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A Switch from Diurnal to Nocturnal Activity in *S. ehrenbergi* Is Accompanied by an Uncoupling of Light Input and the Circadian Clock

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Summary

The subterranean mole rat *Spalax ehrenbergi* superspecies represents an extreme example of adaptive visual and neuronal reorganization [1, 2]. Despite its total visual blindness, its daily activity rhythm is entrainable to light-dark cycles [3], indicating that it can confer light information to the clock. Although most individuals are active during the light phase under laboratory conditions (diurnal animals), some individuals switch their activity period to the night (nocturnal animals) [3, 4]. Similar to other rodents [5], the *Spalax* circadian clock is driven by a set of clock genes, including the *period* (*sPer*) genes [6, 7]. In this work, we show that diurnal mole rats express the *Per* genes *sPer1* and *sPer2* with a peak during the light period. Light can synchronize *sPer* gene expression to an altered light-dark cycle and thereby reset the clock. In contrast, nocturnal *Spalax* express *sPer2* in the dark period and *sPer1* in a biphasic manner, with a light-dependent maximum during the day and a second light-independent maximum during the night. Although *sPer1* expression remains light inducible, this is not sufficient to reset the molecular clockwork. Hence, the strict coupling of light, *Per* expression, and the circadian clock is lost. This indicates that *Spalax* can dissociate the light-driven resetting pathway from the central clock oscillator.

Results and Discussion

The circadian clock coordinates the body's physiological, endocrinological, and behavioral status and enables the organism to maximally benefit from temporally available natural resources [8]. At the molecular level, the clock is based on transcriptional/translational feedback loops (TTLs), a principle that is conserved throughout all *phyla*, even though the single components of the loops vary [9]. In mammals, the master circadian clock is located in the hypothalamic *suprachiasmatic nuclei* (SCN) [10]. From here, subordinated clocks in the peripheral organs of the body are synchronized to generate a concerted rhythm for the whole organism [11]. Among the genes driving the clock in the SCN are the two *Period* genes *Per1* and *Per2* [12–14]. Both homologs show a circadian rhythm of activation within the SCN and can serve as markers for the phase of the circadian clock [15–17].

Recent data indicate that, at both the behavioral and the molecular levels, the blind mole rat *Spalax ehrenbergi* superspecies has a functional circadian clock despite its isolated subterranean ecotope [3, 6, 7]. Twenty-five million years of selective adaptation to this environment have resulted in a radical degeneration of its visual system, leading to atrophied (600 μm wide), totally fur-covered eyes that lack any image-forming ability [18]. Interestingly, the degenerated retina contains opsins and melanopsin, which might be responsible for light detection [19, 20]. The SCN, however, is well developed and receives clock-related signals from the retina via the retinohypothalamic tract [7, 18, 21].

Mole rats show a unique polyphasic activity pattern in that they can switch from day activity (diurnal animals) to night activity (nocturnal animals) depending on environmental conditions [4, 22]. Although the majority of all populations studied are more active during the day, activity patterns seem to be highly influenced by temperature and aridity [23]. Compared with above-ground mammals, for a totally blind subterranean herbivore, a change in the time of activity is less crucial for its ability to find food or for its susceptibility to predators. Therefore, the polyphasic nature of *Spalax* rhythmicity may have been evolutionarily stabilized by balancing the need for social interaction in the mating season on one hand and metabolic economy on the other [24].

In the laboratory, activity of animals can be entrained to shifted light-dark cycles, indicating a sensitivity of the circadian clock to light [3, 25]. At the molecular level, three *Period* genes (*sPer1*, *sPer2*, *and sPer3*) as well as a *Clock* and a *MOP3* homolog have been characterized and show circadian expression rhythms and light inducibility similar to their counterparts in other rodents like mice, rats, and hamsters [6, 7]. Experiments with diurnal species like *Arvicanthis niloticus* and *Spermophilus tridecemlineatus* revealed that clock gene expression in these animals is the same as in nocturnal animals; this indicates that the center managing activity is located downstream from the core pacemaker [26, 27].

The great majority of *Spalax* individuals (~80% of 63 total) used in this study displayed a diurnal activity pattern (see [22] as well). To test whether the behavioral adaptation to a shifted light-dark cycle [3] is reflected at the molecular level, we looked for *sPer* gene expression in the SCN of diurnal animals before and after an inversion (12 hr shift) of a 12 hr light/12 hr dark (LD) cycle. As has been shown before [7], in diurnal animals, *sPer1* expression rises during the early day, with a maximum around noon, and has a steady decline throughout the night (Figures 1A and 2C). The *sPer2* expression peaks at the day-night transition, with low levels during

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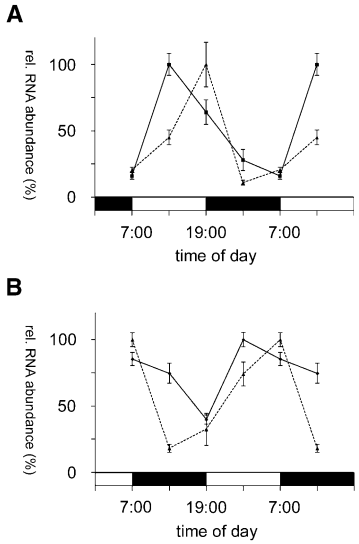

Figure 1. *sPer* Gene Expression in the SCN

(A and B) Expression in (A) diurnal animals and (B) diurnal animals entrained to a 12 hr-shifted LD cycle. Diurnal animals were adapted to a 12 hr light/12 hr dark (LD) cycle and were sacrificed at four different time points throughout the day (7:00, ZT0; 13:00; 19:00; and 1:00). The phase-shift animals were exposed to a 12 hr-shifted LD cycle (19:00, ZT0) and were sacrificed after behavioral adaptation (~1 week, data not shown). Quantitative analysis of *sPer1* (solid line) and *sPer2* expression (dotted line) in the SCN is shown. All values are mean ± SD of three different experiments. Black and white bars indicate dark and light phases, respectively.

the dark period and the early morning (Figures 1A and 2C). Similar expression patterns have been observed in mice [15–17].

After the shift of the LD cycle ("lights on" at 19:00), we examined *sPer1* and *-2* expressions in the SCN after the animals' activity patterns were synchronized to this new light schedule. We found an inverted rhythm in *Period* gene expression, with peaks at 1:00 and 7:00, indicating that the molecular clockwork is reacting to light and adapts readily to new environmental conditions.

Since the *Spalax* clock is light sensitive, what is the explanation for the nocturnal activity pattern of some animals when most are diurnal? To pursue this question, we scanned our colonies for night-active animals. About 20% of all individuals tested displayed a stable nocturnal activity rhythm and were chosen for further experiments (Figures 2A and 2B). We examined *Period* gene expression in these animals to determine whether the observed phenotype was caused by an event in, up-, or downstream of the central oscillator. In LD, *sPer1* shows a biphasic expression — with two maxima, one in the middle of the day and the other at midnight — in nocturnal animals (Figures 2D and 2F). As in diurnal animals, *sPer2* expression shows only one peak of expression, occurring at the end of the activity phase, in nocturnal mole rats (Figures 2C–2F). Maximal *sPer2* expression for nocturnal animals is at *Zeitgeber* time (ZT) 0 (7:00). Hence, in relation to the light/dark cycle, the expression patterns for *sPer1* and *sPer2* are inverted in nocturnal *Spalax* as compared to diurnal *Spalax*, except for the second *sPer1* maximum at ZT6 (13:00).

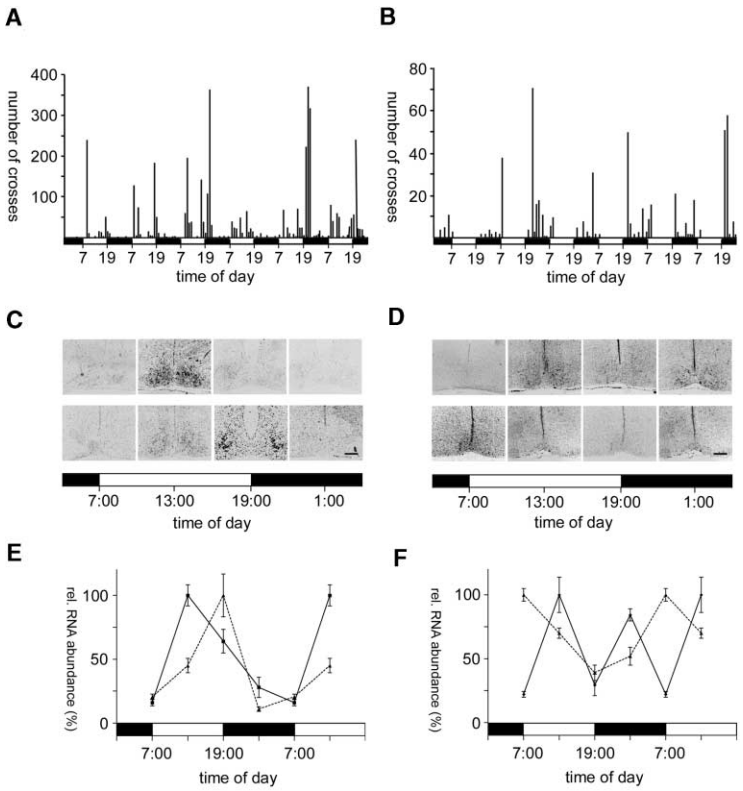

Figure 2. Activity and *sPer* Gene Expression of Nocturnal Animals

(A and B) Typical activity plots of (A) day- and (B) night-active animals measured by crossing events of an infrared beam in the animal's cage.

(C and D) Representative bright-field micrographs of ^{35}S liquid film autoradiographs on coronal brain sections probed for *sPer1* (upper row) and *sPer2* (lower row). (C) Diurnal animals; (D) nocturnal animals in LD. The localization of the SCN was verified by bisbenzimide costaining (not shown).

(E and F) Quantitative analysis (n = 3) of *sPer1* (solid line) and *sPer2* expression (dotted line) in the SCN of (E) day- and (F) night-active animals kept in LD (7:00, ZT0). The scale bar represents 100 μm.

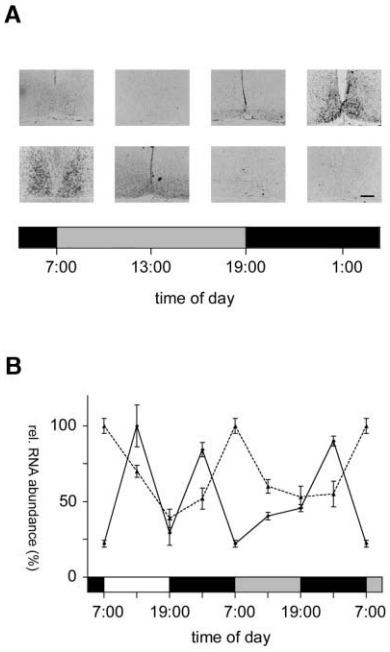

Figure 3. *sPer* Gene Expression in Nocturnal Animals in DD

(A) Night-active animals were released into constant darkness (DD) and were sacrificed every 6 hr of the first day in constant conditions (CT 0, 7:00, with the gray bar approximately indicating subjective day). Upper row: micrographs of *sPer1* in situ hybridization; lower row: *sPer2* probe.

(B) Densitometric analysis (n = 3) of *sPer1* (solid line) and *sPer2* (dotted line) expression in night-active animals released into DD (white bar: light phase of the last LD cycle; gray bar: approximate subjective day of the first DD cycle). The scale bar represents 100 μm.

The two distinct *sPer1* activity maxima in nocturnal animals could be the consequence of an uncoupling of different cell groups within the individual SCN or of an uncoupling of the two SCNs oscillating in anti-phase. Thorough examination of the whole SCN, however, did not reveal any clusters of cells with just one expression maximum or differences between the left and the right nucleus, as has been shown in the mouse SCN [28] and in hamsters with a split activity pattern [29].

To test whether the second *sPer1* peak was clock driven or induced by the LD cycle, we released nocturnal animals into constant darkness (DD) and looked for the *Period* gene expression on the first day in constant conditions (Figure 3). While *sPer2* gene expression continues to oscillate in a circadian manner, the morning peak in *sPer1* expression vanishes in DD. This demonstrates that, in animals with a predominantly nocturnal activity pattern, the molecular clock is shifted for 12 hr, with the activity pattern clearly following gene expression in the SCN. Under normal LD conditions, *sPer1* is regularly induced at dawn. This induction, however, is not sufficient to reset the clock (Figure 2B).

As a rapidly light-inducible gene in mice, *Per1* is thought to integrate light-driven signaling pathways from the retina via the retinohypothalamic tract and the intergeniculate leaflet [30, 31]. The PER proteins would then phase shift the oscillation of the circadian clock, thereby synchronizing it to the environment [5, 32]. Here, we give the first example of a mammalian species in which *Per1* is, under some circumstances, not sufficient to shift the circadian pacemaker. We show that, in the laboratory, the molecular circadian clock of the diurnal *Spalax* is light responsive and that diurnal animals can adapt to changes in the external light cycle. However, in animals with a nocturnal activity pattern, photic signaling can be overruled by factors other than light. We propose that the variability of the mole rat's circadian clock to react to light reflects its subterranean ecotope.

Radiotracking field studies revealed that mole rats are predominantly diurnal during the rainy, short-day winter (mating season) and are predominantly nocturnal during the dry, long-day, hot summer seasons [33]. We tried to simulate this natural ambience by applying different photoperiods, e.g., long day/short night environments, to diurnal animals but failed to induce transition into a nocturnal activity profile by varying only one environmental parameter (light; data not shown). This indicates that other factors like temperature and humidity could have an impact on resetting the *Spalax* clock.

The results indicate that the *Spalax* clock contains a switch controlling the preference for diurnal to nocturnal activity that is located in the input pathway upstream from the core clock mechanism. This is in marked contrast to other mammalian species in which the mechanism that determines the activity phase lies downstream from the clock, because, in all diurnal and nocturnal species studied so far, *Per* gene expression is invariant with maxima during the light phase. We suggest that the *Spalax* clock can vary its sensitivity to light-induced input signals, probably as a response to changes in humidity or temperature. Our findings give a good example for the highly adaptive nature of the clock entrainment mechanism with regard to a species-specific environment.

Experimental Procedures

Animal Handling and Activity Monitoring

We analyzed 63 adults (100–150 g) belonging to *Spalax judaei* (2n = 60) from Anza, Samaria [34]. Field-trapped animals were kept at 22°C–24°C under a 12 hr light/12 hr dark (LD) cycle. Selection of animals was done after monitoring their activity: the animals were kept in 20 × 20 cm cages supplied with two 80 cm, transparent tubes. Each tube was equipped with two infrared-beam sensors that signaled whenever the animal crossed the sensor. For analysis of *sPer* RNA levels in total darkness (DD), light was turned off at *Zeitgeber* time (ZT) 12 for at least 2 days before animals were sacrificed.

In Situ Hybridization

Animals were sacrificed by cervical dislocation under ambient light conditions at 13:00 and 19:00 in LD and under a 15 W safety red light at all other indicated time points. Specimen preparation, ^{35}S-UTP-labeled riboprobe synthesis, and hybridization steps were performed as described in [35]. The probes for *sPer1* and *sPer2* were as described in [7]. Quantification was performed by densitometric analysis of autoradiograph films (Amersham Hyperfilm MP) by using the NIH Image 1.6 program after conversion into the relative optical densities by the ^{14}C-autoradiographic microscale (Amersham). Data from the SCN were normalized with respect to the signal intensities in an equal area of the lateral hypothalamus. Three sections per SCN were analyzed. "Relative mRNA abundance" values were calculated by defining the highest value of each experiment as 100%.

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