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Extraocular Circadian Phototransduction in Humans
Scott S. Campbell, *et al.*
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HA by a human influenza A virus could make a virus lethal (*16*). It remains to be shown whether gene segments other than the HA may also have contributed to the ability of the virus to infect a human.

A pandemic of influenza could begin with isolated cases, in which avian or swine influenza viruses adapt to human hosts or, over time, genetically reassort with circulating human influenza A viruses, or it could begin as a rapid and explosive spread of a pandemic virus derived from a reassortment event in an intermediate host. Increased surveillance efforts have been initiated to identify other cases of illness associated with influenza A (H5N1) viruses. In addition, serosurveys are under way in an effort to identify asymptomatic or mild clinical infections in the region and substantiate the previous report of seroprevalence to H5 viruses (*3*). These studies may determine whether H5 viruses similar to A/Hong Kong/156/97 are circulating in the human population and if the isolation of the A/Hong Kong/156/97 virus is the first step in the recognition of an influenza A virus with pandemic potential or whether this case is simply an isolated event.

Note added in proof: Since the submission of this report, there have been 12 additional confirmed human cases of influenza A (H5N1) infections in Hong Kong, including three fatalities. Sequence analysis of the genes of six of the isolates revealed that all of the genes are of avian origin and are closely related to each other. The HA gene codes for a multiple basic amino acid insertion upstream of the cleavage site, associated with highly pathogenic avian influenza viruses and identical to that seen in the A/Hong Kong/156/97 virus.

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Extraocular Circadian Phototransduction in Humans

Scott S. Campbell* and Patricia J. Murphy

Physiological and behavioral rhythms are governed by an endogenous circadian clock. The response of the human circadian clock to extraocular light exposure was monitored by measurement of body temperature and melatonin concentrations throughout the circadian cycle before and after light pulses presented to the popliteal region (behind the knee). A systematic relation was found between the timing of the light pulse and the magnitude and direction of phase shifts, resulting in the generation of a phase response curve. These findings challenge the belief that mammals are incapable of extraretinal circadian phototransduction and have implications for the development of more effective treatments for sleep and circadian rhythm disorders.

Circadian rhythms are endogenously generated oscillations of about 24 hours that provide temporal structure to a wide range of behavioral and physiological functions. Because the endogenous clock tends to run at a period close to but not exactly 24 hours, a daily adjustment, usually by the natural light-dark cycle, is required to synchronize or entrain circadian rhythms to the external environment. Many vertebrate and nonvertebrate species have multiple photoreceptor systems through which circadian entrainment may be achieved (*1–3*). In the house sparrow, for example, three discrete input pathways for light to act on the circadian system have been identified (*4*). Similarly, a number of fish, amphibian, and reptile species have extraocular and extrapineal pathways for circadian light transduction (*5*).

The photoreceptors responsible for entraining the mammalian biological clock may not be the same cells that mediate vision (*6*). Mice homozygous for the autosomal recessive allele *rd* (retinally degenerate), which have no electrophysiological or behavioral visual responses to light, can be entrained to a light-dark cycle (*7*). Likewise, bright light suppresses melatonin output in some totally blind humans, despite the fact that they have no conscious light perception and no pupillary light reflex (*8*). Such findings support the hypothesis that all vertebrates, including mammals, have specialized nonvisual photoreceptors that mediate circadian responses to the light-dark cycle. It is generally assumed, however, that nonvisual circadian photoreceptors in mammals reside within the retina, and that mammals do not have the capacity for extraocular circadian photoreception (*1, 2, 9*). This conclusion is based on studies showing a failure of several rodent species to entrain to a light-dark cycle or to respond to pulses of light with shifts in circadian phase after complete optic enucleation (*10*). In addition, Czeisler and co-workers found an absence of light-induced melatonin suppression during ocular shielding in two individuals who did show suppression when light fell on their eyes (*8*). A decade earlier, Wehr and co-workers reported a lack of clinical response in seasonal affective disorder when patients' skin (face, neck, arms, legs) was exposed to a bright light stimulus while their eyes were shielded (*11*). However, in that study, no physiological measures of light response, such as melatonin secretion or temperature phase re-

Laboratory of Human Chronobiology, Department of Psychiatry, Cornell University Medical College, 21 Bloomingdale Road, White Plains, NY 10605, USA.

*To whom correspondence should be addressed.

sponse, were obtained.

Here we present results that demonstrate that the human circadian response to light can be mediated through an extraocular route. A total of 33 phase-shifting trials were carried out in 15 healthy individuals (mean age, 35.7 years; range, 22 to 67 years; 13 males, 2 females) (12). Each laboratory session lasted for four consecutive days and nights, during which the participants were assigned randomly to either a control or an active condition. Successive laboratory visits were separated by at least 10 days. During the active sessions (phase delay, n = 13; phase advance, n = 11), light was presented at various times relative to the baseline circadian phase, in order to examine phase response throughout the circadian cycle. The extraocular light stimulus consisted of a 3-hour pulse of light presented to the popliteal region, the area directly behind the knee joint (13). The stimulus was presented in ambient light of less than 20 lux. Throughout their stay in the laboratory, when not sleeping and not involved in the experimental light manipulation, participants were in constant illumination of less than 50 lux.

On the night before (night 1 in the laboratory) and the nights after the light stimulus (nights 3 and 4) participants were required to remain in bed (and were allowed to sleep) from 2400 until noon the following day. On the light exposure night (night 2 in the laboratory), sleep was necessarily displaced to accommodate presentation of the 3-hour light pulse. With the exception of this interval, the participants were in bed from 2400 until noon on night 2 as well. Sleep was not permitted during the light exposure interval, and continuous electroencephalogram and video monitoring of participants throughout the exposure interval ensured compliance.

Body core temperature was recorded continuously (14). In a subset of sessions (n = 18), hourly saliva samples were also collected for melatonin assay (15). The nadir of the temperature rhythm and the dim light melatonin onset (DLMO) were used to evaluate circadian phase before and after presentation of the light pulse (16). The magnitude of the phase shift achieved in each trial was determined by comparing participants' baseline circadian phase (during the first 24 hours in the laboratory) with the phase determined during the final 24 hours in the laboratory.

Examples from single individuals of the phase shifts achieved as a result of light presented before (producing a delay) and after (producing an advance) the minimum of the circadian temperature rhythm (T_{min}) are shown in Figs. 1 and 2. For all active sessions, there was a systematic relation between the timing of the light pulse and the magnitude and direction of the phase shift,

resulting in a classic phase response curve (Fig. 3A). Paired t tests revealed that shifts in both the delay and advance directions were statistically significant (mean delay = 1.43 hours, $P = 0.0001$; mean advance = 0.58 hours, $P = 0.024$). Six of the seven participants who underwent both active and control conditions showed a larger shift in the active condition when compared with their own control condition (mean difference = 1.29 hours, $P = 0.011$). It should be noted, however, that the phase of light presentation was not matched for individual participants under active and control conditions.

In 18 of the 24 active sessions, we assessed the phase response of a second circadian marker, the onset of the endogenous melatonin rhythm under dim light conditions (DLMO) (Fig. 3B). The direction and magnitude of the shifts in DLMO were equivalent to those for temperature. Indeed,

there was a significant correlation between the shift in body core temperature and the shift in DLMO (Spearman rank-order correlation: $\rho = 0.704$; $P = 0.009$). As with temperature, delay and advance shifts in DLMO were statistically significant (mean delay = 0.92 hours, $P = 0.0009$; mean advance = 1.17 hours, $P = 0.021$).

The phase shifts in the active sessions were the consequence of the light administration and not systematically influenced by the experimental procedure itself. In the control condition, participants underwent the identical protocol as in the delay condition, including application of the fiber optic pad and activation of the exhaust fans (13). However, in the control condition, the halogen bulb providing illumination to the optic pad was disconnected. Because in all conditions the light source was not turned on until they were seated and an opaque "skirt" was in place, participants were unaware of
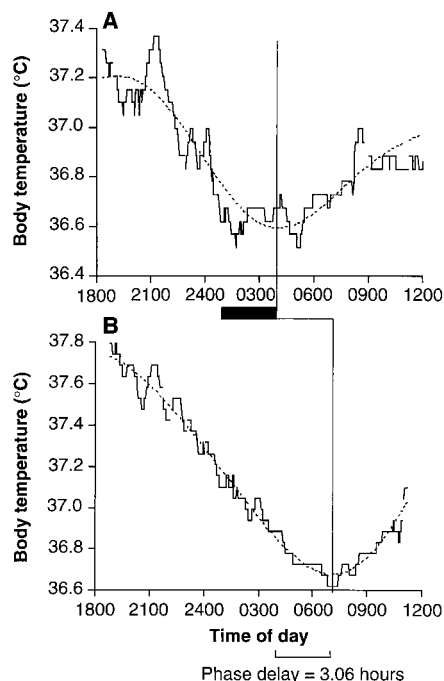
Fig. 1. Example of a delay in circadian phase in response to a 3-hour bright light presentation to the popliteal region. Light was presented on one occasion between 0100 and 0400 on night 2 in the laboratory (black bar) while the participant (a 29-year-old male) remained awake and seated in a dimly lit room (ambient illumination <20 lux). The circadian phase was determined by fitting a complex cosine curve (dotted line) to the raw body core temperature data (solid line). Resulting phase estimates are indicated by vertical lines. The baseline (night 1) circadian phase (**A**) occurred at 0404; the circadian phase after light presentation (**B**) (last 24 hours in the laboratory) occurred at 0708. The phase angle between the midpoint of the light stimulus and the fitted body temperature minimum at baseline was 1.57 hours. The resulting phase delay was 3.06 hours.
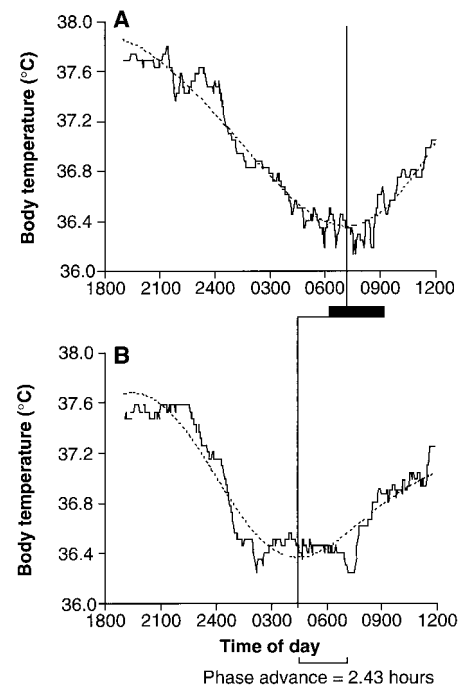

Fig. 2. Example of an advance in circadian phase in response to a 3-hour bright light presentation to the popliteal region. Light was presented on one occasion between 0600 and 0900 after night 2 in the laboratory (black bar) while the participant (a 44-year-old male) remained awake and seated in a dimly lit room (ambient illumination <20 lux). The circadian phase was determined by fitting a complex cosine curve (dotted line) to the raw body core temperature data (solid line). Resulting phase estimates are indicated by vertical lines. The baseline (night 1) circadian phase (**A**) occurred at 0713; the circadian phase after light presentation (**B**) (last 24 hours in the laboratory) occurred at 0453. The phase angle between the midpoint of the light stimulus and the fitted body temperature minimum at baseline was 0.28 hour. The resulting phase advance was 2.34 hours.

whether light was actually being presented during a given session. Comparison of the phase of body temperature at baseline and after the control manipulation revealed no significant shift as a result of exposure to this protocol (mean shift = 0.37 hours, P = 0.103), with five individuals showing delays, three showing advances, and one showing no phase change. There was no relation between the degree or direction of shift and proximity of sham light exposure to T_{min} (Fig. 3C). Repeated measures analysis of variance of the circadian temperature phase across the entire control condition revealed
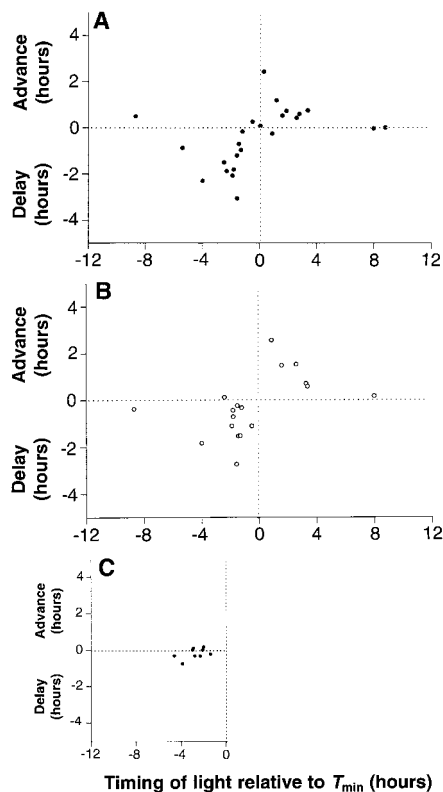

Fig. 3. Response of the endogenous circadian pacemaker, as measured by body core temperature (**A**) and by DLMO (**B**), to a single 3-hour presentation of bright light to the popliteal region. Each point represents the phase shift observed (advances are designated by positive numbers and delays by negative numbers on the y axis) in response to bright light presented at a given time relative to the phase of body core temperature at baseline. "Timing of light relative to T_{min}" (x axis) refers to the interval between the midpoint of light presentation and the fitted temperature minimum. The magnitude of the observed phase shifts varied systematically as a function of this relation. The strong correlation between the two phase markers used (ρ = 0.704, P = 0.009) strongly suggests that the extraocular light stimulus directly influenced the endogenous circadian clock and not simply the output variables. (**C**) The response of the circadian clock, as measured by body temperature, to the no-light control condition. All no-light presentations occurred before T_{min}; therefore, only that portion of the x axis is shown.

no significant change in phase (P = 0.539), confirming the reliability of temperature as a circadian phase marker. The average intra-individual standard deviation in the control condition was 25.2 min.

Our results challenge the widely held belief that mammals are incapable of extraocular circadian phototransduction. The overall temporal profile, as well as the magnitude of the phase shifts achieved with our extraocular light stimulus, is similar to those reported by investigators who used single-pulse, full-spectrum light stimuli presented to the eyes (*17*). The light stimulus we used was composed of a relatively narrow bandwidth (455 to 540 nm). Yet, in a pilot study with an identical protocol but with broad-band white light from commercial fluorescent light boxes (*18*) placed beneath participants' knees, we observed phase delays (no advances were attempted) of similar magnitude to those reported here.

Accurate characterization of the mechanisms of extraocular phototransduction has been difficult, even with respect to the ubiquitous "deep brain photoreceptors" of non-mammalian vertebrates (*19*). Oren has recently proposed a model in which the circulatory system plays a key role (*20*). This "humoral phototransduction" hypothesis posits that light of sufficient intensity, falling on a vascular surface, sets in motion a process by which neuroactive gases transported in and regulated by blood-borne photoreceptors (for example, hemoglobin in erythrocytes) stimulate the neural pathways that entrain biological rhythms. In support of the model is evidence that bright light can dissociate neuroactive gases such as carbon monoxide (CO) and nitric oxide (NO) from heme moieties (*21*); that light exposure can further increase circulating NO concentrations by increasing the activity of NO synthase (*22*); and that NO can shift circadian phase in a manner similar to light (*23*). These facts, when integrated with the vasodilating capability of CO and NO (*24*), constitute a mechanism by which photic cues can be conveyed to the circadian clock. Although the author proposes that humoral phototransduction occurs primarily by means of light falling on retinal vasculature, the hypothesis may apply to extraocular, peripherally mediated circadian phototransduction as well. Whatever the mechanism that underlies extraocular circadian phototransduction, the pathway involved is likely distinct from those currently hypothesized to be responsible for providing the human circadian clock with photic information (*6*).

Timed bright light exposure is an effective treatment for sleep and circadian rhythm disorders including jet lag, shift work sleep disturbance, age-related insomnia, and advanced- and delayed-sleep phase syndromes (*25*). The finding that the endogenous clock can be manipulated through an extraocular route could lead to the development of delivery systems and treatment regimens that may increase the effectiveness of this promising nondrug treatment. For example, treatment regimens that use extraocular light exposure may be able to take advantage of more efficient timing schedules. The nature of the phase response curve to light dictates that the largest shifts, both advances and delays, occur at times during which people are usually asleep.

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12. The protocol was approved by Cornell University Medical College's Committee on Human Rights in Research. All participants gave written consent after being informed of the nature and potential risks of the study. They were paid for their participation.
13. The light source was a BiliBlanket Plus (Ohmeda), a fiber optic phototherapy device commonly used for home treatment of hyperbilirubinemia. The device consists of a halogen lamp in a vented metal housing, which also contains a small fan to disperse heat generated by the lamp. Illumination from the halogen bulb leaves the housing through 2400 optic fibers encased in a flexible plastic tube 1 m in length. The optic fibers terminate in a ~10 cm by ~15 cm woven pad about ~0.64 cm thick. Because the light source is remote, the fiber optic pad generates no heat. A Biliblanket pad was placed over the popliteal area of each leg and secured in place with a polyester athletic knee brace. During the 3-hour light exposure interval, participants remained seated in a reclining chair, with a table positioned over their laps. To ensure that the light stimulus did not reach the retina, we draped a 3 m by 3 m black, opaque, double thickness polyester "skirt" over the table that reached the floor on all sides and secured it with Velcro around the partici-

pant's waist. The lamp housing was placed beneath the table and under the skirt, so that any light escaping through the housing vents was obscured from the participant's eyes. Illumination at the participant's eye level never exceeded 20 lux. Each BiliBlanket provided ~13,000 lux to the popliteal region. An exhaust fan (in addition to those in each BiliBlanket housing) was placed beneath the skirt to evacuate any heat produced by the halogen light source.

14. Body core temperature was recorded in 2-min epochs, with disposable rectal thermistors (Yellow Springs) attached to Mini-logger ambulatory recording devices (Mini-Mitter, Sun River, OR).

15. Saliva samples were collected under dim light from 1800 until 2400 on night 2 (before light exposure) and on night 4. Melatonin concentrations were measured by radioimmunoassay (ALPCO, Windham, NH) with the Kennaway G280 antibody [G. M. Vaughan *et al*., *J. Pineal Res.* **15**, 88 (1993)]. All samples from a given participant during a given laboratory session were analyzed in the same assay. We have calculated an intra-assay coefficient of variation of 2.1%; the interassay precision has been reported as 10.4% (*26*).

16. The raw temperature data set for each participant was divided into 24-hour subsets and demasked {to account for any evoked effect of sleep on body temperature [D. S. Minors and J. M. Waterhouse, *Chronobiol. Int.* **6**, 29 (1989)]}, and then each subset was fit with a complex cosine curve with a 24-hour and a 12-hour harmonic. All temperature curves reached a goodness-of-fit criterion of $r^2 \geq 0.8$ (variance accounted for); one participant whose baseline temperature curve did not meet this requirement was excluded from the analyses. The nadirs (fitted minima) of the fitted curves were used to determine the phase of the body core temperature rhythm. The DLMO was defined as the time at which salivary melatonin concentrations exceeded 3.33 pg/ml. This threshold was based on a conventional definition of a 10 pg/ml threshold for plasma melatonin concentrations [A. J. Lewy and R. L. Sack, *ibid.*, p. 93] and published evidence that the radioimmunoassay used here yields salivary melatonin concentrations ~30% of those obtained in plasma (*26*).

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Postsynaptic Membrane Fusion and Long-Term Potentiation

Pierre-Marie Lledo,* Xiangyang Zhang, Thomas C. Südhof, Robert C. Malenka, Roger A. Nicoll†

The possibility that membrane fusion events in the postsynaptic cell may be required for the change in synaptic strength resulting from long-term potentiation (LTP) was examined. Introducing substances into the postsynaptic cell that block membrane fusion at a number of different steps reduced LTP. Introducing SNAP, a protein that promotes membrane fusion, into cells enhanced synaptic transmission, and this enhancement was significantly less when generated in synapses that expressed LTP. Thus, postsynaptic fusion events, which could be involved either in retrograde signaling or in regulating postsynaptic receptor function or both, contribute to LTP.

Brief repetitive stimulation of excitatory synapses in many regions of the central nervous system results in a long-lasting increase in synaptic strength referred to as long-term potentiation (LTP). Although LTP at most synapses is known to require the activation of the *N*-methyl-D-aspartate (NMDA) subclass of glutamate receptor and a subsequent rise in postsynaptic calcium concentration, the steps involved in generating the persistent increase in synaptic strength are poorly understood (*1*). Thus, it is still unresolved whether the increase in synaptic strength results primarily from a persistent increase in the release of glutamate (the transmitter at excitatory synapses) or from a persistent increase in the sensitivity of the postsynaptic cell to glutamate.

Regardless of which mechanism proves to be correct, an attractive hypothesis is that membrane fusion events in the postsynaptic cell play an important role in LTP. A presynaptic LTP expression mechanism requires the release of retrograde messengers from the postsynaptic cell, a process that could involve either membrane-permeant messengers or the exocytosis of messenger from the postsynaptic cell (*2*). A proposed postsynaptic expression mechanism involves the all-or-none up-regulation of glutamate receptors, possibly by the insertion of membrane containing glutamate receptors (*3*). Thus, membrane fusion events in the postsynaptic cell could be required for generation of both the pre- and postsynaptic modifications that have been proposed to occur during LTP. To test this possibility we examined the effects on LTP of several agents that disrupt membrane fusion by interrupting different steps in the protein-protein interaction cascade involved in membrane fusion. In addition, we examined the effects of introducing a recombinant protein into the postsynaptic cell that promotes fusion.

Standard hippocampal slice and electrophysiological recording techniques were used for all experiments (*4*). All compounds were introduced directly into the postsynaptic cell through sharp, intracellular recording microelectrodes, which were used to prevent the washout of LTP that occurs with whole-cell recording (*5*). In all experiments we compared the responses recorded intracellularly with those recorded simultaneously from an extracellular recording electrode placed nearby in the stratum radiatum. This permitted us to monitor the stability of the preparation and, importantly, the generation of LTP in the cells surrounding the manipulated cell simultaneously.

First, we tested the effects of *N*-ethylmaleimide (NEM), which blocks NEM-sensitive factor (NSF), a cytosolic adenosine triphosphate–binding protein that, by interacting with SNAPs (soluble NSF-attachment proteins), is required for a large number of membrane fusion reactions (*6*). NEM (5 mM) was dissolved in the electrode solution (2 M potassium acetate) and loaded into the postsynaptic cell by diffusion from the intracellular electrode. To allow sufficient time for NEM to diffuse into the cell, we waited 30 to 50 min before attempting to elicit LTP with tetanic stimulation (Fig. 1). Although tetanic stimulation produced a large LTP in the field potential recording,

P.-M. Lledo, Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143, USA.
X. Zhang and T. C. Südhof, Department of Molecular Genetics and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75235, USA.
R. C. Malenka, Departments of Psychiatry and Physiology, University of California, San Francisco, CA 94143, USA.
R. A. Nicoll, Departments of Cellular and Molecular Pharmacology and Physiology, University of California, San Francisco, CA 94143, USA.

*Present address: CNRS, Institut A. Fessard, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France.
†To whom correspondence should be addressed. E-mail: nicoll@phy.ucsf.edu