 °C, 60 °C and 72 °C for 1 min each were performed. ACE was detected after 35 cycles of PCR using primers AC1 (5’-CCT GAT CAA CAA GGA GTT TGC AGA G-3’) and AC2 (5’-GCC AGC CTT CCC AGG CAA ACA GCA C-3’) under the conditions of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and polymerization at 72°C for 2 min, as described by Iwai et al. (1995). For AT1 receptor detection, following primers AT11 (5’-GCA CAA TCG CCA TAA TTA TCC-3’), AT12 (5’-CAC CTA TGT AAG ATC GCT TC-3’) and conditions were used: denaturation at 94° C for 1 min., annealing at 54°C for 1 min. and polymerization at 72°C for 1.5 min (Llorens-Cortes et al. 1994). Primers for AT2 receptors were designed according to Leung et al. (1998) (5’-CAA GAC TTG GTC ACG GGT-3’, 5’-TCT GGC TGT GGC TGA CTT) giving a band at 511 bp. PCR program included 30 cycles of amplification, each consisting of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and polymerization at 72 °C for 1.5 min (Llorens-Cortes et al. 1994). Fragments of brain NOS (599 bp) was amplified as described by Schricker et al. (1996), using the following primers bNOS1 (5’-GAA TAC CAG CCT GATCCA-3’), bNOS2 (5’-TCC AGG AGG GTG TCC ACC GCA-3’) and 33 cycles of denaturation at 94 °C for 1 min, annealing at 60°C for 30 s and polymerization at 72 °C for 1 min. PCR products were analyzed on 2 % agarose gels. Optical density of the individual bands were measured by Kodak camera, quantified using IMAGE software and compared in relation to GAPDH.

Statistics

Results are presented as means ± S.E.M. Statistical differences among groups were determined by one-way analysis of variance (ANOVA). Statistical significance was defined as p < 0.05 and p values were adjusted for multiple comparisons by the Bonferroni method. Linear regression analysis was used to evaluate correlation between variables.

Fig. 2. Gene expression of AT1 receptor and bNOS (A) and relative quantification of mRNA for AT1 receptors (empty columns) and bNOS (filled columns) compared to the housekeeper GAPDH in individual parts of adult rat brain (B). Abbreviations of the structures used are indicated in the legend to Fig. 1. Each column represents mean ± S.E.M. and is an average of measurements in five animals. * statistical significance (p<0.05) of mRNA for AT1 receptor in cerebellum compared to pons, thalamus, septum and cortex. ** statistical significance (p<0.05) of mRNA for AT1 receptor in hippocampus compared to pons and septum. # statistical significance (p<0.01) for mRNA of bNOS in amygdala compared to thalamus, pons and septum. ** statistical significance (p<0.01) for mRNA of bNOS in cerebellum compared to pons, thalamus, cortex and septum. x shows statistical significance (p<0.05) for mRNA of bNOS in the hippocampus compared to cortex, pons, thalamus and septum. C - regression analysis between bNOS and AT1 receptors mRNA levels showed a significant positive correlation (r = 0.525, p = 0.0047).
Results

Renin mRNA was detected in all brain regions studied, with the highest levels in the medulla and lowest ones in the cerebellum (Fig. 1A). Differences between renin mRNA levels in the cortex, hippocampus, amygdala, thalamus and medulla were statistically significant (p<0.05). The distribution pattern of mRNA of other RAS’s enzyme ACE was similar to that of renin mRNA levels (Fig. 1A), with significant differences (p<0.05) between values in the cerebellum and those in the hippocampus, hypothalamus, pons and medulla. ACE mRNA levels in the medulla were significantly higher (p<0.05) compared to those in the septum. Regression analysis showed a significant positive correlation (r = 0.54, p<0.01, Fig. 1B) between renin and ACE mRNA levels.

The highest levels of AT₁ receptor mRNA were observed in the cerebellum (Fig. 2A). Significantly lower levels of AT₁ receptor mRNA compared to those in the cerebellum were found in the cortex, septum, thalamus (p<0.05) and pons (p<0.01). The gene expression of bNOS showed a significant variability among the regions studied (Fig. 2A). The highest levels of bNOS mRNA were found in the cerebellum and these values were comparable to those in the hippocampus and amygdala. The lowest mRNA message for bNOS was observed in the septum and thalamus with values, which were significantly different from those in other brain regions analyzed except the pons and brain cortex. The levels of bNOS mRNA did not correlate with those of renin (r = –0.25, p = 0.201), ACE (r = –0.036, p = 0.858) and AT₂ receptors (r = 0.085, p = 0.675). A significant correlation was found between bNOS mRNA and AT₁ receptor mRNA levels (r = 0.53, p<0.01, Fig. 2B). The gene expression of AT₂ receptors was not detected in the cortex, hippocampus and amygdala (Fig. 3). The highest level of AT₂ gene expression was found in the thalamus and the levels of mRNA in this brain region were significantly different from those in the septum and pons. Measurable amounts of AT₂ receptor mRNA were also observed in the hypothalamus. No correlation was observed between the levels of AT₁ and AT₂ receptor mRNAs (r = 0.11, p = 0.581).

Discussion

Simultaneous measurements of mRNA levels encoding for bNOS and individual components of the
renin-angiotensin system in the present study revealed a similar distribution of bNOS mRNA and AT1 receptor mRNA levels in the rat brain. In contrast, bNOS mRNA levels failed to correlate with gene expression of AT2 receptors, renin or ACE. The expression of bNOS and AT1 receptors in the same brain regions may represent a potential basis for the interaction of NO and angiotensin II at the level of AT1 receptors. This interaction remains to be definitively demonstrated by other approaches at both, mRNA and protein levels.

Since the renin-angiotensin pathway is the only producer of angiotensin II, the presence of angiotensin synthesizing enzymes indicates brain regions with targeted synthesis of angiotensin II. The production of renin and ACE in the brain is well documented (Saavedra, 1992). It was shown on the basis of radioimmununological analysis that renin activity is present in the frontal cortex, striatum, amygdala, hypothalamus, cerebellum and the medulla (Genain et al. 1985). However, detailed studies on brain distribution of renin mRNA are not available. In the present experiments, gene expression of renin was detected not only in the above-mentioned structures but also in other brain areas. The levels of renin mRNA in individual brain regions were comparable with the exception of the cerebellum, in which the levels of both renin and ACE mRNA were low. Interestingly, low ACE activity in the cerebellum was also reported in the human brain (Tani 1991). On the other hand, Whiting et al. (1991) reported higher levels of ACE mRNA in the cerebellum than in the hippocampus. Moreover, Winkler et al. (1998) found high concentrations of ACE mRNA in the striatum, the brain structure that was not included in the present studies. As expected, renin and ACE mRNAs exhibited similar distribution throughout the brain showing a significant correlation.

The distribution of angiotensin II receptor subtypes is of special interest, since several receptors mediate the action of angiotensin II. The brain distribution of both AT1 and AT2 receptors have been studied very intensively (Wright and Harding 1995, Lenkei et al. 1997, Allen et al. 1998). Our results showing high levels of AT1 receptor mRNA in the cerebellum, amygdala, medulla and hypothalamus as well as moderate amounts of the mRNA for AT1 receptors in piriform cortex, thalamus, pons and septum are in good agreement with the results of others (Wright and Harding 1995, Lenkei et al. 1997). In contrast to previously published data, high levels of AT1 receptor mRNA were also observed in the hippocampus.

The pattern of distribution of mRNA levels coding for AT2 receptors is different from that of AT1 receptor mRNA. Our results demonstrating highest AT2 receptor mRNA levels in the thalamus are in agreement with the observation published by Lenkei et al. (1997) and Allen et al. (1998). In general, the expression of AT2 and AT1 mRNAs showed only a limited overlap (Lenkei et al. 1997). Consistently, no correlation between AT1 and AT2 receptor mRNA levels was detected in the present study. Our observation that moderate or high levels of mRNAs coding for angiotensin receptors are associated with low levels of mRNAs for angiotensin synthesizing enzymes in the cerebellum is interesting. It may be speculated that the gene expression of synthesizing enzymes and the synthesis of angiotensin II in the cerebellum would increase under the conditions of greater demands of the body for angiotensinogen, as was suggested by Lippoldt et al. (1994). Nevertheless, in humans, the only site that consistently expresses AT2 receptors is the molecular layer of the cerebellum (MacGregor et al. 1995).

The distribution of bNOS was studied by Iwase et al. (1998) using in situ hybridization. These authors found the mRNA coding for bNOS in several parts of the rat brain, including cerebellum, amygdala, hippocampus and hypothalamus. The present data obtained by a different methodological approach showed a significant variability among the regions studied. Very low amounts of bNOS mRNA were noticed in the thalamus and septum.

The role of centrally produced NO in the regulation of body fluid and blood pressure homeostasis is becoming increasingly apparent. It was shown that increased angiotensin II can stimulate the production of NO in the kidney (Beierwaltes and Sigmon 1996), probably by stimulating the synthesis of endothelial NO synthase (Hemmington et al. 1998). Central or peripheral blockade of NOS was found to potentiate or prolong the pressor response to angiotensin II in conscious rats (Liu et al. 1998).

However, very little is known about the mutual interaction of these two systems in the brain. Since the basic requirement for demonstration of a local protein synthesis is the proof about its gene expression in the tissue, we correlated the mRNA levels for bNOS with mRNA for AT1 and AT2 receptors, respectively. Our data indicate that the quantitative profile of bNOS mRNA in the brain structures studied is similar to that of AT1 receptor mRNA. No correlation was found between the distribution of AT2 receptor and bNOS mRNA levels.
The physiological relevance of the parallel distribution of AT1 receptor and bNOS mRNA is not known, albeit very likely. The brain structures analyzed in the present study were selected as representative of the main brain circuits involved in autonomic, behavioral and neuroendocrine functions. The AT1 receptor site appears to mediate the classic angiotensin functions related to body water balance, maintenance of blood pressure and sexual behavior (Wright and Harding 1995). Moreover, angiotensin II was found to modulate some neuroendocrine functions via this receptor subtype (Ježová et al. 1998). It is known that brain angiotensin II may regulate blood pressure through different mechanisms and several nuclei participating in angiotensin regulation of blood pressure contain AT1A mRNA but are devoid of AT2 mRNA (Lenkei et al. 1997). It has been recognized that NO produced in the central nervous system could act as a neurotransmitter or neuromodulator to influence the impact of vasoconstrictors such as angiotensin II, on the control of cardiovascular system (Zanchi et al. 1995, Persson 1996). The possibility that a dysbalance between NO and AT1 receptor gene expression, particularly at the level of brainstem structures – the pons and medulla, may underlie the development of cardiovascular pathology, remains to be elucidated.

In conclusion, the presented data support the idea that there is an interaction between brain angiotensin II and NO providing the data on a parallel distribution of mRNAs coding for bNOS and AT1 receptors in several brain structures. This suggestion has to be confirmed by further studies evaluating different physiological situations.

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References


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