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Carbon Monoxide and Nitric Oxide: Interacting Messengers in Muscarinic Signaling to the Brain's Circadian Clock

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Within the central nervous system, acetylcholine (ACh) functions as a state-dependent modulator at a range of sites, but its signaling mechanisms are yet unclear. Cholinergic projections from the brain stem and basal forebrain innervate the suprachiasmatic nucleus (SCN), the master circadian clock in mammals, and cholinergic stimuli adjust clock timing. Cholinergic effects on clock state require muscarinic receptor-mediated activation of guanylyl cyclase and cGMP synthesis, although the effect is indirect. Here we evaluate the roles of carbon monoxide (CO) and nitric oxide (NO), major activators of cGMP synthesis. Both heme oxygenase 2 (HO-2) and neuronal nitric oxide synthase (nNOS), enzymes that synthesize CO and NO, respectively, are expressed in rat SCN, with HO-2 localized to the central core of the SCN, whereas nNOS is a punctate plexus. Hemin, an activator of HO-2, but not the NO donor, SNAP, mimicked cholinergic effects on circadian timing. Selective inhibitors of HO fully blocked cholinergic clock resetting, whereas NOS inhibition partially attenuated this effect. Hemoglobin, an extracellular scavenger of both NO and CO, blocked cholinergic stimulation of cGMP synthesis, whereas L-NAME, a specific inhibitor of NOS, had no effect on cholinergic stimulation of cGMP, but decreased the cGMP basal level. We conclude that basal NO production generates cGMP tone that primes the clock for cholinergic signaling, whereas HO/CO transmit muscarinic receptor activation to the cGMP-signaling pathway that modulates clock state. In light of the recently reported inhibitory interaction between HO-2/CO and amyloid-β, a marker of Alzheimer's disease (AD), we speculate that HO-2/CO signaling may be a defective component of cholinergic neurotransmission in the pathophysiology of AD, whose manifestations include disintegration of circadian timing.

INTRODUCTION

Acetylcholine (ACh)[4] is a prominent modulatory neurotransmitter in the brain, controlling selective attention, learning-memory, REM sleep, and arousal. Impairment of cholinergic signals has severe consequences. Cholinergic markers are reduced postmortem in the cerebral cortex of people diagnosed with senile dementia and Alzheimer's disease (AD). This pathology correlates with impairment of learning, remembering, and patterning of circadian rhythms, including severe sleep/wake pattern disturbances, and has led to the cholinergic hypothesis of geriatric neurodegenerative diseases (9). Indeed, acetylcholinesterase inhibitors, which increase synaptic ACh level, are a widely used pharmacotherapy for AD. A role for the cholinergic system in sleep and arousal is well known, but it remains obscure how ACh modifies the biological clock that synchronizes sleep/wake cycles with circadian rhythms in the range of physiological functions.

Cholinergic regulation of the circadian clock originates in the sleep–arousal system of basal forebrain and brain-stem nuclei, whose temporal patterns are themselves under suprachrasmatic nucleus (SCN) control (2, 3). Although ACh was the first neurotransmitter demonstrated to mediate a state change in the SCN, underlying mechanisms are only presently emerging (6, 21, 25, 35). Clock sensitivity to muscarinic agonists *in vivo* and in a brain slice preparation appears at night (2, 21). This process requires activation

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[4] Abbreviations used: SCN, suprachiasmatic nucleus; CT, circadian time; CCh, carbachol; Hb, hemoglobin; SnPP, tin protoporphyrin; CrMP, chromium mesoporphyrin; HO-2, heme oxygenase 2; CO, carbon monoxide; NOS, nitric oxide synthase; NO, nitric oxide; sGC, soluble guanylyl cyclase; PKG, protein kinase G.

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of an M_1-like muscarinic ACh receptor (M_1•R) (21), a soluble guanylyl cyclase-(sGC) and cGMP/protein kinase G (PKG) signaling (20). M_1•Rs are coupled to G proteins that activate phospholipase C, which activates an $InsP_3$/diacylglycerol (DAG)-protein kinase C (PKC) cascade. Thus, the M_1•R does not engage sGC directly. NO, a membrane-permeable product of NOS, is the most ubiquitous activator of sGC. NOS/NO mediates glutamate/NMDA•R signals bearing light information from the eye to the SCN (7).

CO, another gaseous paracrine modulator, has emerged as an activator of sGC alternative to NO (28). CO is a product of degradation of heme by heme oxygenase (HO) (27). HO can be expressed as three homologous isozymes, an inducible form, type 1 (HO-1), and constitutively expressed forms type 2 (HO-2) and type 3 (23). Under physiologic conditions, HO-2 is the major form expressed in brain, with discrete neuronal localization (33). PKC has been implicated in the phosphorylation and activation of HO-2 in several systems, including cerebral cortical cell cultures (28). M_1•Rs activate the G_q-PLCβ-DAG signaling pathway, which can activate PKC. This raises the possibility that HO-2/CO could participate in M_1•R-mediated resetting of the SCN clock. We tested this hypothesis by evaluating whether NOS/NO and/or HO-2/CO are necessary and/or sufficient signaling components of the cholinergic activation of the cGMP pathway and consequent phase advance.

METHODS

Brain Slice Electrophysiology

All experiments with the animals were performed in accordance with institutional and federal guidelines for the humane treatment of animals. Six- to eight-week-old Long–Evans rats from our inbred colony were maintained in 12 h:12 h light/dark cycles. SCN-containing coronal slices were prepared during the subjective day. For single unit recording, 500-μm slices were placed into the brain slice chamber (16), treated on the first day, and evaluated on the second day, as previously described (21). The chamber was constantly perfused with warm (37°C), oxygenated Earle's balanced salt solution (EBSS, GIBCO), supplemented to 24.6 mM glucose, 26.3 mM $NaHCO_3$, and 0.005% gentamicin, and bubbled with 95%O_2/5%CO_2, final pH 7.25.

The SCN brain slice preparation preserves clock properties, including endogenous timekeeping and gating of sensitivity for resetting stimuli, that are observed *in vivo* (12). Circadian clock time in this preparation is expressed as a stable, near 24-h oscillation in the spontaneous neuronal firing rate. In the constant conditions, peak activity appears predictably in the middle of the subjective day, circadian time 7 (CT 7, 7 h after "lights on," which is zeitgeber time 0, ZT 0, in the rat's entraining cycle). The time-of-peak can be used as a reliable marker of clock phase (13).

To study the effects of agonists and antagonists on clock resetting, SCN slices were prepared during the day and treated with drugs at CT 18 of the first cycle *in vitro*. Agonists were applied as a microdrop to the surface of the SCN and allowed to diffuse into the slice during treatment with the perfusion pumps off, after which the surface was rinsed with EBSS and perfusion was resumed. Antagonists were applied by exchange with the medium in the bath; at the end of the treatment, the bath was replaced with EBSS and pumping was resumed. Reagents used were *S*-nitroso-*N*-acetylpenicillamine (SNAP), carbachol (CCh), L-NAME, hemoglobin, hemin (Sigma), metalloporphyrins (Porphyrin Products, Logan, UT). Water-soluble chemicals were dissolved in the oxygenated EBSS. Hemoglobin was dissolved in EBSS and oxygenated with 95%O_2/5% CO_2 gas mixture until it was bright red (oxyhemoglobin). Hemin and metalloporphyrins were dissolved first in Na_3PO_4 (1.7 M) to prepare 1 M solution of stock solutions and then diluted in EBSS (pH 7.3, oxygenated by 95%O_2/5% CO_2 gas mixture) at least 10,000 fold for use.

Extracellular recording of the neuronal firing activity in the SCN was conducted as described previously (21, 26). The time-of-peak in the neuronal activity was determined from the graph by visual analysis for the symmetrically highest point. Effects on phase were determined by comparing the time-of-peak of drug-treated slices with that of vehicle-treated slices. Differences between groups were evaluated with one-way ANOVA analysis with the Tukey *post hoc* test, using "Sigma Stat" software.

Immunocytochemistry

To evaluate HO-2 localization by immunocytochemistry, rats were transferred to a separate light-regulated box with air circulation and free access to food and water. At ZT 18 under dim red light, rats were deeply anesthetized with pentobarbital and perfused through the left ventricle of the heart with 50 ml of cold phosphate-buffered saline (PBS) followed by 350 ml of freshly prepared 4% paraformaldehyde in PBS, pH 7.4. Brains were postfixed for 24 h at 4°C, sectioned by Vibrotome into 50-μm-thick slices, and transferred to multiwell plates as floating sections. Primary antibody for HO-2 (StressGen, Victoria, Canada) was diluted (1:1000) in PBS containing 1% goat serum and 0.3% TX-100. Sections were incubated overnight with primary antibody and then with secondary antibody and ABC solution (Vector Lab) with DAB staining or streptavidin-CY3 with fluorescence microscopy to visualize immunoreactivity. In control experiments, primary antibody was omitted.

Scintillation Proximity Assay (SPA) of cGMP Levels

Hypothalamic slices containing the SCN were dissected to isolate the SCN and adherent optic chiasm. This reduced SCN slice generates the same circadian rhythm of neuronal firing activity as normal slices (14). At the appropriate time in the circadian cycle, reduced SCN slices were transferred into test tubes with 75 μl EBSS with/without inhibitors for 15 min. The cholinerigic agonist carbachol (CCh, 25 μl) was added to achieve 100 μM final concentration. After 3 min, the reaction was stopped by transferring the test tube to dry ice. The cGMP assay was performed with acetylation according to the guidelines of the manufacturer (Amersham). The final assay volume, 100 μl, was mixed with 186 μl of 100% ethanol, supernatant was transferred to the new test tubes, 100 μl of 65% ethanol was added to the pellet for reextraction, and the resulting supernatant was combined with the previous extraction. Extracts were lyophilized and resuspended in 100 μl of 0.05 M acetate buffer containing 0.01% sodium azide. cGMP standard solution, radioactive tracer and primary antibody conjugated to beads were added in equal amounts. Samples were incubated overnight and counted by Beckman scintillation counter. A standard curve was generated by 'SigmaPlot' software (Jandel) with our modifications, and the parameters of the fitting logistic curve were used to calculate the concentration of the unknown sample. Effects of treatment conditions on cGMP levels were compared by ANOVA with Tukey's *post hoc* test.

RESULTS

HO Activator, Hemin, but Not the NO Donor, SNAP, Mimics the Cholinergic Agonist Carbachol and Br-cGMP in Clock Resetting at CT 18

To assess the effects of NO and CO on clock state, donors were studied in the SCN brain slice. Whereas the unperturbed or vehicle-treated peak activity of the neuronal ensemble appears around mid-day (CT 7, Fig. 1a), a microdrop of CCh (100 μM, 10 min), applied to the surface of the SCN at CT 18, induced a robust phase advance of the time-of-peak to CT 1 $\pm$ 0.1 h, ~6 h earlier than in controls (Figs. 1b and 1e). This effect of CCh matches that of cell-permeable analogs of cGMP (21). To examine whether NO release mimics the cholinergic signal, SCN slices were treated by microdrop with the NO donor, SNAP (100 μM, 10 min). At this concentration, SNAP induces a 3.2-h phase delay at CT 14 and a 3.5-h phase delay at CT 20 (7). At CT 18 SNAP caused no significant shift in time-of-peak (Figs. 1c and 1e). To evaluate a role for CO in clock resetting at this time, we added a microdrop of hemin (100 μM, 10 min), a heme-containing molecule cleaved by endogenous HO-2 to yield CO (24). Hemin induced a phase advance equivalent to CCh with the time of peak at CT 1 $\pm$ 0.5 h (Figs. 1d and 1e). This observation supports the notion that CO, but not NO, transmits the signal activated by cholinergic stimulation.

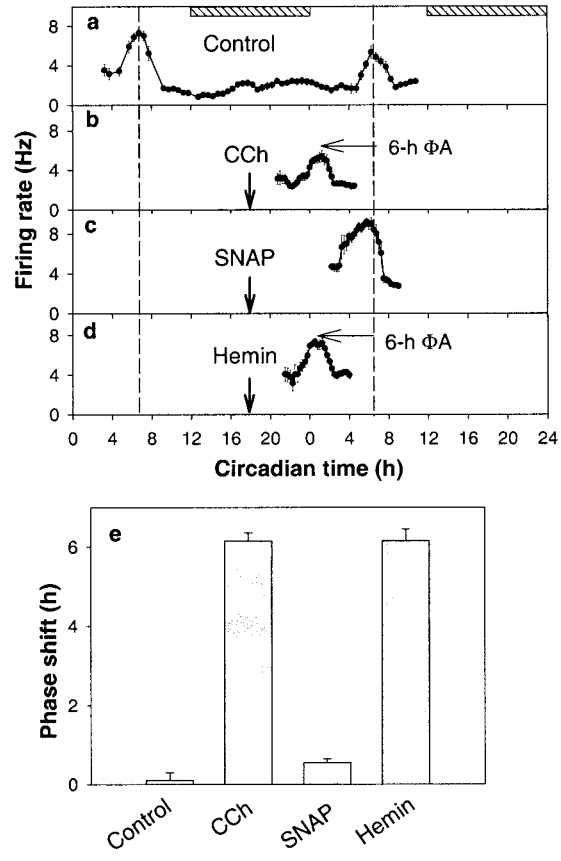

FIG. 1. Clock phase advance, induced by CCh at CT 18 is mimicked by the CO inducer, hemin, but not by the NO donor, SNAP. (a) The circadian rhythm of spontaneous neuronal firing activity recorded over 36 h. Mean activity was continuously recorded on the first and second days in the SCN brain slice. Activity peaked in the mid-subjective day, ~CT 7, on both days. (b) CCh (100 μM, 5 min) advanced the peak of the neuronal activity by ~6 h to near CT 1. (c) The NO donor, SNAP (100 μM, 10 min), did not affect the time-of-peak. (d) Hemin (100 μM, 10 min), the substrate of heme oxygenase reaction, induced CCh-like phase-advance ($n = 3$, $P < 0.001$). The graphs represent the running 2-h means $\pm$ SEM of a single unit activity recorded extracellularly. Dashed vertical lines define the time of the mean peak of the circadian rhythm in the EBSS-treated SCN. Vertical arrows indicate the time of the drug treatment. Horizontal arrows indicate the shift in the time-of-peak. The filled horizontal bars mark the time of the subjective night (CT12–CT24). (e) CCh and hemin induced significant advance of the SCN rhythm ($n = 3$, $P < 0.001$), whereas SNAP did not ($n = 3$, $P > 0.2$). Effects of CCh and hemin were not different from each other. The number of experiments (n) reflects the number of recorded slices, where n slices were taken from n animals and evaluated in n recording experiments. Effects of the treatment on the time of the peak were compared by ANOVA with the Tukey *post hoc* test.

Heme Oxygenase 2 is Expressed in the Rat SCN

HO-2-specific immunoreactivity is concentrated in the central area of the SCN (Fig. 2). Cellular localiza-

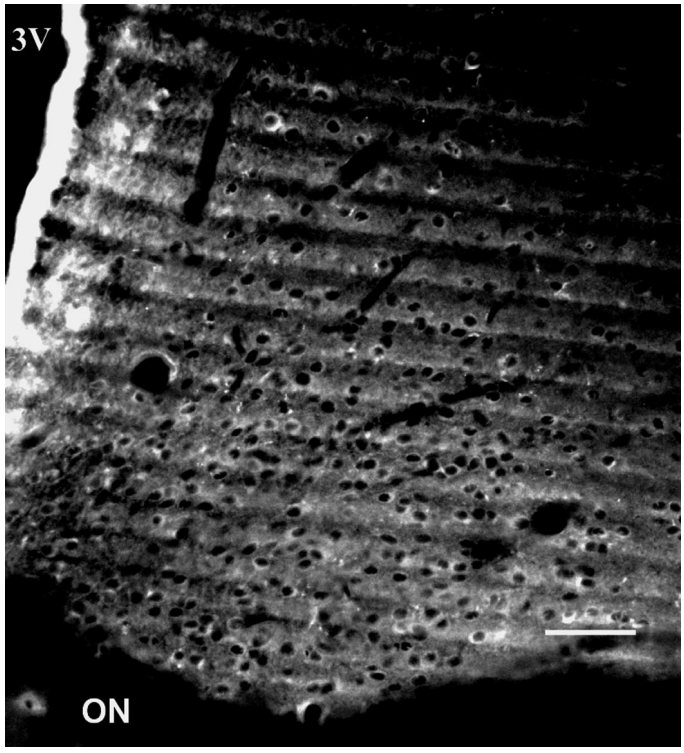

FIG. 2. Heme oxygenase 2 (HO-2) protein is expressed in the core area of the SCN. HO-2 immunoreactivity is concentrated in the central core of the SCN. The SCN boundary is delineated with a virtual diagonal line; 3V, third ventricle; ON, optic nerve. The striped appearance is from 3V (top L) to bar (lower R) product of vibrotome sectioning; bar = 50 μm.

tion within the SCN is represented by diffuse cytoplasmic staining of the tightly packed SCN neurons with distinctive small size, round shape, few processes, and a ring scant cytoplasm. This is in agreement with the reported cellular localization of HO-2 within the endoplasmic reticulum and Golgi system (29). This HO-2 localization is distinct from that of nNOS, which is expressed in a few ventrolateral SCN neurons, but is concentrated presynaptically in puncta of processes that form a plexus throughout the SCN (5).

NOS Inhibition Attenuates Cholinergic Clock Resetting

To evaluate contribution of NOS to circadian clock resetting induced by CCh at CT 18, we used the competitive substrate, L-NAME, to block NOS activation. Bath application of L-NAME (10 μM, 20 min) inhibits NOS activity in the SCN (5) and blocks glutamate-induced phase shifts at CT 14 and CT 20 (7). L-NAME alone did not alter clock phase. Pretreatment with L-NAME reduced the effect of CCh on clock resetting by ~65% to 2.1-h advance (Fig. 3). Increasing concentrations of L-NAME to 50 μM did not cause further inhibition (data not shown).

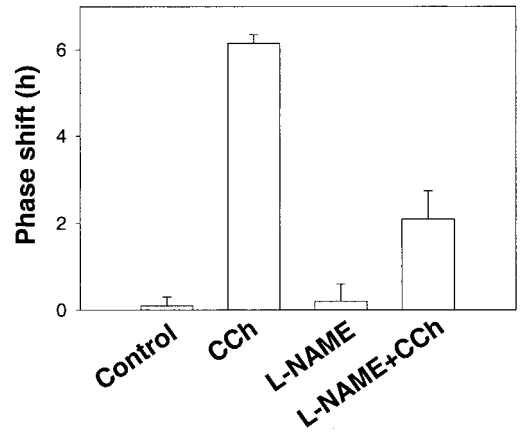

FIG. 3. CCh-induced phase advance is only partially blocked by inhibitor of NOS, L-NAME. CCh-induced ~6-h phase advance (n = 4, $P < 0.001$); L-NAME alone did not effect the phase of the neuronal firing (n = 3, $P > 0.2$); L-NAME attenuated the clock-advancing effect of CCh to 2.0 ± 0.1 h (n = 3, $P < 0.001$). Bars represent mean ± SEM.

NOS Contributes to the Unstimulated, Basal cGMP Level, whereas HO Contributes to CCh-Induced cGMP Accumulation

To compare the potential contribution of NOS and HO to the CCh-stimulated activation of sGC, we measured cGMP in slices reduced to isolate the SCN (Fig. 4). CCh treatment stimulates cGMP level from 20.5 ± 0.9 to 30.8 ± 2.5 fmol/slice, a 50% increase. L-NAME

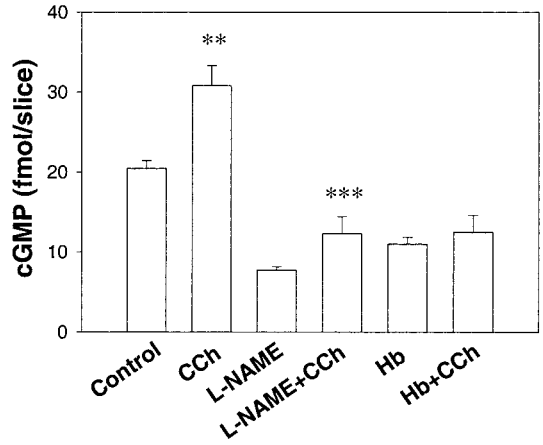

FIG. 4. The NOS inhibitor, L-NAME, and NO/CO scavenger, Hb, alter the level of cGMP in control and CCh-treated SCN slices. CCh significantly increased cGMP when compared with the basal level in control slices. L-NAME (10 μM) inhibited the basal level of cGMP by threefold. CCh plus L-NAME treatment still induced a significant increase when compared with L-NAME-only treated slices, but the overall level of cGMP was less then in the untreated control. Hb (10 mM) decreased basal cGMP by ~50%; this level was unchanged after CCh treatment. Asterisks represent significant increases from the relevant control: two asterisks denote the significance after treatment with CCh in the absence of L-NAME, and three asterisks mark significance after treatment with CCh in the presence of L-NAME. Bars in the graph represent means ± SEM (n = 4–7, $P < 0.001$).

alone decreased the level of cGMP to 7.7 ± 0.4 fmol/slice. This threefold decline indicates that the endogenous cGMP level depends upon basal NOS activity. CCh stimulation after preincubation with L-NAME increased the amount of cGMP from 7.7 ± 0.4 to 12.3 ± 2.1 fmol/slide, a 60% increase. The level of cGMP in L-NAME + CCh-treated samples was not significantly different from that in the control samples where no L-NAME was present. Since there is a similar relative increase in cGMP by CCh in the presence and absence of L-NAME, CCh may be accessing a different pool of sGC from that which contributes to basal cGMP level.

Hemoglobin, an extracellular scavenger of both NO and CO, was used to evaluate the requirement for the intercellular passage of these gaseous messengers to stimulate cGMP production. Hemoglobin (10 mM, 15 min) also significantly decreased the basal level of cGMP in the SCN from 20.5 ± 1 fmol/slice to 11.0 ± 1.2 fmol/slice. Surprisingly, the CCh-stimulated increase in cGMP synthesis did not occur after the pretreatment with hemoglobin (11.0 ± 1.2 fmol/slice vs 12.3 ±2.1 fmol/slice). Since hemoglobin scavenges both NO and CO, and inhibiting NO synthesis failed to block the CCh-induced rise in cGMP, this observation suggests that CO can act as a phasic signaling messenger of CCh-induced cGMP increase. Moreover, the CCh signaling pathway most likely includes intercellular interaction among SCN neurons with CO serving as a paracrine messenger.

HO-2 Inhibitors Block CCh-Induced Phase Advance at CT 18

To further test whether CO could be a messenger of CCh-induced phase advance at CT 18, we employed selective HO inhibitors, tin protoporphyrin IX (SnPP), and chromium mesoporphyrin IX (CrMP). Bath application of either SnPP (10 μM, 20 min) or CrMP (5 μM, 20 min) alone had no effect on time-of-peak of the neuronal firing activity as compared with control slices (Figs. 5c and 5e). However, the CCh-induced 6-h phase advance (Fig. 5b) was diminished to 1 h after pretreatment with SnPP (Fig. 5d) and was completely abolished after pretreatment with CrMP (Fig. 5f). The data summarized in Fig. 5h demonstrate that HO-2 is a necessary component of the signal transduction mechanism of cholinergic clock resetting.

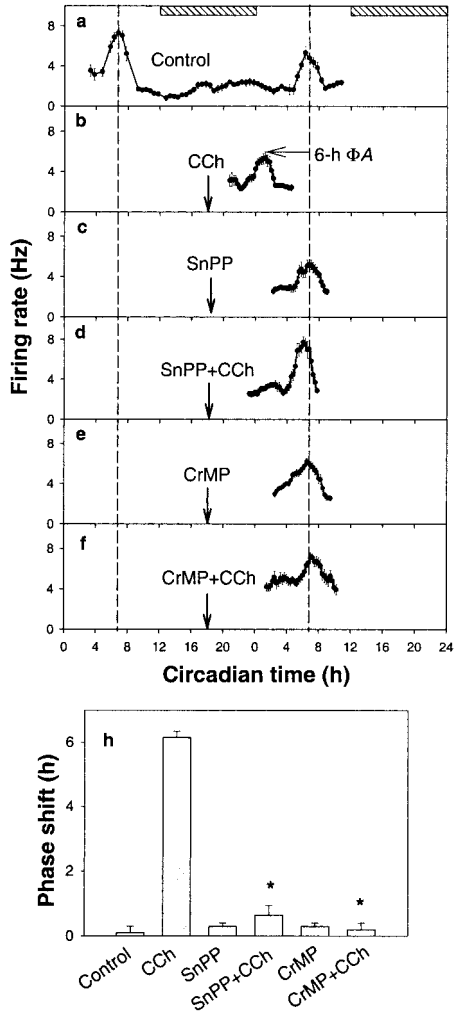

FIG. 5. CCh-induced phase advance was blocked by HO-2 inhibitors. (a) The circadian rhythm of the neuronal firing activity recorded over 36 h. (b) CCh (100 μM, 5 min) applied at CT 18 advanced the peak of the neuronal activity by ~6 h. (c) Application of HO inhibitor, tin protoporphyrin (SnPP, 10 μM, 10 min), had no effect on the clock phase. (d) Pretreatment with SnPP effectively blocked CCh-induced phase advance at CT 18. (e) Another HO inhibitor, chromium mesoporphyrin (CrMP, 5 μM, 10 min), did not effect the clock phase. (f) Pretreatment with CrMP blocked the CCh-induced phase-advance at CT 18. The graphs represent the running 2-h means ± SEM of a single unit activity recorded extracellularly. Dashed vertical lines define the time of the mean peak of the circadian rhythm in the EBSS-treated SCN. Vertical arrows indicate the time of the drug treatment. Horizontal arrows indicate the shift in the time-of-peak. The filled horizontal bars mark the time of the subjective night (CT12-CT24). (h) SnPP and CrMP plus CCh effectively block the CCh-induced phase advance at CT 18 ($n = 3$, $P < 0.001$). Effects of the treatment on the time of the peak were compared by ANOVA with the Tukey *post hoc* test.

DISCUSSION

In evaluating the potential involvement of HO-2/CO and NOS/NO in cholinergic signaling to the SCN clock, we found that HO-2/CO activity is both necessary and sufficient to induce clock resetting, but that the ACh/HO-2 signal requires a permissive clock state that provides significant NOS/NO/cGMP tone. The relation of these findings to previously established cholinergic signaling components is summarized in Fig. 6. This model depicts two different cGMP-producing systems, one NO-dependent and another CO-dependent, that are critical for CCh signaling in the SCN. Such contribution of NOS/NO and HO/CO to the tonic and phasic regulation of cGMP level and cGMP-controlled state,

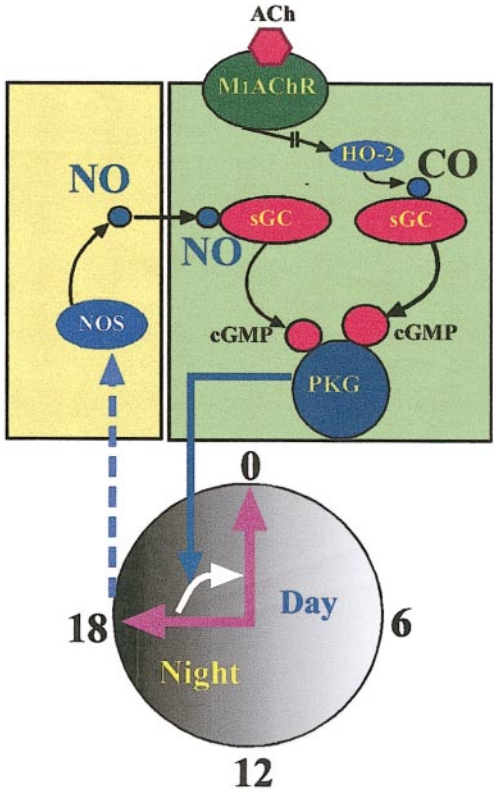

FIG. 6. This model integrates the data to relate putative signaling elements that mediate the modulatory effect of ACh on the circadian clock. NOS and NO provide tonic stimulation of a discrete sGC/cGMP pool at CT 18 that is generated by endogenous clock state, whereas HO-2 and CO are active phasically, when ACh binds to M_1 muscarinic receptors (M_1·Rs, putative activators of a signaling cascade that mediates nocturnal phase advance). The M_1·R couples to $G_{q/11}$, which stimulates phospholipase Cβ (PLCβ) to synthesize $InsP_3$ and DAG. $InsP_3$-induced Ca^{2+} release and DAG stimulate protein kinase C (PKC), the only known activator of HO-2 (an interrupted line represents this putative pathway). Upon HO-2 activation, CO is produced that, in turn, stimulates a distinct pool of sGC/cGMP/PKG, which can engage the clockworks. This cascade leads to advance of the circadian timing by 6 h. The clock-regulated tonic activity of NOS/NO/sGC/cGMP/PKG sets a clock state that is permissive for the critical cholinergic signal, which phasically stimulates a distinct cGMP-producing system and consequent clock resetting.

respectively, has not been described previously. The multilevel gating of the cholinergic signal described here emphasizes the complex nature of muscarinic neuromodulation. Each of the regulatory elements, which converge on the sGC/cGMP system, will be considered in turn.

Previously, we have reported significant NOS-specific activity in the SCN, comparable to cerebellum, but localization was primarily in processes, forming a dense nitrergic plexus throughout the SCN (5). Permissive to the cholinergic response, NO acts as a tonic intercellular signal that determines the basal level of cGMP, and thus the state of cholinergic sensitivity. Resting activity of either eNOS in SCN astrocytes (4) or nNOS in the neural plexus (5) could provide tonic release of NO to maintain the basal state of cGMP and its downstream elements. Analogous tonic activity of eNOS is responsible for the NO-mediated maintenance of basal vascular tone in endothelial cells (18). Tonic NOS activity in the SCN is not required for the normal progression of clock timing during the subjective night, since inhibition of either NOS by L-NAME (7) or of sGC by LY83583 (20) does not reset the clock. Nevertheless, cGMP tone is required for CCh to fully exert its effect. Therefore, the basal level of cGMP must prime the neuronal state to respond to CCh.

Cholinergic clock resetting does not include activation of NO release as the phasic signaling molecule. The NO donor, SNAP, did not induce phase resetting at CT 18. Furthermore, at CT 14, when CCh and Br-cGMP induce a ~4-h phase advance (20, 21), SNAP and other NO donors induce a 3-h phase delay (7), emphasizing the divergence of phasic cGMP and NO effects on the clock at this time of the circadian cycle. Inhibition of NOS at CT 18 reduced the amplitude of the CCh-induced shift by ~3.5 h (~65%); this effect was not amplified by an increased concentration of L-NAME. Likewise, L-NAME failed to block the CCh-induced increased in cGMP. Thus, CCh acts as a phasic signal, stimulating an additional, NOS-independent increase in cGMP that leads to clock resetting.

This partial inhibition of CCh-induced clock resetting by L-NAME is likely a consequence of the strong inhibition by the NOS inhibitor of a specific sGC pool that generates basal cGMP tone. This is supported by our observation that CCh stimulated an increase in cGMP by about the same relative degree, whether NO was inhibited or not. The three-fold decrease in cGMP level upon application of hemoglobin, a putative cell-impermeable extracellular scavenger of both NO and CO, indicates that basal cGMP level is regulated via intercellular communication. Interestingly, stimulation by CCh after pretreatment with hemoglobin did not alter the level of cGMP, supporting the notion that paracrine CO signals activate a sGC pool that accesses the clock mechanism.

NO is an established regulator of clock resetting by nocturnal light/glutamatergic signals. The light signal is transmitted to the SCN via the retinohypothalamic tract (RHT), which releases neurotransmitter glutamate in the ventrolateral SCN (7). nNOS is found in SCN ventrolateral neurons (5), sites of the RHT innervation. The effects of glutamate are mediated via ionotropic NMDA glutamate receptors, consequent influx of Ca^{2+}, and activation of NOS with NO release (7, 34). For metabotropic receptors such as M_1·Rs, $InsP_3$-induced Ca^{2+} release and capacitative Ca^{2+} entry can provide an increase in intracellular Ca^{2+}. However, most likely, the absence of expressed nNOS in the M_1·R-bearing SCN neurons limits the ability of cholinergics to increase endogenous NO.

However, it is surprising that exogenous NO donors fail to mimic the effects of cGMP analogs on clock timing at CT 18. This suggests that at this circadian time the sGC pool mediating clock resetting is insensitive to the activation by NO. A possible explanation for the inability of NO to activate sGC may lie in the susceptibility of sGC activation to redox state. A clock state-dependent oxidation of ferrous iron in the enzyme's heme moiety could render sGC blind to NO. Heme group oxidation to the ferric state leads to the loss of enzyme activity and the consequent dissociation of heme from the sGC, making it insensitive to heme-dependent stimulation (10, 11). Activation of the HO reaction, on the other hand, has the capacity to reduce this heme to the ferrous state by releasing the reducing substance, bilirubin. Bilirubin contributes strong antioxidant activity in the brain (8). Since localization of HO-2 and sGC in the brain is essentially the same (33), bilirubin may be a reducing agent of this sGC's heme moiety. Even though a role for redox-state sensing in mammalian circadian clock regulation has not been reported, several lines of evidence support this hypothesis. For instance, circadian variation in the redox state-controlling enzyme, catalase, has been reported in plants (37), and the redox-potential sensor PAS domain is a highly conserved motif among diverse clock proteins (31). Should the redox state vary in the SCN over circadian time, it is possible that an NO-insensitive but hemin-CO-activatable state of sGC exists in SCN neurons and that this is the form that mediates ACh-induced clock resetting.

A regional colocalization of M_1•R and HO-2 in the SCN neurons is likely the basis of functional linkage. We observed considerable HO-2 immunoreactivity in the SCN, suggesting an important role for this enzyme in the SCN function. A high level of immunoreactivity was detected in neurons in the core central area of the SCN. Localization of the rat's M_1•R expression by immunocytochemistry and *in situ* hybridization analysis includes neurons in the central area of the SCN (19). Thus, both proteins, M_1•R and HO-2, are expressed in central SCN neurons, whereas nNOS expression in the central core area of the SCN is largely absent (5).

Pharmacological analysis with specific inhibitors of HO-2 provides critical support for HO-2/CO as mediators of cholinergic clock resetting at CT 18. The specificity of metalloporphyrins in inhibiting HO over NOS and sGC has been questioned (15, 22), confounding the analysis of HO-2/CO in signal transduction mechanisms. However, a recently published study of the relative potency of metalloporphyrins toward HO, NOS, and sGC in the rat brain revealed that in concentrations $\leq$10 μM, metalloporphyrins inhibit HO without blocking NOS and sGC (1). Chromium mesoporphyrin IX (CrMP) was found to be the most selective inhibitor at a 5 μM concentration. Convincing data supporting the selectivity of SnPP at 10 μM in inhibiting HO-2, but not NOS or sGC, comes from studies on HO-2 knockout mice. In these mice, SnPP failed to block intestinal relaxation and increased cGMP level, whereas NOS inhibitors ablated these effects (36). At the recommended concentrations, both CrMP and SnPP blocked cholinergic clock resetting, supporting the requirement of HO for this effect. Moreover, in experiments where hemin induced CCh-like phase advance, we demonstrated that HO-2 activation is sufficient to induce clock resetting. These data place HO-2/CO downstream from an M_1•R-like activation event that provides specific signals to advance the clock in the subjective night. This is, to our knowledge, the first documented involvement of these molecules in the circadian clock resetting and, more generally, in muscarinic cholinergic neurotransmission in the brain.

The critical role for HO-2 in the cholinergic clock resetting demonstrated in this study, together with recently reported malfunction of this enzyme in neurons expressing amyloid-β peptide (29), may have clinical implications. Amyloid-β depositions in CNS are markers of AD. AD patients exhibit striking abnormalities in the patterning of circadian rhythms, such as irregular day–night rhythms with behavioral restlessness during the night. Changes within the circadian system are a possible source of such dysfunctions (32). Overexpression of amyloid-β in rat SCN disrupted the circadian organization of behavior and was accompanied by unusually high levels of locomotor activity during the day (30). Very low concentrations of the amyloid-β can inhibit various muscarinic functions without significant changes in the number of muscarinic postsynaptic receptors (17). We suggest that HO-2 could be a key defective element in the muscarinic signaling cascade in AD. Our discovery of a role for HO-2 in cholinergic regulation of the circadian clock may shed some light on the pathogenesis of sleep disorders and other circadian rhythm dysfunctions in Alzheimer's patients.

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REFERENCES

1. Appleton, S. D., M. L. Chretien, B. E. McLaughlin, H. J. Vreman, D. K. Stevenson, J. F. Brien, K. Nakatsu, D. H. Maurice, and G. S. Marks. 1999. Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase, by metalloporphyrins at low concentration. *Drug Metab. Dispos.* **27:** 1214–1219.
2. Bina, K. G., and B. Rusak. 1996. Muscarinic receptors mediate carbachol-induced phase shifts of circadian activity rhythms in syrian hamsters. *Brain Res.* **743:** 202–211.
3. Bina, K. G., B. Rusak, and K. Semba. 1993. Localization of cholinergic neurons in the forebrain and brainstem that project

to the suprachiasmatic nucleus of the hypothalamus in rat. *J. Comp. Neurol.* **335:** 295–307.

4. Caillol, M., E. Devinoy, M.-C. Lacroix, and A. Schirar. 2000. Endothelial and neuronal nitric oxide synthases are present in the suprachiasmatic nucleai of Syrian hamsters and rats. *Eur. J. Neurosci.* **12:** 649–661.
5. Chen, D., W. J. Hurst, J. M. Ding, L. E. Faiman, B. Mayer, and M. U. Gillette. 1997. Localization and characterization of nitric oxide synthase in the rat suprachiasmatic nucleus: Evidence for a nitrergic plexus in the biological clock. *J. Neurochem.* **68:** 855–861.
6. Colwell, C. S., C. M. Kaufman, and M. Menaker. 1993. Phase-shifting mechanisms in the mammalian circadian system—New light on the carbachol paradox. *J. Neurosci.* **13:** 1454–1459.
7. Ding, J. M., D. Chen, E. T. Weber, L. E. Faiman, M. A. Rea, and M. U. Gillette. 1994. Resetting the biological clock: Mediation of nocturnal circadian shifts by glutamate and NO. *Science* **266:** 1713–1717.
8. Dore, S., M. Takahashi, C. D. Ferris, R. Zakhary, L. D. Hester, D. Guastella, and S. H. Snyder. 1999. Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. *Proc. Natl. Acad. Sci. USA* **96:** 2445–2450.
9. Everitt, B. J., and T. W. Robbins. 1997. Central cholinergic systems and cognition. *Annu. Rev. Psychol.* **48:** 649–684.
10. Gerzer, R., F. Hofmann, E. Bohme, K. Ivanova, C. Spies, and G. Schultz. 1981. Purification of soluble guanylate cyclase without loss of stimulation by sodium nitroprusside. *Adv. Cyclic Nucleotide Res.* **14:** 255–261.
11. Gerzer, R., F. Hofmann, and G. Schultz. 1981. Purification of a soluble, sodium–nitroprusside-stimulated guanylate cyclase from bovine lung. *Eur. J. Biochem.* **116:** 479–486.
12. Gillette, M. U. 1998. The neurobiology of time: Regulation of the brain's circadian clock. In *Proceedings, New Neuroethology on the Move, 26th Goettingen Neurobiology Conference, Goettingen,* pp. 104–125.
13. Gillette, M. U., M. Medanic, A. J. McArthur, C. Liu, J. M. Ding, L. E. Faiman, E. T. Weber, T. K. Tcheng, and E. A. Gallman. 1995. Electrophysiology of the SCN clock: Intrinsic neuronal rhythms and their adjustment. In *Ciba Foundation Symposium: Circadian Clocks and Their Adjustment* (J. Waterhouse, Ed.), pp. 134–153. Wiley, Chichester.
14. Gillette, M. U., and S. M. Reppert. 1987. The hypothalamic suprachiasmatic nuclei: Circadian patterns of vasopressin secretion and neuronal activity *in vitro. Brain Res. Bull.* **19:** 135–139.
15. Grundemar, L., and L. Ny. 1997. Pitfalls using metalloporphyrins in carbon monoxide research. *Trends Pharmacol. Sci.* **18:** 193–195.
16. Hatton, G. I., A. D. Doran, A. K. Salm, and C. D. Tweedle. 1980. Brain slice preparation: Hypothalamus. *Brain Res. Bull.* **5:** 405–414.
17. Hellstrom-Lindahl, E. 2000. Modulation of beta-amyloid precursor protein processing and tau phosphorylation by acetylcholine receptors. *Eur. J. Pharmacol.* **393:** 255–263.
18. Huang, P., Z. Huang, H. Mashomoto, K. D. Bloch, M. A. Moskowitz, J. A. Beavan, and M. C. Fishman. 1995. Hypertention in mice laking the gene for endothelial nitric oxide synthase. *Nature* **377:** 239–242.
19. Kuriashkina, L. K., J. M. Ding, S. A. Tischkau, and M. U. Gillette. 1997. Expression of m1 muscarinic cholinergic receptors in the rat suprachiasmatic nucleus. *Soc. Neurosci. Abstr.* **23:** 510.
20. Liu, C., J. M. Ding, L. E. Faiman, and M. U. Gillette. 1997. Coupling of muscarinic cholinergic receptors and cGMP in nocturnal regulation of the suprachiasmatic circadian clock. *J. Neurosci.* **17:** 659–666.
21. Liu, C., and M. U. Gillette. 1996. Cholinergic regulation of the suprachiasmatic nucleus circadian rhythm via a muscarinic mechanism at night. *J. Neurosci.* **16:** 744–752.
22. Luo, D., and S. R. Vincent. 1994. Metalloporphyrins inhibit nitric oxide-dependent cGMP formation in vivo. *Eur J. Pharmacol.* **267:** 263–267.
23. Maines, M. D. 2000. The heme oxygenase system and its functions in the brain. *Cell. Mol. Biol.* **46:** 573–585.
24. Mancuso, C., I. Kostoglou-Athanassiou, M. L. Forsling, A. B. Grossman, P. Preziosi, P. Navarra, and G. Minotti. 1997. Activation of heme oxygenase and consequent carbon monoxide formation inhibits the release of arginine vasopressin from rat hypothalamic explants: Molecular linkage between heme catabolism and neuroendocrine function. *Mol. Brain. Res.* **50:** 267–276.
25. Meijer, J. H., E. van der Zee, and M. Dietz. 1988. The effects of intraventricular carbachol injections on free-running activity rhythm of the hamster. *J. Biol. Rhythms* **3:** 333–348.
26. Prosser, R. A., and M. U. Gillette. 1989. The mammalian circadian clock in the suprachiasmatic nuclei is reset in vitro by cAMP. *J. Neurosci.* **9:** 1073–1081.
27. Sjostrand, T. 1949. Endogenous formation of carbon monoxide in man under normal and pathological conditions. *J. Clin. Invest.* **1:** 201–208.
28. Snyder, S. H., S. R. Jaffrey, and R. Zakhary. 1998. Nitric oxide and carbon monoxide: Parallel roles as neural messengers. *Brain Res. Brain Res. Rev.* **26:** 167–175.
29. Takahashi, M., S. Dore, C. D. Ferris, T. Tomita, A. Sawa, H. Wolosker, D. R. Borchelt, T. Iwatsubo, S. Kim, G. Thinakaran, S. S. Sisodia, and S. H. Snyder. 2000. Amyloid precursor proteins inhibit heme oxygenase activity and augment neurotoxicity in Alzheimer's disease. *Neuron* **28:** 461–473.
30. Tate, B., K. S. Aboody-Guterman, A. M. Morris, E. C. Walcott, R. E. Majocha, and C. A. Marotta. 1992. Disruption of circadian regulation by brain grafts that overexpress Alzheimer beta/A4 amyloid. *Proc. Natl. Acad. Sci. USA* **89:** 7090–7094.
31. Taylor, B., and I. Zhilin. 1999. PAS domains: Internal sensors of oxygen, redox potentials, and light. *Microbiol. Mol. Biol. Rev.* **63:** 479–506.
32. Van Someren, E. J., E. J. Scherder, and D. F. Swaab. 1998. Transcutaneous electrical nerve stimulation (TENS) improves circadian rhythm disturbances in Alzheimer disease. *Alzheimer Dis. Assoc. Disord.* **12:** 114–118.
33. Verma, A., D. J. Hirsch, C. E. Glatt, G. V. Ronnett, and S. H. Snyder. 1993. Carbon monoxide: A putative neural messenger. *Science* **259:** 381–384.
34. Weber, E. T., R. L. Gannon, A. M. Michel, M. U. Gillette, and M. A. Rea. 1995. Nitric oxide inhibitor blocks light-induced phase shifts of the circadian activity rhythm, but not c-fos expression in the suprachiasmatic nucleus of the Syrian hamster. *Brain Res.* **692:** 137–142.
35. Wee, B. E. F., K. D. Anderson, N. S. Kouchis, and F. W. Turek. 1992. Administration of carbachol into the lateral ventricle and suprachiasmatic nucleus (SCN) produces dose-dependent phase shifts in the circadian rhythm of locomotor activity. *Neurosci. Lett.* **137:** 211–215.
36. Zakhary, R., K. D. Poss, S. R. Jaffrey, C. D. Ferris, S. Tonegawa, and S. H. Snyder. 1997. Targeted gene deletion of heme oxygenase 2 reveals neuronal role for carbon monoxide. *Proc. Natl. Acad. Sci. USA* **94:** 14848–14953.
37. Zhong, H. H., A. S. Resnick, M. Straume, and C. Robertson McClung. 1997. Effects of synergistic signaling by phytochrome A and cryptochrome1 on circadian clock-regulated catalase expression. *Plant Cell* **9:** 947–955.