Neuropeptide Changes in the Suprachiasmatic Nucleus; cause or consequence of hypertension?

Ajda Yilmaz ¹, Andries Kalsbeek ^{1,2} and Ruud M Buijs ^{1,3*}

¹ Netherlands Institute for Neuroscience (NIN), an Institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam 1105 BA, The Netherlands

²Department of Endocrinology and Metabolism, Amsterdam UMC, University of Amsterdam, Amsterdam 1105 AZ, The Netherlands

³ Present address: Department of Cell Biology and Physiology, Institute for Biomedical Research, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico.

* For Correspondence: Department of Cell Biology and Physiology, Institute for Biomedical Research, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico.
e-mail: ruudbuijs@gmail.com

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ABSTRACT

Human postmortem studies as well as experimental animal studies indicate profound changes in the suprachiasmatic nucleus (SCN) in several pathological conditions. In addition, observations in experimental animals show that the SCN peptides, vasopressin (VP) and vasoactive intestinal peptide (VIP) are essential for adequate rhythmicity. These data prompted us to investigate whether changes in these neuronal populations are the cause or consequence of hypertension. Changes in blood pressure and levels of neuropeptide expression in the SCN were determined during the development of hypertension in spontaneous hypertensive rats (SHR), 2K1C reno-vascular induced hypertensive animals and their respective controls. During the pre-hypertensive stage (5 weeks of age), the VIP and AVP content was higher and the somatostatin (SOM) content was lower in the SHR SCN. At the onset of hypertension (12 weeks of age), when blood pressure levels had just reached ~140 mmHg, AVP and SOM content in the SCN was not different anymore in SHRs compared to control, but VIP was still higher. After 16 weeks, the AVP content was decreased, but SOM was increased and the overall level of VIP in the SCN was still higher in SHRs compared to controls. None of the aforementioned changes in the SCN was observed after induction of hypertension in the 2K1C model. However, while VIP was increased in the NTS projecting medial region of the SCN in SHR animals only after the establishment of hypertension, VIP was decreased in the same region in the 2K1C induced hypertensive rats. Consequently, the present findings confirm previous studies in human and rat indicating that changes in the SCN are strongly associated with the development of hypertension. In addition the changes in peptide content in the 2K1C animals indicate that the SCN is also able to respond to increases in blood pressure.

1. Introduction

The hypothalamic Suprachiasmatic Nucleus (SCN) seems the sole center for the control of bio-behavioral rhythms, including heart rate, blood pressure, sleep –wake, activity and hormone secretion (Janssen et al., 1994; Scheer et al., 2003; Silver and Schwartz, 2005). First, animals bearing bilateral lesions of the SCN become arrhythmic for many rhythms including blood pressure, heart rate, and activity as well as hormone secretion (Janssen et al., 1994; Scheer et al., 2001; Zucker et al., 1983). Secondly, SCN neurons show endogenous rhythmicity in electrical activity and metabolism, both in *in vivo* and *in vitro* conditions (Silver and Schwartz, 2005). Finally, transplantation of embryonic SCN tissue into the animals arrhythmic due to lesions of the SCN or to aging, restores their circadian rhythm of activity (LeSauter and Silver, 1999; Li and Satinoff, 1998; Ralph et al., 1993). Interestingly, in spite of what could be expected on the basis of the restoration of activity by transplantation, input of other areas of the brain to the SCN were shown to be important for the rhythms in temperature and corticosterone secretion (Buijs et al., 2017; Guzmán-Ruiz et al., 2015).

The SCN can be divided in a part that receives direct information from the retina (core) and a part that does not receive this direct input (shell). The retino-recipient part contains vasoactive intestinal peptide (VIP) and gastrin releasing peptide (GRP) as major neurotransmitters, whereas the shell area contains arginine vasopressin (AVP) and more medially somatostatin (SOM) (Romijn et al., 1996, 1997) as its main neurotransmitters. Interestingly also sensory information from other parts of the brain targets the retino-recipient part and therefore this area can be considered as the major entrainment region of the SCN. Intricate interactions between the core and shell region enables the SCN to synchronize its endogenous activity to environmental or bodily changes, such as the light-dark cycle or the daily rhythm in blood pressure, respectively (Brown and Piggins, 2009; Buijs et al., 2014). This is also illustrated by the observation that by disturbing VIP signaling within the SCN, the SCN and the animal loses its daily rhythmicity (Harmar, 2003). Therefore, the core region containing mainly VIP is essential not only for conveying the entrainment signals, but also essential for maintaining the endogenous network oscillatory activity. Developmentally the VIP containing core region can be distinguished as two separate sub-compartments (Ban et al., 1997), these sub-compartments still are detectable in adults functionally and neuro-anatomically (Kawamoto et al., 2003; Mazuski et al., 2018). The early developing medial-VIP region has endogenous oscillatory activity in embryonic and early postnatal life and it lacks light induced *per-1* and GRP expression in adults (Ban et al., 1997). The lateral-VIP region, on the other

hand, expresses light-induced *per-1* but lacks diurnal variation of VIP expression in constant conditions since peptide content of this region is dependent on the changes in environmental light (Kawamoto et al., 2003). This might indicate that especially VIP expression in the lateral-VIP region is most sensitive to sensory information.

A number of human post mortem studies have indicated the involvement of the SCN in pathophysiology and showed alterations of its subdivisions in different conditions. For instance, aging affects the rhythmicity and number of AVP neurons in the SCN and differentially affects the number of VIP cells in male and female subjects (Zhou et al., 1995). In addition, the AVP cell number is decreased in pathological conditions such as depression, Alzheimer disease (Swaab, 2003) and multiple system atrophy (MSA) (Benarroch et al., 2006). Finally, AVP and VIP immunoreactivity was diminished in the SCN of hypertensive patients as compared to normotensive controls (Valeri D. Goncharuk et al., 2001), implying the involvement of the SCN also in the etiology of this disease. In view of the clear evidence from studies in experimental animals that both AVP and VIP are essential for adequate SCN rhythmicity it becomes very attractive to hypothesize that the diminishment of these neuronal populations in these diseases is directly associated with the observed pathology.

Spontaneously hypertensive rats (SHR) are a widely accepted model of essential hypertension in humans. They develop hypertension endogenously with age, and blood pressure rises progressively with maturation (Yamori et al., 1974). They have increased blood pressure, increased heart weight and corticosterone levels which are also observed in human hypertensives (Dunn and Pfeffer, 1999; Eilam et al., 1991; Filipovský et al., 1996; Watlington et al., 1992). Besides, SHRs show neurotransmitter changes, especially in the hypothalamus, which could be the underlying mechanism for the development of hypertension. They have decreased levels of AVP and oxytocin (OXT) (Morris and Keller, 1982), as well as changed corticotrophin releasing hormone (CRH) levels in the hypothalamic paraventricular nucleus (PVN) (Hattori et al., 1986), an important relay center for the regulation of autonomic and endocrine output and target of SCN efferents. Interestingly, SHRs also exhibit abnormalities in their circadian physiology. They show increased phase advances and decreased phase delays in their activity rhythm as a response to nocturnal light as compared to normotensive WKYs (Peters et al., 1994). They have an inverted daily rhythm in blood pressure, but not heart rate after the establishment of hypertension (Minami et al., 1988a; Munakata et al., 1990). The amplitude of their daily locomotor activity as well as their blood pressure rhythm is increased when they get hypertensive (Canal-Corretger et al., 2001; Yilmaz et al., 2018). SHRs have more VIP in their SCN (Peters et al., 1994) and the number of AVP neurons was found

decreased in wild-type hosts 8 months after receiving an SCN graft of hypertensive animals (Eilam et al., 1994). Considering these findings, we aimed to investigate whether the observed neurochemical changes of the SCN are the cause or consequences of hypertension. Therefore, we conducted experiments with SHRs and their normotensive controls, using the SHRs as a model of central nervous system mediated, top down control of hypertension. In addition, we induced hypertension in normotensive animals by using the 2 Kidney 1 Clamp (2K1C) protocol, which is a model for peripherally mediated, bottom up control of hypertension (Navar et al., 1998; Zheng and Patel, 2017). Following this, we evaluated the AVP, SOM and VIP levels in the SCN with semi quantitative immunohistochemistry before, during and after the animals established hypertension by paying special attention to changes in the VIP containing medial and lateral subdivisions. Before sacrifice, intra-carotid blood pressure and heart rate recordings were performed to establish the level of hypertension.

The results indicate that AVP was decreasing and SOM was increasing with the establishment of hypertension, while VIP staining was high in SHRs compared to WKYs at all time points measured. Interestingly, CTB injections into the NTS revealed retrogradely labeled NTS-projecting cells in the SCN corresponding to the medial-VIP region, as well as NTS fibers targeting the lateral-VIP region of the SCN. By analyzing VIP levels in these sub-compartments of the SCN, we showed that VIP increased in this medial-VIP whereas it decreased in the lateral-VIP region in SHRs after the establishment of hypertension. On the contrary, VIP was decreased in the medial but increased in the lateral-VIP region in 2K1C-renovascular hypertensive rats after the development of hypertension.

2. Methods

Quantification of neuropeptide content in different sub-compartments of the SCN in the course of hypertension.

Animals

Experiments were conducted with a total of 36 male SHR and WKY rats (parents from Harlan, UK). Additionally, 8 Wistar Albino rats (10 weeks old, Harlan, Zeist, The Netherlands) were used for the 2K1C induction of hypertension experiments. All SHR and WKY rats used in this experiment were bred in our own animal facility in order to avoid possible interfering effects of stressors (i.e., transportation, changes in housing conditions, etc.) (Tucker and Johnson, 1981) on the development of hypertension in SHRs. The animals were housed as groups, under

a 12:12 h light:dark (LD) cycle (± 100 and 5 lux, respectively; lights on 07:00 h), in a temperature controlled environment ($21 \pm 2^\circ\text{C}$) with *ad libitum* access to tap water and food (Rat Chow).

Two sets of experiments were conducted to reveal the possible neurochemical changes of the SCN after development of hypertension. In the first set of experiments brains of male SHRs and WKYs were collected before, during and after the development of hypertension namely at 5, 12 and 16 weeks of age ($n=6$ per group), respectively. Blood pressure measurements were performed before sacrifice (see below). In the second set of experiments, 8 10-week old Wistar albino rats underwent surgery to induce hypertension ($n=5$) or for sham operations ($n=3$) (for surgery see below). Two weeks after the induction of hypertension or sham surgery, blood pressure measurements were done, animals were sacrificed and brains were removed and tissue processed for further immunohistochemical analyses. 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).

Surgery (2K1C hypertension)

Animals ($n=5$) were operated under Hypnorm (0.05 ml/100g BW, im, Janssen, High Wycombe, Buckinghamshire, UK) and Dormicum (0.04 ml/100g BW, sc, Roche, Almere, The Netherlands) anaesthesia in order to induce hypertension (Okamura et al., 1986). Laparotomy was performed to expose the abdominal aorta by a longitudinal incision on the abdominal midline and intestines in the midline were held aside. The aorta was lifted between the renal arteries to put a surgical thread (4-0 Perma-Hand Said Ethicon) underneath, after separating visible nerve fibres travelling with the aorta from the vessel. The aorta was tied together with a blunted injection needle (25 gauge, $1\frac{1}{4}$ inch needle) on top of it. After removing the needle the aorta was narrowed to the diameter of the needle. The abdominal wall muscles and the skin were sutured separately. Sham operated animals ($n=3$) underwent the same procedure without narrowing the aorta. Animals recovered overnight and were given Temgesic (Schering-Plough, Maarssen, The Netherlands, 0.01 ml / 100 g BW) to reduce post-operative pain.

Blood pressure measurements, tissue collection and data analyses

Blood pressure measurements were performed by the intra-carotid catheterization method, at least one week after the operation for 2K1C and sham-operated rats, in the first half of the animals' inactive period (ZT, *Zeitgeber time*, 4-6). For this procedure animals were gas

anesthetized with isoflurane (Isoflurane; Pharmachemie BV, Haarlem, The Netherlands, 3% in oxygen) followed by sodium pentobarbital (Nembutal, Ceva Sante Animal B.V, Maassluis, The Netherlands, 0.06 ml/100g BW) anaesthesia. Blood pressure and heart rate were measured and recorded for ten minutes via an intra-carotid catheter connected to a computer using HDAS software (Maastricht University). After receiving an overdose of Nembutal, 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.

Immunohistochemistry

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, SCN containing hypothalamic tissue was cut into 40 µm coronal cryostat sections at -20 °C and collected in 6 TBS containing vials. For immunohistochemical analyses six series of adjacent sections - the first for AVP, second for VIP and third for SOM, were taken at 240 µm intervals through the complete SCN. Sets of serial sections of hypertensive and control hypothalamus, containing 3 SCN sections per animal, were stained in parallel, under the same conditions and using the same solutions. For every time period hypertensive (SHR and IndH) and control hypothalami (WKY and sham operated controls) were stained at the same time in order to ensure that all conditions for staining, including solutions, were the same.

Free-floating sections were pretreated with absolute methanol and 3% H₂O₂ for 10 minutes. After thorough washing in TBS, all sections were incubated overnight at 4°C with either AVP (1:4000, Truus, rabbit polyclonal Ab, NIN), VIP (1:4000, Viper, rabbit polyclonal Ab, NIN) or SOM (1:4000, Somar, rabbit polyclonal Ab, NIN) antibodies (Romijn et al., 1997). Following the incubation with the primary antibody overnight, sections were incubated for 1h with a secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, California, USA; 1:400) and then incubated with ABC (Vector; 1:800) 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 gelatin-coated slides, air dried and cover slipped after dehydration with ethanol and xylene.

Quantitative study and data analyses

The quantitative estimation of the density of the AVP, SOM and VIP positive neuronal elements in the hypothalamic SCN sections was performed in two steps, as described

previously (Goncharuk et al., 2011). The bilateral results from all SCN sections (3 per animal) were combined for analysis. 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 the tiled images of the SCN in rat hypothalamus under a 40x NeoFluar objective (Zeiss), which were further combined into one large picture. In order to enhance the contrasts of neuronal elements filled with a final product of the histochemical reaction we used an optical filter conveying a 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 SCN was manually outlined. Within the outlined area, the AVP, SOM or VIP immunoreactivity was analyzed and quantified as integrated optical density with the program ImageJ (NIH; Bethesda, MA, USA) (Saderi et al., 2014). The data obtained from the control and hypertensive groups were analyzed using unpaired *t*-test with $p < 0.05$ considered significant.

Neuronal Tracing and Immunohistochemistry

Animals

Experiments were performed on male Wistar rats (250–300 g) housed individually on a 12:12-h light:dark (LD) cycle (ZT0 being the time of lights on). Rats were provided food and water ad libitum. All experiments were performed in accordance with the committee for ethical evaluation at the Institute for Biomedical Research, Universidad Nacional Autonoma de Mexico and international guidelines for animal handling. All animals undergoing surgery were anesthetized with ketamine (50 mg/kg) and xylazine (2 mg/kg) (Pisa-Agropecuaria S.A. de C.V.; Atitalaia, Mexico).

NTS CtB injections.

After anesthesia, rats were placed in the stereotact with the head fixed at 45°. Dura and arachnoid membranes were dissected exposing the dorsal surface of the medulla at the level of the area postrema. To target the NTS, the tip of the micropipette was aligned perpendicular to the medulla and placed 0.4mm rostral, 0.5mm lateral of the obex and 0.5mm below the surface of the brainstem. Injections were made with 100nl CtB using a glass micropipette as mentioned above (Fig. 1b). Rats were sacrificed after 14 days, allowing optimal transport of CtB.

Immunohistochemistry:

Under an overdose of sodium pentobarbital (Sedal-Vet 65 mg/mL) animals were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS; pH 7.5) (Buijs et al., 1993). Brains were removed, post fixed for 24 h, cryo-protected in 30% sucrose for 3–4 days, frozen and cut in sections of 35 μ m at -20 °C.

In order to visualize projections from the SCN after CtB injections in the NTS, hypothalamic sections were incubated with rabbit anti-CtB (Sigma–Aldrich Corp., MO, St. Louis, USA) at 4°C overnight. After rinsing, sections were incubated in biotinylated donkey-secondary antibody (Jackson ImmunoResearch, West Grove, PO, USA; 1:400) for 1.5 h and then in an avidin–biotin complex (Vector, Burlingame, CA, USA, 1:500) solution. The staining was performed with a solution of 0.025% diaminobenzidine (DAB), 10% NiNH₄SO₄ and 0.01% H₂O₂ (Sigma–Aldrich) in Tris- buffered saline (TBS, 0.01 M, pH 7.6), for 10 min. Sections were mounted on gelatinized slides, dried, dehydrated with graded solutions of ethanol, soaked in xylene, and finally coverslipped in Entellan embedding agent (Merck).

3. Results

At the age of 5 weeks, no significant differences in cardiovascular parameters were observed between SHRs and WKYs. However, at the age of 12 weeks and 16 weeks, all three parameters namely, systolic (12 weeks; $p=0.004$, 16 weeks; $p<0.001$), diastolic (12 weeks; $p=0.002$, 16 weeks; $p<0.001$) and mean arterial (12 weeks; $p=0.002$, 16 weeks; $p<0.001$) blood pressure levels were significantly higher in SHRs compared to WKYs. In a similar manner, systolic ($p=0.009$), diastolic ($p=0.022$) and mean arterial ($p=0.01$) blood pressure levels were significantly higher in the 2K1C hypertensive animals 2 weeks after surgery as compared to their sham operated controls. Table 1 indicates blood pressure and heart rate levels of the animals as measured at the end of the experiments.

In the same animals, AVP immunoreactivity followed an interesting pattern, with SHRs having a significantly higher amount of AVP in their SCN as compared to WKYs at the age of 5 weeks (43.47 ± 3.41 vs 23.56 ± 2.11 ; $p<0.001$). At the age of 12 weeks, however, this difference in integrated optical density (IOD) of AVP staining in the SCN had disappeared (33.76 ± 3.49 vs 41.11 ± 4.52 ; $p=0.204$), while at the age of 16 weeks, after the animals had fully developed hypertension, the IOD for AVP was even lower in SHRs' compared to WKYs' (21.5 ± 1.97 vs

38.02 $\pm$ 4.17; $p < 0.001$). No significant differences were detected between 2K1C hypertensive animals and their sham operated controls for IOD of AVP in the SCN (43.27 $\pm$ 7.7 vs 50.19 $\pm$ 8.64; $p = 0.557$) (Figs. 1A and 2).

A similar analysis was performed for SOM. The IOD of SOM in the SCN of 5 weeks old SHR was significantly lower than that of age matched WKYs (31.06 $\pm$ 3.98 vs 64.85 $\pm$ 6.581 $\times$ 9.08; $p < 0.001$). On the other hand, at the age of 12 weeks when animals started to become hypertensive the difference in SCN SOM between SHR and WKYs had disappeared (51.75 $\pm$ 5.52 vs 45.27 $\pm$ 5.08; $p = 0.394$). At the age of 16 weeks, on the other hand the IOD of SOM in the SHR's SCN was higher than in that of WKYs' (56.31 $\pm$ 6.24 vs 26.52 $\pm$ 2.79; $p < 0.001$). No significant differences were found between 2K1C hypertensive and sham animals for the SOM IOD in the SCN (68.04 $\pm$ 11.16 vs 70.66 $\pm$ 10.78; $p = 0.868$) (Figs. 1B and 3).

VIP immunoreactivity was first measured for the whole SCN. This IOD analysis revealed that SHR had a higher amount of VIP in their SCN compared to WKYs already at the age of 5 weeks, i.e., before they develop hypertension (309.7 $\pm$ 27.67 vs 237.6 $\pm$ 18.73; $p = 0.033$). At the age of 12 weeks hypertensive SHR still had a higher IOD of VIP in their SCN than WKYs' (230.1 $\pm$ 19.11 vs 151.1 $\pm$ 12.56; $p < 0.001$). Also at the age of 16 weeks a significantly higher IOD for VIP was measured in the SCN of SHR compared to WKYs (160.8 $\pm$ 16.06 vs 108 $\pm$ 14.77; $p = 0.02$). Again, similar as with AVP and SOM, no significant differences in total VIP IOD in the SCN were found between 2K1C hypertensive and sham animals (198.8 $\pm$ 25.38 vs 230.1 $\pm$ 23.41; $p = 0.392$) (Figs. 1C and 4).

Next to the total analysis, we also performed IOD analysis at the sub-compartmental level for two VIP subdivisions of the SCN (Abrahamson, 2001), in SHR and WKYs as well as 2K1C hypertensive rats and their controls. The two sub-divisions being the medial- and lateral VIP region of the SCN. The medial VIP region lacks the light-induced *per1* expression (Ban et al., 1997; Kawamoto et al., 2003) and covers the area where NTS projecting SCN neurons are located (Figure 6A and 6B). The lateral-VIP region overlaps with the SCN area where the fibers from the NTS terminate (Buijs et al., 2014). Analyses at the sub-compartmental level revealed interesting changes during the development of hypertension: VIP levels in the medial-SCN were not significantly different at the age of 5 (102.3 $\pm$ 6.61 vs 103.9 $\pm$ 10.77, $p = 0.899$) or 12 weeks (101.2 $\pm$ 19.11 vs 95.26 $\pm$ 12.56, $p = 0.564$), but were significantly higher (78.29 $\pm$ 7.93 vs 50.16 $\pm$ 4.6, $p = 0.003$) well after the establishment of hypertension. On the other hand, in the lateral-VIP region of the SCN the SHR compared to WKYs showed a significantly higher

VIP-IOD at the age of 5 weeks (96.47 ± 9.61 vs 68.10 ± 5.85 , $p=0.029$), while reversing that difference to lower levels in the course of the development of hypertension: 12 weeks (64.87 ± 6.15 vs 48.28 ± 5.92 , $p=0.06$) and 16 weeks (14.69 ± 2.97 vs 26.78 ± 4.08 , $p=0.021$) (Figure 5A and 5B, figures 6C and 6D). Interestingly, the IOD analysis for medial-VIP region in SCN in 2K1C and sham animals revealed significant differences between groups, but in a direction opposite to the results observed in 16 weeks SHR animals. Significantly lower VIP levels were detected in the medial-SCN (58.25 ± 5.474 vs 87.58 ± 14.89 , $p=0.04$), but significantly higher VIP detected in lateral-SCN (51.77 ± 6.74 vs 28.62 ± 7.41 , $p=0.043$) in induced-hypertensive rats compared to aged matched sham-operated controls (Figure 5A and 5B, figures 6E and 6F).

329

330 **4. Discussion**

331 **Discussion**

332 Using immunohistochemical analysis, the present study shows that with respect to its
333 neuropeptide expression, the SCN of spontaneously hypertensive rats (SHRs) is already
334 different from their WKY controls before the animals have established hypertension.
335 Furthermore, the present observations show that after the development of hypertension several
336 of these changes are reversed. These observations are in agreement with our previous
337 observations that the functionality of the SHR SCN had already changed before the animals
338 established hypertension (Yilmaz et al., 2018). Taken together these observations indicate that
339 early changes in the SCN may contribute to the development of hypertension. On the other
340 hand, the reversal of these changes after the development of hypertension indicates that the
341 SCN may not only control, but is also sensitive for the changes in blood pressure, an
342 observation that fits with earlier studies indicating the NTS as a possible source for this
343 feedback (Buijs et al., 2014). The presence of NTS-projecting neurons in the medial-VIP part
344 of the SCN and NTS fibers in the lateral-VIP part of the SCN drew our attention to VIP staining
345 differences in these two sub-divisions of the SCN. Interestingly, both in the SHR and 2K1C
346 animals these two sub compartments showed opposite changes during the development of
347 hypertension. In the 2K1C rats these opposite changes cancelled each other out when analyzing
348 the whole SCN. The presence of these opposite alterations in SCN areas associated with the
349 output to and input from the NTS suggests that these might be the result of the feedback from
350 the NTS and the following adjustment to the increase in blood pressure. In the SHR these two
351 subareas showed changes more in agreement with the development of hypertension, indicating
352 that such counteraction is not present in the SHR.

353 In the current study three SCN neurotransmitter systems, i.e. VIP, SOM and AVP, were studied
354 by means of semi-quantitative IOD analyses of identically and simultaneously stained bilateral
355 SCN sections. The observed changes are therefore not likely due to variations in fixation, age
356 or other factors since the direction of the changes observed were not parallel and differed for
357 each of the three neuropeptides investigated.

358 In agreement with earlier studies (Avidor et al., 1989; Eilam et al., 1991) we observed in the
359 present study that VIP immunoreactivity (VIP-IR) was increased in SHRs. However, our study

provides more elaborate information than these previous studies. Since we included animals at different stages during the development of hypertension, including stages before (5 weeks) and at the onset (12 weeks), as well as after the establishment of hypertension (16 weeks), hence we can conclude that VIP is already elevated before the onset of hypertension, indicating VIP as a possible important participant in the development of essential hypertension.

In addition to the VIP changes in the SCN of hypertensive rats, also changes in SOM and AVP expression were observed. The SCN projects to and receives input from a number of stress-related areas (Buijs et al., 1993). Importantly, the SOM and AVP containing dorsal region of SCN receives mainly stress-related inputs from limbic and other hypothalamic areas (Margaret M Moga and Moore, 1997). Although in normal conditions no differences in SOM and AVP hypothalamic content, including the SCN, were found, after stress exposure the SOM content failed to decrease in the SCN of SHR as compared to WKYs' (Negro-Vilar and Saavedra, 1980). In the current study, we observed that with the increase in blood pressure also the SOM content of the SCN increased. Hence, it is tempting to conclude that SOM in the SCN might be involved in stress-related hypertension in SHRs. Another or additional explanation for the SOM increase in SHRs with the development of hypertension would be that its staining intensity in the SCN increases with aging, since SOM-IR was found to be increased in aged rats (20-26 months) compared to young ones (6 months) (Biemans et al., 2002). However, in the current study the normotensive animals showed an age-related decrease of SOM and only the SHR animals showed an age-related increase. Therefore, in spite of the low levels of SOM-IR before the development of hypertension, the increased amount of SOM-IR, compared to WKY rats, once the animals had become hypertensive, might be an indication of an adaptation within the SCN due to the increased VIP levels.

In stark contrast to SOM-IR in SHRs, we found that with the establishment of hypertension the AVP content in the SCN of SHRs was drastically reduced compared to WKYs, despite the initial increased staining intensity before the development of hypertension. Previously, also AVP in the PVN has been found to be decreased when animals become hypertensive (Morris and Keller, 1982). Moreover, also a reduction of AVP in the SCN and PVN was observed 3 months after transplantation of a hypothalamic graft from SHRs to normotensive WKYs (Eilam et al., 1994). Therefore, the alterations that take place within the AVP (and SOM) cells of the SCN may be a consequence of the changes in VIP, rather than a consequence of the hypertension; otherwise the 2K1C induced hypertension would have induced similar modifications. Our group previously demonstrated in human postmortem tissue that the loss of

AVP content in the SCN correlated with a higher CRF mRNA content in the PVN in the same hypertensive patients (Goncharuk et al., 2002; V D Goncharuk et al., 2001). Besides, Kalsbeek et al. (Kalsbeek et al., 1992) demonstrated that AVP from the SCN provides a strong inhibitory input to the PVN for corticosterone release, which is also related with inhibition of the sympathetic tone to the adrenal (Buijs et al., 1999; Leon-Mercado et al., 2017). ICV injections of CRF or increased amounts of cortisol cause a prominent hypertension in animals and men, respectively (Morimoto et al., 1993; Knowlton et al., 1952). Hence, the decreased AVP content in the SCN of adult SHRs might exacerbate the hypertension in SHR by increasing corticosterone release.

In spite of the resonant aforementioned changes in the SCN of spontaneously hypertensive rats and humans, none of the neurotransmitter systems studied differed at the gross level between 2K1C reno-vascular hypertensive and sham operated rats (Eilam et al., 1994). However, when we started studies at the sub-compartmental level changes could be observed. The medial- and lateral-VIP regions of the SCN showed different changes in neuropeptide expression depending on the type of hypertension. Previous evidence shows that SCN's inputs and outputs are highly specialized (Kreier et al., 2006; Margaret M. Moga and Moore, 1997). As also illustrated here there is a direct input from the NTS to the SCN, which terminates in the lateral-VIP region and is related with receiving blood pressure information (Buijs et al., 2014). In the current study we also identified an adjacent region in the SCN containing SCN neurons projecting to the NTS. As this SCN area containing the NTS-projecting neurons overlaps with the medial-VIP region and is differentially affected by the centrally- or peripherally-mediated hypertension, i.e., SHR or 2K1C, it might also be related with blood pressure regulation. In SHRs, medial-VIP was increased but lateral-VIP was decreased after the establishment of hypertension (16 weeks of age). However, exactly the opposite pattern was observed after induction of hypertension in 2K1C reno-vascular hypertensive rats; the VIP content was decreased in medial- but was increased in lateral-VIP region compared to sham-operated controls (Fig.5 and fig.6). It is tempting to correlate these changes with the changes of running wheel activity, that are increased in SHR in constant dark conditions as compared to normotensive rats, while 2K1C rats' activity level for wheel running was decreased compared to sham-operated controls (Yilmaz et al., 2018). Taken together the results may imply that the VIP levels in the different VIP regions of the SCN are correlated with the level of activity in animals in different hypertension models.

In mammals, the SCN has at least two functionally distinct oscillators (Shinohara et al., 1995). These two different oscillators consist of two different neuronal populations. The core region contains VIP and the dorsal cap region contains mainly AVP (Reghunandanan and Reghunandanan, 2006; van den Pol, 1980). Interestingly, interactions between these two regions and coupling within the SCN is maintained mainly by VIP signaling. The VIP receptor VPAC2 has a major role in signaling between core and shell. VPAC2 knockout mice lose synchrony in dorsal cap region (Aton et al., 2005; Harmar, 2003) and VPAC2 overexpressing mice have shorter rhythms than wild type mice in constant darkness (Shen et al., 2000). Interestingly, VIP receptor knockout mice (*Viper*^{-/-}) also have abnormal blood pressure and heart rate rhythms and abnormal patterns of locomotor activity (Sheward et al., 2010). In addition, the VIP content of the SCN is closely related with the amplitude of activity rhythms both in hypertensive rats (Peters et al., 1994) and aged men (Wang et al., 2015). All these observations imply the importance of VIP signaling for activity-related cardiovascular regulation, besides maintaining the synchrony within the SCN. In accordance with the above literature, SHRs have abnormalities in blood pressure and heart rate rhythms (Minami et al., 1988b; Munakata et al., 1990) and they have shorter activity rhythms and an increased amplitude of their locomotor activity rhythm in constant darkness (Yilmaz et al., 2018). Moreover, by taking into account the neuro-anatomical changes described here it is highly tempting to speculate on a crucial role for the interplay between two different VIP sub-compartments and the AVP subdivision of the SCN for regulation of circadian rhythms in relation to blood pressure control (Figure 7).

Apart from the above described hypertension related changes, changes of the SCN have been reported in many other human pathologies, including aging, dementia, depression, Alzheimer's disease, Parkinson's and MSA (Buijs et al., 2016; Swaab, 2003). Interestingly, high blood pressure or hypertension itself is comorbid to all these pathologies (Fanciulli et al., 2016; Gabin et al., 2017; Nagai et al., 2010; Pinto, 2007; Scalco et al., 2005; Tsukamoto et al., 2013). As also shown here the SCN importantly influences the cardiovascular system (Buijs et al., 2014; Scheer et al., 2001). Interestingly, one of the common findings in most of the aforementioned diseases is a decrease in the number of AVP neurons in the SCN. Taken together the observed changes in the SCN open new possibilities to study the underlying mechanisms of important human pathologies where cardiovascular system changes are comorbid.

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REFERENCES

- Abrahamson EE, Moore RY (2001) Suprachiasmatic nucleus in the mouse : retinal innervation , intrinsic organization and efferent projections *Brain Res* 916:172–191.
- Aton SJ, Colwell CS, Harnmar AJ, Waschek J, Herzog ED (2005) Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nat Neurosci* 8:476–83.
- Avidor R, Eilam R, Malach R, Gozes I (1989) VIP-mRNA is increased in hypertensive rats. *Brain Res* 503:304–307.
- Ban Y, Shigeyoshi Y, Okamura H (1997) Development of vasoactive intestinal peptide mRNA rhythm in the rat suprachiasmatic nucleus. *J Neurosci* 17:3920–31.
- Benarroch EE, Schmeichel AM, Sandroni P, Low PA, Parisi JE (2006) Differential involvement of hypothalamic vasopressin neurons in multiple system atrophy. *Brain* 129:2688–2696.
- Biemans BAM, Gerkema MP, Van der Zee EA (2002) Increase in somatostatin immunoreactivity in the suprachiasmatic nucleus of aged Wistar rats. *Brain Res* 958:463–467.
- Brown TM, Piggins HD (2009) Spatiotemporal Heterogeneity in the Electrical Activity of Suprachiasmatic Nuclei Neurons and their Response to Photoperiod. *J Biol Rhythms* 24:44–54.
- Buijs FN, Cazarez F, Basualdo MC, Scheer FAJL, Perusquía M, Centurion D, Buijs RM (2014) The suprachiasmatic nucleus is part of a neural feedback circuit adapting blood pressure response. *Neuroscience* 266:197–207.
- Buijs FN, Guzmán-Ruiz M, León-Mercado L, Basualdo MC, Escobar C, Kalsbeek A, Buijs

485 RM (2017) Suprachiasmatic Nucleus Interaction with the Arcuate Nucleus; Essential for
486 Organizing Physiological Rhythms. *eneuro* 4:ENEURO.0028-17.2017.

487 Buijs FN, León-Mercado L, Guzmán-Ruiz M, Guerrero-Vargas NN, Romo-Nava F, Buijs
488 RM (2016) The Circadian System: A Regulatory Feedback Network of Periphery and
489 Brain. *Physiology (Bethesda)* 31:170–81.

490 Buijs RM, Markman M, Nunes-Cardoso B, Hou YX, Shinn S (1993) Projections of the
491 suprachiasmatic nucleus to stress-related areas in the rat hypothalamus: a light and
492 electron microscopic study. *J Comp Neurol* 335:42–54.

493 Canal-Corretger MM, Witte K, Lemmer B (2001) Circadian pacemaker function and
494 entrainment during maturation of transgenic hypertensive TGR(mREN2)27 and
495 Sprague-Dawley rats. *Chronobiol Int* 18:627–640.

496 Dunn FG, Pfeffer MA (1999) Left Ventricular Hypertrophy in Hypertension. *N Engl J Med*
497 340:1279–1280.

498 Eilam R, Malach R, Bergmann F, Segal M (1991) Hypertension induced by hypothalamic
499 transplantation from genetically hypertensive to normotensive rats. *J Neurosci* 11:401–
500 411.

501 Eilam R, Malach R, Segal M (1994) Selective elimination of hypothalamic neurons by
502 grafted hypertension-inducing neural tissue. *J Neurosci* 14:4891–4902.

503 Fanciulli A, Göbel G, Ndayisaba JP, Granata R, Duerr S, Strano S, Colosimo C, Poewe W,
504 Pontieri FE, Wenning GK (2016) Supine hypertension in Parkinson's disease and
505 multiple system atrophy. *Clin Auton Res* 26:97–105.

506 Filipovský J, Ducimetière P, Eschwège E, Richard JL, Rosselin G, Claude JR (1996) The
507 relationship of blood pressure with glucose, insulin, heart rate, free fatty acids and
508 plasma cortisol levels according to degree of obesity in middle-aged men. *J Hypertens*
509 14:229–35.

510 Gabin JM, Tambs K, Saltvedt I, Sund E, Holmen J (2017) Association between blood
511 pressure and Alzheimer disease measured up to 27 years prior to diagnosis: the HUNT
512 Study. *Alzheimers Res Ther* 9:37.

513 Goncharuk VD, Buijs RM, Jhamandas JH, Swaab DF (2011) Vasopressin (VP) and

514 neuropeptide FF (NPFF) systems in the normal and hypertensive human brainstem. J
515 Comp Neurol 519:93–124.

516 Goncharuk VD, van Heerikhuize J, Dai JP, Swaab DF, Buijs RM (2001) Neuropeptide
517 changes in the suprachiasmatic nucleus in primary hypertension indicate functional
518 impairment of the biological clock. J Comp Neurol 431:320–30.

519 Goncharuk VD, Van Heerikhuize J, Swaab DF, Buijs RM (2002) Paraventricular nucleus of
520 the human hypothalamus in primary hypertension: Activation of corticotropin-releasing
521 hormone neurons. J Comp Neurol 443:321–331.

522 Guzmán-Ruiz MA, Ramirez-Corona A, Guerrero-Vargas NN, Sabath E, Ramirez-Plascencia
523 OD, Fuentes-Romero R, León-Mercado LA, Basualdo Sigales M, Escobar C, Buijs RM
524 (2015) Role of the Suprachiasmatic and Arcuate Nuclei in Diurnal Temperature
525 Regulation in the Rat. J Neurosci 35:15419–29.

526 Harmar AJ (2003) An essential role for peptidergic signalling in the control of circadian
527 rhythms in the suprachiasmatic nuclei. J Neuroendocrinol 15:335–338.

528 Hattori T, Hashimoto K, Ota Z (1986) Brain corticotropin releasing factor in the
529 spontaneously hypertensive rat. Hypertension 8:1027–1031.

530 Janssen BJA, Tyssen CM, Duindam H, Rietveld WJ (1994) Suprachiasmatic lesions
531 eliminate 24-h blood pressure variability in rats. Physiol Behav 55:307–311.

532 Kalsbeek A, Buijs RM, van Heerikhuize JJ, Arts M, van der Woude TP (1992) Vasopressin-
533 containing neurons of the suprachiasmatic nuclei inhibit corticosterone release. Brain
534 Res 580:62–7.

535 Kawamoto K, Nagano M, Kanda F, Chihara K, Shigeyoshi Y, Okamura H (2003) Two Types
536 of VIP Neuronal Components in Rat Suprachiasmatic Nucleus. J Neurosci Res 74:852–
537 857.

538 Knowlton AI, Loeb EN, Stoerk HC, White JP, Heffernan JF (1952). Induction of arterial
539 hypertnesion in normal and adrenalectomized ratsgiven cortisone acetate. J. Exp. Med.
540 96(3), 187-205.

541 Kreier F, Kap YS, Mettenleiter TC, Van Heijningen C, Van Der Vliet J, Kalsbeek A,
542 Sauerwein HP, Fliers E, Romijn JA, Buijs RM (2006) Tracing from fat tissue, liver, and

543 pancreas: A neuroanatomical framework for the role of the brain in type 2 diabetes.
544 Endocrinology 147:1140–1147.

545 Leon-Mercado L, Herrera Moro Chao D, Basualdo M del C, Kawata M, Escobar C, Buijs
546 RM (2017) The Arcuate Nucleus: A Site of Fast Negative Feedback for Corticosterone
547 Secretion in Male Rats. *eneuro* 4:ENEURO.0350-16.2017.

548 LeSauter J, Silver R (1999) Localization of a suprachiasmatic nucleus subregion regulating
549 locomotor rhythmicity. *J Neurosci* 19:5574–85.

550 Li H, Satinoff E (1998) Fetal tissue containing the suprachiasmatic nucleus restores multiple
551 circadian rhythms in old rats. *Am J Physiol* 275:R1735-44.

552 Mazuski C, Abel JH, Chen SP, Hermansteyne TO, Jones JR, Simon T, Doyle FJ, Herzog ED
553 (2018) Entrainment of Circadian Rhythms Depends on Firing Rates and Neuropeptide
554 Release of VIP SCN Neurons. *Neuron* 99:555–563.e5.

555 Minami N, Imai Y, Munakata M, Sasaki S, Sekino H, Abe K, Yoshinaga K (1988) Reversed
556 circadian rhythm of blood pressure in adult spontaneously hypertensive rats. *J Hypertens*
557 Suppl 6:S70-3.

558 Moga MM, Moore RY (1997) Organization of neural inputs to the suprachiasmatic nucleus in
559 the rat. *J Comp Neurol* 389:508–534.

560 Morimoto A, Nakamori T, Morimoto K, Tan N, Murakami N (1993) The central role of
561 corticotrophin-releasing factor (CRF-41) in psychological stress in rats. *J Physiol*
562 460:221–229.

563 Morris M, Keller M (1982) A specific deficiency in paraventricular vasopressin and oxytocin
564 in the spontaneously hypertensive rat. *Brain Res* 249:173–176.

565 Munakata M, Imai Y, Minami N, Sasaki S, Ichijyo T, Yoshizawa M, Sekino H, Abe K,
566 Yoshinaga K (1990) Cosinor analysis of changes in circadian blood pressure rhythm
567 with aging in spontaneously hypertensive rats. *Tohoku J Exp Med* 161:55–64.

568 Nagai M, Hoshida S, Kario K (2010) Hypertension and Dementia. *Febr Am J Hypertens*
569 23:116–124.

570 Navar LG, Zou L, Von Thun A, Tarng Wang C, Imig JD, Mitchell KD (1998) Unraveling the

571 Mystery of Goldblatt Hypertension. *Union Physiol Sci Am Physiol Soc* 13:170–176.

572 Negro-Vilar A, Saavedra JM (1980) Changes in brain somatostatin and vasopressin levels
573 after stress in spontaneously hypertensive and Wistar-Kyoto rats. *Brain Res Bull* 5:353–
574 358.

575 Nijijima A, Nagai K, Nagai N, Nakagawa H (1992) Light enhances sympathetic and
576 suppresses vagal outflows and lesions including the suprachiasmatic nucleus eliminate
577 these changes in rats. *J Auton Nerv Syst* 40:155–60.

578 Okamura T, Miyazaki M, Inagami T, Toda N (1986) Vascular renin-angiotensin system in
579 two-kidney, one clip hypertensive rats. *Hypertension* 8:560–565.

580 Peters R V., Zoeller RT, Hennessey AC, Stopa EG, Anderson G, Albers HE (1994) The
581 control of circadian rhythms and the levels of vasoactive intestinal peptide mRNA in the
582 suprachiasmatic nucleus are altered in spontaneously hypertensive rats. *Brain Res*
583 639:217–227.

584 Pinto E (2007) Blood pressure and ageing. *Postgrad Med J* 83:109–114.

585 Ralph MR, Joyner AL, Lehman MN (1993) Culture and transplantation of the mammalian
586 circadian pacemaker. *J Biol Rhythms* 8 Suppl:S83-7.

587 Reghunandanan V, Reghunandanan R (2006) Neurotransmitters of the suprachiasmatic
588 nuclei. *J Circadian Rhythms* 4:2.

589 Romijn HJ, Sluiter AA, Pool CW, Wortel J, Buijs RM (1997) Evidence from confocal
590 fluorescence microscopy for a dense, reciprocal innervation between AVP-,
591 somatostatin-, VIP/PHI-, GRP- and VIP/PHI/GRP-immunoreactive neurons in the rat
592 suprachiasmatic nucleus. *Eur J Neurosci* 9:2613–2623.

593 Romijn HJ, Sluiter AA, Pool CW, Wortel J, Buijs RM (1996) Differences in colocalization
594 between Fos and PHI, GRP, VIP and VP in neurons of the rat suprachiasmatic nucleus
595 after a light stimulus during the phase delay versus the phase advance period of the
596 night. *J Comp Neurol* 372:1–8.

597 Saderi N, Buijs FN, Salgado-Delgado R, Merkenstein M, Basualdo MC, Ferri GL, Escobar C,
598 Buijs RM (2014) A role for VGF in the hypothalamic arcuate and paraventricular nuclei
599 in the control of energy homeostasis. *Neuroscience* 265:184–195.

600 Scalco AZ, Scalco MZ, Azul JBS, Lotufo Neto F (2005) Hypertension and depression.
601 Clinics (Sao Paulo) 60:241–250.

602 Scheer FA, Ter Horst GJ, van Der Vliet J, Buijs RM (2001) Physiological and anatomic
603 evidence for regulation of the heart by suprachiasmatic nucleus in rats. *Am J Physiol*
604 *Heart Circ Physiol* 280:H1391–H1399.

605 Scheer FAJL, Kalsbeek A, Buijs RM (2003) Cardiovascular control by the suprachiasmatic
606 nucleus: Neural and neuroendocrine mechanisms in human and rat. *Biol Chem* 384:697–
607 709.

608 Scheer FAJL, Pirovano C, Van Someren EJW, Buijs RM (2005) Environmental light and
609 suprachiasmatic nucleus interact in the regulation of body temperature. *Neuroscience*
610 132:465–477.

611 Shen S, Spratt C, Sheward WJ, Kallo I, West K, Morrison CF, Coen CW, Marston HM,
612 Harmar a J (2000) Overexpression of the human VPAC2 receptor in the
613 suprachiasmatic nucleus alters the circadian phenotype of mice. *Proc Natl Acad Sci U S*
614 *A* 97:11575–11580.

615 Sheward WJ, Naylor E, Knowles-Barley S, Armstrong JD, Brooker GA, Seckl JR, Turek
616 FW, Holmes MC, Zee PC, Harmar AJ (2010) Circadian control of mouse heart rate and
617 blood pressure by the suprachiasmatic nuclei: Behavioral effects are more significant
618 than direct outputs. *PLoS One* 5:1–9.

619 Shinohara K, Honma S, Katsuno Y, Abe H, Honma K (1995) Two distinct oscillators in the
620 rat suprachiasmatic nucleus in vitro. *Proc Natl Acad Sci U S A* 92:7396–400.

621 Silver R, Schwartz WJ (2005) The suprachiasmatic nucleus is a functionally heterogeneous
622 timekeeping organ. *Methods Enzymol* 393:451–465.

623 Swaab D (2003) Suprachiasmatic nucleus (SCN) and pineal gland. *Handb Clin Neurol Vol 79*
624 (3rd Ser Vol 1) Hum Hypothal basic clinical Asp Part 1 79:63–125.

625 Tsukamoto T, Kitano Y, Kuno S (2013) Blood pressure fluctuation and hypertension in
626 patients with Parkinson’s disease. *Brain Behav* 3:710–714.

627 Tucker DC, Johnson AK (1981) Behavioral correlates of spontaneous hypertension. *Neurosci*
628 *Biobehav Rev* 5:463–71.

629 van den Pol AN (1980) The hypothalamic suprachiasmatic nucleus of rat: Intrinsic anatomy.
 630 J Comp Neurol 191:661–702.

631 Wang JL, Lim AS, Chiang W, Lo M, Schneider JA, Buchman AS, Bennett DA (2015)
 632 Suprachiasmatic Neuron Numbers and Rest–Activity Circadian Rhythms in Older
 633 Humans. Ann Neurol 78(2): 317-22.

634 Watlington CO, Kramer LB, Schuetz EG, Zilai J, Grogan WM, Guzelian P, Gizek F,
 635 Schoolwerth AC (1992) Corticosterone 6 beta-hydroxylation correlates with blood
 636 pressure in spontaneously hypertensive rats. Am J Physiol 262:F927-31.

637 Yamori Y, Tomimoto K, Ooshima A, Hazama F, Okamoto K (1974) Proceedings:
 638 Developmental course of hypertension in the SHR-substrains susceptible to hypertensive
 639 cerebrovascular lesions. Jpn Heart J 15:209–10.

640 Yilmaz A, Kalsbeek A, Buijs RM (2018) Functional changes of the SCN in spontaneous
 641 hypertension but not after the induction of hypertension. Chronobiol Int May 22:1–15.

642 Zheng H, Patel KP (2017) Integration of renal sensory afferents at the level of the
 643 paraventricular nucleus dictating sympathetic outflow. Auton Neurosci 204:57-64

644 Zhou JN, Hofman MA, Swaab DF (1995) VIP neurons in the human SCN in relation to sex,
 645 age, and Alzheimers-disease. Neurobiol Aging 16:571–576.

646 Zucker I, Boshes M, Dark J (1983) Suprachiasmatic nuclei influence circannual and circadian
 647 rhythms of ground squirrels. Am J Physiol Integr Comp Physiol 244:R472–R480.

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FIGURE LEGENDS

Figure 1. Quantitative (integrated optical density- IOD) analysis of AVP, SOM and VIP protein immunoreactivity in the bilateral SCNs of hypertensive (HYPT) SHR and 2K1C-induced hypertensive and normotensive (NRMT) WKY and sham- operated rats (A, B, C). AVP immunoreactivity was increased at the age of 5 weeks (5wks- prehypertensive period) and then decreased at the age of 16 weeks (hypertensive period) in SHRs compared to WKYs (A). In contrast to AVP, SOM first was decreased (5wks) and then increased (16wks) in SHRs compared to WKY (B). VIP was significantly higher for all the time points evaluated (5wks, 12wks, 16wks, respectively) in SHRs than WKYs (C). No significant differences were detected between 2K1C- induced hypertensive and their sham-operated controls for any of the neuropeptides examined (A, B, C). * = ($p < 0.05$)

Figure 2. Illustrations of coronal sections of Suprachiasmatic Nucleus (SCN) in hypertensive (A-D) and control (E-H) rats stained for Vasopressin (AVP). All sections show the central part (mid-level) of the SCN containing the largest number of AVP immunostained neurons. It is clear by comparing A to B (5 weeks - 5wks), C to D (12 wks) and E to F (16 wks), respectively, that spontaneously hypertensive rats (SHRs; A, C, E) had first an increased and then a decreased amount of AVP staining intensity compared to normotensive Wistar Kyoto rats (WKYs; B, D, F). However, no significant difference was observed between 2 Kidney 1 Clamp- induced hypertensive rats (2K1C; G) and their sham-operated controls (H) for AVP immunostaining intensity. * Third ventricle.

Figure 3. Illustrations of coronal sections of Suprachiasmatic Nucleus (SCN) in hypertensive (A-D) and control (E-H) rats stained for Somatostatin (SOM). All sections show the central part (mid-level) of the SCN where the highest density of SOM immunostaining was observed. In contrast to AVP (Figure 2), it is clear by comparing A to B (5 weeks- 5wks), C to D (12 wks) and E to F (16 wks), respectively, that spontaneously hypertensive rats (SHRs; A, C, E) had first a decreased and then an increased amount of SOM staining intensity compared to normotensive Wistar Kyoto rats (WKYs; B, D, F). However, no significant difference was detected between 2 Kidney 1 Clamp- induced hypertensive rats (2K1C; G) and their sham-operated controls (H) for SOM immunostaining intensity.

Figure 4. Illustrations of Coronal sections of Suprachiasmatic Nucleus (SCN) in hypertensive (A-D) and control (E-H) rats stained for Vasoactive Intestinal Peptide (VIP). All sections show the central part (mid-level) of the SCN. It is clear by comparing A to B (5 weeks - 5wks), C to D (12 wks) and E to F (16 wks), respectively, that VIP expression was higher at all the time points evaluated in spontaneously hypertensive rats (SHRs; A, C, E) compared to normotensive Wistar Kyoto rats (WKYs; B, D, F). However, no significant difference was observed between 2 Kidney 1 Clamp-induced hypertensive rats (2K1C; G) and their sham-operated controls (H) for VIP immunostaining intensity.

Figure 5. Quantitative (integrated optical density- IOD) analysis of VIP protein expression in the bilateral medial and lateral-VIP regions of SCNs of hypertensive (HYPT; SHR and 2K1C- induced hypertensive) and normotensive (NRMT; WKY and sham- operated) rats. Protein expression of VIP was not significantly different at the age of 5 (5wks- prehypertensive period) and at the onset of hypertension at 12 weeks (12 wks) in SHRs compared to WKYs in the medial-VIP region . However, at the age of 16 weeks (16wks- hypertensive period) SHRs had significantly more VIP compared to WKY in medial-SCN. In contrast to this, 2K1C- induced hypertensive rats significantly lower VIP levels compared to sham- operated animals in medial- VIP region of the SCN (A). On the other hand, SHRs had more VIP in the lateral region of the SCN at the age of 5wks compared to WKYs. However no significant differences observed between SHRs and WKYs at the age of 12 wks. Interestingly, opposite to levels of 2K1C animals and the results in A, SHRs had less VIP expression in lateral VIP- region compared to WKYs' (B).

Figure 6. SCN- NTS interaction after CTB injection into the NTS and illustrations of coronal sections of Suprachiasmatic Nucleus (SCN) in hypertensive and control rats stained for Vasoactive Intestinal Peptide (VIP) indicating differences in staining intensity in medial- VIP region of the SCN. CtB (red) cell bodies are present in the medial- region of the SCN (thick white arrow), together with the fibers in the lateral- region of SCN (white arrows) (A). Site of the CTB injections in the NTS (B). The VIP expression was higher in the medial- but was lower in the lateral-VIP region of the SCN in spontaneously hypertensive rats (SHRs; C) compared to normotensive Wistar Kyoto rats (WKYs; D) at the age of 16 weeks. However, VIP expression was lower in medial- but higher in lateral- VIP region of the SCN in 2 Kidney 1 Clamp-induced hypertensive rats (2K1C; E) compared to their sham-operated controls (sham; F).

Figure 7. A schematic illustration of the proposed functional organization of the SCN and its sub-compartments in the case of blood pressure regulation and light responsiveness.

When the light input reaches the SCN, it effects the VIP neurons and this causes a decrease of the peptide levels in the lateral-VIP region; when peptide levels decrease in the lateral-VIP region this causes a dis-inhibition and further increase in the level or secretion of AVP in the dorsal cap region (Romijn et al., 1996), AVP then inhibits VIP expression in the medial-VIP region and this inhibition results in a dis-inhibition and thereby activation of NTS neurons. Activation of the NTS neurons causes a further activation of vagal efferents (Niiijima et al., 1992) and results in decreased heart rate and blood pressure during the light or animal's rest period (Scheer et al., 2005). However, SCN also regulates short-term blood pressure responses through the NTS (Buijs et al., 2014). When HR and BP reach higher levels, the NTS efferents excite the lateral-VIP neurons and these neurons in turn inhibit the inhibitory medial-VIP neurons. Hence, this mechanism including the activation of NTS neurons and vagal efferents, HR and BP decrease to normal levels. On the other hand, in the case of hypertension the inhibitory signal onto the medial-VIP neurons is lost, either through AVP or NTS afferents to this region. Consequently, the medial-VIP neurons become over-activated (as observed with the IOD results in SHRs in the current study). Ultimately, over-activation of the medial-VIP neurons causes a long-term inhibition of the vagal system and therefore results in increased HR and BP, which characterizes the essential hypertension.

Table 1. Blood pressures and heart rate variables of SHR, WKY, 2K1C and Sham animals during different stages of the experimental protocol. * presents significant difference ($p < .05$, t- test) between comparable groups.

	5 weeks		12 weeks		16 weeks		2K1C (12 weeks)	
	SHR	WKY	SHR	WKY	SHR	WKY	2K1C	Sham
SBP* (mmHg)	101.3 ± 7.03	103.0±5.35	177.8±10.65*	119.7±10.23	185.3±8.65*	107.8±7.29	164.4±8.10*	107.5±4.5
DBP [¶] (mmHg)	74.33±7.95	78.00±4.38	132.4±6.76*	88.17±7.20	145.5±4.37*	84.8±6.28	121.8±7.61*	79.5±3.5
MAP [‡] (mmHg)	81.67±6.65	86.33±4.34	148.6±8.75*	98.5±7.82	158±5.61*	92.4±5.64	137.8± 7.27*	88.5±3.5
HR [§] (bpm)	328.0±15.86	294.3±10.37	318.8±14.19	298±20.31	316.3±11.54	312.6±12.86	333.6±16.4	330±3.0

• SBP; Systolic Blood Pressure, [¶] DBP; Diastolic Blood Pressure, [‡] MAP; Mean Arterial Pressure, [§] HR; Heart Rate