

Circadian Genes in a Blind Subterranean Mammal III: Molecular Cloning and Circadian Regulation of Cryptochrome Genes in the Blind Subterranean Mole Rat, *Spalax ehrenbergi* Superspecies

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Abstract The blind subterranean mole rat superspecies *Spalax ehrenbergi* is an extreme example of mammalian adaptation to life underground. Though this rodent is totally visually blind, harboring a drastically degenerated subcutaneous rudimentary eye, its daily activity rhythm is entrainable to LD cycles. This indicates that it confers light information to the clock, as has been previously shown by the authors in behavioral studies as well as by molecular analyses of its *Clock/MOP3* and its three *Per* genes. The *Cryptochrome* (*Cry*) genes found in animals and plants act both as photoreceptors and as essential components of the negative feedback mechanism of the biological clock. To further understand the circadian system of this unique mammal, the authors cloned and characterized the open reading frame of *Spalax Cry1* and *Cry2*. The *Spalax* CRY1 protein is significantly closer to the human homolog than to the mice one, in contrast to the evolutionary expectations. They have found two isoforms of *Cry2* in *Spalax*, which differ in their 5' end of the open reading frame and defined their expression in *Spalax* populations. They found a large and significant excess of heterozygotes of *sCry2* (*sCry2L/S* genotype). Both *sCry1* and *sCry2* mRNAs were found in the SCN, the eye, the harderian gland, as well as in a wide range of peripheral tissues. Their expression pattern under different LD conditions has also been analyzed. As was already shown for other circadian genes, despite being blind and living in darkness, the *Cry* genes of *Spalax* behave in a similar, though not identical, pattern as in sighted animals. Once again, the results indicate that the uniquely hypertrophied harderian gland of *Spalax* plays a key role in its circadian system.

Key words cryptochrome genes, circadian rhythm, mole rat

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Most organisms, including some prokaryotes and most eukaryotes, have evolved endogenous clock systems synchronizing (entraining) their internal biochemical, physiological, and behavioral processes to the 24-h rhythms of light and dark.

A multiple auto-regulatory transcriptional feedback loop, similar to that described in *Drosophila*, forms the core circadian rhythm-generating mechanism in mammals. Two basic helix-loop helix (bHLH) PAS (PER-ARNT-SIM) transcription factors, CLOCK and BMAL1 (also known as MOP3), form the positive elements of the system and drive transcription of 3 *Period* (*Per 1, 2, 3*) and 2 *Cryptochrome* (*Cry 1, 2*) genes. The *Cryptochromes* were first discovered in plants as blue-light photoreceptors and are widely distributed in both plants and animals (Cashmore et al., 1999).

In animals, *Cry* genes have been characterized in *Drosophila*, where it was shown to act as a circadian photoreceptor involved in the biological clock (Stanewsky et al., 1998), human and mouse (Kobayashi et al., 1998), and zebrafish (Kobayashi et al., 2000). The two murine *Cry* genes are expressed in the SCN and in the retina, but only *Cry1* mRNA levels exhibit clear circadian oscillations (Miyamoto and Sancar, 1998). Unlike in *Drosophila*, where the function of *Cry* is light dependent (Stanewsky et al., 1998), mice *Crys* are light independent.

Despite the accumulating data on the genetics of the mammalian circadian system (reviewed in Albrecht, 2002), not much was known before our studies as to how animals living permanently in darkness, such as blind subterranean mammals, can perceive underground circadian rhythms that occur above ground (Nevo, 1998; Avivi et al., 2001, 2002; Oster et al., 2002).

The blind subterranean *Spalax ehrenbergi* superspecies involve at least 12 allotypes in the Near East and 4 species in Israel, each characterized by a different diploid chromosome number and ranging in a different climatic regime. It has been studied multidisciplinary as an evolutionary model of speciation and adaptation (Nevo, 1999). *Spalax* lives underground in darkness most of its life. Nevertheless, it perceives the daily and seasonal temporal cycles underground (Nevo, 1998). Behaviorally, *Spalax* displays polyphasic and polytypic day-night activity patterns (Tobler et al., 1998) coupled with polymorphic (Ben-Shlomo et al., 1995) and seasonal (Kushnirov et al., 1998) variation.

Spalax is visually blind (Haim et al., 1983) but has morphologically degenerated subcutaneous eyes

(Sanyal et al., 1990) that participate in photoperiodic perception (Nevo, 1998) together with the harderian and pineal glands (Pevet et al., 1984). The structurally and functionally effective but minute (823 ganglion cells) retina projects to all visual structures (Cooper et al., 1993a, 1993b). It expresses *Rhodopsin* (Janssen et al., 2000), *Coneopsin* (Argamaso et al., 1995), and *Melanopsin* (Hannibal et al., 2002) genes. These retinal pigments are effective in photoperiodic perception (David-Gray et al., 1998) and in adaptive spectral tuning by a circadian photopigment (David-Gray et al., 1999).

We initiated a search for biological clock genes in *Spalax* and cloned and unraveled the expression of *Clock* and *MOP3* cDNAs of three species of the *S. ehrenbergi* superspecies in Israel (Avivi et al., 2001). The Q-rich region of *Clock*, assumed to function in circadian rhythmicity, is unique in *Spalax* compared to other mammals, and its CLOCK/MOP3 dimmer is less potent than its human counterpart in driving E-box mediated transcription (Avivi et al., 2001).

We also demonstrated that *Spalax* has a set of three *Per* genes (Avivi et al., 2002), which are light inducible; oscillate with a periodicity of 24 h in the SCN, eye, and harderian gland; and are expressed in peripheral tissues, indicating their role in clock resetting in *Spalax* (Avivi et al., 2002). Moreover, in naturally occurring nocturnally active *Spalax* individuals, we detected a dissociation of the light-driven resetting pathway from the central clock oscillator (Oster et al., 2002). Expression analysis of *sPers* and *sMOP3* in the harderian gland of *Spalax* suggests an important participation of this organ in the stabilization and resetting mechanism of the circadian rhythm as a unique adaptation to life underground (Avivi et al., 2001, 2002; Oster et al., 2002).

Here, we are the first to describe the cloning, sequencing, and expression analysis of the *Spalax Cryptochrome* gene family.

MATERIALS AND METHOD

Animals

Throughout this article, mole rats are defined by their generic name *Spalax*.

All the animals were captured in the field and kept in our animal facility for at least 3 months before use. They were kept under controlled conditions of 22 to 24 °C with seasonal LD hours and supplied with saw-

dust, enough to partially imitate natural conditions. Animals used in this study were adults and of similar weight (100–150 g).

Cloning of the *Spalax Cry* genes (*sCry1* and *sCry2*) was done from the same individual.

For the different expression studies described here, the animals were monitored to define their locomotor activity by which diurnal and nocturnal animals were selected. These animals were kept under 12 h L/12 h D (LD) cycles. While studying the expression of the *sCry* genes in total darkness (DD), the light was turned off at ZT 12, and animals were kept in the dark for at least 2 days before being sacrificed under dim illumination (15-W safety red light). For light-induced experiments, animals were kept in LD for a week followed by a short light pulse (15 min, > 200 Lx) at specified ZT with subsequent release into constant darkness. For gene induction analysis, tissues were prepared 1 h after illumination. Each experiment was done on 3 sets of animals.

Cloning of *Spalax Cry* Genes

Spalax Cry genes were cloned from the *Spalax judaei* species ($2n = 60$) from Anza, Samaria (Nevo, 1999), by RT-PCR. Oligos were synthesized according to known human and mouse homologous sequences (accession numbers: D83702 and XM_051030 for human *Cry1* and *Cry2*, respectively, and AB000777 and XM_203811 for mouse *Cry1* and *Cry2*, respectively). Whole brain total RNA was prepared with Tri Reagent (Molecular Research Center Inc., USA). Total RNA was used for synthesizing 1st-strand copy DNA with oligo(dT) as a primer and SuperScript II reverse transcriptase (GibcoBRL, USA). PCR reaction was carried out using Taq DNA Polymerase (Appligene, USA), under conditions adjusted for each specific reaction. Sequencing was determined by the thermocycling sequencing using di-deoxy nucleotide terminators at the sequencing unit of the Medical School, the Technion (Haifa, Israel).

For testing the expression of *sCrys* in different tissues, a semi-quantitative RT-PCR was employed using *Spalax* authentic oligos. Equal amounts of RNA, spectrophotometrically determined, were taken for cDNA synthesis to which trace amounts of ^{32}p -dCTP were added to enable the use of equal amounts of template cDNA in the PCR reaction. The estimation of relative expression was performed at the log phase (after 21 PCR cycles). The tissues examined were taken from diurnal animals.

For studying the expression of the two isoforms of *sCry2* in different species and populations of *Spalax*, genomic DNA (100 ng), taken from 12 individuals of each of the 4 species of *Spalax* residing in Israel, was used as a template using primers covering the area of the inserted fragment in the longer form.

Evolutionary Analysis

The evolutionary analysis of the *Cry* proteins presented here is based on the absolute number of amino acid differences among the compared species and on distance calculations (Wisconsin package version 10, Genetic Computer Group, Madison, Wisconsin, USA). The protein DISTANCES program applies only to proteins. The formula calculates distances based on the relationship between observed amino acid substitutions and actual (corrected) substitutions. Gap positions are ignored, and only exact matches contribute to the score (Kimura, 1983).

In Situ Hybridization

For in situ hybridization (ISH), tissues taken from the *S. judaei* species were fixed in 4% paraformaldehyde in saline-phosphate buffer for 12 to 18 h at 4 °C before dehydration. The fixed tissue was embedded in paraplast and then sectioned on a microtome, producing slices of 7- μm thickness. ISH was performed as described in Albrecht et al. (1998). The *Spalax Cry1* probe corresponded to nucleotides 1 to 1060; the *sCry2* probe corresponded to nucleotides 1 to 900. Due to the very high similarity of the two *sCry2* isoforms, we were not able to design riboprobes for each of the isoforms, which are usable in ISH, as any riboprobe from the long isoform practically contains the short isoform.

Quantification was performed by densitometric analysis of hybridization signals on X-ray films using Scion Image 4.0.2 software. For each data point, we used three sections of the medial aspect of the SCN from three different animals each, estimated the boundaries of the SCN by comparison with nuclear staining from the same section (Hoechst dye), and integrated the signal intensity (after subtraction of lateral hypothalamic background signal) over the whole area. Statistical analysis was performed using GraphPad Prism 3.03 software. Comparisons between data sets were done using ANOVA with subsequent Bonferroni test for multiple comparisons, with $p < 0.05$ as the criterion of significance.

Nucleotides		
1	ATGGCGGGCGACTGTGGCAGTGGTGGCAGCCGACAGCGCAGCGATGGCGGC	50 <i>sCry2L</i>
1	ATGGCGGGCGACTGTGGCAGTGGTGGCAG.....	29 <i>sCry2S</i>
51	GGCGTCCGTGGTGGTGGCGACGTCGCCGCGGCTATGGCTGTGGACG	100 <i>sCry2L</i>
30CCGCTGCCGCGGCTATGGCTGTGGACG	55 <i>sCry2S</i>
Amino acids:		
1	MAATVAVVAADSAMARAASVVVATSAA?AMAVD	33 <i>sCRY2L</i>
1	MAATVAVVAA.....RA?AMAVD	18 <i>sCRY2S</i>

Figure 1. Nucleotide and amino acid alignment of the 5' area of *sCry2L* and *sCry2S*. Mole rats exhibit two forms of *Cry2* gene, *sCry2L* and *sCry2S*. The shorter isoform, *sCry2s*, lacks 45 nucleotides close to the ATG initiation codon (upper), resulting in a deduced CRY2 peptide shorter in 15 amino acids. Apart from this dramatic structural change, the two isoforms are completely identical.

RESULTS

Cloning and Characterization of the *Spalax Cry1* and *Cry2* cDNA

Analysis of the open reading frame (ORF) of *sCry1* (accession number AJ606298) revealed a cDNA of 1764 nucleotides giving rise to a putative transcript of 587 amino acids. Both the nucleotides and the amino acid sequences are similar to the *Cry1* sequence of other mammals. At the nucleotide level, the relative homology is 89.5% and 93% compared to mice and humans, respectively. At the amino acids level, the homology is 96.8% and 98.1% compared to mice and humans, respectively.

In the case of *sCry2*, we isolated two isoforms, named "short" (*sCry2S*, accession number AJ606299) and "long" (*sCry2L*, accession number AJ606300), differing at the beginning of the coding region (Fig. 1) but otherwise completely homologous. The two *sCry2* isoforms are highly conserved as compared to other mammals. *sCry2S* has an ORF of 1776 nucleotides, giving rise to a putative peptide of 592 amino acids. *sCry2L* has an ORF of 1821 nucleotides, encoding a putative peptide of 607 amino acids. *sCry2* is 91% homologous to mice and 90% homologous to humans at the nucleotide level and 95.9% and 95.3% homologous to mice and humans, respectively, at the amino acid level.

To characterize the two isoforms of *sCry2*, we have analyzed their distribution in genomic DNA of 48 individuals of *Spalax*, a dozen from each of the 4 spe-

Table 1. Matrix of Distances and Amino Acid Differences among CRY Proteins of Mammals

	CRY1	Spalax	Mouse	Monkey	Human	
<i>Spalax</i>			4.38 (25)	2.6 (15)	2.07 (12)	
Mouse				4.03 (24)	3.49 (19)	
Monkey					0.6 (4)	
Human						
	CRY2	Spalax-S	Spalax-L	Mouse	Rat	Human
<i>Spalax-S</i>		0 (0)	5.06 (28)	5.97 (37)	7.09 (43)	
Mouse					2.4 (14)	6.9 (40)
Rat						7.64 (46)
Human						

NOTE: The distances are according to Kimura protein distances calculation (see Materials and Methods). The numbers in parentheses are the absolute number of different amino acids. S, *sCRY2S*; L, *sCRY2L*.

cies residing in Israel. It was found that both *sCry2S* and *sCry2L* variants (heterozygotes) appear in 10 out of 12 individuals of *S. galili*, *S. carmeli*, and *S. judaei* (~80%) and in 9 out of 12 of the *S. golani* species (75%). Two individuals of *S. galili* and *S. judaei* (~20%), 3 individuals of *S. golani* (~25%), and 1 individual of *S. carmeli* (~10%) carried only the short, *sCry2S*, isoform (homozygotes). Only 1 individual from all the 48 animals tested (from *S. carmeli*) carried only the long, *sCry2L*, isoform. Comparing the pattern of expression of the two isoforms, we have found that an identical pattern appears in both the genomic DNA and the mRNA of any specific individual (data not shown).

Evolutionary Analysis

Following the phylogenetic trees of rodents and humans, we expect that the distances between the proteins will correlate with the length of time since the divergence between these taxa. Note that *Primates* diverged 63 million years ago (Mya) (Wildman and Goodman, in press), whereas mole rats, *Spalacidae*, diverged from rodents 40 Mya (Nevo, 1999).

CRY1

The proteins of *Spalax* and humans are more similar than *Spalax* and mice (Table 1). *Spalax* Kimura's protein distance from mice is 4.38 and from humans only 2.07. There are only 12 different amino acids between *Spalax* and humans, while 25 different amino acids were counted between *Spalax* and mice. In addition, the length of the *Spalax* protein is 1 amino acid longer than that of humans, while it is 19 amino acids shorter

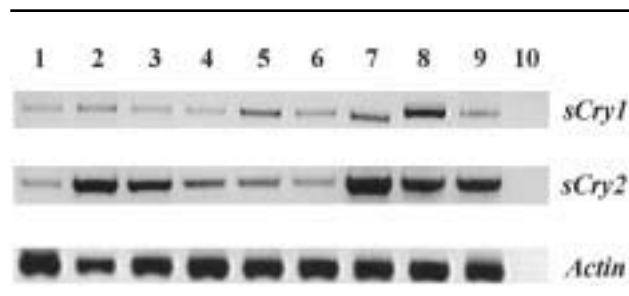


Figure 2. Tissue distribution of *Cry1* and *Cry2* expression in diurnal *Spalax*. The distribution of *Cry1* (upper) and *Cry2* (middle) expression was characterized by RT-PCR tested at the log phase of the reaction, using *Spalax*-specific oligos, of a fragment of each cDNA, on total RNA samples. *Actin* (lower) was used as a control. The following tissues were examined: (1) spleen, (2) liver, (3) heart, (4) skeletal muscle, (5) kidney, (6) intestine, (7) harderian gland, (8) eye, (9) brain, and (10) negative control. No-template negative control. As can be seen, both *sCry* genes are expressed in circadian-associated tissues (eye and brain) and in the harderian gland assumed to play an important circadian role in the blind subterranean *Spalax*. However both *sCry* genes are expressed also in a variety of peripheral tissues.

than that of mice. Therefore, the difference in the number of substitutions between the proteins is negatively correlated with the divergence time.

CRY2

The distance of *Spalax* CRY2 from the human protein is larger than the distance from mice, though it is not as large as expected from the divergence time (Table 1). Moreover, we have also used rat CRY2, which should be similar to mice; however, it differs from mice more than expected.

The Expression of *sCry* Genes

Tissue Distribution

Signals of both *sCry1* and *sCry2* were detected in all 9 tissues studied, though there are some differences in the relative intensity of the expression of the two genes in the different tissues (Fig. 2). A pronounced signal of *sCry1* was detected in the harderian gland, in the eye, and in the kidney. A pronounced signal of *sCry2* was detected in the harderian gland, in the eye, in the brain, as well as in the liver and the heart.

sCry Expression in Diurnal Animals in LD

Diurnal animals were killed under anesthesia at 4 different time points over the LD cycle, and brains,

harderian glands, and eyes were processed for ISH with *sCry1*- and *sCry2*-specific riboprobes. In the SCN, *sCry1* mRNA displays a clear daily rhythm with a maximum at ZT 6 and a minimum in the middle of the night (ZT 18; Fig. 3A). This is similar to *Cry1* expression data observed in mice (Okamura et al., 1999). *sCry2* oscillation is less apparent and does not reach the threshold of significance (i.e., $p > 0.05$) in *Spalax* SCN (Fig. 3A). In mice, results are contradictory whether *mCry2* expression is rhythmic but are in agreement that the amplitude of *mCry2* oscillation is strongly dampened as compared to *mCry1* (Okamura et al., 1999).

In the harderian gland, *sCry1* expression is synchronous to the SCN (Fig. 3B), while in the retina *sCry1* mRNA levels peak at the end of the light phase (ZT 12; Fig. 3C).

sCry2 rhythms appear to be phase delayed in the harderian (maximum at ZT 12; Fig. 3B); however, its oscillation only barely reaches the threshold of significance ($p > 0.05$).

In the retina, *sCry2* exhibits two peaks of expression (Fig. 3C), that is, one at ZT 6 and the second at ZT 18, indicating a specific role for *sCry2* in the *Spalax* retina as compared to the central pacemaker function in the SCN.

sCry Expression in Diurnal Animals in DD and after Light Induction

In line with the hypothesis that mammalian *Cryptochromes* might play a role as circadian photoreceptors like in insects and plants (Sancar, 2000), we investigated the influence of light on *sCry* oscillation by keeping the animals in constantly dark conditions (DD) and by measuring the effect of short light pulses on acute *sCry* mRNA levels during the night.

In DD, *sCry1* oscillation is maintained in the SCN (Fig. 4A) as well as in the harderian gland (Fig. 4B), with synchronous expression maxima at CT 12. Interestingly, the expression phase seems to be delayed compared to LD (Fig. 3). But remarkably, both the SCN and harderian gland retain synchronicity. The amplitude of *sCry1* oscillation in DD is more pronounced in the SCN (Fig. 4A) than in the harderian gland (Fig. 4B), whereas *sCry2* oscillates stronger than *sCry1*. Transcript levels of *sCry2* peak at CT 12 in both tissues.

To test for direct light inducibility of *sCry* transcription, we chose two illumination time points that are known to induce maximal phase delays (ZT 14) or

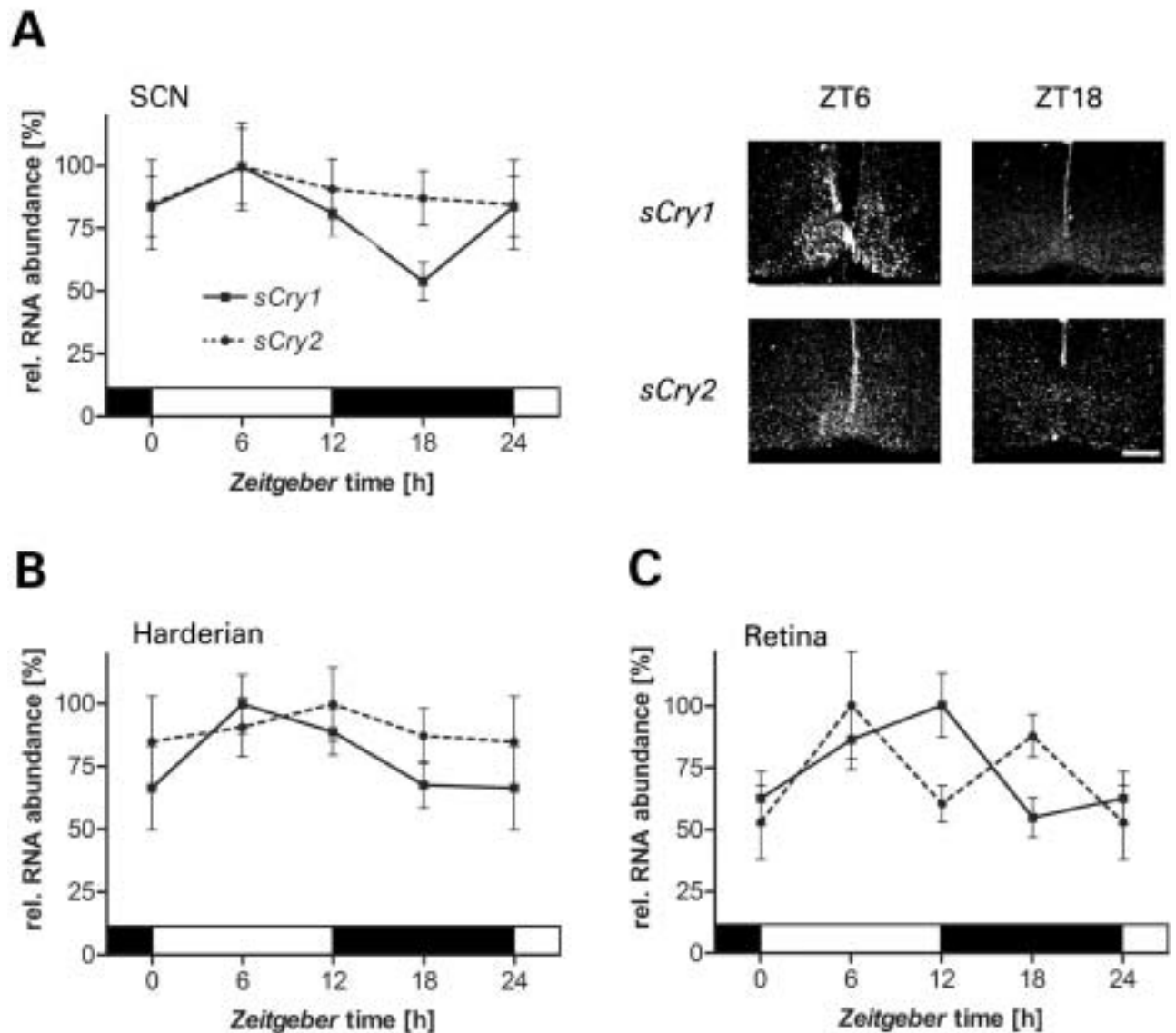


Figure 3. Expression of *Spalax Cry* gene in diurnal animals kept in a 12:12 LD environment. Daily variation of *sCry1* (solid line) and *sCry2* (dashed line) gene expression in the SCN (A), the harderian gland (B), and the eye (C) of diurnally active *Spalax* measured by ^{35}S labeled in situ hybridization. Animals were kept in 12:12 LD (indicated by the white and black bars on the x axis) cycle and sacrificed at 4 different time points (ZT 0/24, 6, 12, and 18 with ZT 0 = "lights-on"). Signal intensity was determined by optic densitometry of x-ray films. The highest signal for each probe and tissue was set as 100%, and all others were calculated accordingly. Values are mean \pm SD, $n = 3$. Right panel shows representative dark field micrographs of the SCN area hybridized with the specified probes at given time points. The length of the white bars in the micrographs correspond to 100 μm .

phase advances (ZT 22) of the circadian clockwork in other rodents (Daan and Pittendrigh, 1976). In an earlier study, we showed that *Spalax Per1* (*sPer1*) is highly light inducible at these time points in both the SCN and the harderian gland (Avivi et al., 2002). Therefore, we included *sPer1* as a positive control. Similar to mice (Okamura et al., 1999) and in accordance with the minor effects of the LD cycle on rhythmic *sCry* expression in *Spalax* (Fig. 3, 4), we did not observe any significant light induction of *sCry1* and *sCry2* expression,

neither in the SCN (Fig. 5A) nor in the harderian gland (Fig. 5B) at both time points. In contrast, *sPer1* expression is highly elevated after light exposure in all experiments (Fig. 5 A, B; $p < 0.001$).

sCry Expression in Nocturnal Animals

A unique feature of *Spalax* is its ability to switch from day activity (diurnal animals) to night activity (nocturnal animals) depending on environmental

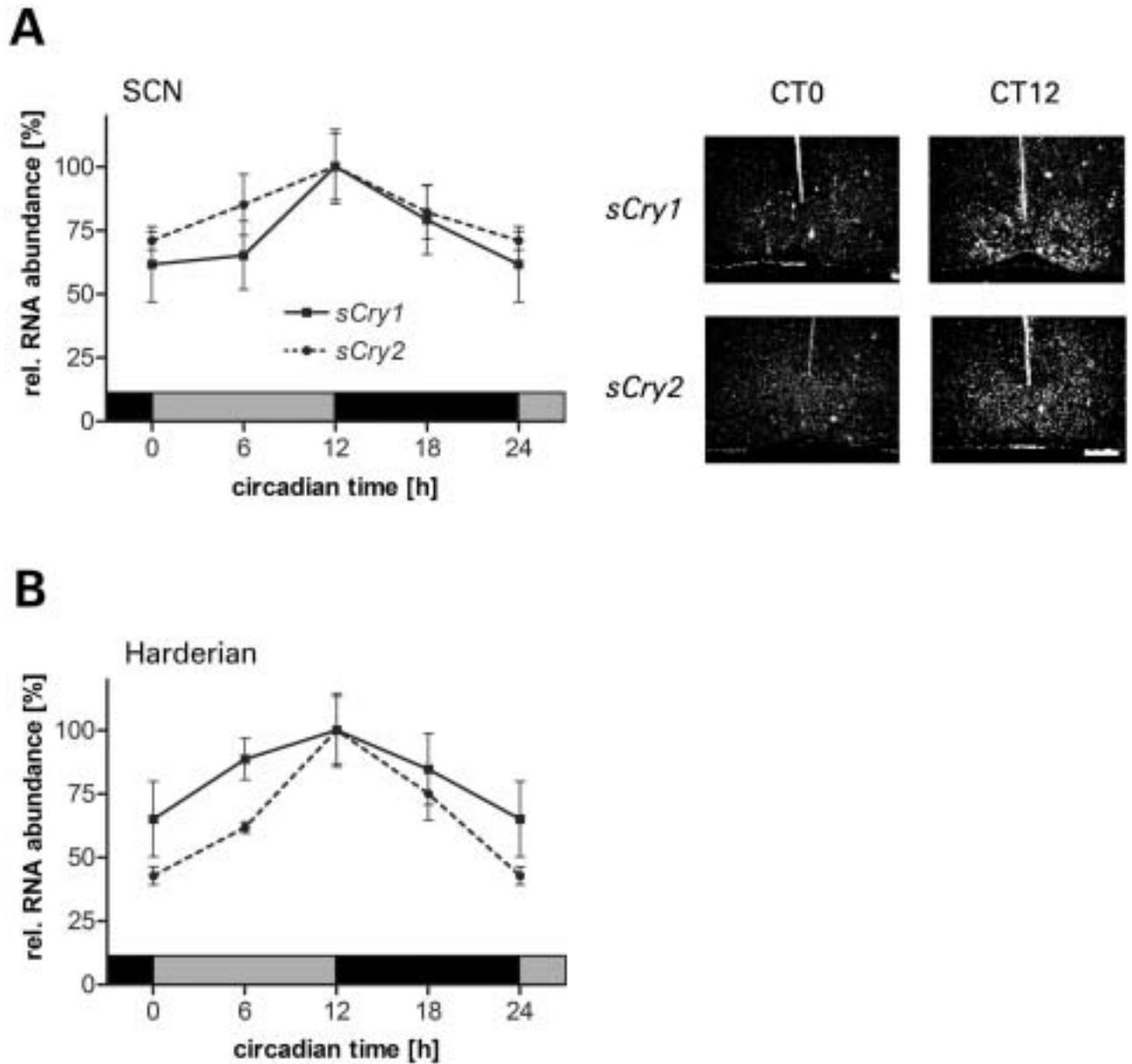


Figure 4. Expression of *Spalax Cry* gene in diurnal animals kept in total darkness. Circadian variation of *sCry1* (solid line) and *sCry2* (dashed line) gene expression in the SCN (A) and the harderian gland (B) of diurnally active *Spalax*. Animals were kept in LD prior to the experiment and sacrificed on the 2nd day in constant darkness (DD, activity and rest phase indicated by the gray and black bars on the x axis, respectively) at 4 different time points (CT 0/24, 6, 12, and 18, with CT 0 depicting the beginning of the activity phase). Signal intensity was determined by optic densitometry of x-ray films. The highest signal for each probe and tissue was set as 100%, and all others were calculated accordingly. Values are mean \pm SD, $n = 3$. Right panel shows representative dark field micrographs of the SCN area hybridized with the specified probes at the time points. The length of the white bars in the micrographs correspond to 100 μ m.

conditions (Ben-Shlomo et al., 1995). We have previously shown (Oster et al., 2002) that *sPer* gene oscillation in the SCN is inverted in nocturnal as compared to diurnal animals. We were interested whether *sCry*

oscillation is also affected in nocturnal animals. In the SCN, both *sCry1* and *sCry2* oscillate in antiphase as compared to diurnal animals in DD (maximum at ZT 0 as opposed to CT 12; Fig. 6A). Interestingly, if com-

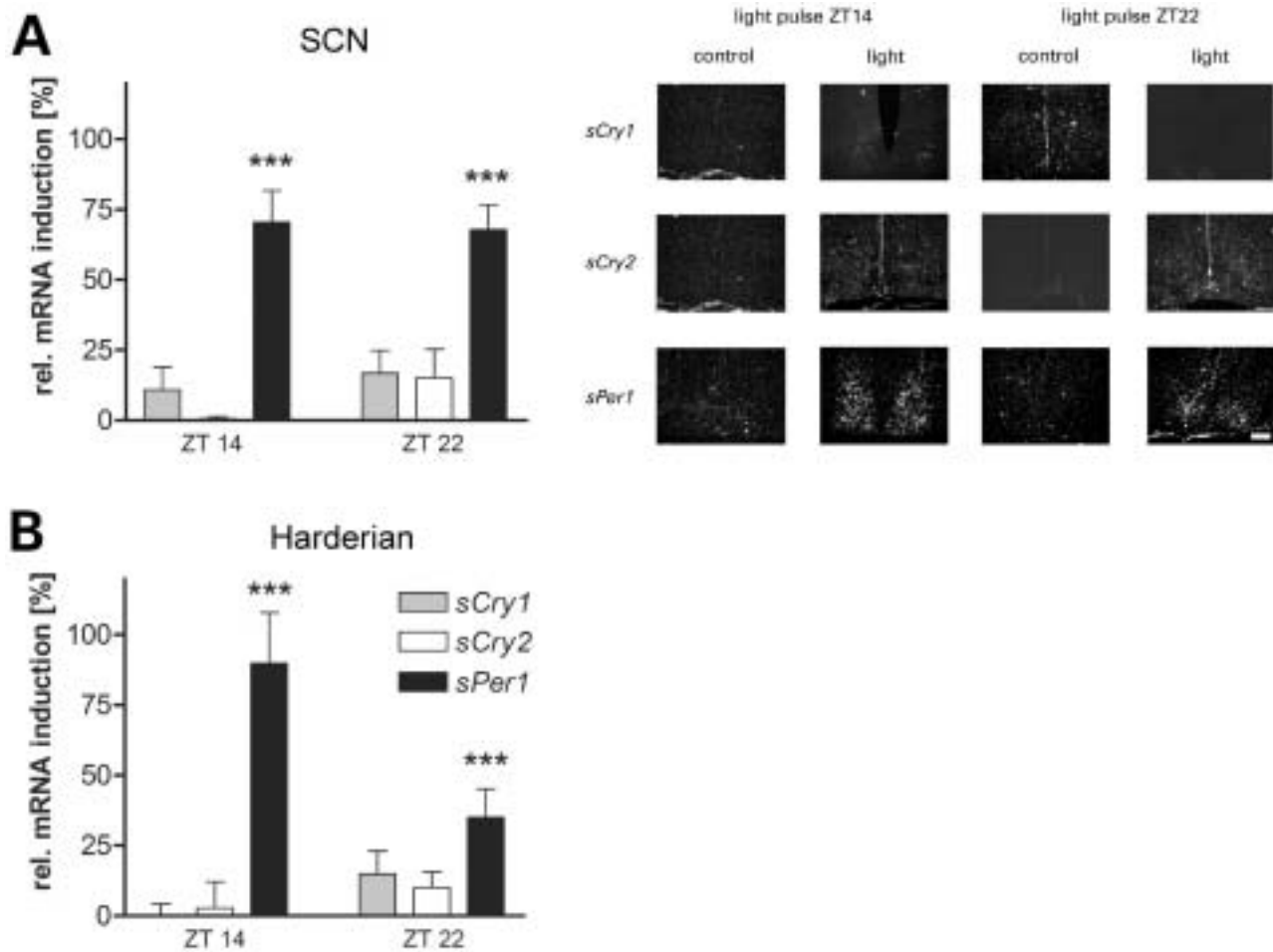


Figure 5. Light inducibility of the *Spalax Cry* gene expression. Dark phase light inducibility of *sCry1* (gray bar), *sCry2* (white bar), and *sPer1* (dark bar) in the SCN (A) and the harderian gland (B) of diurnally active *Spalax*. Animals were kept in an LD cycle prior to the experiment. One group ($n = 3$) of animals was exposed to a 15-min light pulse at the depicted time points (ZT 14 and ZT 22), while a control group ($n = 3$) was kept in darkness. Both groups were sacrificed at ZT 15 or ZT 23, and tissues were used in ^{35}S labeled in situ hybridization. Data shown are relative induction with the average signal intensity of the control group set as 0. All values are mean \pm SD; asterisks depict significant induction with $p < 0.001$ (student's t test). Right panel shows representative dark field micrographs of the SCN area hybridized with the specified probes at the given conditions and time points. The length of the white bars in the micrographs correspond to 100 μm .

pared to diurnal animals in LD, *sCry* oscillation is shifted forward for 6 hours (Fig. 6A). In the harderian gland, we find a similar situation in nocturnal animals with *sCry1* and *sCry2* expression peaks at the night/dark transition (ZT 0; Fig. 6B).

In the harderian gland of nocturnal animals, *sCry2* peaks at ZT 12, which is in antiphase to the expression in diurnal animals in both LD and DD. Notably, since only 4 different time points were chosen along the day cycle, small differences in phase alterations could not be resolved, which might mimic the apparent differences in *sCry* expression delays deduced from some of our data. Since we have shown that both *sCrys* are not directly regulated by light on the transcriptional level

(Fig. 3-5), we do not expect differences in *sCry* expression in nocturnal animals in DD.

DISCUSSION

Mammalian Circadian System in the Dark

The biological clock is localized in the central nervous system in the SCN and is entrained by light signals in the eye. Lightless habitats lead to structural regressive eye evolution (Nevo, 1999); however, the maintenance of a rudimentary eye along millions of years of evolution in the dark suggests preservation of

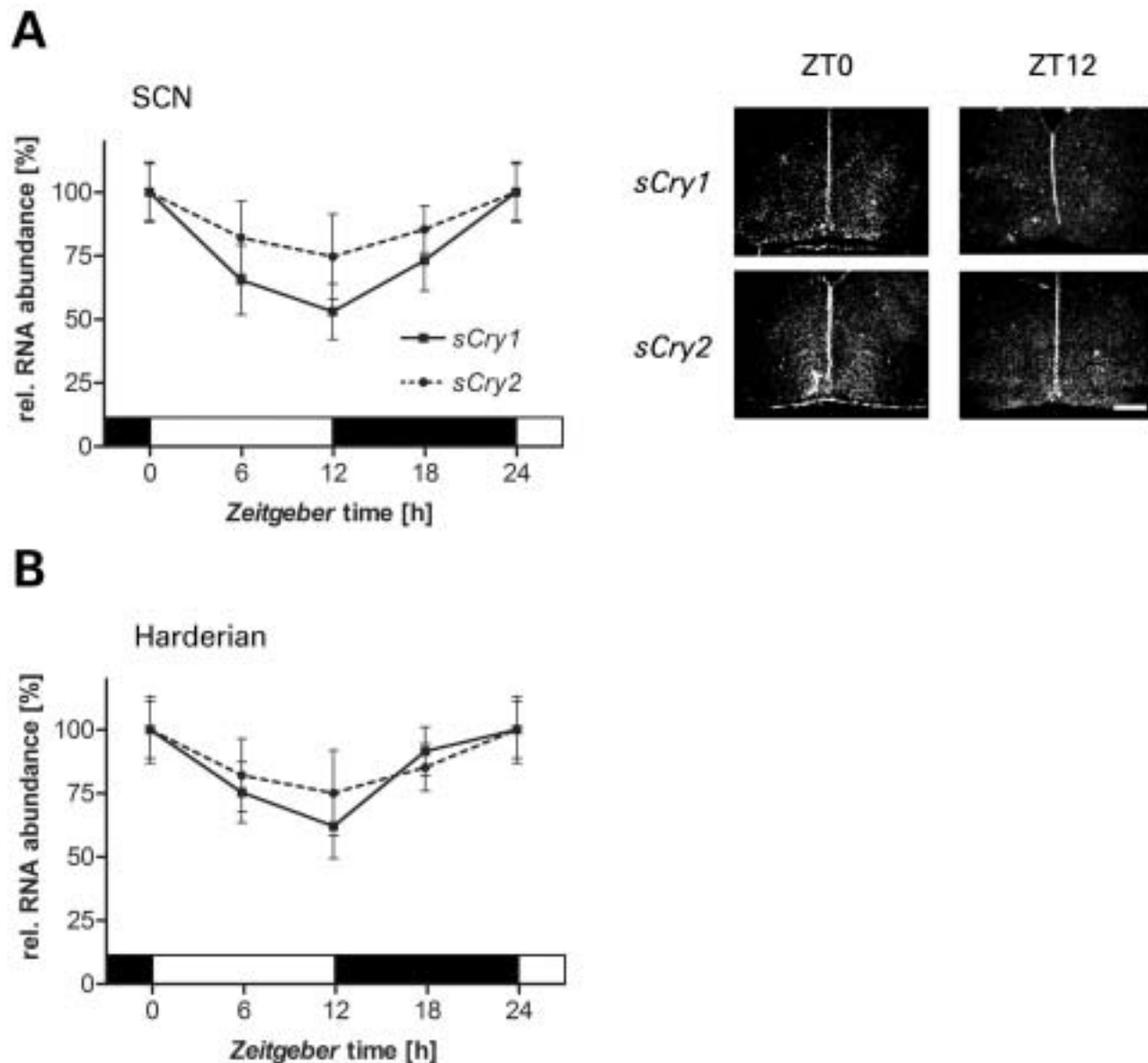


Figure 6. Expression of *Spalax Cry* gene in nocturnal animals kept in a 12:12 LD environment. Daily variation of *sCry1* (solid line) and *sCry2* (dashed line) gene expression in the SCN (A) and the harderian gland (B) of nocturnally active *Spalax* measured by ^{35}S labeled in situ hybridization. Animals were kept under a 12:12 LD (indicated by the white and black bars on the *x* axis) cycle and sacrificed at 4 different time points (ZT 0/24, 6, 12, and 18, with ZT 0 = "lights-on"). Signal intensity was determined by optic densitometry of x-ray films. The highest signal for each probe and tissue was set as 100%, and all others were calculated accordingly. Values are mean \pm SD, $n = 3$. Right panel shows representative dark field micrographs of the SCN area hybridized with the specified probes at the given time points. The length of the white bars in the micrographs correspond to 100 μm .

obligatory functions. That is, while sight is lost, photoperception and circadian function are retained. To understand the molecular mechanisms underlying the circadian clock in naturally blind mammals, we have embarked on a comprehensive study of this system in the blind subterranean mole rat superspecies *S. ehrenbergi* (Spalacidae, Rodentia) in Israel. This superspecies underwent evolutionary tinkering that

has optimized molecular and structural reductions and expansions, in organizing diverse systems, including the photoperiodic system, adapted to life in darkness underground (Nevo, 1999). Similar to sighted mammals, *Spalax* also expresses retinal photopigments that transmit photic signals from the circadian eye to the SCN, the site of the biological clock. To understand the possible role of *Cry* genes in

the circadian system of *Spalax*, we cloned its two *Cry* homologs, named *sCry1* and *sCry2*, determined their nucleotide sequence and the deduced proteins, and studied their expression patterns.

Spalax Cry Genes Structure and Evolution

We isolated cDNA clones of *Spalax Cry1* and *Cry2*. Like other mammals (Kobayashi et al., 1998; Miyamoto and Sancar, 1998), *Spalax* has two active *Cry* genes. Interestingly, *Spalax sCry2* has two isoforms differing in the initiation area of their ORF (Fig. 1). One isoform (*sCry2L*) is longer than the other, *sCry2S*, by 14 amino acids. However, beyond that dramatic difference, the two cDNAs and the deduced proteins are identical. A somewhat similar phenomenon was noticed in the zebrafish (Kobayashi et al., 2000), where six *Cry* genes were cloned and divided into three subclasses: *zCry1a* and *b*, *zCry2a* and *b*, and *zCry3a* and *b*. Similar to what we found in *Spalax Cry2*, most of the coding region and the deduced protein are identical in all six isoforms; however, contradictory to *sCry2*, which varies in the N-terminus of the protein, the zebrafish isoforms vary in the length and sequence of the C-terminal extension.

Heterozygosity

We have shown that 70% to 80% of the individuals are heterozygous (*sCryL/S*), about 10% to 30% contain only the short isoform (*sCry2S/S*), and only 1 individual, from *S. carmeli*, has only the long form (*sCryL/L*) out of the 48 individuals that were tested. From these results, we cannot determine if the *sCryL/L* genotype is just very rare or appears only in *S. carmeli*. Moreover, the genotype found in genomic DNA is always identical with the expressed pattern of *sCry2*.

The heterozygosity level of the *sCry2* (80%) is astounding. The maximum expected heterozygosity ($2pq$, where p and q are the allele frequencies) in a locus with two alleles is 50% when the frequency of the two alleles is equal. Any deviation from equal frequency of the two alleles reduces the expected heterozygosity. If the frequency of the alleles is equal, the probability of having 10 heterozygotes out of 12 animals is 0.019. Thus, the excess of heterozygotes is significant ($p < 0.05$) in *S. galili* ($2n = 52$), *S. carmeli* ($2n = 58$), and *S. judaei* ($2n = 60$). However, in *S. golani* ($2n = 54$), which has 9 heterozygotes out of 12 animals, the deviation from 50% is not statistically significant ($p > 0.05$). Nevertheless, if we test the deviation using the

estimate of the allele frequencies in this species (the L allele appears in 9 out of 24 alleles, while the S allele appears in 15 out of 24), the heterozygosity excess is significant ($p < 0.05$). Moreover, there is no significant difference in the number of heterozygotes among the 4 species of *Spalax*. Therefore, we are allowed to pool the data from the four species. Thus, the deviation from the expected heterozygosity (39 individuals instead of 24) is much more significant ($p < 0.0001$) (binomial probabilities; Aiken, 1955). We may conclude that the presence of such an excess of heterozygotes cannot be the result of just a stochastic process but must have a selective and/or cytological cause. But why should *sCry2* heterozygosity be that high? One possible explanation may be the regulation of the unique circadian rhythm of *Spalax*. Behaviorally, *Spalax* displays polyphasic and polytypic day-night activity patterns (Tobler et al., 1998). In a preliminary radiotracking field study, we found that mole rats were mainly diurnal and polyphasic during the rainy season, but many of them shifted to be nocturnal and monophasic during the dry season (Kushnirov et al., 1998), when they deepen their burrow level to avoid drought and high temperatures (Nevo, 1999). This seasonal shift may select for variation in seasonally dependent circadian rhythmicity. Only future experiments in nature and the laboratory may fully explain the astounding high level of *sCry2* heterozygosity and what is the functional importance of the presence of the two isoforms.

Distances

The distances between sCRY1 and sCRY2 proteins and those of other rodents or humans (Table 1) are expected to be approximately proportional with their divergence times, which are estimated to be 40 Mya and 63 Mya, respectively (Nevo, 1999). It was shown (Margoliash and Smith, 1965) that the rate of amino acid substitution in protein is approximately constant when time is measured in years. According to Kimura's protein distances (Kimura, 1983) used in our studies, the distances are expected to be proportional to the divergence time, if the distance is caused only by genetic drift. If we observe a significant deviation from this expectation, some additional evolutionary forces must be involved. The distance of sCRY1 from that of mice is higher than twice its distance from humans (4.38 vs. 2.07). Counting the actual number of different amino acids among the tested species (Table 1), we found a significantly larger number of substitu-

tions between sCRY1 and mCRY1 than between sCRY1 and hCRY1 ($p < 0.05$, χ^2 test). It must be emphasized that the real significance between *Spalax* and humans is much larger than phylogeny would predict. Noteworthy, similar results have been found for CLOCK and PER3 proteins, where the *Spalax* proteins were also somewhat closer to humans than to other rodents (Avivi et al., 2001, 2002).

The distance of sCRY2 protein from mCRY2 is about 66% of the distance to hCRY2. The distances between *Spalax* or mice and rats to humans are similar, as expected from their divergence time. However, the within-rodent distances are larger than expected from their phylogenesis. Generally, the distances calculated for the CRY2 are somewhat larger than the distances measured for CRY1. The findings that the distance of sCRY1 from mCRY1 is significantly larger than the distance of the sCRY1 from hCRY1, which is opposed to the phylogenetic expectations, may support adaptive selective changes in the evolution of this gene and not just neutral accumulation of amino acid substitutions over time. The sharp deviation from the expected distance between sCRY1 and mCRY1 (4 times), while sCRY2 deviates only moderately from the expected distance, suggests that mere demographic forces are unlikely as such forces should influence all proteins equally. It may reflect that these proteins underwent special adaptations in rodents. As *Spalax* lives in totally different ecologies than other rodents and mammals, we suggest natural selection as the adaptive force, possibly in response to life in darkness underground.

Tissue Distribution of *sCry* Genes

Analysis of the tissue distribution of the *sCry* mRNAs in *Spalax* (Fig. 2) demonstrates that similar to other species, both *sCry1* and *sCry2* are widely expressed. This supports previous findings showing that clock genes are expressed in different organs and indicating the existence of cellular biological clocks throughout the animal body (Balsalobre, 2002; Avivi et al., 2001, 2002). Nevertheless, it is noteworthy that the expression of *Cry* mRNAs, especially *sCry2* in *Spalax*, is not only distinguished in the SCN and the degenerated eye but also in their enlarged harderian gland. This is in line with previous experiments carried out in mice, which indicate a supportive role of CRY proteins in circadian photoperception (reviewed in Sancar, 2000). It also adds to our accumulating data (Pevet et al., 1984; Avivi et al., 2001, 2002; Oster et al.,

2002) that in the blind *Spalax*, the atrophied harderian gland, which occupies the entire eye socket, is a possible photoperiodic organ and/or supports circadian photoperception.

Oscillation of *Spalax Cry* genes

Cryptochrome expression patterns in the SCN of diurnal *Spalax* are comparable to data collected from other nocturnal rodents like mice and hamsters, with peak values in the second half of the light phase (Albrecht, 2002), and nicely correspond to *sPer* gene expression observed in these animals (Avivi et al., 2002). Interestingly, in constant darkness, *sCry* expression is phase delayed as compared to LD (Fig. 3A, 4A), indicating that although both *sCrys* seem not to be directly regulated by light (Fig. 5), the LD regimen may indirectly influence the time course of *sCry* gene activation, probably via activation of *sPer* gene expression and the consecutive modulation of *sCry* transcription activation by interaction of sPER proteins and the CLOCK/MOP3 complex (Avivi et al., 2002).

In the harderian gland, the phase of *sCry1* expression, in both LD and DD (Fig. 3B, 4B), is synchronized to the SCN. This corresponds to our data on *sPer* and *sMOP3* genes in the harderian gland (Avivi et al., 2001, 2002). In contrast, *sCry1* expression in the retina is clearly phase delayed (Fig. 3C), as typically observed for clock gene oscillation in peripheral tissues (Balsalobre, 2002). However, *sCry2* shows two peaks, one synchronized with the SCN and one phase delayed at ZT 18, indicating a specialized role of *Spalax Cry(s)* in the retina.

The synchronicity of SCN and harderian rhythms is maintained even in nocturnal *Spalax* (Fig. 6), where *Per* gene oscillation has been demonstrated to be separated from the light-driven resetting pathways to the clock (Oster et al., 2002). Interestingly, the *sCry* oscillation in nocturnal as compared to diurnal animals is not strictly inverted and thereby not tightly coupled to *sPer* gene oscillation (Oster et al., 2002). This different relationship of *Cry* and *Per* gene expression to the entraining ability of external light has already been demonstrated in mice subjected to shifts of several hours in the LD cycle. It seems that *mPer* expression is rapidly adapting to a new light regimen, while it takes several days for the *mCry* expression to entrain to the new conditions (Reddy et al., 2002). In nocturnal *Spalax*, like in phase-shifting mice, *Cry* oscillation in the SCN is synchronous with activity rhythms, while the strongly light-sensitive *Per* levels only indirectly

modulate the phase of the internal clockwork via CLOCK/MOP3. This is especially important for a subterranean and nocturnal animal, where light exposure is only sporadic—either during brief dwelling above the surface or at dusk and dawn—and while still being important for entrainment of internal rhythmicity to external time, it should not severely disturb the internal pacemaker by direct altering of clock gene activity.

The Harderian Gland

The tremendously hypertrophied harderian gland of *Spalax* surrounds a tiny rudimentary eye (0.7 mm of diameter). It expresses melatonin receptors and itself produces melatonin (Pevet, 2000). It was proposed that it might play a role in circadian photoreception and photoperiodism (Pevet et al., 1984). Our findings that in the harderian gland clock genes are rhythmically expressed and maintain synchronicity with the SCN under different lighting conditions and that the *sPer* genes are highly light inducible during the night strengthen this view (Fig. 5; Avivi et al., 2001, 2002). It is tempting to speculate that similar to the pineal/SCN system in birds (Abraham et al., 2002; Fu et al., 2002), the harderian/SCN dual pacemaker might serve to stabilize circadian rhythmicity in a subterranean ecotope. The two synchronous clockworks residing in the SCN and the harderian are connected neurally and via melatonin signaling and may thereby stabilize each other during long periods of zeitgeber (e.g., light) absence. In the subterranean environment of the *Spalax*, with only very brief exposure to light, the harderian gland is directly activated due to its anatomical position directly beneath the skin and may, therefore, augment light sensation by the rudimentary eye providing an additional gate for zeitgeber input to the core pacemaker of the SCN.

A functional involvement of the harderian gland in the central circadian pacemaker of *Spalax* was already suggested (Pevet et al., 1984) and certainly represents an extreme example of evolutionary progression during the adaptive reorganization for life underground (Nevo, 1999). However, it might also shed new light on the putative role of this organ in other mammalian species. Studies using harderian-ectomized animals may further elucidate this hypothesis, and indeed it is our plan to test the expression of circadian genes in *Spalax* individuals when their eyes and/or harderian glands have been removed.

In conclusion, *sCry1* and *sCry2* are rhythmically and phase synchronously expressed in the SCN and the harderian gland of the blind mole rat. Both genes are not directly influenced by light exposure of the animals during the night, but the external LD cycle indirectly modulates *sCry* expression probably via activation of the *sPers*. In nocturnal animals, *sCry* expression is shifted, but SCN and harderian oscillation remains in phase, indicating a prominent role of the harderian gland in the *Spalax* timekeeping system.

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