Cholinergic Regulation of the Suprachiasmatic Nucleus Circadian Rhythm via a Muscarinic Mechanism at Night

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In mammals, the suprachiasmatic nucleus (SCN) is responsible for the generation of most circadian rhythms and for their entrainment to environmental cues. Carbachol, an agonist of acetylcholine (ACh), has been shown to shift the phase of circadian rhythms in rodents when injected intracerebroventricularly. However, the site and receptor type mediating this action have been unknown. In the present experiments, we used the hypothalamic brain-slice technique to study the regulation of the SCN circadian rhythm of neuronal firing rate by cholinergic agonists and to identify the receptor subtypes involved. We found that the phase of the oscillation in SCN neuronal activity was reset by a 5 min treatment with a carbachol microdrop (1 μl, 100 μM), but only when applied during the subjective night, with the largest phase shift (+6 hr) elicited during the middle of the subjective night. This effect also was produced by ACh and two muscarinic receptor (mAChR) agonists, muscarine and McN-A-343 (M1-selective), but not by nicotine. Furthermore, the effect of carbachol was blocked by the mAChR antagonist atropine (0.1 μM), but not by two nicotinic antagonists, dihydro-β-erythroidine (10 μM) and d-tubocurarine (10 μM). The M1-selective mAChR antagonist pirenzepine completely blocked the carbachol effect at 1 μM, whereas an M3-selective antagonist, 4,2-(4,4'-dialcetoxyphenylmethyl)pyridine, partially blocked the effect at the same concentration. These results demonstrate that carbachol acts directly on the SCN to reset the phase of its firing rhythm during the subjective night via an M1-like mAChR.

Key words: acetylcholine; carbachol; McN-A-343; circadian rhythm; suprachiasmatic nucleus; muscarinic receptor; pirenzepine; atropine; 4-DAMP; nicotine; dihydro-β-erythroidine; d-tubocurarine

The possibility of a direct regulation of the SCN by cholinergics is suggested by neuroanatomical studies. Both the ACh-degrading enzyme acetylcholinesterase and the biosynthetic enzyme choline acetyltransferase are present in the SCN and its surrounding neuropil (Brownstein et al., 1975; van den Pol and Tsujimoto, 1985; Ichikawa and Hirata, 1986; Rao et al., 1987; Tago et al., 1987). Using monoclonal antibodies against the muscarinic and nicotinic ACh receptors (mAChR, nAChR), both antigens were identified within the rat SCN (van der Zee et al., 1991). More recently, the sites of cholinergic neurons that project to the SCN area have been identified (Bina et al., 1993) and include the basal nuclear complex of the forebrain and the laterodorsal tegmental, pedunculopontine tegmental, and parabigeminal nuclei of the brainstem. Although the functional significance of these pathways remains to be determined, it has been speculated that they are part of a feedback loop between the regulation of sleep/arousal and the circadian timing system (Bina et al., 1993).

The receptor mechanisms underlying the effect of carbachol on circadian rhythms have been uncertain (for review, see Rusak and Bina, 1990). Although nicotinic sites appeared to mediate the light-like influence of carbachol on pineal NAT activity, a role for muscarinic receptors was not ruled out (Zatz and Brownstein, 1981). In a recent study using the brain-slice preparation, bath-applied nicotine, but not carbachol, was reported to phase-advance the SCN neuronal activity rhythm (Trachsel et al., 1995). A majority of SCN neurons were excited by iontophoresis of ACh (Nishino and Koizumi, 1977) or by systemic injection of cholinergic agonists (Miller et al., 1987) in rats. However, the effects of ACh on the activity of the SCN neurons recorded in vitro were predominantly inhibitory (Shibata et al., 1983; Kow and Pfaff, 1984).
Animals and brain-slice preparation. Eight-week-old male and female rats were used in this study. Animals were maintained and tissue-prepared in full accord with institutional and federal guidelines for the humane treatment of animals. Animals were killed during the "lights-on" period of the 24 hr cycle. This was necessary to avoid phase-shifting effects, which have been shown to occur with manipulations at night (Gillette, 1986). The animals were decapitated, and the brain was dissected quickly from the skull. The brain then was sectioned manually to form a block of tissue containing the hypothalamic region. This block of tissue was transferred to a mechanical tissue chopper on which 500 μm coronal slices were made.

The hypothalamic slices containing the SCN were transferred to the brain slice dish, in which they were maintained for up to 3 d. The brain-slice dish was modeled after Hatton et al. (1980). The dish consisted of an inner chamber and an outer chamber. The outer chamber was filled with d-H2O warmed to 37°C and bubbled continuously with 95% O2:5% CO2. The slice in the inner chamber was perfused at 35 ml/hr with warmed, oxygenated Earle's balanced salt solution (in mM) 1.8 CaCl2, 5.3 KCl, 0.8 MgSO4, 117 NaCl and 1 NaH2PO4·H2O; Gibco, Gaithersburg, MD supplemented with 24.6 mM glucose, 26.3 mM sodium bicarbonate, and 0.005% gentamycin, pH 7.3–7.4.

Extracellular electrical recording and data analysis. Under these constant conditions of incubation in a brain slice, the SCN generates a near 24 hr oscillation in firing activity of the neuronal ensemble (Green and Gillette, 1982). The unperturbed sinusoidal pattern of ensemble activity is predictably high during the subjective day, peaking mid-day near circadian time 7 (CT 7, or 7 hr after “lights on” in the 24 hr lighting schedule in the rat colony), and low during subjective night (Gillette and Prosser, 1988), so that measurement of the time-of-peak can be used as an accurate assessment of the phase of the oscillation (Gillette et al., 1995).

The details of this sampling method and validation of this technique for determining the phase of the SCN circadian rhythm of ensemble neuronal firing rate have been published previously (Prosser and Gillette, 1989). Briefly, extracellular recordings from single cells were made using glass microelectrodes filled with 5 M NaCl. An electrode was lowered into the SCN using a hydraulic microdrive until the signal from a single cell was encountered. Electrical signals exceeding twice the level of the background noise were isolated using a window discriminator, observed for stability over at least 2 min, and sent to a computer for counting. The firing rate of each cell was monitored over two consecutive 2 min periods, and the values were averaged to determine the mean firing frequency of that cell at that circadian time. The electrode then was repositioned arbitrarily within the SCN until another cell was encountered. An attempt was made to sample all areas of the nucleus. In each experiment, 40–70 cells were recorded over a period of 8–12 hr.

The firing rates of individual SCN neurons recorded during each experiment were grouped into 2 hr bins based on the circadian time at which they were counted. Successive bins were offset by 15 min to provide a running average. This procedure smooths the measurements made on individual neurons, which show a range of activities, and permits better resolution of the circadian time-of-peak in the activity rhythm of the neuronal ensemble. The time-of-peak then was determined by visual inspection of a graph of these values for the symmetrically highest point.

To simplify the illustrations, only the 2 hr mean offset by 1 hr are presented in Figures 1, 3, 5, and 6. The phase shift was determined by comparing the time-of-peak electrical activity in drug-treated slices with that of media- and vehicle-treated control slices. The effect on the time-of-peak of a single treatment on a single SCN, the individual neuronal activities of which were monitored for 8–12 hr, constituted a single n for each experiment presented here. Differences between groups were evaluated using Student's unpaired t test. Because we did not observe any difference between male and female rats, the data from male and female rats were combined.

Experimental treatments. Slices were allowed to equilibrate for at least 2 hr in the recording chamber before any chemical treatment. Cholinergic agonists and antagonists were dissolved in normal incubation medium. Before application, the test solution was adjusted to pH 7.4 and warmed to 37°C and bubbled with 95% O2:5% CO2. At the circadian time selected for evaluation, perfusion was stopped, a 1 μl drop of medium containing carbachol, ACh, or other cholinergic agonist was applied to each SCN for 5 min and then washed away toward the optic chiasm, after which slice perfusion with normal medium was resumed. In the experiment using the antagonists, the normal bathing medium was replaced in <2 min with warmed, oxygenated medium, pH 7.4, containing the inhibitory substance. After 10 min, a drop of control medium or medium containing carbachol was applied as stated above. Five minutes later, the surface of the slice was rinsed with medium containing the antagonist and incubated for an additional 15 min. The bath treatment medium then was exchanged completely for normal medium and perfusion was resumed. Electrical activity then was monitored during the subsequent 1 or 2 circadian cycles to determine the time of the peak in neuronal firing (see data analysis). Carbachol was from Sigma (St. Louis, MO); other agonists and antagonists were from Research Biochemicals (Natick, MA).

RESULTS

Carbachol applied to the SCN resets the neuronal firing rhythm during subjective night

The sensitivity of the isolated pacemaker to cholinergic resetting of its phase was examined by applying microdrops (100 μm, 1 μl) of the cholinergic agonist carbachol directly to each of the paired SCN in a hypothalamic brain slice for 5 min. The rhythm of firing rate was examined the next day. When a microdrop containing 100 μm carbachol in medium was applied in the middle of the subjective day at CT 6 on day 1, the subsequent peak in electrical activity occurred at CT 6.3 ± 0.1 (n = 3) on day 2. An example of the rhythm of one SCN treated at CT 6 appears in Figure 1A. This time-of-peak is not significantly different from media-treated controls (CT 6.8 ± 0.1, p > 0.05; n = 5). In contrast, when SCN were exposed to an identical treatment during mid-subjective night at CT 18 on day 1, the pattern of firing rate in the next cycle (day 2) was altered. Peak activity no longer occurred near CT 7, the normal peak time, or the time in media-treated control; rather, the peak occurred early (Fig. 1B), at CT 0.7 ± 0.2 (n = 3) the next day. Compared with the time-of-peak on day 2 of media-treated control experiments (CT 6.8 ± 0.1, n = 5), this represents a mean advance of the phase (Δt) of 6.1 hr (p < 0.0001). To determine whether these phase advances were stable, we monitored the electrical activity rhythm for a second cycle after treatment (day 3 in vitro). As shown in Figure 1C, the time of peak firing rate was again at CT 1.0 in the second cycle after treatment of carbachol at CT 18.

It is possible that carbachol diffused away from the site of application and the effect measured was attributable to its action on a structure in the hypothalamic slice outside the SCN. Therefore, we used a reduced slice preparation that contained only SCN, the underlying optic chiasm, and a small rim of hypothalamic tissue. The time of peak activity recorded from reduced slices was unchanged from that of the normal slice preparation (Gillette and Reppert, 1987). When applied as a microdrop to
Figure 1. Phase-dependent effects of carbachol on the circadian rhythm of electrical activity. A, Carbachol treatment (100 μM in 1 μl droplet) at CT 6 on day 1 in vitro had little effect on the time-of-peak in neuronal activity on day 2 (peak at CT 6.25). B, The same carbachol treatment at CT 18 on day 1 shifted the electrical activity rhythm so that the time-of-peak activity on the second day in vitro occurred at CT 1.2, which is 5.6 hr earlier than normal. C, Electrical activity recorded in a separate slice treated at CT 18 on day 1 peaked at CT 1.0 on the third day. The time of the shifted peak in activity was consistent across experiments (Δt = +6.3 ± 0.2 hr; n = 3). The 2 hr means ± SEM of all single units recorded (solid circles) are plotted for each 2 hr interval offset by 1 hr to produce a running average that better discriminates the time-of-peak activity. Small open circles in A (omitted in B and C) represent the average firing rate of each neuron sampled. This permits visualization of the data on which 2 hr means are based. Horizontal bars indicate subjective night (CT 12-24), the time of treatment. Dashed lines indicate the mean time-of-peak activity in control slices (CT 6.8 ± 0.1; n = 5).

The effect of carbachol is dose-dependent and mimicked by ACh and by muscarinic agonists, but not by nicotine

Dose–response relationships were determined at CT 18, the time of maximal phase shift to carbachol. Examination of the dose–response relationship for carbachol with the 5 min microdrop treatment shows that the half-maximal response occurred near 1 μM, whereas the maximal response (+6.3 hr) occurred at 10 μM. The neurotransmitter ACh induced a similar phase advance (+5.3 hr) at 0.1 μM. Effects of two muscarinic agonists, muscarine and the M1-selective McN-A-343, also were tested at a range of concentrations. They both induced a phase advance with the largest phase advances (+6.1 ± 0.3 hr; n = 6) occurring when carbachol was applied between CT 17 and CT 19.

However, the SCN circadian rhythms were reset significantly by carbachol throughout subjective night: the largest phase changes (+6.1 ± 0.3 hr; n = 6) occurred when carbachol was applied between CT 17 and CT 19.
A circadian rhythm with different cholinergic agonists applied at CT 18 on the time-of-peak in the SCN electrical activity rhythm. Results of three individual experiments are plotted as the running 2 hr mean firing rates versus circadian time. Both 100 μM muscarine (A) and 10 μM M1-selective agonist McN-A-343 (B) induced phase advances of ~6 hr such that the peak on day 2 occurred at CT 1. Nicotine (100 μM; C) caused a 1 hr phase advance. Symbols are as in Figure 1.

The activation of nAChR is not required in the phase-shifting effect induced by carbachol.

The effect of carbachol is blocked differentially by muscarinic antagonists

To demonstrate further that carbachol acts via an mAChR to induce phase shifts and to explore the mAChR subtypes involved, we used three mAChR antagonists: atropine (nonselective), pirenzepine (M1-selective), and 4,2-(4,4'-diacetoxydiphenylmethyl)pyridine (4-DAMP, M3-selective). When applied in the bath at the concentration of 0.1 μM during the period surrounding carbachol treatment, atropine blocked the phase-advancing effect of carbachol (100 μM) at CT 18 (+1.1 ± 0.3 hr, n = 3; Figs. 6, 7). Atropine alone did not have an effect on the phase of the SCN rhythm at concentrations up to 1 μM. The M1-selective antagonist pirenzepine blocked the phase-advancing effect of carbachol (100 μM) at CT 18 at a concentration of 1 μM (+0.67 ± 0.08 hr, n = 3; Figs. 6, 7), and less effectively at 0.1 μM (+3.9 ± 0.1 hr; n = 3). 4-DAMP, which is M3-selective, partially blocked the effect of carbachol at a concentration of 1 μM (+4.1 ± 0.1 hr, n = 3; Fig. 7). The relative potency of the muscarinic antagonists in blocking the effect of carbachol is as follows: atropine > pirenzepine > 4-DAMP.

DISCUSSION

By evaluating the direct action of carbachol on the circadian rhythm of SCN neuronal activity, we have demonstrated that the SCN is itself sensitive to cholinergic phase resetting in a noctur-
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Finally, restricted pattern and via an mAChR mechanism. The specific findings of our experiments were as follows. (1) The SCN circadian rhythm was phase-advanced up to 6 hr by direct application of the cholinergic agonist carbachol to the SCN in the brain slice, and sensitivity to carbachol was circadian phase-dependent, occurring only during the subjective night. (2) This change in sensitivity is driven by endogenous clock mechanisms because it occurred in the isolated SCN in vitro. (3) The phase-shifting effect of carbachol was reproduced by ACh and two muscarinic agonists, muscarine and McN-A-343 (M1-selective), but not by nicotine. (4) The nAChR antagonist DHP (10 μM) did not affect the carbachol-induced phase advance. (5) Both the muscarinic antagonist atropine (0.1 μM) and the selective antagonist pirenzepine (1 μM) blocked the carbachol phase-shifting effect, whereas the M3-selective antagonist 4-DAMP (1 μM) partially blocked the effect.

Site of action

The site at which carbachol affects circadian rhythms has resisted identification in whole-animal studies. The observation that intracerebroventricular injection of this cholinergic agonist causes phase shifts has suggested that it acts within the SCN or at neurons that project to the SCN. In the present study, we demonstrated that carbachol can induce the same phase shift in the reduced slice preparation (limited to the SCN region) as it does in the hypothalamic slice. Therefore, carbachol must act directly on the SCN or in its immediate vicinity to induce the phase shift of the SCN neuronal activity rhythm. Although we cannot exclude the possibility that carbachol acted on optic nerve terminals that remain in this reduced preparation, the most likely site of action is on SCN neurons, which express both muscarinic and nicotinic receptors (van der Zee et al., 1991).

Our study of the response of the SCN in vitro does not rule out the possibility that, in addition to acting directly on SCN neurons, carbachol injected intracerebroventricularly in vivo acts indirectly at different brain sites on neurons that project to the SCN to exert its effect on the behavioral circadian rhythms. Although early studies showed a light-like effect of intracerebroventricularly injected carbachol on wheel-running activity (Zatz and Herkenham, 1981), this was not repeated by others. Meijer et al. (1988) reported only phase advances, whereas Wee et al. (1997) observed much smaller amplitude of phase delays (1.2 hr) induced by carbachol compared with phase advances (4.3 hr). It is likely that the phase-delaying effect of carbachol seen in vivo is attributable to interaction with the retinohypothalamic tract (RHT) glutamatic regulation...
The muscarinic antagonists atropine (0.1 μM), pirenzepine (PZP, M1-selective; 0.1 and 1.0 μM), and 4-DAMP (M3-selective, 1.0 μM) were tested for their ability to block the carbachol-induced phase shift. Treatments and concentrations (in μM) are labeled above each bar, and the number of replications (n = 3 for each) is noted within each bar. Plotted are the means ± SEM.

**Figure 7. Relative potency of muscarinic antagonists in blocking the effect of carbachol at CT 18.** Microdrop applications of 100 μM carbachol (CARB) for > 30 min induced a 6.5 ± 0.2 hr phase advance at CT 18 (n = 3).Muscarinic antagonists atropine (0.1 μM), pirenzepine (PZP, M1-selective; 0.1 and 1.0 μM), and 4-DAMP (M3-selective, 1.0 μM) were tested for their ability to block the carbachol-induced phase shift. Treatments and concentrations (in μM) are labeled above each bar, and the number of replications (n = 3 for each) is noted within each bar. Plotted are the means ± SEM.

Phase-dependent sensitivity and relationship to light pulses *in vivo*

Although the ACh agonist carbachol is the only agent that has been reported to “mimic” the phase-shifting effects of light when injected *in vivo* (Zatz and Brownstein, 1979; Zatz and Herkenham, 1981; Earnest and Turek, 1983, 1985; Wee et al., 1992), the other evidence for the involvement of ACh in light-induced phase shifts is weak. This has been referred to as “the carbachol paradox” by Colwell et al. (1993). Our results reveal that the SCN clock is sensitive to carbachol only during the subjective night of the circadian cycle. This timing of SCN sensitivity to carbachol is mostly consistent with results from *in vivo* experiments. However, the patterns of response in the two conditions differ. The response to intracerebroventricular injection of carbachol *in vivo* can be biphasic, with phase delays in wheel-running rhythms during early night and phase advances during late night. We did not observe a phase delay *in vivo*, which suggests that a phase delay induced by carbachol *in vitro* is not attributable to the direct effect of carbachol on the SCN. It has been shown that cholinergic fibers in the SCN region originate from cholinergic neurons in the cholinergic basal nuclear complex and in the mesopontine tegmentum. It has been speculated that these pathways provide feedback regulation of circadian timing relative to the state of sleep and wakefulness.
Bina et al. (1993). Therefore, direct stimulation of these areas in vivo would be predicted to cause phase shifts in behavioral circadian rhythms with patterns of sensitivity like the SCN sensitivity to carbachol.

Although cholinergic stimulation of the SCN in vivo does not generate the biphasic phase shifts induced by light, glutamate, the putative neurotransmitter in the retina, does reproduce this effect. Glutamate applied in a similar paradigm to carbachol in the present study induced phase delays early and advances late in the subjective night in vivo (Ding et al., 1994). The pattern is very similar to the light pulse-induced changes in behavioral rhythm in rats (Summers et al., 1984). Because the sensitive periods of the SCN to phase-shifting by carbachol and glutamate are completely overlapping, and the effect of carbachol in vivo was blocked by an NMDA-receptor antagonist (Colwell et al., 1993), it is likely that an interaction exists between the cholinergic and glutamatergic systems within the SCN.

Such an interaction between ACh and glutamate may occur at the level of intracellular and/or intercellular signaling pathways involving nitric oxide (NO). NO donors mimic and NO synthase inhibitors block the effect of glutamate on SCN rhythms (Ding et al., 1994). This suggests the involvement of soluble guanylyl cyclase, which is activated by NO, and of the cGMP/PKG-dependent protein kinase (PKG) system in glutamate-induced phase shifts. Interestingly, the phase-response curves for 8-bromo-cGMP, an analog of cGMP, and for carbachol overlap in terms of sensitivity and arc of the same amplitude (Prosser et al., 1990; Liu and Gillette, 1994). Furthermore, a specific inhibitor of PKG blocked the effect of 8-bromo-cGMP and the effect of carbachol (Liu and Gillette, 1994). This evidence points to the convergence of cholinergic and glutamatergic neurotransmissions at the cGMP–PKG signaling pathway in regulating the SCN response. However, glutamate must act via additional pathways: glutamate induces both delays and advances in the SCN circadian rhythm, whereas carbachol and cGMP analogs induce only advances, and these advances are significantly larger in amplitude than those of glutamate. Our results provide a basis for future study of the interaction between glutamatergic and cholinergic systems at the SCN and may lead to a resolution of the carbachol paradox.

REFERENCES


