Circadian timekeeping: from basic clock function to implications for health

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CHAPTER 7

The suprachiasmatic nucleus controls energy metabolism and insulin sensitivity

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Abstract

Disturbances in the circadian system are associated with the development of type 2 diabetes mellitus. Here, we studied the direct contribution of the suprachiasmatic nucleus (SCN), the central pacemaker in the circadian system, in the development of insulin resistance. Exclusive bilateral SCN lesions in male C57Bl/6J mice, as verified by immunochemistry, showed a small but significant increase in body weight (+17%), which was accounted for by an increase in fat mass. In contrast, mice with collateral damage to the ventromedial hypothalamus and paraventricular nucleus showed severe obesity and insulin resistance. Mice with exclusive SCN ablation revealed a loss of circadian rhythm in activity, oxygen consumption, and food intake. Hyperinsulinemic–euglycemic clamp analysis 8 wks after lesioning showed that the glucose infusion rate was significantly lower in SCN lesioned mice compared with sham-operated mice (-63%). Although insulin potently inhibited endogenous glucose production (-84%), this was greatly reduced in SCN lesioned mice (-7%), indicating severe hepatic insulin resistance. Our data show that SCN malfunctioning plays an important role in the disturbance of energy balance and suggest that an absence of central clock activity, in a genetically intact animal, may lead to the development of insulin resistance.

Introduction

Obesity and type 2 diabetes mellitus have an increasing prevalence in modern society. In the past decade, a strong and potentially causal relationship between metabolic disorders and disturbances of the circadian system has been elucidated.[1] The circadian system is responsible for 24 hr rhythms in a wide variety of physiological and behavioral functions.[2,3] Generation of these rhythms occurs in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus[2,3] and is explained by a transcriptional–translational feedback loop involving the clock genes CLOCK, BMAL1, Period (Per), and Cryptochrome (Cry).[1] The rhythms of the SCN are synchronized to the environmental 24 hr cycle mainly by light–dark information perceived by the eyes. Rhythmic information is transferred from the SCN to the central nervous system and to peripheral organs of the body.[4,5] This output is crucial for synchronization of many metabolic and endocrine factors such as glucose, insulin,[6,7] cortisol,[8] leptin, ghrelin,[9,10] and neuropeptide Y.[11]

Disturbances in circadian rhythms occur as a consequence of shift work and transitions in time zones, but also because of irregular sleep–wake patterns,[12] aging, or neurodegenerative disorders.[13] Animal studies have indicated a link between clock gene mutations and metabolic disturbances. For instance, Clock mutant mice have a greatly attenuated diurnal feeding rhythm, are hyperphagic and obese, and have development of a metabolic syndrome of hyperleptinemia, hyperlipidemia, hepatic steatosis, and hyperglycemia.[14] Because clock gene mutants are not specific to the SCN, the detrimental effects of disturbed rhythms may have their origin in peripheral organs other than the SCN. It is not clear to what extent the SCN itself is involved in metabolic disorders. Given the accumulating evidence for disturbances of SCN cellular organization in aging,[15] neurodegenerative disorders, and dementia,[13,16] this question is also clinically relevant, as it would explain comorbidity between various disorders.

To assess the role of disturbed function of the SCN *per se* in the development of obesity and type 2 diabetes mellitus, we performed bilateral microlesions of the SCN in male C57Bl/6J mice. Because the SCN is anatomically surrounded by areas regulating energy homeostasis, such as the ventromedial hypothalamus (VMH) and paraventricular nucleus (PVN), great care was taken to distinguish between exclusively SCN lesioned mice and mice with collateral damage to surrounding nuclei. We show that selective ablation of the SCN results in complete loss of circadian energy metabolism and, moreover, in the development of severe hepatic insulin resistance, stressing the role of the central clock in the pathophysiology of insulin resistance.

Research design and methods

Animals

All animal experiments were approved by the Animal Ethic Committee from the Leiden University Medical Center (Leiden, the Netherlands). Male C57Bl/6J mice were housed individually in a controlled environment (21°C; 40–50% humidity) under a 12 hr/12 hr light/dark cycle (07:00–19:00 hrs) unless otherwise mentioned. Food (chow, RM3; Special Diet Services, Sussex, U.K.) and tap water were available ad libitum during the entire experiment. Body weight was monitored weekly for all individual mice.

SCN lesions

Bilateral ablation of the SCN was performed in 13-week-old mice as described previously.[17] Mice were anesthetized using a mixture of ketamine (100 mg/kg; Aescoket, Boxtel, the Netherlands), xylazine (10 mg/kg; Bayer AG, Leverkusen, Germany), and atropine (0.1 mg/kg; Pharmachemie, Haarlem, The Netherlands) and mounted in a stereotactic device (Digital Just for Mouse Stereotaxic Instrument; Stoelting, Wood Dale, IL). After identification of bregma, a hole was drilled through which the lesion electrode was inserted into the brain. Lesion needles were made by isolating a 0.3-mm stainless steel insect pin using isolating resin, except for 0.2 mm at the tip. The electrode tip was aimed at the SCN, 0.46 mm posterior to bregma, 0.15 mm lateral to the midline, and 5.2 mm ventral to the surface of the cortex.[21] Bilateral SCN lesions were made by passing a 0.6 mA current through the electrode for duration of 10sec. The sham lesioned mice underwent the same operation, but no current was passed through the electrode.

Circadian rhythm analysis

After SCN lesioning, all mice were housed in constant dark for 10 consecutive days to determine circadian rhythm in behavioral activity of each animal using passive infrared motion detection sensors (Hygrosens, Löffingen, Germany) that were mounted underneath the lid of the cage and connected to a ClockLab data collection system (Actimetrics) that recorded the amount of sensor activation in 1-min bins. The presence of circadian rhythms was determined by F-periodogram analysis based on the algorithm of Dörrscheidt and Beck.[18] Mice were included in the lesion group when no significant rhythm was present.

Positional check of the SCN lesion

The SCN lesions were checked as described previously.[10] To verify the position of the SCN lesions, brains were removed and fixed by immersion with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4°C. For cryoprotection, the brain tissue was equilibrated for 48 hrs with 30% sucrose in 0.1 mol/L Tris-buffered saline before sectioning. Thereafter, the brain tissue was cut into 30-mm sections and divided into two equal vials for

immunocytochemical staining. The two vials of brain sections were incubated overnight at 4°C with either rabbit antivasopressin or rabbit anti-vasoactive intestinal peptide primary antibodies. Sections were then rinsed in 0.1 mol/L Tris-buffered saline, incubated 1 hrs in biotinylated goat anti-rabbit IgG, and subsequently incubated for 1 hrs in avidin– biotin complex (Vector Laboratories, Burlingame, CA). The reaction product was visualized by incubation in 1% diaminobenzidine with 0.01% hydrogen peroxide for 5–7 min. Nickel ammonium sulfate (0.05%) was added to the diaminobenzidine solution to darken the reaction product (diaminobenzidine/ nickel). All sections were mounted on gelatin-coated glass slides, dried, run through ethanol and xylene, and covered for observation by light microscopy. For every animal, we blindly scored the amount of damage to the SCN and surrounding hypothalamic nuclei involved in metabolism (PVN and VMH).

Indirect calorimetry and metabolic cages

Individual measurements by indirect calorimetry were performed for a period of at least 4 consecutive days (Comprehensive Laboratory Animal Monitoring System; Columbus Instruments, Columbus, OH).[19] A period of 24 hrs was included at the start of the experiment to allow acclimatization of the mice to the cages. Experimental analysis started at 09:00. Analyzed parameters included real-time energy, water intake, and activity. Oxygen consumption (energy expenditure) measurements were performed at intervals of 7 min throughout the whole period. Oxygen consumption, activity, and energy intake were analyzed separately for day and night.

Hyperinsulinemic–euglycemic clamp analysis

Eight weeks after SCN lesioning, hyperinsulinemic–euglycemic clamp experiments were performed as previously described.[20] Mice were fasted for 16 hrs with food withdrawn at 17:00 the day before the study. All mice ate within 60 min before the start of the fasting period, minimizing differences in fasting time. During the experiment, mice were anesthetized by intraperitoneal injection with a combination of acepromazin (6.25 mg/kg; Sanofi Sant Nutrition Animale, Libourne Cedex, France), midazolam (6.25 mg/kg; Roche, Mijdrecht, the Netherlands), and fentanyl (0.31 mg/kg; Janssen-Cilag, Tilburg, the Netherlands). Anesthesia and body temperature were maintained throughout the procedure. At the end of the basal and the hyperinsulinemic periods, hematocrit values were determined to ensure that the mice were not anemic. First, basal rates of glucose turnover were determined by administering a primed (0.8 mCi) constant (0.02 mCi/min) intravenous infusion of 3-^{3}H-glucose (specific activity, 9.6 GBq/ mmol; Amersham, Little Chalfont, U.K.) for 60 min. Glucose rate of disposal (Rd; mmol/min/kg) was determined as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of 3-^{3}H-glucose (dpm/mmol) at both time points. Subsequently, insulin (actrapid; Novo Nordisk, Denmark)

was administered intravenously by primed (4.1 mU), constant (6.8 mU/h) infusion to attain steady-state circulating insulin levels of ~4 ng/mL together with 3-^{3}H-glucose. A variable intravenous infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10-min intervals via tail bleeding (<3 mL; Accu-chek, Sensor Comfort; Roche Diagnostics). All mice were clamped at their respective basal glucose levels, because it has been shown that alterations in basal fasting glucose levels alone are sufficient to affect insulin sensitivity.[21] Seventy minutes after the start of the hyperinsulinemic period, when glucose infusion rates (GIRs) for all mice were stable for at least 30 min, blood samples were taken at 10-min intervals for 30 min to determine 3-^{3}H-glucose. Hyperinsulinemic Rd was determined similar to the basal period. The endogenous glucose production (EGP) was calculated as the difference between Rd and the GIR. After the hyperinsulinemic–euglycemic clamp, body composition (lean *vs.* fat mass) was determined by DEXA using the Norland pDEXA Sabre X-Ray Bone Densitometer (Norland, Hampshire, U.K.). All data were analyzed according to the software and recommendations of the manufacturer. Subsequently, the mice were euthanized.

Plasma analysis

Plasma insulin concentrations were measured by ELISA (Crystal Chem). For measurement of plasma 3-^{3}H-glucose, trichloroacetic acid (final concentration 2%) was added to 10 mL plasma to precipitate proteins using centrifugation. The supernatant was dried to remove water and was resuspended in milliQ. The samples were counted using scintillation counting (Packard Instruments).

Statistical analysis

Statistical analysis was performed using SPSS 17 for Windows (SPSS, Chicago, IL). Unpaired *t*-tests were performed for all comparisons, with statistical significance threshold set at $P = 0.05$.

Results

Behavioral and histological verification of SCN lesions.

Periodogram analysis of activity showed that all sham-operated mice retained a strong circadian rhythm (**Fig. 1A**), whereas SCN lesioned mice lost their circadian rhythm in activity after the lesion procedures (**Fig. 1B**). Histological analysis revealed SCN lesions without collateral damage in six mice. In addition to ablation of the SCN, four other mice had unilateral damage to the PVN, 10 other mice had bilateral damage to the PVN, and nine other mice had damage to both the PVN and the VMH. Sham-operated controls ($n = 17$) did not reveal damage to any of the aforementioned brain areas. Representative histological

sections of the hypothalamus, illustrating the histological verification of lesion position and size, are shown in **Suppl. Fig. 1**; 40-mm coronal sections were alternately stained for arginine vasopressin (immunohistochemical staining, **Suppl. Fig. 1A**), vasoactive intestinal peptide (immunohistochemical staining, **Suppl. Fig. 1B**), and cresyl violet (cell nuclei staining, **Suppl. Fig. 1C**).

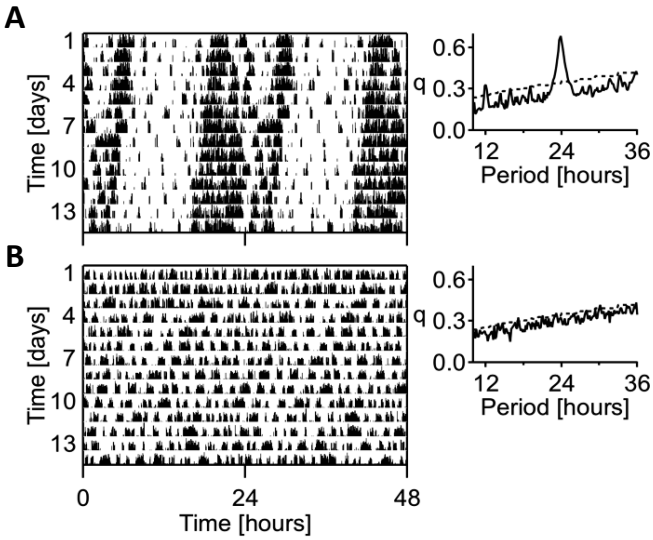
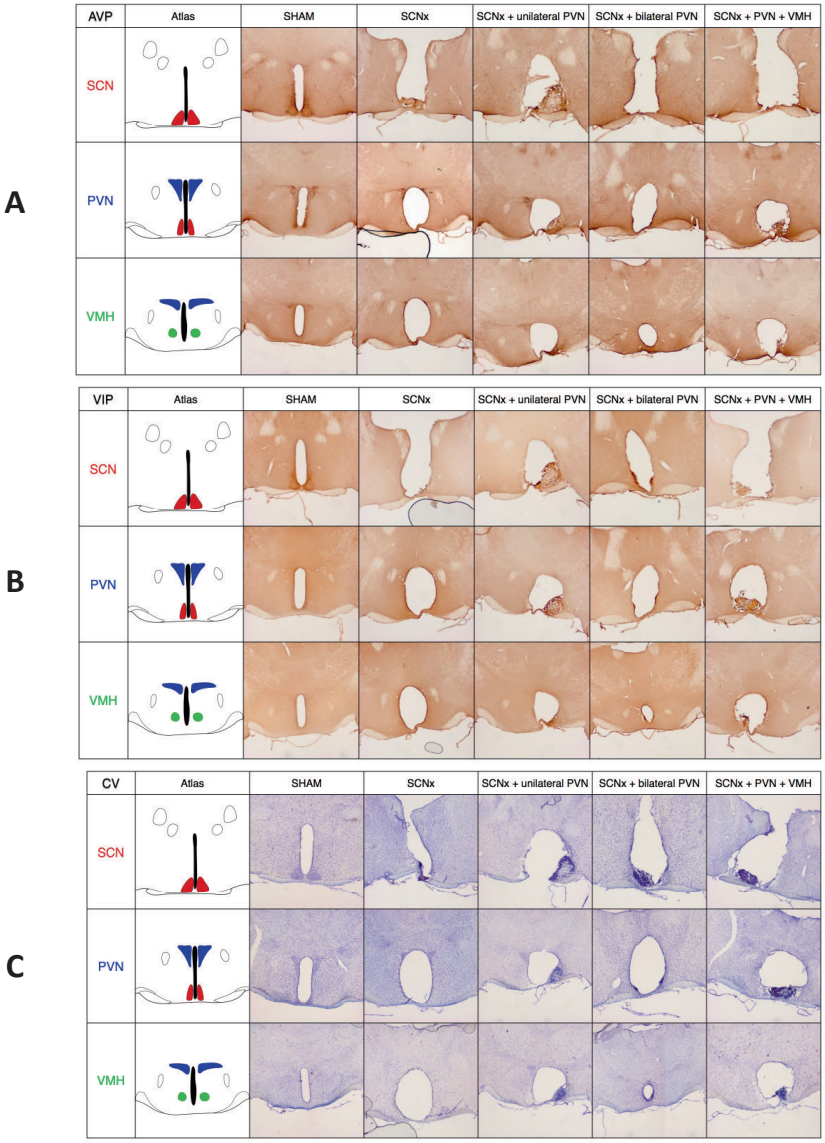


Fig. 1. Representative double-plotted actogram analyses of shamoperated (**A**) and SCN lesioned (**B**) mice under constant dark conditions. Each line of the double-plotted actograms represents 48hrs.

Selective SCN lesions

Body weight and composition

Before the operation and after a recovery period of 8 days, the average body weight of the sham and SCN lesioned mice did not differ (operation day: sham, 26.3 ± 1.5 g, SCN lesion, 26.0 ± 1.5 g, not significant; day 8: sham, 25.9 ± 1.6 g, SCN lesion, 25.3 ± 1.6 g, not significant). Eight weeks after SCN lesion, body mass of SCN lesioned mice was 17% higher compared with that of the sham mice (24.9 ± 1.2 *vs.* 29.2 ± 1.9 g; $P < 0.01$; **Fig. 2**). Assessment of body composition revealed that fat mass was significantly higher in SCN lesioned mice compared with sham (5.8 ± 2.6 *vs.* 1.9 ± 1.0 g; $P < 0.01$), whereas lean mass did not differ (17.7 ± 2.2 *vs.* 17.2 ± 1.7 g; not significant). This indicates that SCN ablation results in only mild overweight status compared with sham mice.



Suppl. Fig. 1. Representative histological sections of the hypothalamus, illustrating the histological verification of lesion position and size. 40 micrometer coronal sections were alternately stained for arginine vasopressin (AVP, immuno-histochemical staining, **A**), vasoactive intestinal peptide (VIP, immuno-histochemical staining, **B**) and cresyl violet (CV, cell-nuclei staining, **C**). For each animal, the location of lesion-induced damage was judged by independent researchers. Animals were classified based on (I) the presence of a bilateral ablation of the suprachiasmatic nucleus (SCN, top row of each panel) and for the presence of additional damage: (II) unilateral or bilateral ablation of the paraventricular nucleus (PVN, middle row in each panel) and (III) damage to the rostral part of the ventromedial hypothalamus (VMH, bottom row in each panel). For comparison, schematic drawings of the mouse hypothalamus (first column) and stainings of sham-operated animals (second column) are included.

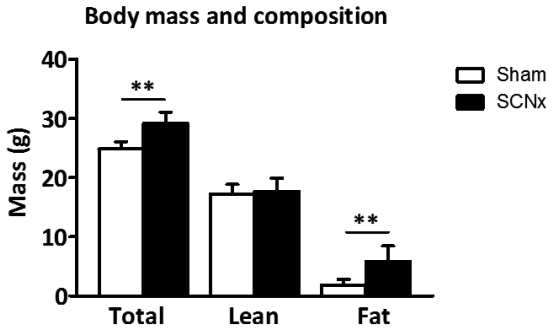


Fig. 2. Body mass and composition of sham (white bars) and SCN lesioned (black bars) mice at the time of the hyperinsulinemic–euglycemic clamp. Total body mass, lean body mass, and fat mass were determined. Data are represented as mean ± SD. $^{**}P < 0.01$.

Indirect calorimetry

Metabolic cage data on oxygen consumption, food intake, and activity were obtained over a period of a minimum of 4 consecutive days and were analyzed separately for day and night. During the night, oxygen consumption was higher compared with the day in sham mice (3,386 ± 174 *vs.* 2,992 ± 155 mL/kg/h; $P < 0.01$; **Fig. 3A**). This circadian rhythm in oxygen consumption was lost in SCN lesioned mice, resulting in higher oxygen consumption during the day in SCN lesioned mice compared with shammice (3,321 ± 252 *vs.* 2,992 ± 155 mL/kg/h; $P < 0.01$). Total 24 hr oxygen consumption was not different between sham and SCN lesioned mice (76,531 ± 3,854 *vs.* 79,518 ± 7,595 mL/kg/day, not significant). During the night, sham mice were more active compared with during the day (195 ± 68 *vs.* 79 ± 33 beam breaks (bb); $P < 0.01$; **Fig. 3B**). In line with the oxygen consumption rates, circadian pattern in activity was lost in SCN lesioned mice (123 ± 48 *vs.* 128 ± 38 bb, not significant). This loss in activity pattern in SCN lesioned mice resulted in increased activity during the day (128 ± 38 *vs.* 79 ± 33 bb; $P < 0.01$) and reduced activity during the night compared with sham mice (123 ± 48 *vs.* 195 ± 68 bb; $P < 0.01$). Total 24 hr activity, however, was not different between sham and SCN lesioned mice (273 ± 96 *vs.* 251 ± 81 bb; not significant). Sham mice consumed 68% of their total food during the night (night *vs.* day: 3.2 ± 0.5 *vs.* 1.5 ± 0.2 g; $P < 0.01$; **Fig. 3C**), whereas SCN lesioned mice consumed only 54% during the night (night *vs.* day: 1.9 ± 0.3 *vs.* 1.6 ± 0.9 g, not significant). This resulted in reduced food intake during the night for SCN lesioned mice compared with sham mice (1.9 ± 0.3 *vs.* 3.2 ± 0.5 g; $P < 0.01$). Furthermore, total 24 hr food intake also was reduced for SCN lesioned mice compared with sham mice (3.5 ± 1.2 *vs.* 4.7 ± 0.5 g; $P < 0.01$). Calculating the respiratory exchange ratio showed that SCN lesioned mice had a lower respiratory exchange ratio compared with sham mice during the day (0.86 ± 0.04 *vs.* 0.90 ± 0.01; $P < 0.05$) as well as during the night (0.87 ± 0.01 *vs.* 0.96 ± 0.02; $P < 0.01$). These data clearly show that ablation of the SCN results in a loss of circadian rhythm in respiratory metabolism and food intake.

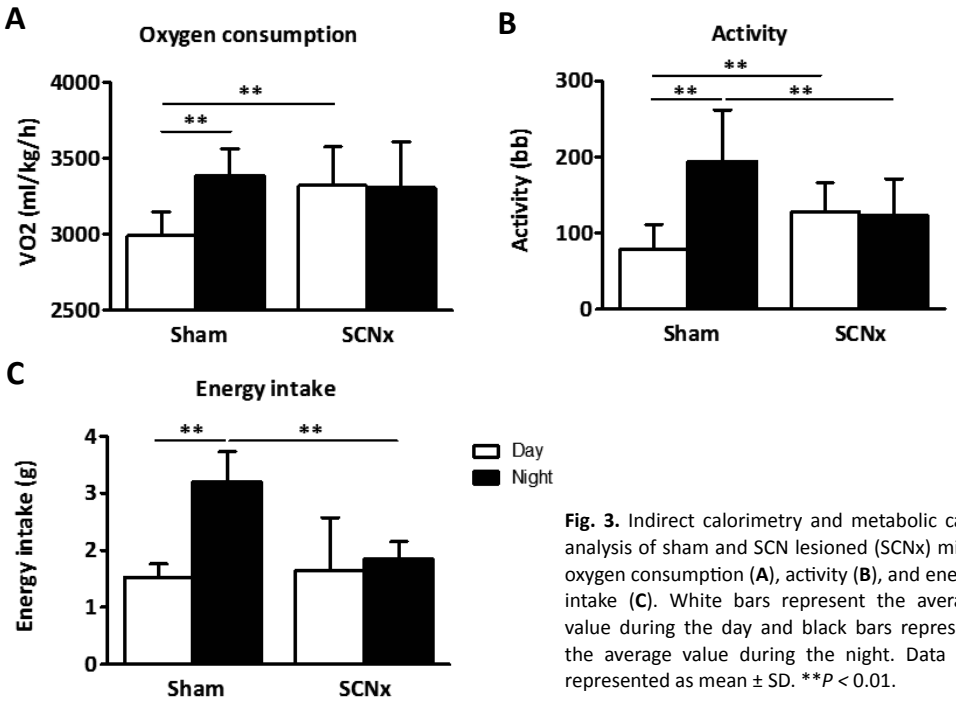


Fig. 3. Indirect calorimetry and metabolic cage analysis of sham and SCN lesioned (SCNx) mice: oxygen consumption (**A**), activity (**B**), and energy intake (**C**). White bars represent the average value during the day and black bars represent the average value during the night. Data are represented as mean ± SD. $^{**}P < 0.01$.

Hyperinsulinemic–euglycemic clamp studies

Hematocrit levels were similar at the basal and the hyperinsulinemic period for sham and SCN lesioned mice, indicating that the mice were not anemic. At the start of the clamp, insulin, glucose, and free fatty acid (FFA) plasma levels were significantly higher in SCN lesioned mice compared with sham controls (**Table 1**). During the hyperinsulinemic period, insulin levels were increased to a similar extent for both the sham and SCN lesioned mice. At the end of the hyperinsulinemic period, circulating glucose levels were isoglycemic compared with fasting levels in sham and SCN lesioned mice, resulting in significantly higher glucose levels in the hyperinsulinemic period for SCN lesioned mice compared with sham mice (**Fig. 4A**). At the end of the clamp, FFA levels were decreased compared with basal plasma levels in sham and SCN lesioned mice, although this failed to reach statistical significance in SCN lesioned mice ($P = 0.08$). GIRs over the final 20 min of the clamp were significantly lower in SCN lesioned mice compared with sham controls (27.6 ± 19.5 *vs.* 74.7 ± 21.1 mmol/min/kg; $P < 0.01$; **Fig. 4B and C**). The glucose-specific activities measured at 10-min intervals indicated the presence of steady-state conditions in all groups (**Suppl. Table 1**).

Table 1. Body mass and hyperinsulinemic–euglycemic clamp parameters of sham and suprachiasmatic nucleus lesioned (SCNx) mice.

	Sham		SCNx	
	Basal	**Clamp**	**Basal**	**Clamp**
Body mass (g)	24.9 ± 1.2		29.2 ± 1.9†	
Insulin (ng/ml)	0.5 ± 0.3	5.2 ± 1.1‡	1.0 ± 0.3†	4.8 ± 1.9‡
Glucose (mmol/l)	4.9 ± 0.8	4.7 ± 0.9	6.7 ± 1.2†	6.8 ± 1.2†
FFA (mmol/l)	0.9 ± 0.2	0.6 ± 0.3‡	1.2 ± 0.3*	0.8 ± 0.2
Hematocrit (%)	41.6 ± 4.7	38.5 ± 2.3	41.7 ± 2.7	40.5 ± 0.7

Data are represented as mean ± SD. *$P < 0.05$ *vs.* sham. †$P < 0.01$ *vs.* sham. ‡$P < 0.05$ basal vs hyperinsulinemic–euglycemic clamp period.

Suppl. Table 1. Glucose (**A**) and tracer specific activity (**B**) data for all groups during the basal and hyperinsulinemic period of the clamp. Data are represented as mean ± SD.

A

	Basal 1 [Glucose] (mmol/l)	**Basal 2 [Glucose] (mmol/l)**	**Hyper 1 [Glucose] (mmol/l)**	**Hyper 2 [Glucose] (mmol/l)**	**Hyper 3 [Glucose] (mmol/l)**
Sham	5.0 ± 0.7	4.9 ± 1.0	4.6 ± 0.8	4.7 ± 1.0	4.7 ± 1.0
SCNx	6.6 ± 1.1	6.8 ± 1.4	7.4 ± 1.3	6.7 ± 1.1	6.8 ± 1.1
SCNx + unilat PVN damage	6.7 ± 0.6	7.3 ± 2.0	7.0 ± 1.1	7.1 ± 1.2	7.1 ± 1.2
SCNx + bilat PVN damage	7.2 ± 0.9	7.4 ± 1.5	7.2 ± 0.7	7.2 ± 0.9	7.2 ± 1.3
SCNx + PVN/VMH damage	7.6 ± 1.7	7.6 ± 1.9	6.8 ±1.0	7.4 ± 1.7	7.4 ± 1.6

B

	Basal 1 SA (DPM/µmol)	**Basal 2 SA (DPM/µmol)**	**Hyper 1 SA (DPM/µmol)**	**Hyper 2 SA (DPM/µmol)**	**Hyper 3 SA (DPM/µmol)**
Sham	12100 ± 3966	12855 ± 4176	7831 ± 2283	7519 ± 2587	7398 ± 1990
SCNx	8849 ± 1828	8891 ± 1803	6066 ± 283	5452 ± 1044	6659 ± 1241
SCNx + unilat PVN damage	9925 ± 2274	9383 ± 2606	6842 ± 1804	7085± 1365	7109 ± 2013
SCNx + bilat PVN damage	6450 ± 1098	6887 ± 1864	5775 ± 1339	6031 ± 1625	6755 ± 2227
SCNx + PVN/VMH damage	7532 ± 4189	8174 ± 5039	5302 ± 1906	6068 ± 2177	5920 ± 2677

In the basal period, EGP, which equals Rd, was not different between sham and SCN lesioned mice (53.6 ± 16.6 *vs.* 65.0 6 8.6 mmol/min/kg, not significant; **Fig. 5A**). In the hyperinsulinemic–euglycemic period, Rd was increasedby 57% in the sham mice compared with the basal period (83.9 ± 23.8 *vs.* 53.6 ± 16.6 mmol/min/kg; $P < 0.01$) and by 27% in the SCN lesioned mice, although this failed to reach statistical significance (82.8 ± 15.9 *vs.* 65.0 ± 8.6 mmol/min/kg; $P = 0.06$). Hyperinsulinemic Rd was not significantly different between sham and SCN lesioned mice. In the hyperinsulinemic–euglycemic period, EGP was decreased by 84% in sham-operated mice compared with the basal period (8.8 ± 12.2 *vs.* 53.6 ± 16.6 mmol/min/kg; $P < 0.01$; **Fig. 5B**), whereas EGP was decreased by only 7% in SCN lesioned mice (60.3 ± 25.8 *vs.* 65.0 ± 8.6 mmol/min/ kg, not significant). These data indicate that bilateral ablation of the SCN induces severe hepatic insulin resistance.

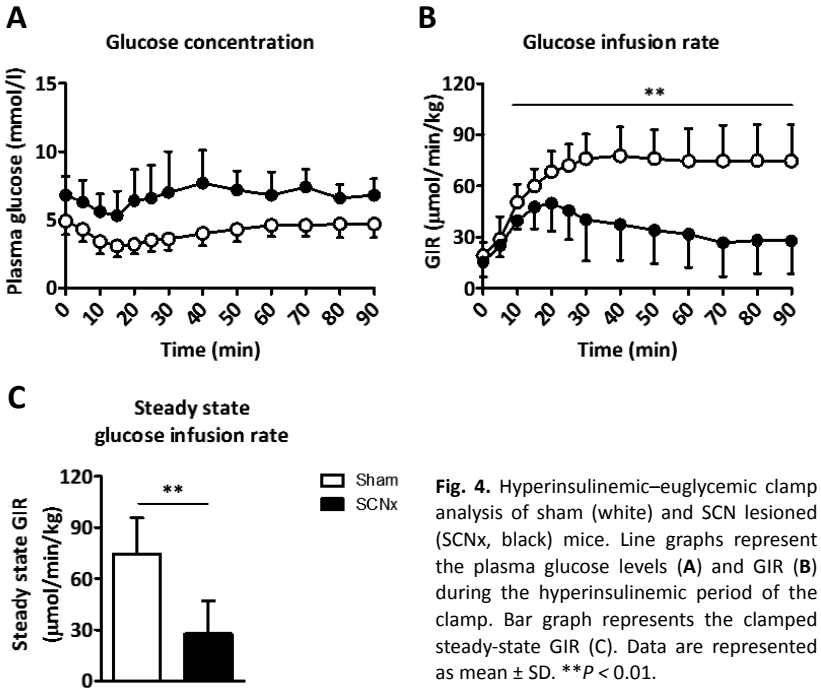


Fig. 4. Hyperinsulinemic–euglycemic clamp analysis of sham (white) and SCN lesioned (SCNx, black) mice. Line graphs represent the plasma glucose levels (**A**) and GIR (**B**) during the hyperinsulinemic period of the clamp. Bar graph represents the clamped steady-state GIR (C). Data are represented as mean ± SD. $^{**}P < 0.01$.

SCN lesion with collateral hypothalamic damage

SCN lesioned mice with collateral damage to hypothalamic nuclei involved in metabolism were analyzed separately. SCN lesioned mice with additional, but selective, unilateral damage to the PVN had modestly increased body mass compared with sham lesioned mice (29.9 ± 3.6 *vs.* 24.9 ± 1.2 g; $P < 0.01$; **Fig. 6A**), which was the result of increased fat mass (7.9 ± 2.5 *vs.* 1.9 ± 1.0 g; $P < 0.01$), but not lean mass (16.2 ± 1.0 *vs.* 17.2 ± 1.7 g, not significant).

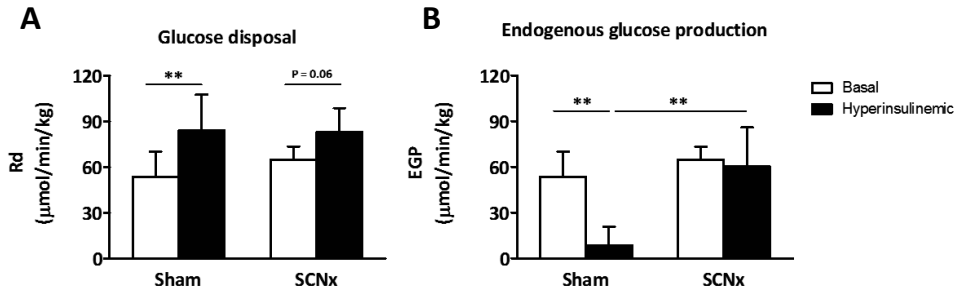


Fig. 5. Glucose Rd (**A**) and EGP (**B**) in basal period (white bars) and hyperinsulinemic–euglycemic clamp period (black bars) of sham and SCN lesioned (SCNx) mice. Data are represented as mean ± SD. $^{**}P < 0.01$.

SCN lesioned mice with bilateral damage to the PVN were considerably heavier than the sham mice (42.5 ± 3.3 *vs.* 24.9 ± 1.2 g; $P < 0.01$), which was the result of increased fat mass (21.3 ± 4.8 *vs.* 1.9 ± 1.0 g; $P < 0.01$), but this also coincided with a decrease in lean mass (13.6 ± 2.2 *vs.* 17.2 ± 1.7 g; $P < 0.01$). SCN lesioned mice with collateral damage to both the PVN and the VMH had development of extreme obesity compared with sham mice (49.3 ± 5.1 *vs.* 24.9 ± 1.2 g; $P < 0.01$), which was the result of an excessive increase in fat mass (24.7 ± 3.6 *vs.* 1.9 ± 1.0 g; $P < 0.01$), whereas lean mass was not different (16.8 ± 3.3 *vs.* 17.2 ± 1.7 g, not significant). Insulin sensitivity in SCN lesioned mice with collateral damage was determined by hyperinsulinemic–euglycemic clamp analysis. GIR needed to maintain euglycemia, as determined over a period of 20 min at stable plasma glucose values, was significantly lower in all SCN lesioned mice with collateral damage (SCN lesion + unilateral PVN damage: 41.3 ±11.0 mmol/min/kg; SCN lesion + bilateral PVN damage: 23.9 ± 13.1 mmol/min/kg; SCN lesion + PVN/VMH damage: 15.4 ± 13.5 mmol/min/kg) compared with sham controls (74.7 ± 21.1 mmol/min/kg; **Fig. 6B**), suggesting insulin resistance. Indirect calorimetry, metabolic cage, EGP, and Rd data and insulin levels of SCN lesioned mice with collateral damage are shown in **Suppl. Table 2**.

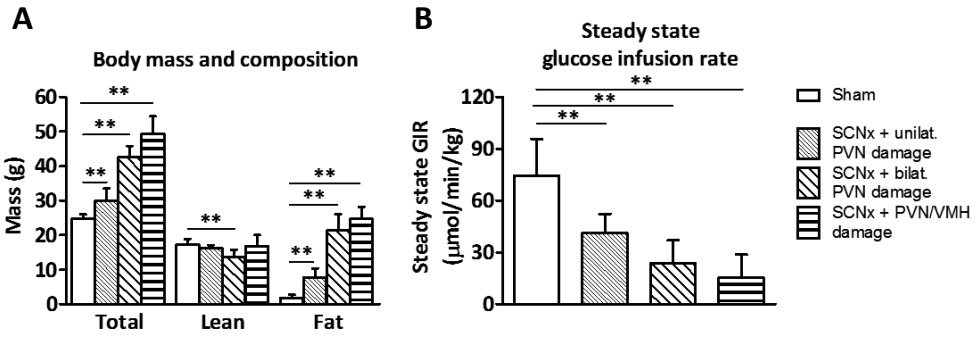


Fig. 6. Body mass and composition (**A**) and hyperinsulinemic–euglycemic clamp analysis (**B**) of sham (white bars) and SCN lesioned (SCNx) mice with collateral damage (striped bars): unilateral PVN, bilateral PVN, and PVN plus VMH. Data are represented as mean ± SD. $^{**}P < 0.01$.

Suppl. Table 2. Indirect calorimetry/metabolic cage (**A**), hyperinsulinemic-euglycemic clamp (**B**) and plasma insulin data (**C**) of sham and SCN lesioned (SCNx) mice with collateral damage. Data are represented as mean ± SD, **P* < 0.05 *vs.* sham, ***P* < 0.01 *vs.* sham, $^{\#}P$ < 0.05 light *vs.* dark, $^{\$}P$ < 0.05 basal *vs.* hyperinsulinemic-euglycemic clamp period.

A

	Dark		
	VO_2 (ml/kg/h)	Activity (bb/12 h)	FI (g/12 h)
Sham	3386 ± 173	195 ± 67	3.20 ± 0.53
SCNx + unilat PVN damage	3107 ± 162*	74 ± 22*	2.24 ± 0.54*
SCNx + bilat PVN damage	2530 ± 196*	61 ± 23*	2.52 ± 0.73*
SCNx + PVN/VMH damage	2594 ± 513*	57 ± 29*	2.42 ± 0.72*
	Light		
	VO_2 (ml/kg/h)	Activity (bb/12 h)	FI (g/12 h)
Sham	2992 ± 154$^{\#}$	79 ± 32$^{\#}$	1.53 ± 0.23$^{\#}$
SCNx + unilat PVN damage	3130 ± 200	92 ± 41	2.27 ± 0.92*
SCNx + bilat PVN damage	2600 ± 167*	93 ± 34$^{\#}$	1.60 ± 0.38
SCNx + PVN/VMH damage	2579 ± 495*	73 ± 45	2.27 ± 0.55*
	24 h		
	VO2 (ml/kg)	Activity (bb)	FI (g)
Sham	76531 ± 3831	273 ± 123	4.73 ± 0.50
SCNx + unilat PVN damage	74849 ± 4302	166 ± 62*	4.51 ± 1.43
SCNx + bilat PVN damage	61561 ± 4327*	154 ± 55*	4.13 ± 1.06
SCNx + PVN/VMH damage	62075 ± 12070*	131 ± 73*	4.69 ± 0.84

B

	Basal Rd (µmol/min/kg)	Hyperinsulinemic Rd (µmol/min/kg)	Hyperinsulinemic EGP (µmol/min/kg)
Sham	53.6 ± 16.6	83.9 ± 23.8	8.8 ± 12.2
SCNx + unilat PVN damage	61.1 ± 17.9	77.5 ± 31.6	36.2 ± 29.3*
SCNx + bilat PVN damage	61.0 ± 9.5	61.7 ± 21.1*	37.8 ± 18.3*
SCNx + PVN/VMH damage	51.2 ± 24.4	55.6 ± 19.8*	40.2 ± 23.1*

C

	Insulin (ng/ml)	
	Basal	Hyperinsulinemic
Sham	0.5 ± 0.3	5.2 ± 1.1$^{\$}$
SCNx + unilat PVN damage	0.8 ± 0.3	4.8 ± 1.1$^{\$}$
SCNx + bilat PVN damage	2.9 ± 1.5**	8.6 ± 2.4$^{\$}$**
SCNx + PVN/VMH damage	1.4 ± 0.6**	4.6 ± 1.7$^{\$}$

Discussion

This study addressed the effects of thermic, bilateral ablation of the SCN on energy metabolism and insulin sensitivity in mice. We show that exclusive lesions of the SCN disrupt the circadian pattern of energy intake, activity, and energy expenditure, as expected. However, although selective SCN ablation resulted in only mild overweight status compared with sham mice, hepatic insulin sensitivity was severely impaired. Therefore, disturbed SCN function has profound metabolic effects.

Anatomically, the SCN is connected with the VMH and the PVN through the subparaventricular zone of the hypothalamus.[22] In line with previous studies, mice with collateral damage to the PVN or VMH developed severe obesity and insulin resistance.[23-28] Interestingly, unilateral PVN damage in SCN lesioned mice did not result in additional overweight status, indicating that PVN damage results in obesity only in the case of bilateral damage. In light of our data, it is of utmost importance to ascertain correct and exclusive lesions of the SCN to study the role of the circadian pacemaker in the context of metabolism. However, the low success rate (~30%) of the operation in a previous study[10] and this study (~20%) limits the potential of this method to study the role of the SCN in metabolism, increasing the value of the current study.

The mild increase in weight gain in mice with exclusive SCN lesions compared with sham mice (+17%) is incontrast to a previous finding in rats.[10] In rats, lesions of the SCN did not induce an increase in body mass, whereas the effect on body composition was not determined. This discrepancy may be attributable to the use of the wellstudied obesogenic C57Bl/6J mouse strain[29-31] in our study, whereas the rat study was performed in Wistar rats. Wistar rats only become obese on hypercaloric food intake.[32]

Previously, it has been shown that the SCN is involved in the regulation of energy homeostasis in mice[33] and rats.[34] In the current study, indirect calorimetry and metabolic cage analysis revealed that ablation of the SCN induced a loss of circadian rhythm in oxygen consumption and activity without affecting the 24 hr average levels. This loss of circadian rhythm in homeostasis is in line with previous findings, in which SCN lesions eliminated a wide range of rhythms, including leptin.[10] Although total food intake over a period of 1 day and 1 night was reduced by 26%, SCN lesioned mice consumed more during the light part of the day compared with sham mice (46% *vs.* 32% of total food intake). Recently, it has been shown that mice and rats fed only during the day gained significantly more weight than mice fed only at night.[35-37] In these studies, obesity resulted from dissociation between the timing of food intake and the intrinsic rhythm of energy expenditure and, thus, animals were eating "against their clock time." In our study, the protocol was essentially different and animals were not forced to eat at the other part of the cycle, but rather their circadian system was impaired. Therefore, it remains unclear how the mice with selective SCN lesions developed mild overweight because they ate less than the controls and showed the same level of overall activity. Interestingly, results comparable with ours were found in continuous light–exposed mice.[38] Continuous light is a strong disruptor of SCN synchrony. When applied for prolonged periods of time, mice become arrhythmic. In continuous light–induced arrhythmic mice, total activity and food intake levels did not differ from that of mice in light/dark. Even though energy balance over 24 hrs was similar, mice exposed to continuous light displayed impaired glucose tolerance. It has been shown that the SCN exerts excitatory effects on thermogenesis by brown adipose tissue,[39] and the mild weight

gain in mice with a selective SCN lesion could be the result of reduced brown adipose tissue activity. Alternatively, changes in time distribution of food intake may play a role, which remains an interesting topic for future studies.

Indirect calorimetry and metabolic cage analysis further revealed that SCN lesioned mice had lower respiratory exchange ratio during the day and during the night as compared with sham mice. This shows that SCN ablation results in lower relative carbohydrate oxidation rates and, conversely, higher fat oxidation rates compared with sham mice. These data suggest that the oxidative response to food intake is less directed to carbohydrate metabolism is lesioned, a hallmark for an impaired metabolic flexibility.[40] Impaired metabolic flexibility has been associated with impaired insulin sensitivity in humans.[41]

Compared with the sham-operated mice, SCN lesioned mice were hyperglycemic and hyperinsulinemic in the postabsorptive state. Furthermore, SCN lesioned mice had increased fasting FFA levels, suggesting a possible removal of inhibitory input from the SCN to the adipose tissue, thereby increasing the basal rate of lipolysis. Increased circulating levels of FFA have been implicated as a possible pathway for development of insulin resistance in obesity.[42] Insulin sensitivity shows a circadian pattern in humans[43] as well as in rodents,[44] with insulin sensitivity being highest during the active period and lowest during the resting period. The protocol used in this study was such that the sham mice were clamped during their resting period, when insulin sensitivity is lowest, opting for conservative conclusions. Severe hepatic insulin resistance, but not peripheral insulin resistance, was present even though body fat mass was increased only minimally. The SCN is crucial for the circadian control of glucose production and glucose uptake.[45-48] Furthermore, there is a direct control of hepatic glucose metabolism resulting from cross-communication of the SCN and PVN, further mediated by innervation of the liver.[49] Therefore, it is likely that the impaired hepatic insulin sensitivity found in the SCN lesioned mice is, at least in part, a direct result of the disrupted SCN-mediated control of glucose and FFA metabolism.

All SCN lesioned mice with collateral damage to PVN or VMH had development of hepatic insulin resistance. Furthermore, SCN lesioned mice with bilateral PVN damage and SCN lesioned mice with PVN or VMH damage showed peripheral insulin resistance, most likely as a result of increased fat mass in these mice.

In conclusion, we demonstrate that exclusive deletion of the SCN induces loss in circadian rhythms in energy metabolism and food intake. Although ablation of the SCN resulted in only mild overweight status, SCN lesioned mice were severely insulin resistant in the liver. Great care was taken to distinguish between exclusive SCN lesioned mice from mice that had collateral damage to the PVN and VMH areas, because the latter resulted in severe obesity. It was previously shown that mice with mutations in clock genes have altered energy homeostasis and glucose metabolism.[14,50] Together, the data from several studies provide solid evidence that the SCN is crucially involved in the maintenance of energy balance and hepatic insulin sensitivity.

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