THE SUPRACHIASMATIC NUCLEUS CONTROLS CIRCADIAN ENERGY METABOLISM AND HEPATIC INSULIN SENSITIVITY

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ABSTRACT

The suprachiasmatic nucleus (SCN) functions as a central pacemaker in the circadian system. Disturbances in the circadian system are associated with the development of type 2 diabetes mellitus (T2DM). Here, we studied the direct contribution of the SCN in the development of insulin resistance. Bilateral microlesions of the SCN in male C57Bl/6J mice resulted in loss of circadian rhythm in locomotor activity. Histological verification by immunocytochemistry was performed to analyze the precision of the lesion. Exclusive SCN lesioned (SCNx) mice 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 (VMH) and paraventricular nucleus (PVN) showed severe obesity and insulin resistance. In mice with exclusive SCN ablation, indirect calorimetry/metabolic cage analysis four weeks after lesioning, revealed a loss of circadian rhythm in oxygen consumption and food intake. Hyperinsulinemic-euglycemic clamp analysis showed that the glucose infusion rate, required to maintain euglycemia, was significantly lower in SCNx mice compared to sham operated mice (-63%). While insulin potently inhibited endogenous glucose production (-84%), this was greatly reduced in SCNx mice (-7%), indicating severe hepatic insulin resistance. Our data indicate that malfunction of the SCN plays an important role in the development of insulin resistance, and underscore a direct role of the central pacemaker in the ontogeny of metabolic disorders.

INTRODUCTION

Obesity and type 2 diabetes mellitus (T2DM) 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 h rhythms in a wide variety of physiological and behavior 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 and BMAL1, Period (Per) and Cryptochrome (Cry) (1). The rhythms of the SCN are synchronized to the environmental 24 h 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 from 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 develop a metabolic syndrome of hyperleptinemia, hyperlipidemia, hepatic steatosis and hyperglycemia (14). As 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, therefore, 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 T2DM, we performed bilateral microlesions of the SCN in male C57Bl/6J mice. Since 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 (SCNx) mice and mice with collateral damage to surrounding nuclei. We studied the effects of SCN ablation on energy expenditure, food intake and locomotor activity by analysis in metabolic cages employing indirect calorimetry. In addition, we assessed changes in body composition by dual energy x-ray absorptiometry (DEXA). Insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamp analysis. We show that selective ablation of the SCN results in the development of severe hepatic insulin resistance.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Animal Ethic Committee from the Leiden University Medical Center (Leiden, the Netherlands) in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Mice.

Male C57Bl/6J mice were obtained from Charles River Laboratories at an age of 9 weeks and acclimatized up to an age of 13 weeks at the Leiden University Medical Center animal facility. Mice were housed individually in a controlled environment (21°C, 40-50% humidity) under a 12 h/12 h light/dark cycle (07:00-19:00) unless otherwise mentioned. Food (chow, RM3, Special Diet Services, Sussex, UK) and tap water were available *ad libitum* during the whole experiment. Body weight was monitored weekly for all individual mice. Monitoring started 2 weeks prior to SCN lesions, and continued throughout the experiment.

SCN lesioning

At the age of 13 weeks, bilateral ablation of the SCN was performed as described before (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 Co, Wood Dale, IL, USA). 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 (Paxinos Mouse Brain Atlas, Franklin 2001). Bilateral SCN lesions were made by passing a 0.6 mA current through the electrode for duration of 10 s. The sham lesioned mice underwent the same operation, but no current was passed through the electrode.

Circadian rhythm analysis

Following SCN lesioning, all mice were housed in constant dark (DD) for 10 consecutive days to determine circadian rhythmicity 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, IL, USA) 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 M phosphate buffer (pH 7.4) at 4°C. For cryoprotection, the brain tissue was equilibrated for 48 h with 30% sucrose in 0.1 M Tris-buffered saline (TBS) before sectioning. Thereafter, the brain tissue was cut into 30 µm sections and divided into two equal vials for immunocytochemical stainings. The two vials of brain sections were incubated overnight at 4°C with either rabbit anti-vasopressin or rabbit anti-VIP primary antibodies. Sections were then rinsed in 0.1 M TBS, incubated 1 h in biotinylated goat anti-rabbit IgG, and subsequently for 1 h in avidin-biotin complex (ABC, Vector Laboratories, Inc., Burlingame, CA, USA). The reaction product was visualized by incubation in 1% diaminobenzidine (DAB) with 0.01% hydrogen peroxide for

5-7 min. Nickel ammonium sulphate (0.05%) was added to the DAB solution to darken the reaction product (DAB/Ni). All sections were mounted on gelatine-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/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 Ohio, USA) (19-21). A period of 24 h 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 and water intake and locomotor 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

Five weeks after SCN lesioning, hyperinsulinemic-euglycemic clamp experiments were performed as previously described (20;22-24) to determine insulin sensitivity. Clamp experiments were performed after an overnight fast. Mice were anesthetized by i.p. 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). Anaesthesia as well as body temperature was maintained throughout the procedure. At the end of the basal and the hyperinsulinemic period, hematocrit values were determined to ensure that the mice were not anemic. During the basal period, 3-^{3}H-glucose was infused in the tail vein at a constant rate of 0.8 µCi/h (specific activity, 9.6 GBq/mmol; Amersham, Little Chalfont, UK). After 50 and 60 min of infusion, basal parameters of glucose and insulin were determined. Glucose rate of disposal (Rd, in µmol/min.kg) was determined as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of 3-^{3}H-glucose (dpm/µmol) at both time points. The hyperinsulinemic period was started by a bolus of insulin (4.5 mU, Actrapid; Novo Nordisk, Chartres, France). Subsequently, insulin was infused at a constant rate (3.5 mU/min). Comparable to the basal period, 3-^{3}H-glucose was infused at a rate of 0.8 µCi/h. A variable infusion of 12.5% D-glucose (in PBS) was also started to maintain euglycemia, as measured by hand every 10 min (AccuCheck, Roche Diagnostics, The Netherlands). After reaching a steady state glucose infusion rate (GIR) 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. Following 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 was analyzed according using the software and recommendations of the manufacturer. Subsequently, the mice were sacrificed.

Plasma analysis

Blood samples were taken from the tail tip into chilled capillaries coated with paraoxon to prevent *ex vivo* lipolysis. The tubes were placed on ice and centrifuged at 4°C. Plasma levels of free fatty acids (FFA) and glucose were determined using commercially available kits and standards according to the instructions of the manufacturer (Instruchemie, Delfzijl, The Netherlands) in 96-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Plasma insulin concentrations were measured by ELISA (Crystal Chem Inc., Downers Grove, USA). For measurement of plasma 3-^{3}H-glucose, trichloroacetic acid (final concentration 2%) was added to 10 µl plasma to precipitate proteins using centrifugation. The supernatant was dried to remove water and resuspended in milliQ. The samples were counted using scintillation counting (Packard Instruments, Downers Grove, USA).

Statistical analysis

Statistical analysis was performed using SPSS 17 for Windows (SPSS Inc, Chicago, IL, USA). 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 locomotor 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 6 mice (example shown in Fig. 2B). In addition to ablation of the SCN, 4 other mice had unilateral damage to the paraventricular nucleus (PVN), 10 other mice had bilateral damage to the PVN and 9 other mice had damage to both the PVN and the ventromedial hypothalamus (VMH). Sham operated controls (n=17) did not reveal damage to any of the aforementioned brain areas (example shown in Fig. 2A).

Selective SCN lesions

Body weight and composition

Five weeks after SCN lesioning, body mass of mice with an exclusive SCN lesion was 17% higher compared to the sham mice (24.9 ± 1.2 *vs.* 29.2 ± 1.9 g, $P < 0.01$, Fig. 3). Assessment of body composition by DEXA scan analysis revealed that fat mass was significantly higher in SCNx mice compared to 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, ns). This indicates that SCN ablation results in only mild overweight compared to sham mice.

Indirect calorimetry

Metabolic cage data on oxidative metabolism, food intake and spontaneous physical activity data were obtained over a period of minimal 4 consecutive days and were analyzed separately for day and night. During the night, oxygen consumption rates were higher compared to the day in sham mice (3386 ± 174 *vs.* 2992 ± 155 ml/kg/h, $P < 0.01$, Fig. 4A). This circadian rhythm in

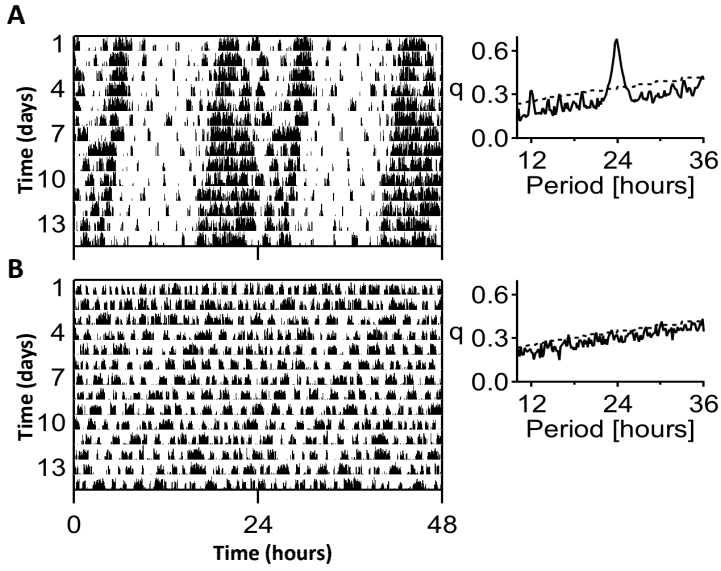

Fig. 1. Representative double-plotted actograms analyses of sham operated (A) and SCN lesioned (SCNx, B) mice under constant dark conditions. Each line of the double-plotted actograms represents 48 h.

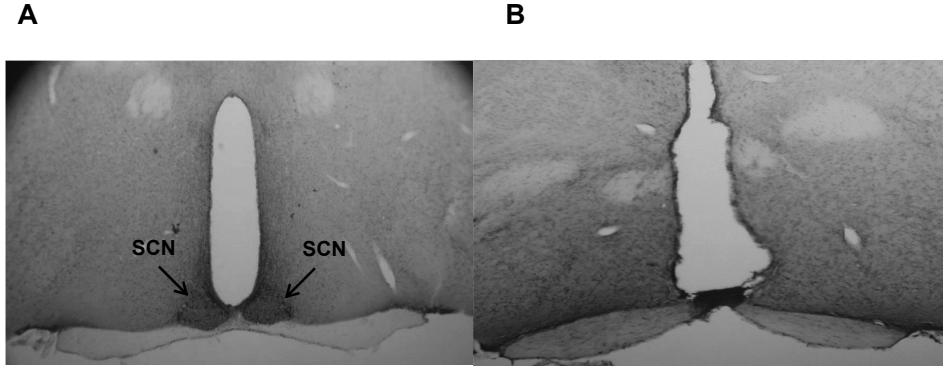

Fig. 2. Microphotographs of mouse brain sections at the level of the SCN. Middle region of the SCN is shown for sham operated (A) and SCN-lesioned (SCNx, B) mice. Note the relatively small size of the SCN lesion, leaving the paraventricular nuclei and ventromedial hypothalamus intact (B).

oxygen consumption was lost in SCNx mice, resulting in higher oxygen consumption during the day in SCNx mice compared to sham mice (3321 ± 252 *vs*. 2992 ± 155 ml/kg/h, $P < 0.01$). Total 24 h oxygen consumption was not different between sham and SCNx mice (76531 ± 3854 *vs*. 79518 ± 7595 ml/kg/day, ns). During the night, sham mice were more active compared to the day (195 ± 68 *vs*. 79 ± 33 beam breaks (bb), $P < 0.01$, Fig. 4B). In line with the oxygen consumption rates, circadian pattern in locomotor activity was lost in SCNx mice (123 ± 48 *vs*. 128 ± 38 bb, ns). This loss in activity

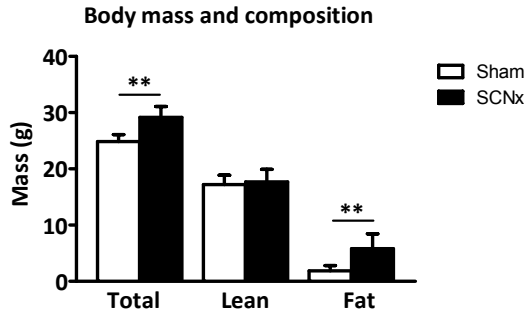

Fig. 3. Body mass and composition of sham (white bars) and SCN lesioned (SCNx, black bars) mice at the time of the hyperinsulinemic-euglycemic clamp. Total body mass, lean body mass and fat mass were determined. Data is represented as mean ± SD, ** $P < 0.01$.

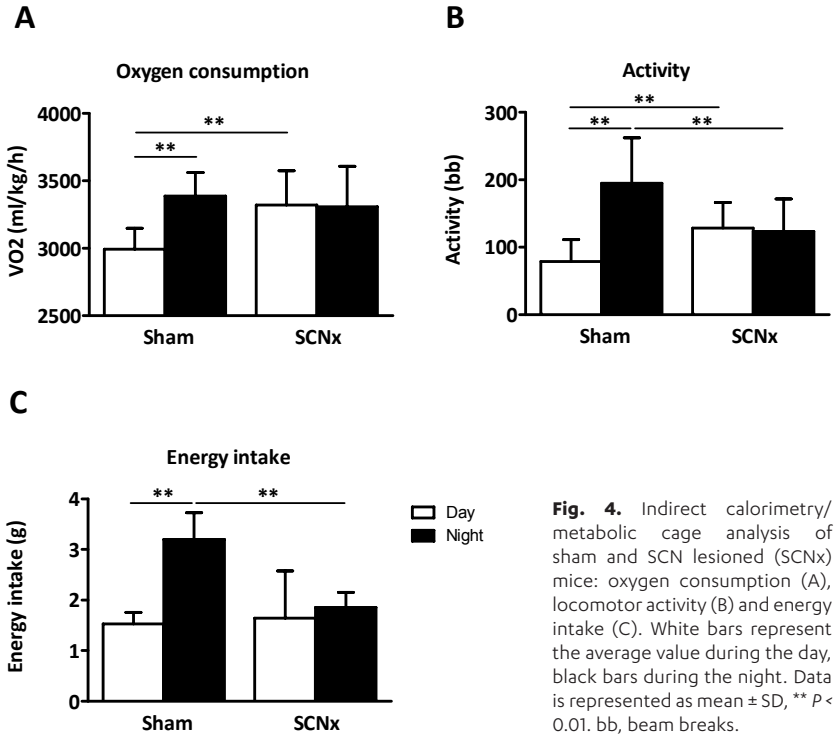

Fig. 4. Indirect calorimetry/ metabolic cage analysis of sham and SCN lesioned (SCNx) mice: oxygen consumption (A), locomotor activity (B) and energy intake (C). White bars represent the average value during the day, black bars during the night. Data is represented as mean ± SD, ** $P < 0.01$. bb, beam breaks.

pattern in SCNx mice, resulted in increased activity levels during the day (128 ± 38 *vs*. 79 ± 33 bb, $P < 0.01$) and reduced activity levels during the night compared to sham mice (123 ± 48 *vs*. 195 ± 68 bb, $P < 0.01$). Total 24 h activity, however, was not different between sham and SCNx mice (273 ± 96 *vs*. 251 ± 81 bb, ns). 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 4C), whereas SCNx mice consumed only 54% during the night (night *vs*. day; 1.9 ± 0.3 *vs*. 1.6 ± 0.9 g, ns). This resulted in reduced food intake during the night for SCNx mice compared to sham mice (1.9 ± 0.3 *vs*. 3.2 ± 0.5 g, $P < 0.01$). Furthermore, total 24 h food intake was also reduced for SCNx mice compared to sham mice (3.5 ± 1.2 *vs*. 4.7 ± 0.5 g, $P < 0.01$). Calculating the respiratory exchange rate (RER) showed that SCNx mice had a lower RER compared to 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.

Hyperinsulinemic-euglycemic clamp studies

Insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp analysis in overnight fasted mice. Hematocrit levels were similar at the basal and the hyperinsulinemic period for sham and SCNx mice, indicating that the mice were not anemic. At the start of the clamp, insulin, glucose and FFA plasma levels were significantly higher in SCNx mice compared to sham controls (table 1). During the hyperinsulinemic period, insulin levels were increased to a similar extent for both the sham and SCNx mice (table 1). All mice were clamped at their individual fasting plasma glucose level. At the end of the hyperinsulinemic period, circulating glucose levels were comparable to the fasting levels for sham as well as for SCNx mice, resulting in significantly higher glucose levels in the hyperinsulinemic period for SCNx mice compared to sham mice (table 1, Fig. 5A). At the end of the clamp, FFA levels were decreased compared to basal plasma levels in sham and SCNx mice, although this failed to reach statistical significance in SCNx mice (P = 0.08). GIR needed to maintain euglycemia were determined over the final 20 min of the clamp studies at stable plasma glucose values and were significantly lower in SCNx mice compared to sham controls (27.6 ± 19.5 *vs*. 74.7 ± 21.1 µmol/min/kg, $P < 0.01$, Fig. 5B, C).

In the basal period, endogenous glucose production (EGP), which equals glucose disposal, was not different between sham and SCNx mice (53.6 ± 16.6 *vs*. 65.0 ± 8.6 µmol/min/kg, ns, Fig. 6A). In the hyperinsulinemic-euglycemic period, glucose disposal was increased by 57% in the sham mice compared to the basal period (83.9 ± 23.8 *vs*. 53.6 ± 16.6 µmol/min/kg, $P < 0.01$) and by 27% in the SCNx mice, although this failed to reach statistical significance (82.8 ± 15.9 *vs*. 65.0 ± 8.6 µmol/min/kg, $P = 0.06$). Hyperinsulinemic glucose disposal rate was not significantly different between sham and SCNx mice. In the hyperinsulinemic-euglycemic period, EGP was

Table 1. Body mass and hyperinsulinemic-euglycemic clamp parameters of sham and SCN lesioned (SCNx) mice. FFA, free fatty acids. Data are represented as mean ± SD, $^{\$}$ $P < 0.05$ basal *vs*. hyperinsulinemic-euglycemic clamp period, * $P < 0.05$ *vs*. sham, ** $P < 0.01$ *vs*. sham.

	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

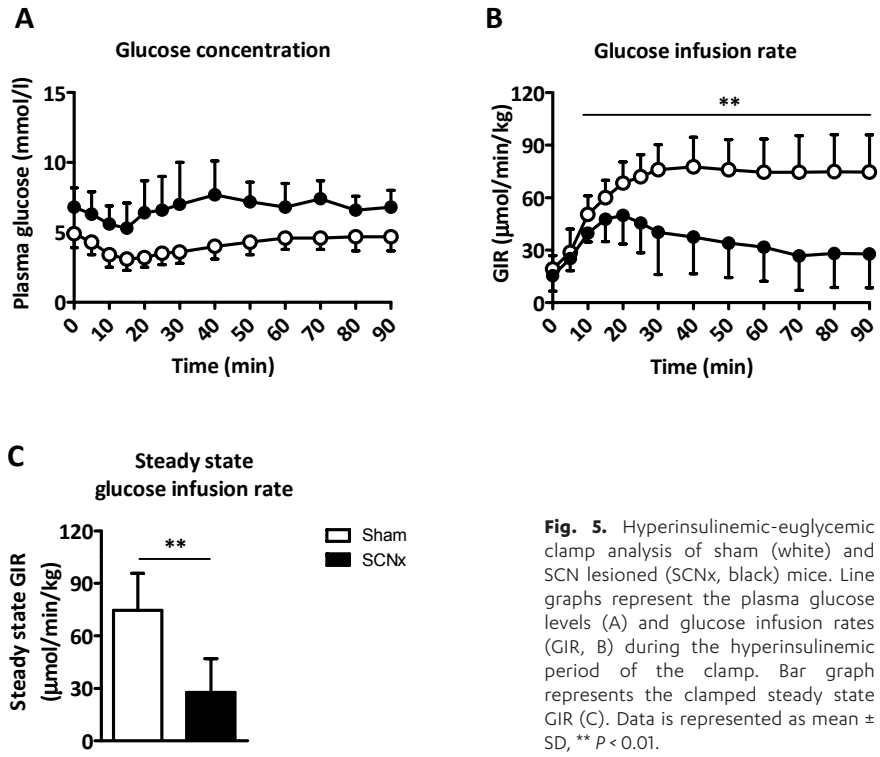

Fig. 5. Hyperinsulinemic-euglycemic clamp analysis of sham (white) and SCN lesioned (SCNx, black) mice. Line graphs represent the plasma glucose levels (A) and glucose infusion rates (GIR, B) during the hyperinsulinemic period of the clamp. Bar graph represents the clamped steady state GIR (C). Data is represented as mean ± SD, ** $P < 0.01$.

decreased by 84% in sham operated mice compared to the basal period (8.8 ± 12.2 *vs*. 53.6 ± 16.6 µmol/min/kg, $P < 0.01$, Fig. 6B), whereas EGP was decreased by only 7% in SCNx mice (60.3 ± 25.8 *vs*. 65.0 ± 8.6 µmol/min/kg, ns). These data indicate that bilateral ablation of the SCN induces severe hepatic insulin resistance.

SCN lesion with collateral hypothalamic damage

SCN lesioned mice with collateral damage to hypothalamic nuclei involved in metabolism were analyzed separately. SCNx mice with additional, but selective unilateral damage to the PVN had modestly increased body mass compared to sham lesioned mice (29.9 ± 3.6 *vs*. 24.9 ± 1.2 g, $P < 0.01$, Fig. 7A), 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, ns). SCNx 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 also coincided with a decrease in lean mass (13.6 ± 2.2 *vs*. 17.2 ± 1.7 g, $P < 0.01$). SCNx mice with collateral damage to both the PVN and the VMH developed extreme obesity compared to 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, ns).

Insulin sensitivity in SCNx mice with collateral damage was determined by hyperinsulinemic-euglycemic clamp analysis. Glucose infusion rates (GIR) needed to maintain euglycemia, as determined over a period of 20 min at stable plasma glucose values, were significantly

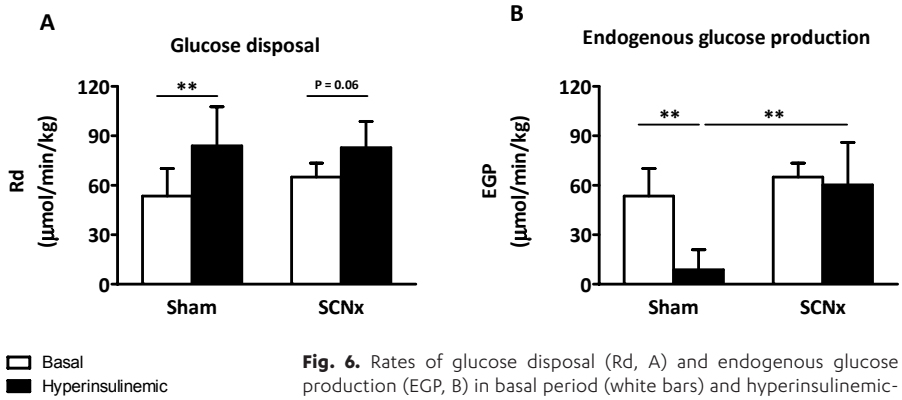

Fig. 6. Rates of glucose disposal (Rd, A) and endogenous glucose production (EGP, B) in basal period (white bars) and hyperinsulinemic-euglycemic clamp period (black bars) of sham and SCN lesioned (SCNx) mice. Data is represented as mean ± SD, ** $P < 0.01$.

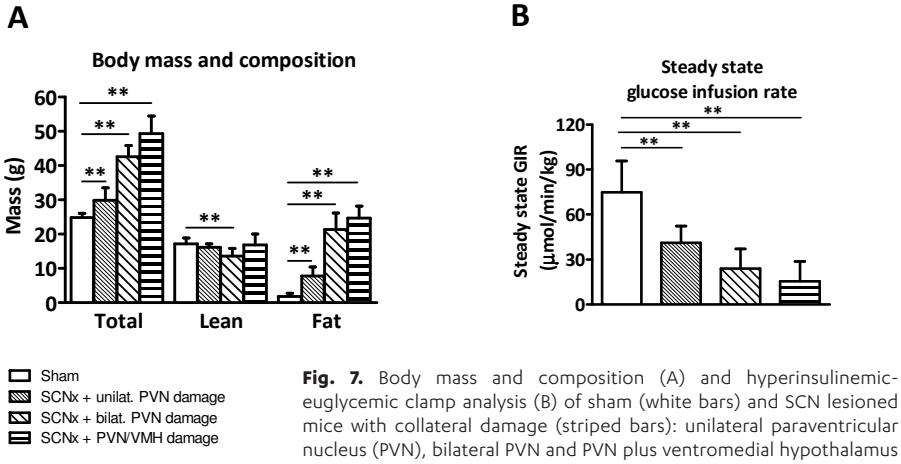

Fig. 7. Body mass and composition (A) and hyperinsulinemic-euglycemic clamp analysis (B) of sham (white bars) and SCN lesioned mice with collateral damage (striped bars): unilateral paraventricular nucleus (PVN), bilateral PVN and PVN plus ventromedial hypothalamus (VMH). Data is represented as mean ± SD, ** $P < 0.01$.

lower in all SCNx mice with collateral damage (SCNx + unilateral PVN damage; 41.3 ± 11.0 µmol/min/kg, SCNx + bilateral PVN damage; 23.9 ± 13.1 µmol/min/kg, SCNx + PVN/VMH damage; 15.4 ± 13.5 µmol/min/kg) compared to sham controls (74.7 ± 21.1 µmol/min/kg, Fig. 7B), suggesting insulin resistance.

DISCUSSION

This study addressed the effects of thermic, bilateral ablation of the SCN on energy metabolism and insulin sensitivity in mice. Since adjacent hypothalamic nuclei, such as PVN and VMH, are involved in energy metabolism, great care was taken to distinguish between SCN lesioned mice and mice with collateral damage to these nuclei. We show that lesioning of the SCN

disrupts the circadian pattern of energy intake, activity and energy expenditure, as expected. However, although selective SCN ablation resulted in only mild overweight compared to 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 (25). In mice with collateral damage to the PVN and/or VMH, the obesity and insulin resistance phenotypes were very profound. This is in line with previous data showing that lesions of the PVN (26;27) and VMH (28-31) result in hyperphagia, obesity and obesity-related hyperinsulinemia. Interestingly, unilateral PVN damage in SCN lesioned mice resulted in only mild overweight, comparable to SCN lesion alone. Apparently, PVN damage results in obesity only when the PVN is damaged bilaterally. In light of our data, it is of utmost importance to ascertain correct and exclusive lesioning of the SCN to study the role of the circadian pacemaker in the context of metabolism. In the current study we included 6 mice with exclusive and total SCN lesions, 23 SCN lesioned mice with collateral damage and 17 sham lesioned mice.

The mild increase in weight gain in mice with exclusive SCN lesions compared to sham mice (+17%) is in contrast to a previous finding in rats (10). In rats, lesioning the SCN did not induce an increase in body mass, whereas the effect on body composition was not determined. This discrepancy in body weight gain between the previous study and our study may be due to the use of the obesogenic C57Bl/6J mouse strain in our study, whereas the rat study was performed in Wistar rats. Wistar rats only become obese upon hypercaloric food intake (32). We studied the contribution of the SCN in the circadian regulation of energy metabolism and insulin sensitivity in C57Bl/6J mice, a mouse strain shown to be an excellent model to study development of obesity en insulin resistance (33-35).

Previously, it has been shown that the SCN is involved in the regulation of energy homeostasisin mice (36) and rats (37). In the present study, indirect calorimetry/metabolic cage analysis revealed that ablation of the SCN induced a loss of circadian rhythm in oxygen consumption and physical activity without affecting the 24 h average level of oxygen consumption or activity. This loss of circadian rhythm in homeostasis is in line with previous findings, where SCN lesions eliminated a wide range of rhythms, including leptin (10). Although total food intake over a period of one day and one night was reduced by 26% in SCN lesioned mice, the SCN lesioned mice consumed more during the light part of the day compared to 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 (38;39). In another study, the time of intake of a high-fat diet was a determinant of weight gain, adiposity, glucose intolerance, hyperinsulinemia and hyperleptinemia (40). In the aforementioned studies, obesity resulted from a 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, on the other hand, the protocol was essentially different, and animals were not eating at the other part of the cycle, but rather, their circadian system was impaired. Therefore, it remains unclear how the mice with selective SCN lesion developed mild overweight. As it has been shown that the SCN has exerts excitatory effects on thermogenesis

by brown adipose tissue (BAT) (41), the mild weight gain in mice with a selective SCN lesion could be the result of reduced BAT activity.

Indirect calorimetry/metabolic cage analysis further revealed that SCN lesioned mice had lower RER during the day as well as during the night as compared to sham mice. This shows that SCN ablation results in lower relative carbohydrate oxidation rates and, conversely, higher fat oxidation rates compared to sham mice. These data suggest that the oxidative response to food intake is less directed to carbohydrate metabolism when SCN is lesioned, a hallmark for an impaired metabolic flexibility (42). Impaired metabolic flexibility has been associated with impaired insulin sensitivity in humans (43).

We assessed insulin sensitivity by hyperinsulinemic-euglycemic clamp analysis. Compared to the sham operated mice, SCN lesioned mice were hyperglycemic and hyperinsulinemic in the postabsorptive state. Furthermore, at the start of the clamp, the SCNx mice had increased FFA levels compared to sham animals, 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 developing insulin resistancein obesity (44). 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). It is, therefore, likely that the impaired hepatic insulin sensitivity found in the SCNx groups is, at least in part, a direct result of the disrupted SCN mediated control of glucose and FFA metabolism.

Assessment of insulin sensitivity by hyperinsulinemic-euglycemic clamp technique revealed that all SCNx mice with collateral damage to PVN and/or VMH were very insulin resistant compared to sham mice. Surprisingly, SCNx with or without collateral damage were insulin resistant to a similar extent, even though SCNx mice with bilateral PVN damage and SCNx mice with PVN and VMH damage were much more obese that SCNx mice with unilateral PVN damage or mice with a clean SCN lesion, i.e., the amount of adiposity is not a direct determinant of the insulin resistance. These data further support the conclusion that, independent of obesity, the SCN output is crucial in determining hepatic insulin sensitivity.

We have clamped all mice at their respective basal glucose levels, as it has been shown that alterations in basal fasting glucose levels by itself are sufficient to affect insulin sensitivity (50). As SCN lesioned mice had increased basal glucose levels compared to the sham operated mice, the hyperinsulinemic glucose levels were also higher in the SCNx mice. However, clamping the mice at similar glucose levels, would have resulted in even larger differences in glucose infusion rates between sham and SCNx mice. Therefore, the degree of insulin resistance present in the SCNx mice may even have been underestimated using our clamp protocol.

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, SCNx 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, as 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;51). Together, the data from the several studies provide solid evidence that the SCN is crucially involved in the maintenance of energy balance and hepatic insulin sensitivity.

ACKNOWLEDGEMENTS

This work was supported by grants from TI Pharma (TIP project T2-105, to J.A. Romijn), the Netherlands Heart Foundation (NHS project 2007B81, to J.A. Romijn), the Dutch Diabetes Research Foundation (DFN project 2007.00.010, to J.A. Romijn), the Center of Medical Systems Biology (CMSB project, to K.W. van Dijk), the Netherlands Consortium for Systems Biology (NCSB project, to K.W. van Dijk.) established by The Netherlands Genomics Initiative/ Netherlands Organization for Scientific Research (NGI/NWO), NWO ZonMW (Top Go grant 81802016, to J.H. Meijer; Top grant 91207036, to A. Kalsbeek) and the Dutch organization for scientific research (Clinical Fellows 90700195, to N.R. Biermasz). We thank H. Duindam, H. van Diepen, H. Post-van Engeldorp Gastelaars and R. van den Berg for excellent technical support.

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