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Buijs, F.N.

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CHAPTER 2

The Suprachiasmatic nucleus is part of a neural feedback circuit adapting blood pressure response.

*Buijs FN
Cazarez F
Basualdo MC
Scheer FA
Perusquía M
Centurion D
Buijs RM.*

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Abstract

The suprachiasmatic nucleus (SCN) is typically considered our autonomous clock synchronizing behavior with physiological parameters such as blood pressure, just transmitting time independent of physiology. Yet several studies show that the SCN is involved in the etiology of hypertension. Here, we demonstrate that the SCN is incorporated in a neuronal feedback circuit arising from the nucleus tractus solitarius (NTS), modulating cardiovascular reactivity. Tracer injections into the SCN of male Wistar rats revealed retrogradely filled neurons in the caudal NTS, where blood pressure (BP) information is integrated. These NTS projections to the SCN were shown to be glutamatergic and to terminate in the ventrolateral part of the SCN where also light information enters. BP elevations not only induced increased neuronal activity as measured by *c-Fos* in the NTS but also in the SCN. Lesioning the caudal NTS prevented this activation. The increase of SCN neuronal activity by hypertensive stimuli suggested involvement of the SCN in counteracting BP elevations. Examining this possibility we observed that elevation of BP, induced by $\alpha 1$ -agonist infusion, was more than twice the magnitude in SCN-lesioned animals as compared to in controls, indicating indeed an active involvement of the SCN in short-term BP regulation. We propose that the SCN receives BP information directly from the NTS enabling it to react to hemodynamic perturbations, suggesting the SCN to be part of a homeostatic circuit adapting BP response. We discuss how these findings could explain why lifestyle conditions violating signals of the biological clock may, in the long-term, result in cardiovascular disease.

Introduction

Numerous aspects of physiological functions such as temperature regulation, hormonal control, autonomic tone, and metabolism show circadian rhythms (Hastings et al., 2003c). These processes are regulated by the suprachiasmatic nucleus (SCN), in interaction with peripheral oscillators (Buijs and Kalsbeek, 2001). Imbalance in the circadian system is thought to contribute to development of disease, *i.e.*, obesity, metabolic syndrome, diabetes and cardiovascular disease (Gangwisch et al., 2006). The latter is a leading cause of death in the world today, though still many questions remain over what pathology gives rise to its development. Recent human studies show that BP is influenced by the circadian system, independent of sleep/wake or fasting/feeding (Scheer et al., 2010c; Shea et al., 2011c). Evidence ranging from animal experimental and clinical data, to post mortem studies, suggests involvement of the SCN in hypertension (Brugger et al., 1995; Goncharuk et al., 2001; Shaw et al., 2001).

Recent studies emphasized a role for peripheral clock genes in cellular processes associated with BP control. Within the adrenal, absence of CRY clock genes has been associated with hyperaldosteronism (Doi et al., 2010b; Okamura et al., 2011). In the kidney, PER1 has been associated with regulation of renal epithelial sodium channels (Gumz et al., 2009), while clock genes have also been implicated in vascular endothelial function (Viswambharan et al., 2007; Anea et al., 2009; Cheng et al., 2011a) and thrombogenesis (Westgate et al., 2008) with potential relevance also in humans (Scheer et al., 2011). Hereby, it is important to note that the SCN synchronizes rhythms of clock gene expression in all organs (Buijs and Kalsbeek, 2001). Disturbance in this function of the SCN could, in time, result in the development of hypertension.

Consequently, a picture has emerged of the circadian system as a regulating entity for body homeostasis, including BP control. In addition to timing rhythmicity of peripheral clock genes, the SCN influences several organs that may affect BP; either by timing autonomic output to, *e.g.*, blood vessels, heart and kidney; or by timing hormonal secretion *e.g.* (nor)adrenalin, cortisol and aldosterone (Buijs and Kalsbeek, 2001; Hastings et al., 2003b). Furthermore, postmortem analysis of hypertensive humans and animals exhibited changes in the SCN and hypothalamus (Peters et al., 1994; Goncharuk et al., 2001; Goncharuk et al., 2002), but have not provided evidence whether such changes are cause or consequence of hypertension. Based on knowledge that the SCN is important for timing cardiovascular control we hypothesized that the SCN, like virtually any structure in the brain, would need feedback to execute its function properly. Therefore, we investigated whether the SCN is incorporated in a brain circuit controlling blood pressure.

Hereto neuronal tracers were injected into the SCN demonstrating retrogradely labeled neurons in the the nucleus tractus solitarius (NTS) suggesting the presence of NTS projections to the SCN. These projections were confirmed by neuronal tracer injection into the NTS revealing anterogradely labeled fibers in the SCN. These fibers were shown

to be glutamatergic and activated the SCN after blood pressure increase. The present data show further that the SCN may use this homeostatic feedback directly from the NTS, to adjust its output resulting in an attenuation of blood pressure increase since lesioning the SCN resulted in a marked increase in blood pressure response.

Materials and Methods

Animals. Experiments were performed on male Wistar rats (250-300g) housed individually on a 12:12h LD cycle (lights on 0700 h). 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.; Atitalaqla, Mexico).

Tracer injections. *SCN CtB injections.* To detect projections to the SCN, Cholera toxin B (CtB) injections labeled with Alexa Fluor 555 fluorescent (Molecular Probes, Eugene, OR, USA) were made unilaterally into the SCN (n=9). After anesthesia, rats were mounted in a stereotact (David Kopf Instruments; Tujunga, USA) using coordinates for SCN injections (Buijs et al., 1993b). The glass micropipette (20-40 μm tip) was aimed at the SCN, with the animal in the stereotactic (toothbar at +5mm) and from bregma, 0.16 anterior; 0.05 lateral and 0.86 ventral from the dura, under an angle of 2°. With the glass micropipette, 0.05 μl , 1% CtB was pressure injected (10 mbar, 5 sec) with the pipette left in place for 5 minutes in order to minimize tracer leakage. Injections were only accepted showing minimal leakage along the injection tract and positioned completely inside the SCN (Fig. 1a).

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 0.4 mm rostral, 0.5 mm lateral of the obex and 0.5 mm below the surface of the brainstem. Injections were made with CtB using a glass micropipette as mentioned above (Fig. 1b).

Ocular CtB injections. Immediately following the injection of CtB in the NTS, animals received an injection of 0.5 μl Alexa Fluor 488 labeled CtB (Molecular Probes) in the vitreous of the eye. Rats were sacrificed after 14 days, allowing optimal transport of CtB.

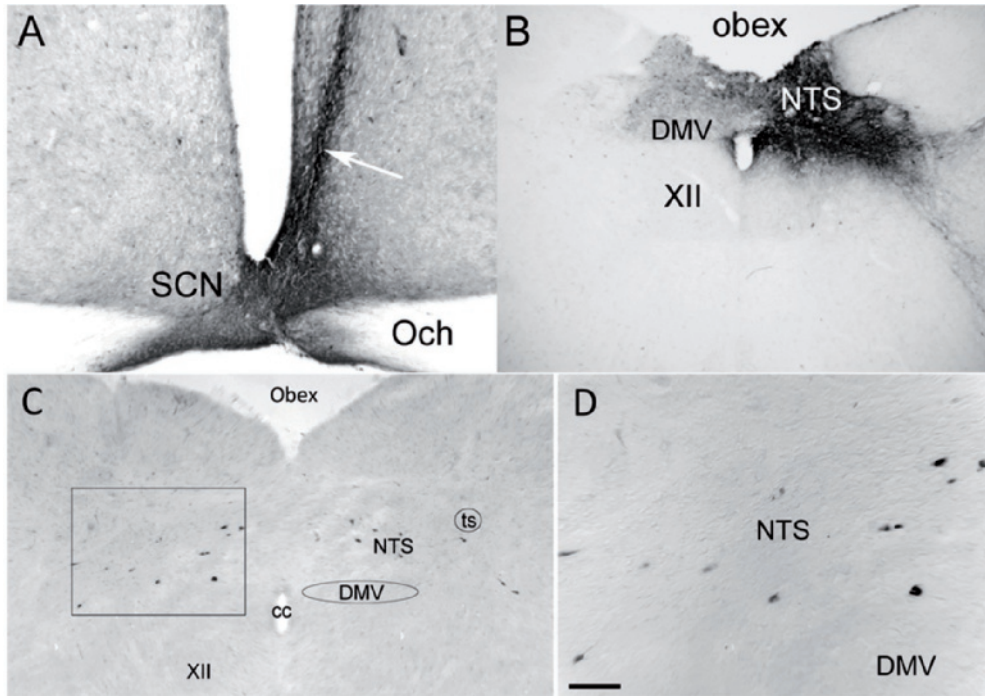


Figure 1. Sites of CtB injections and NTS neurons projecting to the SCN.

A) Representative CtB injection site in the SCN (White arrow showing injection tract). B) Representative picture at the level of the obex demonstrating CtB injection in the NTS. C) Photomicrograph of the caudal NTS after CtB injection into the SCN demonstrating the presence of retrogradely labeled neurons bilaterally at the level of the obex. D) Represents the higher magnification of the square in A which was ipsilateral to the injection site. The black bar represents 140µm in A and 50µm in B. Bregma – 14.35. SCN: suprachiasmatic nucleus, Och: optic chiasm, DMV: dorsal motor nucleus of the vagus, NTS: nucleus tractus solitarius, ts: tractus solitarius, XII: hypoglossal nucleus, cc: central canal.

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., 1993b). Brains were removed, post fixed for 24 h, cryo protected in 30% sucrose for 3 to 4 days, frozen and cut in sections of 35 µm at –20 °C.

Analysis NTS CtB injections. Co-localization of CtB labeled terminals with antibodies to the glutamate transporter VGlut 2 or Glutamate decarboxylase (GAD) (Chemicon (Millipore) Billerica, MA, USA) were examined by double-labeled immuno fluorescence(Acosta-Galvan et al., 2011c). SCN sections were incubated with rabbit VGlut2 or goat GAD-67 (Chemicon) antibody followed by a secondary fluorescent antibody conjugated with Cy-2 or Cy-5 (Jackson Immunoresearch, West Grove, PO, USA). Finally, sections were mounted on gelatinized slides, air-dried and coverslipped with 30% glycerol in PBS.

Analysis Ocular CtB injection. Co-localization of Alexa Fluor 555 labeled CtB terminals originating from the NTS and Alexa Fluor 488 labeled CtB terminals originating from the eye were examined in the SCN. Sections stained with fluorescent dyes were analyzed with the LSM 5 Pascal confocal microscope and the LSM software (Zeiss, Jena, Germany).

Analysis SCN CtB injections. In order to visualize projections from the SCN, NTS sections from these brains were incubated with rabbit anti-CtB (Sigma–Aldrich Corp) at 4°C overnight. After rinsing, sections were incubated in biotinylated donkey-secondary antibody (Jackson Immunoresearch, West Grove, PO, USA; 1:400) for 1.5h 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, 0.01M, pH7.6), for 10 minutes. Sections were mounted on gelatinized slides, dried, dehydrated with graded solutions of ethanol, soaked in xylene, and finally coverslipped in Entellan embedding agent (Merck).

Fos immunoreactivity after increases in BP. We examined the influence of an increase in BP on the neuronal activity in the SCN using c-Fos as activity marker in 4 groups, each containing 7-8 rats. BP was increased by a peripheral vasoconstrictor (Metaraminol, Sigma-Aldrich; St. Louis, USA), acting as a selective agonist of alpha1-adrenoceptors located in arterioles, hence raising BP. Metaraminol was chosen as it does not pass the blood brain barrier and thus cannot directly affect neuronal activity but only indirectly via its action on the periphery (Cunningham et al., 1994b). Groups consisted of non-treated control animals, saline injected intact animals, metaraminol injected SHAM operated animals and metaraminol injected NTS lesioned animals. Subcutaneous 0.2ml metaraminol injections (100µg/kg) were performed twice, 10 min before and at zeitgeber time (ZT)12, to attain a 20-minute increase in BP. Two hours after the injections, animals were anesthetized and sacrificed by 4% paraformaldehyde perfusion at ZT14. Care was taken to keep the animals in complete darkness until sacrifice in order to avoid light disturbing the activity of SCN neurons. In separate experiments a dose-response curve had been determined and the used dose (0.2ml 100µg/kg metaraminol) induced a BP increase of 15-20 mmHg for 20 minutes.

Fos immunohistochemistry. Serial sections were processed for Fos immunohistochemistry with rabbit anti c-Fos antibody (1:4.000, Calbiochem) using the avidin–biotin–peroxidase procedure (Acosta-Galvan et al., 2011b) as mentioned in detail above. Tissues were processed in multiple runs whereby control and experimental tissues were always processed together to avoid the influence of slight differences in processing.

Quantification and statistical analysis. Pictures were taken by using an Axioplan microscope (Zeiss, Jena, Germany) equipped with a digital color camera (Olympus DP25, Olympus, Japan). The SCN was manually outlined, the Fos-positive nuclear profiles were automatically detected by means of size and staining threshold detection using the same parameters for all experimental groups using Image J (NIH; Bethesda, USA). For each rat,

three sections were measured 90 μm apart (between bregma -0.90 to -1.20); the mean number of c-Fos positive nuclear profiles from these sections was calculated. All values are expressed as the mean \pm SEM, and data were analyzed using analysis of variance (ANOVA). When significance was reached for the one-way ANOVA, Tukey multiple comparison *post hoc* test was used with statistical significance set at $P < 0.05$.

Surgery. NTS lesions. The surgical procedure for unilateral NTS lesions was as described for CtB injections. Unilateral lesions were made because in pilot studies they proved to decisively interrupt signal transduction to the SCN while bilateral NTS lesions have been shown to induce fulminant hypertension and limited survivability (Doba and Reis, 1973; Sved, 1986). Unilateral electrolytic lesions were made using a 27 gauge, Teflon coated needle with excoriated tip (0.25 mm). An electrical current of 0.3 mA was passed for 40sec to ensure lesioning of only a small part of the NTS; for sham operations no current was passed. Only small unilateral caudal NTS lesions were accepted.

SCN lesions. A bilateral lesion of the SCN was carried out using above described procedure and coordinates but using electrodes 0.2mm in diameter, using an electrical current of 0.3mA for 40 seconds sufficient to eliminate the SCN bilaterally, but small enough to leave surrounding tissue intact. The following 3 weeks, locomotor activity was recorded to assess the effectiveness of the SCN lesion. Rats without LD rhythm (40 to 60% of their activity during the light period) were considered as SCN-lesioned animals (SCNX) and used for further study. After sacrifice, histological analysis was performed using staining for vasoactive intestinal peptide (Buijs et al., 1993a) to verify the lesion as complete and limited to the SCN. Only animals complying with mentioned criteria were included for analysis.

Blood pressure recordings. Canulation. After obtaining a weight of 300g, animals were anesthetized and implanted with a 100mm silicone catheter (ID 0.020 inch; OD 0.037 inch; Dow Corning; Midland, USA) in the right jugular vein and in the left carotid artery as described earlier (Steffens, 1969).

Arterial pressure recordings. Directly following canulation, BP recordings started and animals were kept under anesthesia. The left carotid artery cannula was connected to a pressure transducer (P23 XL; Grass Instruments, USA), coupled to a computerized data acquisition and analysis system (MP150, BIOPAC Systems, Inc.; Goleta, USA) recording the arterial pressure at 500Hz. Baseline was determined by observing hemodynamic stability for at least 30 min before measurements were started. Mean arterial blood pressure (MAP) was calculated using: $\text{MAP} = [(2 \times \text{diastolic}) + \text{systolic}] / 3$. Xylazine/ketamine anesthesia can have minor influence on BP response, however anesthesia was preferred over freely moving animals since pilot studies demonstrated great variation of BP responses following metaraminol, making analysis arduous. Metaraminol or saline was administered via the jugular vein catheter with consecutive injections given 5-10 min after MAP

had returned to baseline values. Saline was used as a control injection to monitor MAP shifts due to volume change. Increasing doses of metaraminol determined the dose-response in a within-subject design. Injection volumes used for all solutions were 800µl/kg with concentrations metaraminol of 50, 100, 200 and 400mg/kg. For controls, MAP measurements started at ZT 2 (sleep phase) and as experimental group we used animals at ZT 14 (active phase), to assess possible differences in MAP response at different circadian time points. SCN lesioned animals were used to evaluate control of the SCN over MAP. Animals used at ZT14 had their eyes covered in order to prevent activation of the SCN by light. Each group consisted of 7 animals.

Statistical analysis. All MAP data were analyzed using ACQ knowledge software (BIOPAC Systems, Inc.; Goleta, USA). Variables assessed were 1) area under the curve (AUC) of MAP increase, taken from time of injection until return to baseline values, 2) change in MAP and 3) time taken for MAP to return to baseline values after injections. Error bars show the calculated SEM. We used one-way ANOVA comparing control saline injections between groups. For consecutive metaraminol injections a two-way ANOVA for repeated measurements was used analyzing the different BP data sets (Table 1). When significance was reached for ANOVA the *post hoc* Bonferroni test was used for pairwise multiple comparisons and statistical significance was set at *P < 0.05. (** P < 0.01, ***P < 0.001).

Table 1. Blood pressure analysis of outcome measures between experimental groups and interventions.

Injection	Dose	Baseline MAP	Δ MAP	TtB	AUC
ZT 2		88.5 ±4.4			
Saline inj			1.4±0.4	25±5	-65±36
Metaraminol inj	50µg		20.0±3.3	156±20	1455±358
	100µg		27.0±2.3	192±28	2215±417
	200µg		41.8±2.6	288±32	5513±693
	400µg		60.0±2.7	386±19	9759±755
ZT 14		80.2±3.2			
Saline inj			1.7±0.4	44±15	15±38
Metaraminol inj	50µg		14.3±1.9	126±9	722±156
	100µg		19.5±2.1	147±13	1102±214
	200µg		35.2±2.6	215±13	2900±261
	400µg		53.8±4.0	293±18*	6644±917
SCNX		74.8±5.8			
Saline inj			8.8±1.2***	214±29***	1215±364***
Metaraminol inj	50µg		19.3±1.2	355±32***	3119±754
	100µg		26.8±2.3	417±18***	6147±826*
	200µg		37.8±2.6	499±27***	9485±1264*
	400µg		54.0±4.5	600±33***	15254±2658**

MAP: mean arterial pressure (mmHg), TtB: time to baseline (s), AUC: area under the curve (mmHg x s). Analysis: a one-way ANOVA was used comparing baseline MAP and saline injections between groups followed by the Tukey's test if significant. For consecutive metaraminol injections analysis consisted of a two-way repeated measures ANOVA followed by the Bonferroni test. Values are expressed as ±SEM (n = 7 for all groups). * P < 0.05 compared to ZT2. ** P < 0.01 compared to ZT2. *** P < 0.001 compared to ZT2

Results

A reciprocal NTS-SCN connection. To investigate from which potential areas involved in BP control the SCN might receive feedback, retrograde tracer injections of Cholera toxin B (CtB) were placed into the SCN. Unilateral injections resulted in a bilateral distribution of neurons in the NTS with an ipsilateral dominance. Especially injections in the ventral SCN resulted in small but reproducible numbers of retrogradely labeled neurons in the caudal NTS at the level of the obex, continuing up to the medial NTS at the level of the area postrema. The number of labeled neurons ranged from 3-11 per section (totaling 68-86 neurons throughout the NTS, n=3) depending on the level in the NTS and site of injection (Fig. 1c,d). To confirm that the retrogradely labeled neurons were indeed projecting to the SCN and not only to areas surrounding the SCN, injections were placed caudal in the NTS to visualize its input to the SCN area. CtB injections — being both a retrograde and anterograde tracer — in the caudal NTS resulted in a high density of fibers in several areas of the hypothalamus as previously reported (Ter Horst et al., 1989). In addition, we observed the presence of cell bodies dorsomedial in the SCN, implying that the SCN may also be able to project to the NTS area (Fig. 2a,c). The majority of the fibers were found ipsilateral to the injection site, although also contralateral projections were found in concordance with earlier reports showing crossover in NTS efferents (Ter Horst et al., 1989). In the hypothalamus, small and large diameter CtB labeled fibers arising from the NTS could be distinguished, while in the SCN only small diameter fibers were observed (Fig. 2a). The distribution of NTS derived fibers in the SCN was restricted to the ventral and lateral areas of the SCN. This is the same area where light input (Johnson et al., 1988) and input from the dorsomedial nucleus of the hypothalamus (Acosta-Galvan et al., 2011a) also enter the SCN. Fibers originating from the NTS were visible from the most rostral part of the SCN down to the caudal part where the density of NTS fibers was lowest. The combination of retinal tracing with tracing from the NTS revealed that NTS projections completely coincided with the input from the retina suggesting similar target neurons (Fig. 2b).

Identification of the NTS signal. Because numerous NTS efferent neurons involved in cardiovascular function are glutamatergic (Llewellyn-Smith et al., 2007) we stained CtB labeled terminals in the SCN for the glutamate transporter vGlut2, as marker of the neurotransmitter glutamate and for glutamate decarboxylase, as marker of the inhibitory neurotransmitter GABA. These analyses demonstrated that the majority of NTS terminals in the ventrolateral part of the SCN were glutamatergic and not GABA-ergic (Fig. 2c,d), showing the excitatory nature of this connection.

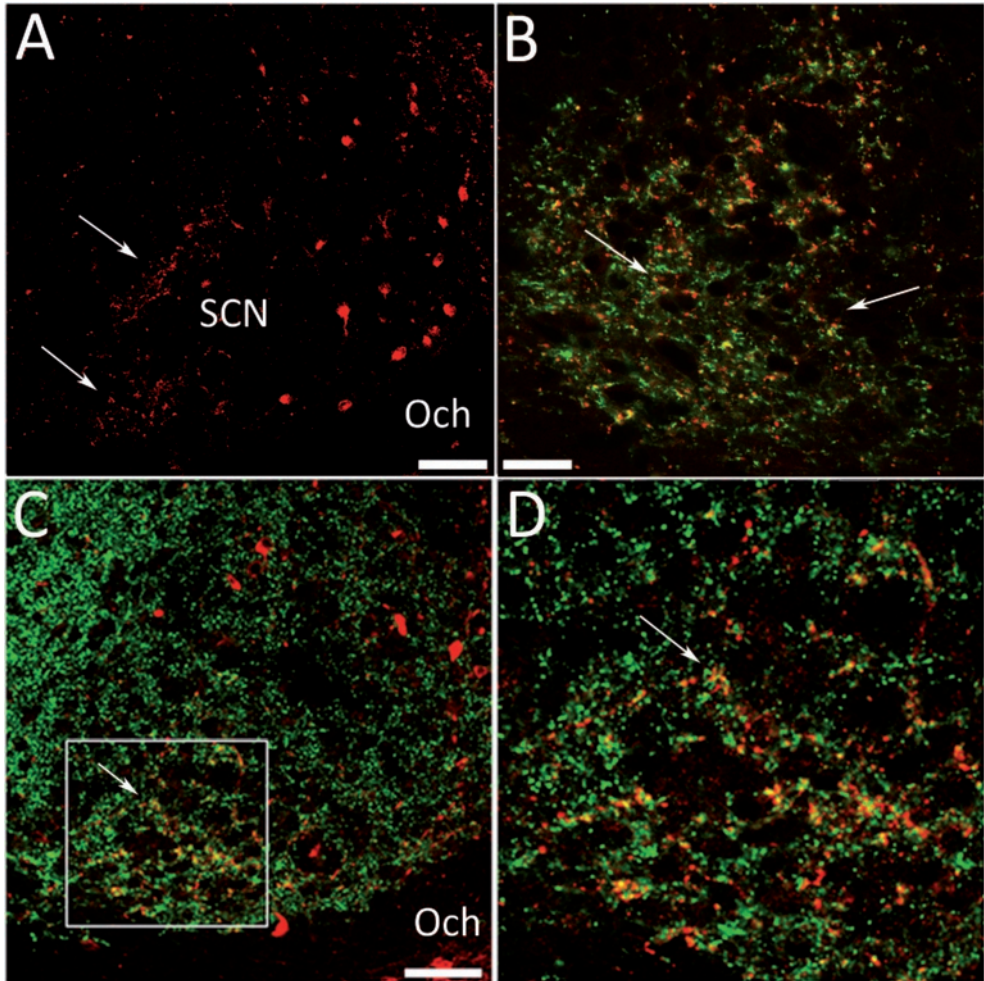


Figure 2. Illustrates the interaction of the NTS with the SCN after a CtB injection into the NTS.

A) CtB (red) fibers are present in the ventro-lateral part of the SCN (arrows), together with cell bodies in the medial SCN. Bar, 50 μ m. B) A magnification of the ventrolateral area of the SCN with retinal input (Green), and CtB labeled fibers from the NTS (red) show that both terminals target the same structures in the SCN (arrows). Bar represents 20 μ m. C) Co-localization of vGlut (green) with CtB (red) labeled neurons and fibers in the medial SCN. D) A higher magnification demonstrates the co-localization (yellow) of CtB labeled fibers arising from the NTS with vGlut2. (arrow in C and D indicate the same structure). Bar represents 50 μ m in C and 15 μ m in D. Och: optic chiasm (Indicated at the side of third ventricle), SCN: suprachiasmatic nucleus.

Signaling blood pressure information to the SCN. Given the glutamatergic NTS projections to the SCN, and that BP elevation activates a large population of glutamatergic NTS neurons, particularly in the caudal NTS (Weston et al., 2003c), we examined the influence of an increase in BP by an $\alpha 1$ -agonist (Metaraminol) on the neuronal activity in the SCN. Initially in pilot studies, we examined c-Fos reactivity 120 minutes after the induction of a transient hypertensive period early in the light period, at Zeitgeber Time 2 (ZT2). In the area of the SCN where the NTS projections terminate, a clear increase in c-Fos was found suggesting increased neuronal activity. However, the endogenous activity of the SCN is already high during the light period and thus did not permit an unambiguous demonstration of enhanced c-Fos activation following BP increase. Therefore we examined c-Fos induction in the SCN in the dark phase (ZT14), when the SCN shows low endogenous activity and little c-Fos expression. Metaraminol given at onset of the dark period resulted in a clear increase of c-Fos positive neurons in the SCN (119.5 ± 15.6 , $n=7$) compared to saline injected animals (54.8 ± 9.2 , $n=8$, $P < 0.001$), whereas non-injected animals barely showed any c-Fos (7.6 ± 2.0 , $n=8$) (Fig. 3a,b). The increase of c-Fos in saline injected animals suggests that the activation is associated with stress of the injection. The significantly higher c-Fos expression in the SCN following metaraminol administration, illustrates its substantial effect on blood pressure and consequent activation of the SCN. The unilateral lesioning of the caudal NTS (between Bregma -14.5 and -13.6; Fig. 4) resulted in a dramatic reduction of SCN activation after metaraminol injection (47.9 ± 7.4 , $n=7$, $P < 0.001$; Fig. 3) indicating that a unilateral lesion of the NTS was sufficient to considerably reduce BP information from reaching the SCN (Fig. 3c,d) and that integral functioning of the NTS is necessary to conduct a plenary cardiovascular signal to the brain. However, blood pressure recordings of such unilateral NTS lesioned animals showed that their response to an increase in blood pressure was comparable to controls. Nonetheless, strong diminishment of c-Fos activation in the contralateral NTS (Fig. 4) suggests the disruption of the integrity of the NTS in relaying BP information to the SCN as shown by the strong reduction in its activity (Fig. 3d).

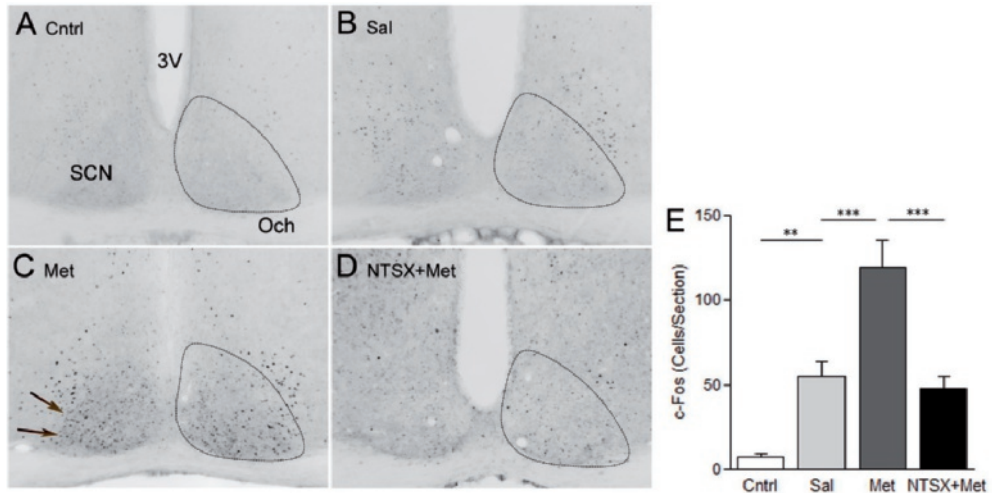


Figure 3. Cross-sections of the SCN showing the presence of activated neurons by means of c-Fos staining. A) Non-treated control animal, B) Saline injected animal, C) SHAM operated, metamaminol injected animal and D) NTSX lesioned, metamaminol injected animal. Arrows in C indicate the high density of c-Fos in the ventrolateral part of the SCN in the area where NTS terminals are present. Bar represents 75µm. E) Non-treated control animals showed a significantly lower amount of c-Fos as compared to saline and metamaminol injected animals at ZT 14. Metamaminol injected animals showed a significantly increased amount of c-Fos compared to metamaminol injected NTSX animals and saline injected animals (n=7-8). Analysis was by one-way ANOVA followed by Tukey's test (* P < 0.05, ** P<0.01, ***P<0.001). Results are expressed as ±SEM. Outlines show an approximation of the borders of the SCN. SCN: suprachiasmatic nucleus, Och: optic chiasm, 3V: third ventricle.

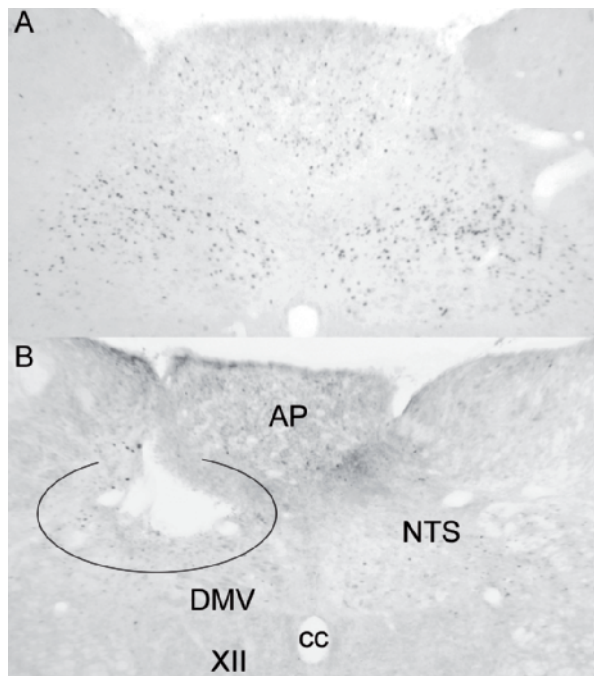


Figure 4. C-Fos activation in the NTS and DMV following s.c. metamaminol injection at ZT 12.

A) An intact animal and an NTS lesioned animal shown in B) with a representative lesion confined to the NTS. A strong decrease of c-Fos was visible in the NTS, also at the non lesioned site and even in the area postrema, compared to intact animals after metamaminol administration. Bregma – 14.05. AP: area postrema, DMV: dorsal motor nucleus of the vagus, NTS: nucleus tractus solitarius, XII: hypoglossal nucleus, cc: central canal.

The involvement of the SCN in blood pressure regulation. In general, increased SCN neuronal activity is associated with light and the associated rest period in nocturnal rodents when BP is at its lowest (Meijer et al., 1998). The neuronal activation of the SCN by NTS input after hypertensive stimuli suggested the direct involvement of the SCN in a BP regulatory system. We thus examined the effect of an increasing dose of metaraminol on the MAP of anesthetized intact animals at two different times of the day/night cycle and in SCN-lesioned animals (Table 1). The MAP response at ZT2 and ZT14 after metaraminol injection differed with regard to the duration of the MAP increase, *i.e.*, the time to bring MAP back to basal levels (Fig. 5). ZT2 (385.8 ± 18.8 sec, $n=7$) started to differ from ZT14 (293.0 ± 18.2 sec, $n=7$, $P < 0.05$) at the injection of $400\mu\text{g}/\text{kg}$ metaraminol, indicating the adaptive response to the increase in MAP was significantly stronger at ZT14. Since HR response did not significantly differ between groups, this could imply a greater centrally regulated plasticity of the cardiovascular system at ZT 14 as compared to ZT2. Lesioning the SCN severely affected the capacity to compensate for hypertensive stimuli (Fig. 6) illustrated by a significant increase in MAP even after saline injection, indicating that even the slight volume increase could not be immediately compensated in contrast with intact animals. Likewise, metaraminol resulted in a significantly greater MAP response in SCN animals as reflected by the AUC ($F(2,18)=18.91$, $P < 0.001$) and duration ($F(2,18)=67$, $P < 0.001$) but not in the crest of MAP increase (Fig. 6); another indication for a change in the central ability to adapt to hypertensive stimuli, and not for the sensitivity to metaraminol. Basal MAP was not different between SCN and control animals.

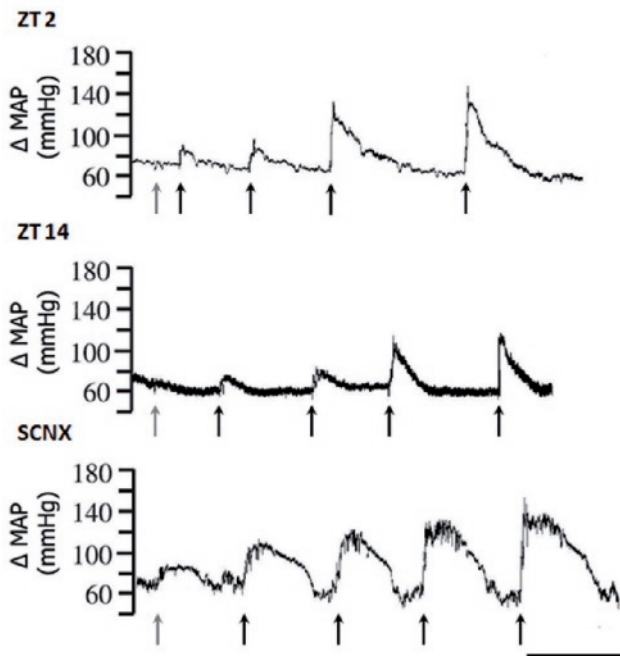


Figure 5. Representative blood pressure responses to saline or metaraminol injections. With saline injections (first grey arrow) followed by consecutive metaraminol injections (50, 100, 200 and $400\mu\text{g}/\text{kg}$; Black arrows) at ZT 2, ZT 14 and in SCN animals. Please note that saline injection induced an increase in blood pressure in the SCN animal only. Bar represents 15min.

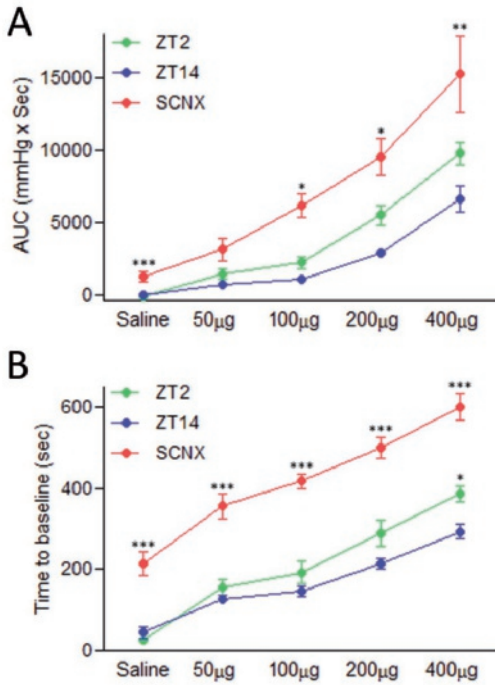


Figure 6. SCNX animals have a significantly increased blood pressure response as compared to controls. A) Area under the curve (AUC in mmHg x sec) after saline or metaraminol injections in control and SCNX animals. AUC of SCNX animals are significantly higher compared to ZT 2 and 14. B) Time in seconds for BP to return to baseline values after saline or metaraminol injections at ZT 2, ZT 14 and in SCNX animals (n=7 for all groups). Analysis was by two-way repeated measures ANOVA followed by Bonferroni test (* P<0.05, ** P<0.01, *** P < 0.001 when compared to ZT2). Results are expressed as \pm SEM.

Discussion

The present data provide evidence that the SCN receives direct projections from the cardiovascular-control area of the NTS, providing a neuroanatomical basis for the involvement of the SCN in the control of the blood pressure. Furthermore, the present data show that the SCN does not influence so much basal BP but rather determines the sensitivity of cardiovascular reactivity as previously suggested (Oosting et al., 1997; Scheer et al., 2010b). Consequently, our data show that the SCN is not merely a clock signaling time, but integrates cardiovascular feedback for an adequate hemodynamic regulation in harmony with the time of day. The present data also demonstrate that the SCN adapts its output based on sensory information relayed by the NTS. Therefore we conclude that, in addition to hypothalamic autonomic nuclei like the paraventricular nucleus receiving sensory cardiovascular feedback, the present study demonstrates that the SCN receives sensory cardiovascular information directly via the NTS. These results add to the accumulating evidence positioning the SCN as integral element in physiological control networks, including as recipient of feedback. In view of the many body functions that are timed by the SCN (Hastings et al., 2003a) and the fact that the NTS is the central relay in forwarding visceral sensory information to the brain, we suspect that the demonstrated pathway between the NTS and the SCN may provide, besides cardiovascular, also other types of visceral information to the SCN essential for homeostatic control. This is also supported by our recent finding that metabolic information may change the neuronal activity of the SCN via pathways that involve the NTS and intergeniculate leaflet (Saderi et al., 2013)

The SCN receives cardiovascular feedback through the NTS. Our present results demonstrate that the caudal NTS has excitatory glutamatergic projections to the SCN, resulting in an increase of SCN neuronal activity following hypertensive stimuli. This is consistent with previous work demonstrating increased activity of glutamatergic neurons in the caudal NTS after BP increases (Chan et al., 2000; Weston et al., 2003a; Weston et al., 2003b). We suggest it is through this NTS-SCN neuroanatomical pathway that cardiovascular feedback reaches the SCN, resulting in an adequate BP control pertaining to the time of day.

We cannot exclude that electrolytic lesioning of the NTS, as executed in the present study, to demonstrate the functionality of this pathway also damaged fibers of passage such as in the commissural NTS. Though trying to prevent this by using kainic acid lesions would bring other disadvantages as the size of such lesions is difficult to control resulting in other non desired side effects. Moreover, following the lesion its size is difficult to establish histologically. The surprising observation, that after a hypertensive stimulus both the NTS and SCN of unilateral NTS lesioned animals show a strong reduction in activity compared to controls, indicates that albeit small NTS lesions, the transmission of blood pressure information to the brain is greatly disrupted. This

agrees with earlier studies showing that damage to the caudal part of the NTS can, in the short-term, dramatically affect the adaptation of blood pressure to pressor stimuli (Sato et al., 1999; Colombari et al., 2002). However, this increased pressor response disappears over time, possibly due to neuronal reorganization within the medulla (Sato et al., 2003) explaining why our unilateral NTS lesioned animals do not show a changed pressor response like in controls. However, we cannot explain why the observed reduction of SCN activity in NTS lesioned animals had no acute effect on BP. Though it does follow our finding that the SCN is incorporated in a compliant feedback circuit regulating BP, it is possible this circuit is able to adapt through a form of neuronal restructuring. This has also been seen for example, in the recovery of temperature rhythmicity, over time, in SCN lesioned rats (Scheer et al 2005). Finally, the fact that sino-aortic denervation (SAD) results in a change in clock gene expression in the SCN (Li et al., 2007) and that SAD animals lose normal rhythmicity in blood pressure (Makino et al., 1997) indicates that disruption of NTS signaling interferes with the regulatory function of the SCN. Therefore we hypothesize that chronic disturbance of SCN activity could in the long-term lead to neuronal changes in the SCN, ultimately resulting in cardiovascular disease. Diminishment of SCN neurotransmitter staining as observed in the post mortem analysis of human hypertensive brains (Goncharuk, 2001) could support this hypothesis.

The SCN incorporated in a physiological feedback circuit. We establish that the area of baroreceptor input in the NTS emits projections to the SCN and targets the same area where also retinal fibers terminate in the SCN. Not surprisingly, in addition to receiving light input, the ventrolateral SCN is also associated with homeostatic and autonomic control (Nakagawa and Okumura, 2010). Neuronal activation of the SCN via these NTS projections by an increase in BP, results in a similar activation pattern as demonstrated with light (Aronin et al., 1990). Since SCN neuronal activity coincides with the inactive period in nocturnal rodents (Meijer et al., 1998), it is assumed that light induced activation of the SCN can be seen as a rest signal as it induces behavioral inactivity and a reduced heart rate, also established by light stimuli given during the active period (Scheer et al., 2005). In this study we establish a role for the SCN in regulating short-term BP variation according to time of day, as seen by a more effective cardiovascular control during the active phase as compared to the rest phase. In part, this may be explained by a change in blood vessel plasticity whereby increased rigidity of the cardiovascular system during the sleep period results in larger BP excursions following a hypertensive stimulus, as compared to controls. The observed variance in BP response amongst groups is not likely the result of divergent metaraminol metabolism since several studies have indicated that it is not rapidly degraded (Anton and Berk, 1977), nor does it pass the blood brain barrier. This is illustrated by the fact that metaraminol only induces a specific compensatory inactivation of vasopressin containing magocellular neurons of the supraoptic nucleus but not those containing oxytocin (Cunningham et al., 1994a).

We show that the SCN has an inhibitory effect on short-term BP regulation through our SCN_X animal study. Here we demonstrate in agreement with earlier studies (Janssen et al., 1994) that an SCN lesion hardly changes basal blood pressure which also agrees with very small changes observed between day and night blood pressure when differences in activity are accounted for (Scheer et al., 2003). We establish a role for the SCN in regulating short-term BP variation according to time of day, as seen by more effective cardiovascular control during the active phase as compared to the rest phase. The fact that SCN_X animals show even larger BP fluctuations indicates that the regulation of cardiovascular plasticity is a dynamic process whereby the SCN sets the tone. A similar role for the SCN has been observed in the control of corticosterone secretion whereby the SCN limits the corticosterone response after a stress stimulus and maintains this response within certain limits that vary between day and night; after an SCN lesion this corticosterone stress response is likewise significantly elevated (Buijs et al., 1993a). Therefore, we suggest that the differential control of short-term BP fluctuations by the SCN according to time of day, i.e., allowing a more flexible regulation to take place during the active period, ultimately could make the SCN responsible for a 24-hour BP rhythm. This conclusion is supported by studies demonstrating that SCN_X animals lack 24h BP rhythms but show higher BP variability (Sano et al., 1995) and, as also presented here, do not exhibit a change in basal BP. Furthermore, recent findings demonstrate that VIPr2 ^{-/-} mice fail to show a circadian BP rhythm in constant conditions showing that an adequate SCN output is essential for regulation of 24h BP rhythmicity (Sheward et al., 2010). We do not know how the output of the SCN may moderate increases in BP; it might be via its projections to pre-autonomic neurons in the hypothalamus or possibly via its projections to the NTS itself, allowing it to alter baroreflex sensitivity (Scheer et al., 2010a; Shea et al., 2011b); therefore more research needs to be done to explore the plurality of this NTS signal and its exact effect within the SCN.

Clinical implications. The prolonged hypertensive response in SCN_X animals and animals in the sleep phase, suggests a greater plasticity of the cardiovascular system expressed by greater adaptability to strong BP fluctuations during activity periods. This is confirmed by recent data reporting that humans, in a constant routine protocol, have activity induced BP peaks which are high just prior to the early rest phase as compared to the early active phase (Shea et al., 2011a), suggesting a more effective adaptive BP response during the activity period. The present observation that the SCN is actively involved in BP regulation provides a possible explanation for the success of treating hypertensive patients focusing on the SCN, i.e., with chronotherapy or melatonin; especially effective in “non-dippers” (Scheer et al., 2004; Grossman et al., 2006) who show suppressed 24-hour BP variation (Pickering, 1990).

The loss of immunoreactivity in the SCN of the hypertensive human postmortem brain (Goncharuk et al., 2001) suggests that a functional SCN lesion has taken place; whether this is cause or consequence of hypertension still needs to be resolved. It

is possible that, via a similar NTS-SCN pathway as demonstrated here, hypertension in humans chronically disrupts the activity of the SCN through perturbing feedback from the NTS, with the possible consequence that the SCN insufficiently prepares a hypertensive individual to cope with sudden BP fluctuations. This is supported by the fact that when hypertension progresses, baroreceptor sensitivity is disrupted (Moreira et al., 1992). On account of this loss of adequate autonomic control, short-term BP changes could aggravate under an insufficient baroreflex. Together with increased platelet aggregation early in the day (Ellis et al., 1991; Scheer et al., 2011) this might clarify why cardiovascular incidents are more frequent in the early activity period than at any other period during the circadian cycle (Zulch and Hossmann, 1967; Muller et al., 1985)

CONCLUSION

We demonstrate that the SCN receives cardiovascular feedback via the NTS emphasizing the importance of the SCN not only as master clock, but also as an integral element in the physiological regulation of BP. Since SCN output also synchronizes peripheral clock genes that have important regulatory functions at the level of organs involved in cardiovascular control, such as blood vessels, kidneys and adrenal (Doi et al., 2010a; Cheng et al., 2011b), it can be inferred that this synchrony between the cardiovascular system and the SCN is essential for body homeostasis; desynchronization between and/or within this system could ultimately result in the development of cardiovascular disease.

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