The cholinergic forebrain arousal system acts directly on the circadian pacemaker

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Sleep and wake states are regulated by a variety of mechanisms. One such important system is the circadian clock, which provides temporal structure to sleep and wake. Conversely, changes in behavioral state, such as sleep deprivation (SD) or arousal, can phase shift the circadian clock. Here we demonstrate that the level of wakefulness is critical for this arousal resetting of the circadian clock. Specifically, drowsy animals with significant power in the 7- to 9-Hz band of their EEGs do not exhibit phase shifts in response to a mild SD procedure. We then show that treatments that both produce arousal and reset the phase of circadian clock activate (i.e., induce Fos expression in) the basal forebrain. Many of the activated cells are cholinergic. Using retrograde tract tracing, we demonstrate that cholinergic cells activated by these arousal procedures project to the circadian clock in the suprachiasmatic nuclei (SCN). We then demonstrate that arousal-induced phase shifts are blocked when animals are pretreated with atropine injections to the SCN, demonstrating that cholinergic activity at the SCN is necessary for arousal-induced phase shifting. Finally, we demonstrate that electrical stimulation of the substantia innominata of the basal forebrain phase shifts the circadian clock in a manner similar to that of our arousal procedures and that these shifts are also blocked by infusions of atropine to the SCN. These results establish a functional link between the major forebrain arousal center and the circadian system.

arousal | phase shift | nonphotic | sleep/wake | brain stimulation

The states of wake and sleep in mammals are regulated by interactions between brainstem and forebrain regions (1, 2), with some areas actively promoting sleep and others promoting wake. The allied circadian clock located in the suprachiasmatic nuclei (SCN) (3) provides temporal structure to behavior and physiology. The sleep/wake systems and circadian systems interact (4), with the circadian system providing temporal input to the sleep and wake systems, thus promoting both states, and ensuring that these behaviors occur at species-specific times of day (5).

The activity of the SCN is endogenously regulated and tightly linked to behavioral state. SCN electrical activity is highest during the day, when nocturnal rodents are asleep, with higher activity during rapid-eye-movement (REM) sleep than during slow wave sleep (6). During the waking phase, SCN activity is suppressed by spontaneous behavior (7). Critically, triggering activity or wakefulness, so-called "nonphotic zeitgebers" (time givers), during the sleep phase can reset the phase of the circadian clock (8, 9). There is an inverse relationship between the size of the phase shift due to sleep deprivation (SD) and the amount of effort required to maintain wakefulness during the procedure (8). Specifically, animals that remain awake and alert on their own exhibit large phase shifts, whereas those that require frequent intervention to maintain wakefulness do not shift. These factors suggest that areas of the brain involved in wakefulness may underlie these nonphotic phase shifts. Some evidence suggests that serotonin from the raphe nuclei may underlie this effect (10), although this conclusion has been questioned (11). Neuropeptide Y from the intergeniculate leaflet appears to be both necessary (12) and sufficient (13) for nonphotic phase shifting; however, this system is not among the areas recognized as part of the sleep/wake system (2). A third option could be acetylcholine. Whereas the SCN receives cholinergic input from brainstem regions (14, 15), the majority of its cholinergic inputs arises from the substantia innominata (SI) of the basal forebrain (16). To date, no function has been ascribed to these inputs. The SCN contains both muscarinic and nicotinic receptors (17). Cholinergic agonists cause phase advances when applied to the SCN in vivo during the subjective day (18, 19), specifically through the M1/4 muscarinic receptors (20). Like other nonphotic neurotransmitters (21, 22), acetylcholine and its agonists suppress SCN firing (23). Cholinergic basal forebrain cells show maximal discharge rates during wake and paradoxical sleep and burst in synchronization with cortical theta-rhythms (24). Stimulation of the basal forebrain leads to desynchrony of cortical EEGs (25, 26). Optogenetic activation of forebrain cholinergic neurons causes animals to transition from slow wave sleep to wake (27, 28). Given these findings, the cholinergic basal forebrain is ideally situated to be the link between the arousal and circadian systems. We hypothesize that arousal, mediated in part by the cholinergic forebrain, underlies nonphotic phase shifts such as those to exercise and SD (8, 9).

Results

Animals That Do Not Phase Shift to Arousal Show Significant Power in the 7- to 9-Hz Band of Their EEGs. First, we asked whether the difference between hamsters that are deemed responders and those

Significance

Sleep and wake states are regulated by a variety of mechanisms. One such important system is the circadian clock, which provides temporal structure to sleep and wake. Conversely, changes in behavioral state can influence the phase of the circadian clock. Here we demonstrate that the level of wakefulness is critical for arousal to reset circadian clock phase. We then show that treatments that produce arousal and reset the circadian clock activate the basal forebrain. Finally, we demonstrate that cholinergic input from the basal forebrain is both necessary and sufficient for eliciting this arousal-induced resetting of the circadian clock. These results establish a functional link between the major forebrain arousal center and the circadian system.

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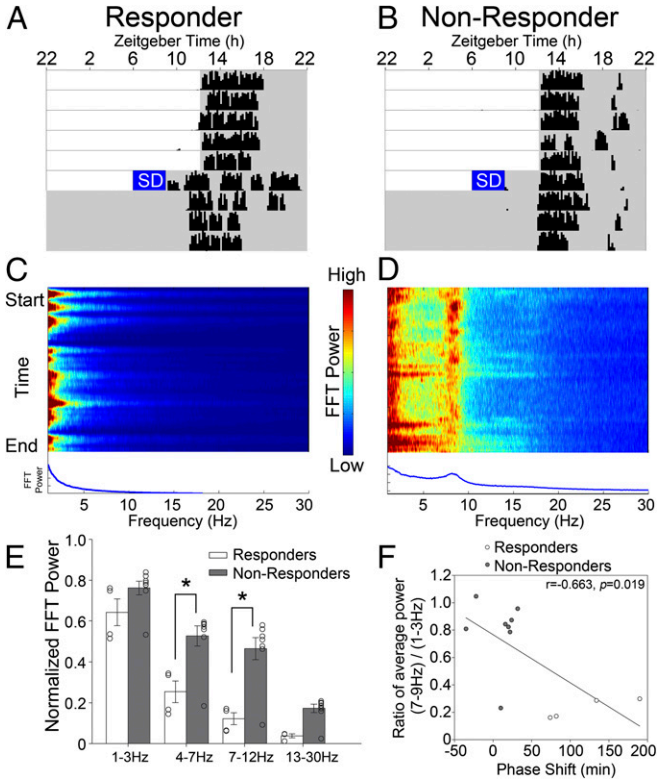

Fig. 1. Actograms depicting the circadian response to a 3-h SD procedure initiated at ZT6 in an animal determined to be a responder (*A*) or a nonresponder (*B*). Every horizontal line represents 1 d of activity, with vertical deflection above each line representing 10-min periods during which activity occurred, and with the height of the deflection being proportional to the amount of activity. Subsequent days are plotted below the previous day. Animals were housed in a 14 h light:10 h dark cycle until the time of the SD, when the lights were dimmed to 1 lx red light until the end of the procedure, at which time the animals were transferred to DD for the duration of the procedure. (*C* and *D*) Heatmaps representing the FFT power from the start (*Top*) to end (*Bottom*) of the SD procedure. The individual overall FFTs for the whole 3-h SD are depicted below their respective heatmaps. (*E*) Nonresponders (gray bars) had a prominent peak in their FFT at about 8 Hz, which led to significantly (*$P < 0.05$) more FFT power over both the 7- to12-Hz and 4- to 7-Hz ranges in the nonresponders than in the responders (white bars). Means are reported ± SEM. (*F*) There was a significant negative correlation between the phase shift and the ratio of power in the 7- to 9-Hz range relative to the power in the 1- to 3-Hz range.

that are nonresponders might be due to a difference in the level of arousal. To examine this question, we looked at the cortical EEGs of animals during the SD procedure, and correlated this activity to their resulting phase shift. Animals that responded (Fig. 1*A*; mean phase shift 2.0 ± 0.4 h) exhibited no prominent peaks in the fast Fourier transform (FFT) (Fig. 1*C*), whereas animals that did not phase shift (Fig. 1*B*; mean phase shift 0.13 ± 0.14 h) exhibited a strong peak in the 7- to 9-Hz range (8.18 ± 0.09 Hz; Fig. 1*D*) throughout the SD procedure. The power integrated over this range was significantly greater in nonresponders [$F_{(4,40)} = 16.8$, $P < 0.001$; Fig. 1*E*] and had a significant negative correlation with the size of the resulting phase shift (Fig. 1*F*).

The Basal Forebrain Is Activated by Arousal Procedures That Shift the Circadian Clock. The peak in EEG power at 8 Hz is similar to hippocampal theta, a brain oscillation observed with movement and sensorimotor integration (29). However, the observation that nonresponders appear more drowsy than responders, in that the nonresponders require earlier and more frequent interventions to maintain wakefulness throughout the procedure (8), argues against this peak being hippocampal theta. An alternative may be alpha-activity. In humans, cortical alpha-activity is indicative of drowsiness (30), thus it is possible that the 8-Hz peak observed might represent a hamster EEG equivalent to the drowsy alpha-activity observed in humans. If the difference between responders and nonresponders is their level of arousal, then a difference in activity in the arousal systems of the brain would be implicated. Of particular interest is the cholinergic system, which promotes wakefulness and arousal (1, 31, 32). The basal forebrain cholinergic system projects to the rat SCN (16). To determine whether these cells were activated by nonphotic manipulations, we examined Fos expression in the basal forebrain following weakly arousing (SD through gentle handling, $n = 4$ experimental and $n = 5$ control) or strongly arousing [confinement to a novel wheel (WC), $n = 6$ experimental and $n = 6$ control] nonphotic manipulations. Relative to their respective control animals, which were just moved from a light/dark cycle (LD) to constant darkness (DD), both procedures induced significant Fos expression in the basal forebrain [SD: $t_{(7)} = 3.31$, $P = 0.013$ and WC: $t_{(10)} = 3.17$, $P = 0.010$; Fig. 2].

Cholinergic Cells in the Basal Forebrain Are Activated by Arousal Procedures That Shift the Circadian Clock. Using double label immunohistochemistry, we confirmed that for the wheel confinement procedures, much of this Fos expression occurred in cholinergic neurons [identified by choline acetyl transferase (ChAT) immunoreactivity]. In the DD control animals, there were very few cells labeled for both ChAT and Fos in the anterior (8.67 ± 4.53), mid (2.00 ± 0.58), and posterior (4.33 ± 1.34) sections of the basal forebrain, whereas animals in the novel WC condition had significantly more cells labeled for both ChAT and Fos in these regions [anterior, 61.33 ± 12.37; mid, 21.33 ± 4.35; posterior, 10.00 ± 2.48; main effect of treatment, $F_{(5,17)} = 15.15$, $P < 0.01$]. Across all levels, animals in the novel WC condition had a significantly greater percentage of ChAT cells containing Fos (33.5 ± 2.7%) than did the control-treated animals [4.5 ± 2.8%, $t_{(4)} = 7.44$, $P = 0.002$]. The

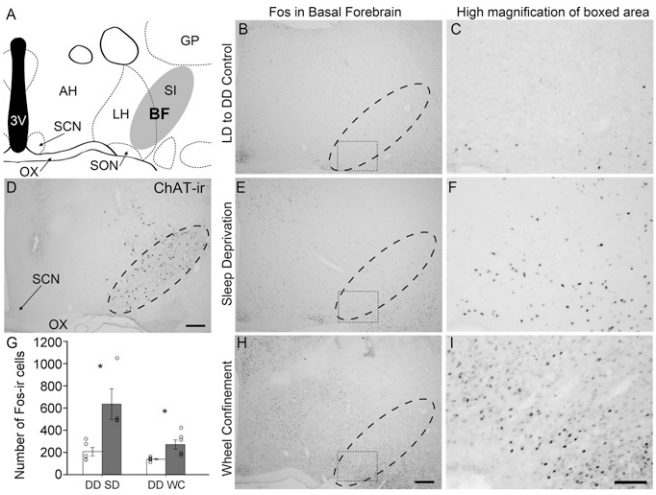

Fig. 2. Fos immunoreactivity was assessed in the cholinergic basal forebrain (*A* and *D*) following a DD control (*B* and *C*), SD (*E* and *F*), or novel WC procedure (*H* and *I*). High magnification photomicrographs (*C*, *F*, and *I*) are of the areas depicted by the dotted box in the low magnification images (*B*, *E*, and *H*). (*G*) Both SD and novel WC led to significantly more Fos-immunoreactive cells (Fos-ir; *$P < 0.05$, gray bars) than their respective controls (white bars). Means are reported ± SEM. [Scale bars, 300 μm for low magnification images (*B*, *E*, and *H*) and 100 μm for high magnification images (*C*, *F*, and *I*)]. Abbreviations are as follows: 3V, third ventricle; AH, anterior hypothalamus; BF, basal forebrain; ChAT-ir, choline acetyltransferase immunoreactive; GP, globus pallidus; LH, lateral hypothalamus; OX, optic chiasm; SI, substantia innominata; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus.

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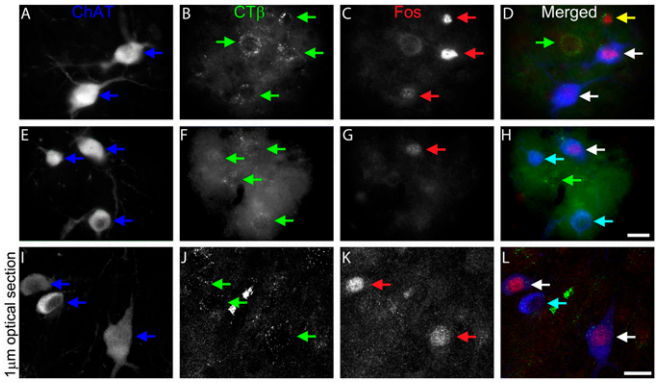

Fig. 3. Two sets of high-resolution photomicrographs from an epifluorescent microscope (*A–H*) depicting immunoreactivity for ChAT (*A* and *E*, blue), CTβ subunit (*B* and *F*, green) from an infusion into the SCN, and Fos (*C* and *G*, red) following 3 h of novel WC. Merged images (*D* and *H*) demonstrate examples of cells containing all three labels (white arrows), as well as SCN-projecting noncholinergic cells not activated by the procedure (green arrows), an SCN-projecting noncholinergic cell activated by the procedure (yellow arrow), and SCN-projecting cholinergic cells not activated by the procedure (teal arrows). Colocalization was confirmed by examining 1-μm optical sections collected with a confocal microscope (*I–L*). (Scale bars, 20 μm.) See also Fig. S1.

percentage of overall Fos cells that were also immunoreactive for ChAT was equivalent between the two conditions [21.4 ± 1.8% for WC, 20.4 ± 7.0% for control, $t_{(4)} = 0.144$, $P = 0.892$], indicating that, whereas there were more Fos cells in the WC condition [$t_{(4)}$=6.399, $P = 0.003$], the percentage of cholinergic versus noncholinergic cells that were activated remained the same.

Cholinergic Cells Activated by Arousal Procedures Project to the SCN. We then asked whether this expression occurred in cholinergic neurons that innervated the SCN. We iontophoretically applied the β-subunit of cholera toxin to the SCN of hamsters (Fig. S1) that were then given access to a novel wheel, and we confirmed that Fos was found in some cholinergic neurons that projected to the SCN (Fig. 3). Additionally, we observed noncholinergic SCN-projecting cells that were activated by the procedure, as well as both cholinergic and noncholinergic SCN-projecting cells that were not activated by the procedure.

Intra-SCN Atropine Block Arousal-Induced Phase Shifts. Given that the cholinergic system was activated in nonphotic manipulations, we next asked whether cholinergic activity at the SCN was necessary for nonphotic phase shifting. To answer this question, animals received an intra-SCN infusion of either saline or the cholinergic antagonist atropine before a strongly arousing nonphotic manipulation (confinement to a novel wheel). Administration of atropine alone into the SCN resulted in a mean phase shift of −0.09 ± 0.1 h ($n = 6$). Animals had significantly smaller phase shifts to the WC procedure when they were pretreated with atropine than they did when pretreated with saline vehicle [$t_{(5)} = 3.06$, $P = 0.028$; Fig. 4 *A–C*]. There was no significant difference found between the number of wheel revolutions recorded during the saline and atropine conditions [Fig. 4*C*; $t_{(5)} = 0.08$, $P = 0.94$].

Basal Forebrain Stimulation Phase Shifts the Circadian Clock. We next asked whether activation of the basal forebrain alone would be sufficient to produce nonphotic phase shifts. To test this question, we used electrical stimulation of the basal forebrain in the midsubjective day to determine whether this alone could elicit a phase shift. Two regions were examined. The rostral basal forebrain contained the largest number of ChAT cells activated by our nonphotic procedure. Stimulation here produced significantly larger phase shifts [$n = 5$, $t_{(9)} = 3.506$, $P = 0.003$; Fig. 5 *B* and *E*] than did sham stimulation ($n = 6$; Fig. 5*A*). The caudal region of the basal forebrain was identified as having the greatest number of ChAT cells that innervated the SCN (16). Stimulation here also elicited large phase shifts (Fig. 5*C*).

Intra-SCN Atropine Blocks Shifts to Basal Forebrain Stimulation. Because electrical stimulation activates all neurons in the vicinity of the electrode tip, we wanted to confirm that it was activation of acetylcholine release at the SCN that was responsible for phase shifts to basal forebrain stimulation. We therefore infused atropine into the SCN before stimulation of the caudal basal forebrain. Atropine pretreatment before basal forebrain stimulation ($n = 4$) significantly attenuated the resulting phase shift [$t_{(5)} = 6.956$, $P < 0.001$; Fig. 5 *C* and *E*] relative to saline controls ($n = 3$). In cases where the cannula was not found to be correctly placed in the SCN but the electrode was in the basal forebrain in the proximity of cholinergic cell groups, hamsters still showed large phase shifts in response to electrical stimulation.

Given that we have previously demonstrated that sleep deprivation/arousal can induce nonphotic phase shifts (8), we examined

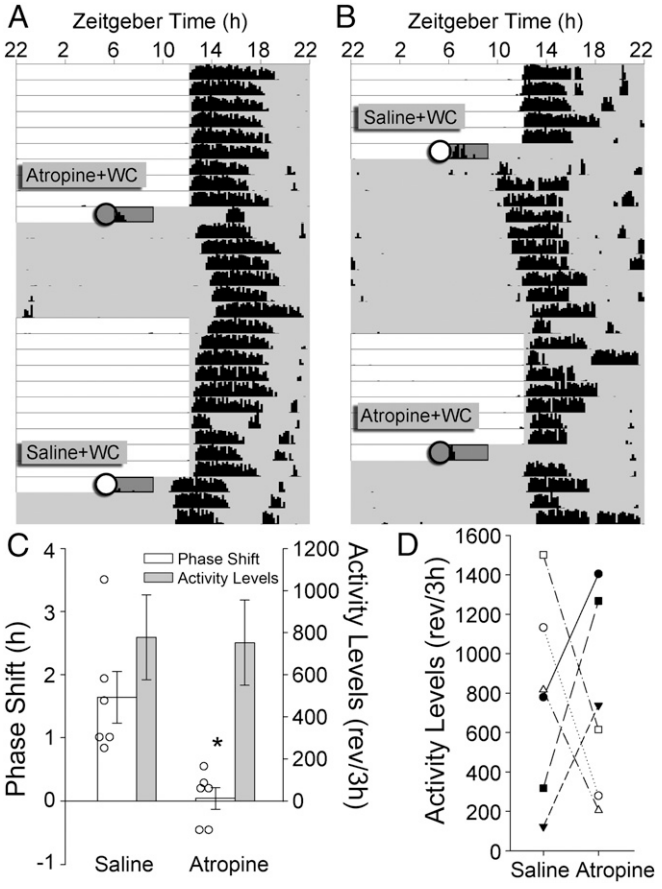

Fig. 4. Animals were pretreated with an injection into their SCN of either saline (0.5 μL, white circles) or atropine (10 mM, gray circles) 10 min before a 3-h confinement to a novel wheel. (*A* and *B*) Two representative actograms. Animals were housed in 14 h light:10 h dark until the time of the manipulation (ZT6), at which point DD began. Animals were reentrained to LD cycle between their counterbalanced treatments. (*C*) Phase shifts (wide white bars) were significantly (*$P < 0.05$) attenuated by atropine pretreatment, whereas average activity levels during the WC (narrow gray bars) were not significantly altered. Means are reported ± SEM. (*D*) Individually, some animals had higher activity with atropine pretreatment (black symbols) whereas others had lower activity (white symbols), and no relationship between activity and the resulting shift was observed.

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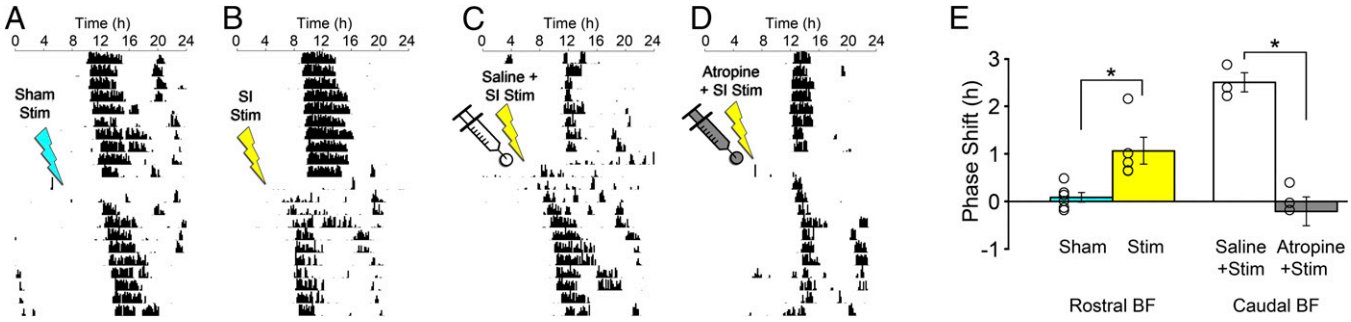

Fig. 5. Bipolar electrodes were placed in the rostral basal forebrain. This portion of the basal forebrain contained the greatest number of Fos-immunoreactive cells depicted in Fig. 2. Sham stimulation (*A*, blue lightning) from CT5 to CT7 produced no effect on circadian phase, whereas stimulation (*B*, yellow lightning, 200 μA, 0.5-ms biphasic constant current pulses, 50 Hz) produced phase advances. Stimulation of the rostral forebrain (*E*, yellow bar) produced significantly (*$P < 0.05$) greater phase shifts than did sham stimulation (*E*, blue bar). Other animals were implanted with a cannula aimed at their SCN and a bipolar electrode in their caudal basal forebrain. This area had the greatest number of SCN-projecting neurons (Fig. 3) (16). Animals received an injection of saline (*C*, white circle and syringe, 0.5 μL) or atropine (*D*, gray circle and syringe, 0.5 μL, 10 mM) before stimulation from CT5 to CT7 (yellow lightning). Atropine (*E*, gray bar) significantly (*$P < 0.05$) attenuated phase shifts to caudal basal forebrain stimulation relative to animals given saline before stimulation (*E*, white bar). Means are reported ± SEM.

activity and wakefulness during the electrical stimulation procedures. Stimulation of the target areas of the basal forebrain did produce initial arousal, but animals returned to a quiet state by the end of the procedure. All but one of these animals exhibited some locomotion throughout the procedure. However, compared with animals where the tip of the electrode missed the target areas (Fig. S2; n = 9), the amounts of activity and wakefulness were similar, yet phase shifts were significantly smaller [0.21 ± 0.21 h; $t_{(15)} = 3.736$, $P = 0.002$], indicating that the arousal alone was not sufficient to cause a shift.

Discussion

Whereas the brain regions regulating sleep and wake are separate and distinct from those regulating circadian rhythmicity (1, 2), behavioral state is determined by interplay between these systems. Notably, the circadian clock both influences (5), and is in turn influenced by (8), arousal. Through the subparaventricular zone, the SCN circadian clock communicates with many of the sleep and wake regulatory regions of the brain, including the basal forebrain (33, 34). The present results show that the cholinergic basal forebrain represents both a necessary and sufficient arousal input to the circadian clock, mediating arousal-induced clock resetting. Recently, a functional link between the brainstem arousal system and the SCN circadian clock has been identified (35), although activation of these areas produces phase delays of the circadian clock no matter which circadian time they are applied, and these responses appear to be due to glutamatergic rather than cholinergic activation.

The phase of the circadian clock can be reset by arousal. Initial reports focused on exercise or activity as the key component (9, 36), but later studies demonstrated that arousal without intense exercise was sufficient to elicit the full-magnitude nonphotic shifts (8), and it is likely that exercise is simply an easy way to achieve sufficient arousal (37). When animals are kept awake without an opportunity to move, phase shifts are not observed. Given the EEG findings here, we would predict that it is not sufficient to simply be awake but rather the animal would need to be awake enough; drowsy animals would not respond. In the present study, we used two approaches to examine how arousal could affect the circadian clock: a weakly arousing procedure (SD through gentle handling) and a strongly arousing procedure (confinement to a novel wheel). Whereas many features differ between these experiences, we believe that the critical common underlying mechanism is activation of the arousal inputs to the circadian clock. The peak EEG at 8 Hz is similar to that for hippocampal theta, which is observed with movement and sensorimotor processing (29). However, this peak was only observed in our drowsy and stationary nonresponders. The lack of power in this frequency in our moving and engaged responders argues against this 8-Hz frequency being hippocampal theta. It is likely that our EEG electrode placements were unable to detect hippocampal theta. More targeted recordings from the hippocampus could help resolve whether theta-oscillation participates in nonphotic phase shifting.

A number of neurotransmitters have been implicated in nonphotic phase shifting (37) including neuropeptide Y (NPY) (12), serotonin (11, 38), and adenosine (39). It was unclear, therefore, whether activation of the basal forebrain alone would be sufficient to elicit nonphotic phase shifts. Agonists for NPY and adenosine produce nonphotic shifts when applied to the SCN (13, 39), as do cholinergic agonists (18–20). However, in each case, the resulting phase shifts are smaller than those observed following exercise or arousal procedures. It is possible that arousal information is communicated to the SCN through a variety of parallel pathways, and that simultaneous activation of these various systems is required to produce full-magnitude phase shifts.

Research into the role of acetylcholine in the circadian system has yielded discordant findings. Initial reports suggested that the cholinergic agonist carbachol produced light-like phase shifts (40), despite acetylcholine not being a component of the input path from the retina (41). This discrepancy led to the so-called "carbachol paradox" (42) and it was suggested that carbachol acted outside the SCN to elicit these responses. When applied to the SCN in vivo, carbachol produced complex phase response curves (PRCs) that appeared to be hybrids of both the photic and nonphotic PRCs (18, 19). Bolus application of a nonselective agonist likely contributed to the confusion, as it lacks site and receptor specificity. The approach used in the present study, of electrical stimulation of the basal forebrain, would only activate SCN cells that were innervated by the cells in the stimulated region, yielding a more coherent activation profile. Looking at this problem with a nonphotic perspective helps resolve some of these discordant findings and suggests a function for the cholinergic input from the basal forebrain. Interestingly, in vitro application of carbachol to the SCN only causes phase shifts during the subjective night (41, 43). The SCN does respond in vitro to carbachol during the day (23), and nicotine causes phase shift when applied in vitro (44). One caveat to this in vitro evidence is that it comes from rat tissue, a species for which nonphotic signals appear weak at best (45). In vitro studies with hamsters may help clarify this inconsistency.

In addition to cholinergic cells, the basal forebrain has a variety of other cell types, including cells containing GABA and glutamate (46). It is likely that some of these projections are activated by both our WC and our electrical stimulation procedures. However, it is unlikely that these other projections and neurotransmitters were involved in bringing about the phase

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resetting that we found, as direct application of atropine to the SCN blocked these phase shifts. This observation provides good evidence that cholinergic activity at the SCN is necessary for arousal-induced resetting of the circadian clock. Our stimulation data highlight two regions of the basal forebrain that mediate nonphotic phase shifts: a rostral region that is strongly activated by arousal manipulations and a caudal region (the SI) that contains SCN-projecting cholinergic cells (16). Stimulation of other basal forebrain regions did not induce phase shifts. These findings may reflect the underlying circuitry in the basal forebrain, with possible connections between the two regions where stimulation yielded phase shifts.

The mechanism at the SCN that underlies arousal-induced phase shifts appears to be inhibition. Chemicals that cause nonphotic phase shifts during the day all inhibit electrical activity of the SCN (21–23). Rhythmic gene expression of the core clock gene *Per1* is inhibited by exercise (47) and SD (37).

Given that behavioral state is regulated through both circadian and homeostatic mechanisms (4), it has been suggested that the function of arousal inputs to the circadian clock could serve two functions. First, given that the circadian clock determines the timing of sleep and wake, receiving feedback from arousal areas could allow for error detection and correction by the circadian clock when arousal occurs at inappropriate times (35). Arousal and exercise may also allow the animal to inhibit light input to the clock (48), thus regulating unwanted phase resetting by light.

In summary, the present study functionally links the basal forebrain arousal system to the circadian system. The circadian system regulates the timing of sleep and wake, and through our findings we have shown how the circadian system can itself be directly influenced by the cholinergic basal forebrain. Given acetylcholine's role in learning and memory, it is possible that the basal forebrain's cholinergic input to the circadian system may contribute to circadian modulation of memory tasks (49–51). People working rotating shifts or traveling across many time zones experience wakefulness during normal sleep phases. It is possible that cholinergic interventions might accelerate adaptation to their new schedule. Finally, loss of cholinergic neurons in the basal forebrain (52) and sleep/circadian disturbances (53, 54) are early events in the progression of Alzheimer's disease. The loss of the cholinergic connection between the basal forebrain and the SCN described here may mediate some of the sleep problems experienced in patients with Alzheimer's disease. Treatments that enhance cholinergic signaling in the SCN in a time-dependent manner may ameliorate these problems and reduce institutionalization.

Materials and Methods

Animals. Adult male Syrian hamsters (Charles River Laboratories) were initially group housed, with two to three per cage in an LD cycle of 14 h light (~300 lx) and 10 h dark (0 lx) in a temperature (21 ± 1 °C) and humidity-controlled room. Food and water were available ad libitum. All procedures were approved by the Life and Environmental Sciences University of Calgary Animal Care Committee and they adhered to the guidelines of the Canadian Council of Animal Care.

Experiment 1: EEG Recordings of Sleep-Deprived Hamsters. Using sterile surgery, hamsters ($n = 12$) were fitted with an EEG assembly and were allowed to recover for 7 d before being given cages equipped with running wheels. Wheel running activity was collected on a desktop computer using ClockLab data collection software (Actimetrics).

After 10 d of stable wheel running in LD, the EEG implanted hamsters were awakened 6 h before dark onset [i.e., at zeitgeber time (ZT) 6, where ZT12 is defined as the time of lights off]. They were given a fresh home cage that was placed in a Faraday cage, and they were then connected to the EEG recording setup (Avatar EEG, Electrical Geodesics). Any time the hamsters became quiescent or attempted to adopt a stereotypical sleeping posture, they were aroused using a gentle touch or puff of air (8). The SD continued for 3 h, and EEG was recorded continuously during this period at 500 Hz via a Bluetooth connection. After the completion of the sleep deprivation and EEG recording protocol, the animal was transferred back to the home cage and the lights were turned off to calculate the phase shift using a modified Aschoff type II procedure. Those hamsters that phase advanced to the SD were determined to be responders ($n = 4$) and those hamsters that did not phase advance were determined to be nonresponders ($n = 8$).

EEG data collected throughout the 3-h SD period were imported into EEGLab (Swartz Center for Computational Neuroscience) through EDFBrowser (open source, www.teuniz.net/). The data were filtered through high (0.5 Hz) and low (100 Hz) band pass filtering to remove noise and partitioned into 10-s epochs. The data were then visually inspected and epochs with excess movement artifacts were rejected. The remaining epochs were then analyzed with a FFT for differences in power and frequency distribution in four frequency ranges (1–3 Hz, 4–7 Hz, 7–12 Hz, and 13–30 Hz). The power for each range was exported to Microsoft Excel and, after normalization with respect to the peak power in the 1–3 Hz range, compared using a 2 (responder/nonresponder) × 4 (frequency bin) factorial ANOVA using SigmaPlot 11.0 (Systat Software).

Experiment 2: Fos Expression in WC and SD Hamsters. Singly housed hamsters ($n = 17$) without access to running wheels were housed in LD for ~2 wk. The hamsters were then transferred to a recording room at ZT6. Animals were given one of three treatments: DD control in their home cage ($n = 6$), WC for 3 h ($n = 6$), or SD ($n = 5$). The lights were turned off at the start of the procedure for both the DD and WC groups, whereas dim red light (~1 lx) was used throughout the SD procedure.

At the conclusion of the DD, WC, and SD at ZT9, the hamsters were administered an overdose of sodium pentobarbital (euthanyl, ~400 mg/kg; Bimeda-MTC) and they were perfused with PBS followed by 4% (wt/vol) paraformaldehyde in PBS. The brains were collected and prepared for immunohistochemistry, and Fos expression throughout the basal forebrain was assessed (*SI Materials and Methods*).

Another set of hamsters was used to examine Fos expression in $ChAT^+$ cells. Singly housed Syrian hamsters ($n = 6$) were initially entrained to an LD cycle without access to a running wheel. At ZT6 on the day of the experiment, hamsters were moved to DD with some ($n = 3$) being confined to novel running wheels and others ($n = 3$) serving as home cage DD controls. The hamsters were perfused at ZT6 and their brains removed and prepared as described above, and Fos expression within $ChAT^+$ cells was assessed (*SI Materials and Methods*).

Experiment 3: Retrograde Tracing from the SCN to the Forebrain During WC. Syrian hamsters ($n = 16$) were give iontophoretic infusions of cholera toxin β (CTβ) using sterile stereotaxic procedure (*SI Materials and Methods*). The hamsters were allowed to recover for 10 d following surgery before being transferred to DD at ZT6. They were confined to a novel running wheel for 3 h and then perfused at ZT9 as described above. The brains were removed, sectioned, and processed for triple label fluorescent immunohistochemistry for CTβ, Fos, and ChAT.

Experiment 4: Saline or Atropine Injection into the SCN Before Novel WC. Hamsters ($n = 18$) were implanted with a cannula aimed at their SCN. Following recovery from surgery, the animals were placed in LD in cages equipped with running wheels. On the day of manipulation animals were given a 0.5-μL injection of either physiological saline ($n = 6$) or atropine sulfate ($n = 6$, 10 mM; Sigma-Aldrich) unilaterally into the SCN 10 min before confinement to a novel wheel (a mesh-covered 24.2-cm diameter wheel). Using an Aschoff type II design, the WC took place as described above starting at ZT6. The lights were turned off at the start of the procedure and remained off for 4–10 d following the manipulation. Following reentrainment, the experiment was repeated with animals receiving the alternate pretreatment before the WC. A control condition was also conducted where animals received only atropine injections into the SCN without a WC ($n = 7$). At the conclusion of the study, brains were examined to assess the placement of the implant.

The phase shifts in the saline condition were compared with the atropine condition using a paired *t* test. The number of revolutions during the wheel confinement in both groups was compared using a paired *t* test to determine whether atropine affected motor activity.

Experiment 5: Electrical Stimulation of the Cholinergic Forebrain. Syrian hamsters ($n = 13$) were surgically implanted with a bipolar electrode aimed at the ventral basal forebrain. Following recovery from surgery (2–4 d), the hamsters were placed in cages equipped with running wheels as described above and released into DD. On the day of the manipulation, the implanted electrodes were connected to the stimulator, and electrical stimulations took place from circadian time (CT) 5 to CT7. The animals were able to freely move about their cages throughout the stimulation, and their behavior was observed with the aid of night-vision goggles (General Starlight Company). A control procedure also took place where animals ($n = 5$) were connected

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to the stimulator from CT5 to CT7 but no current was passed. Following the stimulation, the hamsters were kept in the home cages under DD and the wheel running activity was recorded as described above. The differences in phase shift magnitude were examined using an independent samples *t* test.

Using sterile stereotaxic surgery, another set of hamsters (n = 23) was implanted with both a cannula aimed at the SCN and a bipolar electrode aimed at the dorsocaudal basal forebrain (*SI Materials and Methods*). Following recovery from surgery, the animals were placed in DD in running wheels similar to the other experiments already described. Animals received an intra-SCN injection of either 0.5 μL of physiological saline or atropine (10 mM; Sigma-Aldrich) 10 min before CT5. The hamsters were then attached to the isolated pulse stimulator, the lights were turned off, and stimulation took place from CT5 to CT7 as described above.

All animals with a bipolar electrode were perfused and their brains were collected to assess placement of their implants. Phase shifts were calculated as described above.

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1. Antle MC (2015) *Sleep, Neural Systems. International Encyclopedia of the Social and Behavioral Sciences* (Elsevier, Amsterdam, The Netherlands), 2nd Ed, pp 87–93.
2. Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW (2012) Control of sleep and wakefulness. *Physiol Rev* 92(3):1087–1187.
3. Antle MC, Silver R (2005) Orchestrating time: Arrangements of the brain circadian clock. *Trends Neurosci* 28(3):145–151.
4. Borbély AA (1982) A two process model of sleep regulation. *Hum Neurobiol* 1(3): 195–204.
5. Mistlberger RE (2005) Circadian regulation of sleep in mammals: Role of the suprachiasmatic nucleus. *Brain Res Brain Res Rev* 49(3):429–454.
6. Deboer T, Vansteensel MJ, Détári L, Meijer JH (2003) Sleep states alter activity of suprachiasmatic nucleus neurons. *Nat Neurosci* 6(10):1086–1090.
7. van Oosterhout F, et al. (2012) Amplitude of the SCN clock enhanced by the behavioral activity rhythm. *PLoS One* 7(6):e39693.
8. Antle MC, Mistlberger RE (2000) Circadian clock resetting by sleep deprivation without exercise in the Syrian hamster. *J Neurosci* 20(24):9326–9332.
9. Bobrzynska KJ, Mrosovsky N (1998) Phase shifting by novelty-induced running: Activity dose-response curves at different circadian times. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 182(2):251–258.
10. Edgar DM, Reid MS, Dement WC (1997) Serotonergic afferents mediate activity-dependent entrainment of the mouse circadian clock. *Am J Physiol* 273(1 Pt 2): R265–R269.
11. Mistlberger RE, Antle MC, Glass JD, Miller JD (2000) Behavioral and serotonergic regulation of circadian rhythms. *Biol Rhythm Res* 31(3):240–283.
12. Biello SM, Janik D, Mrosovsky N (1994) Neuropeptide Y and behaviorally induced phase shifts. *Neuroscience* 62(1):273–279.
13. Huhman KL, Albers HE (1994) Neuropeptide Y microinjected into the suprachiasmatic region phase shifts circadian rhythms in constant darkness. *Peptides* 15(8):1475–1478.
14. Castillo-Ruiz A, Nunez AA (2007) Cholinergic projections to the suprachiasmatic nucleus and lower subparaventricular zone of diurnal and nocturnal rodents. *Brain Res* 1151:91–101.
15. Kiss J, Halász B (1996) Synaptic contacts between cholinergic afferents and suprachiasmatic neurones of the rat. *Neuroreport* 7(12):1961–1964.
16. Bina KG, Rusak B, Semba K (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(2):295–307.
17. van der Zee EA, Streefland C, Strosberg AD, Schröder H, Luiten PG (1991) Colocalization of muscarinic and nicotinic receptors in cholinoceptive neurons of the suprachiasmatic region in young and aged rats. *Brain Res* 542(2):348–352.
18. Bina KG, Rusak B (1996) Muscarinic receptors mediate carbachol-induced phase shifts of circadian activity rhythms in Syrian hamsters. *Brain Res* 743(1–2):202–211.
19. Meijer JH, van der Zee E, Dietz M (1988) The effects of intraventricular carbachol injections on the free-running activity rhythm of the hamster. *J Biol Rhythms* 3(4): 333–348.
20. Basu P, Wensel AL, McKibbon R, Lefebvre N, Antle MC (2016) Activation of M1/4 receptors phase advances the hamster circadian clock during the day. *Neurosci Lett* 621:22–27.
21. Liou SY, Albers HE (1991) Single unit response of neurons within the hamster suprachiasmatic nucleus to neuropeptide Y. *Brain Res Bull* 27(6):825–828.
22. Shibata S, Liou SY, Ueki S (1983) Effect of amino acids and monoamines on the neuronal activity of suprachiasmatic nucleus in hypothalamic slice preparations. *Jpn J Pharmacol* 33(6):1225–1231.
23. Yang JJ, Wang YT, Cheng PC, Kuo YJ, Huang RC (2010) Cholinergic modulation of neuronal excitability in the rat suprachiasmatic nucleus. *J Neurophysiol* 103(3):1397–1409.
24. Lee MG, Hassani OK, Alonso A, Jones BE (2005) Cholinergic basal forebrain neurons burst with theta during waking and paradoxical sleep. *J Neurosci* 25(17):4365–4369.
25. Han Y, et al. (2014) Selective activation of cholinergic basal forebrain neurons induces immediate sleep-wake transitions. *Curr Biol* 24(6):693–698.
26. Semba K (1991) The cholinergic basal forebrain: A critical role in cortical arousal. *Adv Exp Med Biol* 295:197–218.
27. Irmak SO, de Lecea L (2014) Basal forebrain cholinergic modulation of sleep transitions. *Sleep* 37(12):1941–1951.
28. Xu M, et al. (2015) Basal forebrain circuit for sleep-wake control. *Nat Neurosci* 18(11): 1641–1647.
29. Bland BH (1986) The physiology and pharmacology of hippocampal formation theta rhythms. *Prog Neurobiol* 26(1):1–54.
30. Cantero JL, Atienza M, Salas RM (2002) Human alpha oscillations in wakefulness, drowsiness period, and REM sleep: Different electroencephalographic phenomena within the alpha band. *Neurophysiol Clin* 32(1):54–71.
31. Jones BE (2004) Activity, modulation and role of basal forebrain cholinergic neurons innervating the cerebral cortex. *Prog Brain Res* 145:157–169.
32. Jones BE (2008) Modulation of cortical activation and behavioral arousal by cholinergic and orexinergic systems. *Ann N Y Acad Sci* 1129:26–34.
33. Saper CB, Lu J, Chou TC, Gooley J (2005) The hypothalamic integrator for circadian rhythms. *Trends Neurosci* 28(3):152–157.
34. Vujovic N, Gooley JJ, Jhou TC, Saper CB (2015) Projections from the subparaventricular zone define four channels of output from the circadian timing system. *J Comp Neurol* 523(18):2714–2737.
35. Abbott SM, et al. (2013) Signals from the brainstem sleep/wake centers regulate behavioral timing via the circadian clock. *PLoS One* 8(8):e70481.
36. Mrosovsky N (1996) Locomotor activity and non-photic influences on circadian clocks. *Biol Rev Camb Philos Soc* 71(3):343–372.
37. Webb IC, Antle MC, Mistlberger RE (2014) Regulation of circadian rhythms in mammals by behavioral arousal. *Behav Neurosci* 128(3):304–325.
38. Yamakawa GR, Antle MC (2010) Phenotype and function of raphe projections to the suprachiasmatic nucleus. *Eur J Neurosci* 31(11):1974–1983.
39. Antle MC, Steen NM, Mistlberger RE (2001) Adenosine and caffeine modulate circadian rhythms in the Syrian hamster. *Neuroreport* 12(13):2901–2905.
40. Zatz M, Herkenham MA (1981) Intraventricular carbachol mimics the phase-shifting effect of light on the circadian rhythm of wheel-running activity. *Brain Res* 212(1): 234–238.
41. Liu C, Gillette MU (1996) Cholinergic regulation of the suprachiasmatic nucleus circadian rhythm via a muscarinic mechanism at night. *J Neurosci* 16(2):744–751.
42. Colwell CS, Kaufman CM, Menaker M (1993) Phase-shifting mechanisms in the mammalian circadian system: New light on the carbachol paradox. *J Neurosci* 13(4):1454–1459.
43. Buchanan GF, Gillette MU (2005) New light on an old paradox: Site-dependent effects of carbachol on circadian rhythms. *Exp Neurol* 193(2):489–496.
44. Trachsel L, Heller HC, Miller JD (1995) Nicotine phase-advances the circadian neuronal activity rhythm in rat suprachiasmatic nuclei explants. *Neuroscience* 65(3):797–803.
45. Mistlberger RE (1991) Effects of daily schedules of forced activity on free-running rhythms in the rat. *J Biol Rhythms* 6(1):71–80.
46. Gritti I, Manns ID, Mainville L, Jones BE (2003) Parvalbumin, calbindin, or calretinin in cortically projecting and GABAergic, cholinergic, or glutamatergic basal forebrain neurons of the rat. *J Comp Neurol* 458(1):11–31.
47. Maywood ES, Mrosovsky N, Field MD, Hastings MH (1999) Rapid down-regulation of mammalian period genes during behavioral resetting of the circadian clock. *Proc Natl Acad Sci USA* 96(26):15211–15216.
48. Mistlberger RE, Antle MC (1998) Behavioral inhibition of light-induced circadian phase resetting is phase and serotonin dependent. *Brain Res* 786(1–2):31–38.
49. Craig LA, Hong NS, Kopp J, McDonald RJ (2009) Cholinergic depletion of the medial septum followed by phase shifting does not impair memory or rest-activity rhythms measured under standard light/dark conditions in rats. *Brain Res Bull* 79(1):53–62.
50. Gritton HJ, Stasiak AM, Sarter M, Lee TM (2013) Cognitive performance as a zeitgeber: Cognitive oscillators and cholinergic modulation of the SCN entrain circadian rhythms. *PLoS One* 8(2):e56206.
51. Hut RA, Van der Zee EA (2011) The cholinergic system, circadian rhythmicity, and time memory. *Behav Brain Res* 221(2):466–480.
52. Auld DS, Kornecook TJ, Bastianetto S, Quirion R (2002) Alzheimer's disease and the basal forebrain cholinergic system: relations to β-amyloid peptides, cognition, and treatment strategies. *Prog Neurobiol* 68(3):209–245.
53. Sterniczuk R, Theou O, Rusak B, Rockwood K (2013) Sleep disturbance is associated with incident dementia and mortality. *Curr Alzheimer Res* 10(7):767–775.
54. Sterniczuk R, Antle MC (2015) Alzheimer's disease and the mistiming of behavior. *Circadian Medicine*, ed Colwell CS (Wiley-Blackwell, Hoboken, NJ), pp 283–294.
55. Antle MC, Marchant EG, Niel L, Mistlberger RE (1998) Serotonin antagonists do not attenuate activity-induced phase shifts of circadian rhythms in the Syrian hamster. *Brain Res* 813(1):139–149.
56. Antle MC, Tse F, Koke SJ, Sterniczuk R, Hagel K (2008) Non-photic phase shifting of the circadian clock: role of the extracellular signal-responsive kinases I/II/mitogen-activated protein kinase pathway. *Eur J Neurosci* 28(12):2511–2518.
57. Mistlberger RE, et al. (2003) Circadian clock resetting by arousal in Syrian hamsters: The role of stress and activity. *Am J Physiol Regul Integr Comp Physiol* 285(4): R917–R925.
58. Mrosovsky N (1988) Phase response curves for social entrainment. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 162(1):35–46.
59. Antle MC, Silver R (2016) Circadian insights into motivated behavior. *Curr Top Behav Neurosci* 27:137–169.
60. Gannon RL, Rea MA (1995) Twelve-hour phase shifts of hamster circadian rhythms elicited by voluntary wheel running. *J Biol Rhythms* 10(3):196–210.
61. Casamenti F, Deffenu G, Abbamondi AL, Pepeu G (1986) Changes in cortical acetylcholine output induced by modulation of the nucleus basalis. *Brain Res Bull* 16(5): 689–695.

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