

The Effects of Intraventricular Carbachol Injections on the Free-Running Activity Rhythm of the Hamster

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Abstract The effects of light on the circadian pacemaker in the suprachiasmatic nucleus (SCN) are mediated by the retinohypothalamic tract (RHT) and by the retinogeniculosuprachiasmatic tract (RGST). The neurotransmitter of the RGST is neuropeptide Y. The RHT may contain glutamate and aspartate. Recent evidence indicates that acetylcholine could also be involved in phase shifting by light. We determined that intraventricular injections with an acetylcholine agonist, carbachol, induces phase advances during the subjective day and phase delays during the early subjective night. No differences were observed between phase shifts induced in constant darkness and those induced in continuous light. A dose–response curve for carbachol was described at circadian time 6 (CT6). Injections at CT14 with various dosages of carbachol indicated the same dose dependency for this circadian time. Finally, carbachol injections in split animals resulted in similar responses of the two components of the split activity rhythm.

The mammalian suprachiasmatic nuclei (SCN) have been identified as a pacemaker for many circadian rhythms (Rusak and Zucker, 1979). These rhythms are entrained to the 24-hr cycle in the environment by the daily light–dark cycle. In mammals, the entrainment pathways originate exclusively in the retina (Underwood and Groos, 1982). Two pathways exist by which light entrains the circadian clock in the SCN: the direct retinohypothalamic tract (RHT) (Hendrickson *et al.*, 1972; Moore, 1973; Pickard, 1982) and the indirect retinogeniculosuprachiasmatic tract (RGST) (Swanson *et al.*, 1974; Ribak and Peters, 1975; Hickey and Spear, 1976).

When the optic nerve of an SCN-containing slice preparation is stimulated electrically, an increase in the concentration of glutamate and aspartate can be detected in the bath (Liou *et al.*, 1986). Therefore, these excitatory amino acids may be the transmitters of the RHT. The geniculoprachiasmatic tract (GST) is characterized by neuropeptide Y immunoreactivity (Harrington *et al.*, 1985). Application of glutamate and neuropeptide Y inside the SCN can shift the phase of the free-running activity rhythm of the hamster (Albers and Ferris, 1984; Meijer *et al.*, 1984, 1988).

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Besides glutamate and neuropeptide Y, other drugs are known to induce phase shifts. Intraventricular injection of carbachol, an agonist of acetylcholine, induces phase shifts of the free-running activity rhythm in mice and hamsters (Zatz and Herkenham, 1981; Earnest and Turek, 1983, 1985). The phase shifts produced by carbachol injections during the subjective night resemble the light-pulse phase response curve (PRC). Injections during the subjective day result in phase advances that are usually not observed following the presentation of a light pulse (Earnest and Turek, 1985). In this study, we compared the carbachol-induced phase shifts of hamsters in continuous darkness with the carbachol-induced shifts of hamsters in continuous light. For hamsters in the illuminated room, we also described a dose-response curve for carbachol.

When hamsters are housed in continuously illuminated cages, their circadian rhythm in drinking, eating, and wheel-running activity and their temperature rhythm may split into two components (Pittendrigh, 1974; Shibuya *et al.*, 1980; Pickard *et al.*, 1984). Following an unstable period, which ranges from several days to a week or more, the two components regain a stable phase relationship when they are about 180° out of phase with one another. This phenomenon has been described in detail by Pittendrigh and Daan (1976b), who proposed a two-oscillator model as underlying the occurrence of splitting.

The anatomical, physiological, or pharmacological correlate of the two-oscillator model is, however, unknown. It remains an open question whether the components of the split rhythm reflect either identical oscillators or two different oscillators. One might expect that different oscillators responding differently to light signals might also exhibit differential responses to pharmacological stimuli. Therefore, as an extension to the above-mentioned experiments, we performed carbachol injections in hamsters with a split activity rhythm. Thus, we determined whether the two components of the split activity rhythm responded differently to carbachol.

METHODS

Male golden hamsters (*Mesocricetus auratus*) were obtained from TNO, Zeist, The Netherlands, at the age of 10 weeks (80–100 g). After the animals had been group-housed under a light–dark regimen (LD 14:10) for 1–3 weeks, they were transferred to individual cages (48 × 27 × 22.5 cm) that were equipped with running wheels (diameter; 14 cm) either in a continuously illuminated (LL) or in a dark (DD) sound-attenuated and temperature-controlled (22°C) room. To enhance the amount of wheel-running activity, the animals received new cages (30 × 22.5 × 29.5 cm) and larger running wheels (diameter, 25 cm) in the course of the experiment (see Meijer *et al.*, 1988). They were allowed free access to food pellets and water and were checked three times a week at random times during the day. The light intensities within the cages in the illuminated room ranged from 20 to 300 lux. Most of these intensities were greater than needed to saturate phase-shifting responses to light (unpublished data).

When the hamsters were 12–15 weeks old, stainless steel homemade cannulas (outer diameter, 0.3 mm; inner diameter, 0.15 mm) were implanted in their lateral

ventricles. The hamsters were anesthetized with an initial dose of 90 mg/kg pentobarbital and were placed in a stereotaxic apparatus with the toothbar 2 mm below the interaural line. The stainless steel cannula was aimed stereotaxically at the lateral ventricle (coordinates: 0.7 mm anterior to bregma, 1.3 mm lateral to the midline, and 3.0 mm ventral to the dura). The cannula was fixed to the skull with dental acrylic and four jeweller screws. A wire was inserted in the cannula to keep the cannula open, and the hole was protected by a removable cap.

Three experiments were performed. In Experiment 1, a PRC for carbachol injections was established for hamsters housed in LL and DD. In Experiment 2, the dose-dependent effect of carbachol was described for injections at CT 6 and 14 (6 hr before and 2 hr after the onset of activity) for hamsters kept in LL. In Experiment 3, the effects of carbachol on the split activity rhythm were investigated.

EXPERIMENT 1

Injections with 2 μ l of 0.01 M carbachol (carbamylcholine chloride, Sigma) dissolved in artificial CSF were performed at CT0, 3, 6, 9, 12, 15, 18, and 21 with a minimal interval between the injections of 8 days. Control injections with 2 μ l of artificial CSF were performed at CT6, 9, 14, and 18. The onset of activity was conventionally defined as CT12. (The injection procedure was described in detail in Meijer *et al.*, 1988). The injections were preceded by a light ether anesthesia during which the Hamilton syringe was attached to the cannula via a plastic tube. The injected volume was estimated from the distance covered by an inkspot inside the tube (and close to the cannula) to correct for changes in pressure (and therefore in volume) that may have occurred in the tube. While the injections were being performed, the animals could move freely in a small cage. Injections in the dark were delivered with the aid of a dim red light with the animal's eyes being occluded by a black cap.

EXPERIMENT 2

At CT6 and 14, injections with 2- μ l carbachol solutions were performed with a carbachol concentration of $\frac{1}{64}$, $\frac{1}{32}$, $\frac{1}{16}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, and 1 times 0.01 M in hamsters housed in LL. The injection procedure was as described above.

EXPERIMENT 3

Carbachol injections (2 μ l; 0.01 M) were performed with intervals of 15–30 days between the injections in hamsters with split activity rhythms in LL. The injections were performed exactly as in Experiment 1.

The location of the cannulas was histologically verified at the end of the experiment by inspection of 40- μ m cresyl violet-stained sections of the brains.

DATA ANALYSIS

EXPERIMENTS 1 AND 2

Carbachol was delivered after a stable free-running rhythm was established for at least 7 days. Straight lines were fit by linear regression through activity onsets prior

to the injection and through the onsets following the injection from the third day after the injection onward. Phase shifts were established by measuring the difference between the pre- and postinjection regression lines at the day of the first activity onset following the injection. The number of transients following an injection was estimated by counting the number of days until a steady-state free-running rhythm was achieved.

EXPERIMENT 3

The E and M component of the split activity rhythm were defined from the initial transients between the unsplit and the split situation in accordance with Pittendrigh and Daan (1976b). The steady-state phase shifts were estimated by measuring the difference between the extrapolated pre- and postinjection regression lines at the first activity onset following an injection for both components of the split rhythm. The preinjection regression line was calculated from the last 10-days of observation before the carbachol application; the postinjection regression line was calculated for the 7th to the 17th day following each injection. The beginning of wheel-running activity was defined as CT12 for both components. In this way, the components were analyzed independently. Immediate phase shifts were estimated for each component by measuring the difference between the extrapolated preinjection activity onset and the actual onset of activity at the day of the first activity onset following the injection.

RESULTS

To obtain a PRC for carbachol, 42 intraventricular carbachol injections were performed in 16 hamsters in DD and 38 injections in 16 hamsters in LL (using a total of 28 hamsters). In Figure 1, examples of carbachol-induced phase shifts are presented. Injections at CT3, 6, and 9 in 19 hamsters consistently produced phase advances of the circadian activity rhythm; injections between CT13 and CT20 predominantly produced delays in 15 hamsters (Fig. 2). Seventeen control injections with artificial CSF were performed in 10 hamsters that were kept in DD, and 15 control injections were delivered in 11 hamsters in LL. Table 1 summarizes the carbachol and artificial CSF-induced phase shifts at CT6, 9, 14, and 18.

In the course of the experiment, all hamsters received new cages and new running wheels. The increment in diameter of the running wheel increased the amount of activity of the animals, which improved the determination of the activity onsets; however, it did not alter the response to carbachol injections. The mean phase shift (*SEM*) at CT9 pooled from LL and DD was 1.18 hr (0.24) for eight animals with small wheels and 1.03 hr (0.15) for eight animals with larger wheels. The period of the free-running activity rhythm was often found to change following an injection. The mean change in period following carbachol injections in the light was 0.04 hr (0.02); in the dark, it was 0.05 hr (0.02). No relation was observed between the phase shift induced by the carbachol injection and the change in period.

The activity during the first cycle following an injection was sometimes mark-

CARBACHOL PHASE-SHIFTS ACTIVITY RHYTHMS

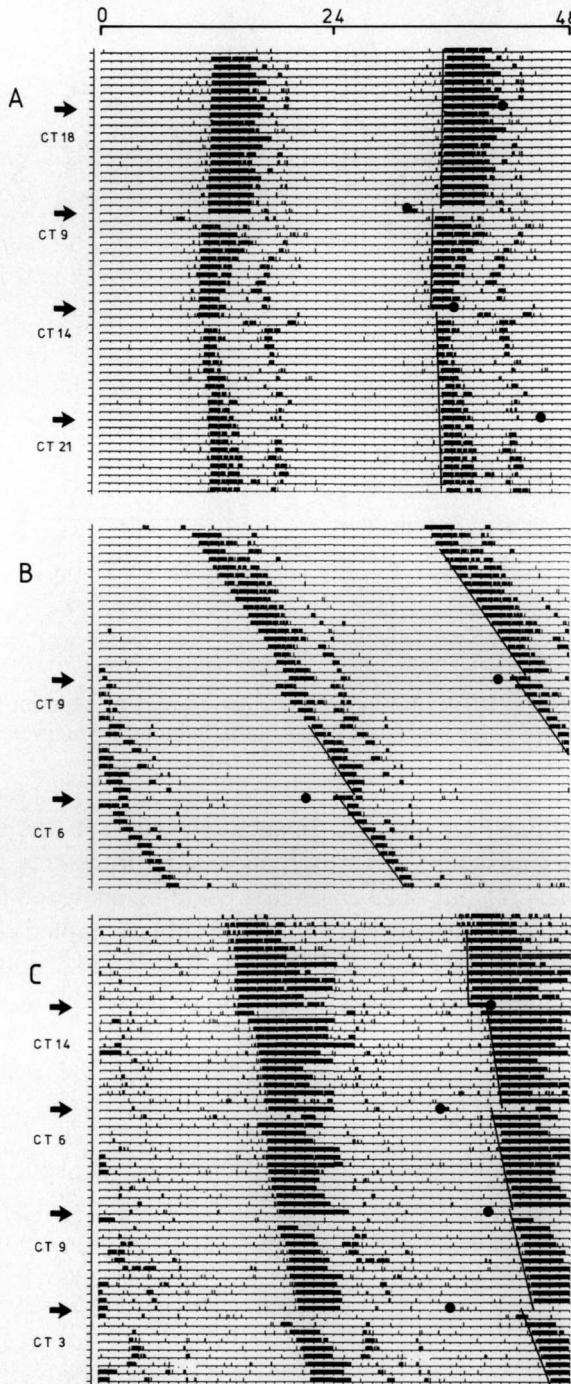


FIGURE 1. Representative examples of carbachol-induced phase shifts of the circadian rhythm in (A) DD and (B, C) LL. The times of the injections are marked by arrows and dots. (A, C) A decrease in activity follows carbachol injections.

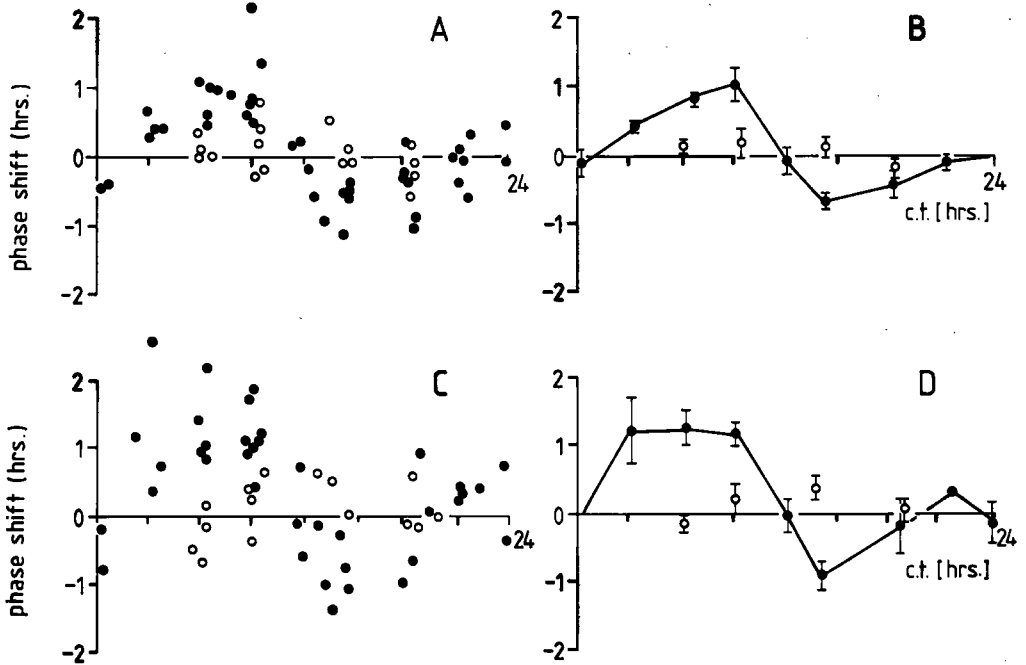


FIGURE 2. Phase response curves for intraventricular carbachol injections in (A, B) DD and in (C, D) LL. The open circles represent phase shifts induced by artificial CSF injections in the lateral ventricle.

edly decreased (see Figs. 1A and 1C). In such instances, it was difficult to assess whether there were transients. At CT6, 9, and 14, no transients were observed in about half of the trials (15 out of 29 cases that could be analyzed for transients). In eight cases, one to three transients were observed at these pooled circadian times. In the remaining seven trials, the phase shift at the first (and second) cycle appeared greater than the eventual phase shift. Examples are presented in Figure 3.

TABLE 1. Carbachol-Induced and ACSF-Induced Phase Shifts

| CT | Carbachol | | | | | ACSF | | | | |
|-------|-----------|----|--------------|------|------|------|----|--------------|------|------|
| | NI | NA | $\Delta\phi$ | SD | SEM | NI | NA | $\Delta\phi$ | SD | SEM |
| Dark | | | | | | | | | | |
| 6 | 6 | 5 | 0.83 | 0.25 | 0.10 | 4 | 4 | 0.11 | 0.17 | 0.08 |
| 9 | 6 | 6 | 1.02 | 0.64 | 0.26 | 5 | 5 | 0.18 | 0.45 | 0.20 |
| 14 | 6 | 6 | -0.68 | 0.29 | 0.12 | 4 | 4 | 0.10 | 0.28 | 0.13 |
| 18 | 6 | 6 | -0.45 | 0.47 | 0.19 | 4 | 4 | -0.20 | 0.31 | 0.16 |
| Light | | | | | | | | | | |
| 6 | 5 | 5 | 1.25 | 0.54 | 0.24 | 4 | 4 | -0.15 | 0.25 | 0.13 |
| 9 | 8 | 8 | 1.15 | 0.47 | 0.17 | 4 | 4 | 0.20 | 0.43 | 0.22 |
| 14 | 5 | 5 | -0.90 | 0.40 | 0.18 | 3 | 3 | 0.36 | 0.31 | 0.18 |
| 18 | 4 | 4 | -0.20 | 0.84 | 0.42 | 4 | 4 | 0.04 | 0.35 | 0.17 |

Note. The carbachol-induced and artificial CSF (ACSF)-induced phase shifts ($\Delta\phi$) with the standard deviation (SD) and the standard error of the mean (SEM) are summarized for CT6, 9, 14, and 18. NI, number of injections; NA, number of animals involved at that circadian time.

CARBACHOL PHASE-SHIFTS ACTIVITY RHYTHMS

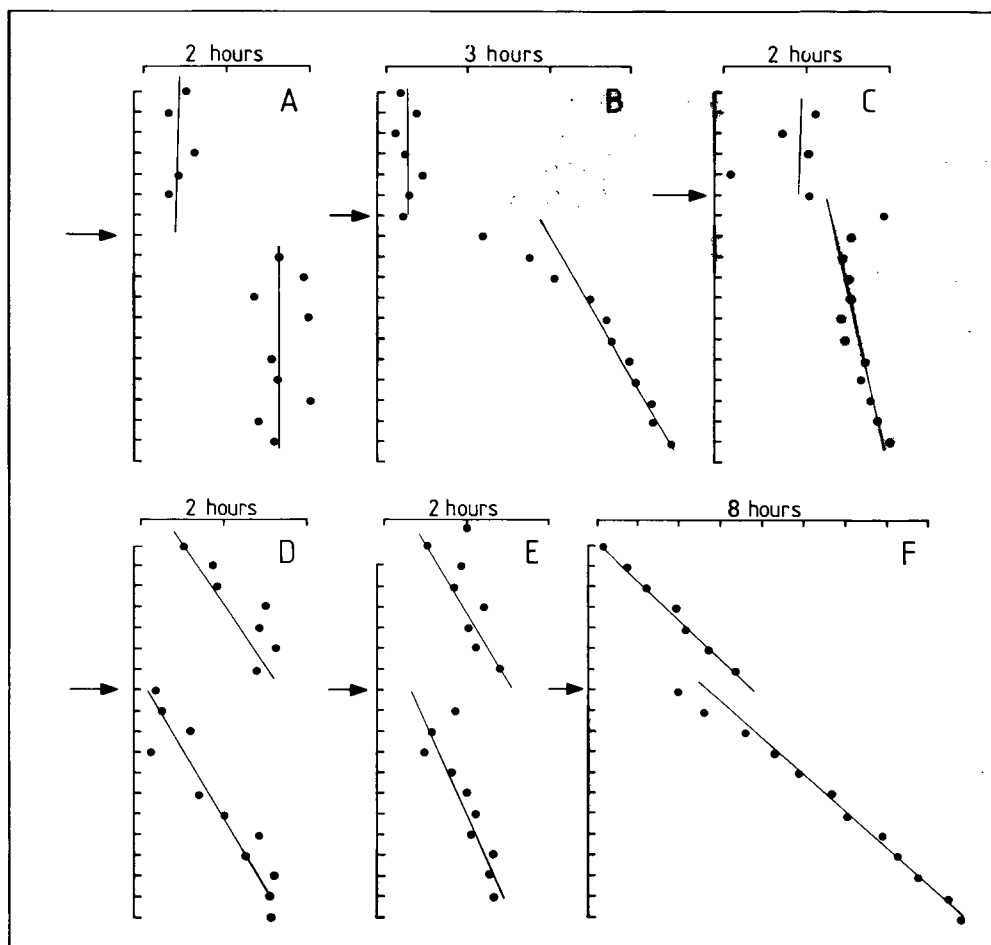


FIGURE 3. Carbachol-induced phase advances (A–C) as well as phase delays (D–F) can be accompanied by both advancing and delaying transients. (A, D) No transients are observed. The starts of activity are marked by dots while the days of the injections are marked by arrows.

DOSE-RESPONSE CURVE

At CT6, 81 carbachol injections were performed in the lateral ventricle of 37 animals with a dose ranging from $\frac{1}{64} \times 0.01$ M carbachol to 0.01 M carbachol. In Figure 4, the carbachol-induced phase shifts of the circadian activity rhythm are shown for different doses of carbachol; the data points that were obtained to construct the PRC are not included. Inspection of the data reveals a great variation in the carbachol-induced phase shifts. The standard deviation (*SD*) for carbachol-induced phase shifts following 0.01 M carbachol (*SD*, 0.39) appears smaller than the *SD* for $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{8} \times 0.01$ M carbachol ($0.62 < SD < 0.72$). This variation does not reflect differences in the response to carbachol between animals; as a matter of fact, this variability could also be observed within animals. The mean phase shifts could be fitted by a Michaelis function with a Hill coefficient of 3.1. Thus, a threshold exists for low

doses of carbachol, and for high dosages of carbachol saturation of the magnitude of phase shift was reached.

An additional 14 injections were performed in 13 animals at CT14 with a dose ranging from $\frac{1}{4} \times 0.01$ M carbachol to $\frac{1}{64} \times 0.01$ M carbachol. The dose dependency of these injections followed the dose response curve of CT6. For $\frac{1}{4}$ and $\frac{1}{8} \times 0.01$ M carbachol, mean phase delays (*SEM*) of -0.9 hr (0.08) and -0.7 hr (0.09) were observed. For $\frac{1}{16}$ to $\frac{1}{64} \times 0.01$ M carbachol, the absolute phase shift was always smaller than 0.1 hr.

RESPONSE OF THE SPLIT ACTIVITY RHYTHM TO CARBACHOL

In 10 animals with a split rhythm, 10 carbachol injections were given 3 hr after the onset of component E and 12 injections 3 hr after the onset of component M (Fig. 5).

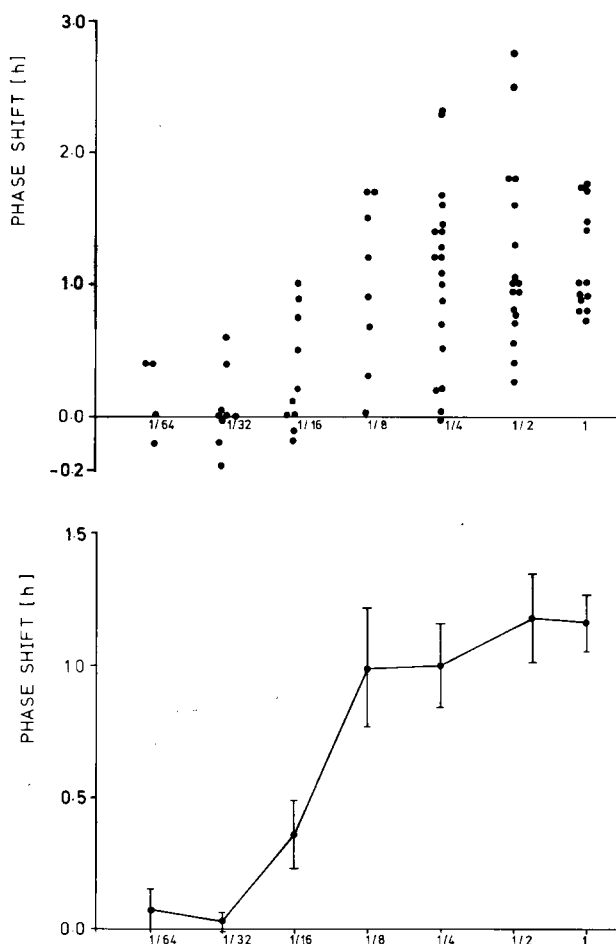


FIGURE 4. (A) The dose-dependent effect of carbachol at CT6 is illustrated. (B) The mean phase shift \pm *SEM* is shown. This curve could also be fitted by a Michaelis function— $\Delta\phi = x^n/(x^n + t_0^n)$ —with a Hill coefficient of 3.1 and a half saturation value (t_0) of 0.08.

CARBACHOL PHASE-SHIFTS ACTIVITY RHYTHMS

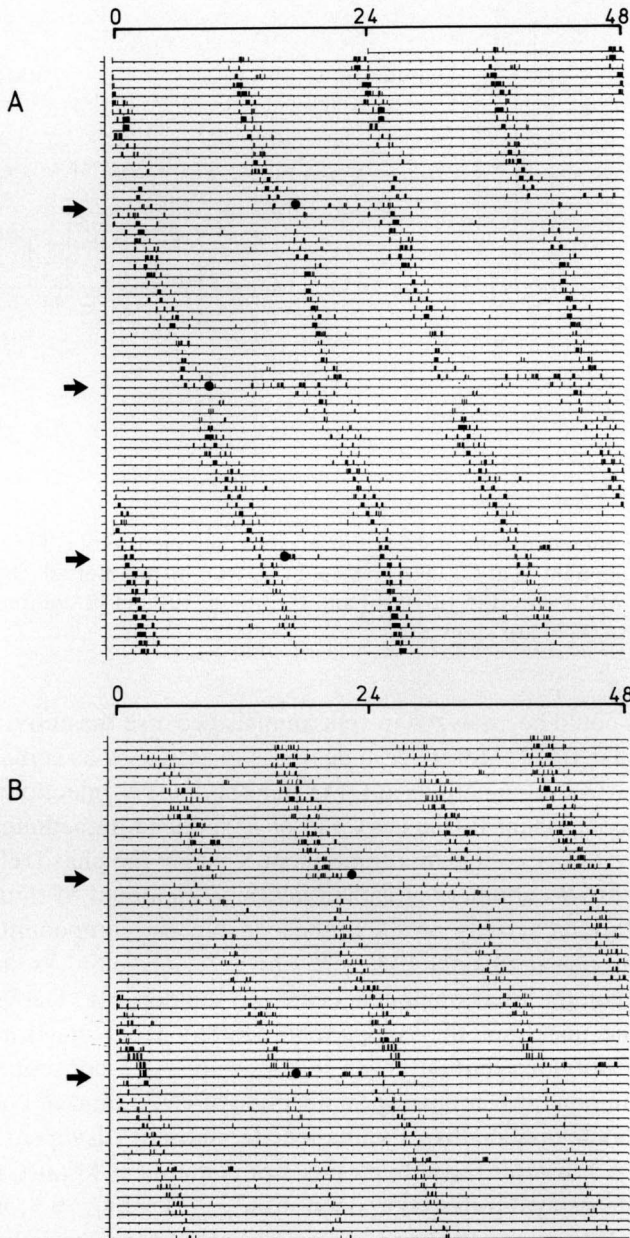


FIGURE 5. Examples of carbachol-induced phase shifts of the circadian activity rhythm for two hamsters housed in LL. The activity onset has been defined as CT12 for both components separately. (A) The first injections at CT14 and at CT2 cause a phase delay and a phase advance, respectively. Although the timing of the second injection is reversed, the response of both components is similar. The third injection represents a control injection with artificial CSF. (B) The response to two carbachol injections is illustrated for another animal: note the lack of activity following an injection at CT14.

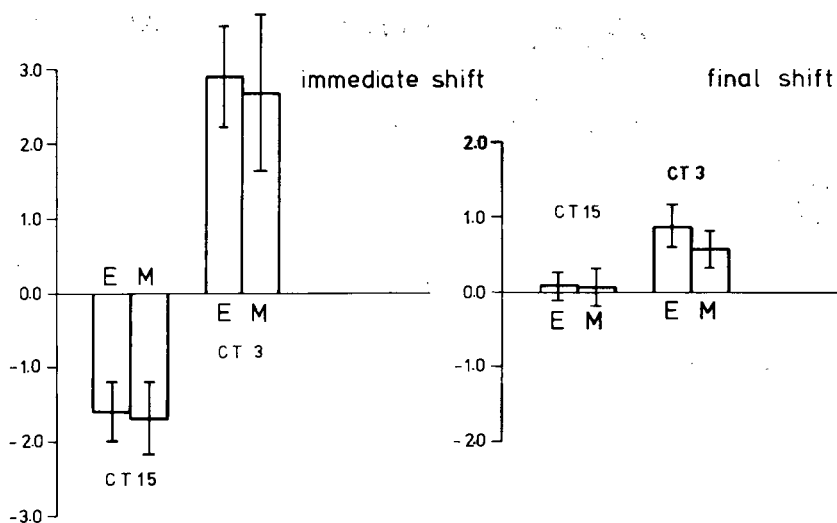


FIGURE 6. (A) The immediate phase shift ($\pm SEM$) for component E at CT15 ($n = 8$) and CT3 ($n = 10$) and of component M at CT15 ($n = 8$) and CT3 ($n = 6$) are plotted. (B) The final shifts ($\pm SEM$) of component E at CT15 ($n = 10$) and CT3 ($n = 10$) and of component M at CT15 ($n = 11$) and CT3 ($n = 6$) are shown.

Not all injections could be analyzed in split animals because the activity onsets were not always clearly defined. Moreover, a decrease in activity was sometimes encountered following carbachol injections at CT15 but not at CT3. Injections 3 hr after the activity onset of component E (thus at CT15 of E) induced immediate delays ($\Delta\phi \pm SEM = -1.6 \text{ hr} \pm 0.4$; $n = 8$) in this component. Usually the phase relation between the two component was about 12 circadian hours. Component M thus received this injection 9 hr before its activity onset. In these instances, component M responded with an immediate phase advance ($\Delta\phi = 2.7 \text{ hr} \pm 1.1$; $n = 6$). As such, the phase relationship between the two components changed temporarily. During the first few days following the injection, the components shifted back, via transients, in the direction of their previous mutual phase relationship. As a consequence, the final phase shifts of component E following an injection at CT15, and of component M at CT3, appeared much smaller than the immediate shifts or disappeared completely (Fig. 6). Injections 3 hr after the activity onset of component M (at CT15 of M) also induced immediate delays in this component ($\Delta\phi = -1.7 \text{ hr} \pm 0.5$; $n = 8$). In this case, component E received the injection mostly at CT3, which induced an immediate phase advance ($\Delta\phi = 2.9 \text{ hr} \pm 0.7$; $n = 10$).

DISCUSSION

A PRC for carbachol injections in the lateral ventricle was obtained for hamsters kept in LL and DD. Phase advances were obtained following carbachol injections during the subjective day, and phase delays were obtained following injections in the early subjective night.

Transients were observed in about half of the delaying and advancing shifts. In some instances, the phase shift on the first few days following an injection exceeded

the steady-state phase shift. These kinds of transients are usually not observed following light pulses although they have been seen in some rare cases (Aschoff, 1965).

In previous experiments, intraventricular carbachol injections have been shown to produce similar shifts as light pulses in the mouse and in the hamster at CT14, 18, and 21 (Zatz and Herkenham, 1981; Earnest and Turek, 1983, 1985). However, carbachol injections at CT2, 4, and 6 induced phase advances, which are commonly not observed following light presentations (Earnest and Turek, 1985). In our series of experiments, phase delays were also encountered following injections at CT14, but phase advances were produced by injections during the subjective day only and not by injections during the late subjective night. Thus, the most important difference between the PRC obtained by Earnest and Turek and our PRC is our lack of phase advances during the late subjective night. Small methodological differences between our study and the prior study (such as the use of artificial CSF instead of saline) are unlikely to account for this difference. It is not impossible, however, that the discrepancy was due to a strain difference between their hamsters, obtained from Lakeview Hamster Colony (Newfield, NJ), and our hamsters (TNO, Zeist, The Netherlands). Since diurnal cycles in choline acetyltransferase activity can differ markedly between two rat lines (Eiermann *et al.*, 1986), such an explanation would not be inconceivable.

Because of the similarity between carbachol-induced phase shifts at CT14 and CT18 and light-induced phase shifts at these circadian times, Zatz and Herkenham (1981) hypothesized that cholinergic receptors in the SCN mediate phase shifting by light. This idea was further substantiated by the observation that intraventricular carbachol injections decrease pineal enzyme activity, resembling the effect of light on the pineal gland (Zatz and Brownstein, 1979). Carbachol also mimics the effects of brief light pulses on gonadal function (Earnest and Turek, 1985). Several other studies are in agreement with the hypothesis that carbachol mediates the light input to the pacemaker. Intraventricular injection of mecamylamine, an anticholinergic drug, blocks the phase-shifting effects of light in the hamster (Keefe *et al.*, 1987). Moreover, photically responsive neurons of the SCN exhibit a similar response to intravenous nicotine as to light, and administration of mecamylamine can eliminate or even reverse the response to light (Miller *et al.*, 1987). Murakami *et al.* (1984) found that the acetylcholine (ACH) concentration in the suprachiasmatic area increases 30 and 60 min after the onset of light. When alpha-bungarotoxin, a putative nicotinic cholinergic antagonist, is injected either in the lateral ventricle or near the suprachiasmatic nucleus, the effects of light on the nocturnal elevation of serotonin *N*-acetyltransferase activity in the rat pineal gland are blocked (Zatz and Brownstein, 1979, 1981; see, however, Miller and Billiar, 1986). Finally, phase delays of pineal enzyme activity are induced by light presentations at CT14. These phase shifts are mimicked by carbachol injections at CT14 (Zatz and Brownstein, 1979).

Although these above-mentioned studies pointed to a role for ACH in mediating phase shifting by light, other studies do not. Depletion of the brain acetylcholine store appears not to interfere with the phase-shifting effect of light (Pauly and Horseman, 1985). When the optic nerve of a hypothalamic slice preparation is stimulated, a response can be recorded in the SCN. Perfusion of the bath with the cholinergic

antagonists atropine, hexamethonium, and curare did not interfere with this response (Shibata *et al.*, 1986). Cahill and Menaker (1987) have shown that SCN responsiveness to optic nerve stimulation is not affected by ACH agents, but it is affected by kynurenic acid, an antagonist of excitatory amino acid transmission. These data, together with the observation that choline acetyltransferase is not present in the optic nerve (Wenthold, 1981) or only in the ventral half of the chiasm (Rao *et al.*, 1987), make it unlikely that ACH functions as a primary transmitter of the RHT.

In our study, it was shown that phase advances were produced by carbachol injections during the subjective day but not at the late subjective night. Because light presentations at CT18 did induce phase advances in our hamsters, our data provide no further evidence for acetylcholine to mediate phase shifting by light. Therefore, the site of action of carbachol remains to be explained. Studies both *in vivo* and *in vitro* have shown a subpopulation of ACH-responsive cells in the suprachiasmatic nucleus. In some of these studies, the proportion of ACH-responsive neurons and the sign of the response was found to be similar to the visual responsiveness of the SCN (Miller *et al.*, 1987). In others, the proportion of the ACH-responsive cells differed markedly from the proportion of visual suprachiasmatic cells (Nishino and Koizumi, 1977). Irrespective of their precise proportion, the existence of ACH-responsive neurons in the SCN suggests that the site of action of intraventricularly injected carbachol is in the SCN. This suggestion is furthermore strengthened by the observation that intraventricular carbachol injections elicit the same response as suprachiasmatic carbachol injections (Zatz and Brownstein, 1979). Thus, carbachol is likely to act on suprachiasmatic pacemaker neurons. Whether carbachol acts through a traditional cholinergic mechanism remains as yet a matter of debate in view of contradictory results on the absence of choline acetyltransferase or ACH receptors in the SCN (Brownstein *et al.*, 1975; Clarke *et al.*, 1985; Ichikawa and Hirata, 1986; Meeker *et al.*, 1986; Miller *et al.*, 1987; Rao *et al.*, 1987; Tago *et al.*, 1987).

The data presented in Figure 4 indicate that carbachol exerts a dose-dependent effect on the circadian pacemaker. Moreover, the data obtained imply that the dosage of carbachol that was used to obtain a PRC was higher than the dose necessary to saturate the phase-shifting effect of carbachol. For lower doses of carbachol, however, the standard deviation increases. This phenomenon has also been described by Anderson and Turek (1985); however, a reduction in the mean response to carbachol was already observed at 0.005 M carbachol by these authors.

We do not know why the standard error increases for lower doses of carbachol. For 0.01 M carbachol, the phase shifts were always greater than 0.7 hr. Dosages smaller than 0.005 M carbachol sometimes induced no phase shift at all while the mean phase shift was still considerable. Probably the amount of carbachol that reaches the SCN following an injection may not be equal for each injection due to diffusion of carbachol to other brain areas. A slight difference in the amount of carbachol that reaches the SCN may have less consequences for the magnitude of the phase shift when the dose is rather high. When the injected dose gets smaller, differences in the amount of carbachol that reaches the SCN result in different magnitudes of phase shift.

Recently, the possibility has been put forward that different neurochemical mechanisms are required to process light information to the pacemaker. This was based on the observation that light-induced phase delays, but not phase advances, are blocked by the γ -aminobutyric acid (GABA) antagonist bicuculline (Ralph and Menaker, 1985). On the other hand, phase advances are blocked by the benzodiazepine, diazepam (Ralph and Menaker, 1986). The similarity between the dose-dependent effects of carbachol at CT6 and at CT14 suggests that a similar neurochemical mechanism may have mediated the carbachol-induced advances and delays.

Phase shifts that were induced by carbachol injections in split animals revealed that both components responded qualitatively similarly to carbachol at CT15 and CT3. Both responded to carbachol with an immediate delay (at CT15) or advance (at CT3) of the free-running activity rhythm. Quantification of the phase shifts was, however, difficult for two reasons. Sometimes the immediate advance of one component and/or the delay of the other was rather large. In those cases, it was difficult to distinguish whether a particular bout of activity resulted from a delay of the previous component or was derived from an advance of the subsequent component (Fig. 5). The immediate phase shift was therefore measured at the following cycle, but this leads to an underestimation of the real shift. Second, a decrease of activity was sometimes observed following an injection (Fig. 5). Decreased wheel-running activity may have increased the estimated phase delay.

As a consequence of the opposite immediate shifts, the phase relation between the components changed. Up to 7 days after the immediate phase shift, the components shifted, often in such a direction as to restore their previous mutual phase relationship of about 12 circadian hours. This behavior of the split circadian activity rhythm was also observed following the presentation of dark pulses (Boulos and Rusak, 1982). Boulos and Rusak attributed the immediate phase shifts to the response of the components to the dark pulse and the transients following the immediate phase shift to coupling forces between the components. A similar interpretation of our data would indicate that carbachol produces a predictable phase shift in either of the two components. Coupling forces between the two oscillators result in a restoration of the previous phase relationship.

Several authors have hypothesized that the two components of the split activity rhythm reflect two underlying oscillators with different properties. The two oscillators of Pittendrigh and Daan's model were thought to be differentially sensitive to light. Since carbachol may not act along the photic input to the pacemaker in the SCN, we do not regard our experiments as a critical test for this particular hypothesis. The two components may be different with respect to their sensitivity to certain transmitters. For instance, Daan *et al.* (1975) suggested that testosterone may selectively affect one component of the multioscillatory system. So far, differences between the two components have not been determined. Split components can be phase advanced as well as phase delayed by dark pulses, and no distinction between the two split components was observed in terms of their responsiveness to light (Boulos and Rusak, 1982; Lees *et al.*, 1983; Boulos and Morin, 1985). The onset of lordosis can be associated with either one of the two components or with both

(Swann and Turek, 1982). We found that both components responded similarly to carbachol injections at CT3 and CT15. Therefore, these data are further evidence for identical responsiveness of the two oscillators to a perturbing stimulus.

ACKNOWLEDGMENT

We thank Dr. S. Daan for critically reading the manuscript and S. Ploeger for his technical assistance. We are also grateful to M. de Rooy for typing the manuscript.

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