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Neurochemical basis for the photic control of circadian rhythms and seasonal reproductive cycles: Role for acetylcholine

(photoperiodism/wheel-running activity/entrainment/carbachol/hamsters)

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ABSTRACT **A pharmacological approach was used to examine the role of acetylcholine in the photic control of circadian rhythms and seasonal reproductive cycles. The experimental protocol was designed to determine whether the administration of carbachol, a cholinergic agonist, could mimic the effects of brief light pulses on gonadal function and/or the circadian rhythm of wheel-running activity in golden hamsters. Intraventricular injections of carbachol, administered singularly at discrete phase points throughout the circadian cycle, induced phase-dependent shifts in the free-running rhythm of activity similar to those caused by a brief light exposure. Injections of carbachol once every 23.33 hr for 9 weeks entrained the activity rhythm and stimulated the neuroendocrine–gonadal axis in a manner similar to that observed after the presentation of 1-hr light pulses at this frequency. In contrast, the administration of carbachol once every 24 hr did not consistently provide an entraining signal for the activity rhythm and did not stimulate reproductive function. Importantly, the effects of carbachol on the seasonal reproductive response were correlated with the timing of the injections relative to the activity rhythm. These findings suggest that acetylcholine may play an important role in the mechanism by which light regulates circadian rhythms and seasonal reproductive cycles.**

The light/dark (L/D) cycle is the primary environmental signal that synchronizes or entrains the internal biological clock(s) underlying circadian rhythms in a variety of physiological and behavioral activities (1). Although the quantitative aspects of the photic control of the circadian system have been examined in great detail (1, 2), little is known about the physiological mechanisms mediating the effects of light on circadian phenomena. The retinohypothalamic tract has been implicated in the transmission of photic information to the circadian timekeeping system by anatomical studies demonstrating that this pathway, which originates in the ganglion cells of the retina, projects to an important component of the circadian system, the suprachiasmatic nucleus of the hypothalamus (3). Since the retinohypothalamic tract presumably mediates the effects of light on the suprachiasmatic nucleus through the release of neurotransmitters, an experimental paradigm utilizing pharmacological agents to mimic the effects of light could provide a useful tool for examining the neurochemical events underlying the photic control of circadian phenomena.

In the present studies, we used a pharmacological approach to examine the role of acetylcholine in the photoentrainment of the circadian rhythm of wheel-running activity and in the circadian-based photoperiodic control of reproduction in the hamster. Acetylcholine provided a focus for these studies because carbachol, a cholinergic agonist, has been reported to mimic the phase-shifting effects of light on both the circadian rhythms of wheel-running activity in mice and pineal *N*-acetyltransferase activity in rats (4, 5). In examining the role of acetylcholine in mediating the effects of light on the circadian organization of the hamster, the experiments that follow took advantage of the fact that very brief periods of photostimulation can affect circadian phenomena in this species (6–8). In the first study, we sought to determine whether intraventricular injections of carbachol could mimic the phase-shifting effects of short light pulses on the activity rhythms of hamsters free-running in constant darkness. In addition, we used what has been referred to as the "T-experiment" paradigm (6) to determine whether carbachol can also mimic the entraining action of light and, if so, whether this pharmacological agent can affect clock-controlled changes in neuroendocrine–gonadal activity as a function of entrainment of the circadian system. The advantage of this experimental approach is that the effects of a particular entraining stimulus on the reproductive axis can be probed at discrete phases throughout the circadian cycle simply by varying the period of the stimulus presentation from its normal value of 24 hr. For example, testicular regression occurs in hamsters exposed to 1 hr of light once every 24 hr (L/D, 1:23), but regression is prevented in hamsters exposed to the same duration of light once every 23.34 hr (L/D, 1:22.34), because the animals entrain such that light falls at different phases of the circadian cycle under these two photoperiods (6). Accordingly, the second study examines the effects of carbachol on wheel-running activity and testicular function in hamsters receiving intraventricular injections of carbachol once every 23.33 hr or once every 24 hr.

MATERIALS AND METHODS

Animals and Housing Conditions. Adult male golden hamsters [*Mesocricetus auratus* LAK: LVG-(SYR)] purchased from Lakeview Hamster Colony (Newfield, NJ) were housed three to five per cage prior to initiation of the experiments. Throughout the studies, hamsters were provided with food (Teklad Hamster Diet) and water ad lib. Under experimental conditions of constant darkness (D/D), periodic animal care was accomplished by using a red safelight (Kodak filter no. 1, 0.5–1.0 lx).

Prior to experimentation, hamsters were rehoused in individual cages, each equipped with a running wheel so that the circadian rhythm of locomotor activity could be recorded. Groups of six such activity cages were enclosed in wooden boxes equipped with ventilating fans and fluorescent lighting (intensity at cage floor, 150–450 lx) controlled by a 24-hr timer.

Intraventricular Cannulation and Injections. As described in an earlier report (9), chronic implantation of a 21-gauge cannula placed in the lateral right ventricle was performed to allow repeated introduction of saline or carbachol into the

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Abbreviations: L/D, light/dark cycle; D/D, constant darkness.

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cerebrospinal fluid. Under light ether anesthesia, doses of either physiological saline or a solution of carbachol were delivered via the guide cannula by a microliter syringe. All intraventricular injections in the following experiments were administered in the dark with the aid of a red safelight.

At the end of each experiment, animals were injected via the cannula with trypan blue (5% solution). Cannula placement within the lateral ventricle was verified in all animals by inspecting the brains for the presence of dye in the ependymal lining.

Experimental Protocol. *Experiment 1.* A group of 17 male hamsters that had experienced testicular regression and recrudescence during a long-term exposure to L/D 6:18 were placed in running wheel cages. Sixteen to 18 days later, the animals were implanted with a ventricular cannula and subsequently transferred from L/D 6:18 to D/D. The animals were allowed to free-run for 10–12 days before the phase-shifting effect of carbachol on the activity rhythm was assessed. At 2–3 week intervals, single injections of saline or 0.01 M carbachol (vol, 2 μl) were administered at various times throughout the circadian cycle. Hamsters were generally injected with saline or carbachol on five different occasions during the course of the experiment, with a maximum of eight injections administered to animals that had patent cannulae and exceptionally stable activity rhythms over a 5-month interval.

Experiment 2. After a 2-week period of acclimation to their running wheels on L/D 14:10 (lights on at 1030 hr), 44 adult male hamsters were either implanted with a ventricular cannula (n = 32) or allowed to continue without surgical intervention (n = 12). Testicular size was measured through the skin covering the scrotal area to ascertain that the gonads were large (i.e., testes width, ≈12.0 mm with an estimated paired testis weight of ≈3000 mg; see ref. 7) and all animals were then exposed to a D/D cycle. Animals with a cannula were randomly divided into four groups and received 1-μl injections of either saline or 0.01 M carbachol according to one of the following protocols: Group 1 (n = 8): saline was administered once every 23.33 hr; group 2 (n = 8): carbachol was administered once every 23.33 hr; group 3 (n = 8): saline was administered once every 24 hr; group 4 (n = 8): carbachol was administered once every 24 hr. Animals without a ventricular cannula were exposed to 1-hr pulses presented either once every 23.33 hr (n = 6; L/D 1:22.33) or once every 24 hr (n = 6; L/D 1:23). The first intraventricular injection or 1-hr light pulse was coincident in each case with the time where lights on would have occurred in the previous L/D 14:10 cycle. Although all animals were housed individually with access to a running wheel, activity was only recorded from 6–7 animals in each of the treatment groups. After 9 weeks, the animals were sacrificed and the testes were removed and weighed.

In both experiments, wheel-running activity was recorded and analyzed (i.e., determination of period and phase shifts) as described by Ellis *et al.* (10). Analysis of variance was used to determine the significance of treatment effects, and differences between treatments were tested for significance using the Student–Newman–Keuls sequential range test.

RESULTS

Experiment 1. After transfer to D/D, all hamsters showed clear free-running activity rhythms (mean τ = 24.11 ± 0.01 hr). Neither the free-running period nor the phase of the activity rhythm was altered by intraventricular injections of saline. While there was no consistent effect of single injections of carbachol on the free-running period, carbachol did evoke shifts in the steady-state phase of the activity rhythm. Fig. 1 (*Top* and *Middle*) depicts both a typical phase delay, caused by an injection of carbachol early in the hamster's active period (i.e., the subjective night), and a typical phase advance, induced by an injection late in the hamster's active period. As characterized in this figure, the activity rhythm returned to steady state within 1–2 cycles after a carbachol-induced phase delay, whereas advancing shifts usually required 4–7 transient cycles before a new steady state was reached.

The responses of hamsters free-running in D/D to intraventricular carbachol are plotted collectively in Fig. 2 as a function of the circadian time of injection. This phase–response curve for intraventricular carbachol is of low amplitude, with maximum phase shifts on the order of 2–4 hr. Furthermore, the phase shift induced by a bolus of carbachol was clearly dependent on the time during the circadian cycle at which the drug was administered. Injections of carbachol coincident with the early subjective night (Ct 12–16) consistently induced discrete phase delays of the activity rhythm while carbachol administered late in the subjective night (Ct 20–24) elicited phase advances of the rhythm. Interestingly, substantial phase advances (1–2 hr) were also elicited by injections of carbachol during the first half of the subjective day. A typical phase advance induced by the administration of carbachol during the subjective day is shown in Fig. 1 (*Bottom*). It is noteworthy that the free-running rhythm depicted here returned to steady state immediately after the carbachol injection; transient cycles following all phase-advancing injections administered during the subjective day were few or absent altogether.

Experiment 2. Fig. 3 depicts the patterns of wheel-running activity from representative animals receiving either saline, carbachol, or 1 hr of light once every 23.33 hr. The activity rhythms of all animals were initially entrained to L/D 14:10, so that the onset of activity occurred every day 0.2–0.5 hr after lights off. After transfer from L/D 14:10 to a 23.33-hr injection cycle, the activity rhythms of all animals receiving intraventricular saline were observed to free run with periodicities that were >24 hr (mean τ = 24.2 ± 0.1 hr). In fact, no sign of period and/or phase modulation was evident in the activity patterns of these animals as different phases of the rhythm passed through the time of saline administration (Fig. 3 *Left*). In contrast, injections of carbachol delivered once every 23.33 hr were effective stimuli for entrainment of the activity rhythm (Fig. 3 *Center*). During steady-state entrainment, wheel-running activity in all animals (n = 6) was initiated every day 8–9 hr before the time of carbachol administration. Four of these animals remained stably entrained throughout the duration of the experiment, whereas entrainment in the other two animals was interrupted by bouts of lability in the activity pattern during which the rhythm drifted out of phase with the injection cycle. The frequency of the rhythm during such intervals, although not synchronized, was modulated by the carbachol injections until the rhythm and the injection cycle were realigned in their former determinate phase relationship, at which time stable entrainment recurred. Exposure to a 1-hr light pulse once every 23.33 hr (L/D, 1:22.33) was similarly marked by entrainment of the activity rhythm such that the daily onset of activity preceded lights on by 8–9 hr in all animals (Fig. 3 *Right*).

The patterns of wheel-running activity shown in Fig. 4 are from representative animals receiving intraventricular injections of saline or carbachol or 1-hr light pulses once every 24 hr. During entrainment to L/D 14:10, daily wheel-running activity in all animals began 0.2–0.5 hr after lights off. Once released from the influence of this entraining light cycle, the activity rhythms of all animals receiving saline on a 24-hr basis were observed to free run with periodicities that were >24 hr (mean τ = 24.2 ± 0.1 hr). Neither the period nor the phase of the free-running activity pattern were altered as the rhythm drifted through the injection time (Fig. 4 *Left*). Injections of carbachol once every 24 hr provided a periodic sig-

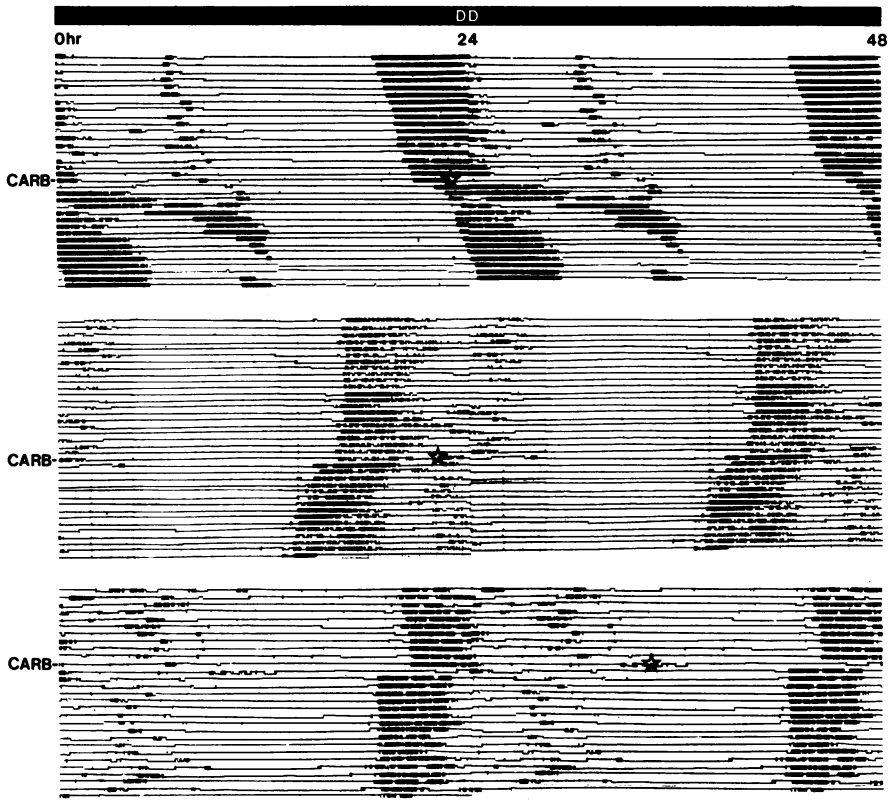

FIG. 1. Daily activity records for three different hamsters exposed to D/D. Successive days are plotted from top to bottom, and the record has been double mounted over a 48-hr time interval. Carbachol was delivered intraventricularly to each animal on the day indicated (CARB), with the actual time of injection shown as an open star.

nal that entrained the activity rhythms of two of the seven animals whose wheel-running behavior was monitored throughout the experiment. The pattern of entrainment in these animals was eventually characterized by a steady-state phase relationship between the activity rhythm and the injection cycle such that the daily onset of activity occurred 2–3 hr after the time of carbachol administration (Fig. 4 *Center*). One of the remaining five animals showed temporary synchronization with the injection cycle, while the other four animals exhibited free-running patterns of activity (mean τ = 24.3 ± 0.1 hr) throughout the period of carbachol treatment. Exposure to 1 hr of light once every 24 hr (L/D 1:23) was accompanied by a series of long-lasting transients before stable entrainment of the activity rhythm occurred. During steady-state entrainment, all animals receiving a 1-hr light pulse on a 24-hr basis initiated daily wheel-running activity 12–13 hr after lights on (Fig. 4 *Right*).

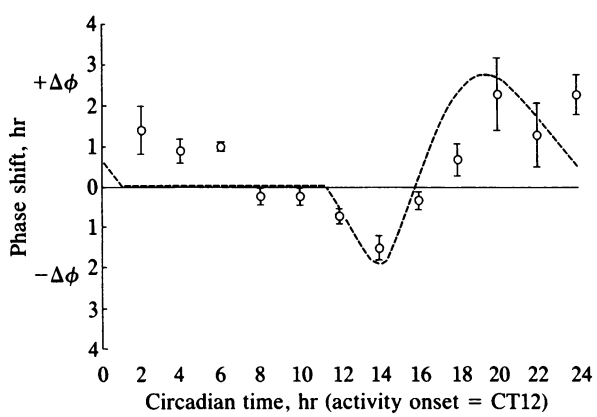

FIG. 2. Open circles depict the response of the free-running circadian rhythm of locomotor activity to intraventricular injections of carbachol administered to hamsters exposed to a D/D cycle. Each point represents the mean phase shift (±SEM), averaged over a 2-hr bin of circadian time, of three to four animals injected with carbachol. A point above the solid line indicates an advance (+Δφ) in the onset of activity; a point below this line indicates a delay (−Δφ) in the onset of activity. For comparative purposes, the phase–response curve to 1-hr light pulses is represented by the dashed line (10).

All animals in this experiment had large testes (mean testis width, 12.3 ± 0.3 mm) prior to transfer to one of the treatment paradigms. Regardless of the timing of the injections (i.e., once every 23.33 hr or once every 24 hr), marked testicular regression occurred in all hamsters receiving intraventricular injections of saline for 9 weeks (Fig. 5; mean paired testis weight, 408.6 ± 38.5 mg and 597.9 ± 171.7 mg, respectively). In contrast, the effects of carbachol and light on testicular function clearly depended on the period of the treatment cycles. Carbachol, when delivered once every 23.33 hr, had a statistically significant effect in maintaining testicular function ($P < 0.01$) relative to saline-injected animals. In fact, the testicular weights of six of the eight animals receiving carbachol once every 23.33 hr are comparable to the paired testis weights of hamsters exposed to 1 hr of light at this frequency. However, treatment with carbachol or 1-hr light pulses on a 24-hr basis failed to prevent the photoinhibition of testicular function as indicated by the mean values for paired testis weight (337.1 ± 42.0 mg and 617.5 ± 205.5 mg, respectively). No significant differences in paired testis weight were observed among groups of hamsters treated with either saline, carbachol, or light on a 24-hr basis.

DISCUSSION

These results indicate that intraventricular injections of carbachol can trigger the cascade of biochemical and neural events underlying the effects of light on the circadian system. In hamsters free running in constant darkness, injections of carbachol yielded phase-dependent shifts of the activity rhythm that were similar in direction and magnitude to those observed after a brief exposure to light. Furthermore, the administration of carbachol on a circadian basis can entrain the activity rhythm and mimic the stimulatory effects of light on clock-controlled changes in neuroendocrine–gonadal activity.

Complete phase–response curves have now been generat-

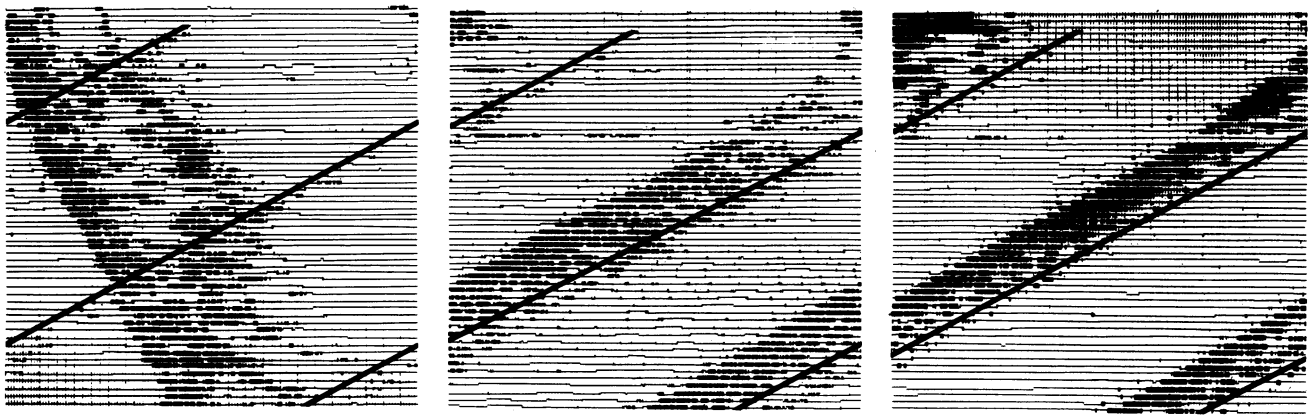

FIG. 3. Continuous records of wheel-running activity from representative male hamsters receiving intraventricular injections (*Left*, saline; *Center*, carbachol) or 1-hr light pulses (*Right*) once every 23.33 hr. With the exception of the first 3 days of each record, activity data collected during stable entrainment to L/D 14:10 has been omitted. Diagonal bar in each record represents the time of injection or the onset of the 1-hr light pulse. Successive 24-hr days are plotted from top to bottom.

ed for three neuropharmacological agents in the hamster: avian pancreatic polypeptide, neuropeptide Y, and carbachol. The phase–response curves for APP and neuropeptide Y resemble the phase–response curve for dark pulses delivered on a background of constant light (11, 12). In contrast, the phase–response curve for intraventricular carbachol is strikingly similar in amplitude and waveform to existing phase–response curves generated with 15- to 60-min light pulses (10, 13), except the advance region of the curve for carbachol extends into the subjective day. This disparity between the phase–response curves for carbachol and light may be related to differences between the pharmacological action of the dose of carbachol used in the present study and the physiological action of light. It has been reported that in *Aplysia* and *Gonyaulax* the amplitude and the waveform of the phase–response curve to a specific chemical or pharmacological agent vary as a function of dose (14, 15); at higher stimulus concentrations, the phase–response curve is generally characterized by an increase in the amplitude of the phase shifts as well as an expansion of the delay and/or advance regions, resulting in little or no unresponsive region. Further experiments, in which phase–response curves are generated for different concentrations of carbachol, are necessary to fully characterize the similarities and differences between the phase shifting effects of carbachol and light on the circadian system.

Carbachol, when injected on a circadian basis, can also mimic the entraining action of light on the hamster circadian clock(s) involved in the generation of the activity rhythm. For the 23.33-hr treatment cycles, the pattern of entrainment in animals receiving carbachol resembled the pattern observed during exposure to 1-hr light pulses (L/D 1:22.33); in both cases, wheel-running activity was initiated every day 8–9 hr after presentation of the stimulus (i.e., the carbachol injection or the onset of light). Injections of carbachol once every 24 hr were less effective than injections recurring on a 23.33-hr basis in entraining the activity rhythm; only two of seven animals were stably entrained to the 24-hr injection cycle. Interestingly, the activity pattern of these animals was clearly different from the pattern observed in animals exposed to 1-hr light cycles with the same period length (L/D 1:23). In these two hamsters, the daily onset of activity occurred a few hours after the injection time, whereas in all of the animals exposed to 1-hr light pulses, wheel-running activity was initiated 12–13 hr after lights on. This difference between the patterns of entrainment to carbachol and to light delivered once every 24 hr may be related to the aforementioned disparity between the advance regions of the phase–response curves for these stimuli. Assuming that the endogenous period of the circadian clock(s) underlying the hamster activity rhythm is slightly greater than the 24-hr periodicity of the treatment cycles, then entrainment to the daily administration of carbachol or a 1-hr light pulse requires that each of these stimuli elicit a small daily phase advance of the clock. It can be determined from the phase–response curves that such "corrective" phase advances will be obtained when the 1-hr light pulses are aligned with the early hr of the subjective day. However, because of the expansion of the advance region of the phase–response curve for carbachol, similar phase adjustments would be expected to occur when

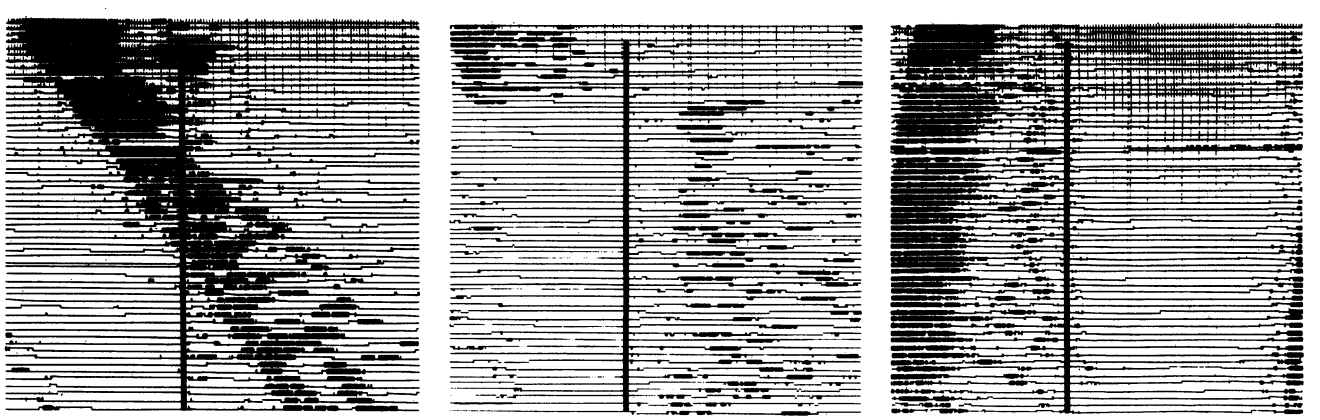

FIG. 4. Continuous records of wheel-running activity from representative male hamsters receiving intraventricular injections (*Left*, saline; *Center*, carbachol) or 1-hr light pulses (*Right*) once every 24 hr.

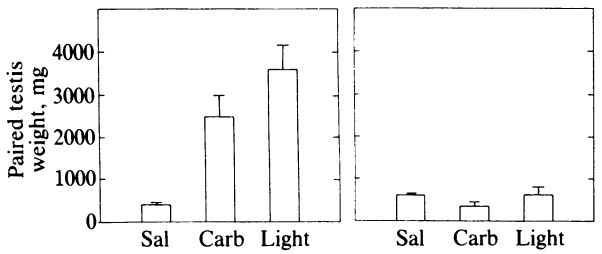

FIG. 5. Mean (±SEM) paired testis weights of male hamsters from experiment 2. Groups of eight hamsters were injected for 9 weeks in constant darkness with either saline (Sal) or carbachol (Carb) on a 23.33-hr (*Left*) or 24.0-hr (*Right*) basis. Two additional groups of hamsters ($n = 6$) were exposed to 1-hr light pulses (Light) every 23.33 hr (*Left*) or every 24.0 hr (*Right*).

the daily carbachol injections are coincident with the middle of the subjective day (i.e., 4–6 hr before activity onset). In the two animals entrained to carbachol administered on a 24-hr basis, the injections preceded the onset of activity by 2–3 hr. More detailed studies on the phase-shifting and entraining action of carbachol are needed before it will be possible to determine how accurately the phase–response curve can predict the pattern of entrainment to this agent.

The present results also suggest that carbachol mimics the effects of light on clock-controlled changes in reproductive activity. The effects of intraventricular carbachol on gonadal function depended markedly on the period of the treatment cycle; maintenance of testicular function occurred under carbachol injection cycles with a 23.33-hr, but not a 24-hr, period length. Furthermore, the effects of circadian injections of carbachol on reproduction are strongly correlated with the phase relationship between the injection time and the activity rhythm. Testicular maintenance in animals receiving carbachol once every 23.33 hr was associated with a pattern of entrainment in which the injections were coincident with the latter portion of the hamsters' subjective night (i.e., active phase of the activity/rest cycle). Although wheel-running activity was only monitored in one of the two hamsters experiencing testicular regression after the administration of carbachol for 9 weeks on a 23.33-hr basis, it is important to note that the entrainment pattern of this animal was characterized by periods of lability in the phase relationship between the onset of activity and the injection time. The correlation between the testicular response to carbachol and the pattern of entrainment is further underscored by the observation that in the two animals entrained to carbachol injections recurring on a 24-hr basis, testicular regression occurred concomitantly with an activity pattern in which the injections were coincident with the latter portion of the hamsters' subjective day. In the remaining animals receiving carbachol once every 24 hr, testicular regression was accompanied by activity patterns in which the injections were not stably aligned with a particular phase of the circadian cycle.

Experiments using brief pulses of light indicate that photostimulation of the hamster reproductive axis depends on the illumination of a particular phase (or set of phase points) of one or more circadian rhythms (6, 7). In the hamster, this light-sensitive phase (ϕi) begins just prior to the onset of wheel-running activity and extends for 10.5–12 hr (6). While we have previously shown that carbachol injections during the night can alter the gonadal response of hamsters exposed to an inhibitory photoperiod (9), the design of that study failed to provide a basis for determining whether carbachol maintained gonadal function as a result of its coincidence with ϕi, or whether the phase-shifting action of carbachol on the circadian system resulted in the illumination of ϕi by the main light period. However, the present finding that carbachol administered once every 23.33 hr entrained the circadian system such that the injections occurred 8–9 hr after the onset of activity indicates that carbachol, in mimicking the effects of light, was coincident with ϕi and was directly responsible for maintaining gonadal function. Carbachol delivered on a 24-hr basis was presumably ineffective in maintaining testicular function in the two animals that entrained to the treatment cycle, because the injections did not coincide with ϕi. An interesting aspect of the present data was the testicular response of carbachol-injected animals that showed either free-running activity patterns or labile entrainment when subjected to a 24-hr treatment cycle. In spite of the periodic coincidence of the carbachol injections with ϕi, all of these animals experienced complete testicular regression. This observation raises the possibility that stimulation of the reproductive axis depends not only on the coincidence of the carbachol injections with ϕi, but also on their stable alignment with a particular portion of ϕi.

At present, the neural site mediating the photomimetic effects of carbachol on circadian rhythms and reproduction has not been defined. The suprachiasmatic nucleus of the hypothalamus represents a potential locus for the phase-shifting, entraining, and photoperiodic actions of carbachol, since this structure is thought to mediate the effects of light on the circadian rhythm of activity and reproductive function in the hamster (16) and cholinergic receptors and choline acetyltransferase have been found in the suprachiasmatic nucleus (17, 18). The pineal gland may also be involved in mediating the stimulatory effects of carbachol on the reproductive system, because the pineal plays an important role in the transduction of photoperiodic information (19) and carbachol can alter pineal enzyme activity (4).

In summary, the present studies demonstrate that carbachol can mimic the effects of brief light pulses on the circadian activity rhythm and the reproductive axis in the hamster. Together with the results of earlier work (4, 5, 9), these data strongly suggest that acetylcholine may play a key role in the mechanism by which light information is transmitted to or within the biological clock involved in the generation of circadian rhythms and photoperiodic time measurement.

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