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Phase shifting the circadian clock with cycloheximide: response of hamsters with an intact or a split rhythm of locomotor activity

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Systemic administration of the protein synthesis inhibitor, cycloheximide, induced both phase advances and phase delays in the circadian rhythm of wheel-running activity in hamsters (*Mesocricetus auratus*) maintained in constant darkness or constant light. The magnitude and direction of the phase shifts were dependent on the circadian time (CT) of drug treatment. The phase response curves in constant darkness and constant light were of similar general shape, but they differed in the overall mean amplitude of the phase shifts. Maximal phase advances were observed after injections around CT 6–8, maximal delays at CT 0–2. Injections of various doses of cycloheximide at CT 0 induced a dose-dependent phase delay in the rhythm with a maximum delay induced by 10 mg cycloheximide. Injections of cycloheximide in animals with a split activity rhythm caused phase shifts of both components in the same direction (20/39) and in different directions (10/39). The results support the hypothesis that 80S ribosomal protein synthesis plays an important role in the biochemical mechanisms of circadian systems.

INTRODUCTION

Although the molecular events involved in the mechanisms of circadian clocks are still poorly understood, there is substantial evidence that protein synthesis on 80S ribosomes is involved in the generation of circadian rhythms. Inhibitors of protein synthesis, such as anisomycin, cycloheximide and puromycin, produce changes in the period or phase of circadian rhythms in a variety of organisms including: *Euglena*[5], *Acetabularia*[12], *Neurospora*[16], *Gonyaulax*[23,26], and *Aplysia*[10,13,19]. In all systems studied so far, treatment with pulses of protein synthesis inhibitors induce phase-dependent shifts (both delays and advances) of circadian rhythms.

While the effects of protein synthesis inhibitors have been extensively studied in micro-organisms and invertebrates, comparable studies in vertebrates have not been undertaken until recently. Recently it was shown that anisomycin can alter the phase of the circadian pacemaker system in the golden hamster[22] and these effects appear to be due to an action of the drug in the suprachiasmatic nucleus region of the hypothalamus[9]. Since anisomycin is known to have a number of other effects on cellular processes, the present study investigated the effect of cycloheximide, a protein synthesis inhibitor with a different mode of action than anisomycin, on the circadian system of hamsters. Cycloheximide is known to be a reversible inhibitor of protein synthesis[27] that acts by apparently reducing the activity of transfer factor II, an enzyme that catalyzes ribosomal translocation along mRNA[7]. The response of the circadian rhythm of wheel-running activity to subcutaneous injections of cycloheximide was determined at various circadian times in hamsters exposed to constant light or constant darkness. In addition, a dose response curve was measured for the phase-shifting effect of cycloheximide at the circadian time of maximum phase delays.

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MATERIALS AND METHODS

Male golden hamsters (*Mesocricetus auratus*, LAK:LVG(SYR)), obtained from Lakeview Hamster Colony (Newfield, NJ) at 9 weeks of age, were initially group housed and maintained on a 14:10 h light–dark cycle before being transferred to constant-darkness or constant light. At the time of transfer to constant darkness or constant light, the animals were housed individually in cages equipped with a running wheel to allow for the continuous recording of the circadian rhythm of locomotor activity. Food and water were available ad libitum. Animals housed in constant dark were kept in light-tight wooden boxes equipped with ventilation fans. Cycloheximide injections and periodic animal care were performed using an infrared viewer.

In the first study, a group of 35 animals maintained in constant darkness received subcutaneous injections of cycloheximide (10 mg in a volume of 0.5 ml saline/animal; weight range of animals was 120–150 g). The animals were allowed to free-run for 10–14 days prior to the first injection. Single injections of cycloheximide were administered at various times throughout the circadian cycle with circadian time (CT) 12 being defined as the onset of locomotor activity. Most animals received more than one injection and consecutive injections were separated by at least 14 days.

In the second study, a group of 35 animals maintained in constant light received single injections of cycloheximide (10 mg in a volume of 0.5 ml saline/animal; weight range of animals was 120–150 g) at various circadian times. After 50 days of exposure to constant light, about 40% of the animals showed 'splitting' of the locomotor activity rhythm, whereby the activity rhythm disassociates into two distinct bouts of activity separated by about 12 h[16,25]. The two components of the split activity record were defined as component 1, corresponding to the dawn or morning component, and component 2, corresponding to the dusk or evening component, using criteria previously described[17,25]. Some of these animals with a split activity rhythm received additional cycloheximide injections that were also separated by at least 14 days.

In the third study, a dose–response curve for the phase-shifting effect of cycloheximide injections was measured in constant light. A total of 20 animals (weight range 120–150 g) were injected either with saline or one of the following doses of cycloheximide: 0.1, 1.0, 2.5, 5.0, 7.5, 10.0 mg. All injections were timed to occur 12 h before the onset of activity (CT 0), a time when maximal phase delays were produced in the second study.

The phase-shifting effects of cycloheximide injections on the circadian rhythm of locomotor activity were assessed by comparing the phase of the onset of locomotor activity before and after the injection by methods previously described[2,4,21]. For animals with intact activity rhythms, steady-state phase shifts were measured after transients had ended. However, phase shifts measured for animals expressing split activity rhythms were determined as immediate phase shifts by comparing the predicted time of activity onset with the actual activity onset after the injection. The phase-shifting effects of cycloheximide were evaluated using a 2-way analysis of variance and Student's *t*-test for small samples. Differences between the two phase response curves obtained in constant darkness and constant light were evaluated using a 2-way analysis of variance and Scheffe's multiple *t*-test.

RESULTS

Single subcutaneous injections of 10 mg cycloheximide induced phase shifts in the free-running activity rhythm that were dependent on the circadian time (CT) of administration. Representative phase shifts induced by cycloheximide injections in animals exposed to constant darkness or constant light are illustrated in Fig. 1. Injections of cycloheximide 4–5 h before the onset of locomotor activity consistently advanced the free-running activity rhythm, while injections made 6–12 h after activity onset consistently delayed the rhythm. Injections of saline had no consistent effect on the phase of the activity rhythm regardless of the time of administration. It is noteworthy that very little activity could be observed for the 1–2 days following cycloheximide administration and that when locomotor activity reappeared after 2–3 days, the phase shifts were completed.

A plot of the phase-shifting effects of cycloheximide as a function of the circadian time of drug administration (i.e. a phase response curve) revealed

that the direction of the cycloheximide-induced phase shifts was strongly dependent on the time at which the injection occurred (Figs. 2 and 3). The general shapes of the phase response curves in constant darkness and constant light were similar, with phase delays being most common when cycloheximide was delivered from CT 20 to CT 2 and phase advances occurring following injections around CT 6–8. The major differences between the phase response curves are: (1) larger phase advances at CT 6 in constant light (mean $\Delta\phi = +80$ min) than in constant darkness (mean $\Delta\phi = +16$ min); and (2) a wider range of circadian times at which phase delays were induced in constant darkness (CT 14–4) than in constant light (CT 0–2). Comparison of the phase response curves by a 2-way ANOVA showed statistically significant variations between phase shifts at various circadian times ($F_{11,113} = 15.46$; $P < 0.01$) and a significant difference ($F_{1,11} = 11.27$; $P < 0.01$) between the phase response curves in constant

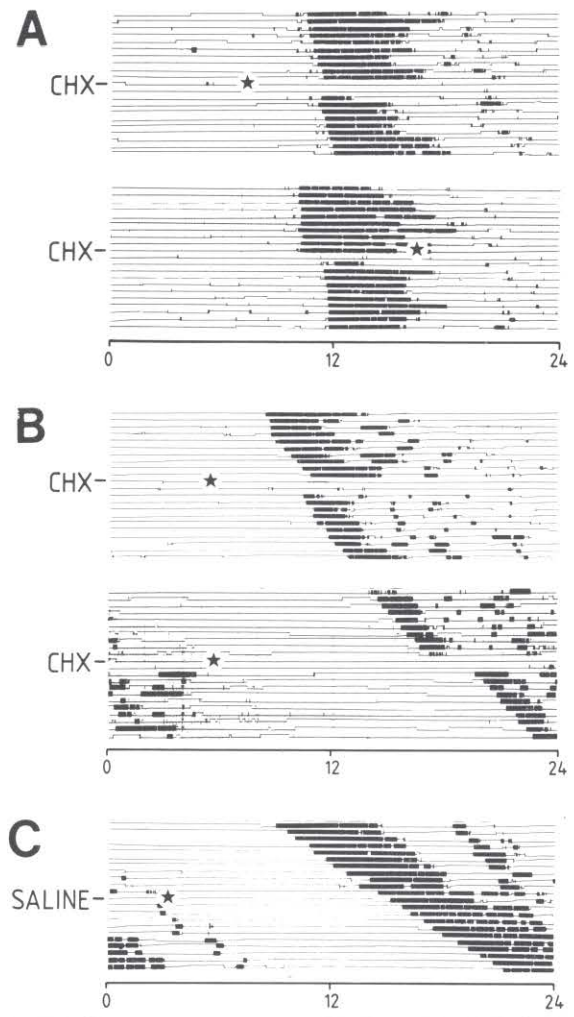

Fig. 1. Effects of subcutaneous injections of cycloheximide (CHX, 10 mg) and saline on the circadian rhythm of wheel-running activity. Each horizontal line represents the activity pattern of one animal over a 24-h period. Successive days are plotted from top to bottom. A star indicates the time of injection. A: cycloheximide injections in two animals exposed to constant darkness. An injection at CT 8 (upper panel) induced a phase advance ($\Delta\phi = +40$ min), an injection at CT 18 (lower panel) produced a phase delay ($\Delta\phi = -75$ min). B: cycloheximide injections in two animals exposed to constant light. While an injection at CT 7 (upper panel) caused a phase advance ($\Delta\phi = +75$ min), an injection at CT 0 (lower panel) caused a phase delay ($\Delta\phi = -100$ min). C: a saline injection at CT 0 in an animal exposed to constant light had no effect on the activity rhythm.

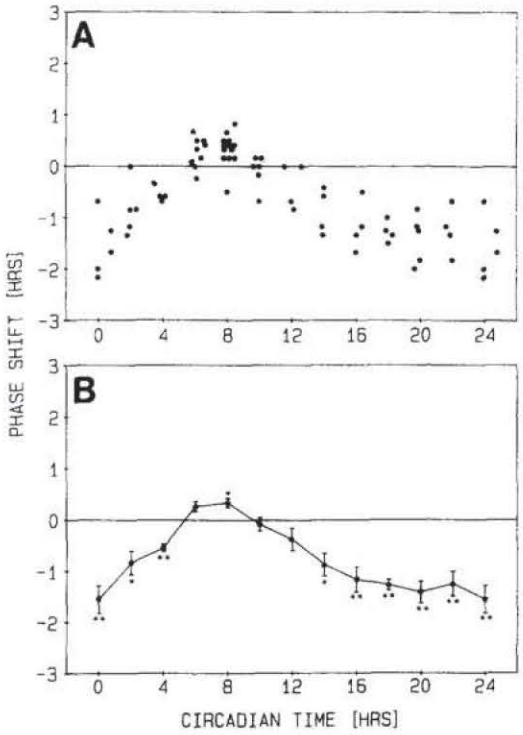

Fig. 2. Phase dependence of phase-shifting effects for cycloheximide injections on the circadian rhythm of locomotor activity in animals maintained in constant darkness. A: each point represents a steady-state phase shift in the activity rhythm induced by a single injection of cycloheximide at a circadian time (CT) corresponding to the position on the abscissa. CT 12 is defined as the onset of activity. Data at CT 0 have been duplicated at CT 24 for clarity. B: phase response curve from data shown in A. Each point represents the mean (± S.E.M.) phase shift determined for 2-h bins throughout the circadian cycle. Mean phase shifts that were statistically significantly different from zero are indicated by asterisks ($^*P < 0.05$, $^{**}P < 0.01$).

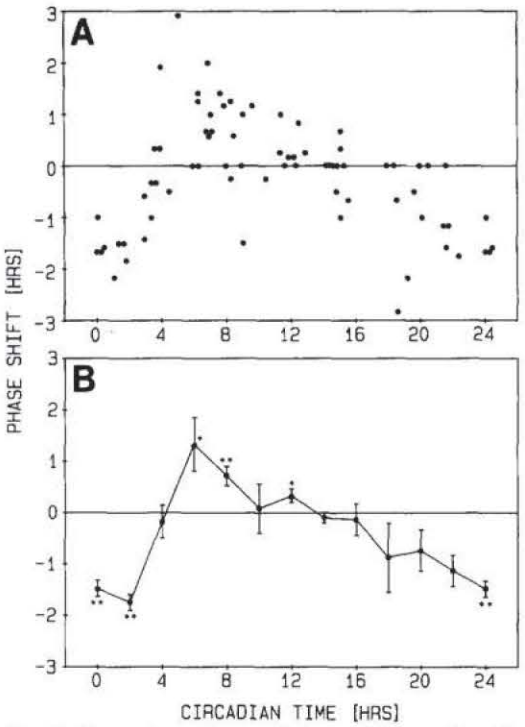

Fig. 3. Phase dependence of phase-shifting effects for cycloheximide injections on the circadian rhythm of locomotor activity of hamsters maintained in constant light. Explanations as in Fig. 2.

darkness and constant light. There was no significant effect of animal or repeated injections on phase shift.

The magnitude of the phase-shifting effects of cycloheximide at CT 0 increased depending on the dose (Fig. 4). Comparison of the dose-dependent phase shifts with a two-way ANOVA showed no significant effect of animal or repeated injections, but a statistically significant variation ($F_{6,33} = 8.99$; $P < 0.01$) between different doses. While injections of 0.1 and 1.0 mg cycloheximide induced phase shifts not significantly different from each other or from phase shifts induced by saline injections alone, injections of 7.5 and 10 mg cycloheximide produced phase shifts that were significantly ($P < 0.05$) greater than the phase shifts produced by saline and 0.1 or 1.0 mg cycloheximide. Injections of 2.5 and 5.0 mg cycloheximide caused phase shifts that were intermediate. Phase delays of at least 25 min were observed in 0/5, 1/7, 2/2, 5/5, 5/5, 5/5, 8/10 animals receiving injections of 0, 0.1, 1.0, 2.5, 5.0, 7.5, and 10 mg cycloheximide, respectively.

About 40% of the animals kept in constant light

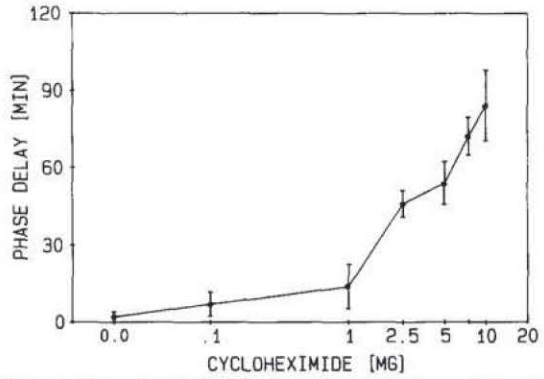

Fig. 4. Mean (± S.E.M.) phase delays in the activity rhythm induced by single injections of either saline or various doses of cycloheximide. All animals were injected 12 h before the onset of activity (CT 0) whilst maintained in constant light.

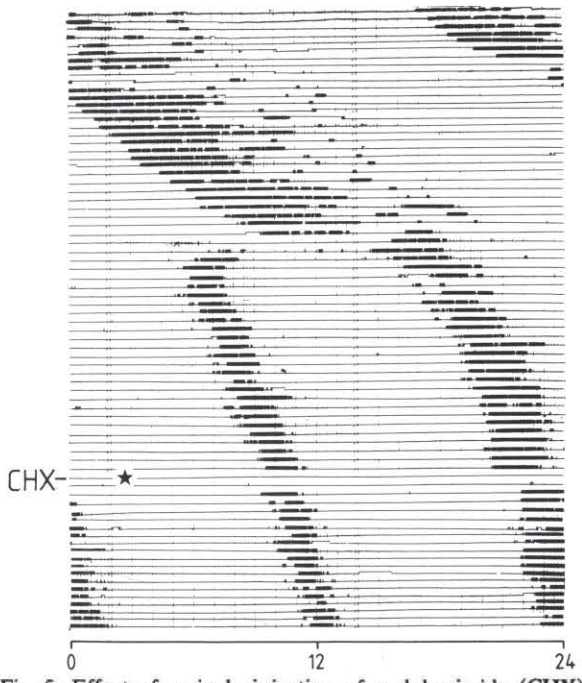

Fig. 5. Effect of a single injection of cycloheximide (CHX) after the activity rhythm had split into two components in a hamster maintained in constant light. The star indicates the time of injection, which occurred at CT 5 for component 1 and at CT 18 for component 2. The onset of activity was defined separately for each component as CT 12. The injection induced a phase advance ($\Delta\phi = +35$ min) of component 1 and a phase delay ($\Delta\phi = -80$ min) of component 2 as determined on the first day the activity reappeared after treatment. For further explanation of the activity plot see Fig. 1.

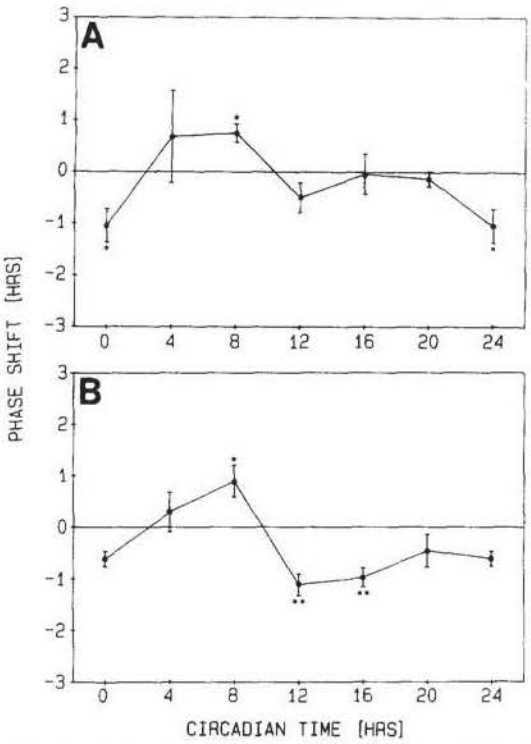

Fig. 6. Comparison of the phase response curves for (A) component 1 and (B) component 2 of a split activity rhythm. Each point represents the mean (± S.E.M.) phase shift calculated for a 4-h interval of the circadian cycle. Mean phase shifts that were statistically significantly different from zero are indicated by asterisks ($^*P < 0.05$, $^{**}P < 0.01$).

showed splitting of the activity rhythm into two components after about 50 days in constant light. Using the common terminology to describe the two components[17,25], component 1 corresponded to the dawn or morning component and component 2 to the dusk or evening component. As described for dark pulses given on a background of constant light[4], the split components rarely exhibited steady state phase shifts. Transient phase shifts in the components were usually observed followed by a return to a stable relationship between the two components of about 180°. Therefore, in the animals expressing split activity rhythms initial, rather than steady state, phase shifts were used to characterize the response of each component. One example of the effect of an injection of 10 mg cycloheximide on both components is shown in Fig. 5. The injection occurred 7 h before the onset of component 1 and induced a phase advance ($\Delta\phi = +35$ min) of that activity component. In the case of component 2, however, the injection occurred 6 h after its onset and caused a phase delay in that component ($\Delta\phi = -80$ min). Phase shifts of different directions were observed after 10/39 injections, phase advances in both components after 3/39 and phase delays in both components after 17/39 injections. An advance in one component with no phase shift of the other component was observed after 5/39 injections and a phase delay of only one component after 4/39 injections. The phase response curves for both components are shown in Fig. 6. A comparison of the phase response curves with a 2-way ANOVA revealed statistically significant variations between the phase shifts at different circadian times ($F_{5,59} = 8.49$; $P < 0.01$), but no significant difference between component 1 and component 2.

DISCUSSION

These results demonstrate that the circadian clock of hamsters can be phase shifted by peripheral administration of cycloheximide and in this way is similar to circadian systems in micro-organisms and invertebrates. Single injections of cycloheximide induced phase advances and phase delays dependent upon the time at which the injection occurred and injections of various concentrations of cycloheximide at CT 0 induced dose-dependent phase delays. Although the phase response curves in constant light and constant darkness differed significantly in their overall mean phase shift, both phase response curves had a similar general shape with maximum phase advances at CT 6–8 and maximum phase delays at CT 0–2.

The phase-shifting effects of cycloheximide on the circadian activity rhythm of hamsters are similar to those observed following treatment with a different protein synthesis inhibitor, anisomycin[22]. The phase response curves for subcutaneous injections of cycloheximide and anisomycin to hamsters free-running in constant darkness are very similar; both inhibitors cause maximal phase delays at CT 0 and maximal phase advances between CT 6 and 8. Since both inhibitors induce phase shifts of similar amplitude and direction, the phase shifting effect of both drugs is probably mediated through a common mechanism on either an input pathway to the circadian clock or on the clock itself.

The specific biochemical mechanisms by which cycloheximide and anisomycin act to inhibit protein synthesis are different[7]. Therefore, the similarities in the effects of cycloheximide and anisomycin on the circadian system of hamsters strongly support the hypothesis that these agents influence the oscillatory system through their effects on protein synthesis. Known side effects of cycloheximide and anisomycin include a decrease of catecholamine biosynthesis by inhibition of tyrosine hydroxylase activity[6,8,20] and inhibition of acetylcholinesterase activity[28,29]. However, administration of α-methyl-*p*-tyrosine, an inhibitor of tyrosine hydroxylase, or physostigmine, an inhibitor of acetylcholinesterase, at CT 0 had no phase shifting effect on the circadian rhythm of hamsters[22]. In *Neurospora,* it has been shown that there is a direct proportionality, across a 100-fold range of cycloheximide concentrations, between inhibition of protein synthesis and phase shifting of the clock[16]. Similar results have been found for anisomycin in *Aplysia,* based on a comparison of the effects of anisomycin and closely related analogs, which had no effect on inhibiting protein synthesis or phase shifting of the circadian rhythm[11]. Taken together, these data strongly support the hypothesis that the effect of protein synthesis inhibitors such as cycloheximide and anisomycin on the circadian system are indeed due to disruption of protein synthesis and are not due to unidentified side effects of the drugs.

Mrosovsky has obtained phase response curves to cage changing and social interactions in hamsters that are similar in shape to those observed for injections of cycloheximide and anisomycin[15]. He has suggested that phase shifts in the circadian oscillator in response to drug treatment may be due in part to an alteration in the general arousal state of the organism. Indeed, exposure to a running wheel for a short period of time by itself can induce phase shifts in the activity rhythm of hamsters that are similar to those observed after treatment with protein synthesis inhibitors (Wickland and Turek, unpublished results). However, it does not appear that phase shifts induced by injections of cycloheximide are mediated through an increase in activity since no increase in locomotor activity following drug treatment was observed. Indeed, as is evident from Fig. 1, treatment with cycloheximide usually resulted in a 24–48 h period of inactivity. Anisomycin treatment also inhibited activity; however, the duration of this effect was shorter and usually less than 24 h[22]. The effects of cycloheximide and anisomycin on other behavioral measures such as feeding and drinking was not evaluated. Therefore, although an effect of protein synthesis inhibitors on the circadian system mediated by changes in behavior cannot be excluded in this study, this explanation appears unlikely.

An important finding of this study is that each component of a split activity rhythm could respond independently to injections of cycloheximide and that each component showed a complete phase response curve to these perturbations with both phase advances and phase delays. These results are consistent with the finding that dark pulses induce qualitatively similar phase shifts in both components of a split activity rhythm[1,3]. These findings are difficult to explain in terms of a single-oscillator model regulating the split activity components, but instead support the hypothesis that the circadian system contains a complex circadian pacemaker which behaves as a mutually coupled dual oscillator system[17].

A possible site for the phase shifting effects of cycloheximide and anisomycin is the suprachiasmatic nucleus of the hypothalamus, since this structure appears to act as a pacemaker for circadian rhythms in a variety of mammalian species[14,18,24]. Indeed, microinjections of anisomycin directly into the suprachiasmatic region have been shown to induce phase shifts in the circadian activity rhythm of hamsters which were comparable to those obtained after subcutaneous injections[9].

Taken together, the results of this and a previous study[22] indicate that the phase response curves for protein synthesis inhibitors in hamsters are remarkably similar to the curves found in invertebrates and micro-organisms. These similarities suggest that common cellular and biochemical mechanisms may be responsible for the generation of circadian rhythms in diverse phylogenetic groups.

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