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Hamster circadian rhythms are phase-shifted by electrical stimulation of the geniculo-hypothalamic tract

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The suprachiasmatic nuclei (SCN) contain the major pacemaker for mammalian circadian rhythms. The SCN receive photic input both directly, via the retinohypothalamic tract (RHT), and indirectly, via the geniculohypothalamic tract (GHT), which originates in cells in the intergeniculate leaflet (IGL) and anterior portions of the ventral lateral geniculate nucleus (vLGN). We tested whether eletrical stimulation of the GHT would induce phase shifts in wheel-running activity rhythms of Syrian hamsters housed in continuous darkness or continuous illumination. In both lighting conditions, electrical stimulation of the GHT induced mainly phase advances when given during the late subjective day and small phase delays when given during the late subjective night and early subjective day. Stimulation in the thalamus outside the GHT failed to produce similar phase shifts. Repeated daily stimulation had only a weak entraining effect on the activity rhythm. Activation of GHT neurons appears to influence the pacemaker for activity rhythms in a phase-dependent manner.

INTRODUCTION

The suprachiasmatic nuclei (SCN) function as a major pacemaker for the mammalian circadian system and as a primary target of photic information relevant to rhythm entrainment[21,31]. There are two major afferents to the SCN that could mediate photic entrainment of rhythms: a direct projection from retinal ganglion cells (the retinohypothalamic tract [RHT][20,25]), and an indirect projection from retino-recipient portions of the intergeniculate leaflet (IGL) and ventral lateral geniculate nucleus (vLGN) (the geniculohypothalamic tract [GHT][3,10,25]). The GHT is characterized in part by the presence of neuropeptide Y (NPY)-immunoreactivity in its cells of origin. These cells are the source of all NPY in the SCN of the rat (*Rattus norvegicus*[3]) and of most of the NPY plexus in the SCN of the Syrian hamster (*Mesocricetus auratus*[10]).

Several lines of evidence suggest that the GHT in hamsters is involved in photic entrainment. Interruption of the optic tracts, which spares the RHT but denervates the geniculate, affected entrainment of hamster circadian rhythms[28]. Destruction of the GHT reduced the size of advance shifts to light pulses applied during the late subjective night while delay shifts were minimally affected[11,26]. Activity rhythms recorded in constant illumination (LL) showed shortened freerunning periods in geniculate-ablated animals[12,26], and the tendency for hamster activity rhythms to split into two components in LL was drastically reduced[12].

Activation of the GHT and consequent release of NPY into the SCN may phase shift circadian rhythms. Microinjections of NPY into the SCN area caused phase-dependent shifts of hamster activity

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rhythms with advances during the late subjective day and smaller delays during the late subjective night[1]. Injection of the excitatory neurotoxin *N*-methyl-DL-aspartate (NMA) into the geniculate area caused a similar pattern of phase shifts[14].

The results of these studies are consistent with the idea that GHT activation affects entrainment, but other interpretations are possible. The effects of NPY injections near the SCN of hamsters may indicate a role for NPY in regulating rhythms, but this does not necessarily implicate the GHT since NPY in the hamster SCN comes from both the GHT and another, unknown source[10]. In addition, the excitotoxic lesions that generated phase shifts damaged large portions of the thalamus and hippocampus and sometimes induced seizures[14], so the specificity of these effects is uncertain.

We approached the question of whether the GHT affects circadian rhythms using electrical stimulation of thalamic nuclei that give rise to the GHT. Electrical stimulation induced phase shifts in hamster activity rhythms with a pattern of phase-dependence similar to that generated by dark pulses and by injections of NPY into the SCN or NMA into the geniculate area. Because of the suggestion that some or all of these effects may be mediated by increases in activity and arousal[23], we also assessed the intensity of wheel-running activity during electrical stimulation.

MATERIALS AND METHODS

Adult male Syrian hamsters (LAK:1vg) were used in this study. Nineteen hamsters received stimulations, of which 11 animals provided usable behavioral data as well as histological results on electrode placements. Hamsters were housed in individual Plexiglas cages (24 × 22 × 20 cm) with free access to Purina rodent chow and tap water. Each cage was equipped with a commutator switch and a bipolar lead that permitted animals to reach all parts of the cage while the lead was attached to an implanted intracranial electrode. Each cage also had an 18 cm diameter running wheel, rotation of which activated a microswitch and recorded a single pen deflection on an Esterline-Angus operations recorder in another room.

For studies in LL, up to 6 cages were kept on open shelves in a room maintained in dim constant illumination (~5 lux) and exposed to continuous white noise (~70 dB). Because cages were shielded individually from direct overhead illumination, light intensities within the cages were variable and considerably below 5 lux. The room was always entered from a darkened outer room for animal maintenance and to turn on the stimulation equipment. Hamsters who were asleep did not usually awake in response to entry to or departure from the room. The room was subsequently darkened to test animals freerunning in continuous darkness (DD). Other animals were tested in identical cages placed in individual, light-tight boxes.

All hamsters were implanted permanently with twisted-wire bipolar stainless steel electrodes (Plastic Products, MS303/2 or 303/3) insulated except for the cross-sectional tip of each wire (0.125–0.20 mm); tips were separated by 0.1–0.2 mm. Hamsters were anesthetized with sodium pentobarbital (initial dose of 80 mg/kg body wt., with supplementary doses of 10–15 mg/kg as needed), and placed in a stereotaxic instrument with the incisor bar 2 mm below the interaural line. A drill bit was used to make a hole in the skull through which an electrode was aimed at the area of the IGL (1.5–1.8 mm caudal to bregma, 3.1–3.2 mm lateral to the sagittal suture, and 4.3–4.4 mm ventral to the surface of the cortex). Three or 4 stainless steel screws were attached to the skull around the electrode and the screws and electrode were cemented into place using dental acrylic cement.

After recovery from anesthesia, hamsters were placed in individual cages and connected to the electrical leads, and via the commutator to a Grass S9 or S44 stimulator. The stimulator could be activated manually or via a remote timer to provide stimulation to any single hamster. When freerunning activity rhythms appeared stable, hamsters received stimulation trains consisting of 20 Hz, 0.5 ms bipolar pulses of 50–100 V for 1–4 h. Pulses were delivered through an intervening 0.5 MΩ resistor to stabilize the current. Most stimulations involved 2 or 4 h of stimulation at 100 V (~200 μA). Hamsters studied in LL were frequently observed as the stimulation was turned on. For some animals that showed motor artifacts associated with stimulation, the voltage was gradually increased from 0 to the target value over

5–25 min. Hamsters were also stimulated without being observed by programming a timer to activate the stimulator.

At the end of the experiment, hamsters were killed with a barbiturate overdose and perfused transcardially with 0.9% saline followed by 10% formalin. Some hamsters received anodal direct current (10 μA for 10 s) through the electrode immediately before perfusion in order to deposit iron near the electrode tip. Brains were removed and postfixed, then 40-μm frozen sections were collected through the site of electrode placement. Alternate sections through the lateral geniculate area were stained for cell bodies and myelinated fibers using the method of Klüver and Barrera[15]. The Prussian blue reaction was used to identify the stimulation site in animals that received DC lesions. In other animals, tissue was examined microscopically to identify the most ventral site to which electrode tracks penetrated.

Phase shifts were analyzed by eye-fitting straight lines to activity onsets for 10 days immediately before and for 10 days after stimulation. In order to estimate the steady-state phase shift produced, one or two days of transient shifts after the stimulation day were sometimes excluded from the analysis and fewer than 10 days were used if there was an obvious change in the freerunning period late in the 10-day segment. The two fitted lines were extrapolated to the day following stimulation; the horizontal displacement between them on that day was converted to hours and used as the estimate of the steady-state phase shift. The phase of the stimulation was calculated in circadian hours with Circadian Time (CT) 12 defined as the time of activity onset[4]. The amount of phase shift was plotted relative to the midpoint of the stimulation train.

Seven stimulations were excluded from the analysis because of instability in the records; 5 additional stimulations were excluded because histological results were unavailable for 4 animals. A total of 36 stimulations were analysed, 26 in LL and 10 in DD. The time between CT 6 and CT 10 appeared to yield the largest shifts, so initial stimulations for each animal were usually given at that phase. Since many animals received only one stimulation, this strategy resulted in a clustering of points in that interval, although we attempted to sample a variety of circadian phases across animals.

One observer scored each stimulation for the intensity of wheel-running recorded on the activity chart during the length of the stimulation train. The observer used a scale with 0 = no activity and 3.0 = very intense activity throughout the stimulation.

For two animals that responded with reliable phase shifts to stimulation, daily sessions of stimulation were programmed to attempt to entrain the freerunning rhythm. These continued for 18 days (02.15–06.15) for one animal (No. 9), and for 6 days (16.00–18.00) and 17 days (00.00–02.00) during two attempts using a second animal (No. 5). Because of a programming error, there was a single 2-h period of stimulation at 14.00–16.00 on the day immediately preceding the first entrainment attempt for animal No. 5.

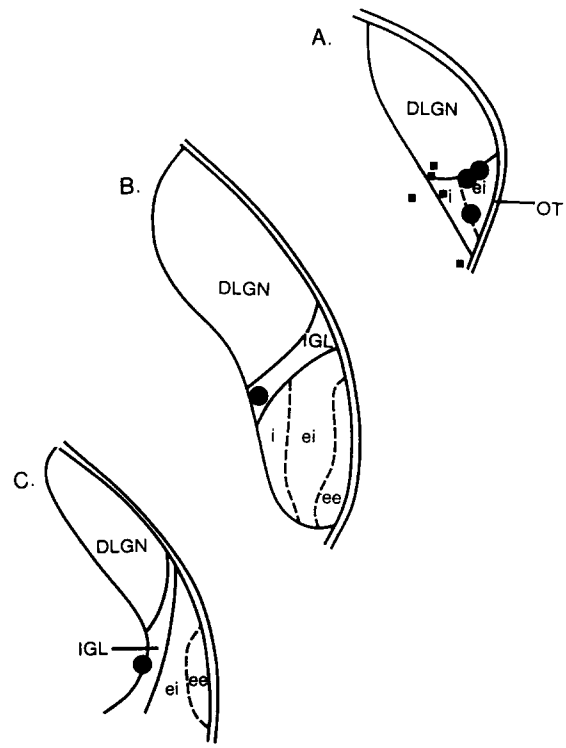

Fig. 1. Histological reconstruction of the brain sites at which stimulation was delivered. Circles indicate stimulation sites in 5 animals that fell within the regions known to give rise to the GHT. Squares indicate stimulation sites in 6 animals that were outside the regions known to give rise to the GHT. One non-GHT stimulation site is not shown because it was in the caudate nucleus outside the region illustrated. Sections run from the rostral vLGN (A) to the caudal portion of the IGL (C). The small letters indicate the divisions of the vLGN; i, internal division; ei, internal lamina of the external division; ee, external lamina of the external division.

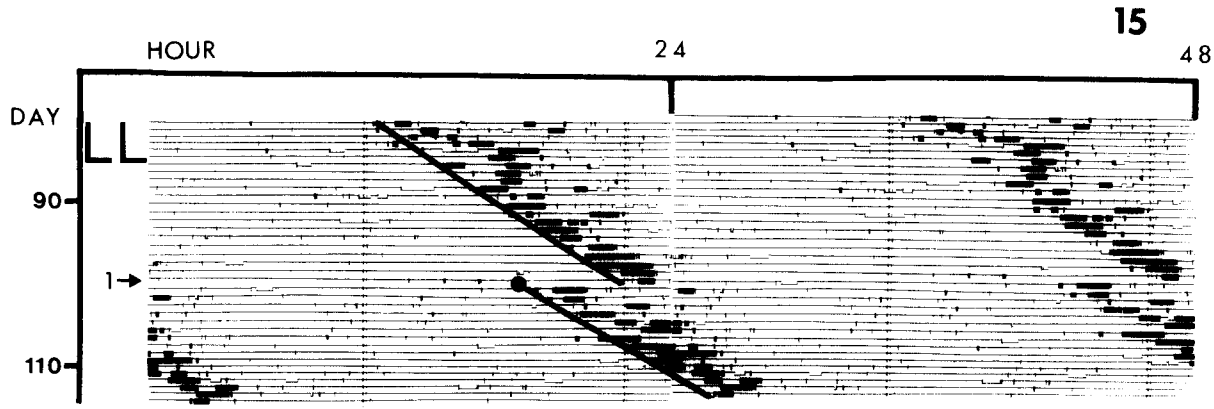

Fig. 2. A portion of the running-wheel activity record of hamster No. 15 showing the response of the rhythm to electrical stimulation of GHT neurons centered on CT 7.4 (indicated by a black circle) on the day indicated by the arrow. The record is double-plotted (48 h) and lines are fitted to indicate the estimated times of CT 12 (activity onset) used for assessing phase shifts. LL indicates constant dim illumination.

RESULTS

Histological results indicated that 26 of the usable stimulations (from 5 hamsters) involved electrodes that were in the IGL or in portions of the vLGN that contain NPY-immunoreactive neurons and project to the SCN, according to our previous mapping of neurons in this area that project to the SCN[10]. These are referred to as GHT stimulations. Three of these animals had electrodes in the external division of the anterior vLGN (Fig. 1A), one had an electrode in mid-IGL (Fig. 1B), and one had an electrode that impinged only on the most caudal portion of the IGL (Fig. 1C). Stimulation of the GHT during the middle to late subjective day caused advance phase shifts in both LL and DD (Figs. 2, 3 and 5). The animal with the most caudal IGL placement (Fig. 1C) contributed only one shift; this was the only GHT stimulation between CT 7 and CT 12 that failed to elicit a phase advance (Fig. 5).

The largest advance shift was a 3.9 h shift following stimulation near CT 7 (Fig. 2). Delay shifts occurred primarily in response to stimulation in the late subjective night and early subjective day (Fig. 4). The largest delay shifts were just under 1 h and occurred in response to stimulation near CT 0 (Fig.

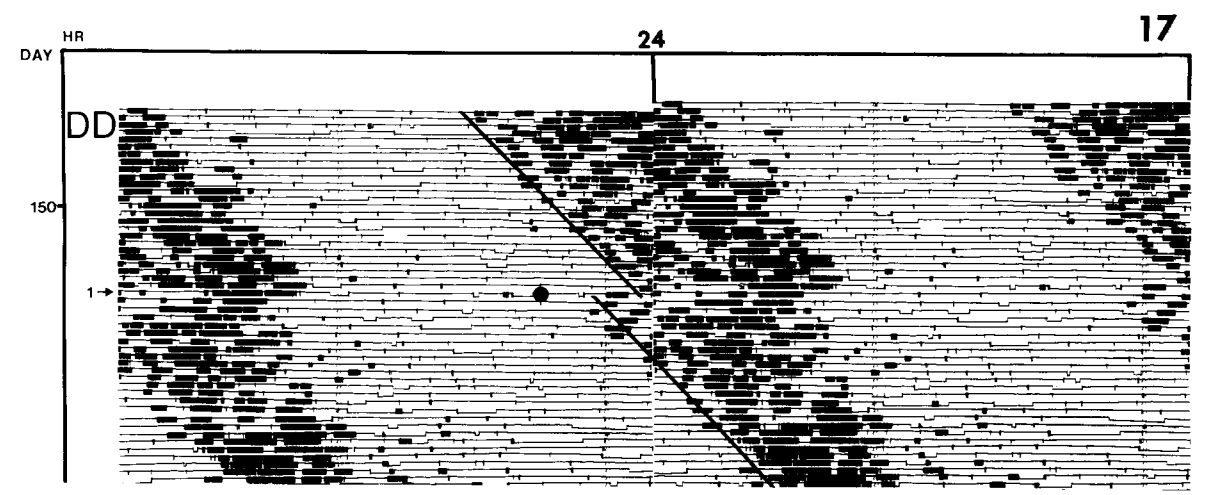

Fig. 3. A portion of the running-wheel activity record of hamster No. 17 showing the response to GHT stimulation centered on CT 7.7 in continuous darkness (DD). See Fig. 1 for further details.

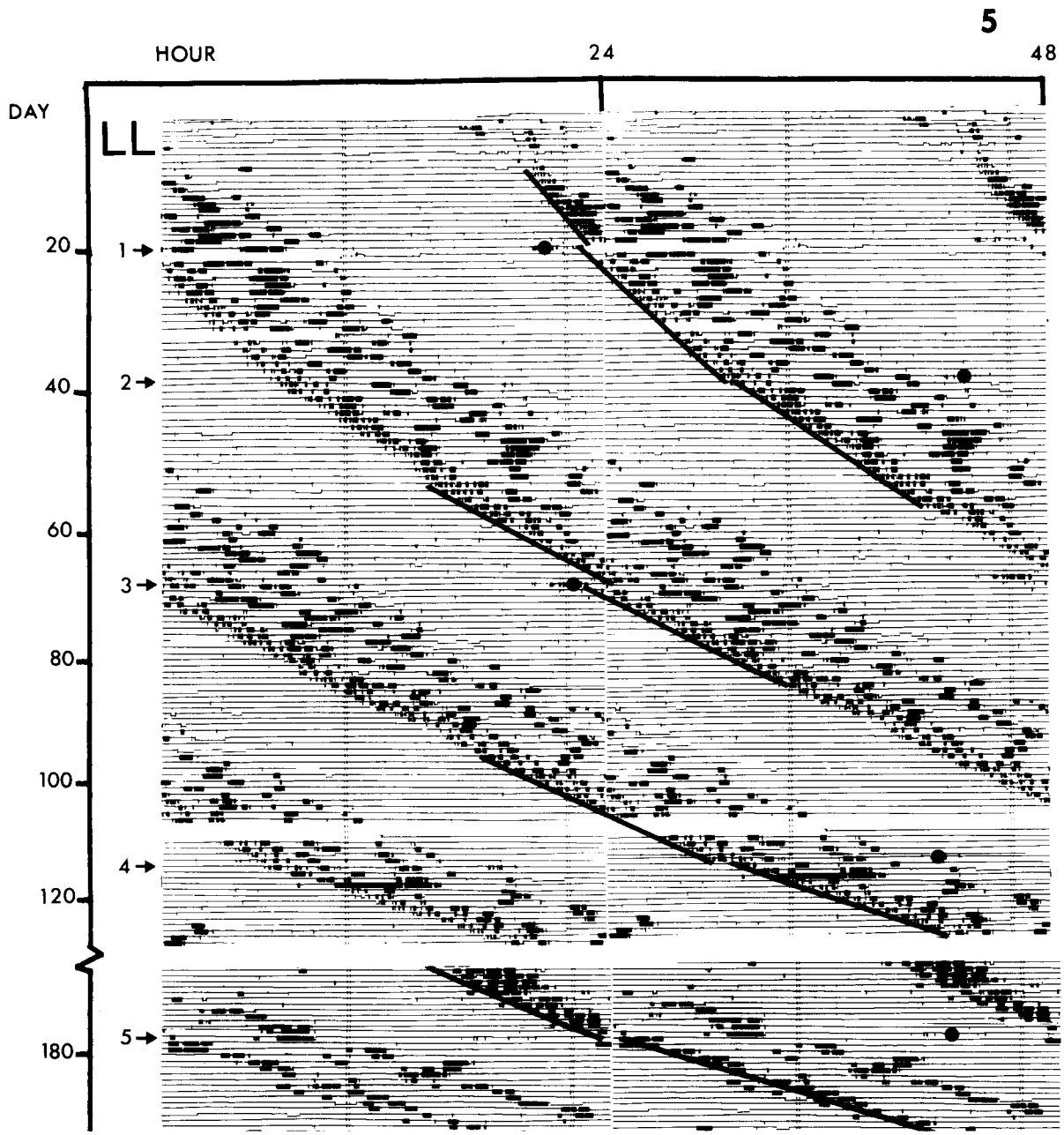

Fig. 4. A portion of the running-wheel activity record of hamster No. 5 showing responses to GHT stimulation at several circadian phases. The midpoints of the stimulations were: 1, CT 9.1; 2, CT 0.8; 3, CT 9.4; 4, CT 23.3; 5, CT 0.1. See Fig. 1 for further details.

5). Delay shifts were sometimes accompanied by lengthening of the subsequent freerunning period, which may have obscured the true size of the shifts. The two GHT stimulations between CT 12 and CT 20 yielded only small shifts, but there were too few data available to reliably assess sensitivity during that phase (Fig. 5).

Ten stimulations (from 6 hamsters) were at sites in various portions of the thalamus outside the areas that give rise to projections to the SCN (Fig. 1). These stimulations yielded shifts up to 0.4 h, although most caused no measurable shifts. The most substantial shifts induced by stimulation outside the GHT were small *delays* that occurred near

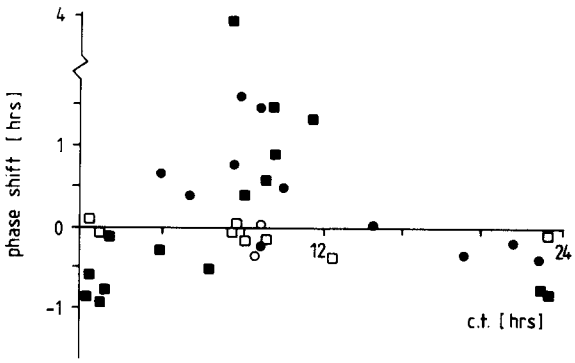

Fig. 5. Phase shifts induced by electrical stimulation in the hamster lateral geniculate nucleus. The size and direction of phase shift is plotted on the ordinate against the midpoint of the stimulation time on the abscissa, with circadian time (c.t.) 12 defined as activity onset. Advance shifts are shown as positive values, delay shifts as negative values. Filled symbols indicate stimulations through electrodes in the GHT; open symbols indicate stimulations through electrodes outside the GHT (see Fig. 1). Square symbols indicate stimulations in continuous dim illumination and circles indicate stimulations in continuous darkness.

CT 8 and CT 12, phases which yielded *advance* shifts to GHT stimulation; no advance shifts were produced by stimulation outside the GHT (Fig. 5).

There were no striking differences between results obtained in the two lighting conditions. It is clear that GHT stimulation during CT 7–12 can yield substantial advance shifts in either DD or LL, and the sizes of the shifts do not appear to be systematically related to lighting (Fig. 5).

Since only 3 hamsters with electrodes in the GHT contributed multiple phase shifts, no statistical assessment of the relation between induced activity level and phase shifting was attempted. Qualitatively, two animals ran vigorously during stimulations that resulted in large phase advances, and less so after other stimulations. But for one hamster, there was clearly no relation between shift size and activity level. Nine shifts were recorded for this animal, ranging in size from a 0.75 h delay to a 0.9 h advance, and all activity ratings were 0 or 0.5. This hamster (No. 9) showed quite pronounced motor artifacts (head-turning and rearing) which habituated during the course of stimulation; these might have affected the tendency to show wheel-running during stimulation.

Of the 3 attempts to entrain hamsters to daily geniculate stimulation, one (animal No. 9) was clearly unsuccessful, and resulted in little change in the freerunning rhythm. Two attempts using animal No. 5 yielded some suggestive evidence of entrainment (Fig. 6). In the first attempt, 6 days of daily stimulation resulted in a dramatic phase shift of the subsequent freerun relative to the predicted phase

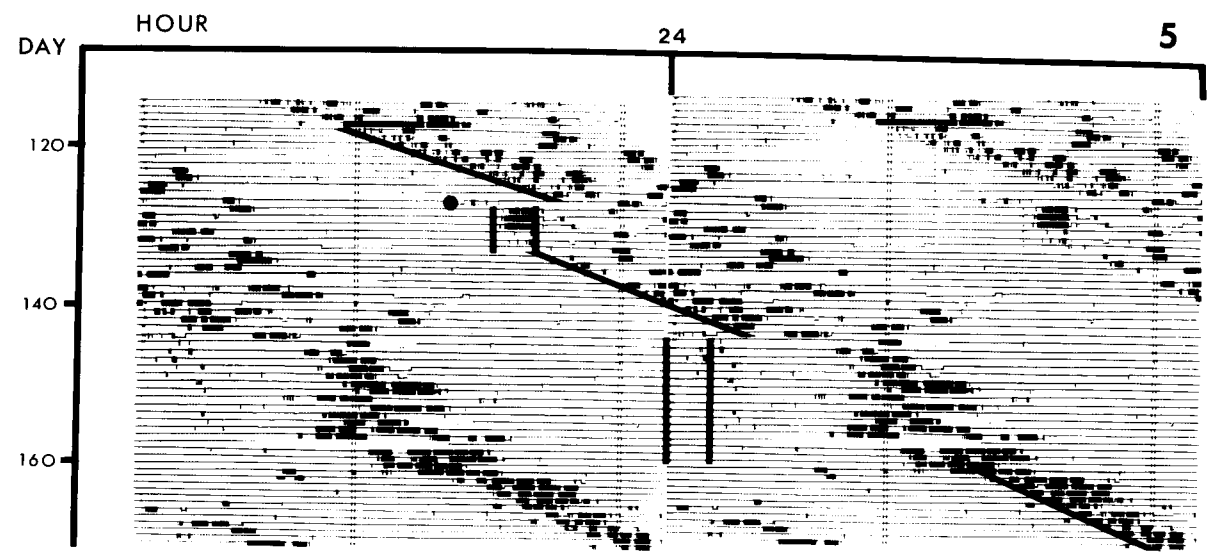

Fig. 6. A portion of the wheel-running activity record for hamster No. 5 showing two attempts at entrainment to repeated daily stimulation of the GHT. The times at which daily 2-h stimulations were delivered are enclosed by parallel vertical lines. The black circle indicates the midpoint of a 2-h stimulation delivered accidentally before the first entrainment attempt. The animal was in continuous dim illimunation. See Fig. 1 for further details.

based on extrapolation of the freerun that preceded the stimulation schedule. During a second attempt, daily stimulation was given for 17 days and activity appeared to become unstably entrained to a 24 h period for the first two weeks. Activity shifted to a later time for about 3 days before the schedule ended. Activity onset then showed several large delays before the rhythm settled into a stable freerun, suggesting that the expression of activity had been masked to some degree by the scheduled stimulation. Extrapolations of the freeruns that preceded and followed the stimulation schedule indicated that a small phase shift had occurred.

DISCUSSION

Electrical activation of the cells of origin of the GHT produced phase shifts with a phase–advance zone in the late subjective day and a phase-delay zone centered on subjective dawn. This phase–response curve (PRC) is similar in shape to a number of others that have been generated in Syrian hamsters using various agents, such as dark pulses[2,5], injections of NPY or glutamate into the SCN[1,18], injections of NMA into the geniculate area[14], injections of the benzodiazepine triazolam[36], and exposure to various arousing stimuli[23].

Mrosovsky[23] has proposed that these events produce similar phase shifts because all have the property of activating hamsters. A recent report indicated that triazolam, which both activates and phase-advances hamsters when injected in the late subjective day, had no phase-shifting effect when hamsters were restrained after the injection to prevent activity increases[37]. Our data suggest a correlation between increased wheel running and large advance shifts in two animals, but substantial phase shifts occurred in an animal that showed very little wheel-running. Since we had no measure of general activity outside the wheel, this observation does not rule out the possibility that geniculate stimulation causes phase shifts because it activates animals in ways not reflected in wheel-running.

It is possible that a variety of environmental stimuli that increase activity, or the increased activity itself, affect NPY-containing cells in the geniculate region that project to the SCN. The vLGN is known to receive projections from the raphe nuclei, locus coeruleus and reticular formation[16,24], which play a role in the regulation of behavioral arousal. The GHT may form a common pathway by which several kinds of stimuli, in addition to photic cues, can influence the SCN-based pacemaker. In support of this hypothesis, one study has reported a loss of phase shifts induced by systemic triazolam injections after geniculate area lesions[14].

Delay shifts were typically smaller and less convincing than advance shifts. This asymmetry between advance and delay shifts is also seen following NPY injections in the SCN[1], and it is consistent with the finding that GHT ablation reduced the amplitude of advance shifts to light pulses or dark pulses but had minimal effects on delay shifts[11,26]. The GHT may be particularly involved in mediating phase-advancing effects of environmental stimuli on the circadian pacemaker.

The simplest explanation for the occurrence of phase shifts after GHT stimulation is that stimulation activates GHT neurons which then release NPY onto their target cells in the SCN. NPY may alter the firing rates of SCN neurons that comprise the circadian pacemaker, thereby shifting the phase of the activity rhythm. There is independent evidence that NPY can both alter the firing rates of SCN cells and phase-shift the SCN pacemaker. SCN cells in hamsters[17,33] and rats[6,7] show a spontaneous circadian rhythm of firing rate in vitro, which is apparently phase-shifted by bath application of NPY to the hypothalamic slice (S. Shibata and R.Y. Moore, personal communication, 1988). Micropressure ejection of NPY onto SCN cells in a hamster hypothalamic slice preparation generated increases in firing rates during the subjective day, but cells were relatively insensitive during the subjective night[17]. Bath application of NPY to the rat SCN slice generated biphasic responses, with primarily suppressive effects on firing rates, again with greatest sensitivity during the subjective day[34]. Photic input, which has potent phase-shifting effects on overt rhythms, also alters firing rates of SCN cells in hamsters and rats[8,19].

It is not certain, however, that firing rate is a direct reflection of the pacemaker's timekeeping activity. Treatment of the rat SCN with chronic infusions of tetrodotoxin should prevent the generation of the presumably sodium-dependent action

potentials[35] that are recorded in neurophysiological studies, yet the circadian clock continues to run during such infusions[32]. Phase shifts caused by NPY injections or activation of the GHT may, therefore, be a consequence of their effects on a cellular process (e.g. membrane potential) which is integral to the timing mechanism. Changes in this process may be manifest secondarily in altered firing rates of these pacemaker cells or of SCN efferents that are regulated by the pacemakers.

The responses of animal No. 5 to the first entrainment attempt using repeated daily stimulation were consistent with entrainment having taken place; the second attempt gave an ambiguous result, possibly because of tissue damage from repeated stimulations (Fig. 6). The second animal (No. 9) freeran through the stimulation schedule with no sign of entrainment (data not shown). Taken together, these results suggest that repeated daily activation of the GHT has a weak entraining effect on the pacemaker. Non-photic stimuli, some of which might exert their effects through the GHT, tend also to be relatively weak entraining cues compared to photic stimuli[23,27,30].

One previous study examined the effects of electrical stimulation on rodent circadian rhythms. Rusak and Groos[29] reported that electrical stimulation of the SCN in rats and hamsters phase-shifted freerunning behavioral rhythms. The phase–response curve for SCN stimulation was similar to that for light pulses and quite different from the one for GHT stimulation. The differences between the effects of SCN and GHT stimulation, and the lack of effect of geniculate stimulation outside the GHT suggest that the phase shifts reported here are specific to activation of the GHT.

GHT stimulation has been assumed to work by activating neurons to release NPY in the SCN. Since most GHT neurons, including those that can be demonstrated to project to the SCN, are activated by increasing light intensity[9,13,38], such activation should have the same effect as increasing light intensity. Instead, GHT stimulation mimics the effects of *decreasing* light intensity (dark pulses), as does NPY injection into the SCN and excitotoxic activation of the geniculate. This inconsistency is compounded by uncertainty as to the neurophysiological effects of NPY in the SCN[17,34]. It is clear that the GHT plays a significant role in the circadian system, but our current knowledge does not permit a coherent account of its effects on the circadian pacemaker in the SCN.

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