Integration and saturation within the circadian photic entrainment pathway of hamsters

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Nelson, Dwight E., and Joseph S. Takahashi. Integration and saturation within the circadian photic entrainment pathway of hamsters. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1351–R1361, 1999.—The sensitivity of the visual pathway that subserves circadian entrainment was measured in hamsters after prior stimulation and using trains of multiple pulses. Immediately after subsaturating stimulation in the late subjective night, there was a significant decrease in responsiveness that persisted for at least 1 h. The reduced responsiveness was not due to light adaptation (shifting of the stimulus-response curve) but rather to response saturation, which appeared to reduce the sensitivity to subsequent stimulation and limit the maximum response of the pacemaker. The system, therefore, integrates the total number of photons delivered in two light stimuli separated in time by up to 1 h. The responsiveness was also measured using stimulus trains containing 10–1,000 individual pulses of equal irradiance and equal total photons. Results suggest that this pathway is responsive to the total photons delivered in all of the stimuli and is not responsive to light onsets or offsets associated with individual stimuli. These data outline several fundamental characteristics of phase shifting for the circadian photic entrainment pathway in hamsters. Knowledge of these characteristics is important for designing and interpreting results of future studies to dissect the cellular and molecular nature of the mammalian circadian clock and for understanding how visual information affects the cellular clock during entrainment.

phase shifts; biological clock; suprachiasmatic nuclei; activity rhythms

CIRCADIAN RHYTHMS are a common feature of physiological systems and provide organisms with an internal temporal framework for coordinating physiological processes with periodic environmental events (23, 24). To be useful for predicting periodic events, however, circadian rhythms must be entrained or synchronized to environmental time. The daily light-dark cycle is the major environmental signal that entrains endogenous circadian rhythms to the environmental day. The experiments described here focus on several aspects of the sensitivity of the photic entrainment pathway in the golden hamster using a functional behavioral assay. Specifically, these experiments examine the changes in the sensitivity and responsiveness of this visual pathway after prior stimulation.

The golden hamster has been a model for studying the effects of light on mammalian circadian rhythms. The relative insensitivity of the hamster’s photic entrainment pathway and its ability to integrate light input over time both suggest that this visual pathway has evolved to transform the light information for circadian entrainment without responding to photic “noise” in the environment that may disrupt circadian entrainment (20, 30). The ability to integrate light input over quite long durations of time suggests that light adaptation may not decrease the sensitivity of this pathway in a manner that is common in other visual pathways. Although numerous experiments have examined the sensitivity of the hamster photic entrainment pathway using single pulses, few studies have carefully examined the photic sensitivity to two or more light pulses or investigated how the photic sensitivity changes after an initial stimulus.

We have performed a series of three experiments to examine the visual sensitivity of the photic entrainment pathway to multiple pulses. First, we determined whether this visual system light adapts after prior stimulation. Second, we sought to determine whether response saturation completely blocks additional phase shifts of the pacemaker. Finally, we attempted to determine whether the number of light/dark transitions within a series of light stimuli affects the response of the photic entrainment pathway. Together these experiments support the hypothesis that the photic entrainment pathway in the hamster is a physiologically unique visual pathway and quite distinct from “image forming” visual pathways that mediate mammalian pattern vision. The hamster circadian pacemaker and the visual pathway that subserves it seem to integrate even multiple distinct light pulses delivered over extended durations of up to 1 h and respond to these pulses as a single stimulus.

GENERAL METHODS

Male golden hamsters [Mesocricetus auratus, Lak:LVG (SYR); 3–5 wk of age] were group housed in a 24-h light-dark cycle (14 h light, 10 h darkness). Illuminance inside the cages during the light phase was adjusted to ~250 lx (lumen/m²) using General Electric “cool white” fluorescent tubes (model F40CW). After at least 2 wk, hamsters were moved to individual cages equipped with running wheels and microswitches to monitor wheel running activity as described previously (20). After 7 days, the light-dark cycle was discontinued and the hamsters were kept in constant darkness. On the 7th day of darkness, a stimulus was delivered to each hamster at the appropriate phase of the rhythm. After stimulation, each hamster was returned to constant darkness for 2 wk, and the steady-state phase shift was measured.

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Phase advances of the running wheel activity rhythm at circadian time 19 were used to measure the light sensitivity of the circadian system's phase-shifting mechanism after prior stimulation. Circadian time is determined by normalizing the endogenous period of an animal to 24 h using the onset of activity as a phase reference point (arbitrarily designated as circadian time 12 for the nocturnal hamster). The procedures for stimulating and measuring responses to light pulses have been detailed previously (20). The clock time for delivery of each light pulse was determined for each hamster on the day of stimulation. The mean, standard deviation (SD), and range of the actual circadian times of stimulus onset are reported for each experiment. The hamsters ranged from 6 to 12 wk of age at the time of photic stimulation and were used only once for these experiments.

### Photic Stimulation

Light stimuli were provided by a Kodak projector module (EC-6) mounted within a ventilated, light-tight enclosure. This module included a tungsten halogen source (GE:FHS), an infrared passing mirror, an infrared absorbing filter, and a condensing lens. A Kodak projection lens directed the output of this module through infrared absorbing filters (Schott KG-1 and KG-4) and neutral density filters (Schott NG). The light was then projected through an interference filter (Schott AL; peak transmission wavelength of 503 nm and a one-half bandwidth of 20 nm) and onto a flashed-opal diffusing screen that formed the top of the stimulus chamber. The duration of light stimulation was controlled by a manual or electronic shutter (Uniblitz; Vincent Associates).

For stimulation, each hamster was transferred to a cylindrical (5.5 cm radius; 10.5 cm height) white-plastic stimulus chamber. All transfers were made without visible light using an infrared viewer (FJ W Optical Systems, Palatine, IL). Irradiance inside the chamber was measured before and after each stimulus with a radiometer/photometer (model S350, probe 248, United Detector Technologies, Hawthorne, CA), with the probe inside the chamber centered 5 cm below the diffusing screen. The stimulus irradiance was the average of these two measurements, and the mean stimulus irradiance (±SD) is reported for each group of animals.

### Data Analyses

The magnitude of the phase shift is reported for each group of animals in minutes of circadian time ± SE (that is, the response is normalized to the free running rhythm of each hamster). The procedure for measuring the phase shift responses to light for hamsters is detailed in Nelson and Takahashi (20). For analysis of the visual sensitivity, mean phase shift responses are plotted as a function of the total photons/cm² in the light pulses. The stimulus-response data in experiments 1, 2, and 3 were analyzed using ALLFIT (11). This program fits a form of the Naka-Rushton equation needed to fit many physiological stimulus-response curves (6). Statistical differences between the parameters (Rmin, N, σ, and Rmax) for different experimental manipulations were measured using ALLFIT (significance level of P < 0.05).

Statistical comparisons between the responses of individual groups were made using ANOVA or Student's t-test. When more than two groups were involved in a test, the responses of the groups in question were compared using the Tukey-Kramer method (ANOVA). A significance level of P < 0.05 was used to determine all significant differences between mean responses. SYSTAT (version 4.0) was used to perform both the ANOVA and the post hoc tests.

### Terminology

For this discussion, "light adaptation" will refer to a specific type of sensitivity decrease after light stimulation of a visual pathway. Light adaptation is evident as a shifting the operational range of the pathway's response (shifting of the stimulus-response function) to higher irradiance levels. The presence of light adaptation is detected as a shift in the half-saturation constant (σ) for the curve to higher irradiance levels. "Response saturation" will refer to a compression of the stimulus-response function that arises from moving up a nonadapting stimulus-response curve to higher irradiance levels and responses. Response saturation will be determined as the change in the maximum response for the curve (Rmax) after an initial stimulus.

Light adaptation changes the sensitivity in other visual pathways within milliseconds after the onset of a stimulus (1). We have tested for light adaptation over a much longer time course because of two unique characteristics of the hamster photic entrainment pathway (20). First, this pathway is very insensitive to brief light pulses of 30 s or less. Second, this pathway can fully or partially integrate the total photons in a single stimulus over durations of 300 s or more. For these reasons, the present study generally used stimuli of 300-s durations. A limitation of these experiments, therefore, is that only light adaptation with a long time course (300 s to 1 h) can be detected using these stimulus parameters. Light-adapting changes with a time course of milliseconds to seconds or hours to days would not be detected using the methods in this study. Light adaptation that is "functionally significant" to the hamster phase-shifting mechanism, however, would be detected using our methods.

"Initial stimulus" will be defined as the first of two stimuli when the second stimulus is used to measure the responsiveness after a prior stimulus. The "response" to this initial stimulus is the response to this stimulus presented alone (as a control). The "increment" or "test stimulus" will be defined as a subsequent light pulse used to measure the responsiveness or sensitivity of the pathway after the initial pulse. The "total response" will be the response to both the initial and the test stimulus. The "increment response" will be defined as the additional response induced by a test stimulus (the response to the initial stimulus subtracted from the total response).

### SPECIFIC EXPERIMENTS

#### Part I: Assessing Light Adaptation and Response Saturation

**Methods.** Three experiments were performed to characterize the sensitivity of the photic entrainment pathway after prior stimulation. These experiments measured 1) the sensitivity immediately after a subsaturating stimulus, 2) the sensitivity 1 h after a
saturating stimulus, and 3) the sensitivity immediately after a "weak" initial stimulus. In each case, the stimulus-response curve for behavioral phase shifts was compared with the stimulus-response curve measured without prior stimulation (20). In this prior study, 300-s light stimuli were presented to 21 groups of hamsters (3–8 per group; total n = 77). The mean circadian time of stimulation for this experiment was 19:01 (h:min; range = 18:40–19:30). Light pulses delivered without prior stimulation induced statistically significant phase advances for stimulus levels >2.2 × 10^{13} photons/cm^2 [Tukey-Kramer: degrees of freedom (df) = 88; P < 0.01]. The responses reached a maximum at −3 × 10^{15} photons/cm^2 with no response increase above this stimulus level. The magnitudes of the light-induced phase shifts are plotted as a function of the total photons (log_{10}) in the stimulus in Fig. 1 and are shown fit by equation 1.

For animals receiving two light pulses, the total phase advance and the phase advance attributable to the test stimulus (the increment-phase advance) were determined to measure the system's light sensitivity after the initial stimulus. To determine the parameters of the function describing the stimulus-response relationship, ALLFIT was used to statistically fit equation 1 to the data. Together these experiments quantify the changes in sensitivity that occur after an initial stimulus.

The appearance of specific changes in the stimulus-response curve during light adaptation is dependent on the plotting convention used to plot the stimulus-response data (1, 21, 31). After an initial stimulus, the half-saturation constant for the stimulus response curves appears to shift to the right during light adaptation if the data are plotted as total stimulus vs. total response or increment response vs. increment stimulus. The apparent half-saturation constant may also shift without light-adaptive changes if the responses are plotted as a function of the increment stimulus. To circumvent this problem, we first plot the increment response as a function of the photons in the test stimulus to determine the minimum and maximum response of the curve using the ALLFIT curve-fitting program. Once these values are estimated, the total responses are plotted as a function of the total stimulus using the minimum and maximums for the curve determined in the initial fit. The resulting analysis compared the half-saturation constants and slopes of the curves to determine whether these parameters had changed significantly.

**EXPERIMENT 1.** To determine the light sensitivity immediately after an initial stimulus, test pulses were presented to five groups of hamsters (4–10 per group) that received a stimulus (300 s) immediately before the test stimulus. The initial stimulus was started at circadian time 19 [19:04 ± 16 min (mean ± SD); range = 18:28–19:32]. The irradiance of the initial stimulus was 1.5 (±0.06) × 10^{12} photons·cm^{-2}·s^{-1} (4.5 × 10^{14} total photons/cm^2) and, on the basis of the stimulus-response curve for single pulses, was estimated to induce a phase advance of ~75 min when delivered alone (see Fig. 1). As a control, an additional group of animals (n = 5) received only the first stimulus. For these experiments, test stimuli (300 s) were substituted for the first stimulus by removing or adding neutral density filters to the light path of the stimulus. This substitution changed the irradiance of the initial stimulus to that of the test pulse. No interruption of the stimulus occurred during this substitution.

**EXPERIMENT 2.** The sensitivity to light 1 h after a prior stimulus was determined by delivering test stimuli to...
five groups of hamsters (5–7 per group; total n = 22) that had received a 300-s stimulus 1 h earlier (64 ± 7 min). The initial stimulus was delivered at circadian time 19 (19:04 ± 10 min; range = 18:53–19:22) and was composed of 1.5 (±0.02) × 10^{12} photons/cm². The initial stimulus was delivered alone to one group to determine its affect without the test stimulus. The total phase advance and the increment response were measured to quantify the sensitivity of the system to light 1 h after this initial stimulus.

**Experiment 1.** The sensitivity was also measured immediately after a “weak” stimulus (a pulse that induced < 25% of the maximum response). Test stimuli (300 s) were presented to groups of hamsters that also received a stimulus immediately before the test stimulus (4–8 per group; total n = 32). The initial stimulus was 300 s in duration and started at circadian time 19 (18:59 ± 8 min; range = 18:40–19:29). The irradiance of this initial pulse was 3.5 (±0.18) × 10^{10} photons·cm⁻²·s⁻¹ and was chosen to induce a phase advance of ~20 min when delivered alone. The initial stimulus was also delivered to two groups of hamsters to determine the phase shift induced without the test stimulus (n = 4 and 7 per group). The total responses and the increment responses were estimated for each animal to measure the sensitivity of the system after this initial low-irradiance stimulus.

**Results. Experiment 1: Sensitivity measurement immediately after subsaturating photic stimuli.** The response range of the photic entrainment pathway was reduced immediately after the initial stimulation (Fig. 1). The initial stimulus induced a phase advance of 80 ± 5 min when delivered without a test stimulus. The additional test stimuli induced phase advances that increased the magnitude of the total phase shift in those animals receiving both pulses. The largest average response to both pulses was 113 ± 13 min.

The average response to the initial pulse was subtracted from the total phase shift induced by both pulses to estimate increment responses. When increment responses are plotted as a function of the photons per square centimeter in the test stimuli (Fig. 1A), the minimum response (~3 min) was not significantly different from the minimum response to pulses delivered without prior stimulation (ALLFIT). The slope of the curve (3.9) was also not different from the slope for the curve measured without prior stimulus. The half-saturation constant for the fit was 6.3 × 10^{15} photons/cm² and was significantly different from the one-half maximum for 300-s stimuli at circadian time 19 (ALLFIT; F = 24.65; df = 20). The maximum increment response after prior stimulation was only 33 min and was significantly smaller than that measured using single pulses at this phase (ALLFIT; F = 54.13; df = 20). Immediately after the first stimulus, therefore, there was an effective decrease in the response range available for the responses to the test stimulus. The decrease in the responsiveness range, however, can be almost entirely explained by the initial response to the test stimulus.

To compare the half-saturation constants for the stimulus-response curve measured immediately after prior stimulation and without prior stimulation, the increment responses were plotted as a function of the total number of photons in both the initial and the test stimuli (Fig. 1B). To analyze these data using ALLFIT, the minimum response for the Naka-Rushton curve fit to the data was held at ~80 min (to correct for the response induced by the initial stimulus). The maximum was held constant at 33 min (the maximum increment response fit to the data as previously described). The half-saturation constant for the curve fit with these constraints was 1.4 × 10^{14} photons/cm², which was not different from that measured without prior stimulation. The slope (0.5) was also not significantly different. These data suggest that the half-saturation constant for the stimulus-response curve remained unchanged by the initial stimulation. There is no evidence for light adaptation (shifting of the stimulus-response curve on the stimulus irradiance axis).

**Experiment 2: Sensitivity measurement 1 h after a subsaturating stimulus.** Even 1 h after the initial stimulus, the responsiveness of the system to light remained reduced (Fig. 2). The initial stimulus alone induced a response of 80 ± 5 min. The test stimuli delivered 1 h after this initial pulse induced additional phase shifts in animals that received both pulses. The maximum total response to both pulses was 104 ± 10 min. The maximum increment response 1 h after the initial pulse was, therefore, 24 min. When a sigmoidal curve was fitted to the data, the maximum increment response for the curve was 38 min (Fig. 2A). The minimum response was zero and not significantly different from that measured for stimuli delivered without prior stimulation. The slope, 0.4, and half-saturation constant, 3.0 × 10^{16} photons/cm², were also not significantly different from those measured without prior stimulation. The effective decrease in the response range observed immediately after the first stimulus continued for at least 1 h.

The increment responses measured 1 h after prior stimulation are also shown plotted as a function of the total photons in both the initial and test stimuli (Fig. 2B). The fit of equation 1 to the data was fixed at ~80 min for the minimum and 38 min for the maximum. The half-saturation constant for the resulting fit, 1.2 × 10^{13} photons/cm², was not significantly different from that measured without prior stimulation. The slope (0.2) appeared somewhat smaller than that for 300-s stimuli delivered without a prior light pulse. The large decrease in the maximum response (from 114 to 38 min) without significant changes in the half-saturation constant indicates that the contributions of light adaption (curve shifting) to reductions in sensitivity are quite small relative to the decrease in responsiveness caused by response saturation.

It is important to consider that the response to a second stimulus delivered 1 h after an initial stimulus may be smaller due to a phase advance of the pace-
The average phase shift induced by the initial stimulus, 18 min, was subtracted from the total response to determine the increment responses to the test stimuli for those groups that received both pulses. The minimum response (4 min) and the slope (1.4) were not different from those measured for 300-s stimuli delivered without a prior stimulus. The half-saturation constant, \(2.1 \times 10^{14}\) photons/cm\(^2\), was significantly different (ALLFIT; \(F = 7.77; df = 21\)). The maximum response was also reduced to 78 min and was significantly different from that for stimuli delivered without a prior light pulse (ALLFIT; \(F = 5.29; df = 21\)). This reduction, however, was not as pronounced as that reduction seen after an initial stimulus of greater magnitude.

The stimulus-response curve measured immediately after the weak initial stimulus is also presented as the increment response to the total photons per square
centimeter delivered in both the initial and test stimuli (Fig. 3B). The minimum response for this fit was fixed at −18 min (to correct for the response to the initial pulse). There was a significant decrease in the maximum response and no significant shift in the half-saturation constant \((2.2 \times 10^{14} \text{photons/cm}^2)\) or the slope \((0.6)\) after the small initial stimulus. The maximum increment response was 84 min and was significantly different from that measured in pulses without prior stimulation (ALLFIT; \(F = 4.28; \text{df} = 22; \text{P} = 0.050\)). This indicates that there is no evidence for light adaptation (curve shifting) in these experiments and the reduced responsiveness to light is caused primarily by response saturation.

Part II: Responsiveness Measurement After a Saturating Stimulus

Experiment 4: is there a maximum phase advance limiting the total response of the system? METHODS. To determine whether this photic entrainment pathway can show any response after a "saturating" stimulus, we performed an experiment to assess the responsiveness after an initial high-irradiance stimulus. One group of animals \((n = 5)\) received \(2.6 \times 10^{14} \text{photons} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}\) \((300\text{s})\) at circadian time 19 \((19:01 \pm 3\text{ min})\). The irradiance and duration of this pulse were chosen to induce a maximum response of −2 h at circadian time 19. A second group of animals \((n = 11)\) was given the same irradiance and duration of stimulation at circadian time 19 but was also given a test stimulus of \(2.5 \times 10^{14} \text{photons} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}\) \((300\text{s})\) after an interstimulus interval of −10 min in darkness \((9 \pm 1\text{ min}; \text{range} = 7–11\text{ min})\). On the basis of the results of Part I, we hypothesized that the test pulse would not induce an additional phase shift.

An important control for this experiment was also performed. If the initial stimulus had induced a phase advance of 2 h, then the phase of the oscillator may be approximately circadian time 21 \((\text{circadian time 19} + 2\text{ h} = \text{circadian time 21})\) at the time of the second pulse. To test the responsiveness of the hamster circadian system at this later phase without prior stimulation, a third group of hamsters \((n = 6)\) was given a saturating pulse at circadian time 21 \((21:06 \pm 3\text{ min})\). The irradiance of this 300-s light pulse was also \(2.6 \times 10^{14} \text{photons} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}\).

RESULTS. The single light pulse delivered at circadian time 19 induced a steady-state phase advance of \(113 \pm 7\text{ min}\) \((\text{Fig. 4})\). The average phase shift induced in those hamsters that received a light pulse at circadian time 21 was \(70 \pm 4\text{ min}\). Each of these saturating pulses alone induced the maximum possible shift at their respective phases of delivery \((20)\). Animals that received a light pulse at circadian time 19 and again after 10 min of darkness displayed phase advances averaging \(109 \pm 5\text{ min}\). This shift was not significantly different from the phase advances induced by single pulses delivered at circadian time 19. There was no significant additional response induced by the second light stimulus. It is important to note that the system was not less responsive to light simply because the phase-response curve had already shifted to a later phase \((\text{circadian time 21})\) of reduced responsiveness due to the initial pulse. The phase shift expected at circadian time 21 is −70 min, and our data demonstrate that there was no additional response due to the second stimulus. This result, coupled with the results of Part 1, suggests that the circadian photic entrainment pathway of the golden hamster may only be capable of phase advancing −2 h within a single circadian cycle. If an initial stimulus induces a maximum phase advance of −2 h, then subsequent stimuli will probably not induce a phase advance. Similarly, an initial light pulse that induces a half-saturating phase shift probably reduces the maximum response to subsequent stimuli by this amount (see experiments 1–3).

Part III: Responsiveness to Multiple Stimuli Containing Multiple Light/ Dark Transitions

Experiment 5: are the number of onsets and offsets associated with light stimuli important for determining the response of the circadian system to light? METHODS. In a final experiment, we measured the responsiveness to single and multiple \((10–1,000)\) stimuli to determine whether the multiple pulses or the multiple transitions from dark to light associated with these stimuli influenced the photic responsiveness of the hamster pacemaker. For this experiment, a light stimulus was delivered to hamsters at circadian time 19 \((19:08 \pm 14\text{ min}; \text{range} = 18:43–19:46\text{); this stimulation was presented as either a single 300-s light pulse \((n = 5)\) or as 300 s of light divided into multiple pulses \((10–1,000)\) of shorter duration pulses. These "trains" of multiple stimuli were 10 30-s pulses, each separated by 30 s of darkness \((n = 10)\); 100 3-s pulses separated by 3 s of darkness \((n = 14)\); or 1,000 pulses of 300 ms in duration, each separated by 300 ms of darkness \((n = 8)\). The irradiance of each of the pulses, \(2.7 \pm 0.02 \times 10^{11} \text{photons} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}\), was equivalent for all stimuli. The
total number of photons in each series of pulses was also constant \((8.1 \times 10^{13} \text{ photons/cm}^2)\) and would induce a response of approximately one-half the maximum response if delivered in a single 300-s light pulse at this phase (20).

Although the total duration of the light stimulation was equal for each group, the duration of the entire stimulus train (from the onset of the first stimulus to the offset of the last) was slightly different. For example, for the 3-s stimuli, \((3 \text{s/stimulus} \times 100 \text{stimuli}) + (3 \text{s between stimuli} \times 99 \text{interstimulus intervals}) = 597 \text{s}\) of duration for the stimulus train. For the 30-s pulses, the total duration of the stimulus train was 570 s. None of the durations of stimulation exceeded 600 s of stimulation. As a control for these different stimulus durations, one group of hamsters \((n = 8)\) received a stimulus of the same irradiance but with a duration of 600 s.

We also measured the responsiveness to multiple stimuli using a saturating total number photons in the stimulus train. For this test, a stimulus irradiance of \(3.8 \pm 0.01 \times 10^{14} \text{ photons·cm}^{-2}·\text{s}^{-1}\) was presented as either a single 300-s stimulus \((n = 5)\) or as 100 stimuli \((n = 6)\) of 3 s in duration. The total photons delivered in these stimulus trains was \(1.1 \times 10^{17} \text{ photons/cm}^2\).

RESULTS. The phase shifts induced by \(8.1 \times 10^{13} \text{ photons/cm}^2\) divided into different numbers of individual pulses were not significantly different from one another (Fig. 5). The average phase advance induced by the single 300-s pulse was \(56 \pm 3\) min and was approximately one-half of the maximum response that can be induced by a 5-min pulse at this phase. The mean response to the 600-s stimulus of the same irradiance was \(68 \pm 6\) min and was not significantly different from the response to the 300-s pulse. The responses to this total number of photons divided into 10, 100, or 1,000 pulses were \(40 \pm 4, 45 \pm 6,\) and \(49 \pm 8\) min, respectively.

The phase advance to the total number of photons in the saturating stimulus of \(1.1 \times 10^{17} \text{ photons/cm}^2\) was also unrelated to the number of individual stimuli (or dark-to-light and light-to-dark transitions) in the stimulus train. The phase advance induced by the single 300-s pulse was \(113 \pm 8\) min. The response to the train of 100 stimuli with the identical total number of photons was also \(113 \pm 8\) min and not significantly different from the response to the single pulse (Student’s t-test). Neither the number of light pulses in a stimulus train nor the number of dark/light transitions associated with the stimulus (at least within a duration 600 s) appear to be significant parameters of the stimulus for influencing the magnitude of phase advances.

DISCUSSION

In prior experiments, we defined the ability of the hamster circadian entrainment pathway to integrate the total photons in single light pulses (20). This pathway appears to be capable of integrating the total photons in single pulses for extended durations as large as 300 s or more (with a maximum sensitivity to 300-s pulses). Experiments outlined here extend our prior results to reveal how this photic entrainment pathway responds to multiple light pulses. These results demonstrate that the hamster photic entrainment pathway does not respond independently to multiple light pulses delivered within 1-h durations. Instead, this system appears to integrate the photons within even multiple pulses over durations of up to 1 h. This temporal integration of inputs appears to be accompanied by a saturation of the photic entrainment pathway (or specifically the phase-shifting mechanism) without evidence for adaptation-like changes in light sensitivity. These results define several intriguing and fundamental characteristics of the hamster circadian pacemaker and the visual pathway that carries light information to it. Knowledge of these characteristics is important for designing and interpreting results from future studies to dissect the cellular and molecular nature of the mammalian circadian clock and how visual information affects this cellular clock during entrainment.

Temporal Integration and Saturation Without Light Adaptation

Immediately after a light pulse there is a significant reduction in the responsiveness of the hamster photic entrainment pathway. Our results suggest that this responsiveness decrease is not due to light adaptation (stimulus-response curve shifting) but that another mechanism limits the responsiveness to light after an initial pulse. This reduced sensitivity is most easily explained by response-saturation of the circadian phase-shifting mechanism or saturation of the photoreceptive pathway that transmits light information to the pacemaker. The form of this decreased responsiveness can best be visualized by plotting the total response as a
function of the strength of the test stimulus for experiments 1, 2, and 3 (Fig. 6A). The maximum response for the hamster photic entrainment pathway is visible as a limit of ~2 h for all of the stimulus-response curves. The phase shift induced by the first stimulus increases the minimum response without increasing the maximum total response. The result is a saturation (response compression) of the response range available for the second light pulse. Figure 6B shows a saturation model (1, 21, 31) that appears to match the data quite well. The data provide a clear example of response saturation: a decrease in the response range and a “compression” of the stimulus-response curve. No evidence for significant “curve shifting” was observed, suggesting that the system does not light adapt in a manner similar to other visual systems. Within the 1-h time course tested here the hamster circadian pacemaker and the photoreceptive pathway that subserves it appear incapable of reducing their sensitivity through a mechanism similar to light adaptation. Instead, multiple pulses appear to be integrated within this duration and response saturation or compression limits the total response to multiple pulses presented.

Light adaptation has been discussed in the context of circadian photic entrainment in several previous studies. It has been suggested that the circadian systems of animals may light adapt when maintained in constant light for long periods of time (7, 22, 33). Without some type of light adaptation, it is reasoned a circadian pacemaker would effectively “stop” during constant light because it would experience continuous phase delays in the delay region of the phase response curve. Our results suggest that response saturation may be responsible for the reduced responsiveness of the pacemaker in constant light. The pacemaker may be responsive to light until the response saturates (the maximum shift has been achieved). After saturation, the pacemaker may be insensitive to additional light and continue its forward motion out of the phase delay region of the phase response curve. Additional light would be effectively “ignored” by the saturated pacemaker.

If light adaptation cannot be detected in this functional output of the hamster circadian pacemaker, it is unlikely that light adaptation changes the sensitivity at any point along the pathway between the photoreceptor to the pacemaker’s phase-shifting mechanism over the durations (300 s to 1 h) tested here. On the other hand, light adaption may be occurring with a time course that is much slower or more rapid than our experiments can detect. For example, if light adaptation occurred in <1 s, a time course for light adaptation measured for the rat electroretinogram (12), then the resolution of our test for light adaptation may be inadequate to identify true light adaptation. If light adaptation occurred rapidly, then the 300-s “dark-adapted” stimulus-response curve measured without prior stimulation (Fig. 1) may actually represent already light-adapted responses. However, because this photic entrainment pathway is relatively insensitive to brief stimuli (20) it is unlikely that adaptation occurring with time course of <1 s would be functionally important for changing the sensitivity of this circadian oscillation to light. Light adaptation, therefore, does not appear to be functionally important for the induction of phase advances in hamsters.

The absence of functional light adaptation in this pathway may provide an additional means of physiologically identifying the elements (photoreceptors and ganglion cells) of the neural pathway that delivers photic information from the eye to the pacemaker. For example, few retinal ganglion cells possess the physiological properties that are required of this photic pathway. “Luminance units” (4) and “tonic W-cells” (29) appear to possess physiological properties that would enable these cells to mediate the affects of light to the mammalian circadian pacemaker. Luminance units respond to maintained illumination with a sustained change in firing.

Fig. 6. A: total responses (means ± SE) and their associated best-fit curves (experiments 1 and 3) have been plotted as total phase shift (response to both first stimulus and test stimulus) as a function of number of photons in test stimulus. ●, Total responses from experiment 3 (weak initial stimulus: $1.1 \times 10^{13}$ photons/cm$^2$). ▲, Total responses from experiment 1 (strong initial stimulus: $4.5 \times 10^{13}$ photons/cm$^2$). □, Responses obtained without prior stimulation. Dashed and thin solid lines are best-fit curves (from experiments 3 and 1, respectively) adjusted to represent total response to both pulses. Thick solid line is equation 1 fit to responses measured without prior stimulation (from Fig. 1). B: curves represent a model for saturation of hamster photic entrainment pathway by light. Thick solid line represents a stimulus-response curve from animals receiving a single light pulse. Dotted line represents a curve expected after an initial stimulus of $1.1 \times 10^{13}$ photons/cm$^2$ that induced an initial 18-min response ($R_{\text{min}} = 18$ min; $N = 0.6; \sigma = 9.3 \times 10^{13}$ photons/cm$^2$; $R_{\text{max}} = 114$ min). Thin solid line represents a curve expected after an initial stimulus of $4.5 \times 10^{14}$ photons/cm$^2$ that induced an initial response of 80 min ($R_{\text{min}} = 80$ min; $N = 0.6; \sigma = 9.5 \times 10^{13}$ photons/cm$^2$; $R_{\text{max}} = 114$ min). Initial light pulse induces a phase shift that effectively reduces response available to second light stimulus. There is an obvious increase in $R_{\text{min}}$ with increasing levels of initial stimulation, whereas there is no increase in $R_{\text{max}}$. 


rate and make up a very small proportion of the retinal ganglion cells in the cat (<1%). Interestingly, these cells also have a relatively high threshold for light-induced changes in mean firing rate compared with that measured for other retinal ganglion cells. This high threshold for stimulation of luminescence cells is similar to the high thresholds measured for electrophysiological responses and phase shifts in the photic entrainment pathway (14, 17–20). Interestingly, there is little evidence for either light adaptation or temporal integration in light-induced electrophysiological responses recorded from the hypothalamic suprachiasmatic nucleus (SCN), which is the site of the mammalian circadian pacemaker. Many cells in this nucleus demonstrate a transient response component at the onset of a photic stimulus, but a large sustained component is also present (14, 18). In addition, the photic sensitivity of SCN neurons is not reduced by prior illumination in a manner consistent with light adaptation (13, 18, 19).

Implications of Temporal Integration for Circadian Systems

Recently it has been reported that the mouse and hamster photic entrainment pathways can respond independently (with apparently complete phase shifts) to two or more light pulses delivered with interstimulus intervals of 2 h (5). Our results suggest that a maximum response ceiling (a maximum phase shift) may limit the hamster photic entrainment pathway’s response to multiple pulses presented within intervals of up to 1 h. A phase advance of ~2 h was induced in those animals that received a single saturating pulse at circadian time 19, whereas no additional response was induced by a stimulus delivered 10 min after a saturating pulse (experiment 4). If the system responded equally to both stimuli we would have expected a 226-min phase shift in response to the two pulses (113 + 113 = 226 min). Because the phase of the oscillator may have been changed immediately by the initial stimulus, the circadian phase of the of the oscillator at the time of the second pulse may be closer to 21 than to circadian time 19 (circadian time 19 + 113 min = circadian time 21). A single pulse delivered at circadian time 21 induced a phase advance of 77 min. If these responses were summed “linearly” by the pacemaker then a phase advance of 190 min may be expected (113 + 77 min = 190 min). The second pulse, however, induced no additional shift. This experiment indicates that the pacemaker was quite unresponsive to light stimulation 10 min after an initial pulse at circadian time 19, even though the system would have been responsive to light at either circadian time 19 or 21. Similarly, the total maximum response to two pulses delivered up to 1 h apart in experiments 1–3 also appears to be ~2 h (Fig. 6). Finally, the total response induced by 100 brief light pulses in experiment 4 was also ~2 h. Recent studies in mice have also suggested that within short periods of time the circadian system can integrate and respond to very brief multiple light pulses as if they were actually a single stimulus (32). Our data suggest that, within durations of up to 1 h, the hamster circadian system appears not to respond to each stimulus in an independent manner, but instead the system may integrate the total light input over time and respond to it as it would respond a single light pulse. Although our data do not address the question of a temporal limit for integration of multiple light pulses by the photic entrainment pathway, recent results from mouse and hamster suggest that two or more pulses delivered with interstimulus intervals of 2 h may be interpreted as discrete stimuli by the circadian system (5).

Single light pulses have been found to induce a maximum phase advance of 2–3 h in the golden hamster in a number of studies (8, 9, 30), and our current results suggest that this maximum may also exist for multiple light pulses delivered within durations up to 1 h. DeCoursey (10) has also suggested that a response ceiling may also limit phase delay responses to multiple pulses in the flying squirrel. Larger light-induced shifts are observed in hamsters after pretreatments such as administration of 5-hydroxytryptamine 1A receptor antagonists (29) or dim constant light (Nelson and Takahashi, unpublished). In tau-mutant hamsters, pretreatments of constant darkness for long durations (49 days) cause the light responsiveness to increase from ~2 to 12 h or more (28). Because two or more pulses falling outside the limit for temporal integration may induce larger total shifts (5), it is possible that pretreatments that cause the photic entrainment pathway to be more responsive may act by shortening the duration of temporal integration within this pathway in addition to increasing the sensitivity of the pathway to irradiance.

In the future, it will be interesting to use the responsiveness to multiple light pulses to dissect the mechanism and function of the mammalian photic entrainment pathway. For example, the apparent insensitivity to a second pulse after a saturating stimulus probably represents response saturation at the level of the phase-shifting mechanism and not saturation of the retinal visual system that subserves it. Behavioral phase shifts have been compared with electrophysiological SCN responses using trains of light pulses (18). Stimulus trains of 2, 4, and 8 light pulses (1-min pulses of light, each separated by 1 min of darkness) induced increasing magnitudes of phase advances at circadian time 18. The total shifts observed, however, were smaller than predicted by simply linear addition of the responses to individual pulses. This result is consistent with the evidence for stimulus integration and response saturation of the phase-shifting mechanism demonstrated in our study. Interestingly, electrophysiological results suggested that the responsiveness of SCN neurons to retinal illumination did not change over the course of a stimulus train. SCN neurons responded similarly to each pulse of the stimulus train. These findings suggest that the decreased responsiveness after an initial stimulus probably originates “downstream” from the electrophysiological responses of SCN neurons to light.

One mechanism that may account for the integrating ability of this system and the maximum response...
ceiling may be the “motion” of the circadian pacemaker itself. The pacemaker presumably moves through the phase advance region in the late subjective night with a time course that is determined by the normal forward motion of the clock. The time course of this movement may also change due to the phase advance of the pacemaker by light. The motion of the pacemaker through the subjective night could act as a sort of “memory” for light stimulation of the pacemaker and allow for the temporal integration of single and multiple stimuli presented to the animal. In addition, this movement through the phase advance region of the phase-response curve may also limit the maximum response to light stimuli delivered during this time because the pacemaker could be shifted (by light) out of the region of responsiveness and into an unresponsive region during the subjective day. On the other hand, our results in experiment 4 show that even though the pacemaker should be sensitive to light after the initial light pulse (whether the second stimulus is delivered at circadian time 19 or 21), the second light pulse is ineffective at inducing an additional phase shift. There is clearly more to the response saturation than the motion of the pacemaker through the phase advance region. The pacemaker’s motion could allow temporal integration of stimuli, but the maximum response ceiling of 2 h is not due to the simple movement of the pacemaker out of the phase advance region of the phase-response curve. Another mechanism must be reducing the responsiveness of the photic entrainment pathway to subsequent simulation.

Finally, the behavioral results presented here also reveal a great deal about the nature of molecular and cellular events that must mediate the effects of light to the clock. At the cellular level, it is interesting to speculate that photic stimulation causes some cellular process (such as the induction of an intracellular signal) to be less responsive to stimulation after an initial photic stimulus. Recent studies have demonstrated that visual stimulation induces the expression of immediate early genes (3, 15, 16, 26) and putative clock genes (2, 27) within SCN neurons. The most recent of these results have demonstrated that mPer1 message is induced in mouse SCN neurons by light stimulation that also causes phase shifts in the mouse circadian pacemaker. The photic threshold for inducing mPer1 message is very similar to the threshold for inducing phase delays in the mouse behavioral rhythm (27). There is also a rhythm of mPer3 production in constant darkness, which can be shifted by a light pulse (2, 27). Taken together, these results suggest that mPer1 may be a central component of the circadian oscillation mechanism and also mediate the effects of light to the circadian oscillator in the mouse. It is possible that a gene such as mPer1 may play a role in the temporal integration of photic information observed for single and multiple light pulses. The kinetics for light induction of mPer1 or a similar gene could explain the extended time course for integration of single and multiple photic pulses observed in the hamster entrainment pathway. In mouse, mPer1 message is rapidly induced by light at circadian time 16, and the peak of mPer1 is reached ~60 min after stimulus initiation. Levels return to near baseline by 180 min after stimulus onset. This time course for mPer1 induction may explain the time course of 300 s to 1 h for temporal integration of single light pulses observed for the hamster photic entrainment pathway (20). The kinetics of mPer1 induction by light may also explain the apparent integration of multiple light pulses observed in the present study. Additional studies are needed to determine whether the characteristics of light sensitivity for mPer1 induction may explain the unique characteristics of response saturation and temporal integration of multiple stimuli observed in behavioral studies of the mammalian circadian system. Behavioral studies continue to show that the photic entrainment pathway, which mediates the effects of light to the mammalian circadian pacemaker, is functionally very different from pathways that mediate pattern vision. These unique functional characteristics, in turn, provide useful tools to further dissect the cellular and molecular mechanism of the mammalian photic entrainment pathway.

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