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New light on an old paradox: Site-dependent effects of carbachol on circadian rhythms

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Abstract

Acetylcholine (ACh) was the first neurotransmitter identified as a regulator of mammalian circadian rhythms. When injected in vivo, cholinergics induced biphasic clock resetting at night, similar to nocturnal light exposure. However, the retinohypothalamic tract connecting the eye to the suprachiasmatic nucleus (SCN) uses glutamate (GLU) to transmit light signals. We here resolve this long-standing paradox. Whereas injection of the cholinergic agonist, carbachol, into the mouse ventricular system in vivo induced light-like effects, direct application to the SCN in vitro or in vivo induced a distinct response pattern: phase advance of circadian rhythms throughout the nighttime. These results indicate that a new regulatory pathway, involving an extra-SCN cholinergic synapse accessible via ventricular injection, mediates the light-like cholinergic clock resetting reported previously.

Keywords: Acetylcholine; Carbachol; Circadian clock; Glutamate; Mouse; Suprachiasmatic nucleus

Introduction

The circadian clock in the hypothalamic suprachiasmatic nucleus (SCN) is sensitive to regulation by multiple neurochemical systems (Gillette and Mitchell, 2002; Watts, 1991), including glutamatergic projections from the retina (Ebling, 1996; Moore and Lenn, 1972) and cholinergic innervation from the brain stem and basal forebrain (Bina et al., 1993). These various pathways are hypothesized to communicate time-of-day information from the day–night cycle as well as brain and body systems in order to adaptively synchronize the SCN and, thus, the internal milieu (van Esseveldt et al., 2000). Whereas glutamate relays information concerning environmental light (Ebling, 1996), the role of cholinergic influence has been unknown. The nocturnal sensitivity and biphasic effects of cholinergics injected at a range of peripheral and central sites made ACh the prime candidate mediator of the effects of light on circadian rhythms (Bina and Rusak, 1996; Earnest and Turek, 1983; Mistlberger and Rusak, 1986; Wee et al., 1992; Zatz and Brownstein, 1979; Zatz and Herkenham, 1981). Furthermore, the anticholinergic agent, mecamylamine, was able to block the effects of light when injected intracerebroventricularly (Keefe et al., 1987; Zhang et al., 1993). However, there has been no direct evidence that ACh communicates the light signal from the retina to the SCN. When applied directly to the SCN isolated in a brain slice, cholinergic stimulation during subjective night did not cause the light-like response, but rather induced only phase advances (Liu and Gillette, 1996). Importantly, optic nerve stimulation causes release of GLU, which also has been localized within terminals of the

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retinal ganglion cells that invest the SCN (Hannibal, 2002). Numerous studies, both in vitro and in vivo, have demonstrated that glutamate, working through the *N*-methyl-D-aspartate receptor, is the primary messenger of light to the SCN (Colwell et al., 1991, 1993; Ding et al., 1994; Mintz et al., 1999). The question then remains, if ACh is not the mediator of light signals, why do cholinergics induce light-like effects when injected in vivo at non-SCN sites?

Here, we test the hypothesis that effects of extra-SCN injections are not through direct cholinergic action on the SCN, but rather via an alternative anatomical pathway that indirectly stimulates the light/GLU response in the SCN. We assessed both the pattern of temporal sensitivity to cholinergics within the context of the near 24-h circadian cycle and the direction of the response, phase advance or delay. First, we characterized the effect of carbachol applied in vitro to the mouse SCN isolated in a hypothalamic brain slice by examining the phasing of the spontaneous neural activity rhythm. Second, we tested the response to carbachol injected directly into the SCN in vivo; effects on phasing of mouse wheel-running activity rhythms were evaluated. Third, we examined responses in phasing of this behavioral rhythm to carbachol injected into the ventricular system of the mouse, as has been done previously in hamster and rat. We found that in all cases, sensitivity to carbachol was limited to subjective night. Direct SCN stimulation, whether in vitro or in vivo, caused phase advance of circadian rhythms, whereas extra-SCN application of carbachol generated biphasic responses, like those of light. This identifies two distinct sites of cholinergic action that can differentially alter the phasing of the circadian clock.

Materials and methods

Animals and circadian time

Male B6129PF1/J mice at approximately 4 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). The B6129PF1/J mice are first generation progeny from crosses between C57BL/6J females and 129P3/J males. Mice were placed individually into cages (27 × 21 × 14 cm) equipped with running-wheels (12 cm diameter). Wheels were locked for the first day, to prevent novelty-induced phase shifts, which can delay entrainment (Bobrzynska and Mrosovsky, 1998). Two cages were housed within a circadian activity monitoring system (CAMS). Initially, mice were entrained in a 12-h light (100 lx):12-h dark (LD) cycle. After 10–14 days in LD, they were released into constant darkness (DD). Thereafter, the time of activity-onset was designated as circadian time (CT) 12, according to convention. CT for subsequent behavioral and brain slice experiments was reckoned from this point. Units of the circadian cycle were corrected for the free-running period, *tau*, which is <24 h in mouse. All experiments were conducted under protocols approved by the UIUC Institutional Animal Care and Use Committee of the Vice Chancellor for Research, and under continuous supervision of the campus veterinarian staff.

Preparation and treatment of brain slices

To prepare brain slices, mice were euthanized by decapitation with a guillotine no later than 2 h before subjective night. The brain was quickly removed, blocked, and sliced with a mechanical chopper into 1 cm^2 by 500-μm-thick coronal sections containing the SCN. The slice was maintained in a brain slice chamber at 33–34°C and perifused with Earle's Balanced Salt Solution (EBSS; Sigma, St. Louis) supplemented with 24.6 mM glucose, 26.2 mM bicarbonate, and 2.5 mg/L gentamycin, pH 7.4, and bubbled with 95%O_2/5%CO_2.

At the time of treatment, pumps were disabled and the media level lowered slightly. A 0.5-μl drop of either vehicle (supplemented EBSS) or 100 μM carbachol in EBSS was applied to the surface of each SCN. After 10 min, the surface of the brain slice was rinsed with EBSS and perifusion resumed. Effect of treatment vs. control on the time of peak in spontaneous activity of SCN neurons was assessed (Ding et al., 1994).

Single-unit recordings of SCN neuronal activity

Spontaneous activity of SCN neurons was recorded extracellularly by glass microelectrode, as described previously (Ding et al., 1994). After sampling the activity over 4 min, the electrode was advanced by micromanipulator and the next neuron encountered was sampled. Care was taken to record through the normal peak time: Recording began at least 3 h prior to predicted peak and continued for at least 3 h past the peak. Two-hour running averages of firing rate data with a 15-min sliding window were calculated and plotted on the same axes as the single cell averages to determine the circadian time of peak activity of the population of SCN neurons. The time of peak activity in the circadian rhythm of neural activity is a reliable phase marker in this system (Prosser and Gillette, 1989).

Cannula implantation

A guide cannula (26 ga; extending 3.2 mm below the pedestal; Plastics One, Inc., Roanoke, VA) was stereotaxically implanted under sodium pentobarbital anesthesia (80 mg/kg) with post-surgical anesthesia (buprenorphine 0.05 mg/kg) using a stereotaxic apparatus (Stoelting; Wood Dale, IL). Coordinates were determined experimentally with the aid of a mouse brain atlas (Franklin and Paxinos, 1997). For implantation within one SCN (*i*SCN), surgical coordinates from bregma were −0.3 mm

AP, −0.1 mm ML, and −1.8 mm DV (from the surface of dura). For cannula implantation into the third cerebroventricle (icv), surgical coordinates from bregma were −0.5 mm AP, 0.0 mm ML, and −2.1 mm DV. Each cannula was secured with small machine screws inserted into the skull and cranioplastic cement (Plastics One, Inc., Roanoke, VA). Each mouse was returned to its CAMS and allowed to recover for 7–10 days.

Injection of test substances

Upon entrainment to LD, mice were released to DD. Following establishment of a well-defined, free-running rhythm for at least 10 days, drug treatments were performed under dim (<1 lx) red light. A 1-μl Hamilton syringe (Hamilton Co., Reno, NV) fitted with 1.5-cm polyethylene tubing and a 33-ga infusion cannula (Plastics One, Inc., Roanoke, VA) was employed to administer either saline (0.9% NaCl) or 50 μM carbachol in saline (carbamylcholine chloride; Sigma, St. Louis) in a 0.3 μl volume through the guide cannula and into the SCN at the appropriate CT. Injection cannulae were designed to extend 3.4 mm beyond the tip of the guide cannulae, situating the tip of the injector inside of the dorsal SCN border. To ensure cannula patency, a stylet fashioned from 33-ga stainless steel hypodermic tubing (Small Parts, Inc., Miami Lakes, FL) remained in the guide until injection time. The stylet was replaced after the injection. Injection sites were verified histologically and data only from those animals in which the tip of the injection cannula penetrated the dorsal border of the SCN were used for analyses of *intra*-SCN injections.

Data analysis

Circadian rhythms of behavior were assessed by monitoring wheel running of individually-housed mice. Each wheel revolution moved an attached neodinium magnet (Magnetic Energies, Inc., San Antonio, TX) past a hermetically-sealed reed switch (Hermetic Switch, Inc., Chickasha, OK), completing a circuit. Circuit closure, signifying a wheel revolution, was conveyed to a computer equipped with Clocklab Acquisition software (Actimetrics, Inc., Evanston, IL), which plotted the resulting rhythm in an actogram. Clocklab Analysis software was also used to calculate the period, *tau* (τ), of the animal's free-running circadian rhythm and the onset of activity within a circadian cycle. *Tau* was determined from the slope of the regression line drawn through the activity onsets for at least 5 consecutive days. Using CT 12 as the reference point, τ was then used to calculate CT of treatment. Phase shifts were quantified by measuring the distance between eye-fitted lines drawn through activity onsets for 5 days immediately prior to treatment and 5 days post-treatment, following establishment of a stable free-running rhythm. Measurements were made on the day following the treatment. The phase of activity onset post-injection was determined "blind," without knowledge of the treatment condition of that animal.

Statistics

Data were subjected to statistical testing using analysis of variance (ANOVA) with Tukey post hoc test or Student's *t* test, as appropriate.

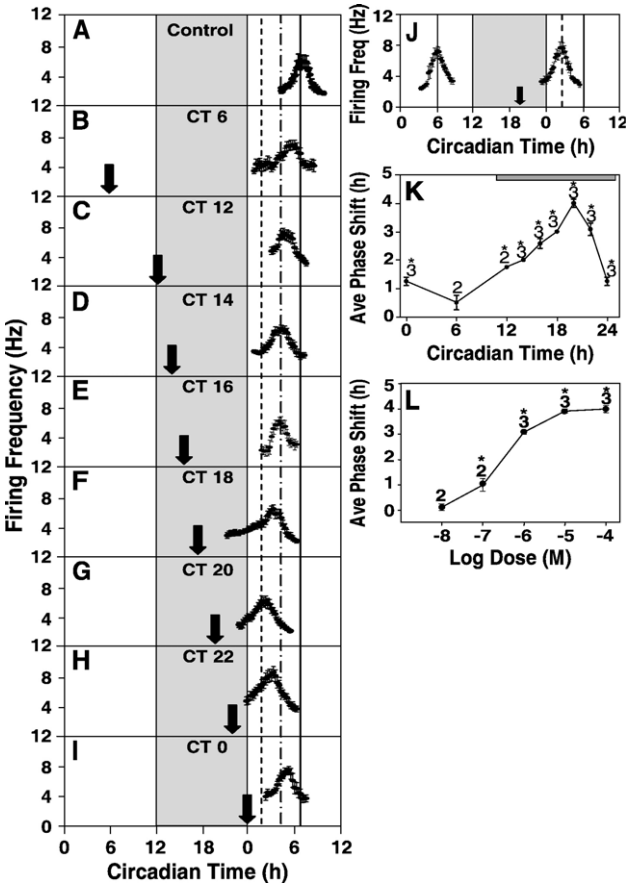

Fig. 1. Carbachol, applied directly to the SCN in vitro, induces shifts in circadian rhythms of SCN neuronal firing that are phase- and dose-dependent. Carbachol sensitivity was studied by monitoring spontaneous activity of the SCN neuronal ensemble in coronal brain slices from the B6129PF1/J mouse. (A–I) Electrophysiological records of peak activity after saline vs. carbachol (100 μM, 0.5 μl, 10 min) treatment at various times across the circadian cycle. Closed circles are 2-h averages ± SEM with a 15-min sliding window. Arrows indicate treatment time. Shaded area represents subjective night. Solid vertical line indicates time of peak neural activity in control (CT 7); broken vertical lines indicate time of peak following treatment at CT 14 (CT 4 – – – –) and 20 (CT 2 – · – · –), respectively. (J) Tandem recording from one SCN slice shows the unperturbed peak on day 1 (CT 6) and, on day 2 in vitro, the early peak (CT 2.5, +3.5 h) after a 10-min carbachol application at CT 20 on day 1. Symbols are as above. (K) Phase–response relationship relating the clock phase (circadian time, CT) of carbachol treatment to the mean response measured as time of the peak in the neural activity rhythm. Numbers are sample sizes; *$P < 0.001$, ANOVA with Tukey post hoc test. Horizontal bar represents subjective night. (L) Dose–response curve to carbachol applied by microdrop to the SCN in vitro at CT 20 (concentration, 0.5 μl drop). Symbols as in K.

Results

Direct cholinergic stimulation of the SCN in vitro causes phase- and dose-dependent phase advance of circadian rhythms of neuronal activity

To evaluate the response of the isolated mouse SCN to direct cholinergic stimulation, we examined the relationship between the CT at which carbachol was applied and the effect on phasing of the spontaneous rhythm of neuronal activity in brain slices. In unperturbed brain slices from the B6129PF1/J mouse, the spontaneous firing rate of the SCN neural ensemble peaked at CT 6.25 ± 0.25 h (n = 3). The average maximum firing rate at peak time was 10.21 ± 1.47 Hz (n = 3). In response to brief exposure of the SCN to carbachol in vitro, carbachol application resulted in an advance of the phase of this intrinsic rhythm at all points tested during subjective night (CT 12, 14, 16, 18, 20, 22, 24/0); exposure during subjective daytime at CT 6 had no significant effect on phasing (Figs. 1A–K). The maximal

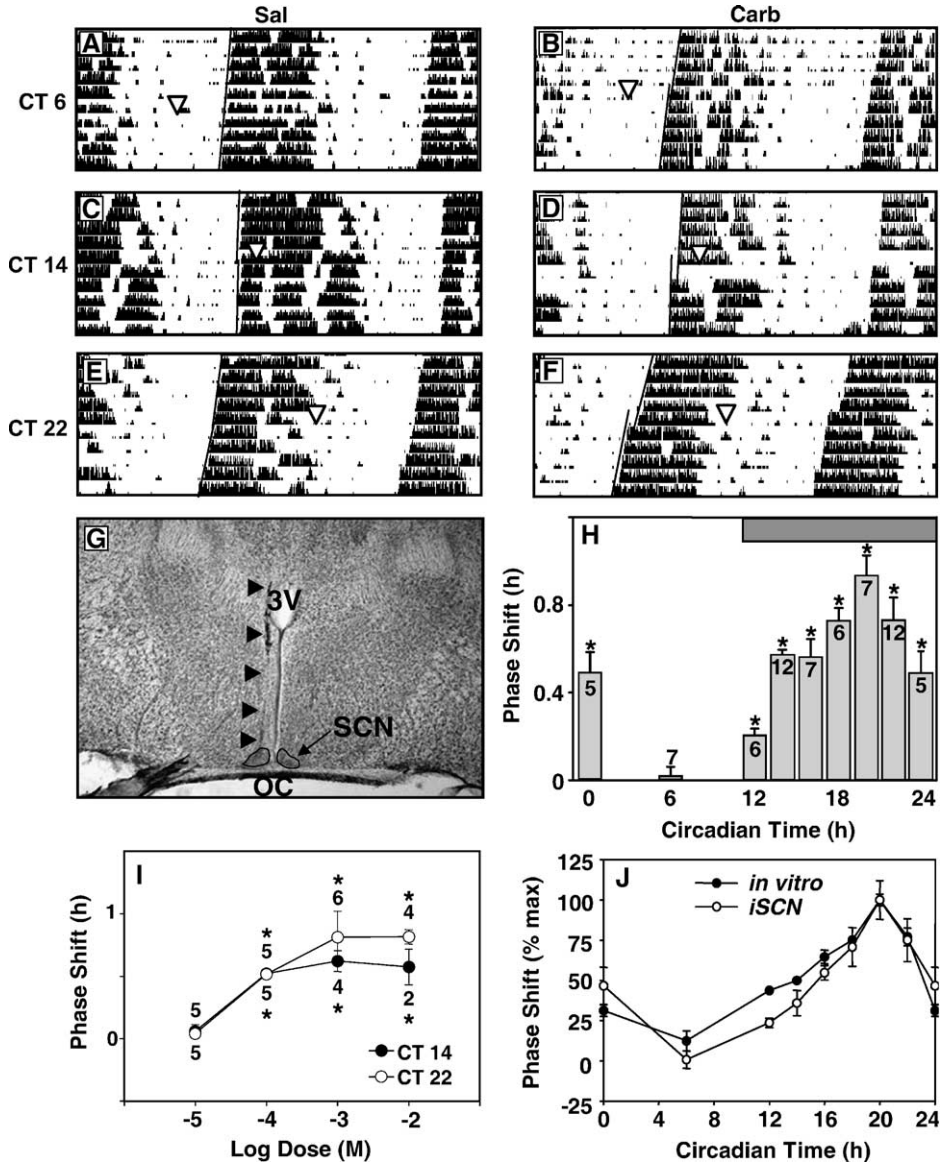

Fig. 2. *Intra-SCN* (*iSCN*) carbachol injection in vivo during subjective night phase-advances circadian rhythms of mouse wheel-running activity in a phase- and dose-dependent manner. (A–F) Actograms of wheel-running activity before and after *iSCN* injection of saline (A, C, E) or carbachol (B, D, F) at CT 6, 14, and 22. Animals were free-running in constant darkness during the records. Each horizontal line represents 48 h of wheel-running data. This double plot, with the second 24 h of each line replotted as the first 24 h of the following line, facilitates visualization of activity patterns. Inverted open triangle indicates treatment time. A line is drawn through the 5-day activity onsets, pre- and post-treatment to aid in visualizing phase changes. (G) Nissl-stained coronal section showing *iSCN* cannula placement, with the tip penetrating the dorsal SCN. 3V: third ventricle; OC: optic chiasm. Arrowheads indicate cannula tract. (H) Phase–response relationship for carbachol (50 μM, 0.3 μl) injected *iSCN* vs. effect on behavioral rhythms. Error bars are ±SEM. Numbers are sample sizes; *P < 0.001, ANOVA with Tukey post hoc test. Horizontal bar represents subjective night. (I) Dose–response curves to carbachol at CT 14 (○) and 22 (●). Symbols as in H; *significance compared to 10 μM carbachol. (J) Normalized line graphs demonstrate that the relative phase response profiles are similar when carbachol is applied in vitro (●) and *iSCN* in vivo (○).

phase advance (4.00 ± 0.14 h, $n = 3$) occurred in response to treatment at CT 20 (Fig. 1K). The stimulus–response relationship was dose-dependent and linear over three orders of magnitude (Fig. 1L). The amplitude of the response plateaued with microdrops containing 10^{-5} M carbachol; at each condition, the effective concentration may be 10- to 100-fold lower due to dilution upon application of the microdrop to the brain slice.

During subjective night, direct cholinergic stimulation of the SCN in vivo causes phase advance of behavioral rhythms

To assess the effect of direct cholinergic stimulation of the SCN in vivo, we injected carbachol through a cannula implanted so that the tip projected through the dorsal border of one SCN (*iSCN*, Fig. 2G), and examined effects on time of onset of wheel-running behavior, the conventional phase marker for locomotor activity rhythms, under DD conditions (Figs. 2A–F). This form of direct cholinergic stimulation of the SCN resulted in phase advance of subsequent cycles when applied during the subjective night, but not daytime. Phase shifts following injection at the various CT tested were significant except for CT 6 ($P < 0.001$, ANOVA with Tukey post hoc) (Fig. 2H). The shape of this phase–response curve for direct carbachol stimulation of the SCN in vivo, generated by plotting the relative effect on behavioral rhythms against clock phase of carbachol stimulation via injection *iSCN*, was not significantly different from that elicited via application in vitro (ANOVA, Fig. 2J). In both conditions, the maximum effect occurred following carbachol treatment at CT 20.

Mean amplitude of carbachol-stimulated phase advance was smaller in vivo than in vitro; the maximal change was +0.93 ± 0.09 h ($n = 7$, Fig. 2H). This was the largest amplitude shift that was elicited by this cholinergic applied *iSCN*. It was generated by injections of $\geq 10^{-4}$ M carbachol, whereas treatments of 10^{-5} M had no effect on rhythms (Fig. 2I). Amplitude of the phase advance induced by injection of a bolus ranging from 10^{-4} to 10^{-2} M carbachol was greater at CT 22 than CT 14, indicating that absolute sensitivity changes with circadian time. While carbachol injection during subjective night had profound effects on the phasing of subsequent rhythms, it had no effect on the endogenous period, τ, of the B6129PF1/J mouse compared with τ pre-treatment (ANOVA, $n = 179$).

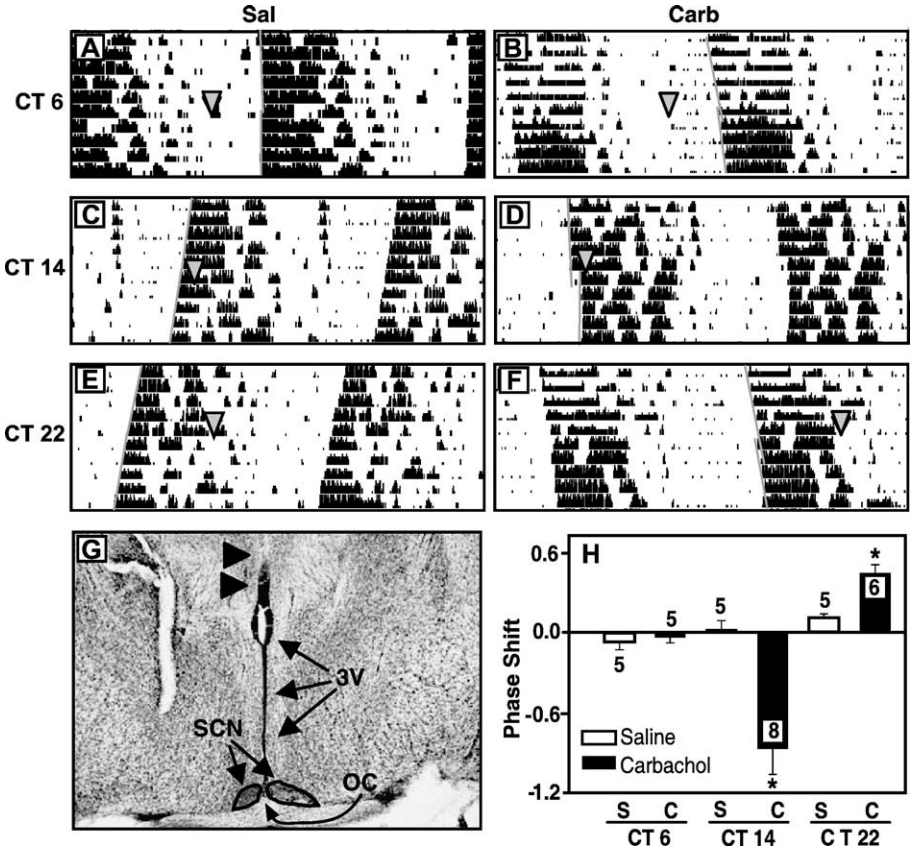

Fig. 3. Icv injection of carbachol in vivo resets behavioral rhythms with a light-like pattern: no change in subjective day, phase delay in early night, and phase advance in late night. (A–F) Actograms of wheel-running after icv injection of saline (A, C, E) or carbachol (B, D, F) at CT 6, 14, or 22 permit comparisons of effects on phase. (G) Nissl-stained coronal section of mouse hypothalamus showing icv cannula placement. (H) Phase–response relationships for saline vs. carbachol (50 μM, 0.3 μl) injected icv and effects on phasing of onset of wheel-running activity rhythms. Symbols as in Fig. 2.

During subjective night, indirect cholinergic stimulation of the SCN in vivo causes biphasic, light-like effects on behavioral rhythms

To investigate whether the site of cholinergic stimulation in vivo could affect the circadian behavioral outcome, mice were cannulated into the third ventricle (icv) (Fig. 3G). When carbachol was injected via the icv cannula, nocturnal stimulation elicited a biphasic response in altering phasing of wheel-running rhythms. As with direct SCN stimulation, icv carbachol injection midday at CT 6 had no effect on subsequent rhythms (-0.04 ± 0.04 h, $n = 5$, Fig. 3). However, stimulation during subjective night had differential effects such that in early night at CT 14 it caused phase delay (-0.88 ± 0.19 h, $n = 5$), whereas in late night at CT 22, it induced phase advance (0.45 ± 0.06, $n = 6$; Fig. 3). The temporal sensitivity as well as the shape and amplitude of this phase–response relationship is comparable to that produced by light stimulation in this strain of mice (Buchanan, 2003), as well as the C57BL/6J and 129P3/J parental mouse strains (Buchanan, 2003; Schwartz and Zimmerman, 1990; Tischkau et al., 2003).

Discussion

The notable finding of the present study is that the site of cholinergic stimulation has profound consequences for the phasing of circadian rhythms. During subjective night, direct application of carbachol to the mouse SCN, either in vitro or in vivo, results in phase advance of circadian rhythms, as does carbachol application to the rat SCN brain slice (Liu and Gillette, 1996). However, when injected into the third ventricle or dorsal to the SCN of hamster (Bina and Rusak, 1996; Wee et al., 1992), or mouse (this study), nocturnal carbachol elicits a biphasic phase–response. This stimulus–response relationship is characteristic of a nocturnal pulse of light to the eyes or glutamate to the SCN (DeCoursey, 1964; Ding et al., 1994; Ebling, 1996).

Comparison of the responses to direct cholinergic stimulation of the SCN in vivo with those in vitro in the brain slice revealed fundamental similarities in the temporal domain of SCN sensitivity and directionality of the response, even though quantitative differences in sensitivity and in the amplitude of the response were observed. Application of $\geq 10^{-5}$ M carbachol in vitro induced a larger maximal shift ($+4.00 \pm 0.14$ h) than in vivo (0.93 ± 0.09 h). Furthermore, responsiveness in vitro takes place over a broader range of concentrations. Nevertheless, complete overlap of the relative phase–response curves (Fig. 2J) indicates that clock-controlled timing of SCN sensitivity and response characteristics in vitro are the same as in vivo. This suggests that the increased amplitude of the shift in clock phase in vitro compared to in vivo may reflect loss of damping by inputs from extrinsic circuits removed during surgical preparation of the brain slice. However, the temporal sensitivity and directionality of the shift must be governed by factors intrinsic to the SCN.

The early observations on cholinergic sensitivity of the circadian system have remained enigmatic since the initial prediction that they communicate light information to the clock failed to be proven. Indeed, these data comprise the 'carbachol paradox': carbachol induces light-like responses when injected systemically or centrally, within the ventricles or hypothalamus, yet there is no evidence that acetylcholine is the neural signal from the eye to the SCN (Colwell et al., 1993). Glutamate and pituitary adenylyate cyclase-activating peptide (PACAP) colocalize in terminals of the melanopsin-bearing retinal ganglion cells (Hannibal et al., 2000) that innervate the SCN and fulfill most criteria for neural messengers of light from the eye to the SCN circadian clock (Berson et al., 2002; Chen et al., 1999; Ding et al., 1994; Ebling, 1996; Hattar et al., 2002; Mintz et al., 1999; Provencio et al., 1998).

A common feature of the earlier studies is that cholinergic agents were not administered directly to the SCN. Effects attributed to cholinergic involvement in the light signal were observed after carbachol injection via icv in hamster, rat and mouse (Bina and Rusak, 1996; Earnest and Turek, 1983; Mistlberger and Rusak, 1986; Zatz and Brownstein, 1979; Zatz and Herkenham, 1981) or into the medial hypothalamus of hamster, dorsal to the SCN (Wee et al., 1992). Generally, it has been inferred that cholinergics introduced at these sites act within the SCN to alter phasing of circadian rhythms. However, lack of correlation between the distance of the carbachol injection site from the SCN (up to 600 μm) and the amplitude of the resulting phase shift in locomotory rhythms was interpreted as carbachol acting at a non-SCN site to induce phase shifts (Wee et al., 1992). Indeed, effects of dorsomedial hypothalamic injections were not significantly different from icv injections of carbachol into the lateral ventricle of hamsters in that study, causing biphasic changes like nocturnal light. It is noteworthy that our results in mouse subjected to icv injection of carbachol into the third ventricle are so similar to the earlier findings with injection at non-SCN sites in both rat and hamster, despite differences in vehicle, concentration and species. All extra-SCN cholinergic stimulation has resulted in biphasic, light-like phase shifts, suggesting a fundamental similarity in the relation of this extra-SCN cholinoceptive site to the SCN.

Our results also extend evidence of the cholinoceptive nature of the SCN itself. Previously, report of direct cholinoceptive responsiveness was limited to observations on the rat SCN brain slice. In this paradigm, responses to bath-applied nicotine have been reported to produce modest phase advances across the circadian cycle (Trachsel et al., 1995). Under conditions where glutamate stimulates light-like responses in the rat brain slice (Ding et al., 1994), large amplitude phase advances in vitro were elicited upon brief nocturnal cholinergic stimulation localized by microdrop to the SCN (Liu and Gillette, 1996). At the time of peak

responsiveness, acetylcholine (0.01–0.1 μM) and a range of muscarinics were effective even at relatively low doses (0.01–10 μM), while nicotine was effective only at a relatively high concentration (100 μM) (Artinian et al., 2001; Liu et al., 1997). Cholinergic projections from basal forebrain and brain stem to the SCN have been reported (Bina et al., 1993). While the full complement of SCN cholinergic receptors has not been established, there is evidence that the rat SCN expresses an α7-based nicotinic receptor (Trachsel et al., 1995; van der Zee et al., 1991) and several types of muscarinic cholinergic receptors (Gillette et al., 2001; van der Zee et al., 1999). The functional context of these cholinergic pathways is as yet unknown.

The distinct responses elicited by direct vs. indirect cholinergic stimulation of the SCN suggest that an extra-SCN cholinergic site is stimulated by systemic, icv, or intramedial hypothalamic carbachol injection. Because this effect differs strikingly from that induced when carbachol is applied directly to the SCN, we infer that this extra-SCN site may then access the circadian clock via a non-cholinergic signal. This site may lie within the hypothalamus in close proximity to the cerebral ventricles, enabling activation by icv as well as intrahypothalamic cholinergic injection. Possible extra-SCN sites include the retina and/or fibers of the retinohypothalamic tract. Cholinergic receptors are expressed on optic nerve fibers (Zhang et al., 2004), and the optic nerve is covered by dura matter so carbachol injected into the ventricles would readily be communicated there via cerebrospinal fluid in the subarachnoid space. The extra-SCN site is predicted to express cholinergic receptors, receive cholinergic input that stimulates these receptors, and send relay projections to the SCN. Neurotransmitter release from these terminals would be predicted to impinge upon the photo-transduction system within the SCN, either by activating key glutamatergic receptors directly, or by stimulating glutamate release from terminals of the retino-hypothalamic tract. Indeed, the effects of icv carbachol are blocked by antagonists of NMDA receptors, key mediators of the light signaling pathway (Colwell et al., 1991, 1993; Ding et al., 1994).

Retrograde labeling using cholera toxin B and pseudorabies virus has demonstrated the presence of juxta-ventricular sites that project to the SCN (Krout et al., 2002). Such SCN afferents arise from the peri-ventricular and arcuate nuclei. Other peri-ventricular regions that project to the SCN include the median preoptic nuclei and medial preoptic area, as well as the dorsomedial and ventromedial hypothalamic nuclei (Krout et al., 2002). Of these, the median preoptic nucleus contributes to sleep–wake cycle regulation (Aston-Jones et al., 2001) and the dorsomedial hypothalamic nucleus is intermediary in the arousal pathway from the locus caeruleus to the SCN (Aston-Jones et al., 2001). Little is known about whether or how these loci modulate circadian rhythms, or the neurochemical nature of synapses they make at the SCN.

The central cholinergic system is a powerful regulator of sleep–arousal states. Furthermore, day–night patterning of sleep and arousal are under tight control of the circadian clock (Borbely, 1982). Brain regions that regulate behavioral states comprising sleep and arousal are likely to feed back upon the circadian system that times them (Abbott et al., 2003; Deboer et al., 2003). This feedback could be communicated through direct cholinergic innervation of the SCN, or via the extra-SCN cholinergic circuit we here predict. It is significant that there may be feed-forward into the glutamatergic phototransduction pathway, as no other intrinsic activator of this system has been described. Understanding the contexts in which both the direct and indirect cholinergic circuits act on the SCN will provide fundamental insights to integrative brain functions that regulate circadian clock timing in response to internal changes of brain state.

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