Early changes of immunoreactivity to orexin in hypothalamus and to RFamide peptides in brainstem during the development of hypertension

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Running Head: Neuropeptides, NTS, Vagus, hypertension
Hypertension is a condition with multifactorial facets in which the interaction between kidney, heart, and brain plays a central role. Enhanced sympathetic output to kidney and heart, as occurs for example, after stress or high salt intake, is the basis for an increase in blood pressure (BP). BP increase in turn, alerts a series of feedback mechanisms to bring it back to a lower level. To investigate what might happen in the central nervous system, we examined three different peptide systems in the rat brainstem after the development of hypertension. We used an experimental model, in which hypertension is induced by a clamp around the left renal artery. In the brainstem, we examined the expression of neuropeptide FF (NPFF) and prolactin releasing peptide (PrRP); two neuropeptides with cardiovascular regulating properties, as well as calcitonin gene related peptide (CGRP). The level of all three neuropeptides correlated negatively with the level of blood pressure. Although NPFF immunoreactivity was not significantly changed in the NTS after the development of hypertension, PrRP and CGRP expression were reduced in the NTS and AMB, respectively.

Finally, voluntary exercise restored the diminished levels of PrRP and CGRP in respectively the NTS and AMB. The changes in neuropeptide expression in the brainstem and the effect of exercise are discussed with regard to their possible involvement in the etiology and treatment of hypertension.

Key words: Parasympathetic, Vagus, hypertension, and exercise.
1. INTRODUCTION

Hypertension is one of the most prevalent conditions associated with cardiovascular disease leading to premature death worldwide. There are two types of hypertension, primary (or essential) and secondary hypertension. While the primary form of hypertension is characterized by its unknown cause, the secondary hypertension is mostly related to other conditions such as kidney dysfunction (30). Recent evidence shows the involvement of the biological clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, in the development of hypertension. The SCN showed alterations in its neuropeptide content not only before, but also after the development of hypertension (12, 40). The neuropeptide changes were especially significant in the part of the SCN that sends to and receives information from the nucleus of the solitary tract (NTS) in the brainstem(40), indicating the importance of peripheral feedback related to blood pressure fluctuations (4).

The NTS is the first synaptic relay station and integration center in the neural arc of baroreflex (NABR), using negative feedback regulation to dampen blood pressure fluctuations (18). The NABR consists of the afferents to the NTS, vagal efferents, as well as sympathetic (modulatory) centers in the caudal and rostral ventrolateral medulla (CVLM and RVLM, respectively) (8,13). The peripheral afferents carrying systemic blood pressure information terminate in the NTS. In turn, the NTS sends integrated signals to the vagal neurons located in the dorsal motor nucleus of the Vagus (DMV) and nucleus ambiguus (AMB) to dampen circulatory variables (i.e., cardiac output or heart rate) in the case of high arterial pressure (7,34), which simply defines the cardiac baroreflex gain/sensitivity (BRS). In hypertension, the BRS decreases, and blood pressure increases both in humans and rats (2, 14, 17, 35).
Presumably, this is not only because of the increased sympathetic, but also because of a reduced parasympathetic tone (15,26).

Interestingly, it seems that the decreased gain of the cardiac vagal baroreflex precedes sympathetic overactivation before the onset of both primary and secondary hypertension in animals (15, 26). Previously, we have shown early changes of RFamide peptides in the NTS and the DMV that occurring before the development of hypertension in spontaneously hypertensive rats (SHRs) (39), which might underly the early changes of BRS in essential hypertension. Since a decreased gain of the cardiac vagal baroreflex is also found in the acute phase of renovascular hypertension (26) we hypothesized that the neuropeptide changes in the NABR might also occur during the initial phase of 2 Kidney 1 Clamp of (2K1C) (renovascular) hypertension (39). Therefore, in this study we investigated neurotransmitter changes in three NABR structures, namely the NTS, DMV and AMB two weeks after left renal artery clipping. For this, we quantified the levels of NPFF and PrRP, two peptides belonging to the RFamide peptide family (19, 20, 32) in the NTS and DMV and Calcitonin Gene Related Peptide (CGRP) that is expressed by cardiac vagal neurons of AMB (36).

Exercise is well known to decrease blood pressure and heart rate and improve baroreflex sensitivity, both in hypertensive humans and rats (3, 21, 37, 42). Therefore, we investigated the possible ameliorating effects of exercise on cardiovascular variables and neuropeptide expression in the NABR structures. For this, we compared hypertensive and sham-operated control rats 2 weeks after 2K1C hypertension with hypertensive and sham groups that had access to a running wheel 5 weeks after 2K1C-induced hypertension.

Finally, we injected the retrogradely transported neuronal tracer Cholera Toxin B (CTB) into the left kidney to investigate its possible vagal innervation.
2. METHODS

2.1. Ethical Approval

All experiments were conducted under the approval of the ethical committee of the Royal Netherlands Academy of Art and Sciences (KNAW) and the Netherlands Institute for Neuroscience (NIN).

2.2. Animals

Experiments were conducted with a total of 24 male Wistar Albino rats (8 weeks old, Harlan, Zeist, The Netherlands). Three sets of experiments were conducted. In the first set of experiments, after 2 weeks of acclimatization, 10-weeks old male Wistar albino rats underwent 2K1C surgery to induce hypertension (n=5) or received sham operations (n=4) as described elsewhere (40, 41). Blood pressure and heart rate measurements were performed two weeks after the induction of hypertension or sham surgery. In the second set of experiments, animals (n=5 for 2K1C surgery, n=4 for the sham-operated group) were housed individually in light- and sound-isolated so-called “circadian cages” (39x 38x 38 cm), where they had free access to a running wheel, food and water and were handled twice a week throughout the experiments. Final blood pressure measurements were performed 5 weeks after the induction of hypertension or sham surgery. All blood pressure and heart rate measurements were performed at Zeitgeber Time 5 (ZT5) in the first half of the animals’ inactive period, by the intra-carotid catheterization method as reported earlier (40, 41). Subsequently, animals were terminated, and brains were removed, and tissue processed for further immunohistochemical analyses.
In the third experiment, a neuronal tracer, CTB (*Cholera Toxin B*), was injected in the left kidney (see Kidney CTB injections). These animals (*n*=6) were housed individually.

All animals undergoing surgery, either for induction of hypertension or CTB injections, were anesthetized with Hypnorm (0.05 ml/100g BW, i.m., Janssen, High Wycombe, Buckinghamshire, UK) and Dormicum (0.04 ml/100g BW, s.c., Roche, Almere, The Netherlands).

### 2.4. Kidney CTB injections and tissue collection

To perform kidney CTB injections, a midline laparotomy was performed under anaesthesia. After that, two microliters of CTB- Alexa Fluor 647 (1%, Molecular Probes, Eugene, OR; no. C22844) was injected into the left kidney (*n*=4) at a single spot using a 30-gauge needle connected to a Hamilton syringe. In the control experiment, CTB was applied on top of the intact organ (*n*=2). Animals recovered overnight and were given Temgesic (Schering-Plough, Maarssen, The Netherlands, 0.01 ml/100 g BW) to reduce post-operative pain. Rats were terminated 5 days after the injection, allowing optimal transport of CTB.

### 2.5. Immunohistochemistry

After receiving an overdose of Nembutal, all animals were transcardially perfused with saline followed by solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. For further anatomical evaluations, brains were removed and post-fixed in paraformaldehyde solution overnight. After overnight post-fixation in paraformaldehyde, brain tissue was further incubated for 3 days with 30% sucrose in 0.1 M Tris-buffered saline (TBS, pH 7.4). After this, brainstem tissue was cut into 40 µm coronal cryostat sections at -20°C and collected in TBS containing vials. The sections were taken at 240 µm intervals through the whole NTS, DMV and AMB nuclei from the most caudal to the rostral extent of the medulla oblongata.
Sets of serial brainstem sections of hypertensive and control animals were stained in parallel, under the same conditions and using the same solutions. Free-floating sections were pre-treated with absolute methanol and 3% H$_2$O$_2$ for 10 minutes. After thorough washing in TBS, all sections were incubated overnight at 4°C with NPFF (1:5000; for specificity see figure 1 in 22), PrRP (1: 2000; for the specificity see 39) or CGRP (Calcitonin gene-related peptide, 1:6000; generous donation from Robert K. Kui while he was affiliated at UCLA, Los Angeles, California, USA; for the specificity see 5) antibodies. Following the incubation with the primary antibody overnight, sections were incubated for 1h with a secondary antibody, biotinylated goat anti-rabbit IgG (1:400; Vector Laboratories Inc., Burlingame, California, USA) for the NPFF and PrRP antibodies and biotinylated goat anti-mouse IgG (1:200; Vector Laboratories Inc., Burlingame, California, USA) for the CGRP antibody, and then incubated with ABC (1:800; Vector) for 1h. Finally, sections were incubated with 0.05% 3,3’- diaminobenzidine tetrachloride and 0.01% hydrogen peroxide for 7 minutes. Sections were mounted on gelatine-coated slides, air-dried and cover slipped after dehydration with ethanol and xylene.

Brain sections with CTB-Alexa Fluor were rinsed extensively with PBS (pH 7.2) and cover-slipped with 50% PBS glycerol for examination under a Philipps (Eindhoven, The Netherlands) confocal laser-scanning microscope (LSM 410/510).

**2.6. Quantitative study and data analyses**

According to the procedure described previously (10), the quantitative estimation of the density of the NPFF-, PrRP- and CGRP–positive neuronal elements in the brainstem sections, was performed in two steps. First, using a microscope from Axioskop (Zeiss, Germany) equipped with a black-and-white CCD camera (Sony XC-77, Japan) and run by the computer program Image-Pro Plus 6.3 (Media Cybernetics, Bethesda, MD), we recorded tiled images of the brainstem under a 40x NeoFluar objective (Zeiss), which were further combined into one
large picture. To enhance the contrasts of neuronal elements filled with the final product of 
the histochemical reaction we used an optical filter conveying light with a wavelength of 430–
490 nm. To measure the optical density in the images, special attention was paid to adjusting 
the saturation of the light current such that the non-stained areas of all animals had the same 
level of intensity. The second step involved projecting the large image onto the computer 
screen where the NTS, DMV and AMB were manually outlined. Within the outlined areas, the 
NPFF, PrRP or CGRP immunoreactivity was analysed and quantified as integrated optical 
density by using the computer program Image-Pro Plus 6.3 (10). For NPFF and PrRP 
immunoreactivity, the analyses were performed throughout the NTS and DMV. For CGRP, the 
analyses were performed on all immunopositive AMB sections in the medulla oblongata. The 
bilateral results from all sections were combined for analysis. The data obtained from the 
control and hypertensive groups were analysed using one-way ANOVA. Simple regression 
analysis was performed to analyse the relationship between parameters obtained from 
neuropeptide, heart rate and blood pressure measurements. Significance was determined at 
p<0.05 for all tests performed. All data presented as mean ± S.E.M.

3. RESULTS

3.1. Brainstem changes - 2K1C-induced hypertensive vs. sham-operated rats and the effect 
of exercise

NPFF immunoreactivity in the NTS showed no significant difference when 2K1C animals were 
compared to sham-operated rats 2 weeks after 2K1C-induced hypertension (849.2 ± 103.1 vs. 
720.7 ± 65.44 (p=0.27)) or 5 weeks after 2K1C-induced hypertension plus exercise (1772 ± 
209.1 vs. 2034 ± 183.6 (p=0.35)) (Figure 1A, 1E and Figure 2).

The IOD of PrRP in the NTS of 2K1C animals, however, was significantly lower than that of 
sham-operated rats 2 weeks after 2K1C-induced hypertension (x55.71 ± 23.59 vs. 44.78 ±
15.29; p< 0.001), but in animals that had access to running wheels for 5 weeks, this significant
difference in PrRP IOD had disappeared (265.1 ± 12.89 vs. 227.9 ± 14.95; p=0.07) (Figure 1B, 1F and Figure 3).

2K1C animals also had a lower amount of CGRP in the nucleus ambiguus (AMB) compared to
sham-animals 2 weeks after induced hypertension (121.6 ± 14.14 vs 79.18 ± 7.38; p=0.004)
and similar to PrRP in the NTS, this difference for CGRP IOD in the AMB was not found after 5
weeks exercise (480.9 ± 62.46 vs 450.3 ± 45.47; p=0.68) (Figure 1C, 1G and Figure 4).

Apart from these, IOD analysis revealed a significant difference for NPFF IOD in the DMV
between 2K1C hypertensive and sham animals (813.1±72.15 vs. 630.4±48.72; p=0.034)
(Figure 1D).

### 3.2. The cardiovascular parameters and neuropeptide interactions

SBP, DBP and MAP were significantly higher in 2-weeks and 5-weeks 2K1C animals as
compared to their sham-operated controls (Table 1). In 2K1C rats, SBP (p=0.33), DBP (p=0.94),
MAP (p=0.69) and HR (p=0.16) values did not differ significantly 2 weeks and 5 weeks after
induction of hypertension. The same was true for the sham-operated control animals when
comparing 2 weeks and 5 weeks (SBP; p=0.32, DBP; p=0.30, MAP; p=0.34 , HR; p=1.00) (Table
1).

Secondly, although the correlation did not reach significance, there was a negative correlation
between NPFF levels in NTS and SBP levels (Pearson’s r=-0.65, P=0.06)(Figure 5A). On the
other hand, PrRP expression in the NTS was negatively correlated with the levels of SBP in 2-
weeks 2K1C hypertension and sham rats (r=-0.8, P=0.01)(Figure 5B). Furthermore, SBP levels
were negatively correlated with CGRP expression in the AMB in 2-weeks 2K1C hypertensive
and sham rats \( (r=-0.8, \ P=0.006) \) (Figure 5C) and PrRP levels in the NTS were positively correlated with levels of CGRP in the AMB in the same animals \( (r=0.8, \ P=0.008) \) (Figure 5D).

I guess somewhere you should mention why in Figure 1 the numbers of the Y-axis are so much higher for the animals with a running wheel.

3.3. CTB Tracing from the Left Kidney

All animals injected into the left kidney showed 2-3 labelled cells per section (at least 15 cells per animal) in the ipsilateral caudal DMV at the level of the area postrema (Figure 1H).

4. DISCUSSION

The present experiments indicate that neuropeptides expression in the brainstem (vagal) components of the NABR are involved in the development of renovascular hypertension as measured by quantitative immunohistochemistry. Sympathoexcitation is thought to be the major mechanism leading to hypertension since it was observed before the onset of renovascular hypertension (i.e., three weeks after renal artery clipping). However, a decreased gain of cardiac vagal baroreflex was observed even before the sympathoexcitation (i.e. first week after the clipping) (26). In our current study, NPFF immunoreactivity was decreased in the DMV of 2K1C hypertensive rats compared to sham animals. Also PrRP immunoreactivity in the NTS and CGRP levels in the AMB were decreased in 2K1C hypertensive rats relative to sham control animals. The reduced in PrRP levels in the NTS and CGRP levels in the AMB correlated significantly with the rise of SBP. Together, these observations indicate early changes in the vagal component of the NABR, during the initial phase of renovascular hypertension. In addition, the decreased neuropeptide expression in the vagal nuclei induced by 2K1C-hypertension seems to be modulated by exercise since we
found no such decrements in neuropeptide content when animals had access to a running wheel.

**4.2. Brainstem changes - 2K1C-induced hypertensive vs. sham-operated rats**

Previously three theoretical phases (initial, development and maintenance phases) and related mechanisms have been reported for 2K1C-induced renovascular hypertension (23, 26). Although an early increase of plasma renin activity and increased circulating angiotensin II concentrations seem to trigger the blood pressure changes in renovascular hypertension, still the neurogenic mechanisms are key for development of hypertensive pathology (1). Sympathetic overactivity was proposed as the major mechanism for the rise of blood pressure in the initial phase of 2K1C hypertension, however, a decreased cardiac vagal baroreflex sensitivity seems to precede the sympathetic overactivity (26). Accordingly, previously we showed decreased levels of NPFF and PrRP -both members of the RFamide peptide family with hemodynamic activity (19, 20, 32, 38)- in NABR structures during the development of spontaneous hypertension (39). In the current study, decreased PrRP levels in the NTS were also detected in 2K1C-induced hypertensive rats. Importantly, the reduced PrRP expression in the NTS was correlated with the increase in blood pressure, corroborating our previous findings (39).

The decreased NPFF levels in the DMV and CGRP levels in the AMB of 2K1C animals in the initial phase of hypertension suggest a reduced vagal motor output to circulatory organs. Remarkably, the decreased NPFF levels in the DMV of hypertensive rats, both in SHR rats (39) and now in 2K1C rats, resembles the decrease previously observed in human hypertensives (10). Notably, in this study, we showed the existence of vagal motor neurons in the DMV that directly innervate the rat kidney (figure 1H). Previously, cardiac vagal motor neurons in the
AMB were shown to express CGRP (36). Therefore, the reduced neuropeptide content in the DMV and AMB might explain the reduced parasympathetic tone suggested in the initial phase of 2K1C hypertension (26). The NTS is one of the major sites where the baroreceptor and renal afferent signals terminate (25). Activation of NTS neurons, which may contain NPFF or PrRP, might inhibit the GABAergic inputs converging on the vagal motor nuclei of DMV and AMB (34) and in turn, this may result in a diminished baroreflex function due to decreased vagal activity on target organs such as heart and kidney. Interestingly, we observed a lateralized reduction of NPFF content in the left NTS as early as 4 days after left renal artery clipping (Yilmaz et al unpublished observations). This reduced NPFF expression might underly the early decrease of the BRS, which later results in sympathetic overexcitation and renovascular hypertension (22).

The parallel changes of NPFF and PrRP levels in the NTS of SHR (39) and 2K1C animals (current study) indicate that with the development of hypertension, the changes in the NABR become more prominent because of peripheral feedback. This indicates that failure of the baroreceptor reflex is not only because of increased sympathetic activity, but could also be due to reduced vagal control (24, 27, 31, 33) of the heart and the kidney.

4.3. Brainstem changes after exercise in animals with 2K1C-induced hypertension

It is well known that the baroreflex sensitivity can be modulated by exercise (14), probably mediated via afferent input to barosensitive NTS neurons from skeletal muscle ergoreceptors (28, 29). Therefore, we investigated the impact of voluntary exercise on the neuropeptide content of NTS and AMB during 2K1C-induced hypertension. We hypothesized that in view of the improved baroreflex sensitivity by voluntary exercise (3) one or more of the changes
found in peptide content in NABR structures in 2K1C hypertensive rats would be reversed, as previously also observed in hypertensive rats (37, 42).

Indeed, in the current study, voluntary exercise restored NPFF and PrRP levels in the NTS and CGRP levels in the AMB. Thus, although voluntary exercise did not eliminate the established hypertension in 2K1C rats (possibly because of the mechanical obstruction to the left kidney), it did restore and improve PrRP levels in the NTS and CGRP levels in the AMB (Figure 1B and 1C vs 1F and 1G, respectively) and blood pressure levels did not increase further (table1).

5. CONCLUSION

Therefore, instead of only an activation of the sympathetic nervous system, also an initial failure of the parasympathetic nervous system may contribute to the onset and establishment of hypertension (26, 39). The changes observed in the NABR structures might lead to abnormal baroreflex control and development of hypertension both in animal and man (16). These make a role for the parasympathetic nervous system in blood pressure regulation and related pathologies become more obvious. In addition, this also means that modulating its activity (for instance by voluntary exercise) could be a possible target for new treatment strategies of hypertension.

ADDITIONAL INFORMATION

Author Contributions

Ajda Yilmaz: Conceptualization, Methodology, Investigation, Formal Analysis, Visualization, Writing – Original Draft, Review & Editing
Ramon A. Piñol: Investigation, Formal Analysis, Visualization, Writing – Review & Editing
Andries Kalsbeek: Resources, Investigation,
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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Figure 1. Quantitative integrated optical density (IOD) analysis of NPFF (A), PrRP (B) and CRGP (C) expressions in 2K1C-hypertensive (HYPT; 2 weeks or 5 weeks after the clipping) and 2K1C- normotensive (NRMT; 2 weeks or 5 weeks after sham surgery) rat brainstems. After 2 weeks of 2K1C induction of hypertension no difference for NPFF expression in the NTS (A), but significant reductions in PrRP content in the NTS (B), CGRP content in the AMB (C) and NPFF content in DMV (D) were detected in hypertensive rats compared to sham rats. Five weeks of voluntary use of running wheels after the 2K1C procedure, expression levels of NPFF, PrRP and CGRP had increased in the aforementioned structures and the differences indicated above had disappeared (E, F and G). Renal vagal motor neurons identified after the CTB injection into the left rat kidney (H). * indicates significant difference (p< 0.05, one-way ANOVA) between hypertensive and normotensive rats.

Figure 2. Coronal sections of the caudal brainstem in 2K1C-hypertensive (HYPT) and 2K1C-normotensive (NRMT) rats with or without a running wheel stained for NPFF. All sections show the mid-caudal level of NTS at the level of the area postrema (AP). The staining intensity for NPFF in the NTS was increased in both HYPT and NRMT rats after 5 weeks of voluntary running wheel use (B and D) as compared to HYPT and NRMT rats 2 weeks after the 2K1C procedure (A and C) in which the both HYPT and NRMT rats did not have access to running wheels.
Figure 3. Coronal sections of caudal brainstem in 2K1C-hypertensive (2K1C-HYPT) and 2K1C-normotensive (2K1C-NRMT) rats stained for PrRP. All sections show the caudal level of NTS just before the area postrema (AP). The difference in staining intensity for PrRP in the NTS between hypertensive and normotensive rats after 2 weeks of 2K1C-induced hypertension (A and C) had disappeared in rats that had had 5 weeks access to a running wheel, i.e. 5 weeks of voluntary exercise (B and D).

Figure 4. Coronal sections of brainstem in 2K1C-hypertensive (2K1C-HYPT) and 2K1C-normotensive (2K1C-NRMT) rats stained for CGRP. All sections showing the CGRP cell bodies located in the compact formation of the AMB (dashed outline). The difference in staining intensity for CGRP in the AMB between hypertensive and normotensive rats after 2 weeks of 2K1C-induced hypertension (A and C) had disappeared in rats which had had 5 weeks access to a running wheel, i.e. 5 weeks of voluntary exercise (B and D).

Figure 5. Interactions between cardiovascular parameters and neuropeptide expressions 2 weeks after left renal artery clipping. (A) Negative correlation (r=-0.8, P=0.03) between the amount of NPFF in the NTS and SBP in 2K1C animals. (B) Negative correlation (r=-0.8, P=0.03) between the amount of PrRP in the NTS and SBP in 2K1C animals. (C) Negative correlation (r=-0.8, P=0.02) between amount of CGRP in the AMB and SBP levels in 2K1C animals. (H) Positive correlation (r= 0.9, P=0.008) between the amount of CGRP in the AMB and amount of PrRP in the NTS in 2K1C animals. Dashed lines indicate the upper and lower confidence limit (95%).

HPT = 2K1C hypertensive animals; NRMT = 2K1C sham animals.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

A. NPFF in NTS vs SBP

- HYPT
- NRMT

B. PrRP in NTS vs SBP

C. CGRP in AMB vs SBP

D. PrRP in NTS vs CGRP in AMB

Statistical details:

- r = 0.65, p = 0.06
- r = -0.79, p = 0.01*
- r = 0.82, p = 0.007*
- r = 0.81, p = 0.009*