

SHORT COMMUNICATION

Evening exposure to blue light stimulates the expression of the clock gene *PER2* in humans

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Abstract

We developed a non-invasive method to measure and quantify human circadian *PER2* gene expression in oral mucosa samples and show that this gene oscillates in a circadian (= about a day) fashion. We also have the first evidence that induction of human *PER2* expression is stimulated by exposing subjects to 2 h of light in the evening. This increase in *PER2* expression was statistically significant in comparison to a non-light control condition only after light at 460 nm (blue) but not after light exposure at 550 nm (green). Our results indicate that the non-image-forming visual system is involved in human circadian gene expression. The demonstration of a functional circadian machinery in human buccal samples and its response to light opens the door for investigation of human circadian rhythms at the gene level and their associated disorders.

Introduction

Our light-sensing systems' familiar function is to collect and process light to generate an image of the world. Although light primarily serves vision, it is now known that it also exerts powerful non-visual effects on a number of physiological variables in humans, among them synchronization of circadian rhythms (the circa-24-h rhythms) (Lockley *et al.*, 2003; Warman *et al.*, 2003), suppression of the hormone melatonin (Brainard *et al.*, 2001a,b; Thapan *et al.*, 2001; Lockley *et al.*, 2003; Cajochen *et al.*, 2005), an increase in cortisol and heart rate (Scheer & Buijs, 1999; Scheer *et al.*, 1999; Cajochen *et al.*, 2005), and an acute alerting response (Badia *et al.*, 1991; Cajochen *et al.*, 2005). In mammals, light activates signalling pathways in a brain region where the central circadian pacemaker is located – the suprachiasmatic nuclei (SCN) (Klein *et al.*, 1991) – that coordinates the daily temporal organization of physiology and behaviour. The molecular mechanisms underlying circadian rhythmicity involve self-sustaining transcriptional/translational feedback loops based on rhythmic expression of the mRNA and proteins of clock components. Synchronization of the SCN to light ultimately leads to expression of a set of clock genes, among them *Per1* and *Per2* (Albrecht *et al.*, 1997). Thus, light exposure acutely activates expression of *Per1* and *Per2* in the SCN of mice (Albrecht *et al.*, 1997; Zylka *et al.*, 1998; Yan *et al.*, 1999).

Reports about circadian regulation of clock genes in humans are rare. Two recent studies provide evidence that clock genes (*PER1*, *PER2*, *PER3* and *DECI*) in human peripheral blood mononuclear cells (Boivin *et al.*, 2003) and human oral mucosa (*PER1*, *CRY1* and *BMAL1*) are expressed in a circadian manner (Bjarnason *et al.*, 2001). In the present study we aimed at developing a method to measure circadian expression of *PER2* expression in a non-invasive way via collection of oral mucosa samples. In a second step, we tested the hypothesis that evening light exposure induces an immediate increase in *PER2* expression in human mucosa. Furthermore, because part of light's non-visual effects are mediated by non-classical photoreceptors

with melanopsin acting as photopigment with a maximum sensitivity in the 'blue' part of the visible electromagnetic spectrum (Lucas *et al.*, 1999; Melyan *et al.*, 2005; Panda *et al.*, 2005; Qiu *et al.*, 2005), we applied light in the 460 nm range and compared its effect to light at a different wavelength (550 nm) to test to what extent the novel photoreceptor system is involved in the regulation of *PER2*.

Materials and methods

Study participants

Twelve male volunteers were studied in the light study (mean age \pm SD, 25.3 \pm 3.6 years), and 12 female and male volunteers in the circadian study (28.4 \pm 9.9 years). All study participants were free from medical, psychiatric and sleep disorders, as assessed by history, a physical examination and questionnaires. For the participants in the light study an ophthalmological examination was carried out prior to and after completion of the study in order to exclude volunteers with visual impairments as well as to be certain that our light application was not harmful. The volunteers were instructed to abstain from excessive caffeine and alcohol consumption for 1 week before the study. They were asked to keep a regular sleep-wake schedule (bedtimes and waketimes within \pm 30 min of self-selected target time) during 1 week prior to their admission to the laboratory. Adherence to this regular schedule was verified with a wrist actigraph (Cambridge Neurotechnologies®, UK) and daily sleep diaries for the participants in the light study. All volunteers gave written informed consent. The protocol, screening questionnaires and consent form were approved by the Ethical Committee of Basel, Switzerland, and were in agreement with the Declaration of Helsinki.

Study protocol

Light study

The study consisted of three arms, performed in a balanced order, separated by a 1-week intervening period (Fig. 1). Based on the

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volunteers' habitual bedtimes, a constant posture (CP) protocol started 10 h after usual waketime in the early evening (e.g. 18.00 h) and ended the next day, 2 h after usual waketime (e.g. 10.00 h). Under CP conditions, the volunteers experienced a controlled lying down episode of 1.5 h under 2 lux, followed by a 2-h dark adaptation episode under complete darkness (0 lux). After that, light exposure was initiated for the next 2 h. During this 2-h episode, the volunteers received monochromatic light at 460 nm, or monochromatic light at 550 nm, or no-light (0 lux). After this, the volunteers remained awake for another 1.5-h episode under 2 lux (polychromatic white light) before they were allowed to sleep for 7.75 h. One study participant developed a mild cold during one of the study arms and was therefore excluded from further analysis. To quantify *PER2* expression, oral mucosa samples were collected at 18.30 h before light exposure, at 24.00 h after light exposure and the next morning at 10.00 h. For two study participants not enough mucosa material was available for a reliable quantification of *PER2*, resulting in a total of nine subjects for statistical analysis.

Circadian study

The study comprised a 30-h episode, which the volunteers spent in one of our temperature-controlled chronobiology suites. The volunteers were allowed to read, work on a laptop and listen to music. Ambient light levels were kept low, <15 lux at the angle of gaze, and meals were provided by the clinic (lunch, dinner and breakfast). An 8-h sleep episode was scheduled according to the volunteers' habitual bedtime. The mucosa and the saliva samples for melatonin were taken in 3-hourly intervals, starting 3 h after habitual bedtime (on average at 9.00, 12.00, 15.00, 18.00, 21.00, 24.00, 3.00, 6.00, 9.00, 12.00 h). For double-plotting, the last two data points at 9.00 and 12.00 h were omitted. In order to take the 3.00 and 6.00 h samples, volunteers had to wake up, take the sample and go back to sleep. One subject discontinued the study after 12 h because of difficulties in taking mucosa samples, and two subjects had to be excluded because not enough mucosa material was available for a reliable quantification of *PER2*. The circadian study was conducted 3 months after the light study was completed. Because the experimental conditions of the subjects in the two studies were different it is not possible to compare the *PER2* data of the two studies in their absolute dimension, as the Q-PCR method used was based on relative measures in each experiment.

Light exposure

Monochromatic 2-h light exposure was scheduled at a circadian phase at which polychromatic white light exposure induces robust phase delays and alerting effects (Cajochen *et al.*, 1992, 1998; Khalsa *et al.*, 2003). The monochromatic light was generated by a 300 Watt arc-ozone-free Xenon lamp (Thermo Oriel, Spectra Physics, Statford, CT, USA), filtered by either 460 nm or 550 nm (Interference filter, ± 10 nm half-peak bandwidth, Spectra Physics, Statford, CT, USA) and transmitted via two glass fibre bundles (L.O.T. Oriel-Suisse, Romanel-sur-Morges, Switzerland) through the wall into the sound-proof and temperature-controlled chronobiology suite on to the goggles, which covered the volunteers' eyes. The custom-built goggles (K. Haug AG, Basel, Switzerland) consisted of two spheres (27.5 mm inner radius) coated with white reflectance paint (two component polyurethan-acryl anti-fading paint, Lachenmeier & Co. AG, Basel, Switzerland). Each sphere was illuminated via three branches of the main fibre optic cable in order to provide constant uniform illumination. Equal photon densities (2.8×10^{13} photons/cm²/s) for the 460 nm and 550 nm wavelength

light were administered. This irradiance level ($12.1 \mu\text{W}/\text{cm}^2$ for 460 nm and $10.05 \mu\text{W}/\text{cm}^2$ for 550 nm) was chosen according to recently reported results on monochromatic light on the human circadian timing system (Lockley *et al.*, 2003). Irradiances were measured with a laser power meter (Laser Check, Coherent, Auburn, CA, USA) before the beginning and at the end of each light exposure. During light exposure as well as during the non-light condition, volunteers were asked to keep their eyes open and to fix their gaze on the middle of the spheres. A technician checked the latter by on-line monitoring the polysomnographic recordings and also verifying that the subjects remained awake. The volunteer's pupils were not dilated in order to avoid possible repercussions of the dilation agent *per se* on thermoregulation, heart rate and alertness. However, we tested the effects of the light stimulus on pupil constriction by applying monocular light exposure (light via the goggle of the right eye) and concomitantly measuring the pupil size on the left eye via an infrared camera. The entire control protocol was conducted at the same time of day (evening) and with the same light intensity in six subjects. Results from the control experiment revealed a significantly smaller pupil size after the short wavelength light at 460 nm than after light at 550 nm in comparison to the dark condition ($P < 0.01$), Duncan's multiple range test performed after a one-way ANOVA for repeated measures with the factor 'light condition' ($P < 0.02$; dark, 460 nm and 550 nm).

Salivary melatonin

Saliva was collected at 30-min intervals during scheduled wakefulness. A direct double-antibody radioimmunoassay was used for the melatonin assay validated by gas-chromatography-mass spectroscopy (Bühlmann Laboratories, Allschwil, Switzerland). The minimum detectable dose of melatonin (analytical sensitivity) was determined to be 0.2 pg/mL. The functional least detectable dose using the <20% coefficient of interassay variation criterion was below 0.65 pg/mL, and individual serum and saliva melatonin profiles showed excellent parallelism ($r = 0.977\text{--}0.999$, slopes = 0.21–0.63).

Human oral mucosa collection and quantification of *PER2*

Human oral mucosa was collected using blue pipette tips (Treff AG, 2 mm of tissue into the tip) and then was dissolved in lysis buffer containing β -mercaptoethanol provided in the Absolutely RNA Nano-prep Kit (Stratagene). Subsequently, the samples were frozen at -80°C . The RNA was isolated according to the manufacturer's instructions of the kit using 200 μL of lysis buffer followed by a DNase treatment of 30 min. The cDNA was generated using SuperScriptTM II Reverse Transcriptase (Invitrogen) and poly(dT)₁₅ primers (Roche). Subsequently, TaqMan quantitative real-time RT-PCR was performed using iQ Supermix (Bio-Rad) to determine *GAPDH* and *PER2* levels for each sample. The following primers and probes were used:

PER2 TaqMan probe:

5'-[FAM]ATGCCTTCAGCGATGCCAAGTT[BHQ]-3'

PER2 sense primer:

5'-GCATCCATATTTCACTGTAAAAGA-3'

PER2 anti-sense primer:

5'-AGTAAAAGAATCTGCTCCACTG-3'

GAPDH TaqMan probe:

5'-[FAM]AGCCTCAAGATCATCAGCAATGCC [BHQ]-3'

GAPDH sense primer:

5'-TGTGAACCATGAGAAGTATGAC-3'

GAPDH anti-sense primer:

5'-ATGAGTCCTCCACGATACC-3'.

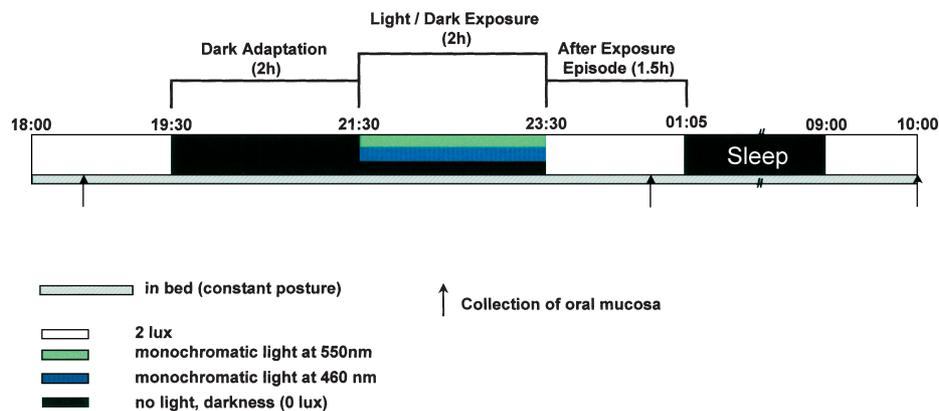
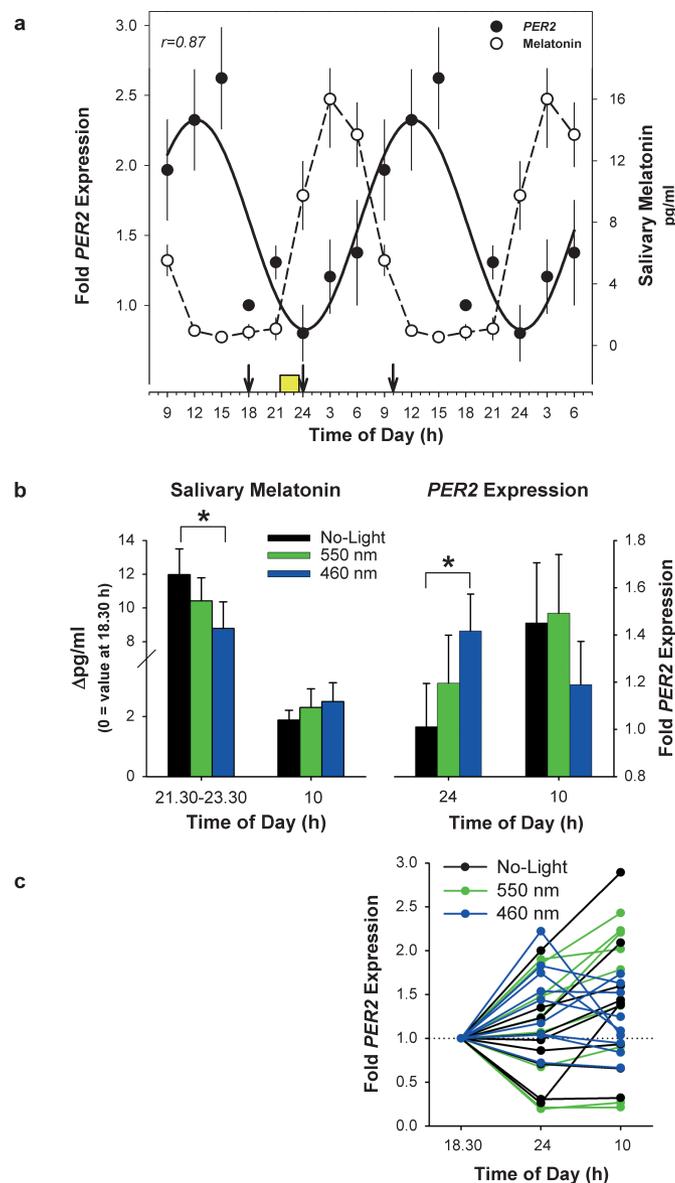


FIG. 1. Overview of the light study protocol design. After 1.5 h under 2 lux, subjects were dark adapted for 2 h, followed by another 2 h in darkness or light exposure at 460 nm or 550 nm (for details about the light exposures, see Materials and methods). Subsequently, subjects spent 1.5 h under 2 lux before they were allowed to sleep for 8 h. The entire protocol was carried out under constant recumbent posture conditions in bed.



The final primer concentration was 300 nM, fluorescent oligomeric probe concentration was 100 nM and $MgCl_2$ concentration was 5 mM. The amplification was carried out at 55 °C for 1 min followed by a denaturation at 94 °C for 15 s using the iCycler (Bio-Rad). After 55 cycles, ΔC_T was determined. The samples of one subject (all time points) in the reaction mix were applied with filter tips (Axigen) onto a 0.2 mL semiskirted thermo-fast 96-well plate (ABgene AB-0900), which was covered with an optically clear adhesive seal sheet (ABgene AB-1170). Additionally to the single *GAPDH* controls, a dilution curve for *GAPDH* was run on each plate for one time point to control for reaction efficiency.

GAPDH was used as standard because of its constant expression in mucosa tissue (Pan *et al.*, 2002) and its validation as a standard gene for this tissue under injury and repair conditions (Warburton *et al.*, 2005).

Data analysis

For both the circadian and the light study three reference *GAPDH* values and three or four *PER2* values were obtained for each mucosa sample (triplets or quadruplets a, b, c, d). A ΔC_T value per mucosa sample was calculated according to the comparative C_T ($2^{-\Delta\Delta C_T}$) method, which could be applied because the efficiencies of the

FIG. 2. (a) Twenty-four-hour profile of *PER2* expression in human oral mucosa (●) and of salivary melatonin levels (○). Samples were taken in 3-h intervals under dim light conditions (< 15 lux) throughout a 30-h period ($n = 9$, mean values \pm SEM). For better visualization of the 24-h rhythms, all values were double-plotted and *PER2* values fitted with a sine function. The r -value, an estimate of the goodness of fit, was 0.87 with $P = 0.0001$ for *PER2*. Arrows on the abscissa indicate the timing of mucosa collection and the timing of the 2-h light exposure (yellow bar) in the experiment in (b). (b) Left-hand side: Salivary melatonin levels during 2-h light exposure at 460 nm and 550 nm and during the control no-light condition. Right hand side: *PER2* expression in oral mucosa after the 2-h light exposure (460 and 550 nm) and no-light exposure at 24.00 h and 10.00 h the next morning. Values were expressed as percentages to the value before light treatment at 18.30 h (0 = value at 18.30 h; $n = 9$, mean values \pm SEM; * $P < 0.05$, Duncan's multiple range test). (c) Scatter plot of the individual normalized data. After blue light at 460 nm, for most of the subjects values > 1.0 can be observed at 24 h, but also for some subjects after green light at 550 nm and for two subjects in the dark condition. Only in the blue light condition did the values at 24.00 h differ significantly from the 18.30 h values (paired t -test, $P = 0.017$). For most of the subjects in the blue light condition, the values at 10.00 h the next morning decreased, statistical analyses revealed a trend for lower values after blue light, when compared with the green condition (paired t -test; $P = 0.09$).

GAPDH and *PER2* assays were optimized to be comparable (Livak & Schmittgen, 2001). A validation assay was performed where serial dilutions were assayed for both genes. This method minimizes the variation in performance of the iCycler apparatus. Another advantage of this relative quantification compared with the absolute method is that it corrects for variance in the efficiency of cDNA synthesis.

The formula below was used to calculate the ΔC_T values:

$$\Delta C_T = (2^{GAPDHa-PER2a} + 2^{GAPDHa-PER2b} + 2^{GAPDHa-PER2c} + 2^{GAPDhb-PER2a} + 2^{GAPDhb-PER2b} + 2^{GAPDhb-PER2c} + 2^{GAPDhc-PER2a} + 2^{GAPDhc-PER2b} + 2^{GAPDhc-PER2c})/9$$

In the case of quadruplets, the formula was changed accordingly. This method allowed the use of parametric statistics repeated-measure ANOVA (rANOVA) with the factor 'light condition' and 'time of day'. For each subject, we expressed the transformed values relative to the value obtained at 18.00 h in the circadian study and to the value obtained at 18.30 h in the light study, as in the light study the sample taken at 18.30 h was the only sample taken under the same lighting conditions and was the closest in terms of time to the 18.00 h sample taken in the circadian study. All *P*-values derived from rANOVAs were based on Huynh-Feldt's (H-F) corrected degrees of freedom, but the original degrees of freedom are reported. For *post-hoc* comparisons the Duncan's multiple range test was used.

The time course of *PER2* expression in the circadian study was fitted with a sinusoidal function comprising the fundamental oscillation (24-h component):

$$f(t) = y_0 + A \sin[(2\pi t/\tau_{24}) + c].$$

Data were fit with a non-linear least-square fitting analysis based upon the Marquardt-Levenberg algorithm to find the coefficients (parameters) of the independent variable(s) that give the best fit between the equation and the data (SigmaPlot for Windows, Version 7.0, Richmond, CA, USA, Systat Software). The goodness-of-fit of each sine fit was assessed by calculating the adjusted correlation coefficient and the power, or the probability that the model correctly describes the relationship of the variables, if there is a relationship. The statistical package Statistica® (StatSoft 2004, Statistica for Windows, Tulsa, OK, USA, Version 6.0) was used.

Results

PER2 gene expression in the circadian study showed a clear diurnal rhythm (Fig. 2a). The sine fit to the data points yielded a good fit with a *r*-value of 0.87, and the power that the model correctly describes the relationship between the variables was 0.99. Maximal *PER2* gene expression in oral mucosa, sampled in 3-h intervals throughout a 30-h period under controlled dim lighting conditions, occurred between 12.00 and 15.00 h during the biological day, while minimal *PER2* expression was found between 21.00 and 03.00 h during the biological night, defined as the period of melatonin secretion. The circadian profile of the salivary melatonin exhibited the well-known time course (Fig. 2a), with maximal values during night-time hours and low values during daytime. The phase and amplitude of the melatonin curve was very similar to the one we have recently reported (Knoblauch *et al.*, 2003).

Figure 2b illustrates changes in *PER2* expression and salivary melatonin in the light study. A rANOVA with the factor 'light condition' and 'time of day' yielded a significant effect of 'time of day' ($F_{1,8} = 8.4$, $P = 0.020$) and a significant interaction between

'light condition' and 'time of day' ($F_{2,16} = 4.6$, $P = 0.027$). *Post-hoc* comparisons revealed a significant increase in *PER2* expression after blue light at 460 nm at 24.00 h ($P = 0.032$), but not after green light at 550 nm ($P = 0.28$). The comparison between blue and green light at this time point (24.00 h) did not reach significance ($P = 0.18$). Melatonin was significantly suppressed after blue light at 460 nm (from 12.0 pg/mL to 8.8 pg/mL, $P = 0.021$), but not after green light at 550 nm. At this time point *PER2* levels are usually near minimal levels (Fig. 2a).

PER2 gene expression the next morning at 10.00 h no longer differed significantly, nor did melatonin levels. Nonetheless, a tendency for a lower *PER2* gene expression was found after blue light at 460 nm when compared with green light at 550 nm ($P = 0.098$).

In the scatter plot showing individual data (Fig. 2c), for most of the subjects values > 1.0 can be observed at 24 h after blue light, but also for some subjects after green light and for two subjects in the dark condition. However, only in the blue light condition did the values at 24.00 h differ significantly from the 18.30 h values (paired *t*-test $P = 0.017$). For most of the subjects in the blue light condition, the values at 10.00 h the next morning decreased and a trend for lower values after blue light when compared with the green light was found (see paragraph above).

Discussion

We were able to show rhythmic expression of a clock gene *PER2* in peripheral human tissue collected non-invasively from the oral mucosa. In addition, our findings provide the first evidence in humans that a circadian regulator gene, *PER2*, acutely responds to light. Importantly, this response to light was clearly wavelength-dependent, such that the increase in *PER2* after light is blue-shifted relative to the three-cone visual photopic system, supporting a role of the novel 'non-photopic' photoreceptors at the level of human clock gene expression.

Circadian expression of *PER2* in our study peaked on average 12 h later, during the biological day at 15.00 h, with respect to maximal melatonin expression at 03.00 h. Although a bit later into the day, it is in accordance with Boivin *et al.* (2003), who reported peak *PER2* expression mostly during the habitual time of activity (around noon) in human peripheral blood mononuclear cells.

We have evidence that the induction of *PER2* is acutely stimulated by evening light exposure in the human mucosa. Studies in rats show that the clock of peripheral tissues, such as liver and kidney, takes more time to adjust its phase to a shifted light-dark cycle compared with the SCN (Yamazaki *et al.*, 2000). In contrast, our study indicates that oral mucosa might be a tissue that responds rather quickly to such changes as shown by the rapid response of the *PER2* gene to light in this tissue. Whether this represents an acute stimulation of *PER2* in the central circadian clock in the SCN can not be conclusively proven in this study. However, the light-evoked reduction of melatonin and increase in core body temperature and heart rate published in the same subject sample (Cajochen *et al.*, 2005) are clear indications for non-visual circadian effects of light, which are mediated by the SCN. Therefore, we assume that the light effects reported here on *PER2* were most likely mediated via the circadian system. Further support for this comes from the fact that only the short-wavelength light (i.e. blue light) evoked this effect. It is known that the human circadian system is particularly sensitive to non-visual effects of ocular light at short wavelengths via novel photoreceptors (Provencio *et al.*, 2000; Hankins & Lucas, 2002; Hattar *et al.*, 2002; Gooley *et al.*, 2003).

The demonstration of a functional circadian machinery in human buccal samples and its response to the most important Zeitgeber light suggests that this method may be useful for the investigation of human circadian rhythms at the gene level and their associated disorders.

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Abbreviations

CP, constant posture; SCN, suprachiasmatic nuclei.

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