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Research paper

Circadian phase-shifting by light: Beyond photons

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ABSTRACT

Circadian entrainment to the solar light:dark schedule is thought to be maintained by a simple photon counting method. According to this hypothesis, the pacemaker adjusts the phase of the body's endogenous rhythms in accordance to the intensity and duration with which it encounters a perceived twilight signal. While previous data have generally supported the hypothesis, more recent analysis has codified other factors besides irradiance that influence the magnitude of resetting responses to light delivered within the same phase of the circadian cycle. In particular, the frequency with which light is alternated with darkness, or whether it's packaged in millisecond flashes versus continuous blocks, can significantly alter the dose-response relationship. Here, we used a drosophilid model to test whether circadian photon-counting trends can be broken with light administration protocols spanning just 15 minutes. In the early part of the delay zone, a 15-min continuous light pulse was fragmented until it could no longer produce a full-magnitude shift of the flies' locomotor activity rhythms. The remaining exposure was then reorganized along various fractionation schemes that employed pulses with different widths and interstimulus intervals. Our results suggest that the pacemaker integrates the phase-shifting effects of equiluminous light differently depending on the stimulus pattern with which light is made available. For example, despite having fewer photons, certain ratios of light and darkness could be optimized on a timescale of seconds and minutes so as to achieve pacemaker resetting close to par with steady luminance. These data provide further evidence that the circadian pacemaker's responses to light entail more than photon counting and motivate continued discussion on how phototherapy can be best optimized in clinical practice to improve conditions linked to circadian impairment.

1. Introduction

The reciprocity hypothesis summarizes much of the dogma surrounding the study of light's effects on the circadian pacemaker. It postulates that any timekeeping shift made to light at a given phase of the subjective evening is based solely on the number of photons registered by the pacemaker: the brighter or longer the pulse, the greater the resulting phase jump one should see up to some pseudosaturation level (Takahashi et al., 1984). Given the complexities of photoentrainment, not the least of which are the various signal-to-noise problems encountered within the dynamic light environment of the twilight zones, the reciprocity hypothesis might appear at first glance to be ill-suited to explain the process by which light information gets translated into phase-shifting drive. However, to a first approximation, reciprocity trends appear to hold when conventional artificial lighting is shone on animals for periods exceeding 5 min up to about an hour. The pacemaker integrates photic input the same way over this span such that different trains and durations of non-saturating pulses from ~5 to 60 min will elicit the same final phase shift as long as the overall photon flux is conserved (Best et al., 1999; Dkhissi-Benyahya et al., 2000; Nelson and Takahashi, 1991, 1999; Takahashi et al., 1984). In a long-running series of experiments, Czeisler and colleagues have shown that the human pacemaker also tracks reciprocity trends; volunteers exposed to higher illuminance or longer pulses in the early and late biological night exhibit greater delay and advance resetting, respectively (Boivin et al., 1996; Chang et al., 2012; Gronfier et al., 2004; Rimmer et al., 2000; Zeitzer et al., 2000; 2005).

A novel feature of photic resetting that fell out of the Czeisler experimental series was the added observation that, after a particular threshold of exposure, further introduction of light produced diminishing returns on phase movement (i.e., the more the light was shown, the less the photic information got translated into the magnitude of the

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phase shift; Boivin et al., 1996; Chang et al., 2012; Rahman et al., 2017). This nonlinear duration-efficacy relationship prompted Kronauer to develop a mathematical model for how pacemaker photosensitivity changes throughout continued light exposure (Kronauer et al., 1999). Broadly construed, the Kronauer model proposes that light stimulation always prompts an initial response by the pacemaker that persists in decaying fashion for a period of time after the stimulation has stopped (like the initial pedals of a bicycle wheel). In order to sustain phase-shifting drive, the onsets of the pulse must be long enough to reach full phase-shifting strength and then be balanced with periods of darkness so that steady activation of the pacemaker can occur without triggering competing processes that curb photosensitivity. Maximal phase-shifts are achieved when the rate constants for drive build-up and decay are optimized against the rate constant by which the pacemaker loses photosensitivity (Jewett et al., 1999; Kronauer et al., 1999). Upon testing these assumptions, the Czeisler group found that light delivered intermittently could, indeed, elicit circadian responses almost as effective as those seen after constant light despite the sizable difference in overall exposure (Gronfier et al., 2004; Rimmer et al., 2000).

The pacemaker's reaction to intermittent light has been explored mainly in the context of protocols where stimulation is delivered in recurring millisecond bursts over an hour (Najjar and Zeitzer, 2016; Van Den Pol et al., 1998; Vidal and Morin, 2007) or within wider segments (5–45 min) that alternate with an hour or half-hour of darkness throughout a large portion of the subjective night (Gronfier et al., 2004; Rimmer et al., 2000). In the current paper, we have asked whether reciprocity trends are "broken" with simple 15-min protocols that do not take advantage of flash perturbation strategies or longer stimulation windows more conducive to building phase-shifting momentum. From circadian time 13 (CT13) to CT13.25, a continuous 15-min light pulse was whittled down until it could no longer produce a full-magnitude delay shift. The remaining exposure was then rearranged into a sequence of patterns involving second and minute-long episodes of light and darkness to see if certain combinations of stimulation and rest could overcome the exposure deficit to reinstate full pacemaker resetting. Our results suggest that the pacemaker is impacted by the pace at which light is introduced on the order of *seconds and minutes*. When reconciled with the observations that have been made with intermittent light at other time scales, these results hint at a Matryoshka or "nesting doll" operational logic for the metazoan circadian system. They raise the possibility that the pacemaker calculates phase-shifting drive based on Kronauer-like principles functioning at several decreasing temporal resolutions (placed one inside another), where drive is a running computation of pacemaker sensitization and desensitization moving from milliseconds and seconds, seconds to minutes, and minutes to an hour.

2. Materials and methods

To obtain the clearest picture possible on light-induced phase resetting, we tracked the locomotor activity rhythms of *Drosophila ananassae*, a particular cosmopolitan species of fruit fly that co-evolved with human society. *Ananassae* show a unimodal pattern of locomotor activity during the day—and consolidated sleep at night—that mimics the diurnal sleep/wake patterns of people and offer a realistic model of human circadian behavior (Prabhakaran and Sheeba, 2012, 2013, 2014). *Ananassae* were purchased and regularly replenished from an isofemale line maintained at the Drosophila Species Stock Center (DSSC) at the University of California, San Diego (since relocated to Cornell University; stock # 14024-0371.16; NSF Award #1351502). The animals were reared at 25 °C in DigiTherm® incubators (Tritech Research, Inc., Los Angeles, CA), entrained to a 12:12 LD cycle (600 lx, compact white fluorescent lighting, lights-on at 0700 h, MST), and transferred daily to generate a steady supply of offspring. For phase-shifting experiments, female flies were selected as late-stage, "pharate-adult" pupae, moved onto fresh food, and housed in groups of 5 to 6. A few days post-eclosion, individual animals were placed into Pyrex glass chambers (5 mm outside diameter, 65 mm long) containing a plug of corn flour-nutritional yeast-agar medium on one end (0.8% agar, 3.5% sucrose, 1.7% glucose, 6% fine-grained masa, 1% yeast) and a cotton fitting on the other, and loaded into Trikinetics DAM2 Drosophila Activity Monitors (TriKinetics, Inc., Waltham, MA). Motion was detected and counted by cross-sectioned infrared beams, which transmitted movement information over modem/USB to a computer acquisition software (DAMSystem-308) every 30 s. DAM2 units were situated in climate-controlled vivariums identical to the ones used in colony management and under the same ambient conditions. Two independent environmental trackers (TriKinetics DEnM Drosophila Environment Monitor and the Tritech DeviceCom3 log) continuously measured the temperature and relative humidity of each enclosure's surrounding air, and archived the intensity of visible-band illumination, providing a quality control record for all the experiments.

An Aschoff Type II paradigm was used to generate an *ananassae* PRC to broad spectrum fluorescent light, to establish a proof of concept that flies have the capacity to integrate photic information presented across a series of millisecond xenon flashes (just as rodents and humans), and to quantify the effects of pulse fractionation on phase resetting of locomotor activity rhythms (Aschoff, 1965). This procedure offers an accurate assessment of the natural field shape of the PRC vis-à-vis photoentrainment by avoiding the amplitude inflation that develops after long-term housing in DD (Johnson, 1999; Mrosovsky, 1996) and might be especially relevant for applying data across animal and human models (Mistlberger et al., 1996). For the PRC experiment, flies continued entrainment to the 12:12 LD schedule under which they were reared for 3 days. After lights-off on the last day of the schedule, independent groups received a single 15-min pulse at one of the hourly intervals of the subjective night (i.e., CT13, CT14, etc) or within half-hour increments near the previous LD schedule's transitions (CT12.5, 13.5, 22.5, or 23.5). This was accomplished by software-controlled activation of the house lamp (600 lx, white fluorescent light; Tritech Research, DeviceCom3™). Post-pulse, animals were left to free-run in DD for 5 days. The millisecond flash and second/minute long fractionation experiments were carried out using the same general steps described above, except that all stimuli were delivered at CT13. For the flash experiment, animals were temporarily removed from their vivarium, placed onto a titanium dioxide paint-coated platform, and exposed to 4ms pulses of xenon light (205 lx) delivered at 1 Hz for 15 min with a ColorDome Ganzfeld lamp (Diagnosys LLC, Lowell, MA). For the fractionation experiment, separate cohorts of flies were administered 1 of the following 11 light regimens (**A-K**) with the house lamp in the 15 min between CT13 and CT13.25:

A. A uniform, uninterrupted light pulse delivered over 15 min.
B. Intermittent delivery of light for 45 s each minute on the minute (referred to as a 45 s duty cycle).
C. Intermittent delivery of light for 30 s each minute on the minute (referred to as a 30 s duty cycle).
D. Intermittent delivery of light for 15 s each minute on the minute (referred to as a 15 s duty cycle).
E. A series of fifteen 15 s light pulses separated by an interstimulus interval of 30 s (centered in the middle of the CT13-CT13.25 timeframe).
F. A series of fifteen 15 s light pulses separated by an interstimulus interval of 15 s (centered in the middle of the CT13-CT13.25 timeframe).
G. Intermittent delivery of light for 30 s every 2 min.
H. Intermittent delivery of light for 45 s every 3 min.
I. A 225 s light stimulus delivered within two symmetrical 112.5 s blocks distributed at the tail-ends of CT13 and CT13.25. The first bookend pulse began precisely at CT13, while the second pulse ended precisely at CT13.25.