

Vasopressin and oxytocin gene expression in the supraoptic and paraventricular nucleus of the spontaneously hypertensive rat (SHR) during development of hypertension*

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To study the regulation of hypothalamic vasopressin (VP) and oxytocin (OT) gene expression in relation to the development of hypertension, levels of VP mRNA and OT mRNA were determined in spontaneously hypertensive rats (SHR). Differences in VP and OT mRNA content of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of 4- and 10-week-old SHR and Wistar-Kyoto controls (WKY) were quantitated by dot-blot and Northern blot analysis. VP and OT pituitary content and VP plasma levels were measured by radioimmunoassays. VP mRNA levels were approximately 2-fold and 3-fold higher in the SON and PVN of 4-week-old SHR, respectively, as compared to controls. The OT mRNA levels were approximately 35% lower in both nuclei of the SHR. There was no difference in VP and OT pituitary content between 4-week-old SHR and WKY, but VP plasma levels were higher in SHR. In the 10-week-old SHR VP mRNA levels were still approximately 30–40% higher and the OT mRNA levels were approximately 40% lower in both nuclei when compared to age-matched WKY. Pituitary VP and OT contents were respectively 1.5-fold higher and 20% lower in the 10-week-old SHR than in 10-week-old WKY. VP plasma levels were still elevated in the SHR. The data indicate that in the hypothalamo-neurohypophyseal system of the SHR the VP system is in a higher state of activity, while the OT system is lower in activity.

INTRODUCTION

The spontaneously hypertensive rat (SHR) is characterized by a rise in blood pressure with age. During the first month after birth blood pressure is only slightly higher in the SHR when compared to Wistar-Kyoto controls (WKY), but at an age of approximately 2 months the SHR is clearly hypertensive³⁰. Several reports have suggested that vasopressin (VP) is involved in the development of high blood pressure of the SHR^{20,42}. This suggestion is based on the vasoconstricting properties of VP^{20,41} and the observed alterations in the VP system of the SHR. In the SHR higher plasma VP levels have been reported^{7,}

^{16,20,22,25,36,41} as well as pituitary VP contents as compared to WKY^{7,8,16,23,36}. Furthermore, there are indications that the SHR is hyper-responsive in its VP release following several stimuli^{6,8,25–27,35}. Besides changes in the VP system, alterations in the oxytocin (OT) system have also been reported for the SHR. The SHR has lower plasma OT levels and pituitary contents^{23,26,36,41}. The magnitude of the changes may not be sufficient to explain the degree of hypertension^{16,20} and may be related to strain differences. However, the SHR and the genetically related WKY rat may provide an animal model for studies on VP and OT gene expression, biosynthesis and release.

In this paper we have investigated whether

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changes in the expression of the VP and OT genes of the SHR underlie the altered pituitary and plasma levels of the neurohypophyseal hormones. The major site of synthesis of VP and OT in the pituitary gland is in the magnocellular neurons of the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus. The PVN also innervates extra-hypothalamic brain areas^{12,33,46}. Therefore, VP and OT mRNA levels were measured in the SON and PVN of SHR and WKY during the period in which hypertension develops. The data indicate that the SHR is more active in expressing the VP gene and less active in OT gene expression than the WKY. The greatest difference in VP and OT gene expression between the strains occurs before hypertension is fully developed. It demonstrates that the alterations in the VP and OT systems are constitutive and not caused by increased blood pressure per se.

MATERIAL AND METHODS

Animals and tissue preparation

Male SHR and WKY of 4 and 10 weeks of age (own breeding of the strains provided by TNO-CPB, Zeist, The Netherlands) were used. The brain and pituitary gland were immediately removed from the skull after decapitation, frozen on dry ice and stored at -70°C . Plasma was obtained from trunk blood and stored at -20°C . The SON and PVN were isolated from the brains by the punch technique of Palkovits³². A constant number of punches containing the whole nucleus (10 punches from 5 slices of $300\text{ }\mu\text{m}$ per nucleus) was used. Equal amounts of cortex tissue were isolated. Furthermore, *in situ* hybridization showed that VP mRNA and OT mRNA is present only in the soma of the VP- and OT-producing neurons^{4,19}. Therefore the data were expressed per whole nucleus instead of per protein content.

Blood pressure was measured in age-matched groups of freely moving SHR and WKY via an indwelling carotid cannula which was implanted one day before under ether anesthesia.

RNA extraction

The isolated SON, PVN and cortex tissue were homogenized in $200\text{ }\mu\text{l}$ 4 M guanidine thiocyanate, 0.1 M β -mercaptoethanol, 25 mM sodium citrate, pH 7.0 by suction through a 21-gauge needle. The homoge-

nate was extracted with $200\text{ }\mu\text{l}$ phenol-chloroform-isoamylalcohol (25:24:1, v/v/v), pH 8.0. The aqueous phase was isolated and 0.6 volumes of 45 mM acetic acid in ethanol were added. After an incubation period of 16 h at -20°C , RNA was precipitated by centrifugation at $10,000\text{ g}$ for 20 min. RNA was dissolved in $50\text{ }\mu\text{l}$ of 50 $\mu\text{g/ml}$ proteinase K, 10 mM Tris-HCl, 5 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), pH 7.8 (preincubated for 15 min at 37°C), and incubated for 1 h at 37°C . Next, $50\text{ }\mu\text{l}$ water was added and the solution was extracted with $100\text{ }\mu\text{l}$ phenol-chloroform-isoamylalcohol (25:24:1, v/v/v), pH 8.0. The aqueous phase was isolated and 0.1 vol 3 M sodium acetate, pH 5.2 and 3 vol ethanol were added. After 30 min at -70°C RNA was precipitated by centrifugation at $10,000\text{ g}$ for 20 min. The yield was $1.7 \pm 0.1\text{ }\mu\text{g}$ RNA per mg tissue².

Northern- and dot-blot analysis

For Northern blot analysis RNA of the SON and PVN pooled from 4 animals was denatured with glyoxal and dimethylsulfoxide, fractionated on 1.4% agarose gels and transferred to a nylon membrane (Hybond-N, Amersham, U.K.)². For dot-blot analysis one-fourth of the RNA isolated from SON, PVN and cortex tissue was dissolved in $50\text{ }\mu\text{l}$ $6.7 \times \text{SSC}$ ($1 \times \text{SSC} = 150\text{ mM}$ sodium chloride– 15 mM sodium citrate, pH 7.0), 7.4% formaldehyde at 65°C for 15 min. The samples, which were in duplicates, were cooled to 0°C and spotted with a dot-blot apparatus (Bio-Dot microfiltration apparatus, Bio-Rad, CA, U.S.A.) on Hybond-N membrane. The blots are shortly washed in $2 \times \text{SSC}$, dried, and baked at 85°C for 2 h.

The blots were hybridized with the ^{32}P -labeled single-stranded OT- and VP-specific probes, MPB-5 and MPB-1 respectively², at 55°C in the presence of 10% dextran sulfate. After hybridization, the blots were washed twice in $2 \times \text{SSC}$ –0.1% SDS for 5 min at room temperature, once in $0.5 \times \text{SSC}$ –0.1% SDS at 50 – 60°C for 15 min, and once in $0.1 \times \text{SSC}$ –0.1% SDS at 65°C for 15 min. The blots were exposed to X-ray film (Kodak XAR-5 (Eastman Kodak, Rochester, NY)) with an intensifying screen at -70°C for various lengths of time. For rehybridization the probe was removed from the blots by washing at 60°C in 50% formamide, 10 mM Tris-HCl–10 mM EDTA, pH 8.0 for 1 h. Completeness of probe re-

moval was checked by autoradiography of the blots.

Image analysis

The autoradiographic signals from the Northern and dot-blot were quantitated by determination of the optical density (OD) using a VIPER image analysis system (Gesotec, Darmstadt, F.R.G.) equipped with a Hitachi CCD-videocamera (Hitachi, Tokyo, Japan). The area with the specific hybridization signal is automatically contoured using a preset OD limit which is kept constant throughout the measurements. The average OD of these contoured areas is determined. The OD of the background next to the measured areas is subtracted from the OD value obtained from these areas.

Measurement of the OD of the signals from the Northern blots provided a relative index for the changes in mRNA levels. The percentual changes in mRNA levels are based on the average ODs of the total specific RNA bands on the autoradiograms. These ODs were converted to arbitrary units of mRNA using a film response curve which was obtained from dot-blot with known amounts of MPB-4 and MPB-2 which are the DNA complements of the ^{32}P -labeled probes MPB-5 and MPB-1 respectively¹. These dot-blot were hybridized and exposed together with the Northern blots.

Quantitative measurements of the VP mRNA and OT mRNA contents of the samples were done by dot-blot using known amounts of MPB-2 and MPB-4 DNA as standards which were spotted on the same blot. The RNA samples on the dot-blot were diluted such that their ODs were in the linear part of the standard curve. The OD values are converted by the standard curves and corrected for the dilution factor of the sample. The cortex values were subtracted from the values obtained for the different nuclei resulting in values which represents the total amount of OT or VP mRNA per SON.

Radioimmunoassay

OT and VP were extracted from the pituitary gland⁵ and measured by radioimmunoassay (RIA) according to Dogterom^{9,10}, using the VP-antiserum W1E and the OT-antiserum 02¹⁷. Each pituitary extract was measured in two dilutions. The final dilutions for the VP-antiserum was 1:240,000 and for the OT antiserum was 1:16,000. The amount of iodi-

nated tracer in the RIA was 4000 cpm. Sensitivity of the VP-RIA was 0.5 pg VP/tube (90% tracer displacement). Fifty percent displacement was obtained with 8 pg VP/tube. Intra- and interassay coefficients of variation are 10% and 20%, respectively. The OT-RIA had a sensitivity of 4 pg OT/tube (90% tracer displacement) and 50% displacement was obtained at 40 pg OT/tube. Intra- and interassay coefficients of variation of the OT-RIA are 10% and 20%, respectively.

RESULTS

Blood pressure

In 4-week-old SHR blood pressure was significantly higher than in 4-week-old WKY. The mean arterial pressure (\pm S.E.M.) of WKY of 4 weeks of age was 101 ± 5 mm Hg ($n = 6$) and of SHR 117 ± 4 mm Hg ($n = 6$) ($P < 0.05$; Student's *t*-test). This difference in blood pressure was more pronounced at 10 weeks when the WKY had a mean arterial pressure (\pm S.E.M.) of 124 ± 6 mm Hg ($n = 8$) and that of the SHR was 168 ± 3 mm Hg ($n = 9$; $P < 0.05$, Student's *t*-test).

VP mRNA and OT mRNA in the SON and PVN

Northern blot analysis of VP mRNA from the SON and PVN showed that the 4- and 10-week-old SHR had higher VP mRNA levels in both nuclei than age-matched WKY (Table I and Fig. 1). Dot-blot analysis, which is more precisely standardized, showed that at 4 weeks of age the SHR contained significantly (ANOVA) 1.8- and 2.7-fold more VP mRNA than the WKY in the SON and PVN respectively. At 10 weeks of age, VP mRNA levels were still higher in the SON and PVN of the SHR, but the difference between the two strains was reduced. The SON of the SHR contained significantly 1.3-fold more VP mRNA, and 1.4-fold higher levels (non-significant) were present in the PVN as compared to WKY. During the development from 4 to 10 weeks the VP mRNA levels in the SON and PVN of the WKY increased 1.4- and 2.4-fold. The VP mRNA levels in the SHR did not change during the developmental period studied according to dot-blot analysis (Fig. 2), but a developmental increase in VP mRNA was still observed by Northern blot analysis (Table I).

TABLE I

Relative VP and OT mRNA content of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of 4- and 10-week-old SHR determined by Northern blot analysis

The VP and OT mRNA contents are expressed as the mean percentage of the optical density value obtained from two Northern blots in which the VP and OT mRNA content in the SON of the 10-week-old WKY is set as 100%.

| Brain area | Age (weeks) | VP mRNA (%) | | |
|------------|-------------|-------------|-----|---------|
| | | WKY | SHR | WKY/SHR |
| SON | 4 | 80 | 100 | 0.8 |
| SON | 10 | 100 | 155 | 0.6 |
| PVN | 4 | 15 | 30 | 0.5 |
| PVN | 10 | 50 | 75 | 0.7 |

| Brain area | Age (weeks) | OT mRNA (%) | | |
|------------|-------------|-------------|-----|---------|
| | | WKY | SHR | WKY/SHR |
| SON | 4 | 50 | 25 | 2.0 |
| SON | 10 | 100 | 45 | 2.2 |
| PVN | 4 | 55 | 35 | 1.6 |
| PVN | 10 | 80 | 60 | 1.3 |

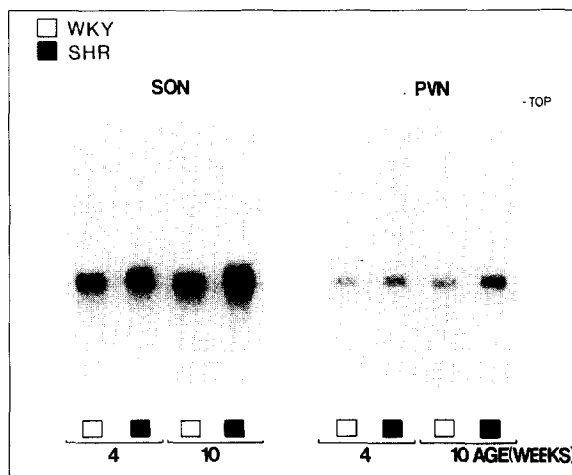


Fig. 1. VP mRNA detected on Northern blots of RNA isolated from the SON (left panel) and PVN (right panel) of 4- and 10-week-old SHR and WKY. Each lane contained total RNA pooled from 4 rats. The approximate length of VP mRNA was 700 bases as determined by M13 *HpaII* restricted DNA size markers.

Rehybridization of the dot-blot and Northern blots with an OT mRNA specific probe showed that OT mRNA levels of the SON were significantly (ANOVA) 35% lower in the SHR of 4 weeks and 10

weeks of age than in the age-matched WKY (Fig. 3). Determined by Northern blot analysis, PVN OT mRNA levels of the SHR of 4 and 10 weeks of age were respectively 40% and 25% lower than in the

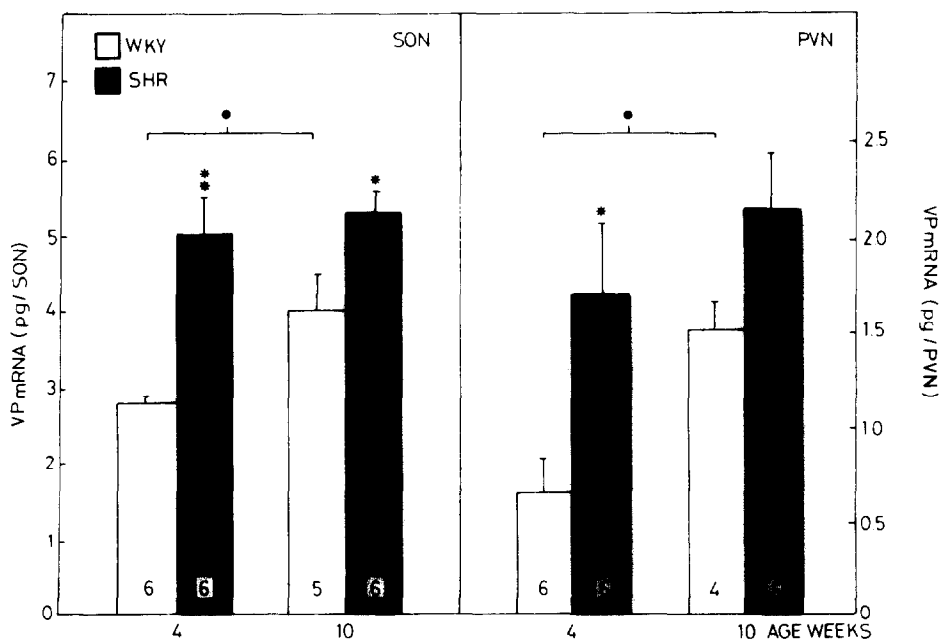


Fig. 2. VP mRNA content of the SON and PVN of 4- and 10-week-old SHR and WKY as determined by dot-blot analysis. VP mRNA levels are expressed in pg as the mean \pm S.E.M. The number of animals is indicated at the bottom of the column. Statistical significance was tested by ANOVA and Student's *t*-test. Age-matched SHR versus WKY, * $P < 0.05$, ** $P < 0.01$; 4- versus 10-week-old rats, • $P < 0.05$ (Student's *t*-test).

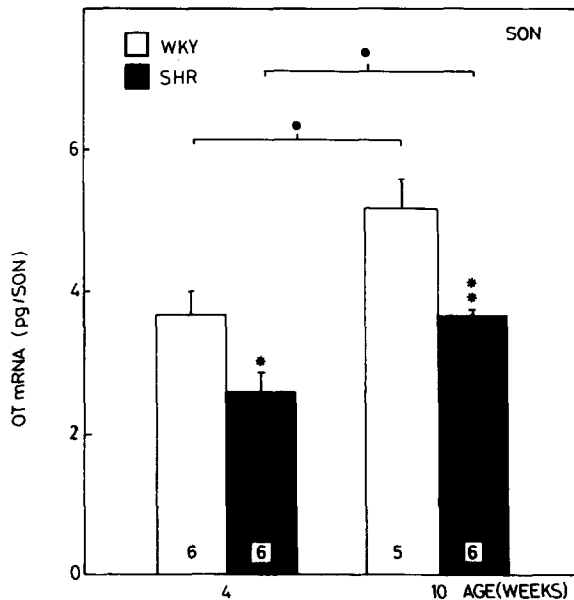


Fig. 3. OT mRNA content of the SON of 4- and 10-week-old SHR and WKY rats as determined by dot-blot analysis. Values are expressed in pg of OT mRNA as the mean \pm S.E.M. and the number of animals is indicated at the bottom of the column. Statistical significance was tested by ANOVA and Student's *t*-test. Age-matched SHR versus WKY, * $P < 0.05$, ** $P < 0.01$; 4- versus 10-week-old rats, • $P < 0.05$ (Student's *t*-test).

WKY (Table I and Fig. 4). Furthermore, both SHR and WKY showed a 1.4-fold increase in OT mRNA content of the SON during aging from 4 to 10 weeks (Fig. 3). This was in agreement with Northern blot analysis which indicated a 2- and 1.5-fold increase in the SON and PVN respectively of SHR and WKY (Table I and Fig. 4).

VP and OT content of the pituitary gland and VP plasma level

At 4 weeks of age, pituitary VP and OT contents were not different between SHR and WKY. However, at 10 weeks of age, the SHR had a 1.5-fold higher pituitary VP content than the WKY, while the OT content was 1.3-fold lower in the SHR (Fig. 5). In both 4- and 10-week-old SHR the VP plasma levels were approximately 1.5-fold higher (ANOVA) than in WKY (Fig. 6).

DISCUSSION

The higher levels of VP mRNA and lower levels of OT mRNA in the SON and PVN of the SHR com-

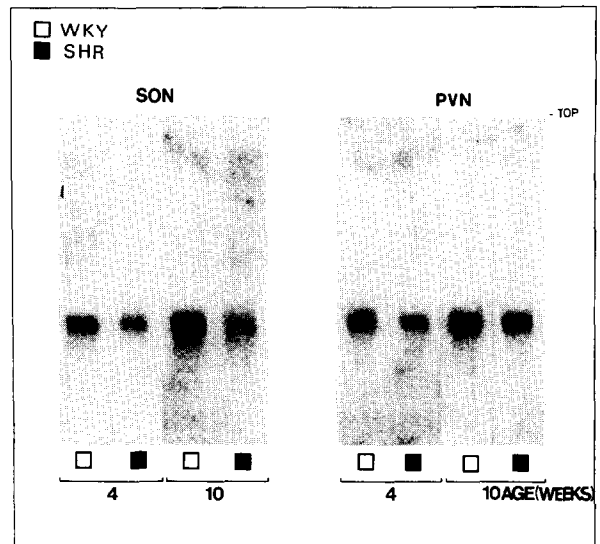


Fig. 4. OT mRNA detected on Northern blots of total RNA preparations from the SON (left panel) and PVN (right panel) of 4- and 10-week-old SHR and WKY. The lanes contained total RNA preparations pooled from 4 animals. The length of OT mRNA was approximately 650 bases as determined by DNA size markers (cf. Fig. 1).

pared to the WKY are indicative for a higher level of expression of the VP gene and a lower expression of the OT gene in the SHR. These changes in VP and OT mRNA levels cannot be caused by cell division and migration during development since these processes already stop before birth^{11,15,38,47,48}. There are no differences between the SHR and the WKY with respect to the number of vasopressinergic neurons in the SON⁴⁰ and the total number of magnocellular neurons in the SON²⁸. It has been reported that the PVN of the SHR has a reduced amount of magnocellular neurons²⁸. However, it has not been determined if this is in the vasopressinergic and/or oxytocinergic cell population and whether the number of parvocellular neurons is different. This means that differences in VP and OT mRNAs in the SON are not caused by differences in the number of vasopressinergic and oxytocinergic neurons. The reduced amount of magnocellular neurons in the PVN indicate that the higher VP mRNA levels in this nucleus are caused by a higher level of VP gene expression, but the lower OT mRNA levels in the PVN could be the result of a lesser amount of oxytocinergic neurons. Alternatively, differences in mRNA stability cannot yet be excluded. By using the punch technique of Palkovits³²

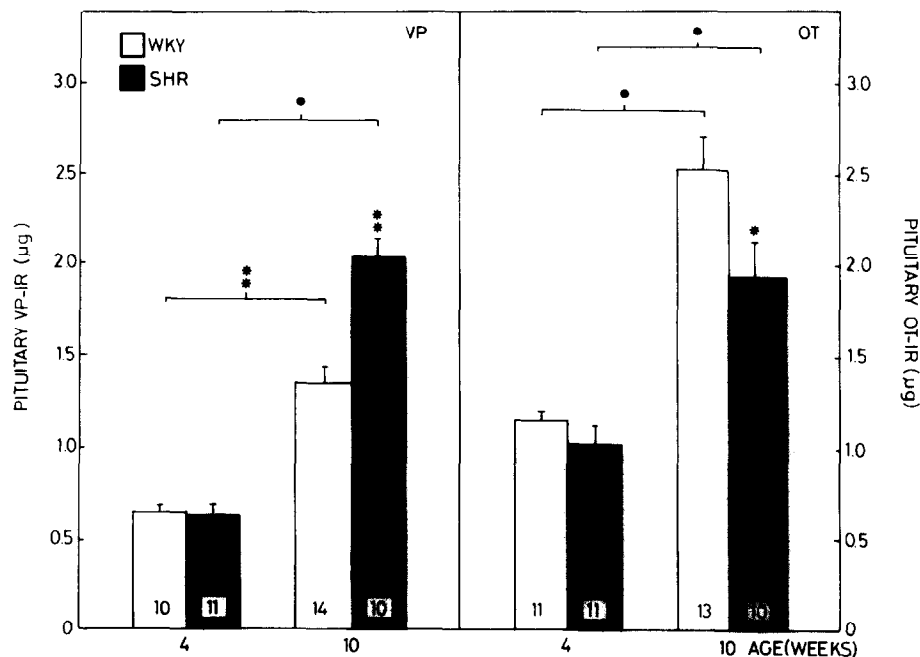


Fig. 5. The pituitary contents of VP (left panel) and OT (right panel) in 4- and 10-week-old SHR and WKY. The VP and OT immuno-reactivity (IR) are expressed as the mean (μg) \pm S.E.M., and the number of rats is indicated at the bottom of the column. Statistical significance was tested by ANOVA and Student's *t*-test. Age-matched SHR versus WKY, * $P < 0.05$; 4- versus 10-week-old rats, • $P < 0.01$ (Student's *t*-test).

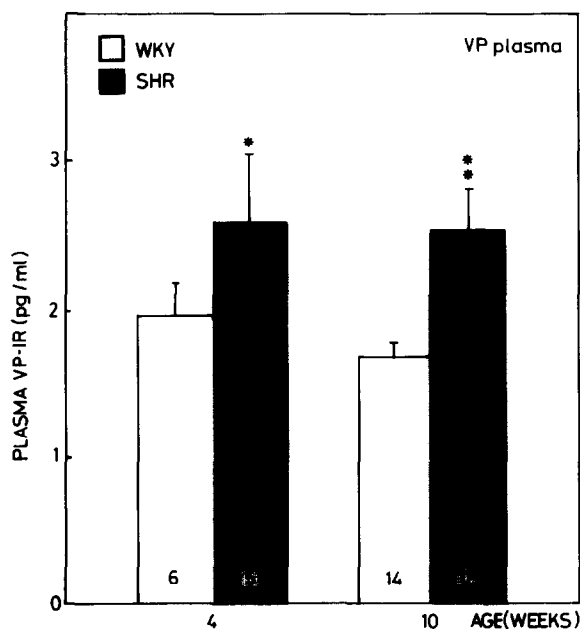


Fig. 6. Plasma levels of VP in 4- and 10-week-old SHR and WKY as determined by radioimmunoassay. The levels are expressed in pg VP/ml VP as the mean \pm S.E.M. The number of animals is indicated at the bottom of the column. Statistical significance between WKY and SHR was tested by ANOVA. * $P < 0.05$, ** $P < 0.01$.

we obtained the average level of the VP mRNA and OT mRNA content per total nucleus. However, a change or difference does not need to occur evenly in all the neurons of the SON and PVN, but could be limited to a certain population of neurons as has been found for example following osmotic stimulation by *in situ* hybridization¹⁹.

Several reports on VP levels in plasma and in the hypothalamo-neurohypophyseal system indicate that the SHR has a higher basal VP release as compared to WKY^{7,16,20,22,25,36,41}. On the other hand in other reports differences in VP plasma levels were not found^{31,34}. This may be due to strain differences. A higher level of VP gene expression in the hypothalamo-neurohypophyseal system of 4-week-old SHR accompanied by unchanged VP pituitary levels and higher plasma levels indicate that VP biosynthesis and release are both higher in the 4-week-old SHR. In the 10-week-old SHR VP mRNA levels remain higher although the difference with the WKY is less prominent. This is accompanied by higher VP pituitary and plasma levels indicating that a higher rate of biosynthesis and release of VP is still present in the SHR later in development. In itself the elevation of

plasma VP levels in the SHR is insufficient to increase blood pressure²⁰.

The VP pituitary content is the net result of VP synthesis and release. The net result of synthesis and release of VP in young SHR and WKY is similar since VP pituitary contents are not different between both strains. However, the VP mRNA and VP plasma levels are higher in the SHR. In SHR of 10 weeks of age the VP pituitary content also is higher, suggesting that, compared to young SHR and WKY, there is a different balance in VP synthesis and release between 10-week-old SHR and WKY. Studies on VP release support the view that increased release is due to hyper-responsiveness of the VP system in young SHR³⁷, which may stimulate VP gene expression. Later in the hypertensive phase this hyper-responsiveness disappears³⁷ which suggests that the high VP gene expression has become constitutive.

The level of OT gene expression of the hypothalamo-neurohypophyseal system of the SHR changed in a direction opposite to that of VP gene expression. In 4- and 10-week-old SHR OT mRNA levels are lower than in the WKY. OT pituitary levels are the same at 4 weeks but lower in 10-week-old SHR. These lower OT pituitary levels at 10 weeks are accompanied by lower OT plasma levels, as has been measured in 17-week-old animals³⁶. This again indicates that young SHR and WKY have a similar balance in synthesis and release while the older SHR and WKY have a different balance and suggests that the lower expression of the OT gene has become constitutive in the old SHR, resulting in decreased OT pituitary and plasma levels.

The VP systems primarily to brainstem areas are of interest since baroreceptor responses and cardiovascular functions are mediated via brainstem areas, primarily via the nucleus tractus solitarius^{13,21,50}. Both vasopressinergic and oxytocinergic neurons from the PVN project to brainstem areas^{29,46,51}. Furthermore, VP administration via the cisterna magna, spinal cord or into the nucleus tractus solitarius increases blood pressure in rats^{14,18,43}. On the other hand it has been reported that low doses of i.c.v. given vasopressin decreases the sensitivity of the baroreceptor heart reflex¹. Moreover, intracisternal injected VP can result in a reduction of pressor responses induced by stimulation of the afferent nervus vagus in dogs²⁴, and that intracerebroventricular administration of

VP and OT in rats inhibited a pressor response induced by electrical stimulation of the mesencephalic reticular formation^{44,45}. These data indicate that endogenous VP and OT of the brainstem may participate in blood pressure regulation. Mohring et al.²³ found that there was no difference in brainstem VP and OT levels in 3-week-old stroke-prone SHR (SHRSP), but that at 7 weeks and later VP and OT levels were lower. Although the subset of neurons from the PVN that project to the lower brainstem could have a different level of gene expression than indicated by the overall mRNA levels of the PVN which were measured here, the higher VP mRNA levels in the PVN and lower VP content in the brainstem of old SHRSP suggest that VP release is enhanced in the brainstem of old SHR(SP). Furthermore, the higher VP mRNA levels in the PVN of the young SHR and the similar levels of VP in the brainstem of young SHRSP suggest that VP release is already enhanced in young SHR(SP). The lower OT levels in older rats could then be a result of increased release and/or decreased biosynthesis. If the overall mRNA levels of the PVN reflect the mRNA levels of those neurons projecting to the brainstem than the VP mRNA and OT mRNA content of the PVN suggests that the biosynthetic capacity of the vasopressinergic neurons is higher and of oxytocinergic neurons is lower in this extra-hypothalamic system compared to controls.

Arguments against a role of VP in hypertension of SHR were put forward by Lang et al.¹⁶ who made a cross-breeding of VP-deficient Brattleboro rats (DI) with SHRSP. These SHRSP-DI rats, which are VP-deficient, still develop hypertension. However, in view of the blood pressure increasing effect of OT after application to the nucleus tractus solitarius¹⁸ it should be noted that the OT system of the Brattleboro rat is markedly activated, as indicated by high OT mRNA levels in SON and PVN^{3,49} and high OT plasma levels¹⁰. The decreased OT levels in the pituitary gland and the hypothalamus of the SHRSP-DI¹⁶ could reflect a similar activation of the OT system in this hybrid strain and may have consequences for the development of hypertension.

From the present observations it is concluded that VP gene expression and release are enhanced in the hypothalamo-neurohypophyseal system of the SHR during the development of hypertension, while OT

gene expression is reduced. The data suggest that these strain differences may be primarily caused by differences in release of the neurohypophyseal hormones. Later in development differences in VP and OT gene expression between SHR and WKY may be the main cause for the observed alterations. The observations in this study are not conclusive whether VP and/or OT relate to the cause of hypertension development. They rather indicate that the enhanced activity of the VP system and reduced activity of the OT system are not a consequence of the development of hypertension. OT, may play a role in the development of hypertension in the SHR.

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