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Photic Sensitivity Ranges of Hamster Pupillary and Circadian Phase Responses Do Not Overlap

Roelof A. Hut,[*,†,‡,1,2] Malgorzata Oklejewicz,[*,†,‡,3] Camille Rieux,[*,†,‡] and Howard M. Cooper[*,†,‡]

*INSERM, Bron, France, †Stem Cell and Brain Research Institute, Department of Chronobiology, Bron, France, ‡University of Lyon, France

Abstract   Mammalian retinal photoreceptors form an irradiance detection system that drives many nonvisual responses to light such as pupil reflex and resetting of the circadian clock. To understand the role of pupil size in circadian light responses, pupil diameter was pharmacologically manipulated and the effect on behavioral phase shifts at different irradiance levels was studied in the Syrian hamster. Dose-response curves for steady-state pupil size and for behavioral phase shifts were constructed for 3 pupil conditions (dilated, constricted, and control). Retinal irradiance was calculated from corneal irradiance, pupil size, retinal surface area, and absorption of ocular media. The sensitivity of photic responses to retinal irradiance is approximately 1.5 log units higher than to corneal irradiance. When plotted against corneal irradiance, pharmacological pupil constriction reduces the light sensitivity of the circadian system, but pupil dilation has no effect. As expected, when plotted against retinal irradiance all dose-response curves superimposed, confirming that the circadian system responds to photon flux on the retina. Pupil dilation does not increase the circadian response to increasing irradiance, since the response of the circadian system attains saturation at irradiance levels lower than those required to induce pupil constriction. The main finding shows that due to the different response sensitivities, the effect of pupil constriction on the light sensitivity of the circadian system in the hamster under natural conditions is virtually negligible. We further suggest the existence of distinct modulating mechanisms for the differential retinal irradiance sensitivity of the pupil system and the circadian system, which enables the different responses to be tuned to their specific tasks while using similar photoreceptive input.

Key words   pupillary light reflex, circadian system, Syrian hamster, photoreception, photic system

Environmental light intensity affects many biological parameters in mammals. Irradiance is detected by the retina and this photic information is relayed by the retinohypothalamic and optic tracts to brain structures that regulate the circadian system, melatonin secretion, pupil constriction, and other physiological, behavioral, and cognitive functions (Lucas et al., 1999; Thapan et al., 2001; Brainard et al., 2001;

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1. Present address: University of Groningen, Center for Life Sciences, Department of Chronobiology, Haren, The Netherlands.

2. To whom all correspondence should be addressed: R.A. Hut, University of Groningen, Center for Life Sciences, Department of Chronobiology, PO Box 14, 9750 AA, Haren, The Netherlands; e-mail: r.a.hut@rug.nl.

3. Present address: Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands.

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Panda et al., 2002, 2003; Ruby et al., 2002; Hattar et al., 2003; Mrosovsky and Hattar, 2003; Cajochen et al., 2005). The precise involvement of different photoreceptor types in irradiance detection responses is not yet entirely clear, but major advances have been made over the last few years. Although classical photoreceptors (rods, cones) play a role in detecting environmental light intensity, intrinsically photosensitive retinal ganglion cells (ipRGCs) appear to be highly specialized for this task (Provencio et al., 2002; Berson et al., 2002; Hattar et al., 2002). IpRGCs express the photopigment melanopsin but also receive synaptic inputs from rods and cones (Belenky et al., 2003; Sollars et al., 2003; Wong et al., 2007; Dkhissi-Benyahya et al., 2007; Drouyer et al., 2007; Viney et al, 2007). IpRGCs innervate different brain areas (Hattar et al., 2002, 2006) among which are the SCN, residence of the circadian pacemaker, and the olivary pretectal nucleus (OPN) that controls pupillary light response (PLR).

Since structures of both the circadian and the pupillary control systems receive input by the same photoreceptor types, one might expect similar response sensitivities to light. Indeed, action spectra in wild-type rodents for PLR, melatonin suppression, and circadian phase shifts show roughly the same relative peak spectral sensitivity around 500 nm (Takahashi et al., 1984; Provencio and Foster, 1995; Yoshimura and Ebihara, 1996), while in mice lacking rods and cones both these responses show a shift in peak sensitivity to 480 nm (Yoshimura and Ebihara, 1996; Lucas et al., 2001; Hattar et al., 2003) that matches the sensitivity of melanopsin ipRGCs (Berson et al., 2002). However, the absolute sensitivities in terms of retinal irradiance for these different responses have not been directly compared. This question is not trivial, since the pupil acts as an aperture regulating the amount of light falling on the retinal surface. Thus, as pupil size dynamically adjusts to changes in irradiance, the photon flux available to retinal photoreceptors involved in circadian responses may potentially be modified. In theory, similar retinal irradiance sensitivity in both systems should widen the dynamic range of the circadian response to environmental irradiance, since as corneal irradiance increases the constricting pupil aperture would progressively shield off much of the light impinging on the retina. Therefore, we explored the hypothesis that the PLR functions to expand the dynamic range of photic circadian responses to changing environmental light conditions.

PLR interaction with circadian light response was quantified in Syrian hamsters, using PLR dynamics, action spectrum, and dose-response curves in pharmacologically dilated, constricted, and control-treated pupils. Sensitivity of both systems was compared by calculating retinal irradiance from corneal irradiance using eye anatomy, pupil size, and wavelength specific absorption of ocular media. The measured absolute retinal irradiance sensitivity was different for circadian and steady-state PLR responses, leading to the finding that manipulated pupil constriction, but not dilation, affects circadian light sensitivity. We conclude that PLR in the hamster has minor effects on widening of the circadian dynamic response range, while different sensitivities of both systems indicates system specific tuning of light responses within the dynamic range offered by the nonvisual photoreceptive channels involved in these functions.

MATERIALS AND METHODS

Animals

Four- to 8-month-old male Syrian hamsters were kept in individual running wheel cages. Food and water were available ad libitum. Animals were maintained in compliance with current international regulations on animal care, housing, breeding, and experimentation.
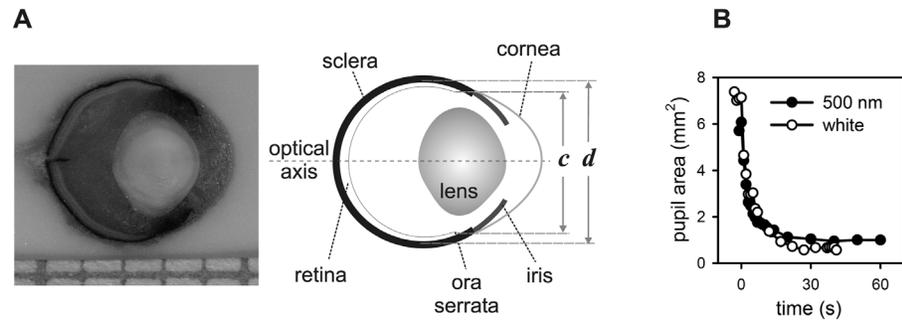
Light Stimulation Procedures

All light stimulations were performed by placing an individual hamster in a closed white reflecting chamber covered by a white diffuser. The diffuser was illuminated by a light source equipped with a tungsten-halogen light bulb (24 V/150 W; Osram, Rosny-Sous-Bois, Paris, France), mounted with a parabolic reflector, infrared filter, collimating lenses, and interchangeable monochromatic interference filters (half band width = 10 nm; Corion, Franklin, MA). The lamp's light intensity was regulated using a calibrated computer driven voltage regulator. Intensity and spectrum were measured with a photometer (IL700; International Light, Newburyport, MA) and a spectrophotometer (Ocean Optics, Dunedin, FL).

Quantification of Behavioral Phase Shifts

For behavioral phase shifts, light stimulations were applied at ZT19 during the first cycle in continuous darkness (31 h) following entrainment under a 12-h light/12-h dark (LD) cycle (Aschoff type 2 protocol). At this circadian phase, hamsters show maximal advances

in behavioral phase shifts (Nelson and Takahashi, 1991). The animals were removed from their home cages at 2 time points (30 and 15 min) before light stimulation and a drop of aceclidine (a parasympathetic cholinergic agonist; glaucostat, 2%; MSD Laboratories, Paris, France), tropicamide (a parasympathetic cholinergic antagonist; 0.5%; CIBA Vision Ophthalmics, Toulouse, France), or saline (NaCl 0.9% in sterile, demineralized water) was placed on both eyes at each time point in total darkness using an infrared night visor (Unitec, GSCI, Richmond Hill, Ontario, Canada). Five minutes before the time of light

Figure 1. Anatomical parameters for retinal irradiance calculation and PLR dynamics. (A) Hamster eye cross section along the equator of the eye. Corneal to retinal irradiance transformation (equations 2 and 3) is based upon transmission of ocular media (in particular the lens), pupil size, and 2 anatomical measures used to calculate retinal surface: corneal diameter (*c*) and retinal diameter (*d*). Scale marks are millimeters (mm). (B) Dynamics of the pupil response over time. Maximum light intensity of the light source was used either with the 500-nm monochromatic filter (3.6×10^{13} photons/cm²/sec) or without (bright white light, 86.1 μW/cm²). The pupil reached a stable size after roughly 30 sec.

stimulation, animals were placed in the stimulation chamber in complete darkness. After acclimatization in the stimulator, a 15-min light pulse was delivered at a calibrated irradiance level, after which the animals were removed from the stimulator in complete darkness and placed back in their home cages.

Animals (*n* = 48) were stimulated for 15 min at 500 nm using 6 irradiances from 1.0×10^8 to 1.0×10^{14} photons/cm²/sec. Light stimulations were applied using a crossover design in which all individuals received treatment with tropicamide, aceclidine, or saline in a pseudo-random order. For each light intensity, a different group of hamsters was used (*n* = 8). Individual behavioral activity patterns were measured with passive infrared motion detectors directed at the running wheels. Activity data were continuously recorded and collected in 1-min bins (CAMS; Circadian Activity Monitoring System, INSERM, France). Phase shifts were quantified using an objective activity onset detection template procedure (ClockLab; Actimetrics, Evanston, IL). A regression line through 10-14 onsets before the light pulse was used to determine the expected phase on the first cycle after the light pulse. After the light pulse the phase of the circadian rhythm was found to be unstable for 3-5 days. These transient days were not used in the calculation of phase after the light pulse. A regression line through 10-14 onsets after the transient days was extrapolated to determine circadian phase at the first cycle after the light pulse. The difference between the first expected onset after the light pulse and the observed first onset was used to determine the circadian phase shift.

Quantification of Steady-State Pupillary Light Responses

For measures of steady-state pupillary light responses, the same light stimulator was used, but now the animal was exposed to a range of irradiances, using continuous light stimulation with stepwise increments. In each session, 4 hamsters were measured around ZT19 and each session lasted 1-2 h. At 30 min and 15 min before a series of light stimulations, these individuals were treated with tropicamide, aceclidine, or saline in a pseudo-random order. After 30-sec exposure to a certain light intensity (when pupil constriction was found to be stable; Fig. 1B), the side door of the animal compartment in the stimulator box was opened to restrain the hamster. With the head of the animal in the center of the stimulation chamber, the hamster's pupil was placed into camera focus and a series of 5-10 digital images was taken using a software-triggered infrared CCD (charge-coupled device) camera with a long-focus macro objective and a short-range depth of field. The eye was illuminated by an array of infra-red light emitting diodes (LEDs), which did not affect pupil constriction (Hattar et al., 2003). The animal was subsequently placed back, the lid was closed, and the light intensity was raised to the next level.

The restraining procedure had no measurable effect on light intensity at the level of the cornea because the animal's head was placed in the center of the chamber and pupil size measured though an small window in the side of the chamber. To minimize the effect of restraining stress on pupil size, 4 hamsters underwent a series of 4 separate training ses-

sions before the actual pupil response measurements were taken. After training the hamsters were found to be at ease while being manually restrained for ~10 sec and their pupil showed repeatable responses to a light stimulation. During 10-15 min, a full irradiance dose-response curve was measured in each hamster using 10 different increasing irradiance levels ranging from 6.3×10^7 to 2.5×10^{15} photons/cm^2/sec. To avoid under-estimation of pupil size, the perpendicular position of the eye to the camera's optical axis was judged by online visual inspection (circularity of the pupil) before an image was taken. Pupil size was quantified by digital image analyses on pixel numbers within the outlined pupil circumference. The short ranged depth of field enabled precise calibration of pixel size, which was recalibrated before every measurement session.

Dose-response curve parameters were estimated by nonlinear regression using a 4-parameter sigmoidal function (equation 1; modified after Naka and Rushton, 1966):
in which,

P_{I_c} = pupil area (in mm^2) at I_c,

$$P_{I_c} = P_{min} + \frac{P_{max} - P_{min}}{1 + \left(\frac{10 \log I_c}{-a}\right)^{-b}} \qquad (1)$$

I_c = corneal irradiance (in photons/cm^2/sec),
P_{min} = minimal asymptotic pupil area (in mm^2),
P_{max} = maximal asymptotic pupil area (in mm^2),
a = log sensitivity,
b = steepness.

Retinal Irradiance Calculation

Retinal irradiance was calculated from corneal irradiance, average steady-state pupil size, retinal area, average corneal transmittance, and average lens transmittance (Hut et al., 2000), using equation 2:
in which,

$$I_r = \frac{P_{I_c} \times I_c \times T_c \times T_l}{A} \qquad (2)$$

I_r = retinal irradiance (in photons/cm^2/sec),
I_c = corneal irradiance (in photons/cm^2/sec),
P_{I_c} = pupil area (in cm^2) at I_c,
T_c = corneal transmittance (as fraction),
T_l = lens transmittance (as fraction),
A = outer retinal surface area (in cm^2).

Before photons attain the retinal surface, they must pass through absorbing ocular media and the aperture of the pupil. The transmittance of aqueous humor in the anterior chamber and the vitreous humor is negligible and was considered to be 1, whereas photon absorption is most prominent in the lens and cornea. Therefore, the actual number of photons per time unit (flux) that is received by the retina equals the photon flux on the cornea (I_c) multiplied by pupil aperture (P_{I_c}) and the transmittance of the cornea (T_c), and lens (T_l). Thus, the total amount of photons projected to the retina will be $P_{I_c} \times I_c \times T_c \times T_l$, which is the numerator in equation 1. For the calculation of the outer retinal surface area (A), we assumed a spherically shaped retina. However, this spherical shape is not completely covered by retinal tissue: the retina ends at the ora serrata, which forms a circle on the retinal sphere, close to circle where the cornea attaches to the sclera. The distance between the ora serrata in a cross section of the eye equals the diameter of this circle (Fig. 1A). This distance could also be estimated by the diameter of the cornea in the intact eye. The area of the sphere that is not occupied by retinal tissue must be subtracted from the total surface area of the sphere to calculate the actual area occupied by retinal tissue. This can be done by using equation 3, which was deduced for this purpose:

$$A = \pi d \left\{ \frac{d}{2} + \sqrt{\left(\frac{d}{2}\right)^2 - \left(\frac{c}{2}\right)^2} \right\} \qquad (3)$$

in which,
A = outer retinal surface area (in cm^2),
d = outer retinal diameter (in cm),
c = diameter of the ora serrata or corneal diameter (in cm).

Classically, outer retinal surface area (A) is calculated using the "spherical dome" equation, $A = \pi h d$, in which d is the outer retinal diameter and h is the height of the retinal dome (i.e., the distance along the optical axis between the outer retina and the perpendicular line connecting both ora serrata points on a cross section). Although this approach seems simpler, it needs an additional determination of the optical axis, which is not needed in our approach. In dissected eyes, we found that equation 3 yields more reliable results than the classical approach because fewer anatomical points must be determined.

Although c and d can in principle be estimated on whole eyes, these values were measured more

accurately from freshly sectioned eyes by embedding the eye in mounting gel (Tissue-Tek, Sakura, Bayer Diagnostics, Cergy-Pontoise, France; 2008, distributed by Sakura, Lille, France) on a freezing sliding microtome, with the optical axis parallel to the cutting plane of the knife. During sectioning the eye was digitally photographed (along with a calibration ruler) and the image showing the largest eye diameter was used for digital measurement of c and d using image analyses software (ImageJ, NIH; http://rsbweb .nih.gov/ij/download.html). When the eye was positioned correctly, this image should also show the nervus opticus, as can be seen in Figure 1A.

RESULTS

Retinal Surface Area

To calculate retinal irradiance from corneal irradiance, the ora serrata diameter (c) and outer retinal diameter (d) was obtained from 4 eyes from 4 different individuals following completion of the behavioral experiments (Fig. 1A). Outer retinal surface area (A) was found to be 0.4457 (SD = 0.01) cm^2 using equation 3. This value was subsequently used to calculate the retinal irradiance from corneal irradiance depending on pupil size.

Pupillary Light Response in Dilated, Constricted, and Control Hamsters

The dynamics of hamster pupil constriction were first assayed to optimize the experimental design for measuring light-induced pupil constriction and behavioral responses of the circadian system. This initial set of experiments aimed to assay the time required to attain a stable pupillary constriction state in an untreated animal. Stable constriction was reached after 30 sec using either bright white light (86.1 μW/cm^2) or 500-nm monochromatic light at 2.5×10^{15} photons/ cm^2/sec corneal irradiance (Fig. 1B). The 500-nm stimulus was used, since this value is close to peak sensitivity for the pupil response in mouse (Lucas et al., 2001), in hamster (personal observations), for the action spectrum for hamster behavioral phase shifts (502 nm, Takahashi et al., 1984), and c-Fos induction in the SCN (496 nm; Dkhissi-Benyahya O and Cooper HM, personal observation).

Measures of steady-state pupil sizes at different corneal irradiances (P_{I_c}) were obtained to calculate retinal irradiance (I_r) from corneal irradiance (I_c) using

equation 2. Pupil size was measured in animals with pharmacologically constricted (aceclidine treated) or dilated (tropicamide treated) pupils, and in the control (saline treated) animals. Tropicamide and aceclidine were found to be very potent in affecting pupil size (Fig. 2A) and maintained either a fully dilated (tropicamide) or constricted (aceclidine) pupil around the dynamic range of the saline-treated control animals. The 500-nm monochromatic light dynamic response range for saline-treated pupil area when plotted on a linear scale, as commonly used, extends from 6.0×10^{10} to 1.0×10^{13} photons/cm^2/sec corneal irradiance. For this study, however, the logarithm of pupil area is used, since it is more relevant for calculating retinal irradiance. The dynamic range of log pupil area extended from 3.0×10^{11} to 2.2×10^{14} photons/cm^2/sec corneal irradiance. With the 2 pharmacological treatments, the maximum (tropicamide) and minimum (aceclidine) pupil sizes were greater or less than in saline-treated animals, which is a known phenomenon of these topical treatments in humans. The parameters of the curves obtained from these data are presented in the legend of Figure 2A and were used for calculating retinal irradiances in Figure 2B.

Effects of Pupil Size Manipulation on Circadian Behavioral Phase Shifts

To study the effect of pupil size on behavioral phase shifts of the circadian rhythm, pupil aperture was manipulated by application of tropicamide (dilated) and aceclidine (constricted, Fig. 2B). Control animals (saline treated) showed increased phase-shift responses over 1.4 log units corneal irradiance (dynamic range: 3.31×10^{10} to 9.12×10^{11} photons/cm^2/sec), which is slightly smaller than previously reported (Takahashi et al., 1984; Nelson and Takahashi, 1991). Absolute sensitivity of the response as defined by the half saturation value was found at 1.62×10^{11} photons/cm^2/sec, which is slightly more sensitive than 3.1×10^{11} photons/ cm^2/sec as described by Nelson and Takahashi (1991) for 5-min stimulation at 503 nm. There was no effect of pupil dilation on the behavioral phase-shift response, because both corneal irradiance dose-response curves superimpose (Fig. 2B, left panel; t test, $p = 0.89$). In contrast, pupil constriction was found to decrease light sensitivity of this circadian response since the dose-response curve was shifted to higher corneal irradiances by 2.31 log units (Fig. 2B, left panel; t test, $p = 0.004$).

To determine why pupil constriction, but not pupil dilation, affects circadian responses, it is necessary to

A

B
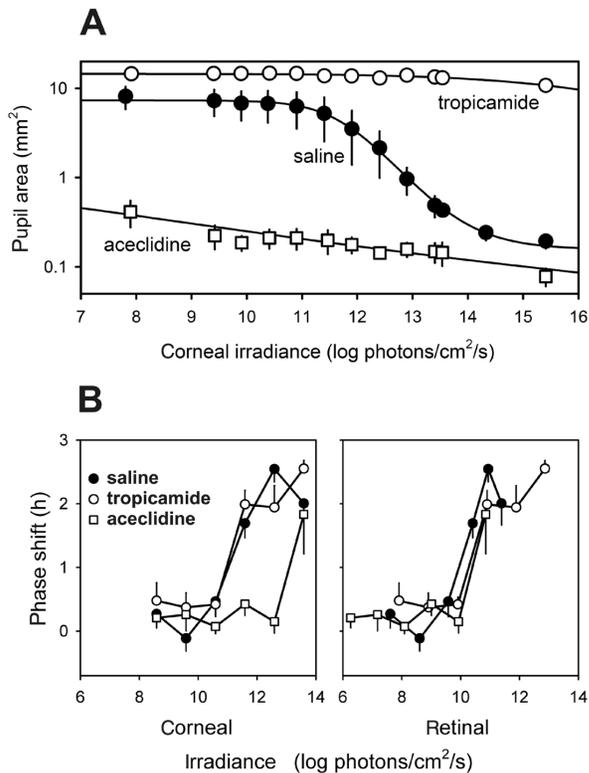
Figure 2.   Pharmacologically manipulated and normal PLR and circadian phase-shift responses to a 500-nm monochromatic light stimulation. (A) PLR, plotted on a logarithmic scale. All data were collected in the same 4 animals that were either treated with tropicamide (dilation, ○), aceclidine (constriction, □), or saline (control, ●). Dose-response curves were estimated by non-linear curve fitting on the linear response data using equation 1. Estimated curve parameters for saline: $P_{min} = 0.16$, $P_{max} = 7.33$, $a = -11.84$, $b = -23.29$; tropicamide: $P_{min} = 0.08$, $P_{max} = 14.52$, $a = -17.20$, $b = -9.59$; and aceclidine: $P_{min} = 0.00$, $P_{max} = 0.91$, $a = -7.00$, $b = -2.73$. Each data point is the mean for 4 individuals and the same individuals were used in all 3 treatments. Error bars indicate SEM. In the tropicamide group, as in some other points, the symbols may cover the error bars. (B) Behavioral phase shifts of the circadian activity rhythm induced by different light levels for hamsters with dilated (tropicamide, ○), constricted (aceclidine, □), and control (saline, ●) pupils. Phase shifts were plotted against corneal irradiance (left) and retinal irradiance (right). Four parameter sigmoidal curves (equation 4) fits were estimated (corneal irradiance: saline, $P_{min} = 0.16$, $P_{max} = 2.25$, $a = -11.21$, $b = 34.19$; tropicamide, $P_{min} = 0.42$, $P_{max} = 2.28$, $a = -11.32$, $b = 71.29$, aceclidine, $P_{min} = 0.22$, $P_{max} = 2.14$, $a = -13.52$, $b = 339.9$; and retinal irradiance: saline, $P_{min} = 0.16$, $P_{max} = 2.25$, $a = -10.11$, $b = 38.54$; tropicamide, $P_{min} = 0.42$, $P_{max} = 2.27$, $a = -10.64$, $b = 66.78$; aceclidine, $P_{min} = 0.22$, $P_{max} = 2.14$, $a = -10.80$, $b = 267.7$). When plotted as corneal irradiance (left) the response sensitivity of the aceclidine-treated animals was lower than animals treated with saline (t test, $p = 0.004$) or tropicamide (t test, $p = 0.038$).

plot the phase-shift responses under dilated, constricted, and control conditions against retinal irradiance representing the actual photon flux attaining the

retina. Equations 2 and 3 were used to calculate retinal irradiances from corneal irradiances, assuming Syrian hamster corneal and lens transmittance fractions to be respectively 0.73 and 0.91 at 500 nm (Hut et al., 2000), and the average hamster retinal surface area (A), which was found to be 0.4457 cm^2 (Fig. 1A). When plotted against retinal irradiance, dose-response curves for tropicamide-, aceclidine-, and saline-treated animals superimpose and no significant differences in sensitivities were detected (Fig. 2B, right panel; t tests, $p > 0.87$). In both the saline and tropicamide dataset, our analysis indicates saturation in circadian phase advance responses around 2 h, which is similar to the finding of Nelson and Takahashi (1991).

To illustrate the effect of manipulated pupil size on light-induced behavioral phase shifts, a comparison is shown for an intermediate corneal irradiance (4.0×10^{11} photons/cm^2/sec at 500 nm). Pupil photographs show a clear effect of treatment on pupil aperture (Fig. 3, top panels). In this example, pupil constriction strongly reduces retinal irradiance by roughly 2.5 log units, whereas the effect of pupil dilation does not notably alter retinal irradiance compared to the control state. (Fig. 3, middle panels). The effect of pupil manipulation on the phase shift in the circadian activity rhythm is illustrated in the bottom panels. The activity rhythm advances when a light pulse is applied at ZT19 and, as expected, the amount of advance correlates well with retinal irradiance rather than corneal irradiance, which is equivalent in all 3 groups.

The dose-response curves calculated from the data presented in Figure 2B allow assessment of the dynamic range of the circadian phase-shift response. When only the control (saline treated) phase-shift curves are plotted against retinal or corneal irradiance (Fig. 4A), a decrease in sensitivity by 1.1 log unit is observed. This is partly due to absorption at 500 nm by the cornea (0.13 log unit) and the lens (0.04 log unit; Hut et al., 2000), but the main factor results from the small pupil aperture projecting to a relatively large retinal surface area. The steady-state surface area of saline-treated pupil may range between 0.0015 and 0.0732 cm^2 (Fig. 2A) and combined with a retinal surface area of 0.4457 cm^2, the reduction in light intensity per surface area (equation 2) potentially ranges between 1.00 (normal dilated pupil) and 2.45 (normal constricted pupil) log units.

To illustrate the effect of pupil function on circadian behavioral responses, a dynamic range for the circadian system was calculated for the corneal and the retinal dose-response curves as the range
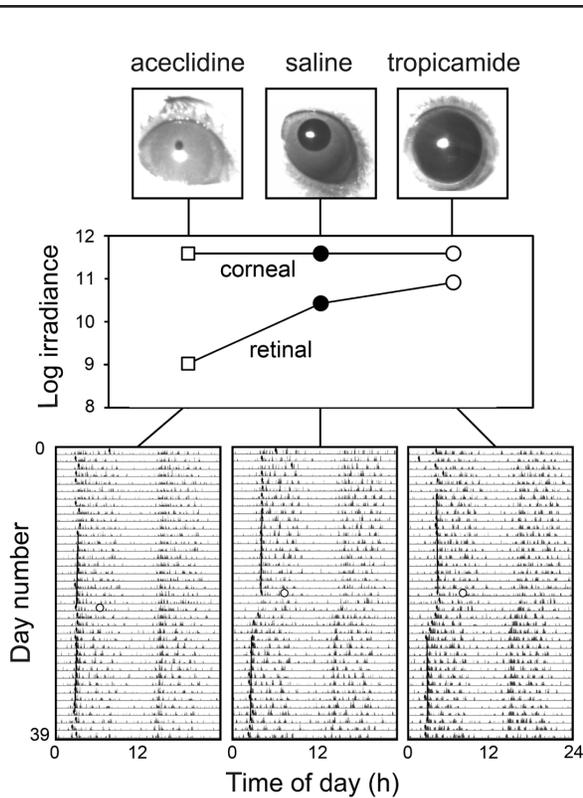
Figure 3.   Representative examples of the effect of aceclidine (☐), saline (●), and tropicamide (○) treatment on behavioral phase shifts. (Top panels) Pupil sizes at intermediate corneal light levels (4.0 × 10¹¹ photons/cm²/sec). (Middle panels) Effect of pupil manipulation on retinal irradiance at equal corneal irradiance (irradiances in photons/cm²/sec). (Bottom panels) Representative actograms of 3 hamsters receiving the light conditions represented in the middle panel, timed at ZT19 (white circle dot). Actograms are double-plotted activity records over 39 days. Onsets of activity are marked by a black oval dot. To estimate the phase shift, 10-14 days before and after the light pulse were analyzed by linear regression lines through the stable onsets (3-5 days after the light pulse were skipped to account for transient days).

between 10% and 90% of the response. This range in irradiances is indicated by the hatched areas in Figure 4A. These dynamic ranges in irradiance can be projected on curves that indicate the relationship between retinal and corneal irradiance (transformation curves; Fig. 4B). In the aceclidine- and tropicamide-treated pupils, the transformation curve is almost a straight line since the pupil has almost a fixed size under these conditions (see also Fig. 2A). Both lines are below the line $y = x$; thus retinal irradiance in the Syrian hamster is always lower than corneal irradiance (overall ~0.5-2.5 log unit reduction), even when the pupil is extremely dilated by tropicamide. When

the pupil is constricted by aceclidine, retinal irradiance is reduced by ~2.5 log units. In the case of saline treatment, the pupil can exert its normal dynamics in size, following pupil size of the dilated tropicamide curve at the lower irradiances and at the higher irradiances than of the constricted aceclidine pupil. Following the previously described dynamic range between 10% and 90% response, it was calculated that the steady-state pupil constriction (expressed as log pupil area) occurs between 2.95×10^{11} and 2.19×10^{14} photons/cm²/sec corneal irradiance, as indicated in Figure 4B by the horizontal bar. The curves in Figure 4A describing the dynamic range for corneal and retinal irradiances indicate that the steady-state pupil constriction occurs at higher corneal irradiances (dark gray bar in Fig. 4B) than the response range of the circadian system (light gray shaded area in Fig. 4B).

DISCUSSION

A first result of our study on the effects of pupil size manipulation shows that light-induced circadian phase shifts in the hamster depend on retinal irradiance (Fig. 2B). Although this observation appears rather evident, all previous studies of photic phase-shift behaviors in rodents use corneal irradiance to express dose-response relationships. Second, pupil dilation has no detectable effect on circadian phase-shifting responses (Fig. 2B). Finally, the hamster circadian system was found to be more sensitive to light than the steady-state pupillary light response (data respectively in Figs. 2 and 5). When plotted against corneal irradiance, the dynamic range of the circadian system (3.3×10^{10} to 9.12×10^{11} photons/cm²/sec) shows little overlap with the dynamic range of steady-state pupil constriction (2.95×10^{11} to 2.19×10^{14} photons/cm²/sec; Fig. 4B). The relative difference remains essentially similar when plotted against retinal irradiance (Fig. 5). Due to this disparity in sensitivity, an essential, somewhat surprising conclusion is that in normal conditions circadian phase-shifting responses are not significantly affected by steady-state pupil size. There are 2 caveats to this conclusion. First, it is unknown whether this disparity in response sensitivity is valid for other species (rodent or nonrodent); and second, steady-state pupil measures were measured after 30 sec, whereas phase shifts represent the integrated response over 15 min of light stimulation. It is possible that use of shorter light pulses for phase shifts or longer exposures of the pupil could potentially modify the results. However, we find that
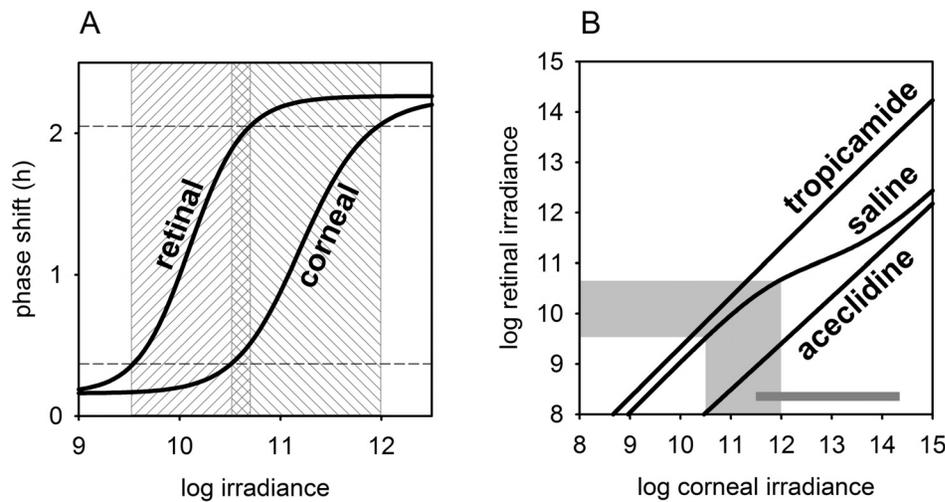
Figure 4. Comparison of corneal and retinal dose-response curves. (A) Dose-response curves for behavioral phase shifts, deduced from the data in Figure 2, for saline-treated animals plotted as a function of retinal irradiance and corneal irradiance. The hatched areas indicate the dynamic response range between 10% and 90% of the response (lower and upper dashed lines) for retinal and corneal irradiance. (B) Relationship between corneal and retinal irradiance as described by the transition curves for tropicamide-, aceclidine-, and saline-treated animals. The light gray area indicates the dynamic response range for behavioral phase shifts as a function of corneal and retinal irradiance, obtained from (A). The dark gray bar indicates the dynamic response range for saline-treated pupil constriction obtained from Figure 2A.

for longer pupillary light exposure (>5 min) in the mouse, steady-state pupil size does not vary (Mure L, personal communication). In addition, reciprocity (response amplitude as a function of total photon number) for single pulse phase shifts and FOS induction in hamsters holds within the boundaries of 30 sec to 15 min (Nelson and Takahashi, 1991; Dkhissi-Benyahya et al., 2000). In contrast, for very short light pulses or pulse sequences this relation no longer holds (Vidal and Morin, 2007). Finally, our test situation differs considerably from the dynamic changes of light exposure that occur in natural conditions.

Within the dynamic range of the circadian system, pharmacological pupil dilation (tropicamide) has almost no effect on circadian responses, since the untreated pupil (saline) is also in a dilated state in this range. Constricting pupil size (aceclidine) in this range of irradiances reduces light-induced phase shifts considerably (Fig. 4B). It was thus initially expected that pupil constriction would exert an effect on circadian responses when the untreated pupil starts to constrict at high irradiances (>2.95 × 10^{11} photons/cm^2/sec, corneal irradiance). However, at these irradiance levels, the circadian system is close to saturation and therefore unable to further increase the response (at

least in the present experimental conditions). Pharmacological manipulation of the pupil thus provides critical support for the contention that the pupillary system is less sensitive than the circadian system, since under the matched stimulus conditions in the different experiments, pupil dilation under bright light conditions does not produce a larger phase shift than that obtained with a control pupil diameter.

Our results on the normal pupillary light reflex in the hamster may at a first glance appear to show less sensitivity to corneal irradiance than previous findings in other rodents. We find half-saturating responses in the hamster at 11.84 log photons/cm^2/sec, whereas in the mouse it was found at ~10.1 (Lucas et al., 2001), ~10.5 (Lucas et al., 2003), and ~11.5 (Tu et al., 2006; Van Gelder et al., 2003). This may be due to species differences and/or experimental protocols. In the mouse studies, the animals were fully dark adapted, while in our study hamsters were exposed to stepwise increases of light during which the continuous exposure could lead to saturation of the rod response, which is reported to occur between 10^{11} and 10^{12} photons/cm^2/sec (Nathan et al., 2006). Nonetheless, the main effect seems to be attributable to the use of pharmacological dilation in the stimulated eye of the mouse by Lucas and colleagues, in which the response was found to be ~1 log unit more sensitive than determined by Van Gelder et al. (2003) and Tu et al. (2006) using no dilation. The results of the above studies by the groups of Lucas, Van Gelder, and our study all differ from that of Pennesi et al. (1998) who found pupil constriction at very low levels of retinal illumination prompting the suggestion that the mouse pupil response is controlled almost exclusively by rod signals. These discrepancies with the results of Pennesi et al. (1998) may be due not only to the specific experimental conditions of dark adaptation in anesthetized animals but also to age factors, since the authors report that animals
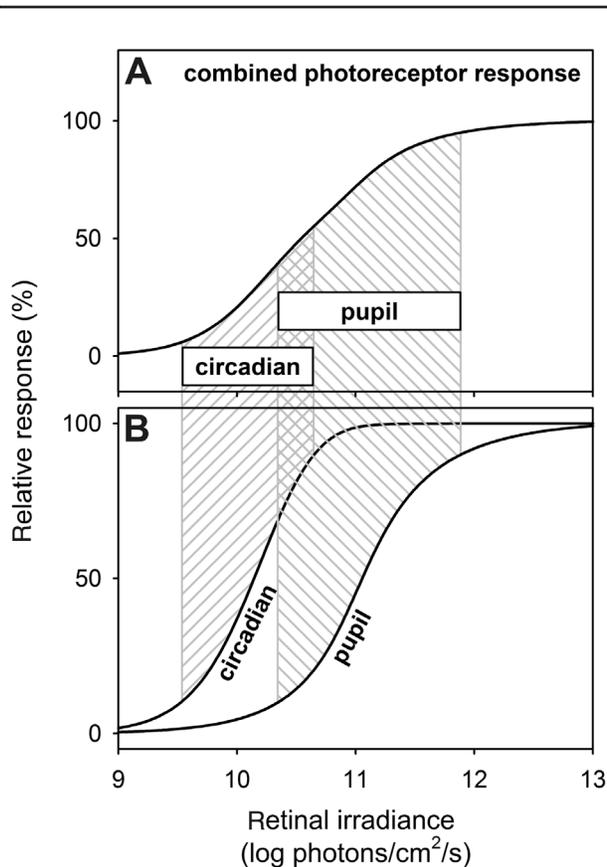
Figure 5. The combined (hypothetical) photoreceptor response curve with a wide dynamic range (A) may affect neuronal tissues that translate this wide range into a more narrow dynamic range with different sensitivities. For the hamster data presented in this study the relative dose-response curves for pupil response and circadian response are plotted against retinal irradiance (B). Both response curves were added to calculate the combined hypothetical photoreceptor response curve in A. The use of retinal irradiance instead of corneal irradiance reduced the dynamic ranges by ~0.5 log unit for the circadian response and ~1.5 log unit for the pupil response. Dynamic ranges (10%-90% of response) for pupil response and circadian response are indicated by hatched areas (circadian response: 3.4×10^9 to 4.5×10^{10} photons/cm²/sec; pupil response: 2.5×10^{10} to 7.9×10^{11} photons/cm²/sec). Note that pupil responses were plotted as the percentage response of log (pupil area); when calculated as pupil area without the log transformation, the dynamic range is 5.9×10^9 to 1.3×10^{11} photons/cm²/sec.

aged older than 4 months showed significant decreases in sensitivity compare to younger mice.

The recent study by Gamlin et al. (2007) in monkey and human subjects shows that the phasic response components to light onset and offset are highly sensitive and are derived from rod and cone inputs, while the sustained pupillary response component is predominantly driven by melanopsin input. The sustained melanopsin-driven response is less sensitive to light than the phasic response, and monkeys, humans, and hamsters all show similar threshold sensitivities in

the range of 10^{10} to 10^{11} photons/cm²/sec in terms of retinal irradiance.

The PLR and the circadian responses are considered to rely on the same photoreceptor inputs that involve rods, cones, and melanopsin ipRGCs (Berson et al., 2002; Hattar et al., 2002, 2003; Lucas et al., 2003; Dacey et al., 2005; Dkhissi-Benyahya et al., 2007; Gamlin et al., 2007). The sensitivity difference between the circadian system and the pupil system described here was therefore unexpected. Several possible (nonexclusive) explanations can be invoked.

A first possibility is that the projections to the circadian system and the pupillary system originate from different subpopulations of melanopsin-expressing and non-melanopsin-expressing ganglion cells. In the hamster, it was originally suggested that 10% to 30% of the ganglion cells that project to the SCN do not express melanopsin and that the OPN receives projections from a much higher proportion of non-melanopsin-expressing cells (Morin et al., 2003; Sollars et al., 2003). Hattar et al. (2006) have shown that the mouse SCN receives an almost exclusive innervation from melanopsin-expressing ganglion cells, whereas the OPN receives a significant projection from nonmelanopsin ipRGCs. Furthermore, several studies have shown that the ipRGC population is composed of 2 to 3 cell types that display different sensitivities and temporal responses to light (Sekaran et al., 2003; Tu et al., 2005; Drouyer et al., 2007). It is presently unknown whether the projections to the SCN and OPN arise from the same or different populations of ipRGCs.

Second, the difference in sensitivities may be related to downstream properties and/or feedback mechanisms of each of the 2 systems rather than to the properties of the photoreceptor cells themselves. For example, the nonoverlapping dynamic ranges of the 2 nonvisual responses may result from differences in the responsiveness of the SCN and OPN to the light signal transmitted by the same sets of retinal photoreceptors. This is suggested by the similarity in the shape of the dose-response curves for pupillary and circadian responses (Fig. 5B). Circadian responses measured at a behavioral or physiological level may involve additional signal treatment involving motor (locomotor activity) or hormonal (pineal) feedback influences to the SCN via neuronal projections and hormone receptors. Pupil size depends on a push-pull system balancing pupil constriction and pupil dilation, involving sympathetic and parasympathetic control via the ciliary and superior cervical ganglions as well as additional cortical controls. Thus, this feedback control to the circadian system, as well as to the pupillary system may tune the

dynamic range of each system to meet the specific needs of both nonvisual and visual responses to light.

Although photic input to the PLR and circadian systems may originate from the same inner and outer retinal photoreceptor types that set the irradiance limits of the response, the dynamic range of each system may be tuned to specific functional requirements. The lower boundary of the dynamic range is likely to be set by the threshold sensitivity of dark adapted rods (highest sensitivity), while the upper boundary is more difficult to define and may depend on the balance between bleaching and chromophore regeneration of the different photopigments. Cones play a role in circadian entrainment and SCN responses (Aggelopoulos and Meissl, 2000; Hattar et al., 2003; Dkhissi-Benyahya et al., 2007; Drouyer et al., 2007) as well as in pupil constriction (Lucas et al., 2003). Because of their capacity for a rapid retinoid cycle turnover rate involving Müller cells, cones may not suffer from saturated responses to high light levels (Rodieck, 1998; Arshavsky, 2002). The responses of melanopsin ipRGCs are more complex and display a high resistance to saturation by light even at extremely high light levels (Berson et al., 2002; Dacey et al., 2005). The steady-state response of the pupil to sustained light exposure may also reflect the underlying photoequilibrium state of melanopsin resulting from chromophore photoregeneration (Koyanagi et al., 2005; Melyan et al., 2005; Mure et al., 2007).

Our findings may have important implications for understanding normal photic responses in a wide variety of behavioral and physiological systems (e.g., activity, alertness, sleep). Each of these response systems may have a different sensitivity to light that may be linked to the response function of the pupil as a limit-setting aperture for light. In particular, the pupil serves the dual function of modulating light input for both nonvisual and visual functions. The involvement of less sensitive (ipRGCs), medium sensitive (cones), and highly sensitive (rods) photoreceptors in the photic pathway may be explained by the wide functional range of neural responses to an extensive range of environmental light intensities. At high light levels, pupil constriction may be of greater importance for the photopic visual responses and for protection against retinal photodamage rather than for nonvisual responses. The important task for non-image-forming irradiance detection in the retina is to use a variety of photoreceptor signals to provide sufficient dynamic range, which enables different recipient brain areas to adjust their responses to specific functional requirements.

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