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The Suprachiasmatic Nucleus Generates the Diurnal Changes in Plasma Leptin Levels

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ABSTRACT

At present it is not clear which factors are responsible for the diurnal pattern of plasma leptin levels, although the timing of food intake and circulating hormones such as glucocorticoids and insulin have both been proposed as independent determinants. In this study we show that ablation of the biological clock by thermal lesions of the hypothalamic suprachiasmatic nucleus (SCN) completely eliminates the diurnal pattern of plasma leptin levels. By contrast, removal of the diurnal corticosterone signal by adrenalectomy and corticosterone replacement did not affect diurnal plasma leptin levels. More importantly, removal of the nocturnal feeding signal by submitting the animals to a regular feeding schedule of six meals per day did not abolish the diurnal plasma leptin levels. However, both SCN lesions and the regular feeding schedule did cause an increase in the 24-h mean plasma leptin levels. As neither rhythmic feeding, insulin, or corticosterone signals can completely explain the diurnal plasma leptin rhythm, we conclude that biological clock control of the sympathetic input to the adipocyte is essential for regulation of the daily rhythm in leptin release. (*Endocrinology* **142:** 2677–2685, 2001)

LEPTIN IS SECRETED by adipose tissue and circulates as a hormone in the blood in proportion to the amount of body fat. Moreover, in addition to its role as a lipostatic signal contributing to body weight regulation, recent energy intake is another determinant of circulating leptin levels (1–3). The discovery of the food intake-reducing properties of leptin (4, 5) boosted the research into the (neural) pathways underlying leptin signaling. It became clear that the lipostatic, behavioral, neuroendocrine, and autonomic effects of leptin are predominantly mediated by the brain through binding of leptin to its hypothalamic receptors (6–8). Despite the major interest in afferent leptin signaling to the brain, efferent signals from the central nervous system that mediate leptin release have not been elucidated.

Blood levels of leptin follow a diurnal rhythm in healthy lean, obese, and type II diabetic subjects (9–12). The genesis of this diurnal pattern of leptin levels has not been elucidated. In fact, it is not even clear whether, like many other hormonal rhythms, the diurnal plasma leptin rhythm is under direct control of the biological clock or, conversely, whether it is a mere consequence of circadian rhythms in hormone release and/or behavior. Meal shifts entrain the diurnal rhythm of leptin secretion, suggesting that daytime feeding contributes to the nocturnal increase in plasma leptin levels (13–16). This feeding effect would be in accordance with the acute effect of fasting and insulin on leptin secretion (1, 17). On the other hand, diurnal leptin release was maintained during prolonged hyperinsulinemia in normal human subjects (18), indicating that factors other than feeding behavior must also be involved. The inverse relationship between diurnal rhythms of leptin, on the one hand, and ACTH and cortisol, on the other, was apparent from the discovery of leptin (9, 19) and led to the suggestion that the circadian changes in cortisol might account for the circadian rhythm in leptin release (20, 21). However, experiments employing hydrocortisone infusions and observations in patients with Cushing's syndrome showed persistent leptin rhythms despite constant (high or low) levels of cortisol (22, 23). Another argument against a predominant role of either endogenous hormone or feeding rhythms in the control of diurnal leptin rhythmicity is that in both rodents and humans the highest leptin levels are found at night despite the fact that these species have contrasting patterns of feeding and adrenal activity.

In mammals (including humans) the biological clock is located at the base of the anterior hypothalamus in the suprachiasmatic nuclei (SCN) (24, 25). The SCN plays a key role in the induction, synchronization, and entrainment to the environmental light/dark cycle of circadian rhythms in behavioral, metabolic, and endocrine functions (24, 26). Disappearance, however, of the diurnal leptin rhythm after ablation of the SCN does not prove a direct clock control of leptin release, because in these SCN-ablated animals other rhythmic signals (*e.g.* feeding and glucocorticoids) have also become arrhythmic. Therefore, to investigate separately the respective roles of the SCN, adrenal glucocorticoids, and feeding rhythms in the control of leptin rhythmicity, we determined daily plasma leptin rhythms in four experimental groups of rats, *i.e.* unoperated intact animals, SCN-lesioned animals, adrenalectomized animals that had received a chronic corticosterone-releasing pellet, and animals on a regular feeding schedule.

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Materials and Methods

Animals

Male Wistar rats were obtained from a commercial supplier (Harlan, Horst, The Netherlands) and housed in a temperature-controlled environment (20−22 C) on a 12-h light/12-h dark schedule (lights on at 0700 h). Before the start of the experiments animals were allowed to acclimatize to the lighting schedule for several weeks with four animals in a cage. At least 1 week before the experiments started animals were moved to individual cages (38 × 26 × 16 cm). Water was available *ad libitum*. Food was available *ad libitum* too, except for the animals on the six meals per day feeding schedule. Postoperative care was provided after each surgical procedure by sc injection of Temgesic (Reckitt & Colman, Kingston-upon-Hull, UK; 0.3 ml/kg) after the animals awakened from anesthesia. The body weights of the animals were registered on a regular basis. Blood sampling was performed in four experimental groups of animals: 1) unoperated control animals, 2) SCN-lesioned animals, 3) adrenalectomized animals provided with corticosterone pellets, and 4) animals on a regular feeding schedule. All of the following experiments were conducted under the approval of the animal care committee of the Royal Netherlands Academy of Sciences.

SCN lesions

For SCN lesions 36 animals, 180–200 g, anesthetized with Hypnorm (Duphar, The Netherlands; 0.6 ml/kg, im), were mounted with their heads in a David Kopf Stereotact (Tujunga, CA) with the toothbar set at +5.0 mm, and sustained a bilateral lesion of the SCN (coordinates: 1.4 mm rostral to bregma; 1.1 lateral to the midline; 8.3 mm below the brain surface) using bilateral lesion electrodes, 0.2 mm in diameter, with temperature set at 85 C for 1 min (lesion generator, Radionics, Burlington, MA). This temperature was found empirically to result in lesions large enough to eliminate the SCN bilaterally, but small enough to leave surrounding hypothalamic brain structures, such as the paraventricular (PVN) and supraoptic (SON) nuclei intact. A drawback of this restricted lesion size is the limited yield of completely lesioned animals (*i.e.* ~30%). To restrict the number of (false positive) animals to be operated upon, initially the effectiveness of the lesions was checked by measuring daily water intake. After a rest period of 2 weeks to recover from the trauma of anesthesia and brain trauma, the daily water intake of the animals was measured during the middle 8 h of the light period. Only animals showing a daytime water intake of more than 30% of the total daily intake (in intact control animals this value is typically <10%) were assumed to have complete lesions of the SCN until the final immunocytochemical analysis and were allowed to enter the experiment. After the experiments, SCN lesions were checked histologically by immunocytochemical staining of hypothalamic sections for the presence of vasopressin (VP) and/or vasoactive intestinal polypeptide-containing cell bodies or fibers in the SCN area. If animals had cell bodies that stained positively for either VP or vasoactive intestinal polypeptide at the border of the lesion, or for immunoreactive fibers in SCN target areas such as the PVN, they were excluded from the data analysis (27–29).

Adrenalectomy (ADX)

Before ADX (via the flank approach) animals were anesthetized with a combination of Hypnorm and Dormicum (Roche, Mÿdrecht, The Netherlands; 0.4 ml/kg, sc). Together with bilateral adrenalectomy the animals received a jugular vein catheter (see below), and 100-mg slow release corticosterone pellets (Innovative Research of America, Sarasota Beach, FL) were implanted sc. The corticosterone pellets provided stable plasma concentrations of corticosterone for up to 60 days. Adrenalectomized animals (*i.e.* ADX+Cort animals) had free access to fresh water and 0.9% saline to compensate for mineral loss.

Regular feeding

For the rats entrained to the six meals per day feeding schedule (*i.e.* regular-fed animals) food pellets were available in metal food hoppers. A rat could gnaw off pieces of food through vertical stainless steel bars. Access to food could be prevented by a sliding door situated in front of the food hopper. Door opening and door closing were activated by an electrical motor and controlled by a clock. The rats were entrained to a feeding schedule of six 10-min meals spaced equally over the day-night cycle. Food became available at zeitgeber time (ZT) 2, ZT6, ZT10, ZT14, ZT18, and ZT22 (ZT12 being defined as the onset of the dark period).

Door-opening time was determined empirically in previous experiments, *i.e.* with longer door-opening times animals would start to skip one or more of the daytime meals. Animals did not save food for consumption during the 4-h intermeal period. Adaptation was considered completed when animals had learned to consume approximately 3 g at every meal. Despite the equally distributed feeding activity, general (locomotor) activity still showed a clear day-night rhythm, with the major part of activity occurring during the dark period. During the light period animals would wake up, eat, and resume sleeping. No anticipatory activity was observed. Diurnal glucose, insulin, and corticosterone profiles as a result of this feeding pattern have been reported previously (27, 30).

Blood sampling

For undisturbed blood sampling intact, regular-fed, SCN-lesioned, and ADX+Cort animals were provided with a permanent silicon heart cannula (id, 0.5 mm; od, 1.0 mm) at the entrance of the right atrium (vena cava) via an external jugular venotomy as described previously (27). The cannula was externalized and fixed on top of the skull with three screws and dental cement. Animals were operated on when their body weight was more than 325 g. After the operation a 10-day recovery period was included to allow complete reinstatement of circadian rhythms in activity, body temperature, and plasma corticosterone. During this period animals became accustomed to the experimental conditions in the Plexiglass cages designed to allow blood sampling under unrestrained conditions. All experiments were performed in the animal's own home cage. Tubings were threaded through a stainless steel support spring that was attached to the dental cement on the skull. The entire assembly was suspended from the animal by a counterbalanced beam and did not influence the animal's posture or motion. Daily changes in the plasma levels of leptin and corticosterone were assessed in three experimental sessions of 8 or 10 h, each 1 week apart, by taking a 0.6-ml blood sample every 2 h. Within one session sampling was initiated at different ZTs for the different animals.

Hormone assays

Blood samples were collected in heparinized tubes, placed on ice, and centrifuged, and plasma was stored at −20 C until assay. Plasma corticosterone was measured directly, without extraction, using a RIA from ICN Biomedicals, Inc. (Costa Mesa, CA), with iodinated corticosterone. From the samples 10 μl plasma were taken and diluted in 4 ml assay buffer. The interassay coefficient of variation for corticosterone was less than 4%, and the detection limit was 1 ng/ml. Plasma leptin concentrations were determined by RIA (Linco Research, Inc., St. Charles, MO) in 100 μl plasma. The detection limit was 0.5 ng/ml, and inter- and intraassay coefficients were 6% or less. The samples of the intact and SCN-lesioned animals were analyzed in one assay, as were the samples of the ADX+Cort and regular-fed animals.

RNA isolation and quantification

Experimental animals were killed at either ZT2 or at ZT14. Material from the regular-fed groups was lost. In a follow-up experiment, six groups of control rats (n = 8–10) were killed at 4-h intervals through a 24-h period starting at ZT2. From all animals epididymal fat depots were removed, snap-frozen in liquid N_2, and stored at −80 C. Total RNA was purified using TriPu; from this complementary DNA was synthesized using AMV reverse transcriptase and random primers (Roche). Leptin messenger RNA (mRNA) was quantified using real-time PCR in a LightCycler (SYBRgreen format, Roche). The primers used were 5′-gtgcctatccacaaagtccag-3′ (forward) and 5′-tcagcattcagggctaaggtc-3′ (reverse), giving a fragment of 441 bp. The homologous fragment was used as a standard. All mRNA levels were corrected for β-actin expression measured with the LightCycler as well, using the primer sequences 5′-gggtcagaaggattcctatg-3′ (forward) and 5′-ggtctcaaacatgatctggg-3′ (reverse).

Statistics

The mean ± SEM were calculated for plasma leptin, plasma corticosterone, and adipocyte mRNA concentrations for each sampling point. ANOVA with repeated measures was used to test for significant effects of ZT, group, or ZT *vs.* group interaction. ANOVA was followed by Student's *t* test (paired) to establish which time points differed significantly from trough values. The diurnal rhythm of each leptin profile was quantitatively described by cosinor analysis, as described previously (10, 11). In short, a cosine curve with a fixed period of 24 h (because of the 12-h light, 12-h dark schedule) was fitted through all data, and the following chronobiological parameters were obtained: acrophase (time of peak value), amplitude (half of the total predictable change in a rhythm), and mesor (the average value of a cosine curve fitted to the data; the mesor and 24-h mean leptin concentration are equivalent). Because of a considerable individual variation in the 24-h means of SCN-lesioned and regular-fed animals, leptin levels were first normalized by calculating the absolute change at different time points in relation to the 24-h mean. Due to its nonuniform distribution leptin mRNA values were compared using the nonparametric Kruskal-Wallis test. $P < 0.05$ was considered significant.

Results

Control animals

Complete 24-h plasma leptin profiles (*i.e.* 12 samples) were obtained in 7 intact control animals. Plasma leptin profiles showed a clear diurnal rhythm, with peak levels attained shortly after lights off (Fig. 1A). During the remainder of the dark period plasma leptin concentrations slowly decreased, but remained elevated compared with mean daytime levels (*i.e.* 1.3 ± 0.2 *vs.* 1.9 ± 0.2 ng/ml; light *vs.* dark, respectively). The diurnal trough occurred during the first half of the light period. ANOVA showed a very significant effect of time [$F(11,66) = 9.9$; $P < 0.001$]. Plasma corticosterone values in intact control animals varied between 5.1 ± 0.1 (at dawn) and 109.8 ± 40.0 ng/ml (at dusk).

Effect of SCN-lesions on diurnal plasma leptin levels

Thermal ablation of the SCN resulted in the absence of a diurnal drinking pattern in 12 of the 35 operated animals (1 animal died shortly after the operation). During the middle 8 h of the light period mean water intake in these animals was 39 ± 2% and 40 ± 1% of the total 24-h water intake during the third and fourth weeks after their operation, respectively. The 12 effectively SCN-lesioned animals were provided with jugular cannulas.

Complete 24-h plasma leptin profiles were obtained from 10 SCN-lesioned animals. Upon histological verification (Fig. 2) one more animal of the SCN-lesioned group had to be discarded due to an incomplete lesion. Mean plasma leptin concentrations in the 9 remaining SCN-lesioned animals ranged between 2.6 ± 0.2 and 3.7 ± 0.4 ng/ml (Fig. 1A), with individual samples varying between 0.5 and 5.4 ng/ml. ANOVA did not detect a significant effect of time [$F(11,88) = 1.4$; $P = 0.185$]. The lack of a significant diurnal fluctuation is also reflected in the absence of a day/night difference, *i.e.* mean day and night values were 3.3 ± 0.3 and 2.9 ± 0.2 ng/ml, respectively. When comparing SCN-lesioned and control animals, ANOVA indicated clear effects of group and time × group [$F(1,14) = 22.89$; $P < 0.001$ and $F(11,154) = 3.54$; $P < 0.001$], confirming the overall higher mean levels in SCN-lesioned animals and the changed diurnal profile, respectively. Plasma corticosterone values in SCN-lesioned animals varied between 13.7 ± 2.1 and 187.0 ± 50.7 ng/ml; the overall 24-h mean value (80.3 ± 10.8 ng/ml) was almost twice as high as that in control animals (39.5 ± 3.4 ng/ml; $P < 0.005$).

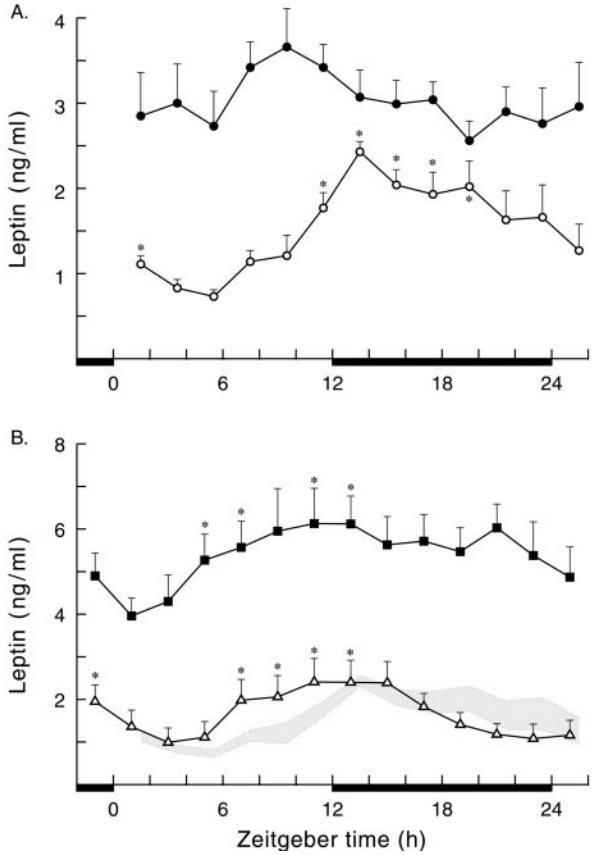

FIG. 1. Diurnal plasma leptin profiles in different experimental conditions. The diurnal plasma leptin profile (mean ± SEM) of nonoperated control animals (n = 7; ○) is compared with that in SCN-lesioned (n = 9; ●; A) or ADX+Cort (n = 8; △) and regular-fed (n = 6; ■) animals (B). *Asterisks* indicate plasma leptin values significantly different ($P < 0.01$) from trough values between ZT0 and ZT6. In B, the *shaded area* indicates the mean ± SEM for the nonoperated control animals.

Effect of feeding behavior on diurnal leptin levels

The daily food intake in the animals subjected to the six meals per day feeding schedule was 20.7 ± 0.6 g, which is approximately 80% of the daily food intake in control animals (*i.e.* 24.8 ± 0.8 g). Food intake was equally distributed over the six feeding opportunities offered; mean intakes during the ZT2, ZT6, and ZT10 meals were 3.6 ± 0.1, 3.2 ± 0.1, and 3.5 ± 0.2 g, respectively. Nocturnal intake was 11.2 ± 0.4 g, *i.e.* about 3.7 g/meal. Spillage was about 0.1 g/meal. During the six meals per day schedule, nocturnal food intake varied between 49–55% of the total food intake. When *ad libitum* feeding was reinstated at the end of the experiments, nocturnal feeding rapidly returned to approximately 85% of the total food intake.

Complete 24-h profiles of plasma leptin were obtained in six animals in the regular-fed group. Plasma leptin levels varied between 4.0 ± 0.4 and 6.0 ± 0.6 ng/ml (Fig. 1B), with

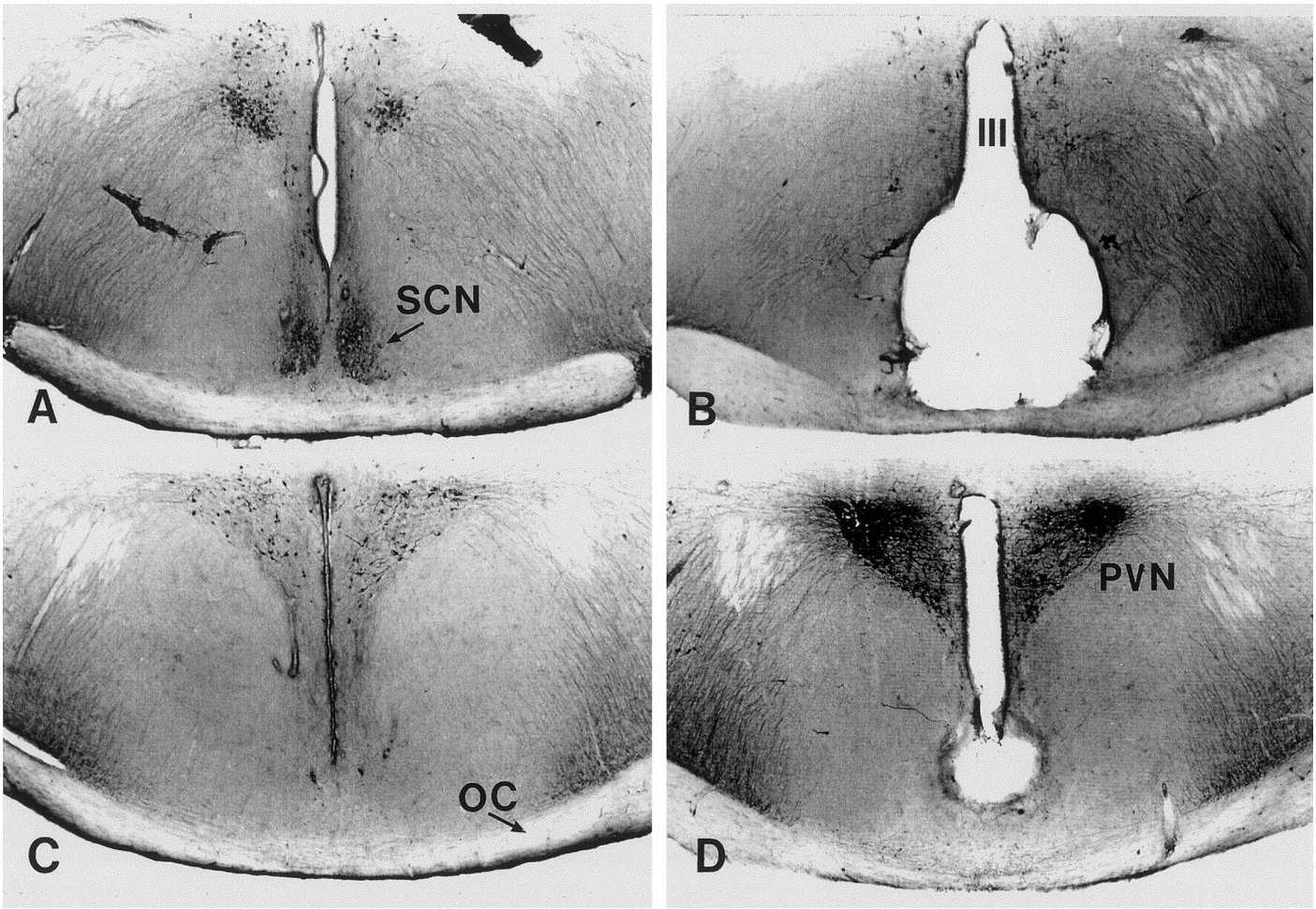

FIG. 2. Microphotographs of coronal rat brain sections at the level of the SCN stained for VP. Middle (A and B) and caudal (C and D) regions of the SCN are shown for nonoperated control (A and C) and SCN-lesioned (B and D) animals. Note the relatively small size of the SCN lesion, leaving the VP-containing supraoptic and paraventricular nuclei intact (B). Also at the caudal end of the SCN collateral damage is limited, leaving intact the (leptin receptor-containing) retrochiasmatic area (C). III, Third ventricle; OC, optic chiasm.

individual data varying between 2.2–10.3 ng/ml. ANOVA detected a significant effect of time [$F(14,70) = 3.87$; $P < 0.001$]. When comparing the 24-h plasma leptin profiles of the regular-fed animals with those of intact and SCN-lesioned animals, ANOVA indicated significant differences for both comparisons [intact: $F(11,121) = 2.83$; $P = 0.002$ and $F(1,11) = 44.64$; $P < 0.001$; SCN lesion: $F(11,143) = 2.32$; $P = 0.012$ and $F(1,13) = 17.70$; $P = 0.001$; effects of group *vs.* time and group, respectively]. Plasma corticosterone values in the regular-fed animals varied between 5.0 ± 0.1 and 153.5 ± 46.2 ng/ml; 24-h mean values (39.5 ± 2.1 ng/ml) were comparable to those in control animals.

Effect of (the lack of) diurnal changes in plasma corticosterone on plasma leptin levels

In the adrenalectomized animals complete 24-h plasma corticosterone and leptin profiles were obtained in eight of the nine animals. Plasma corticosterone values in the ADX+Cort animals varied between 13.8 ± 3.2 and 23.2 ± 6.0 ng/ml, with no apparent diurnal rhythmicity. Thus, 24-h plasma corticosterone levels in ADX+Cort animals were higher than the trough levels in control animals, but were well below the daily peak values, as also indicated by their lower 24-h means (17.8 ± 3.8 ng/ml). Mean plasma leptin levels varied between 1.1 ± 0.5 and 2.3 ± 0.5 ng/ml (Fig. 1B), with individual values varying between 0.5 and 6.1 ng/ml. In the ADX+Cort group ANOVA detected a significant effect of time [$F(13,91) = 10.00$; $P < 0.001$] for plasma leptin levels. The overall plasma leptin levels in the ADX+Cort animals differed from those in SCN-lesioned and regular-fed animals, but not from those in the control animals [$F(1,13) = 0.11$; $P = 0.75$]. However, ANOVA did indicate a significant interaction of group *vs.* time [$F(11,143) = 4.19$; $P < 0.001$], probably due to the earlier nocturnal decline in plasma leptin levels in the ADX+Cort group.

Diurnal rhythmicity

All groups, except that with SCN lesions, showed trough leptin levels during the first part of the light period and an acrophase during the light/dark transition (Figs. 1 and 3). Cosinor analysis of the data from the SCN-intact group confirmed a clear daily rhythm (explaining 73% of the variation; $P < 0.01$), with an acrophase at ZT16.5 and an amplitude of 42%. Cosinor analysis of the data from SCN-lesioned animals

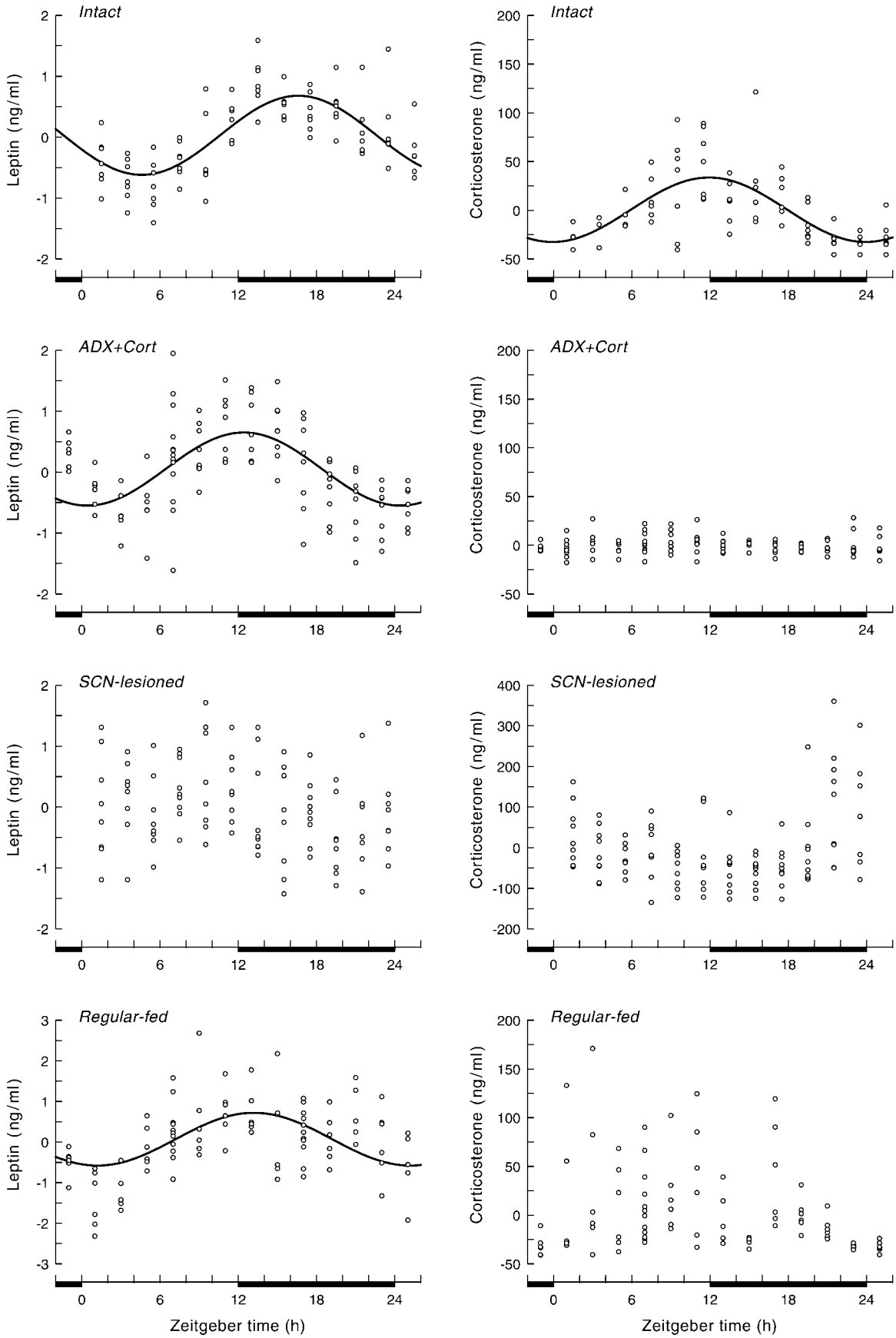

FIG. 3. Individual (normalized) plasma leptin and corticosterone values and the cosine fits for the different treatment groups. The cosine curve corresponds to the best-fit significant model obtained by the population mean cosinor. Data were normalized by expressing each of the 12 data points of an animal as the difference from the 24-h mean (*i.e.* the mean of the 12 samples) of that animal. Only significant cosine fits are shown.

did not produce a significant correlation ($P > 0.05$); introducing a daily rhythmicity could at best explain 28% of the variation. On the other hand, cosinor analysis of the data from the regular-fed and ADX+Cort animals produced significant fits ($P < 0.01$), and the daily rhythmicity could explain 48% and 60% of the variation, respectively. In both groups the acrophase was slightly phase-advanced compared with that in intact animals (ZT13½ and ZT12½, respectively). Whereas ADX+Cort animals also showed an approximately 40% amplitude, the (relative) amplitude was clearly reduced in the regular-fed animals, *i.e.* about 10%, due to the higher 24-h means of this group. Expressed as absolute deviations from the 24-h means, however, all three groups showed very similar daily variations in their plasma leptin levels, with the difference between trough and peak values amounting to approximately 1.3 ng/ml. Cosinor analysis of the plasma corticosterone data of the intact animals also produced a significant fit that explained 68% of the variation. No significant fits were detected in the corticosterone data of the three remaining groups, although the normal circadian corticosterone pattern could be recognized in the data of the regular-fed group (Fig. 3).

Correlative analysis

The mean body weights of the four treatment groups showed no significant differences [$F(3,32) = 0.69$; $P = 0.57$]. The body weight of the individual animals in the four treatment groups combined was positively, but weakly, related to the 24-h mean plasma leptin values (Fig. 4A; $r = 0.37$; $P = 0.04$), with 24-h mean plasma leptin levels increasing by 2 ng/ml with every 100 g in body weight. This positive relation was not found when the different treatment groups were analyzed separately. Therefore, SCN-lesioned and, even more so, regular-fed animals show higher 24-h mean plasma leptin levels compared with control and ADX+Cort animals despite comparable body weights.

The 24-h mean plasma corticosterone values of the treatment groups differed significantly [$F(3,32) = 15.15$; $P < 0.001$], with SCN-lesioned animals showing significantly higher (80.3 ± 10.8 ng/ml) and ADX+Cort animals showing significantly lower (17.8 ± 3.8 ng/ml) means compared with intact and regular-fed animals (39.5 ± 3.4 and 39.5 ± 2.1 ng/ml, respectively). Comparing the 24-h mean plasma corticosterone and 24-h mean plasma leptin levels of individual animals again indicated the distinct position of the regular-fed animals (Fig. 4B). Taking together the data from the intact, ADX+Cort, and SCN-lesioned animals showed a very significant positive relation between plasma corticosterone and plasma leptin levels ($P < 0.0001$), indicating that the differences in 24-h mean plasma corticosterone levels could explain 73% of the variation in 24-h mean plasma leptin levels. Although the grouping of intact, ADX+Cort, and regular-fed animals resulted in a positive relation ($P = 0.03$), in this case the changes in corticosterone levels could explain only 47% of the variation in plasma leptin levels. Analyzing the different treatment groups separately or taking together the data from all four treatment groups did not produce a significant linear regression.

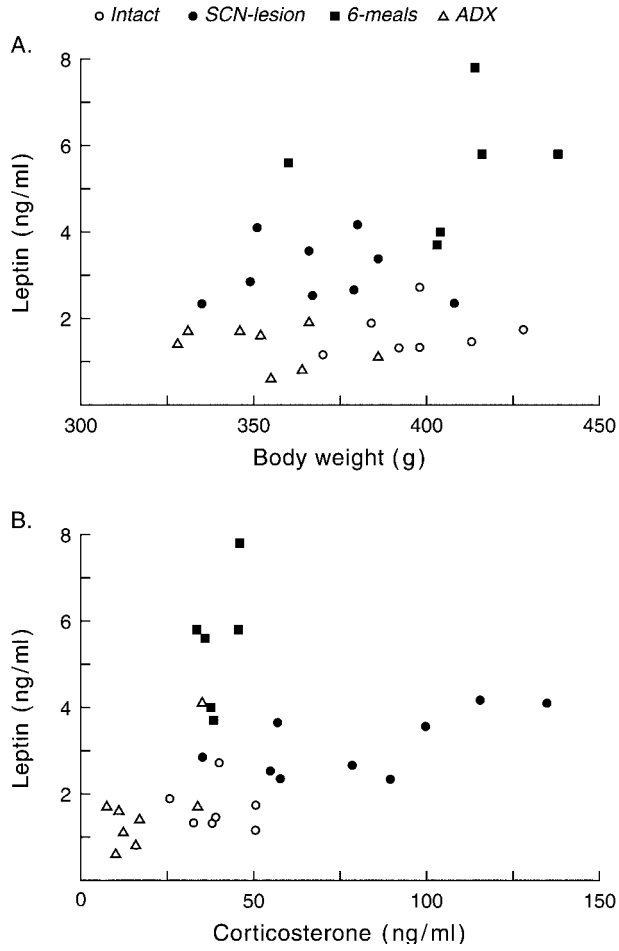

FIG. 4. The relation between body weight (A) or 24-h mean corticosterone concentrations (B) and 24-h mean leptin concentrations in individual animals of the four different treatment groups.

Adipocyte leptin mRNA

Leptin expression in the epididymal fat depots of none of the three experimental groups used for the blood-sampling experiments (*i.e.* intact, ADX+CORT, and SCN-lesioned) showed a significant light/dark difference. The mean values of the three groups also did not differ (2.3 ± 0.4, 4.3 ± 0.9, and 3.5 ± 0.5 relative arbitrary units for intact, ADX+CORT, and SCN-lesioned groups, respectively; $P = 0.165$). As the absence of significant differences was probably due to the limited number of time points sampled (*i.e.* two), we performed an additional experiment. With more frequent sampling a diurnal difference in leptin mRNA levels in *ad libitum*-fed control animals just escaped significance ($P = 0.068$; Fig. 5). Excluding the two outliers at ZT22, however, produced a very significant diurnal difference ($P = 0.003$). Trough values were found during the end of the dark period and the beginning of the light period, as also observed previously (31). Leptin mRNA values in SCN-lesioned animals showed less variation and were restricted to the median ranges of control animals.

Discussion

The present study provides the first clear evidence for a direct control of the mammalian biological clock, located in

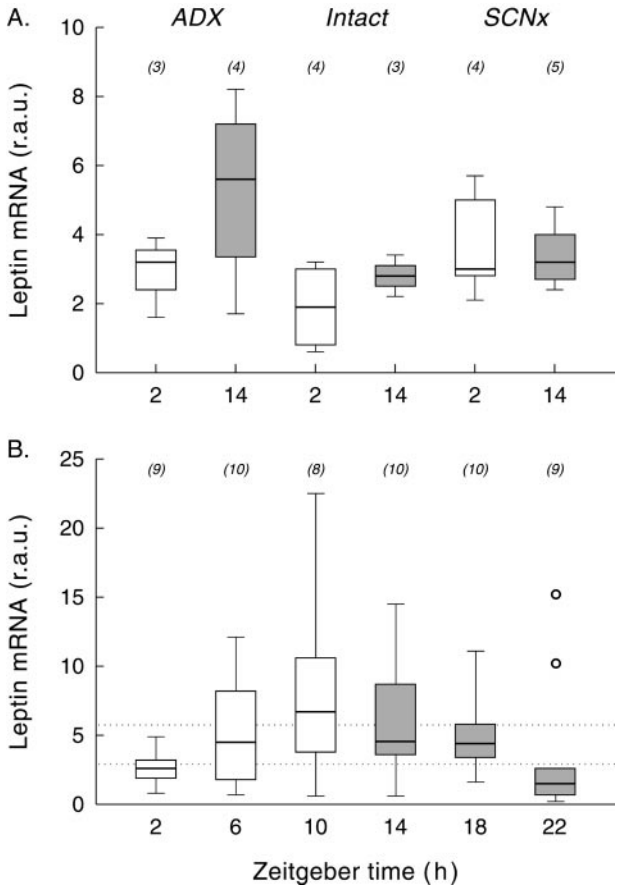

FIG. 5. Diurnal variation of leptin mRNA levels in adipocyte tissue of the different experimental groups (A) and a group of control animals (B). Values of the SCN-lesioned animals show little variation and are restricted to the intermediate range of control animals (*i.e. dashed lines* in B represent upper and lower values of the SCN-lesioned group). The *box* represents the interquartile range, which contains 50% of the values. The "whiskers" are lines that extend from the box to the highest and lowest values, excluding outliers (outliers being defined as values more than three box lengths from the upper or lower edge of the box; *i.e.* the two *circles* at ZT22). The *line across the box* indicates the median. Numbers in *brackets* denote the number of animals. Note the different scales of the *y*-axes in A and B.

the SCN, on the diurnal rhythm of plasma leptin levels in the rat. Contrary to the two main explanations that were put forward shortly after the discovery of leptin, the diurnal rhythm of plasma leptin is not merely a consequence of diurnal rhythms in circulating glucocorticoids or feeding behavior. The present data demonstrate that the biological clock controls the diurnal rhythm of plasma leptin levels directly, independently of other clock-controlled rhythms. It is shown that the plasma leptin rhythm in adrenalectomized animals with constant corticosterone release pellets is identical to that observed in control animals with a diurnal corticosterone peak. Moreover, modification of the normal nocturnal feeding pattern of animals by introducing a fixed 24-h feeding schedule (*i.e.* one meal every 4 h) does not remove the diurnal cyclicity of plasma leptin levels, although it causes a pronounced increase in mean 24-h values. Only ablation of the locus of the biological clock, *i.e.* SCN lesions, eliminated the diurnal rhythmicity of circulating plasma leptin levels.

The diurnal rhythm of plasma leptin and leptin mRNA levels described in the present study corresponds with the rhythms described recently in three other reports (31–33), with a daytime trough and peak levels in the early dark period. The recent descriptions of plasma leptin rhythms in the rat clearly show the similarity between leptin rhythms in rodents and humans. The similar phasing of the leptin rhythm in humans and rodents despite their opposite, *i.e.* nocturnal *vs.* daytime, feeding patterns indicates that it is unlikely that the rhythmic features of 24-h plasma leptin levels are the sole consequence of their feeding behavior. Nevertheless, a number of experiments have indicated that changes in the daily pattern of food intake can affect plasma leptin levels (1, 13, 14, 16, 17). For instance, it was shown that a 6.5-h phase delay in the timing of the meals also induces a phase delay in the nocturnal leptin peak (14). In addition, Ahima *et al.* (16) showed that mice on a feeding schedule consisting of one daytime meal restricted to 4 h, exhibited an extra leptin surge during the daytime. However, during continuous enteral feeding in humans the plasma leptin rhythm remained intact (13). Together with our present results, these data indicate that in addition to food intake, other factors are involved in the diurnal regulation of leptin secretion.

Soon after the discovery of the pronounced diurnal rhythmicity of plasma leptin levels (9, 12), a number of reports indicated a stimulatory effect of glucocorticoids on leptin release (20, 21, 34, 35). In combination with the inverse relationship between plasma leptin levels and adrenal glucocorticoid activity (9, 19), the above-mentioned results led to the proposition that the diurnal rhythm in plasma leptin levels might be due to a 12-h lag in the stimulatory effect of circulating glucocorticoids on the fat cell (22). In the meantime, however, a number of observations in humans have indicated that the circadian rhythm in plasma leptin levels cannot be accounted for by the diurnal variations in circulating plasma cortisol levels (9, 22, 23). In accordance with that observation, in our experiments in rats the absence of a diurnal fluctuation in circulating corticosterone levels in the ADX+Cort animals did not prevent a clear diurnal variation in plasma leptin levels comparable to that in intact control animals. Therefore, the present experiments provide further evidence for control of the daily leptin rhythm independently of the hypothalamo-pituitary-adrenal axis. Furthermore, the persistence of diurnal leptin rhythms in patients with perinatal stalk transsection syndrome also makes it unlikely that other pituitary hormones are involved in the diurnal control of plasma leptin levels (36).

Our finding of increased mean 24-h plasma leptin levels but unchanged daily rhythmicity of plasma leptin in the regular-fed animals is very much in line with the recent observations by Boden *et al.* (18) during 72-h euglycemic-hyperinsulinemic clamp studies. Although these prolonged euglycemic-hyperinsulinemic clamps caused a dose-dependent increase in serum leptin levels, the circadian rhythmicity remained intact. Therefore, data from our regular-fed animals and the results of Boden *et al.* (18) clearly show that a rhythm in insulin release cannot be the primary cause of the diurnal leptin rhythm. As the six meals per day feeding schedule does not result in increased body weight or increased plasma insulin levels (27, 30, 37), it is not clear what

causes the increased plasma leptin levels in our regular-fed group. In fact, as these animals are slightly food-deprived, a lowering of plasma leptin levels would be expected. As it has been suggested that leptin acts as a sensor of nutrient flux in adipose tissue (38, 39), we are currently testing the hypothesis that the aberrant feeding schedule causes an increased flux of nutrients through the adipocyte, resulting in increased leptin release. Indeed, it has been reported that rats on a food restriction schedule develop high rates of lipolysis in adipose tissue and liver (40).

The question then remains which SCN-generated mechanism is responsible for the diurnal rhythm in plasma leptin levels if it is not the rhythm in feeding behavior or in the activity of the hypothalamo-pituitary-adrenal axis. Bearing in mind the reports that glucose metabolism regulates insulin-mediated leptin expression and secretion by adipocytes (38), the meal-independent circadian rhythm of leptin could also result from daily fluctuations in adipocyte glucose utilization via changes in insulin sensitivity (18). Both glucocorticoids and GH are known to reduce insulin-mediated glucose uptake, but in view of the evidence presented previously (36) it is unlikely that the rhythmic release of either of these hormones is responsible for a (insulin sensitivity-mediated) leptin rhythm. A number of experiments have indicated an important role for the autonomic nervous system in the regulation of leptin secretion. Reduction of noradrenaline release from sympathetic nerve endings by administration of α-methyl-*p*-tyrosine or 6-hydroxydopamine induces hyperleptinemia (41, 42). Conversely, β-adrenergic receptor agonists decrease plasma leptin levels and leptin mRNA expression (43, 44). This regulation of leptin secretion by sympathetic activity resembles the regulation of melatonin release by the pineal gland. The pronounced day/night rhythm of plasma melatonin levels is due to the nocturnal release of noradrenaline from the sympathetic nerve endings in the pineal gland (45). Another appealing similarity between leptin and melatonin rhythms is the nocturnality of its acrophase, *i.e.* peak levels of both leptin and melatonin are found at night, independently of the diurnal or nocturnal preference of a species. Both anatomical and (electro)physiological techniques have demonstrated that the SCN controls the sympathetic input to the pineal gland by contacting those PVN neurons that possess descending projections to the preganglionic neurons in the spinal cord (46–49). We hypothesize that a similar neural pathway underlies the circadian control of plasma leptin levels. This idea is fully supported by the observation that retrograde virus tracing from the white adipose tissue labels neurons in spinal cord, PVN, and SCN (50), and the fact that insulin sensitivity can be changed via the autonomic nervous system (51).

Finally, SCN-lesioned animals showed increased 24-h mean plasma leptin levels compared with intact control animals. In view of the restricted size of our lesions we are confident that this increase is not due to collateral damage to other brain areas, such as the paraventricular, ventromedial, or arcuate nuclei (52, 53). Moreover, in these lesion models the increased plasma leptin levels are accompanied by obesity, which is certainly not the case in our SCN-lesioned animals. At present, however, we cannot completely exclude some additional effects of damage to the medial preoptic area on the observed changes. The preoptic area has been implicated in glucose metabolism (54–56), but we are not aware of any data on the effects of lesions in the preoptic area on plasma leptin levels. Therefore, it is possible that, similar to the rhythm in melatonin release, SCN control of leptin release is mainly inhibitory (46, 57), and thus removal of this inhibitory SCN control results in increased leptin levels. However, at least two other explanations cannot be excluded at present. First, the SCN-lesioned animals also show a regular feeding pattern somewhat comparable to that of the regular-fed animals. Therefore, if the regular feeding pattern itself induces increased plasma leptin levels, similar changes might be expected in SCN-lesioned animals, although the effect is more pronounced in the regular-fed group. A second alternate explanation is found in the positive relation between 24-h mean leptin and 24-h mean corticosterone levels. This correlation is in agreement with several previous observations (20, 21, 34, 35) and indicates that the higher plasma leptin levels in the SCN-lesioned animals might be due to the higher corticosterone levels in these animals. Future experiments using SCN transmitter (ant)agonist administration at the level of the PVN, SCN-lesioned animals with an enforced nocturnal feeding pattern, and SCN lesions combined with ADX+CORT, respectively, will allow further investigation of these possibilities. In addition, the data from the SCN-lesioned animals indicate an effect on leptin release and not so much leptin synthesis. Previous experiments have also shown differential effects on leptin release and synthesis (3, 32, 35). Although the present data suggest that leptin secretion can be stimulated from a preexisting intracellular pool, more information about the cellular and molecular biology of leptin is needed before a justified interpretation can be made.

In conclusion, the present study shows that the SCN directly orchestrates the diurnal changes in plasma leptin levels. This study therefore indicates the presence of an efferent pathway, probably mediated by the autonomous nervous system, between the central nervous system and the adipose tissue involved in the control of leptin secretion.

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References

1. **Dallongeville J, Hecquet B, Lebel P, Edme JL, Le Fur C, Fruchart JC, Auwerx J, Romon M** 1998 Short term response of circulating leptin to feeding and fasting in man: influence of circadian cycle. Int J Obesity 22:728–733
2. **Boden G, Chen X, Mozzoli M, Ryan I** 1996 Effect of fasting on serum leptin in normal human subjects. J Clin Endocrinol Metab 81:3419–3423
3. **Sivitz WI, Walsh S, Morgan D, Donohoue P, Haynes W, Leibel RL** 1998 Plasma leptin in diabetic and insulin-treated diabetic and normal rats. Metabolism 47:584–591
4. **Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P** 1995 Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 269:546–549
5. **Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F** 1995 Effects of the *obese* gene product on body weight regulation in *ob/ob* mice. Science 269:540–543
6. **Glaum SR, Hara M, Bindokas VP, Lee CC, Polonsky KS, Bell GI, Miller RJ** 1996 Leptin, the *Obese* gene product, rapidly modulates synaptic transmission in the hypothalamus. Mol Pharmacol 50:230–235

7. **Tang-Christensen M, Holst JJ, Hartmann B, Vrang N** 1999 The arcuate nucleus is pivotal in mediating the anorectic effects of centrally administered leptin. NeuroReport 10:1183–1187
8. **Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK** 1998 Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron 21:1375–1385
9. **Licinio J, Mantzoros C, Negrao AB, Cizza G, Wong ML, Bongiorno PB, Chrousos GP, Karp B, Allen C, Flier JS, Gold PW** 1997 Human leptin levels are pulsatile and inversely related to pituitary-adrenal function. Nat Med 3:575–579
10. **Saad MF, Riad-Gabriel MG, Khan A, Sharma A, Michael R, Jinagouda SD, Boyadjian R, Steil GM** 1998 Diurnal and ultradian rhythmicity of plasma leptin: effects of gender and adiposity. J Clin Endocrinol Metab 83:453–459
11. **Langendonk JG, Pijl H, Toornvliet AC, Burggraaf J, Frolich M, Schoemaker RC, Doornbos J, Cohen AF, Meinders AE** 1998 Circadian rhythm of plasma leptin levels in upper and lower body obese women: influence of body fat distribution and weight loss. J Clin Endocrinol Metab 83:1706–1712
12. **Sinha MK, Ohannesian JP, Heiman ML, Kriauciunas A, Stephens TW, Magosin S, Marco C, Caro JF** 1996 Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. J Clin Invest 97:1344–1347
13. **Simon C, Gronfier C, Schlienger JL, Brandenberger G** 1998 Circadian and ultradian variations of leptin in normal man under continuous enteral nutrition: relationship to sleep and body temperature. J Clin Endocrinol Metab 83:1893–1899
14. **Schoeller DA, Cella LK, Sinha MK, Caro JF** 1997 Entrainment of the diurnal rhythm of plasma leptin to meal timing. J Clin Invest 100:1882–1887
15. **Coleman RA, Herrmann TS** 1999 Nutritional regulation of leptin in humans. Diabetologia 42:639–646
16. **Ahima RS, Prabakaran D, Flier JS** 1998 Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding: implications for energy homeostasis and neuroendocrine function. J Clin Invest 101:1020–1027
17. **Romon M, Lebel P, Velly C, Marecaux N, Fruchart JC, Dallongeville J** 1999 Leptin response to carbohydrate or fat meal and association with subsequent satiety and energy intake. Am J Physiol 277:E855–E861
18. **Boden G, Chen XH, Kolaczynski JW, Polansky M** 1997 Effects of prolonged hyperinsulinemia on serum leptin in normal human subjects. J Clin Invest 100:1107–1113
19. **Korbonits M, Trainer PJ, Little JA, Edwards R, Kopelman PG, Besser GM, Svec F, Grossman AB** 1997 Leptin levels do not change acutely with food administration in normal or obese subjects, but are negatively correlated with pituitary-adrenal activity. Clin Endocrinol (Oxf) 46:751–757
20. **Newcomer JW, Selke G, Melson AK, Gross J, Vogler GP, Dagogo-Jack S** 1998 Dose-dependent cortisol-induced increases in plasma leptin concentration in healthy humans. Arch Gen Psychiatry 55:995–1000
21. **Dagogo-Jack S, Selke G, Melson AK, Newcomer JW** 1997 Robust leptin secretory responses to dexamethasone in obese subjects. J Clin Endocrinol Metab 82:3230–3233
22. **Purnell JQ, Samuels MH** 1999 Levels of leptin during hydrocortisone infusions that mimic normal and reversed diurnal cortisol levels in subjects with adrenal insufficiency. J Clin Endocrinol Metab 84:3125–3128
23. **Weise M, Abad V, Considine RV, Nieman L, Rother KI** 1999 Leptin secretion in Cushing's syndrome: preservation of diurnal rhythm and absent response to corticotropin-releasing hormone. J Clin Endocrinol Metab 84:2075–2079
24. **Rusak B, Zucker I** 1979 Neural regulation of circadian rhythms. Physiol Rev 59:449–526
25. **Swaab DF, Fliers E, Partiman TS** 1985 The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. Brain Res 342:37–44
26. **Van Cauter E, Refetoff S** 1985 Multifactorial control of the 24-hour secretory profiles of pituitary hormones. J Endocr Invest 8:381–391
27. **La Fleur SE, Kalsbeek A, Wortel J, Buijs RM** 1999 An SCN generated rhythm in basal glucose levels. J Neuroendocrinol 11:643–652
28. **Palm IF, Van Der Beek EM, Wiegant VM, Buijs RM, Kalsbeek A** 1999 Vasopressin induces an LH surge in ovariectomized, estradiol-treated rats with lesion of the suprachiasmatic nucleus. Neuroscience 93:659–666
29. **Buijs RM, Kalsbeek A, Van Der Woude TP, Van Heerikhuize JJ, Shinn S** 1993 Suprachiasmatic nucleus lesion increases corticosterone secretion. Am J Physiol 264:R1186–R1192
30. **Kalsbeek A, Strubbe JH** 1998 Circadian control of insulin secretion is independent of the temporal distribution of feeding. Physiol Behav 63:553–560
31. **Dallman MF, Akana SF, Bhatnagar S, Bell ME, Choi S, Chu A, Horsley C, Levin N, Meijer O, Soriano LR, Strack AM, Viau V** 1999 Starvation: early signals, sensors, and sequelae. Endocrinology 140:4015–4023
32. **Xu B, Kalra PS, Farmerie WG, Kalra SP** 1999 Daily changes in hypothalamic gene expression of neuropeptide Y, galanin, proopiomelanocortin, and adipocyte leptin gene expression and secretion: effects of food restriction. Endocrinology 140:2868–2875
33. **Mastronardi CA, Walczewska A, Yu WH, Karanth S, Parlow AF, McCann SM** 2000 The possible role of prolactin in the circadian rhythm of leptin secretion in male rats. Proc Soc Exp Biol Med 224:152–158
34. **Van Dijk G, Donahey JCK, Thiele TE, Scheurink AJW, Steffens AB, Wilkinson CW, Tenenbaum R, Campfield LA, Burn P, Seeley RJ, Woods SC** 1997 Central leptin stimulates corticosterone secretion at the onset of the dark phase. Diabetes 46:1911–1914
35. **Bradley RL, Cheatham B** 1999 Regulation of *ob* gene expression and leptin secretion by insulin and dexamethasone in rat adipocytes. Diabetes 48:272–278
36. **Pombo M, Herrera-Justiniano E, Considine RV, Hermida RC, Galvez MJ, Martin T, Barreiro J, Casanueva FF, Dieguez C** 1997 Nocturnal rise of leptin in normal prepubertal and pubertal children and in patients with perinatal stalk-transection syndrome. J Clin Endocrinol Metab 82:2751–2754
37. **Strubbe JH, Alingh Prins AJ, Bruggink J, Steffens AB** 1987 Daily variation of food-induced changes in blood glucose and insulin in the rat and the control by the suprachiasmatic nucleus and the vagus nerve. J Autonom Nerv Syst 20:113–119
38. **Mueller WM, Gregoire FM, Stanhope KL, Mobbs CV, Mizuno TM, Warden GH, Stern JS, Havel PJ** 1998 Evidence that glucose metabolism regulates leptin secretion from cultured rat adipocytes. Endocrinology 139:551–558
39. **Wang J, Liu R, Hawkins M, Barzilai N, Rossetti L** 1998 A nutrient-sensing pathway regulates leptin gene expression in musle and fat. Nature 393:684–688
40. **Leveille GA** 1967 *In vivo* fatty acid synthesis in adipose tissue and liver of meal-fed rats. Proc Soc Exp Biol Med 125:85–88
41. **Sivitz WI, Fink BD, Morgan DA, Fox JM, Donohoue PA, Haynes WG** 1999 Sympathetic inhibition, leptin, and uncoupling protein subtype expression in normal fasting rats. Am J Physiol 277:E668–E677
42. **Rayner DV, Simon E, Duncan JS, Trayhurn P** 1998 Hyperleptinaemia in mice induced by adminstration of the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine. FEBS Lett 429:395–398
43. **Trayhurn P, Duncan JS, Rayner DV** 1995 Acute cold-induced suppression of *ob* (obese) gene expression in white adipose tissue of mice: mediation by the sympathetic system. Biochem J 311:729–733
44. **Li H, Matheny M, Scarpage PJ** 1997 β_3-Adrenergic-mediated suppression of leptin gene expression in rats. Am J Physiol 272:E1031–E1036
45. **Roseboom PH, Coon SL, Baler R, McCune SK, Weller JL, Klein DC** 1996 Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. Endocrinology 137:3033–3044
46. **Kalsbeek A, Garidou ML, Palm IF, Van Der Vliet J, Simonneaux V, Pévet P, Buijs RM** 2000 Melatonin sees the light: blocking GABA-ergic transmission in the paraventricular nucleus induces daytime secretion of melatonin. Eur J Neurosci 12:3146–3154
47. **Kalsbeek A, Cutrera RA, Van Heerikhuize JJ, Van Der Vliet J, Buijs RM** 1999 GABA release from SCN terminals is necessary for the light-induced inhibition of nocturnal melatonin release in the rat. Neuroscience 91:453–461
48. **Teclemariam-Mesbah R, Ter Horst GJ, Postema F, Wortel J, Buijs RM** 1999 Anatomical demonstration of the suprachiasmatic nucleus-pineal pathway. J Comp Neurol 406:171–182
49. **Hermes MLHJ, Coderre EM, Buijs RM, Renaud LP** 1996 GABA and glutamate mediate rapid neurotransmission from suprachiasmatic nucleus to hypothalamic paraventricular nucleus in rat. J Physiol 496:749–757
50. **Bamshad M, Aoki VT, Adkison MG, Warren WS, Bartness TJ** 1998 Central nervous system origins of the sympathetic nervous system outflow to white adipose tissue. Am J Physiol 275:R291–R299
51. **Nonogaki K** 2000 New insights into sympathetic regulation of glucose and fat metabolism. Diabetologia 43:533–549
52. **Bell ME, Bhatnagar S, Akana SF, Choi SJ, Dallman MF** 2000 Disruption of arcuate/paraventricular nucleus connections changes body energy balance and response to acute stress. J Neurosci 20:6707–6713
53. **Choi SJ, Dallman MF** 1999 Hypothalamic obesity: multiple routes mediated by loss of function in medial cell groups. Endocrinology 140:4081–4088
54. **Babcock AM, Barton C, Gunion MW, Rosenthal MJ** 1992 Bombesin-induced hypothermia and hypophagia are associated with plasma metabolic fuel alterations in the rat. Physiol Behav 51:933–938
55. **De Castro MGB, Foscolo RB, Reis AM, Coimbra CC** 1997 Modulation of plasma glucose by the medial preoptic area in freely moving rats. Physiol Behav 61:215–220
56. **Coimbra CC, Migliorini RH** 1988 Cold-induced free fatty acid mobilization is impaired in rats with lesions in the preoptic area. Neurosci Lett 88:1–5
57. **Kalsbeek A, Drijfhout WJ, Westerink BHC, Van Heerikhuize JJ, Van Der Woude T, Van Der Vliet J, Buijs RM** 1996 GABA receptors in the region of the dorsomedial hypothalamus of rats are implicated in the control of melatonin. Neuroendocrinology 63:69–78