Large phase-shifts of circadian rhythms caused by induced running in a re-entrainment paradigm: The role of pulse duration and light

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Summary. Bouts of induced wheel-running, 3 h long, accelerate the rate of re-entrainment of hamsters' activity rhythms to light-dark (LD) cycles that have been phase-advanced by 8 h (Mrosovsky and Salmon 1987). The bouts of running are given early in the first night of the new LD cycle, and by the second night the phase advance in activity onset already averages 7 h. Such large shifts contrast with the mean phase advance of <1 h at the peak of the phase response curve when hamsters in constant darkness (DD) experience 2-h pulses of induced activity (Reebs and Mrosovsky 1989). The present paper investigates pulse duration and light as possible causes for the discrepancy in shift amplitude between these two studies. In a first experiment, pulses of induced wheel-running 1 h, 3 h, or 5 h long were given at circadian times (CT) 6 and 22–2 to hamsters free-running in DD. Pulses given at CT 6 caused phase-advances of up to 2.8 h, whereas pulses at CT 22–2 resulted in delays of up to 1.0 h. Shifts after 3-h and 5-h pulses did not differ, but were larger than after 1-h pulses, and larger than after the 2-h pulses given in DD by Reebs and Mrosovsky (1989). Thus 3 h appears to be the minimum pulse duration necessary to obtain maximum phase-shifting effects. In a second experiment, the re-entrainment design of Mrosovsky and Salmon (1987) was repeated with the light portion of the shifted LD cycle eliminated. Hamsters exercised for 3 h phase-advanced 2.9 h on average (excluding 2 animals who ran poorly). When the same hamsters were exposed 7 days later to a 14-h light pulse starting 5 h after their activity onset, they advanced by an average of 3.3 h. Adding the average values for activity-induced shifts and light-induced shifts gives a total of about 6 h. Possible synergism between the effects of induced activity and those of light may account for the remaining small difference between this total and the 7-h advances previously reported.

Introduction

This paper investigates an apparent discrepancy in the recent literature on nonphotic influences on circadian rhythms. The discrepancy is between two studies in which phase shifts of very different amplitude were obtained in response to induced wheel-running.

In the first study, Mrosovsky and Salmon (1987) discovered that behavioural events accelerated the rate at which hamsters' activity rhythms re-entrained to a shifted light-dark (LD) cycle. They subjected male Syrian hamsters (*Mesocricetus auratus*) to an 8-h phase-advance of the LD cycle, beginning with an advance in dark onset; one group was left undisturbed, while another group was induced to run for 3 h on the first night of the new LD cycle, starting 1 h after the new dark onset. Hamsters ran after being transferred from their home cages to novel running wheels from which there was no exit (hamsters usually run on their own volition under such circumstances). Whereas the undisturbed animals took 8.5 days to re-adjust their activity rhythms to the new LD cycle, the exercised hamsters took only 1.6 days. While the activity onsets of undisturbed animals re-entrained via a series of gradually advancing transients, the exercised hamsters had already, by the second night of the new LD cycle, begun activity 7 h before the time of their old activity onset. These 7-h advances reflected the true

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state of the internal clock and were not the result of masking. This has been demonstrated by releasing hamsters into constant darkness (DD) after the first complete cycle of the shifted LD regime (Mrosovsky, in press).

Working with a different paradigm, Reebs and Mrosovsky (1989) let hamsters free-run in DD and then subjected them to single 2-h bouts of running activity, also by confining them to novel wheels. This was done at different circadian times (CT) to establish a phase response curve (PRC). The largest advances were observed at CT 4.5–7.5, which is similar to the time at which hamsters experienced the 3-h bouts of activity in the re-entrainment experiments described above. The advances, however, were well short of 7 h; in fact, the mean shift at CT 4.5–7.5 was only 0.6 h (range: −0.34–2.3 h).

We propose two nonmutually exclusive hypotheses to explain the discrepancy in shift amplitude between the two studies. The first hypothesis concerns pulse duration: the bouts of activity were 1 h longer in the re-entrainment experiments than in the PRC experiment, and it may be that longer pulses have larger effects. The second hypothesis is that light contributed to the large shifts observed in the re-entrainment studies; the shifts may have been smaller in the PRC experiment because light was entirely absent.

The present paper addresses these two hypotheses. In a first experiment conducted in DD, we compared the effects of exercise bouts of different durations. Although we were primarily interested in phase-advancing effects, we also tested for phase-delaying and report these results as well. In a second experiment, we modified the LD-shift-and-re-entrainment paradigm to temporally dissociate induced activity and light, and to measure their phase-shifting effects separately.

Experiment 1: Effect of pulse duration in DD

Material and methods

Thirty male hamsters (LAK:LVG, from Charles River Quebec, 60 days old at beginning of experiment) were housed in metal-walled cages equipped with running wheels connected to an Esterline-Angus recorder. These cages were similar to those used by Mrosovsky and Salmon (1987) and by Reebs and Mrosovsky (1989). The hamsters were kept in DD. Room temperature was about 21 °C. After an initial 15 days, the animals were matched for free-running period (τ) and assigned to 3 different groups (10 hamsters per group). The 3 groups then received a single pulse of induced activity lasting 1, 3, and 5 h, respectively. The pulse was given at CT 6, the approximate time at which maximum advances were observed with 2-h pulses (Reebs and

Mrosovsky 1989). The procedure to give pulses was the same as in Reebs and Mrosovsky (1989): with the aid of an infrared scope, the hamsters were individually transferred and confined to a clean novel running wheel, observed there at intervals of <4 min, and gently blown upon if they showed any sign of sleepiness or inactivity. The number of times each animal was thus prompted to run was recorded. Running in the novel wheel was also recorded on the Esterline-Angus. Hamsters assigned to the 5-h pulse were allowed a 10-min break during which they could drink and eat in their home cages; the break started 3 h after the beginning of the pulse.

After free-running for 19–21 days following the first pulse, the hamsters were given a second pulse, again either 1, 3, or 5 h long. Each original group was divided into 3 subgroups, and each of the 3 subgroups was assigned a different pulse duration. All pulses were administered so that they *ended* at CT 3 (thus 1-h pulses began at CT 2, 3-h pulses at CT 0, and 5-h pulses at CT 22). These times were chosen because they ensured that the full duration of all pulses coincided with the delay portion of Reebs and Mrosovsky's (1989) PRC. After this second pulse, the animals were allowed to free-run for at least 14 days.

We mounted actograms by pasting successive 24-h records below each other. The phase-shifts caused by the pulses were quantified as in Reebs and Mrosovsky (1989). Briefly, regression lines were calculated for the 7 activity onsets immediately preceding the pulse, and for the 7 onsets following it (excluding the very first one). The two lines were extrapolated to the time following the pulse, and the difference between the extrapolations was taken as the phase-shift. All CTs were calculated with reference to the extrapolation of the pre-pulse regression line. Results are reported as means ± SD. The statistical analyses followed Conover (1980) and all appropriate tests were two-tailed.

Results

Figure 1 shows the amount of phase-shifting caused by the various pulses ($n=9$ in one of the groups because one hamster escaped from his cage after a pulse and was not included in the analysis). Figure 2 presents a few typical actograms. For the pulses starting at CT 6, pulse duration had an effect on shift amplitude (Kruskall-Wallis $T=6.658$, $P<0.05$). Shifts following 3-h and 5-h pulses were not significantly different from each other, but were larger than shifts following 1-h pulses (multiple comparison test, $P<0.05$). The average shifts were 1.8 ± 1.2 h and 1.9 ± 1.1 h for the 3-h and 5-h groups, respectively, and 0.5 ± 0.8 h for the 1-h group.

Much of the variance in the 3-h and 5-h groups was caused by 5 animals who showed small (<0.4 h) shifts (see Fig. 1). Four of these 5 animals were the only hamsters who needed to be prompted more than 4 (between 7 and 27) times during their respective pulses. Their general behaviour was sluggish: they mostly walked in the wheel, often stopping to curl up and apparently try to go to sleep. In contrast, all other animals in this experiment needed less than 4 prompts and generally

ran vigorously throughout the pulses. If all 4 sluggish animals in the 3-h and 5-h groups are excluded from the analysis, then the average shifts become 2.2 ± 0.9 h and 2.5 ± 0.4 h, respectively, for these
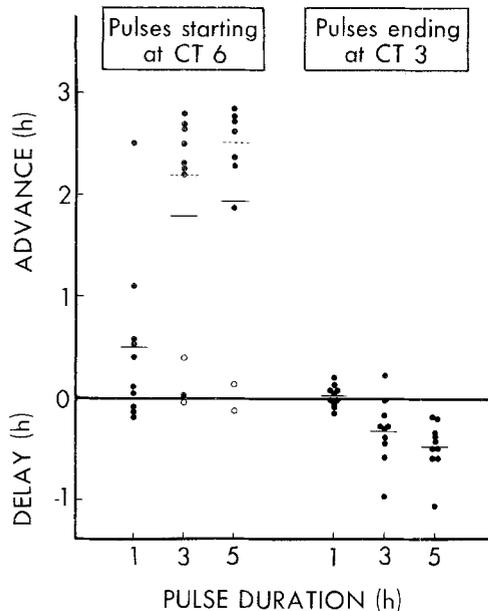

Fig. 1. Phase-shifts of activity rhythms resulting from pulses of induced exercise given to hamsters in constant darkness. Open circles from animals that did not run vigorously during the exercise pulse. Horizontal solid lines show means for the entire groups; horizontal dotted lines show means when the open circles are excluded

two groups. The difference is still not significant (Mann-Whitney $T = 71$, $P > 0.1$).

For the pulses ending at CT 3, phase-shift amplitude also depended on pulse duration (Kruskall-Wallis $T = 20.43$, $P < 0.001$). Shifts following 3-h and 5-h pulses did not differ but were larger than shifts following 1-h pulses (multiple comparison test, $P < 0.05$). The average shifts were 0.0 ± 0.1 h, -0.3 ± 0.3 h, and -0.5 ± 0.3 h for the 1, 3, and 5-h groups, respectively. All animals ran vigorously throughout the pulses; none needed to be prompted more than 4 times.

Discussion

The data show that the amount of phase-shifting resulting from bouts of induced activity depends on the duration of these bouts. Pulses 1 h long do not produce shifts as large as pulses 3 h or 5 h long. On the other hand, 3-h pulses have the same effect as 5-h pulses, at least when both start at CT 6 or end at CT 3. In the PRC experiment of Reebs and Mrosovsky (1989), 2-h pulses starting around CT 6 (CT 4.5–7.5) resulted in 4 animals shifting by 1.1–2.3 h, and 8 animals giving shifts between -0.3 and 0.4 h (no sluggish animals). These values are intermediate between those found here for 1-h and 3-h pulses. Thus 3 h seems to be the minimum pulse duration necessary to obtain maximum phase-shifting effects, at least at CT 6
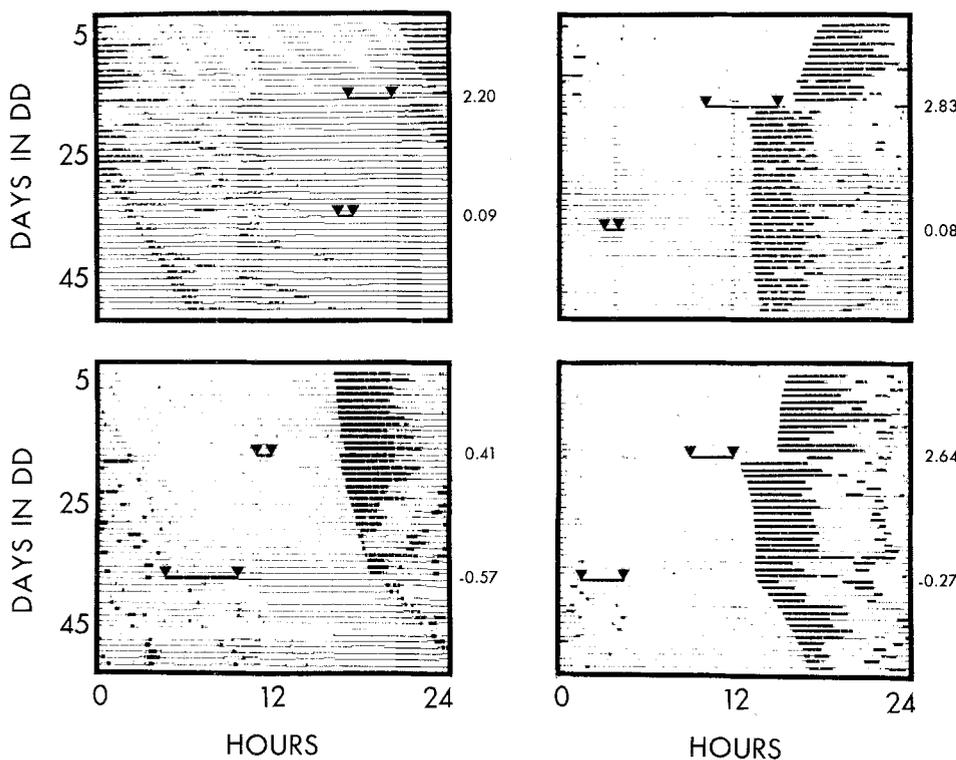

Fig. 2. Actograms of 4 hamsters given pulses of induced running (time between tips of triangles) in DD. Numbers on the right show the calculated phase-shifts (in h)

and in DD. Whether this conclusion is valid under other conditions (constant light, other CTs, pulses > 5 h long) is an open question.

For a given pulse duration, delays were smaller than advances (see Fig. 1). This may be attributable to a number of factors, such as our choice of CTs (CT 3 might not be appropriate for maximum delays) and order effects (all animals received the delaying pulses last). However, in Reebs and Mrosovsky's (in press) PRC experiment, in which pulses were given at various CTs and order effects minimized, the largest delays were also much smaller than the largest advances. Therefore, at least for pulses of novelty-induced wheel-running, there is an asymmetry in amplitude between phase-advances and phase-delays, and this asymmetry seems to persist over a wide range of pulse duration.

The few animals that ran much less in the novel wheels also shifted much less. This is consistent with the previous observation that less active hamsters entrained poorly to 2-h pulses of induced activity repeated daily (Reebs and Mrosovsky 1989). It remains an open question whether more novel stimuli could be used to arouse such animals, and whether this would in turn lead to increased phase-shifting. It is also interesting to note that we did not observe sluggish animals when pulses ended at CT 3, which suggests that sensitivity to novel stimuli depends on the time of day.

In the 3-h and 5-h groups, the average phase-advances varied between 1.8 and 2.5 h, depending on whether the few sluggards are included or not. These values are clearly more than the average 0.6 h observed in the PRC experiment of Reebs and Mrosovsky (1989), but they are still far less than the 7-h shifts observed in Mrosovsky and Salmon's (1987) and Mrosovsky's (in press) rate of re-entrainment experiments. In the following attempt to find out whether light also contributes to the 7-h shifts, we modified the rate of re-entrainment paradigm and temporally dissociated activity-induced and light-induced shifts.

Experiment 2: Contribution of light to accelerated re-entrainment

Material and methods

Twenty male hamsters (LAK:LVG, from Charles River Quebec, 63 days old at the start of the experiment) were kept in metal-walled cages as in Experiment 1. They were first kept in LD 14:10 for 25 days. Light intensity inside the cages was 5–30 lux (Gossen Lunasix 3 lightmeter). Room temperature was about 21 °C. On the 26th day, the hamsters were put into DD, starting 8 h before the normal dark onset. Starting 1 h

after initiation of DD, 10 hamsters were induced to run for 3 h in novel running wheels. The animals were neither observed nor prompted to run during the pulse. The other 10 hamsters were left undisturbed in their home cages. This experimental design was the same as that used by Mrosovsky and Salmon (1987) and by Mrosovsky (in press) except that the hamsters did not experience any light after the initial advance in dark onset. Records were mounted and phase-shifts measured as in Experiment 1.

Seven days after initiation of DD (the time needed to measure the phase-shifts that followed the above manipulation), all hamsters received a 14-h pulse of light starting 5 h after their activity onsets on the 7th day. To give the light pulse, we moved each hamster individually at the appropriate times to and from an adjacent room that was lit. Light intensity within the cages inside the lit room approximated 20 lux. Except for the physical transfer (which we assume had only marginal, if any, phase-shifting effects), this manipulation simulated the change in photic input the exercised hamsters would have experienced on the first day of the shifted LD cycle if we had maintained the light portion of the shifted LD cycle rather than using DD. The light would then have appeared 5 h after activity onset because most of the exercised hamsters phase-advanced by about 3 h relative to the old dark onset (see below), and light appears about 2 h after the old dark onset when a LD 14:10 cycle is advanced by 8 h. The phase-shifts caused by light were measured as above, with one exception: the post-regression line excluded the first 5 onsets following the pulse because transients were observed. Thus only onsets 6–12 post-pulse were used to obtain a steady-state shift value. The statistical methods used were the same as in Experiment 1.

Results

After the initiation of DD, the rhythms of undisturbed hamsters hardly shifted (mean shift: 0.1 ± 0.1 h, Fig. 3A). Exercised animals, on the other hand, advanced by 2.5 ± 0.9 h (Fig. 3B, C, D). Much of the variance in the exercised group was caused by 2 individuals who ran for about half of the pulse only (as opposed to the other 8 hamsters who ran for virtually the full duration, see Fig. 3B, C). The 2 sluggish hamsters shifted less (0.15 and 1.61 h, Fig. 3D). If these 2 animals are excluded, the average shift for the exercised group becomes 2.9 ± 0.3 h. Post-pulse τ (as determined by the slope of the regression line) did not differ between exercised and undisturbed group (exercised: 24.1 ± 0.1 h; undisturbed: 24.0 ± 0.1 h; Mann-Whitney $T = 83$, $P > 0.05$).

After the 14-h light pulse, all hamsters phase-advanced with 3–5 transients present (Fig. 3). The steady-state phase-shift for the group that had received a pulse of exercise 7 days earlier averaged 3.3 ± 0.7 h, while for the previously undisturbed group it averaged 2.9 ± 0.8 h. The difference is not significant (Mann-Whitney $T = 88$, $P > 0.1$). If the results from both groups are pooled, then the mean shift becomes 3.1 ± 0.8 h. There was no difference between the groups in their τ after the light pulse
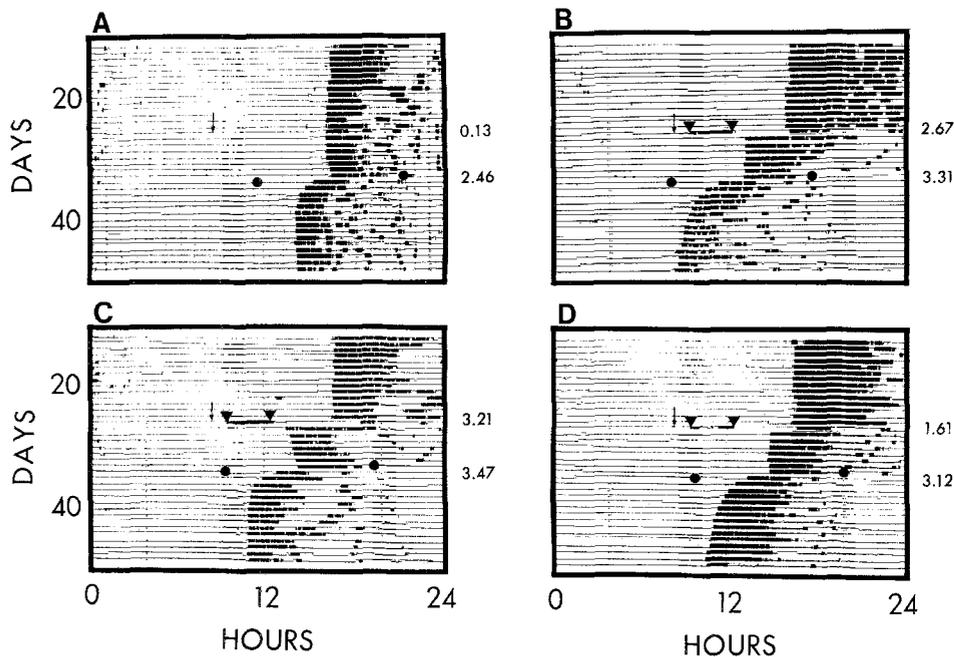
Fig. 3A–D. Actograms of 4 hamsters originally kept in LD 14:10 (dark onset at 1610 h), then put into constant darkness (arrow), confined to a novel wheel for 3 h (time between tips of triangles) or left undisturbed in their home cages, and finally given a 14-h pulse of light (time between circles) 7 days later. Numbers on the right indicate the calculated phase-shifts (in h). A Hamster left undisturbed after DD initiation. B and C Hamsters that ran continuously in the novel wheel. D One of 2 hamsters that ran during about half of the time spent in the novel wheel

(previously exercised: 23.9 ± 0.1 h; previously undisturbed: 23.9 ± 0.1 h; Mann-Whitney $T_1 = 0.2424$, $P > 0.1$).

Discussion

After initiation of DD, undisturbed animals essentially did not phase-shift relative to their pre-DD activity onsets. This indicates that the pre-DD activity onsets constituted an appropriate baseline for the measurement of post-pulse shifts in the exercised animals. With this in mind, the data from the exercised animals in Experiment 2 lead to the following interpretation of the 7-h shifts previously reported (Mrosovsky and Salmon 1987; Mrosovsky, in press). A first component of the 7-h shifts is an advance of 2.5–2.9 h resulting from the entirely nonphotic 3-h exercise pulse. (2.9 h is the shift value when 2 hamsters who stood out as poor runners are excluded; this is the only value we will consider hereafter because poor runners were not present in the previous rate of re-entrainment experiments.) Although large, this shift is obviously less than the 7-h shifts previously reported. The conclusion therefore is that the light portion of shifted LD cycles contributes to the 7-h shifts. The experiment of Mrosovsky (in press) shows that the first light episode of the shifted cycle is by itself sufficient to produce this contribution. Exercised animals advancing 2.9 h on the first night of a new LD 14:10 cycle that is itself advanced by 8 h would receive their first 14 h of light about 5 h after activ-

ity onset. Thus our manipulation on the 7th day in DD simulated the photic input an exercised hamster would experience on the first day of the shifted LD cycle. The steady-state shift to light was 3.1 h and this value can be added to that of the average shift to exercise (2.9 h) in an attempt to account for the large 7-h shifts. The total (6 h) is encouragingly close to 7 h.

The idea that the two pulses are additive in a simple arithmetical way (no synergism) involves two assumptions: (1) the PRC of sensitivity to light is the same on the first and 7th day, in terms of both its shape and its phase relative to activity onset, and (2) the shifts to both light and exercise should be instantaneous, as hamsters show 7-h shifts immediately after the first light episode of a shifted LD cycle (Mrosovsky, in press). The first of the above assumptions is untested, and the second appears to be borne out only for exercise-induced pulses (see Fig. 3). It is now necessary to test these two assumptions, and to consider the possibility of synergism between exercise pulses and light pulses. Such synergism might in fact account for the comparatively small 1-h difference between expected and observed results. For example (looking at the first assumption), the activity pulse may alter the shape and the phase of the light-sensitive PRC on the first day in such a way that larger light-induced shifts would occur. Experimental designs involving double pulses (see Pittendrigh 1967; Binkley and Mosher 1987) may prove useful for testing this idea. Furthermore (looking at the second assumption), investigating the condi-

tions in which transients are or are not expressed might show that a first, activity-induced, shift places the circadian system in such a state that the components of the system are more firmly coupled, thereby preventing the occurrence of transients during subsequent, light-induced, shifts. Reversing the order in which exercise and light pulses are presented is a possible approach (for example, one could give the exercise bout on the *second* night of the shifted LD cycle, and then release the hamsters into DD). These ideas await further study. The conclusion, at this point, is that both exercise and light contribute to the 7-h shifts and that their effects are in some way additive.

A few additional points are worth discussing here. Our exercise-inducing manipulation resulted in large effects only when animals became fully active. In both Experiment 1 and 2, a minority of hamsters were less active and shifted less. The reasons why some animals were less responsive to the exercise wheel are unknown. An increased predisposition to sleep may be involved, as suggested by the numerous attempts to adopt a sleep posture on the part of sluggish animals in Experiment 1. Whether the long (>1 h, see Fig. 3) bouts of no running observed in 2 animals in Experiment 2 corresponded to bouts of sleep cannot be ascertained, although it is hard to conceive what other activity would leave the running wheel stationary for such long periods of time. The conclusion, at any rate, must be that activity has to be sustained (or maybe wakefulness uninterrupted) to result in full phase shifts.

The smaller shifts resulting from sluggish animals and from pulses <3 h long, together with our interpretation of the mechanism of 7-h shifts following LD shifts, leads to the prediction that hamsters would not re-adjust faster to a shifted LD cycle if the nonphotic pulse they are given is too short or fails to arouse them for a long period of time. This prediction appears to be borne out (Honrado and Mrosovsky, unpublished results; Reebs and Mrosovsky, unpublished results).

The phase-shifting effects of *sustained* running can be large. When sluggish animals were excluded, the 3-h exercise pulses yielded 2.2-h shifts in Experiment 1, and 2.9-h shifts in Experiment 2. These nonphotically induced advances are superior to the advances (ca. 2 h) observed when hamsters of the strain used here are given 1-h pulses of light up to 150 lux (Elliott 1974; Pohl 1983). They are also comparable to the advances (ca. 3 h) we obtained here after 14-h pulses of light (20 lux). As opposed to light however, exercise did not result in transients during the phase-advances (Figs. 2

and 3). Examples of large phase-advances (>2 h) without transients are rare in the literature on vertebrates, and seem limited to studies of drug effects (e.g. glucocorticosteroid in rats, Horseman and Ehret 1982; serotonergic agonist/neurotoxin in sparrows, Cassone and Menaker 1985; benzodiazepine in hamsters, Turek and Losee-Olson 1986) and brain stimulation (Rusak et al. 1989). Thus, in terms of the presence of transients, the effects of exercise are qualitatively closer to those of drug administration and brain stimulation than to those of light.

There is, however, one type of photic manipulation that results in phase-advances without transients. In hamsters, DD initiation (Albers 1986) and dark pulses given in constant light (Boulos and Rusak 1982) can result in large phase-advances without advancing transients. However, it is unclear whether the effects of darkness are truly photic. In another paper (Reebs et al. 1989) we argue that darkness has phase-shifting effects not because of a change in photic input to the clock but because it is given in such conditions that it arouses the animals and makes them run. Darkness-induced shifts would therefore be a special case of nonphotic effects, and the absence of transients would be consistent. As a corollary to this view, darkness would not be expected to cause phase-shifts if it fails to induce running. Our Experiment 2 provides some evidence: in undisturbed hamsters, DD initiation around CT 4 failed to induce running, and no phase-shift took place (Fig. 3A). Clearly, nonphotic effects are interesting not only in their own right but also for their contribution to manipulations that, at first, appear exclusively photic.

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