Placing ocular mutants into a functional context: 
a chronobiological approach

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Abstract

The behavior of mammals is characterized by a 24-h cycle of rest and activity which is a fundamental adaption to the solar cycle of light and darkness. The pacemaker of this circadian clock is localized in the ventral part of the hypothalamus, the so-called suprachiasmatic nuclei (SCN), and is entrained by light signals mediated by the eye. The eye is directly connected via the retinohypothalamic tract (RHT) to the SCN. Light that reaches the retina elicits glutamate release at the synaptic terminals of the RHT and influences the neurons in the SCN in a manner that alters the behavioral state of the animal. A light pulse that reaches the retina at the beginning of the night elicits a delay of the clock phase, whereas a light pulse that reaches the retina at the end of the dark period leads to an advance of the clock phase. This advance or delay can be quantified by measuring the change in onset of wheel-running activity. Such measurements have, and continue to provide, a remarkably powerful assay of how light is detected and transduced to regulate circadian rhythms. The methods used for such measurements in mice are described in the following article.

1. Introduction

The circadian system “fine-tunes” physiology and behavior to the varying demands of activity and rest. However, the circadian system cannot confer any selective advantage unless internal biological time is phase-locked to environmental time. Most organisms use the changing quality of light at dawn and dusk to provide time of day information to set the phase of the clock, a process known as photoentrainment [1]. In mammals the photoreceptors that detect this change in twilight are located in the eye [2], but unraveling the mechanisms whereby the eye brings about photoentrainment have been surprisingly difficult. In recent years genetic lesions combined with behavioral analysis have provided significant advances in our understanding of these mechanisms. For example, mice in which the rod and cone photoreceptors have been genetically ablated are still able to shift their circadian rhythms in response to light, showing that uncharacterized ocular photoreceptors mediate the effects of light on the clock [3,4]. In addition, by manipulating both the irradiance and the wavelength of light stimuli the photopigment can be defined using action spectrum techniques [5]. Furthermore, behavioral observations in mice with a mutation in a phosphorylation site of the transcription factor CREB have shown that phosphorylation of CREB is responsible for light-induced phase shifts and that phosphorylation of CREB forms a critical part of the signal transduction pathway that translates light signals into a behavioral response [6]. Although we know that novel ocular photoreceptors play an important role in the regulation of the circadian system, the identity of the photopigment genes and genes of the signaling pathway remains poorly understood. One approach to identify these components is to screen mouse mutants for abnormal clock resetting behaviors. Here we describe the methodology of how to measure...
the influence of light on mouse circadian resetting behavior.

2. Material

2.1. Isolation cabinets

Diverse environmental cues including light, temperature, and behavioral or social signals such as activity, feeding, and pheromones can affect the circadian rhythm of a mouse. Therefore the study of rodent circadian rhythms requires measurement of circadian physiology or behavior under defined environmental conditions. Hence, isolation of an animal from other individuals and from lighting, noise, and activity of the workspace is important. Custom-designed isolation cabinets are widely used for a broad range of circadian experiments. They allow the animals to be singly housed in individual cages under independently controlled lighting regimes. However, isolation cabinets do not have independent temperature control and it is therefore important that air temperature is stable in the room in which the cabinets are housed. The size and proportions of cabinets can be altered to suit specific requirements. A standard design is depicted in Fig. 1 and consists of separate modules that house up to 12 cages. These are then stacked for space efficiency. Isolation cabinets are often connected directly to an externally vented air-handling system to avoid a buildup of pheromones. A description of the construction of isolation cabinets that house approximately six mice is given in Appendix A.

Fig. 1. The composition of an isolation cabinet containing running-wheel cages is schematically shown. Note that the setup can be adjusted to specific needs (see Appendix A).
2.2. Cages and wheel running

Running wheels provide a remarkably reliable measure of circadian behavior in rodents. Animals are housed individually in transparent plastic cages (e.g., 280 mm long × 105 mm wide × 125 mm high; Techniplast 1155M) that are equipped with a steel running wheel of 115 mm in diameter (Trixie GmbH; www.trixie.de, Article No. 6083) (Fig. 2). The axis of the wheel is held by Teflon fittings and is equipped with a plastic disk holding a small magnet (Fehrenkemper Magnetsysteme; www.fehrenkemper.de, Article No. 34.6401300702; see Fig. 2D). The magnet opens and closes a magnetic switch (Reed-Relais 60; Conrad Electronic AG; www.conrad.ch, No. 503835-22; see Fig. 2C) on rotation of the running wheel (Fig. 2; similar cages with a mechanical switch can be bought from Actimetrics; www.actimetrics.com). The switch is connected to a computer that counts the revolutions of the wheel (Appendices B and C). Twelve cages of this type can be placed in one isolation cabinet with the dimensions shown in Fig. 1 (length = 180 cm; height = 54 cm; depth = 69 cm). A larger type of cage is described in Appendix B; six large cages fit into the box described in Appendix A. This cage allows mice to be maintained for longer time intervals before cage cleaning becomes necessary. The different setups shown in Figs. 1 and 2 and described in Appendices A and B will not cause profound differences in the wheel-running behavior of mice, although the number of wheel rotations shown by individual mice will vary. Because the type of running wheel may influence the overall amount of wheel-running activity [7], it is important to state the type of running-wheel setup that is used in any circadian experiment.

2.3. Lighting

In circadian experiments light is used as a stimulus to elicit a response from the clock, i.e., phase delays and phase advances. In the isolation cabinets, mice are exposed to “bright” artificial light controlled by a timer which regulates exposure by turning lights either “on” or “off.” These conditions bear little resemblance to the natural photoperiod, where increase or decrease in light intensity occurs gradually in the morning and evening. For experiments designed to look at basic responses of the circadian system to light it is sufficient to provide this crude representation of the day and night cycle. The artificial light source in the isolation cabinet should...
provide a broad emission spectrum. For example, we use standard 18-W fluorescent light bulbs (e.g., Mazdafluor Symphony Azura 965 SF 18W/AZU) of which two are mounted in an isolation cabinet (Fig. 1; alternatively one longer bulb with 36W can be used). Painting the interior of the isolation chamber black minimizes reflection of light. The amount of light from all directions over a 180° field of view (illuminance), measured with a luxmeter (e.g., Testo 0500; Testo GmbH, D-79849 Lenzkirch, Germany), is between 200 and 300 lux at the bottom of the isolation chamber. Note that a luxmeter provides a measure of brightness as it would appear to a human observer. Thus the spectral response of the detector is not flat but attempts to reproduce the spectral sensitivity of the average human eye [8]. As a result a luxmeter is useful only for providing a crude measure of brightness and cannot be used to quantify defined wavelength or monochromatic light sources.

Examining the effect of specific wavelengths of light on resetting of the circadian clock is more complex [9] (for commercial light sources see Appendix C). The light emitted from the light source has to be monochromatic, which requires specific light sources and spectral filter systems [5]. Furthermore, measurements of monochromatic light demand the use of a specialized radiometric detector which measures all electromagnetic energy within the optical spectrum (see Macam Photometrics; http://www.macam.com). An ideal radiometric detector has a flat spectral response (unlike the luxmeter). A range of radiometric quantities and units are used, but the most commonly used is irradiance. Irradiance is a radiometric measure of the amount of light falling on a known surface area. The international unit of measure of irradiance is W/m². However, most detectors are calibrated in W/cm². Biologists use this measure to quantify the incident light coming from all directions over a 180° field of view. Because photopigments act as photon counters, a comparison of the effect of different monochromatic light stimuli demands that the stimuli contain the same number of photons (photon flux). Because the standard measurements of light are assessments of energy and not of photon number, the values of energy measurements have to be adjusted for equality in photon content. Thus Watts must be converted to photons. The energy of one photon is described by the equation 

\[ E = \frac{(6.626 \times 10^{-34} \text{Ws}^2) \times (3.00 \times 10^{17} \text{nm/s})}{500 \text{nm}} \]

\[ = 3.976 \times 10^{-19} \text{Ws}^2/\text{photon} \]

\[ = 3.976 \times 10^{-13} \mu\text{Ws}^2/\text{photon}. \]

The conversion of a light value from Watts into photons proceeds as follows. If, for example, the irradiance of the stimulus was 3 x 10⁻² µWs² at a λ of 500 nm light then one would need to divide the measured value in µWs² by the energy of a 500-nm photon in µWs². Thus, 3 x 10⁻² µWs²/3.975 x 10⁻¹³ µWs²/photons = 7.5 x 10¹⁰ photons/cm²/s. This calculation can be repeated using the same irradiance (3 x 10⁻² µWs²) but substituting different λ values. By doing this the relationship between Watts and photons and the fact that an equal number of Watts does not contain the same number of photon can be appreciated.

3. Light and wheel-running assays

3.1. Entrainment to a light–dark (LD) cycle

Before experiments can begin, stable entrainment must be achieved in the isolation cabinet. Wheel-running activity is monitored under a LD cycle that usually consists of 12 h of light and 12 h of darkness. Note that the activity duration of mice (z) is usually longer than 12 h. As a result 16L:8D h light–dark cycles are sometimes used for mice. It is common practice to match the light phase of cycles to the working day; lights in the isolation cabinet are programmed for “on” at 06:00 h and “off” at 18:00 h on a 12/12 h LD cycle. This makes it easier to check and care for the animals. If samples need to be collected after 18:00 h, then it may be convenient to entrain the animals to a reversed lighting regime (“lights on” at 18:00 h and “lights off” at 06:00 h) and sample during the normal day. The isolation cabinets can be used to entrain animals so that tissue can be collected at specific times of the light–dark cycle or to measure behavioral or physiological parameters at specific times.

Mice are transferred from the breeding colony and placed into cages containing a running wheel just before lights off (e.g., at 18:00 h) when mice are beginning their active period. Transfer at this time causes less disruption of the clock in response to the novel environment [10,11]. The individual running-wheel cages should have plenty of food, water, and “high-absorbance” bedding. The cage is fitted into an isolation cabinet bay and connected for data collection, and a unique file is created for data acquisition. Before the start of any experiment it is important to check the condition of the running wheel, as these may become distorted during the cage-washing process, and the connections of the wheel with the computer. Fixing problems is much more complicated after animals have been loaded into the isolation cabinet and after the experiment has started. If adjustments are necessary, it is best to do them just before lights off. This is also the best time to change food, water, and bedding (necessary every 3–4 weeks, depending on the size of the cage). After 7–10 days in the LD cycle the animals are
usually "entrained" with a stable phase relationship and experiments can be performed (see Fig. 3).

3.2. Determination of the circadian period length (τ)

After 7 days of stable entrainment (see above) the lights in the isolation cabinets are switched onto constant darkness (DD) and the animals are allowed to "free run." Free running rhythms have been particularly valuable in identifying clock mutants, and hence clock genes, in rodents [12,13]. The period of the circadian clock is not exactly 24h, and depending on species it will run a little slower or faster. The free running period of a mouse is typically between 23.5 and 23.8h but can vary between strains considerably. It is critical that the cage and the wheel should not be replaced during the free running phase of the experiment as another cage or wheel represents a novel stimulus which can phase shift the clock [10]. When exposed to LD cycles the mouse will be entrained, and the phase of activity onset will occur at more or less the same time every day. Activity onset in a nocturnal mammal such as a mouse is defined as zeitgeber time (ZT) 12. Under conditions of constant darkness activity onset will be determined by the free

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**Fig. 3.** Locomotor activity record for a mouse. Wheel-running activity (bold black line) is shown for 23 days (1 line = 1 day). The mouse was housed in a wheel-running cage in an isolation cabinet. For the first 7 days, the mouse was exposed to a light–dark cycle, dawn at 06:00h and dusk at 18:00h. Zeitgeber time is indicated (see text for details). The LD cycle was then replaced by constant darkness (DD). On the 7th day in DD, the mouse was exposed to a 15-min light pulse at circadian time 16. In this case the animal was removed from the isolation cabinet and transported in constant darkness, using an infrared viewer, and placed in a device that provided a 15-min monochromatic light pulse [9] (note gap in the recording of that day). After an additional 9 days the phase shift was determined (see text for details). Assays such as this have proved invaluable in examining the impact of retinal mutants on the ability of mice to response to light [18].
running period or \( t \) of the mouse and is termed circadian time (CT) 12. Note that activity onset for a diurnal animal is designated as either ZT0 or CT0. Wheel running is most frequently represented as an actogram, an example of which is shown in Fig. 3. About 7–10 days of uninterrupted recording are required to reliably measure the free running phase. The circadian data are analyzed mathematically with \( (\psi) \) periodogram analysis (e.g., with the Clocklab software from Actimetrics). Period length \( (\tau) \) is determined by the CT for activity onset on consecutive days. The individual variation in period length for mice of the same strain is not very large, and the CT based on the average period length of that strain is often used for all individuals. However, if a pulse of light is to be administered to a single animal at a specific circadian time then the phase of the individual animal must be determined (see below). Mouse mutants that loose the circadian rhythmicity in DD, such as the Per1/Per2 and the Cry1/Cry2 double-mutant mice [14,15], have no functional clock and so cannot be classified on the basis of their CT times. However, these animals often show overt rhythmic activity under LD cycles. Under these conditions behavior is being “forced” by the LD cycle and is not driven by an endogenous clock. Such apparent rhythmicity is called masking [16].

3.3. Measurement of light-mediated phase shifts

Phase shifts in circadian rhythms are the consequence of a disturbance of the clock by an environmental cue, such as light, noise, or food availability. As light is the most powerful zeitgeber, we will confine our discussion to the impact of light on the circadian system. Single, short-duration light pulses applied to the animals at the beginning or the end of the dark period either advance or delay the clock, respectively [17]. In this section we describe how phase advances and phase delays can be generated and measured.

Before exposure to a single pulse of light it is essential that mice are fully adjusted to the isolation cabinet and are fully equilibrated as described above. Once a stable free running period has been established the effect of a single light pulse can be examined. Most light pulse experiments use pulse durations between 15 and 30 min. A light pulse presented during the resting period of a mouse (its subjective day) does not influence the phase of the clock. Light is effective only when given during the activity phase of the mouse, i.e., between CT12 and CT24 (subjective night). In Fig. 3 the mouse has been entrained to an LD 12:12 h cycle and then released into DD to free run. A single pulse of light presented to the mouse given between the second part of the night CT18 and CT24 will induce activity to start earlier the next day; the mouse will show a phase advance, while light given between CT12 and CT18–20 will cause the mouse to start its activity later the next day (phase delay). Note that in most mouse strains phase delays are larger than phase advances. The largest phase shift is centered around CT16. In Fig. 3 the phase-delaying effect of light is seen after the presentation of a single light pulse given at CT16 (4 h after activity onset). Following the light pulse animals are left in DD. After at least 7 days (for phase delays 10 days is recommended) the size of the phase shift can be measured. To quantify the magnitude of phase shift, regression lines are drawn through all the activity onsets of the pretreatment free running activity onsets (Fig. 3, red line) and the posttreatment free running onsets (Fig. 3, blue line). The phase shift in onset on the first day after treatment is included; however, the first day after the treatment is not used in fitting the regression lines because the clock is usually still in transience and moving toward its final phase. The two regression lines are compared on the day following the pulse and the difference between them reveals the phase difference of the clock before and after the light pulse (Fig. 3, blue and red lines). Note that after a light pulse the period of the freerunning rhythm may change slightly. As a result the regression lines prior to and after the light pulse are frequently not parallel. Hence the reason for comparing the difference in regression lines on the day following the light pulse. If the value is positive this indicates a phase advance and when it is negative a phase delay (shown in Fig. 3).

4. Concluding remarks

The methods described above are extremely useful for the investigation of mouse mutants that do not show an obvious phenotype but that have a defect in the pathways that sense and transmit light information to the circadian pacemaker in the suprachiasmatic nuclei. Defects in the retina, the retinohypothalamic tract, and the signaling pathways that release and convert glutamate signals into cellular responses can be investigated. A critical point in behavioral experiments is the number of animals that are observed. In the assay described above the number of animals should not be below six to obtain a statistically significant representation of the population of a particular mouse strain. Additionally, mutant mice should be compared to littermate wild-type or congenic mice to ensure similarity in the mixed genetic background.

Appendix A. Isolation cabinets: structure, construction, and circuitry

All measurements are stated in centimeters and are purely guidelines.

The cabinet consists of a main box with a large front access door and two air circulation vents with light-tight baffles. The cabinet framework must be (1) sufficiently strong to bear the weight of being stacked and in constant use, (2) structurally rigid to ensure the integrity of
the light-tight junctions, and (3) finished in a water-resistant matte black for a nonreflective, cleanable surface. We selected 12-mm-thick marine medium-density fiberboard (mMDF) as the primary material for strength, rigidity, workability, water resistance, and cost.

The design is based on a central box with a large open frame in the front panel and light-tight seals in all joins (Fig. 4). The front opening includes staged pine framework with a central vertical strut to add rigidity to the structure.

The door is built from a single piece of mMDF with a pine framework attached to create tight junctions with the framework of the cabinet front opening (Fig. 5). It is hinged at the bottom to open downward and is fixed closed using durable “fitch” fasteners. Poor light-tight junctions can be rectified using Velcro tape layers along the joints of the framework, which compress to close any gaps.

Air circulation vents are built in each end of the modules (Fig. 6). Light-tight baffles are fitted over these vents. The baffles consist of a box structure with integrated partial panels to block light passage while allowing air circulation (Fig. 7). To reduce the module weight, 3-mm plyboard is used for the baffle panels.

Each cabinet has 7-day programmable lighting and a fan-driven constant air circulation system (Fig. 8).

To reduce the effects of electromagnetic radiation on data acquisition systems, both the fan and lighting cir-
cuits should be fitted through one end of the cabinet using shielded wiring wherever possible. The voltage specifications of the fans and lights should be changed to conform to the local standard mains power.

To drive air circulation, a 120-mm² 240-V AC sleeve-bearing axial fan is fitted over one of the internal air vents. This is connected to an external main supply using a manufacturer’s plug-cable, which is passed through a light-tight cable junction (Fig. 9). The four fans in a stack are connected to a common 240-V main power source.

For the programmable lighting control system a 200-mm² 240-V AC strip light is fitted centrally into the ceiling of each cabinet using ~8-mm-thick spacing blocks. This is connected to an external 7-day programmable timer via a light-tight junction, which is then connected to a 240-V AC main power supply.

Light-tight cable junctions are commercially available. The sleeve of the junction is fitted into a core, which has been drilled through the wall of the cabinet. The cable is fed through the junction and the sealing ring tightens around the cable as the cap is screwed down onto the junction sleeve, creating a light-tight seal.

The completed modules can then be stacked together on a custom-built wheeled base (Fig. 10).

Appendix B. Automated measurement of wheel running activity

This registers current change in a specific circuit, which is regulated by wheel rotations and can be broken down into a sequence of processes (Fig. 11).

B.1. Cages

Medium-sized rodent cages were modified to house a freely rotating running wheel with a cam fitted to the wheel axle (Fig. 12). The cage lid is extended to protect and enclose the wheel. The side panels of this extension are sheet metal joined by serial parallel metal bars; this provides strength while allowing normal cleaning. The wheel axle is fitted as a free bearing on one side and with an extended arm on the other. The cam is fitted to the extended arm. Mounting pins are located on the side panel so that the microswitch can be attached with its activating arm resting on the cam. Rotation of the wheel/cam depresses the switch arm.

Specialized cages of similar design are available commercially from Minimitter.

B.2. Circuitry

The microswitches and a light-dependent resistor (LDR) connect to cabinet circuitry with terminals in an
external junction box. The terminals are linked to a data acquisition board, which in turn connects to a computer, which caches count data in the appropriate bins for the specific channel and time.

B.3. Switches

The microswitch is connected to a pair of 4-mm male banana plugs/bunch pin plugs.

B.4. Cabinet circuitry

The cabinet contains six pairs of 4-mm female insulated head panel sockets, each pair corresponding to a numbered cage bay (1 to 6 from left to right) that are mounted in a plastic conduit running the length of the cabinet. This is fitted to the internal door framework at the top of the cabinet (Fig. 13). A two-ply (red and black) shielded data cable runs from each terminal pair, along the conduit to the end of the cabinet opposite to the fan and light wiring. The LDR is fitted to the inside wall of the cabinet near to the junction box end of the conduit.

The cage bay and LDR cable are fed to an external junction box. The junction box is secured onto the external cabinet wall and a port is drilled through both the back of the junction box and the cabinet wall for the cables. A light seal is created when the junction box
Fig. 10. Isolation cabinet modules with a wheeled base.

Fig. 11. The processes and equipment in automated running wheel activity measurement.
lid is secured. In the junction box each black wire is attached to a corresponding numbered port on a terminal block and the red wires to commoning block (Fig. 14).

The terminals should be connected to the appropriate color-coded wires of a shielded eight-way data cable (Fig. 14). The other end of the cable is then fitted with a shielded 8/8 RJ45 plug with the gray line on the left when looking at the flat side of the plug.

B.5. Computer hardware and software

All of these components can be purchased as a ready to go package from Actimetrics.
An IBM-compatible desktop computer is located remote from the experimental room (in an adjacent room) to reduce the effects of dust on performance and reliability. An off-line UPS is used to ameliorate the potential interference of power surges and short power cuts on this system.

An AMUX 64-T (National Instruments) data acquisition board is fitted with pull-down 10-kΩ resistors, secured in a protective shielded case with the channels connected to specific RJ45 sockets. The RJ45 plug for a cabinet corresponds to one of nine sockets in the modified housing.

A PCI 6503 card (National Instruments) is fitted into a vacant slot and the AMUX 64-T is connected using specified cables to the PCI 6503. The computer is then configured using the manufacturers’ NI-DAQ software to accommodate the PCI 6503 and the number of AMUX 64 T boards being supported.

The ClockLab software (Actimetrics) is installed into the computer and the channels are programmed as
count or light channels. When animals are placed into cabinets for experiments a unique file is raised in the specific channel of the ClockLab window.

The internal light data are recorded as on or off over time. The running wheel data are recorded as counts over time. The light data for a cabinet are integrated with the count data files for the cabinet.

These files can then be analyzed using the ClockLab data analysis program, which allows a range of tests on the effect of the experimental conditions on circadian behavior.

Appendix C. Suppliers of hardware and software for circadian experiments

<table>
<thead>
<tr>
<th>Company</th>
<th>Products</th>
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<tbody>
<tr>
<td>Actimetrics http://</td>
<td>Computerized technology to monitor circadian wheel running and other</td>
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<td><a href="http://www.actimetrics.com">www.actimetrics.com</a></td>
<td>aspects of behavior. Clocklab is a specialized software system for</td>
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<td></td>
<td>circadian research, which is compatible with technology from other</td>
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<td></td>
<td>companies</td>
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<tr>
<td>Data Sciences</td>
<td>Computerized implanted radiotelemetry technology to measure physiological</td>
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<tr>
<td>International http://</td>
<td>parameters and gross motor activity</td>
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<tr>
<td><a href="http://www.datasci.com">www.datasci.com</a></td>
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<tr>
<td>Minimitter http://</td>
<td>Computerized technology and accessories to monitor temperature, heart</td>
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<tr>
<td><a href="http://www.minimitter.com">www.minimitter.com</a></td>
<td>rate, gross motor activity, wheel running activity, metabolism, and feeding</td>
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<tr>
<td><a href="http://www.trikinetics.com">www.trikinetics.com</a></td>
<td>Commercial light sources for circadian experiments</td>
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<td>PLM illumination</td>
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