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Phase-shifting Mechanisms in the Mammalian Circadian System: New Light on the Carbachol Paradox

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A variety of evidence now suggests that excitatory amino acid receptors mediate the effects of light on the circadian system of mammals. However, the ACh agonist carbachol is the only agent that has been reported to "mimic" the phase-shifting effects of light *in vivo*. Because the other published evidence for the involvement of ACh in light-mediated phase shifts is weak, we have referred to this situation as "the carbachol paradox." In the present study, we found that the administration of NMDA receptor antagonists could prevent carbachol-induced phase shifts of the circadian rhythm of wheel-running activity recorded from the hamster. In addition, we found that carbachol-induced phase shifts, unlike those produced by light, are not accompanied by induction of Fos-like immunoreactivity in the suprachiasmatic nucleus (SCN). Our data are simply explained by the assumption that the intraventricular administration of carbachol causes phase shifts through a pathway distinct from that of light. Alternatively, if carbachol is acting via the light input pathway, then it must do so by a mechanism independent of Fos induction in the SCN. In either case, elucidating the mechanisms by which carbachol acts in the circadian system may provide novel insights into the cellular events by which phase shifts are generated.

[Key words: hamster, circadian rhythm, excitatory amino acids, ACh, CCP (2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid), dizocilpine, carbachol]

Circadian rhythms are endogenously generated oscillations with periods of approximately 24 hr. The physiological system responsible for these rhythms can be modeled in three parts: (1) an input pathway by which environmental signals are transmitted to (2) a pacemaker, which is coupled to the processes it controls by (3) an output pathway. In order to function adaptively, the circadian system must be synchronized (or entrained) with appropriate phase to the 24 hr period of the environment in which the organism lives. The daily light–dark cycle, acting through light-induced advances and delays of the endogenous oscillation, is the dominant environmental signal responsible for this entrainment. A major goal of circadian rhythm research is to understand the cellular mechanisms by which light signals phase shift endogenous circadian oscillators.

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In mammals, the suprachiasmatic nucleus (SCN) functions as the dominant pacemaker of the circadian system. The effects of light on the SCN are mediated by unknown photoreceptors located in the retina (Nelson and Zucker, 1981; Foster et al., 1991) that project to the hypothalamus, at least in part, via a monosynaptic fiber tract known as the retinohypothalamic tract (RHT) (Moore, 1983).

A variety of evidence suggests an excitatory amino acid (EAA) is a transmitter at the RHT/SCN synaptic connection. There is evidence that both the NMDA and non-NMDA receptor subclasses of EAA receptors are involved. Electrophysiological studies using an *in vitro* hypothalamic slice preparation that contains the SCN suggest a role for both receptor types (Shibata et al., 1986; Cahill et al., 1989; Mason and Rusak, 1990; Ito et al., 1991; Kim and Dudek, 1991). For example, Kim and Dudek (1991) found that EPSPs recorded intracellularly in the SCN in response to optic nerve stimulation were blocked by both NMDA and non-NMDA receptor antagonists. Recent anatomical evidence suggests that EAA-immunoreactive fibers innervate the SCN (Moffett et al., 1990; Van den Pol, 1991). Finally, pharmacological studies have implicated both NMDA and non-NMDA receptor types in the photic regulation of the circadian system of rodents (Colwell et al., 1990, 1991; Rea et al., 1991; Takeuchi et al., 1991; Colwell and Menaker, 1992).

It has been suggested that ACh may also play a role in the light-input pathway. The intraventricular administration of the ACh agonist carbachol causes phases shifts that have been reported to mimic the effects of light on the mammalian circadian system (Zatz and Brownstein, 1979; Zatz and Herkenham, 1981; Earnest and Turek, 1983, 1985; Wee et al., 1992; but also see Meijer et al., 1988, for alternative view). Additionally, mecamylamine, an antagonist to the ion channels activated by ACh, has been reported to prevent light-induced phase shifts (Keefe et al., 1987). Further, electrophysiological studies indicate that some SCN neurons show similar responses to light and cholinergic agents (Nishino and Koizumi, 1977; Miller et al., 1987). Finally, the ACh content of the SCN increases after a light pulse (Murakami et al., 1984). Nevertheless, the neuroanatomical site of action and pharmacological specificity of carbachol remain unclear (see Rusak and Bina, 1990, for discussion).

The protein Fos (the product of the c-*fos* gene) is induced in neurons in response to a number of stimuli including EAAs and carbachol (Morgan and Curran, 1991). Recent work has shown that light causes an increase in Fos-like immunoreactivity (Fos-LI) and c-*fos* mRNA in the SCN (Rea, 1989; Aronin et al., 1990; Earnest et al., 1990; Kornhauser et al., 1990; Rusak et al., 1990; Colwell and Foster, 1992). In addition, the induction of Fos in the SCN by light is correlated in both its phase dependence and threshold with the ability of the same light treatment to phase shift the circadian oscillator (Kornhauser et al., 1990). Fur-

thermore, EAA receptor antagonists inhibit the photic induction of Fos in the SCN (Abe et el., 1991, 1992; Colwell and Foster, 1991; Rea et al., 1991) while the administration of the EAA agonist NMDA can cause an induction of Fos in the SCN (Ebling et al., 1991). Although it is not clear whether Fos plays a causal role in light-induced phase shifts of the circadian system, previous work does suggest that Fos-induction can be used as a cellular marker of photic input to the SCN.

To elucidate the effects of carbachol and the possible role of ACh in mediating light-induced phase shifts of the circadian system, we conducted experiments to determine whether the administration of the NMDA receptor antagonists dizocilpine (MK-801) and 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) could prevent carbachol-induced phase shifts of the circadian rhythm of wheel-running activity. We then asked whether intraventricular injections of carbachol caused induction of Fos-LI in the SCN. We reasoned that if ACh acts at any point along the light input pathway to the circadian system then EAA receptor antagonists should prevent carbachol-induced phase shifts and carbachol itself should cause an induction of Fos-LI in the SCN.

Materials and Methods

Animals. Male golden hamsters (*Mesocricetus auratus*, LVG-outbred), obtained from Lakeview, Charles River at 10 weeks of age, were housed individually and their wheel-running activity recorded. The animals were exposed to a 14 hr:10 hr light–dark cycle for 2 weeks. A cannula was then stereotaxically placed into the third ventricle under sodium pentobarbital anesthesia (coordinates: 0.6 mm anterior to bregma, on midline, 8.1 mm ventral). The cannula was fixed to the skull with dental acrylic and three screws. A wire was inserted in the cannula to keep it open, and the hole was protected by a removable cap. The placement of the cannula was later verified histologically.

Two to three weeks after cannulation, the animals were placed in constant dark (DD) for 10 d to assess their free-running activity pattern. Hamsters remained in DD and were subjected to one of four treatments: (1) injection of vehicle alone, (2) injection of carbachol, (3) injection of EAA receptor antagonist alone, (4) injection of EAA receptor antagonist plus carbachol. The carbachol treatments were delivered 8 hr after the onset of activity [i.e., circadian time (CT) 20.0; onset of activity is defined as CT 12 for nocturnal animals] when light would normally induce a phase advance. Animals used for immunocytochemistry were killed 60 min after treatment. Otherwise, following each treatment, the animals were allowed to free-run undisturbed in DD for 10 d before receiving another treatment. No animal was treated more than twice.

Carbachol (an acetylcholine agonist; 0.05 or 0.005 M) and CPP (an NMDA receptor antagonist; 0.1 mM) were solubilized in artificial cerebral spinal fluid and were administered via the cannula at CT 20. Injection volumes were 1.0 μl of solution. Dizocilpine (MK-801; an NMDA receptor antagonist; 4.8 mg/kg) in dimethyl sulfoxide was administered by intraperitoneal injection at CT 19. All drugs were purchased from Research Biochemicals Incorporated, Natick, MA. The light stimulus used to induce Fos-LI was a 15 min pulse of monochromatic light (515 nm) at an intensity of 1.5×10^{-1} μW/cm² at CT 20. The apparatus used to produce the stimulus has been previously described (Foster et al., 1991). The stimulus parameters (duration, irradiance, and wavelength) were chosen to produce submaximal phase shifts. Stimulus intensity (irradiance) was measured before each trial with a radiometer (United Detector Technologies, Hawthorne, CA). Following the light pulse, the hamsters were returned to constant darkness until the time of perfusion. All handling and treatment of animals was carried out in complete darkness with the aid of an infrared viewer (FJW Industries, Elgin, IL).

Analysis of wheel-running behavior. Phase shifts in the activity rhythm were determined by measuring the phase difference between eye-fitted lines connecting the onset of activity for a period of 7 d before and 10 d after an experimental manipulation. In order to estimate the steady state phase shifts produced, 4 d of data after treatments that caused phase advances were excluded from the analysis. In other respects, the method for calculating phase shifts was the same as has been reported elsewhere (Takahashi et al., 1984). The phase-shifting effects of a treatment were considered to be significant when the 95% confidence interval of the group mean did not overlap zero. Differences between treatment groups were evaluated using a Kruskal–Wallis one-way analysis of variance, followed by a Mann–Whitney U test where appropriate. Values were considered significantly different if $P < 0.05$. In the text, values are shown as means ± SEM; and phase advances are shown as positive values while phase delays are shown as negative values.

Tissue preparation and immunocytochemistry. For perfusion, animals were removed from their light-tight boxes and anesthetized with a lethal dose of halothane by inhalation in darkness. Then, under dim red light, the hamsters were perfused intracardially first with 30–40 ml of physiological saline (0.9% w/v) containing 150 IU of heparin/10 ml, followed by 300 ml of 2% w/v formaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.2–7.4) with picric acid (15%). After perfusion, the brains were removed and postfixed overnight at 4°C. Serial frontal sections were cut at 50 μm into PBS using a cryostat.

Free-floating sections were transferred to a solution of normal goat serum (1:30 in PBS with 2% Triton) for 30 min then directly into primary antiserum. The Fos antiserum used in this study was an anti-Fos (4-17) rabbit polyclonal antiserum purchased from Oncogene Science (Uniondale, NY). The antibody was used at a dilution of 1:250 for 72 hr at 4°C. The sites of the antibody:antigen binding were visualized with an avidin–biotin–peroxidase procedure (Elite ABC kit, Vector Labs, Burlingame, CA). Sections were washed in Tris buffer (pH 7.2, 15 min) before incubation for 6 min in 0.025% diaminobenzidine (DAB) containing 0.003% (w/v) peroxide. Sections mounted on gelatin-coated slides were dehydrated through graded alcohols into xylene and left overnight before being rehydrated to distilled water and immersed in 0.2% (w/v) osmium tetroxide solution (2 min) to intensify the DAB reaction. Histological analysis and photomicrographs utilized a Zeiss Axiophot photomicrographic system.

To minimize variability, we used a standard immunocytochemical protocol, which kept incubation times constant, and used a single batch of antisera and other reagents. In addition, we processed hamster tissue for immunocytochemistry in large groups of 10 or 12 brains, which therefore constituted a self-contained experiment. Two types of immunocytochemical controls were performed. Tissue sections were processed for immunocytochemistry; however, the primary antiserum was replaced with normal nonimmune rabbit serum. Sections were also incubated with "preabsorbed" primary antibody. To preabsorb the antibody, 1 μg of peptide (obtained from the suppliers of the antisera) was added to 1 ml of diluted antibody and incubated at 4°C overnight before being added to the brain sections.

Analysis of Fos immunostaining. A series of immunostained sections was counterstained with toluidine blue in order to define Fos immunostaining within the histological borders of the SCN. Noncounterstained sections were observed by phase-contrast microscopy to confirm the distribution of Fos staining within the SCN. Camera lucida drawings of Fos-stained perikarya within the SCN were made. It was possible to identify and count immunostained perikarya accurately.

Results

The intraventricular administration of carbachol (0.05 M) at CT 20 caused a phase advance of 194.4 ± 36.7 min ($N = 9$; range, 45–365 min, with six of nine values > 180 min. Examples of carbachol-induced phase advances are shown in Figure 1, *A* and *D*. All of the phase shifts appeared to be completed by the second cycle after administration of the drug. This same dose of carbachol (0.05 M) administered at CT 18 caused a smaller phase advance (38.6 ± 19.0 min, $N = 7$, two of seven values > 90 min). Neither a lower dose of carbachol (0.005 M) nor saline alone administered at CT 20 produced significant phase shifts (−13.3 ± 22.3 min, $N = 6$; −10.0 ± 5.5 min, $N = 5$; respectively).

The intraperitoneal administration of the NMDA receptor antagonist dizocilpine (4.8 mg/kg) prevented carbachol-induced phase advances in the circadian rhythm of wheel-running activity (Figs. 1*C*, 2). Experimental animals that received an injection of dizocilpine 30 min prior to the administration of

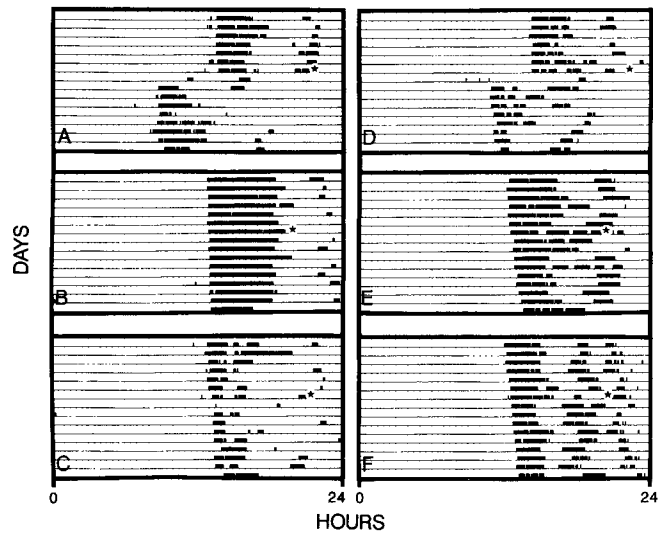

Figure 1. Locomotor activity records from experimental and control animals maintained in DD. Each *horizontal line* represents the activity record for a 24 hr day, and successive days are plotted *from top to bottom*. *Stars* represent the time of light and/or drug treatment. *A* and *D*, Activity records illustrating the phase-advancing effects of an intraventricular injection of carbachol at CT 20. *B*, Activity record illustrating the lack of effect of an injection of dizocilpine (4.8 mg/kg) at CT 19.5 on the phase of the circadian rhythm in locomotor activity. *E*, Activity record illustrating that an injection of CPP (1 μl of 0.1 mM solution) at CT 20 had no effect on the phase of the circadian rhythm of locomotor activity. *C*, Activity record illustrating the blockade of light-induced phase advances by an injection of dizocilpine (4.8 mg/kg) 30 min prior to a carbachol treatment at CT 20. *F*, Activity record illustrating the blockage of carbachol-induced phase advance by the coinjection of CPP (1 μl of 0.1 mM solution) at CT 20.

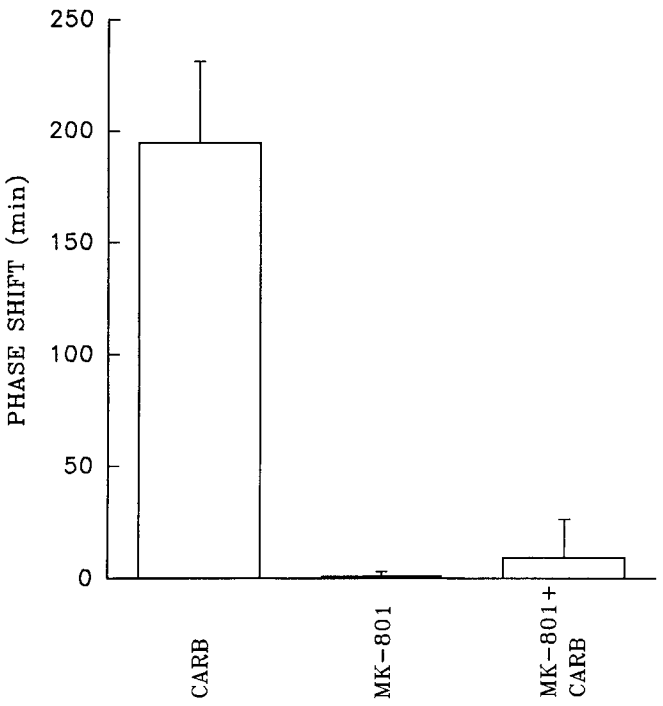

Figure 2. Mean phase shift in the rhythm of locomotor activity of hamsters in DD that received a treatment of either carbachol (0.05 mM), dizocilpine (4.8 mg/kg), or dizocilpine + carbachol. Carbachol treatments were delivered at CT 20, 8 hr after the onset of activity. The vehicle or drug treatments (1 μl vol) were delivered 30 min prior to the carbachol treatment. $N = 6$–8 for all points; error bars represent SEM.

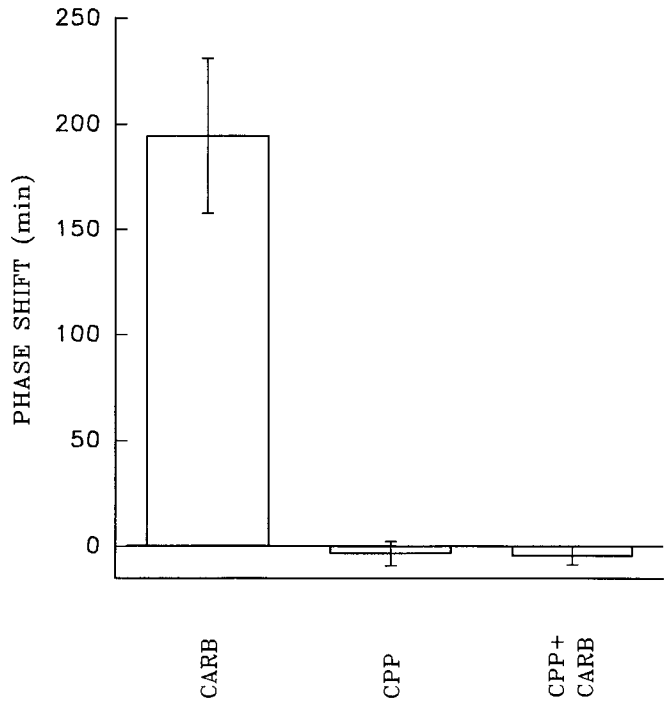

Figure 3. Mean phase shift in the rhythm of locomotor activity in hamsters in DD that received a treatment of either carbachol (0.05 mM), CPP (0.1 mM), or CPP + carbachol. The treatments were delivered 8 hr after the onset of activity at CT 20. For the experimental treatment, CPP and carbachol were coinjected (1 μl vol). $N = 6$–8 for all points; error bars represent SEM.

carbachol did not show a significant phase shift (9.2 ± 17.2 min, $N = 6$). This value was significantly different from that of the group treated with carbachol alone ($P < 0.001$). Examples of activity records from experimental and control animals are shown in Figure 1*A–C*. Control injections of saline or dizocilpine alone did not cause significant phase shifts of the circadian rhythm of locomotor activity (−10.0 ± 5.5 min, $N = 5$; 0.8 ± 2.3 min, $N = 6$, respectively).

The intraventricular administration of the NMDA receptor antagonist CPP (0.1 mM) also prevented carbachol-induced phase advances in the circadian rhythm of wheel-running activity in the hamster (Figs. 1*F*, 3). Control intraventricular injections of CPP alone did not cause significant phase shifts of the circadian rhythm of locomotor activity (−3.3 ± 5.7 min, $N = 6$). Experimental animals that received a coinjection of CPP and carbachol did not show a significant phase shift (−4.3 ± 4.3 min, $N = 7$). This value was significantly different from that of the group treated with carbachol alone ($P < 0.001$). Examples of activity records from experimental and control animals are shown in Figure 1*D–F*.

Unlike light, carbachol did not cause an induction of Fos-LI in the SCN of the hamster (Fig. 4). Figure 5 shows a schematic diagram comparing the pattern of Fos-LI in the SCN of a hamster exposed to a 15 min light pulse at CT 20 with that of a hamster that received an intraventricular injection of carbachol at the same phase. The carbachol injection, which does cause a phase shift in the rhythm of wheel-running activity (Figs. 2, 3), did not cause an induction of Fos-LI in the SCN; the light pulse caused a robust induction of Fos-LI. We also examined Fos-LI in the SCN of animals sampled at various intervals after a carbachol injection at CT 20: 30 min ($N = 3$), 60 min ($N = 6$), 90 min ($N = 3$), and 120 min ($N = 4$). In no case did we find

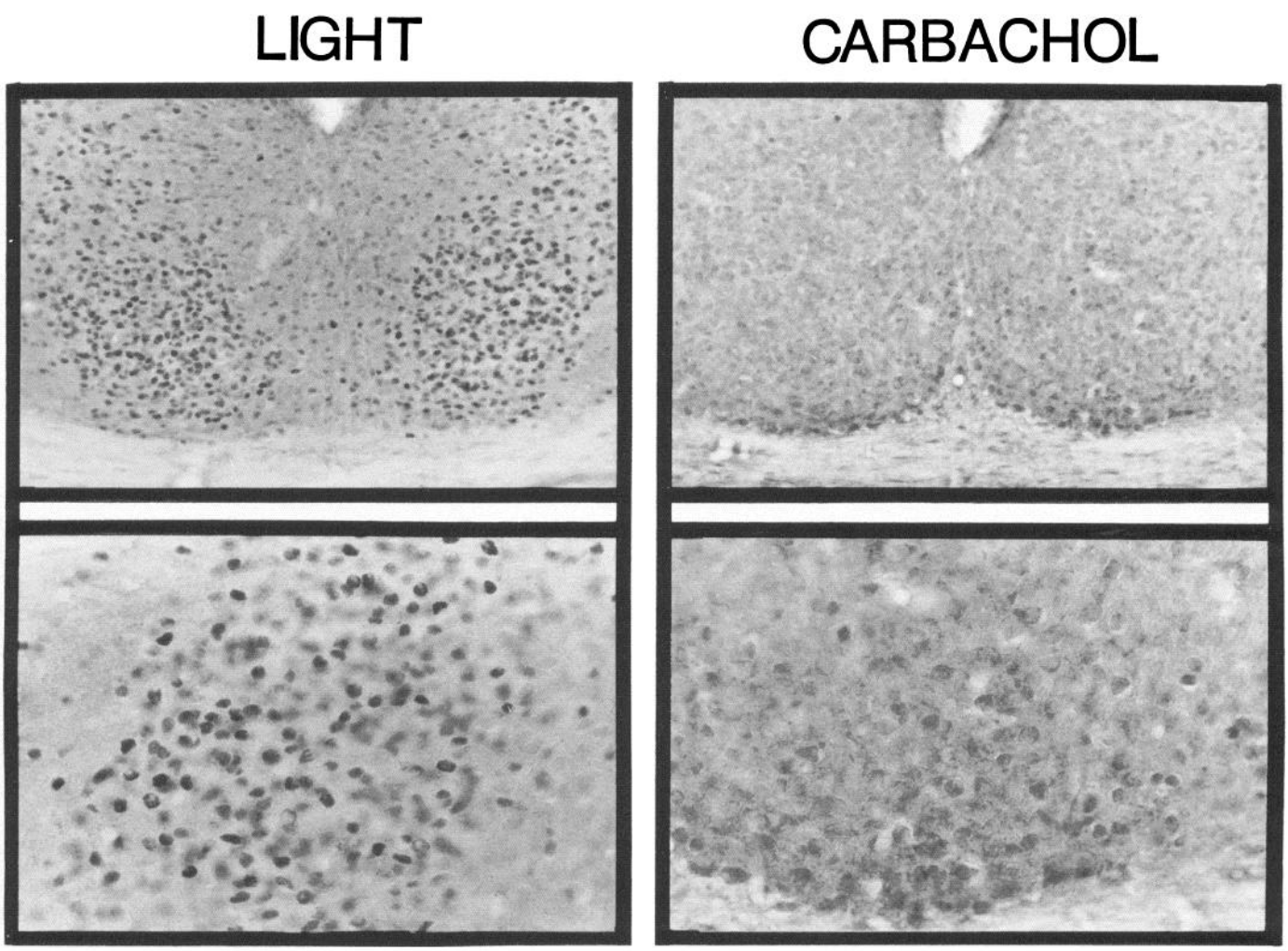

Figure 4. Photomicrographs of coronal sections through the SCN region of hamsters that have been stained for Fos-LI. Hamsters in DD either were treated with an intraventricular injection of carbachol 8 hr after activity onset at CT 20 (*right*) or were exposed to 15 min of light (*left*). Animals were perfused 60 min after the treatment. Magnification: *top*, 200×; *bottom*, 400×.

levels of Fos-LI that were higher than those found in the untreated controls.

Discussion

Carbachol is a nonselective cholinergic agonist that activates both muscarinic and nicotinic ACh receptor subclasses. In mammals, this agonist has been reported to mimic light-induced phase shifts *in vivo* (Zatz and Brownstein, 1979; Zatz and Herkenham, 1981; Earnest and Turek, 1983, 1985; Wee et al., 1992; also see Meijer et al., 1988, for alternative view). Both the intraventricular injection of carbachol and exposure to brief pulses of light cause phase-dependent phase shifts of the circadian system. The phase dependence of these two treatments is roughly similar; both light and carbachol applied during the early subjective night cause phase delays, whereas the same treatments administered during the late subjective night cause phase advances (DeCoursey, 1964; Earnest and Turek, 1985; Meijer et al., 1988). This similarity has led to the suggestion that carbachol acts on the light-input pathway. However, during other phases of the circadian cycle, the effects of intraventricular injection of carbachol are clearly different from those of light. Carbachol injections have consistently been found to cause phase advances at phases during the subjective day at which light pulses are without effect. Thus, although the phase shifts caused by carbachol show some similarities to those produced by light pulses during the subjective night, there are also clear differences.

Earnest and Turek (1985) and Meijer et al. (1988) have obtained different results in the phase shifts generated by carbachol treatment at CT 18. Earnest and Turek found that carbachol caused phase advances at CT 18, while Meijer and coworkers did not. In our hands, carbachol injections at CT 18 resulted in phase shifts of variable amplitude. While some animals clearly responded with phase advances, the overall group mean phase shift caused by this treatment was not significant. However, at CT 20, we found consistent phase advances to carbachol injections. Interestingly, these carbachol-induced phase shifts lacked the transients normally associated with light-induced phase advances. All of the carbachol-induced phase advances were completed by the second cycle after treatment, while completion of light-induced phase advances typically requires four or five cycles. This result is consistent with a difference in the mechanism of action between light and carbachol treatments.

A number of previous studies have shown that light causes induction of Fos protein and message in the SCN (Rea, 1989; Aronin et al., 1990; Earnest et al., 1990; Kornhauser et al., 1990;

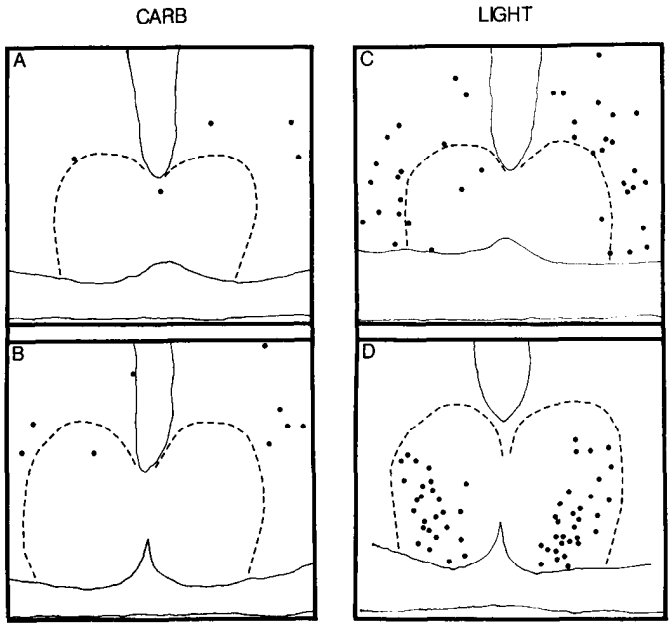

Figure 5. Schematic diagrams illustrating the distribution of Fos-LI through rostral (*A, C*) and (*B, D*) caudal aspects of the hamster SCN. The animal on the right (*LIGHT*) was exposed to 15 min of light at CT 20 while the animal on the left (*CARB*) was treated with an intraventricular injection of carbachol at the same phase. The animals were perfused 60 min after treatment. Immunoreactive perikarya are represented by *solid dots*, and the Nissl-defined SCN is shown with the *dashed line*.

Rusak et al., 1990; Colwell and Foster, 1991). Although the functional role of this Fos induction is not known, it does appear to be a good marker of photic stimulation of the SCN. If carbachol were acting via the light-input pathway to cause phase shifts of the circadian system, one would expect that, like light, it would cause an increase in Fos-LI in the SCN. However, this does not seem to be the case.

Our data demonstrate that it is possible to cause phase shifts of the circadian system without concomitant Fos-induction. A recent study also indicated that a nonphotic stimulus could cause phase shifts during the subjective day without also causing Fos-induction (Mead et al., 1992). The lack of effect of carbachol on Fos-LI is particularly interesting in that, like light, it causes phase shifts during the subjective night. This dissociation suggests that Fos induction may not be generally involved in phase shifting.

The neuroanatomical site of action of carbachol is unknown. The observation that this cholinergic agonist causes phase shifts suggests that it acts within the SCN or at neurons that project to the SCN. Electrophysiological studies indicate that ACh is not a transmitter at the RHT/SCN synaptic connection (Shibata et al., 1986; Cahill and Menaker, 1987). Nevertheless, there is evidence that carbachol does affect the firing rate of SCN neurons (Nishino and Koizumi, 1977; Miller et al., 1982). Anatomical studies have found some evidence for both muscarinic and nicotinic cholinergic receptors being present in the SCN (e.g., van der Zee et al., 1991; but also see Clarke et al., 1985; Rusak and Bina, 1990). However, it is currently unclear whether carbachol acts directly to stimulate cholinoceptive neurons within the SCN or indirectly on neurons that project to the SCN.

The pharmacological similarities that exist between the ion channels activated by nicotinic ACh and NMDA receptors complicate the present study. Previous studies have shown that under certain conditions dizocilpine can antagonize both nicotinic and NMDA receptor channels (Ramoa et al., 1990; Aizenman et al., 1991; Amador and Dani, 1991). However, the receptors themselves are distinct. Competitive receptor antagonists, such as CPP, are highly selective for the NMDA receptor, having little to no effect on other receptors studied (Davies and Watkins, 1982; Lehmann et al, 1987; Childs et al., 1988). The observation that the competitive NMDA receptor antagonist CPP can block carbachol-induced phase shifts makes it unlikely that the actions of the NMDA receptor antagonists could be explained through a nonselective blockade of ACh receptors.

Our results with dizocilpine and CPP demonstrate that NMDA receptor antagonists can block the phase-shifting effects of carbachol. Previous studies have found that these antagonists block light-induced phase shifts and suggest that the RHT/SCN synaptic connection is the likely site of action (Colwell et al., 1990, 1991; Mason and Rusak, 1990; Kim and Dudek, 1991; Colwell and Menaker, 1992). In a simple model of the circadian system, light causes a linear cascade of events resulting in a phase shift of the circadian pacemaker. If carbachol acts via this light-input pathway to cause phase shifts, then our data indicate that it must act at a point in such a cascade that occurs before the step(s) blocked by the EAA receptor antagonists. Alternatively, carbachol-induced phase shifts may be mediated by an entirely different pathway than that which underlies light-induced phase shifts. This is perhaps the simplest explanation for the data presented in this article. It is supported by (1) the difference in phase dependence of the two treatments, (2) the difference in observed transients after treatment, and (3) the observation that carbachol does not cause induction of Fos-LI in the SCN. Alternatively, if carbachol is acting via the light-input pathway, then it must do so by a mechanism independent of Fos induction in the SCN. In either case, elucidating the mechanisms by which carbachol acts in the circadian system may provide novel insights into the cellular mechanisms by which phase shifts are generated.

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