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Effects of Suprachiasmatic Transplants on Circadian Rhythms of Neuroendocrine Function in Golden Hamsters*

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ABSTRACT

Grafts of fetal tissue including the suprachiasmatic nucleus (SCN) of the hypothalamus restore locomotor rhythmicity to behaviorally arrhythmic, SCN-lesioned Syrian hamsters. We sought to determine whether such transplants also reinstate endocrine rhythms in SCN-lesioned hamsters. In Exp 1, SCN lesions interrupted estrous cycles in a 14 h light, 10 h dark photoperiod and locomotor rhythms in constant dim red light (DD). SCN grafts that reinstated behavioral circadian rhythms consistently failed to reestablish estrous cycles. After ovariectomy, estradiol implants triggered LH surges at approximately circadian time 8 in 10 of 12 brain-intact control females and 0 of 9 SCN-lesioned, grafted females. Daily rhythms of the principal urinary melatonin metabolite, 6α-sulfatoxymelatonin, were not reestablished by behaviorally functional grafts. In Exp 2, SCN lesions eliminated locomotor rhythmicity in adult male hamsters maintained in DD. Seven to 12 weeks after restoration of locomotor activity rhythms by fetal grafts, hosts and sham-lesioned controls were decapitated at circadian times 4, 8, 12, 16, 20, or 24. Clear circadian rhythms of both serum corticosterone and cortisol were seen in sham-lesioned males, with peaks in late subjective day. No circadian rhythms in either adrenal hormone were evident in serum from lesioned-grafted males. Testicular regression, observed in intact and sham-lesioned males maintained in DD, was absent not only in arrhythmic SCN-lesioned hamsters given grafts of cerebral cortex, but also in animals in which hypothalamic grafts had reinstated locomotor rhythmicity. The pineal melatonin concentration rose sharply during the late subjective night in control hamsters, but not in SCN-lesioned animals bearing behaviorally effective transplants.

Even though circadian rhythms of locomotor activity are restored by SCN transplants, circadian endocrine rhythms are not reestablished. Endocrine rhythms may require qualitatively different or more extensive SCN outputs than those established by fetal grafts. (*Endocrinology* **140:** 207–218, 1999)

THE SUPRACHIASMATIC nucleus (SCN) of the hypothalamus functions as a master pacemaker for the mammalian circadian system (1, 2). Ablation of this structure not only prevents the expression of endogenous daily rhythms of locomotor activity, feeding, drinking, and body temperature in hamsters and rats, but also eliminates circadian oscillations in serum concentrations of PRL, melatonin, GH, ACTH, and cortisol (3–8). Furthermore, the estrous cycles of hamsters and rats are controlled by the circadian system (9–14); the estrogen-induced surge of LH that precipitates ovulation is restricted to a particular time of day and is delayed by 24 h if barbiturate is injected during a critical period on the afternoon of proestrus (15, 16). Estradiol (E_2) treatment of ovariectomized rodents induces daily surges of LH, whose period and phase are dictated by the circadian system (13, 14). Moreover, either destruction of the SCN by electrolytic lesions or its surgical isolation by knife cuts abolishes the estrous cycle and eliminates E_2-induced LH surges (17–19).

Transplantation of the SCN can reinstate behavioral circadian rhythms in rats and hamsters previously made arrhythmic by SCN lesions (19–25). It is not known whether such grafts can also reinstate circadian rhythms of neuroendocrine function in SCN-ablated animals. The present studies were undertaken to determine whether SCN transplants adequate to reinstate locomotor rhythms in SCN-lesioned hamsters also restore estrous cycles, E_2-induced LH surges, or circadian rhythms of serum glucocorticoids, pineal melatonin content, or the urinary melatonin metabolite, 6α-sulfatoxymelatonin (6αMTS).

Materials and Methods

General

Adult Syrian hamsters (LVG strain) were obtained from the Lakeview hamstery (Wilmington, MA) or bred locally from that source. Animals

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were maintained in plastic tub cages (26 × 48 × 21 cm) under a 14-h light, 10-h dark photoperiod (14L:10D; lights on, 0730 Eastern Standard Time) unless otherwise noted. Hamsters had continuous access to running wheels (17 cm) throughout the experiment except where indicated otherwise. Revolutions were recorded in 10-min bins using Dataquest II software on an IBM PC computer. Rhythmicity was assessed by visual inspection of actograms. All animals had continuous access to food (Purina chow, Ralston Purina Co., St. Louis, MO) and water throughout the experiment. Protocols were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Surgery

Lesions. Hamsters were anesthetized with sodium pentobarbital (65 mg/kg BW) and placed in a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Bilateral electrolytic lesions of the SCN were made using a tungsten electrode (0.5-mm diameter; A & M Systems, Everett, WA), insulated with Epoxylite resin except at the tip. Each lesion was made by passing a direct positive current (1.75 mA) for 20 sec using a Grass LM-5 lesionmaker (Grass Instrument Co., Quincy, MA). The electrode was positioned 0.35 mm on either side of the midline, 0.8 mm rostral to bregma, and 7.8 mm ventral to the dura.

Grafting. Animals displaying an absence of circadian rhythmicity for a period of at least 6 weeks of constant darkness (DD) were selected as transplant recipients. Fetal tissue containing the SCN or cerebral cortex (as a control) was obtained from embryonic day 15 hamster pups (two per host) and transplanted into the third ventricle as previously described (22, 25). A drop of trypan blue was placed on the chiasmatic region to facilitate visualization of the SCN area during dissection and preparation of the tissue to be grafted. The injection cannula (Wiretrol, Fisher Lab Products, Pittsburgh, PA) containing the graft tissue (~5 μl) was lowered at the midline to the same coordinates as the lesion. As the injection cannula was raised, the graft was slowly ejected.

RIAs

After collection from males, whole blood was allowed to clot at 4 C for several hours before harvesting of serum by centrifugation at 1000 × *g*. Serum was stored at −20 C until RIAs were performed. Cortisol and corticosterone concentrations were measured in male hamsters using kits from ICN Biomedicals, Inc. (Costa Mesa, CA). Serum aliquots were heated to 98 C for 10 min and allowed to cool before addition of antibody and trace. Serial dilutions of extracted hamster serum generated a slope that was parallel to standard, and serum from adrenalectomized hamsters had undetectable concentrations of immunoreactive cortisol and corticosterone. All samples were run in a single RIA for each hormone; the intraassay coefficients of variation (CVs) were 7.9% for cortisol and 7.4% for corticosterone.

Pineal glands were sonicated, and melatonin was extracted using methylene chloride as described previously (26, 27). Pineal and serum samples were assayed for melatonin using previously described methods (27). The mean intraassay CV (six assays) was 9%, and the interassay CV was 14.9% at 50% binding. Urinary fractions were frozen at −20 C until 6αMTS was assayed by Stockgrand (Surrey, UK) using the method of Arendt *et al.* (28).

LH was assayed in blood plasma collected from female hamsters, using a rat kit from the National Hormone and Pituitary Program. Reference preparation RP-3 was used as standard, NIDDK rLH I-9 (AFP-10250C) was used for iodination, and NIDDK S-10 anti-LH was used at a dilution of 1:200,000. Bound LH was precipitated using goat antirabbit IgG (1:20; Antibodies, Inc., Davis, CA). Standard and hamster serum produced parallel inhibition curves in this assay system. The interassay coefficient of variation was 8.4%, and the intraassay coefficient of variation was 7.0% (five assays).

Exp 1: female hamsters

Figure 1 illustrates the procedures used in this experiment. To assess ovarian cyclicity, all animals were inspected daily for a vaginal discharge 2–6 h after light onset during all phases of the experiment in which the hamsters were housed in a light-dark (LD) cycle (29). Hamsters exhibiting a minimum of three regular estrous cycles (n = 47) received bilateral SCN lesions. After 6–8 weeks in 14L:10D, hamsters were transferred to DD for 3 weeks to confirm lesion efficacy. Hamsters whose behavior remained rhythmic despite hypothalamic lesions were discarded. Subsequent histology confirmed that complete destruction of the SCN resulted in arrhythmicity (see below). Animals that continued to show an absence of circadian rhythmicity of locomotor activity in DD (n = 34) were returned to 14L:10D and grafted with fetal tissue. Immediately before grafting, seven of these hamsters were ovariectomized to rule out the possibility that the fetal grafts might be exposed to gonadal steroid hormones that might influence their subsequent capacity to sustain LH surges. Hamsters recovered for 2 weeks before they were returned to DD. The recovery of locomotor rhythms was assessed over the ensuing 8–13 weeks. Hamsters were returned to 14L:10D for the remainder of the experiment, and those that had not been ovariectomized were inspected daily for vaginal discharge to assess estrous cyclicity. Four weeks later, the remaining recipients of SCN grafts were ovariectomized. An additional 12 hamsters were ovariectomized at this point to serve as brain-intact controls.

Urine collection. A subset of the original group was analyzed for diurnal rhythms of urinary 6αMTS while housed in 14L:10D. Hourly urine samples were collected from female hamsters (n = 45) housed in metabolic cages (Nalgene, Rochester, NY) for 48–72 h. Urine samples were taken from 13 experimental animals both after the lesion was made and after the graft had reinstated locomotor activity rhythms. In 4 additional cases, samples were collected both after SCN lesions and after transplantation of fetal hypothalamic tissue that failed to restore locomotor rhythms. The interval between the production of complete SCN lesions and the onset of urine collection ranged from 10 days to 14 weeks, and the interval between neurotransplantation and collection of urine samples ranged between 9–13 weeks. In addition, 48–72 h of urine samples were collected from 8 SCN-lesioned hamsters between 4–14 weeks after surgery; these females either received no fetal grafts or recovered behavioral rhythmicity after transplantation but died before they were due to be returned to the LD cycle. Urine samples were also collected from

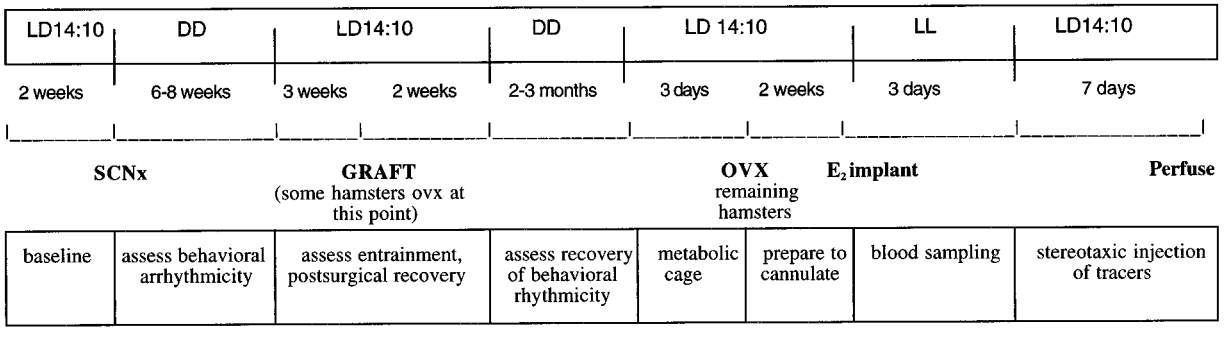


FIG. 1. Design of Exp 1. Adult female Syrian hamsters were maintained in the photoperiods designated *above* the time line. Hamsters were housed in individual cages with access to running wheels for recording of locomotor activity throughout the experiment, except during the 72-h intervals when they were housed in metabolic cages as indicated. Serum was collected at 2- to 3-h intervals on the third day after sc implantation of SILASTIC E_2 capsules as described in text. SCNx, Bilateral electrolytic lesion of the SCN; OVX, ovariectomy.

10 intact control females. As animals did not urinate every hour, fractions were pooled in 3-h bins for statistical analysis.

Blood collection. Chronically ovariectomized females were anesthetized for implantation of a SILASTIC brand jugular cannula (Dow Corning Corp., Midland, MI) to permit blood sampling. On the following day, they were anesthetized with methoxyflurane (Metofane, Mallinckrodt Veterinary, Inc., Mendelein, IL) and implanted sc with three SILASTIC capsules (Dow Corning Corp.; 5-mm length, 1.45-mm id, 1.93-mm od) containing 1% crystalline E_2 mixed in cholesterol (Sigma Chemical Co., St. Louis, MO). Such capsules provide E_2 concentrations comparable to those found at noon on the day of proestrus (~100 pg/ml serum; Jetton, A. E., Meyer, and E. L. Bittman, unpublished data). Approximately 48 h after implantation of E_2 capsules, hamsters were transferred to constant dim illumination (~50 lux), and blood sampling was begun. In 6 grafted and 12 unlesioned control animals, blood samples (~0.5 ml) were collected every 2–3 h for a 24- to 36-h period. In 1 additional control and 5 additional grafted hamsters, the cannulas became occluded, but 2–3 samples were withdrawn by cardiac puncture under light methoxyflurane anesthesia.

Blood cells were separated from plasma by centrifugation (1000 × *g*) at 4 C. Plasma was frozen at −20 C until assay. Cells were mixed with a corresponding volume of solution containing 0.015% human plasma substitute (Plasmanate, Cutter Laboratories, Berkeley, CA), 10 U heparin, and 0.5 mg dextrose/ml and slowly returned to the animal. Hamsters were returned to 14L:10D at the conclusion of the bleeding session.

Tract tracing and histological procedures. Approximately 2 weeks after blood sampling, grafted hamsters (n = 11) were anesthetized with pentobarbital and placed in a stereotaxic apparatus. Eight of these hamsters received a pressure injection of fluorescent microspheres (0.5 μl; Lumafluour, NY) directed at either the preoptic area (POA; 6.1 mm rostral to λ, 0.2 mm lateral to the midsagittal sinus, and 7.7 mm below the dural surface) or the paraventricular nucleus (PVN; 4.5 mm rostral to λ, 0.6 mm lateral, 7.3 mm ventral).

In three additional cases, grafted animals were given iontophoretic injections of the anterograde tracer, *Phaseolus vulgaris* leucoagglutinin (2.5% PHA-L; Vector Laboratories, Inc., Burlingame, CA; 0.1 M PBS, pH 8.0) applied through a glass micropipette (50-μm diameter) as described by Gerfen and Sawchenko (30). The micropipette was lowered at a 10° angle and aimed at the graft. A current of 7 μA was applied for 10 min, alternating between 7 sec on and 7 sec off.

One to 3 weeks later, all hamsters were deeply anesthetized with sodium pentobarbital and injected intracardially with 5000 U sodium heparin (Rugby Pharmaceuticals, Norcross, GA). Animals were transcardially perfused with 200 ml 0.01 M PBS followed by 350 ml 4% phosphate-buffered paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). Brains were removed, postfixed overnight, and transferred to 30% sucrose for 1–3 days. Four series of coronal sections (40 μm) were cut on a rotary freezing microtome. Sections were stored in cryoprotectant (31) until the time of immunocytochemical processing.

Series of frozen sections through the graft and adjacent POA/hypothalamus were processed for immunocytochemical detection of vasoactive polypeptide (VIP; Incstar Corp., Stillwater, MN) and PHA-L (Vector Laboratories, Inc.) using previously described procedures (22, 32). Antigens were detected using a modified avidin-biotin-horseradish peroxidase procedure (22) with diaminobenzidine as the chromagen. Sections were mounted, dehydrated, cleared, coverslipped, and examined under bright- and darkfield illumination. Fluorescent microspheres were visualized using fluorescein and rhodamine filter cubes.

Exp 2: male hamsters

Figure 2 illustrates the animal treatments used in this experiment. Male hamsters were entrained to 14L:10D for at least 2 weeks before they were subjected to SCN or sham lesions. Two weeks after surgery, each hamster was transferred to DD for 11 weeks, during which time lesion efficacy was assessed. Constant dim red light (<0.5 lux at the cage level) was present to permit animal maintenance and cage changes at approximately 3-week intervals. Experimental animals exhibiting an absence of circadian rhythmicity in their running wheel behavior were returned to 14L:10D for transplantation of fetal hypothalamic tissue as described above. The present report is based on 56 of these animals that became arrhythmic after SCN lesions, recovered circadian rhythms after fetal hypothalamic transplant, and survived to the end of the experiment. Control data were obtained from animals with SCN lesions that received either no graft (n = 8) or a temporal cortex graft (n = 7). Testes were palpated at the time of transplantation to confirm that the SCN lesion had blocked the gonadal regression that occurs in intact hamsters maintained in DD (33). Two weeks postgraft, hamsters were returned to DD for 11–13 weeks to assess the ability of the graft to reinstate locomotor rhythmicity.

Each SCN-lesioned animal whose locomotor rhythmicity was restored by hypothalamic tissue grafts was rapidly decapitated using a guillotine at one of six circadian times (n = 8–11 at each time point; CT4, 8, 12, 16, 20, or 24, where CT12 designates activity onset). Each sham-lesioned or intact control hamster was killed at the one of the same six circadian times (n = 7–10/time point). As SCN-lesioned cortex-grafted and nongrafted hamsters did not regain circadian rhythmicity, their times of death were arbitrarily determined. The pineal gland and brain from each animal were rapidly frozen on dry ice and stored at −80 C. Trunk blood was stored at 4 C overnight, after which serum was collected by centrifugation and stored at −20 C. Testes were removed and weighed.

Coronal brain sections (20 μm) were cut on a cryostat and mounted onto subbed slides. These sections were processed for assessment of 2-[^{125}I]iodomelatonin and [^{3}H]DAMGO binding to evaluate rhythmicity of melatonin and opiate binding, respectively. The results of these analyzes will be reported elsewhere. After exposure of autoradiograms, sections were stained with cresyl violet and inspected to verify the completeness of lesions and the presence of viable grafts.

Results

Exp 1: female hamsters

Locomotor rhythmicity. Twenty-one hamsters that had become arrhythmic in their behavior after SCN lesions were performed recovered clear circadian rhythms after transplantation of fetal anterior hypothalamic tissue containing the SCN (Fig. 3). Three additional animals showed a transient recovery or an increase in rhythmic organization without a clear circadian pattern, and 10 others remained arrhythmic after the transplant.

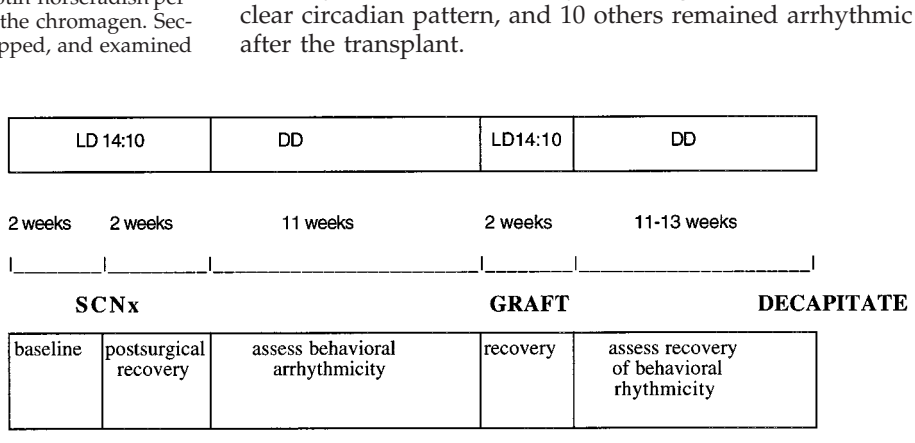


FIG. 2. Design of Exp 2. Adult male Syrian hamsters were maintained in the photoperiods designated *above* the time line. Hamsters were housed in individual cages with access to running wheels for recording of locomotor activity throughout the experiment.

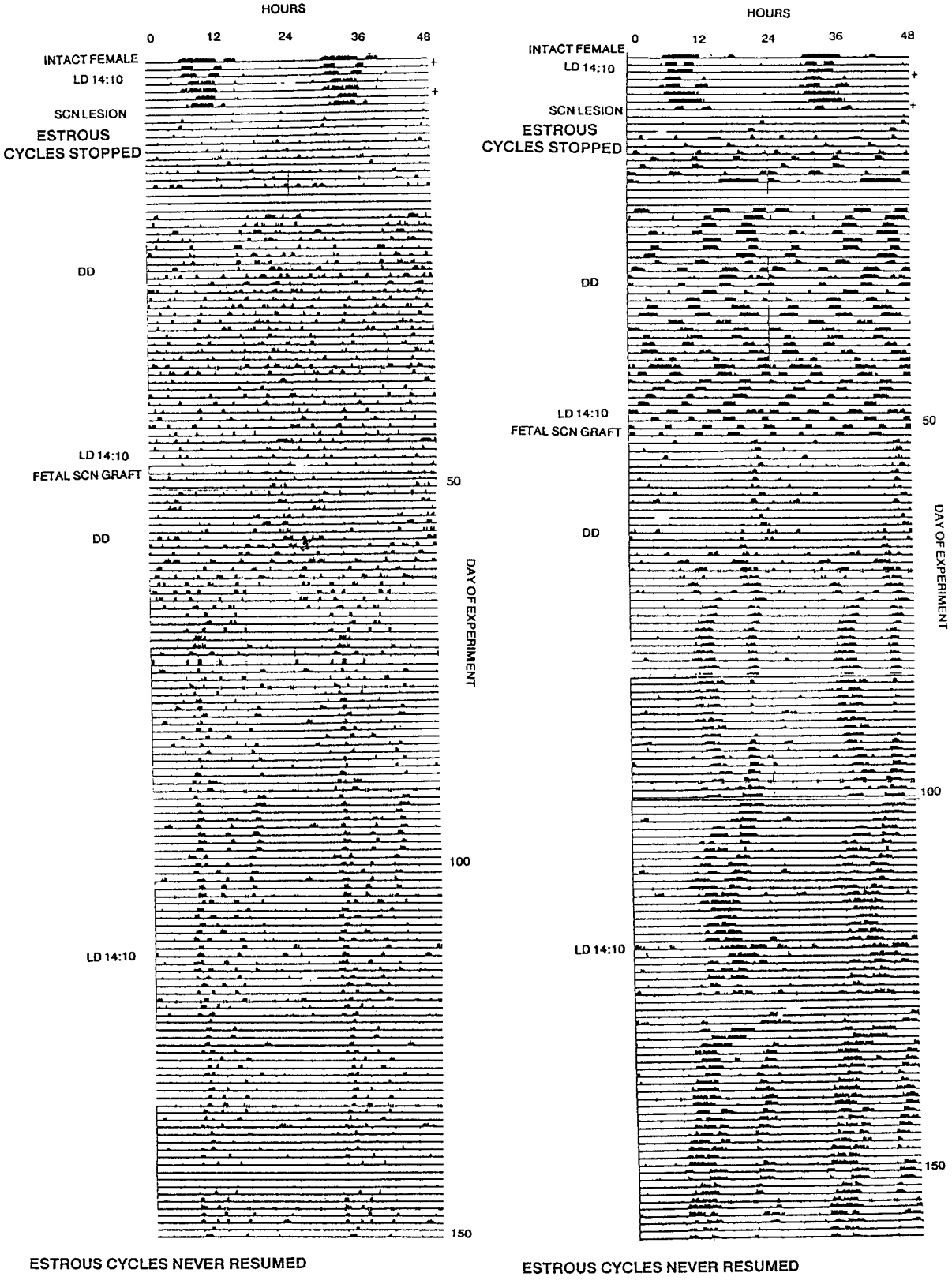


FIG. 3. Double plotted locomotor activity and estrous smear records of two female hamsters that received SCN transplants. Estrous cycles were monitored daily while animals were in LD cycles. Estrous vaginal discharge is indicated by + at the *right* of activity record. Electrolytic lesions of the SCN (performed on day 7) eliminated not only estrous cycles, but also circadian locomotor rhythms in both 14L:10D and DD. Although recovery of locomotor rhythms took place at intervals ranging from a few days (*right*) to several weeks (*left*) after the SCN transplant and persisted for the duration of the experiment, none of the animals recovered estrous cyclicity.

Estrous cycles. Each of the intact control hamsters exhibited regular 4-day estrous cycles throughout their maintenance in 14L:10D. As expected, SCN lesions eliminated estrous cycles in each of the hamsters that sustained complete ablation of the nucleus and experienced locomotor arrhythmicity. Seven of these hamsters were ovariectomized at the time of transplantation to avoid the possibility that ovarian hormones might masculinize the graft; thus, no analysis of the ability of SCN grafts to restore estrous cycles was possible for these individuals. None of the remaining ovary-intact animals subsequently exhibited estrous cycles, even in the 11 cases in which clear recovery of locomotor rhythms occurred after SCN transplantation (Fig. 3). In most cases, the vaginal smear record indicated a condition of continuous diestrus without any evidence of a discharge over a period of at least 6 weeks after reintroduction to 14L:10D following verification of free running locomotor rhythmicity in DD.

LH surges. Twelve control hamsters were successfully cannulated and bled for a period of at least 24 h, and 10 of these animals experienced unambiguous LH surges (Fig. 4, *solid lines*). The mean amplitude of these surges was 10.32 ± 2.30 ng/ml (mean ± SEM, nadir to peak). LH surges uniformly reached a sharp peak and never lasted more than 2 sampling intervals (4 h). Without exception, these LH surges occurred during the mid- to late subjective day in LL, with the LH peak falling at zeitgeber time 7.8 ± 0.3 h (mean ± SEM).

No evidence of a LH surge was seen in 10 of the 11 animals that had complete SCN lesions and behaviorally functional hypothalamic grafts (Fig. 4, *dashed line*). Although low amplitude excursions (<2 ng/ml, nadir to peak) of LH were evident in blood samples obtained by serial bleeding from several of the 6 cannulated, SCN-lesioned female hamsters bearing grafts that reinstated locomotor rhythms, none of these animals experienced a LH surge at CT8. Five of these animals exhibited no LH surge at any time during the sampling period. The remaining grafted hamster exhibited an aberrant pattern of LH secretion: basal levels were higher than any of the control levels at all of the bleeding times, suggesting a disruption of the negative feedback effects of estradiol. Although there was no surge during the subjective day, LH levels rose during the subjective night and surged at CT24 (Fig. 4, *dotted line*; animal 249).

In addition, LH surges were absent in blood samples collected by cardiac puncture from each of the hamsters whose cannulas became occluded during sampling. In three cases, cardiac puncture was performed at CT8, when LH surges occurred in the controls. The patterns of LH secretion in hamsters that were ovariectomized at the time of cannulation did not differ from those that were gonadectomized at the time the SCN transplant was performed. Thus, it is unlikely that the absence of LH surges is attributable to a masculinizing effect of ovarian hormones on the transplant.

Urinary 6αMTS rhythms. Urinary concentrations of 6αMTS exhibited robust diurnal rhythms in intact controls, with concentrations showing a 6-fold rise during the mid-dark period and declining through the day (Fig. 5a). SCN lesions eliminated the nocturnal rise in 6αMTS, and this effect was not reversed by SCN grafts that reinstated locomotor rhythms, either in individual animals or in the mean values of the group (Fig. 5, b and c). Urinary 6αMTS concentrations in the lesioned animals remained near the nadir values of intact controls throughout the 2-day sampling period.

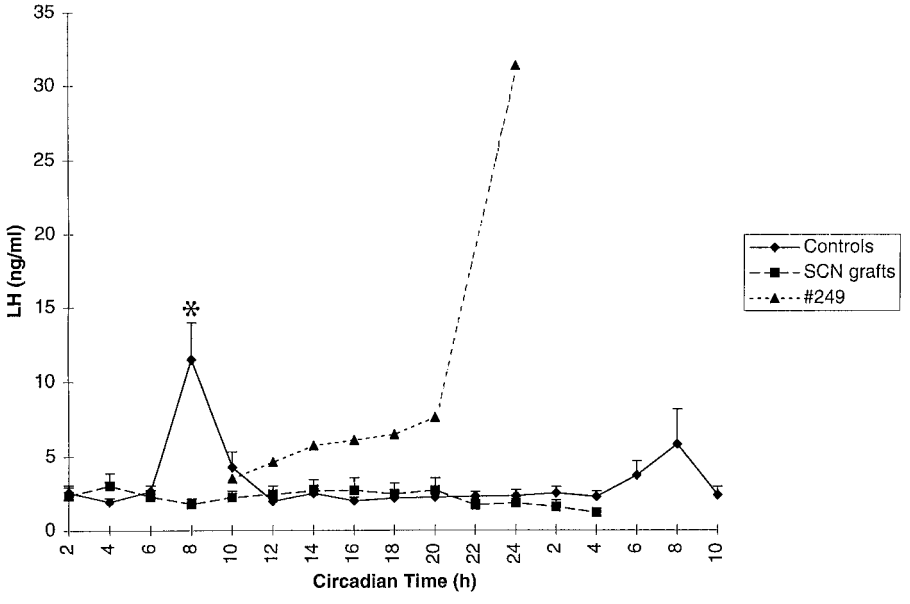


FIG. 4. LH values (mean ± SEM) in ovariectomized female hamsters on the third day after implantation of estradiol capsules and placement of indwelling jugular cannulas. *Filled diamonds*, brain-intact controls; *filled squares*, SCN-lesioned hamsters that had received transplants that reinstated circadian rhythms of locomotor activity. The CT at which each sample was collected is indicated on the *abscissa*, with the time of running onset set at CT12 according to convention. For the intact controls, running onset reflects the phase of the preceding LD cycle, but the locomotor activity of grafted animals does not entrain to the LD cycle so that blood sampling began at different circadian phases for the different individuals as indicated by their preceding wheel-running records. Individual data from an exceptional SCN-lesioned, transplanted hamster (no. 249) are also shown (*solid triangles*). The *asterisk* indicates a significantly significant ($P < 0.01$) difference from basal LH concentrations.

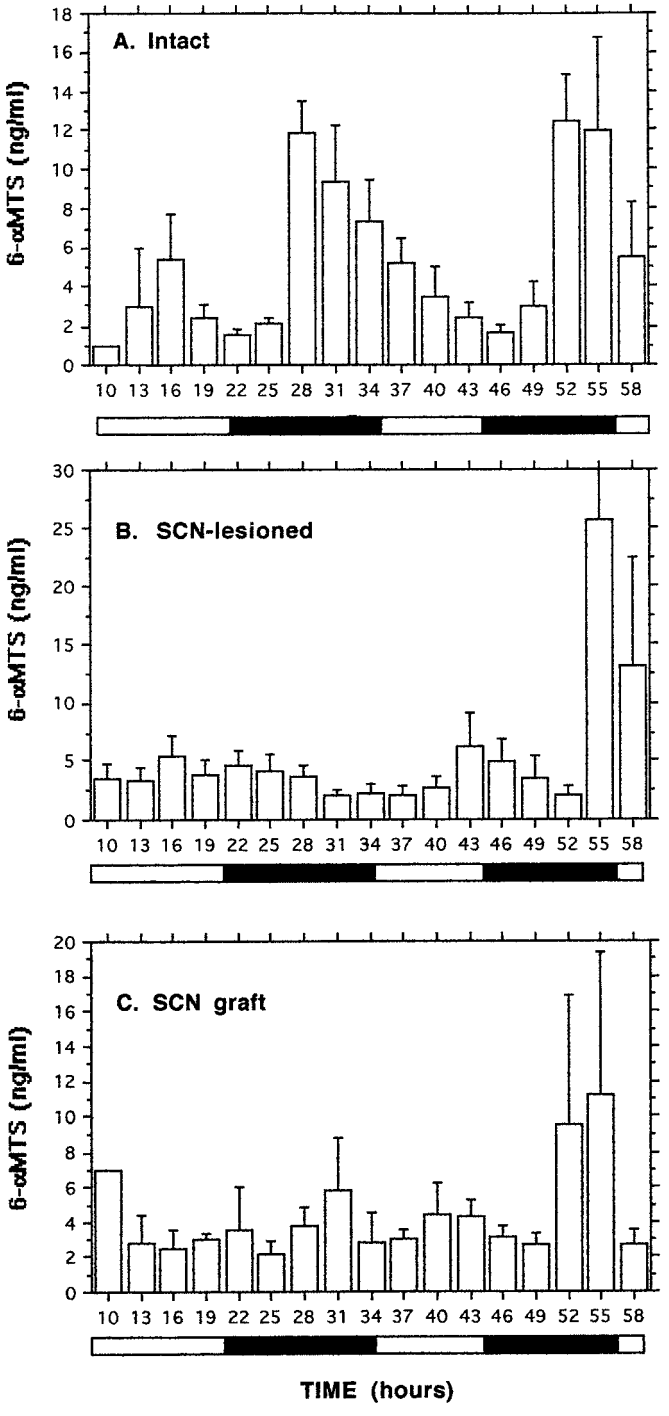


FIG. 5. Mean (±SEM) urinary 6αMTS concentrations in groups of brain-intact control (*top*), SCN-lesioned (*middle*), and SCN-lesioned (*bottom*) hamsters that had received SCN grafts that reinstated locomotor activity (n = 6/group). Hamsters were maintained in metabolic cages in 14L:10D, and values were pooled in 3-h bins. The *shaded bar* on the *abscissa* indicates the hours of darkness. The pattern of data from individual animals resembled group means, indicating that arrhythmicity after SCN lesion is not attributable to asynchrony of rhythmic individuals within the group.

Histological analysis of lesions and grafts. Histological examination confirmed SCN lesions in animals that exhibited locomotor arrhythmicity. Each lesion reported in this study included the entire SCN, but most also impinged upon adjacent portions of the anterior hypothalamus and the optic chiasm. Fetal hypothalamic grafts were localized to the third ventricle in the rostral hypothalamus. All fetal transplants included VIP-immunoreactive cell clusters, but nonimmunostained tissue reflecting adjacent portions of the SCN and neighboring regions of the hypothalamus was also present in each of the grafts. Fluorescent tracers and PHA-L immunocytochemistry revealed only sparse graft-host connections, including some innervation of the transplant from the surrounding hypothalamus of the recipient.

Of the eight animals that received retrograde tracer injections, only three injections were restricted to the host POA. In these animals, numerous retrogradely labeled cells were seen in the anterior and medial hypothalamus of the host brain, but very few cells (two or three per graft) were detected in the graft itself (Fig. 6, D and E). In the five remaining cases, tracer from the injection site also spread into the graft (Fig. 6, A and B). In these cases, in addition to retrogradely labeled cells in the medial hypothalamus of the host, numerous labeled neurons were found in the graft (Fig. 6, A, B, and F). Retrogradely labeled cells were seen in all portions of the graft, including those corresponding to the location of VIP cells characteristic of the donor SCN (Fig. 6C).

Three animals also received iontophoretic injections of the anterograde tracer PHA-L, directed toward the intraventricular graft. In all cases, however, injections missed the graft and were restricted instead to the adjacent host POA (Fig. 7A). Numerous anterogradely labeled fibers could be traced from PHA-L deposits in the POA to other areas of the host brain, including the lateral septum and bed nucleus of the stria terminalis bilaterally, and the contralateral POA. By contrast, relatively few host fibers entered the graft (Fig. 7B). When they did, anterogradely labeled fibers crossed the graft-host border at attachment sites along the wall of the third ventricle where the ependymal cell layer was absent.

Exp 2: male hamsters

Locomotor rhythmicity. Each animal exhibited an absence of circadian rhythmicity in wheel-running behavior after SCN lesions were performed (Fig. 8). Hamsters were included only in cases in which fetal hypothalamic transplants reinstated circadian locomotor rhythms and in which running onsets were sufficiently clear and reliable to allow determination of circadian phase of death. None of the hamsters that received no graft or an equivalent volume of fetal cortex experienced a restoration of locomotor rhythms.

Gonadal condition. After approximately 11 weeks of maintenance in DD, sham-lesioned control hamsters experienced gonadal regression (mean combined testis weight, 1.27 ± 0.15 g, mean ± SEM; Fig. 9). SCN-lesioned hamsters bearing behaviorally functional SCN grafts had significantly heavier testes at the time of death, as did SCN-lesioned controls that received grafts of temporal cortex (combined testis weight, 3.35 ± 0.11 and 3.40 ± 0.26 g, respectively, mean ± SEM; both $P < 0.001$ *vs.* sham-lesioned controls; Fig. 9).

Pineal rhythm. ANOVA of pineal melatonin content indicated significant main effects of surgical treatment and circadian

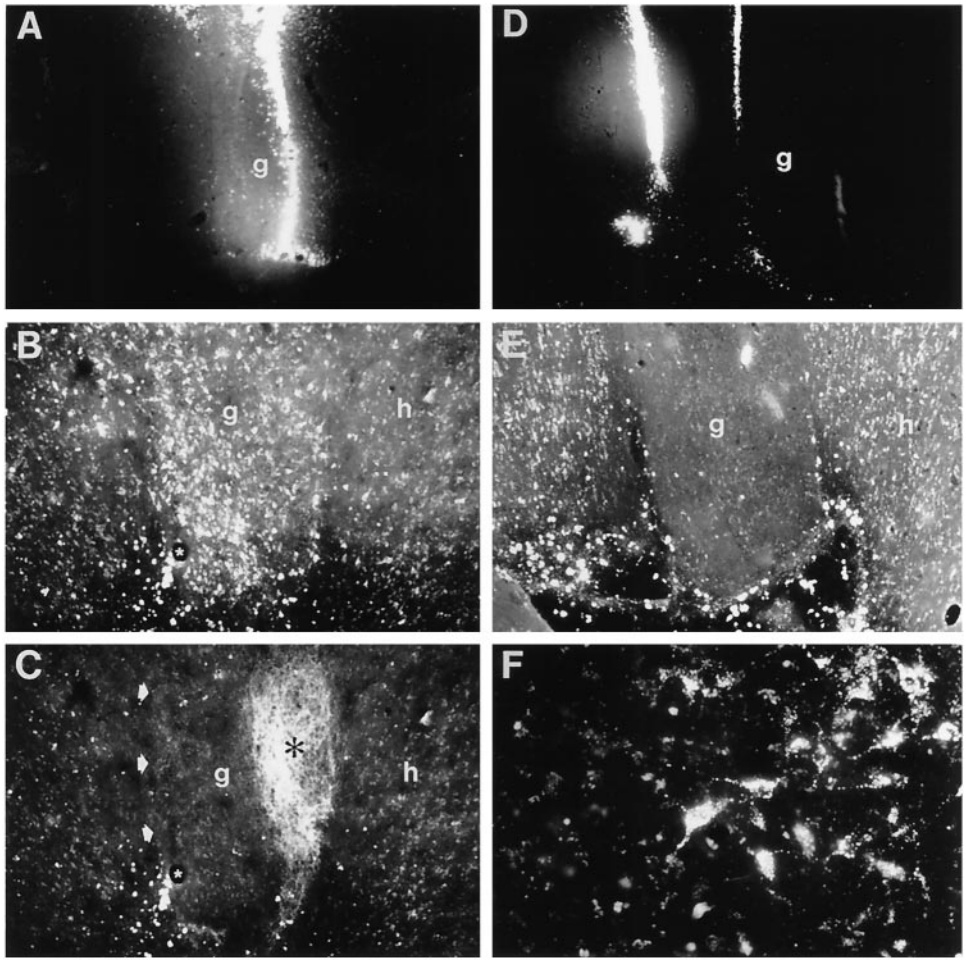


FIG. 6. Photomicrographs of brain sections from grafted animals in which injections of rhodamine-labeled fluorescent microspheres were located either predominantly in the graft (A–C) or in the adjacent POA of the host (D and E). A, B, D, E, and F were taken under rhodamine excitation filters to reveal the fluorescent beads. A and D show the tracer injection sites; B and E show portions of the same grafts seen in A and D, respectively, which were caudal to the injection site. When injections spread into the graft (A), retrogradely labeled cells were found throughout other portions of the graft (B). In contrast, when injections were located in the host POA, very few graft neurons were labeled (D), although many labeled cells were seen in the medial hypothalamus of the host. C is the same section shown in B and is viewed under a fluorescein excitation filter to reveal the presence of immunofluorescent VIP cells and fibers (*asterisk*) in the graft. The same blood vessel is marked by a *small asterisk* in B and C to facilitate orientation; *arrows* in C indicate the border between host and graft. F is a higher power view of retrogradely labeled graft neurons shown in B. g, Graft; h, host. Magnification: A–E, approximately ×50; F, approximately ×200.

time, and an interaction between these two factors ($P < 0.0001$). Sham-lesioned hamsters maintained in DD showed a sustained elevation during the subjective night (Fig. 10). The pineal melatonin content in control hamsters was significantly higher at CT16, 20, and 24 than at CT4 ($P < 0.001$). A modest (2-fold) rise in serum melatonin concentrations occurred during the subjective night in control hamsters, but did not achieve statistical significance.

Six of the 11 SCN-lesioned hamsters killed at CT20, 11 weeks after receiving SCN transplants that reinstated behavioral rhythms, showed evidence of elevated pineal melatonin synthesis (values >100 pg/gland). Nevertheless, the pineal melatonin content in SCN-grafted hamsters was not significantly elevated at any time during subjective night ($P > 0.05$ *vs.* subjective day values) and was significantly lower than that in sham-lesioned and unoperated controls at each point during the subjective night ($P < 0.001$). SCN-lesioned hamsters bearing cortex grafts had uniformly low pineal content and serum melatonin concentrations. Individual hamsters with SCN grafts that experienced a partial rise in melatonin were no more likely to have regressed testes than those that did not; the correlation between pineal melatonin content during the subjective night and paired testis weight was not significant ($r^2 = 0.006$; $P = 0.71$).

Glucocorticoid rhythms. Assay of serum cortisol and corticosterone collected from groups of intact animals killed at various circadian times indicates that both hormones display free running rhythms that are phase locked to that of locomotor activity (Fig. 11, *top*). Peak concentrations of these hormones occurred during the late subjective day. Corticosterone values at CT8 and 12 were significantly higher than those at CT24, and values at CT8 were significantly higher than those at CT4 ($P < 0.05$, by Duncan's multiple range test). Cortisol values at CT4 were significantly lower than those at CT12 and 20 ($P < 0.05$). In contrast, mean values for both cortisol and corticosterone showed no significant effects of circadian time among hamsters whose locomotor rhythms had been restored by SCN transplants (Fig. 11, *bottom*).

Discussion

The present experiment confirms previous observations that ablation of the SCN eliminates circadian rhythms of locomotor activity and that transplants containing this nucleus reverse this effect (2, 20–25). Our findings are also consistent with previous observations that the persistence of rhythmic secretion of adrenal, pineal, and pituitary hormones depends upon the integrity of the SCN (3, 4, 8, 17–19). The present results establish for the first time that rhythms of endocrine secretion differ from those of behavior, in that after anterior hypothalamic lesions, transplantation of the SCN is not sufficient to reinstate their expression. These findings suggest significant differences between the circuitry

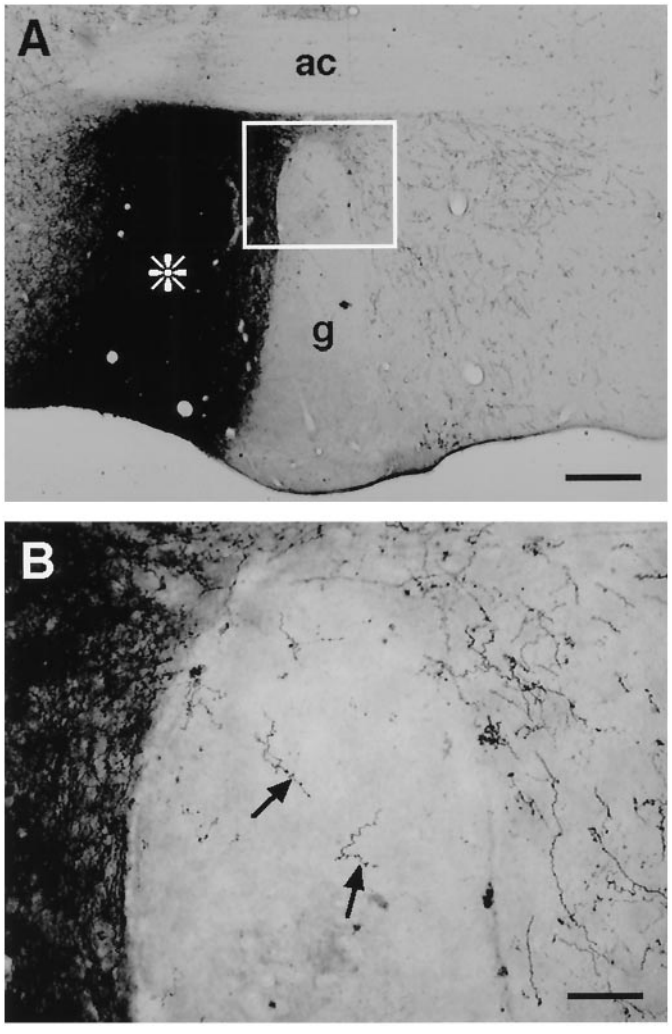


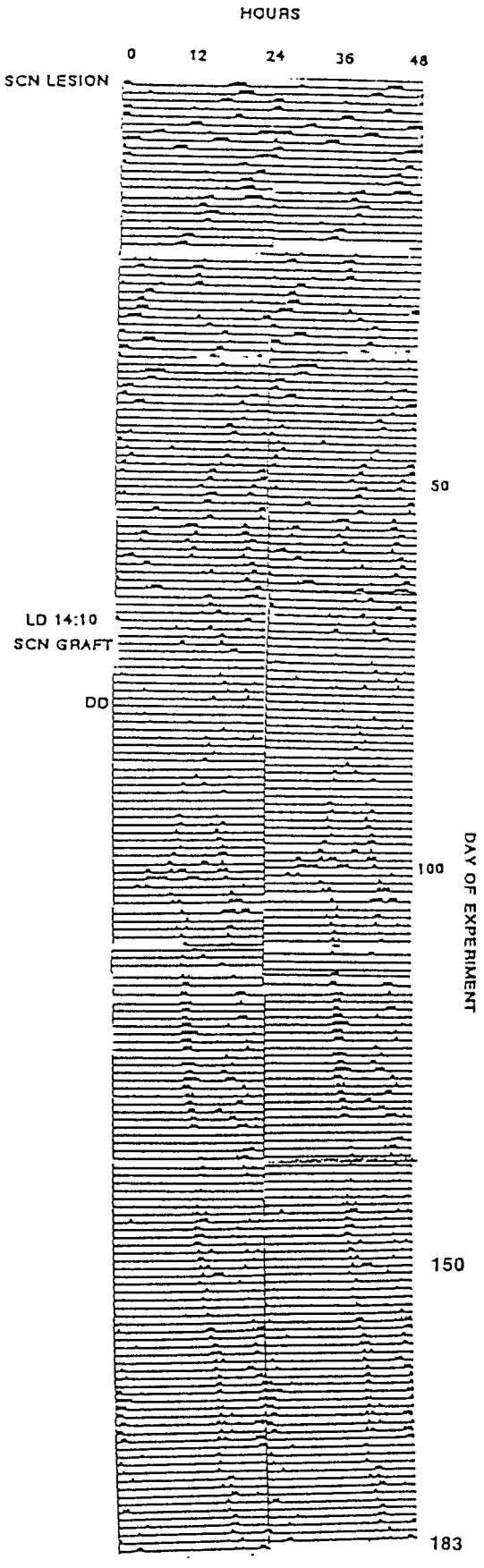
FIG. 7. The graft receives relatively sparse input from the host POA. A, Low power brightfield photomicrograph showing an injection (*asterisk*) of the anterograde tracer, PHA-L, located in the host POA adjacent to a SCN graft (g). The *boxed area* in A is shown in B at higher magnification. Labeled fibers course around the graft to innervate the contralateral host POA, but relatively few fibers actually enter the graft (*arrows*). ac, Anterior commissure. *Bar* in A = 100 μm; *bar* in B = 50 μm.

that sustains circadian rhythms of behavior *vs.* those of neuroendocrine function.

Locomotor rhythmicity has provided a useful set of "hands" of the circadian clock in many previous studies (10–13). Observation of the running activity records of intact hamsters enables one to predict with great accuracy the timing of the LH surge, ovulation, sexual behavior, pineal melatonin secretion, and adrenal corticosteroid release. The finding that hamsters in which exposure to constant light induces "splitting" of the activity rhythm have two LH surges, each phase-locked to one of the activity bouts (13), indicates the linkage of endocrine oscillators to the circadian clock that regulates activity. In light of such findings, the dissociation between the ability of grafts to reinstate rhythms of activity and endocrine secretion is surprising. The condition of the SCN-grafted hamster may be most comparable to that of animals sustaining knife cuts in the vicinity of the SCN that selectively interrupt particular sets of afferent or efferent projections of this nucleus. For example, semicircular knife cuts posterior to the SCN that eliminate estrous cycles spare circadian rhythms of drinking (34), and horizontal cuts that compromise photoperiodic responses and E_2-induced LH surges may leave oscillations of locomotor activity intact (35, 36).

The observation that SCN grafts reinstate locomotor rhythms has raised the question of the source and nature of the signals by which the SCN expresses its pacemaker function. Our present tract tracing results are consistent with earlier indications that the graft establishes relatively few afferent or efferent connections with the host POA. Instead, graft neurons appear to project predominantly to other portions of the graft (22, 37, 38). Such evidence along with the ability of SCN grafts placed far from the basal anterior hypothalamus to restore activity rhythms and the effectiveness of interstitial transplants of dissociated SCN cells (39) argue for the existence of a humoral signal in the regulation of locomotor activity rhythms. The SCN releases neuropeptides rhythmically *in vivo* (40) and *in vitro* (41, 42), and the persistence of locomotor rhythmicity in hamsters after isolation of the SCN (43) and placement of encapsulated grafts (44) provides the most conclusive evidence for humoral output. However, neither the studies of hypothalamic islands nor those of encapsulated grafts used neuroendocrine rhythms as an end point. The present data indicate that if the output of the SCN graft responsible for locomotor activity is indeed humoral, it is qualitatively or quantitatively inadequate to restore neuroendocrine rhythms.

Recent studies are consistent with the hypothesis that SCN outputs important for neuroendocrine rhythms are axonal rather than humoral. Pharmacological evidence indicates that vasopressinergic projections to the paraventricular and dorsomedial hypothalamus suppress ACTH secretion, and that the rhythmicity of serum corticosterone results from fluctuations in activity in this pathway (45). The preovulatory surge of LH in hamsters and rats may be triggered by direct inputs of SCN efferents to GnRH- and/or estrogen receptor-immunoreactive cells (33, 46), and VIPergic SCN cells have been suggested to play an important role in phasic GnRH release. Similarly, rhythms of melatonin secretion in hamsters appear to depend upon SCN efferents to the region of the PVN, which, in turn, project to thoracic preganglionic neurons of the sympathetic nervous system (36). The failure of grafts to restore endocrine rhythms may reflect the inability of the grafted SCN to establish appropriate connections with neuroendocrine targets in the host, such as GnRH neurons and/or estrogen receptor-immunopositive cells (32, 46, 47). Some of these projections may need to be reciprocated for proper coordination of the LH surge or other endocrine rhythms. Although efferents from anterior hypothalamic xenografts form close contacts with GnRH cells in the host medial septum (48), it is not clear whether such outgrowth arises from the donor SCN in these grafts or instead from other anterior hypothalamic tissue (49). The POA and anterior hypothalamus, in which many hypophysiotropic cells reside, also receive dense input from the subparaventricular region, which is a target of the SCN (32, 50–52). It is likely that variable portions of the sub-PVN were included in the



transplants, but we lack immunocytochemical markers with which to assess this. Furthermore, efferents of SCN grafts may travel short distances into the host brain, including the sub-PVN region. Although such input might have been expected to permit some relay of circadian information to neurosecretory cells, it must be insufficient to reinstate statistically significant neuroendocrine rhythmicity.

It is possible that the SCN lesions performed in this study eliminated not only the circadian pacemaker of the host, but also descending neurosecretory fibers that pass through the suprachiasmatic region *en route* to the median eminence. In this event, grafts that replace the pacemaker might have failed to repair damage to critical fibers of passage that may have arisen from either the electrolytic lesion or deformation of the host tissue by introduction of the transplant to the third ventricle. This explanation for the failure of SCN grafts to restore endocrine rhythms is unlikely for several reasons. First, fibers other than those of SCN cells that may be important to glucocorticoid and pineal rhythms are unlikely to have been damaged by the lesion. Second, the GnRH fibers that reach the rat median eminence from the POA traverse a variety of routes, including lateral trajectories that would be unlikely to have been compromised by the lesions (53, 54). The observation that GnRH-immunoreactive fibers are normally present in the intact SCN suggests, however, that reciprocal input between the POA and the pacemaker may be important to generation of the surge (46) (de la Iglesia, H. O., and E. L. Bittman, unpublished observations). As we did not examine the grafts for such contacts, we cannot evaluate the possibility that relevant host projections to the SCN were compromised by the surgery.

We have also considered the possibility that fetal grafts may have been masculinized by E_2 released from polycystic ovaries of the host, thus eliminating the capacity of the graft to sustain GnRH surges. Although sexual dimorphism of the SCN has been reported (55), it is not established whether the male SCN is adequate to support LH surges. In an attempt to address this issue, we included in our study hosts in which ovariectomy was performed at the time of the graft to ensure that the transplant was not exposed to gonadal steroids. Hamsters treated in this way were no more likely to recover E_2-induced LH surges than were animals that retained their ovaries until the time of cannulation a few days before the E_2 challenge. Thus, it seems unlikely that masculinization of the SCN in the grafts can account for the failure of estrogen-induced LH surges to recover after transplantation.

Photoperiodic control of testicular function is clearly dependent upon the SCN. Melatonin receptors reside in the SCN of some photoperiodic species, and infusions of melatonin are particularly effective at regulating gonadal function when directed at structures in the SCN region of Siberian hamsters (56). In Syrian hamsters, however, injections or

FIG. 8. Locomotor activity record of the grafted male hamster with the highest pineal melatonin content in Exp 2. The SCN lesion was performed on the day before the start of the record. Transplantation of fetal anterior hypothalamic tissue, performed on day 67, reinstated locomotor rhythmicity within 3 weeks. The animal was killed 118 days after transplantation. Despite exposure to DD, testes were nonregressed, weighing 4.27 g at the time of death. Pineal melatonin content was 261.69 pg at CT20.

infusions of melatonin regulate gonadal function even after destruction of the SCN (57, 58). The present observation that SCN grafts were rarely if ever effective in reinstating nocturnal elevations of melatonin in the host pineal gland are consistent with the conclusion, drawn from earlier studies, that the role of the SCN in regulation of photoperiodic responses is attributable to its control of melatonin synthesis and secretion. Both the studies of females, in which the pattern of urinary melatonin metabolites was recorded in serial samples from individuals, and the data from males, in which grouped data were obtained by the death of free running hamsters at various circadian times, indicate that the endogenous melatonin patterns of the hosts were inadequate to trigger gonadal regression in DD.

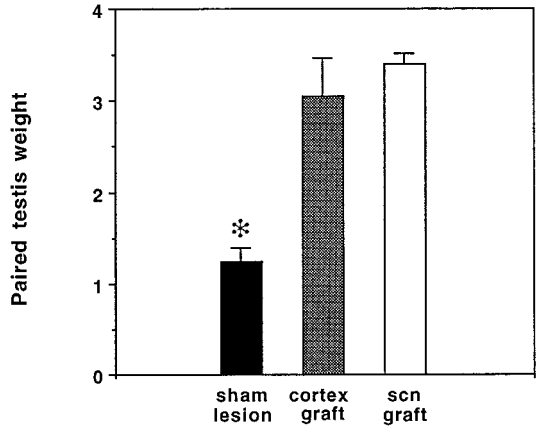


FIG. 9. Combined mean (±SEM) testis weight of intact hamsters (*filled bar*) or SCN-lesioned hamsters bearing hypothalamic transplants (*open bar*) or cortical transplant (*stippled bar*) after extended exposure to DD. Transplants reinstated locomotor activity rhythms in each of the SCN-grafted hamsters, but in none of the cortex-grafted hamsters. The *asterisk* indicates significant gonadal regression ($P < 0.05$) of sham-lesioned animals relative to that in the two grafted groups, which did not differ from each other.

The restoration of circadian rhythms by SCN grafts may not be an all or none phenomenon. Even the behavioral rhythms restored in this study, as in previous experiments, tend to show more variable onsets and less stable periods than those of intact hamsters. The melatonin contents of the pineal glands in the SCN-grafted male hamsters suggests that a partial restoration of this rhythm might have occurred; more than half of the hamsters killed at CT20 exhibited a partial rise in melatonin content. Even though the melatonin concentration in this group was significantly less than that in intact hamsters and did not differ from that of pineals collected during the subjective day, it is possible that the graft was adequate to induce a small nocturnal rise in melatonin in some of the hamsters. Similarly, one of the SCN grafted female hamsters (no. 249) exhibited a rise in LH. This apparent surge occurred at an inappropriate circadian time and was accompanied by apparent derangement of basal LH secretion. Furthermore, SCN grafts were uniformly incapable of reinstating estrous cycles. Nevertheless, data from this exceptional animal suggests that partial restoration of endocrine rhythmicity may occur, although it is insufficient to reinstate the functional response (ovarian cycles or gonadal regression in short days) upon transplantation of the SCN. Additional studies would be required to establish surgical procedures that might improve or maximize restoration of neuroendocrine rhythmicity.

We chose to study glucocorticoid rhythms in part because they seem most tightly linked to arousal and general activity. Thus, the inability of behaviorally effective grafts to reinstate adrenocortical rhythms was unexpected. On the other hand, it is rare that circadian rhythms of behavior in hamsters bearing SCN grafts are as regular and consistent as those in intact animals. Perhaps the absence of endocrine rhythms in

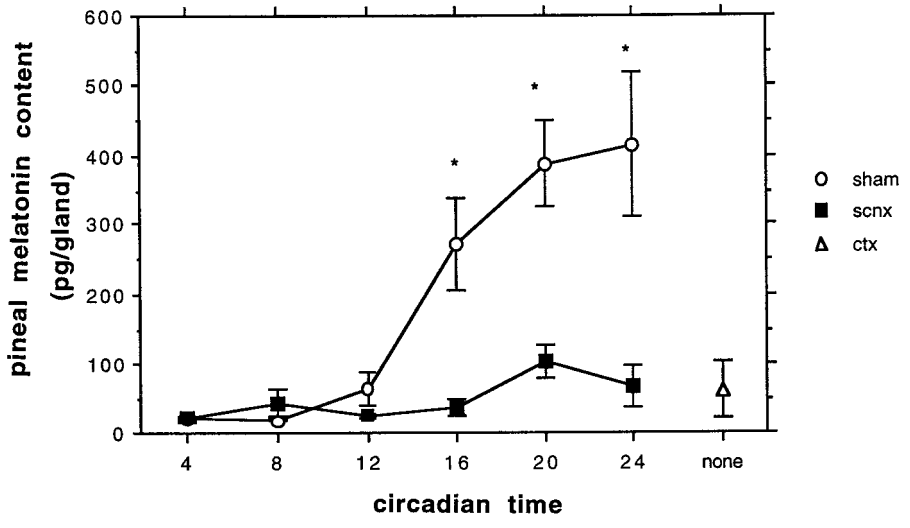


FIG. 10. Mean (±SEM) pineal melatonin content of intact (*open symbols*) and SCN-lesioned (*closed symbols*) hamsters bearing transplants that reinstated locomotor activity rhythms (n = 7–11/group). The melatonin content of the pineals of SCN-lesioned control hamsters transplanted with an equivalent volume of fetal cerebral cortex (n = 7) is indicated by the *triangle*; as these grafts did not reinstate circadian rhythms of locomotor behavior, the time of death of these animals was determined arbitrarily, and no circadian time could be assigned. Hamsters were maintained in DD for 7–11 weeks and were killed at circadian times determined on the basis of locomotor activity onset. *Asterisks* indicate a statistically significant increase in the melatonin content of intact hamsters over basal (CT4, -8, and -12) values ($P < 0.001$). Hamsters given SCN grafts did not exhibit a statistically significant rise over basal values at any circadian time.

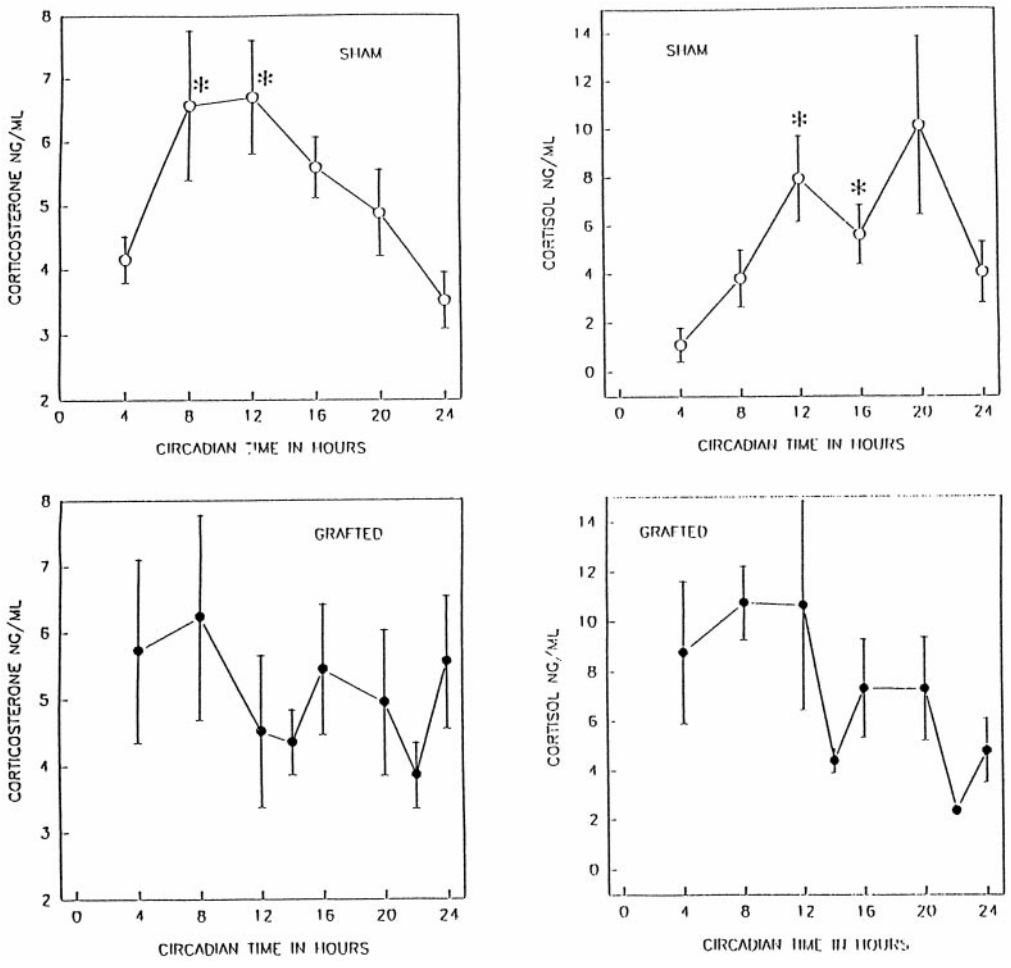


FIG. 11. Mean (±SEM) serum concentrations of corticosterone (*left*) and cortisol (*right*) in male hamsters that were intact (*open circles, top*) or that had received SCN lesions and transplants (*filled circles, bottom*; n = 7–10/group). An *asterisk* indicates a significant elevation of serum hormone values compared with CT4 levels ($P < 0.05$).

SCN-grafted animals contributes to the imprecision of the restored behavioral oscillations. Projections or humoral signals of the graft that influence arousal or eating could generate periodic feedback signals (*e.g.* blood glucose, fatty acids, osmolality, pH, *etc.*) that could regulate endocrine secretion even if central pacemaker control over hypophysiotropic neurons was not restored. The absence of endocrine rhythms in hamsters bearing behaviorally effective grafts, however, suggests that such control by activity-generated feedback signals is weak. It will be interesting to examine the ability of SCN grafts to reinstate temperature rhythms, whose elimination by SCN lesions has been controversial (6, 7) and which may rely in part upon oscillations of thyroid hormone and interleukins. The contributions of gut hormones, insulin, thyroid hormones, GH, and vasopressin secretion to drinking and eating suggest that endocrine rhythmicity might facilitate the reappearance of behavioral rhythms after transplantation of the SCN. Additional experiments would be required to establish whether SCN grafts reinstate circadian patterns of secretion of these hormones.

In conclusion, the elimination of LH surges, melatonin, and glucocorticoid rhythms by SCN lesions is consistent with the findings of many previous studies in hamsters and rats and supports the role of this master pacemaker in regulation of rhythmic endocrine function. The inability of grafts adequate to restore locomotor rhythms to reverse the elimination of ovarian, adrenal, pituitary, and pineal rhythms and DD-induced testicular regression by SCN lesions suggests that SCN outputs necessary for neuroendocrine rhythms differ quantitatively or qualitatively from those that maintain locomotor behavior. For example, targets of SCN efferents that regulate endocrine function may require more extensive innervation than they receive from the transplants. In contrast, the maintenance of locomotor rhythmicity may require only a sparse, local neuronal outgrowth or a humoral signal from the graft.

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References

1. **Miller JD, Morin LP, Schwartz WJ, Moore RY** 1996 New insights into the mammalian circadian clock. Sleep 19:641–667

2. **Ralph MR, Foster RG, Davis FC, Menaker M** 1990 Transplanted suprachiasmatic nucleus determines circadian period. Science 247:975–978
3. **Moore RY, Eichler VB** 1972 Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res 42:201–206
4. **Abe K, Kroning J, Greer MA, Critchlow V** 1979 Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body temperature, feeding, and plasma thyrotropin. Neuroendocrinology 29:119–131
5. **Bethea CL, Neill JD** 1980 Lesions of the suprachiasmatic nuclei abolish the cervically stimulated prolactin surges in rat. Endocrinology 107:1–5
6. **Kittrell EM** 1991 The suprachiasmatic nucleus and temperature rhythms. In: Klein DC, Moore RY, Reppert SM (eds) Suprachiasmatic Nucleus: The Mind's Clock. Oxford University Press, New York, pp 233–245
7. **Refinetti, R, Kaufman, CM, Menaker M** 1994 Complete suprachiasmatic lesions eliminate circadian rhythmicity of body temperature and locomotor activity in golden hamsters. J Comp Physiol [A] 175:223–232
8. **Moore RY, Klein DC** 1974 Visual pathways and the central neural control of a circadian rhythm in the pineal serotonin *N*-acetyltransferase activity. Brain Res 71:17–33
9. **Alleva JJ, Waleski MV, Alleva FR** 1971 A biological clock controlling the estrous cycle of the hamster. Endocrinology 88:1368–1379
10. **Fitzgerald KM, Zucker I** 1978 Circadian organization of the estrous cycle of the golden hamster. Proc Natl Acad Sci USA 73:2923–2927
11. **Carmichael MS, Nelson RJ, Zucker I** 1981 Hamster activity and estrous cycles: control by a single versus multiple circadian oscillator(s). Proc Natl Acad Sci 78:7830–7834
12. **Moline ML, Albers HE, Todd RB, Moore Ede MC** 1981 Light-dark entrainment of proestrous LH surges and circadian locomotor activity in female hamsters. Horm Behav 15:451–458
13. **Swann J, Turek, FW** 1985 Multiple circadian oscillators regulate the timing of behavioral and endocrine rhythms in female golden hamsters. Science 228:898–900
14. **Stetson MH, Gibson JT** 1977 The estrous cycle in golden hamsters: a circadian pacemaker times preovulatory gonadotropin release. J Exp Zool 201:289–294
15. **Everitt JW, Sawyer, CH** 1950 24-hour periodicity in the "LH-release apparatus" of female rats, disclosed by barbiturate sedation. Endocrinology 47:198–218
16. **Norman R, Spies HG** 1974 Neural control of the estrogen-dependent twenty-four hour periodicity of LH release in the golden hamster. Endocrinology 95:1367–1372
17. **Brown-Grant K, Raisman G** 1977 Abnormalities in reproductive function associated with the destruction of the suprachiasmatic nuclei in female rats. Proc R Soc Lond [B] 198:279–286
18. **Gray GD, Sodersten P, Tallentire D, Davidson JM** 1978 Effects of lesions in various structures of the suprachiasmatic-preoptic region on LH regulation and sexual behavior in female rats. Neuroendocrinology 25:174–191
19. **Kawakami M, Arita J, Yoshioka E** 1980 Loss of estrogen-induced daily surges of PRL and gonadotropins by SCN lesions in ovariectomized rats. Endocrinology 106:1087–1092
20. **Drucker-Colin R, Aguilar-Roblero R, Garcia-Hernandez F, Fernandez-Cancion F, Rattoni FB** 1984 Fetal suprachiasmatic nucleus transplants: diurnal rhythm recovery of lesioned rats. Brain Res 311:353–357
21. **Sawaki Y, Nihonmatsu I, Kawamura H** 1984 Transplanation of the neonatal suprachiasmatic nucleus into rats with complete bilateral SCN lesion. Neurosci Res 1:67–72
22. **Lehman MN, Silver R, Gladstone WR, Kahn RM, Gibson M, Bittman EL** 1987 Circadian rhythmicity restored by neural transplant: immunocytochemical characterization of the graft and its integration with the host brain. J. Neurosci 7:1626–1638
23. **De Coursey PJ, Buggy J** 1989 Circadian rhythmicity after neural transplant to hamster third ventricle: specificity of suprachiasmatic nuclei. Brain Res 500:263–275
24. **Sollars PJ, Pickard GE** 1993 Time course of fiber outgrowth from fetal anterior hypothalamic heterografts. Brain Res 614:212–219
25. **Matsumoto S-I, Basil J, Jetton AE, Lehman MN, Bittman EL** 1996 Regulation of the phase and period of circadian rhythms restored by suprachiasmatic transplants. J Biol Rhythms 11:145–162
26. **Aldhous M, Arendt J** 1988 Radioimmunoassay for 6-sulphatoxymelatonin in urine using an iodinated tracer. Ann Clin Biochem 25:298–303
27. **Maywood ES, Hastings MH, Max M, Ampleford E, Menaker M, Loudon ASI** 1993 Circadian and daily rhythms of melatonin in the blood and pineal gland of free-running and entrained Syrian hamsters. J Endocrinol 136:65–73
28. **Arendt J, Bojkowski C, Franey C** 1985 Immunoassay of 6-hydroxymelatonin sulphate in human plasma and urine: abolition of the urinary rhythm with atenolol. J Clin Endocrinol Metab 60:1166–1172
29. **Orsini MW** 1961 The external vaginal phenomena charaterizing the stages of the estrous cycle, pregnancy, pseudopregnancy, lactation and the anestrous hamster, *Mesocricetus auratus* Waterhouse. Proc Anim Care Panel 11:193–206
30. **Gerfen CR, Sawchenko PE** 1984 An anterograde neuroanatomical tract tracing method that allows the detailed morphology of neurons, their axons and terminals: immunohistochemical localization of an axonally transported plant lectin, *Phaseolus vulgaris* leucoagglutinin (PHA-L). Brain Res 290:219–238
31. **Watson RE, Wiegand SJ, Clough RW, Hoffman GE** 1986 Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides 7:155–159
32. **De la Iglesia HO, Blaustein JD, Bittman EL** 1995 The suprachiasmatic area in the female hamster projects to neurons containing estrogen receptors and GnRH. NeuroReport 6:1715–1722
33. **Elliott JA, Tamarkin L** 1994 Complex circadian regulation of pineal melatonin and wheel-running in Syrian hamsters. J Comp Physiol 174:469–484
34. **Nunez AA, Stephan FK** 1977 The effects of hypothalamic knife cuts on drinking rhythms and the estrous cycle of the rat. Behav Biol 20:224–234
35. **Watts AG, Sheward WJ, Whale D, Fink G** 1989 The effects of knife cuts in the sub-paraventricular zone of the female rat hypothalamus on estrogen-induced diurnal surges of plasma prolactin and LH, and circadian wheel-running activity. J Endocrinol 122:593–604
36. **Badura LL, Sisk CL, Nunez AA** 1977 Neural pathways involved in the photoperiodic control of reproductive physiology and behavior in female hamsters (*Mesocricetus auratus*). Neuroendocrinology 46:339–344
37. **Lehman MN, Silver R, Bittman EL** 1991 Anatomy of suprachiasmatic nucleus grafts. In: Klein DC, Moore RY, Reppert SM (eds) Suprachiasmatic Nucleus: The Mind's Clock. Oxford University Press, New York, pp 349–374
38. **Canbelyi RS, Lehman MN, Silver R** 1991 Tracing SCN graft efferents with DiI. Brain Res 554:40–45
39. **Silver R, Lehman MN, Gibson M, Gladstone WR, Bittman EL** 1990 Dispersed cell suspensions of fetal SCN restore circadian rhythmicity in SCN-lesioned adult hamsters. Brain Res 525:45–58
40. **Schwartz WJ, Reppert SM** 1985 Neural regulation of the circadian vasopressin rhythm in cerebrospinal fluid: a pre-eminent role for the suprachiasmatic nuclei. J Neurosci 5:2771–2778
41. **Earnest DJ, Sladek CD** 1986 Circadian rhythms of vasopressin release from individual rat suprachiasmatic explants *in vitro*. Brain Res 382:129–133
42. **Murakami N, Takamure M, Takahashi K, Utunomiya, K, Kuroda H, Etoh T** 1991 Long term cultured neurons from rat suprachiasmatic nucleus retain the capacity for circadian oscillation of vasopressin release. Brain Res 545:347–350
43. **Hakim H, DeBernardo AP, Silver R** 1991 Circadian locomotor rhythms, but not photoperiodic responses, survive surgical isolation of the SCN in hamsters. J Biol Rhythms 6:97–114
44. **Silver R, LeSauter J, Tresco PA, Lehman MN** 1996 A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. Nature 382:810–813
45. **Kalsbeek A, van Heerikhuize JJ, Wortel J, Buijs RM** 1996 A diurnal rhythm of stimulatory input to the hypothalamo-pituitary adrenal system as revealed by timed intrahypothalamic administration of the vasopressin V_1 antagonist. J Neurosci 16:5555–5565
46. **van der Beek EM, Wiegant VM, van der Donk HA, van den Hurk R, Buijs RM** 1993 Lesions of the SCN indicate the presence of a direct vasoactive intestinal polypeptide-containing projection to gonadotropin releasing hormone neurons in the female rat. J Neuroendocrinol 5:137–144
47. **van der Beek EM** 1996 Circadian control of reproduction in the female rat. In: Buijs RM, Kalsbeek, A, Romijn HJ, Pennartz CMA, Mirmiram M (eds) Progress in Brain Research. Elsevier, Amsterdam, pp 293–318
48. **Lehman MN, LeSauter J, Kim C, Berriman SJ, Tresco PA, Silver R** 1995 How do fetal grafts of the suprachiasmatic nucleus communicate with the host brain? Cell Transplant 4:75–81
49. **Lehman MN, LeSauter J, Silver R** 1997 Fiber outgrowth from anterior hypothalamic and cortical xenografts implanted into the third ventricle, J Comp Neurol 44:445–457
50. **Watts AG, Swanson LW, Sanchez Watts G** 1987 Efferent projections of the suprachiasmatic nucleus. I. Studies using anterograde transport of *Phaseolus vulgaris* leucoagglutinin in the rat. J Comp Neurol 258:204–229
51. **Kalsbeek A, Teclemariam-Mesbah R, Pevet P** 1993 Efferent projections of the suprachiasmatic nucleus of the goden hamster (*Mesocricetus auratus*). J Comp Neurol 332:293–314
52. **Morin LP, Goodless-Sanchez N, Smale L, Moore RY** 1994 Projections of the suprachiasmatic nuclei, subparaventricualr zone and retrochiasmatic area in the golden hamster. Neuroscience 61:391–410
53. **Merchanthaler I, Kovacs G, Lovasz G, Setalo G** 1980 The preoptico-infundibular LH-RH tract of the rat. Brain Res 198:63–74
54. **Hoffman GE, Gibbs FP** 1982 LHRH pathways in rat brain: 'deafferentation' spares a sub-chiasmatic LHRH projection to the median eminence. Neuroscience 7:1979–1993
55. **Guldner F-H** 1976 Sexual dimorphisms of axo-spine synapses and postsynaptic density material in the suprachiasmatic nucleus of the rat. Neurosci Let 28:145–150
56. **Badura L, Goldman BD** 1993 Central cites mediating reproductive responses to melatonin in juvenile male Siberian hamsters. Brain Res 598:98–106
57. **Bittman EL, Goldman BD, Zucker I** 1979 Testicular responses to melatonin are altered by lesions of the suprachiasmatic nuceli in golden hamsters. Biol Reprod 21:647–656
58. **Maywood ES, Buttery RC, Vance GHS, Herbert J, Hastings MH** 1990 Gonadal responses of the male Syrian hamster to programmed infusions of melatonin are sensitive to signal duration and frequency but not to signal phase nor to lesions of the suprachiasmatic nuclei. Biol Reprod 43:174–182