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The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior

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DBP, a PAR leucine zipper transcription factor, accumulates according to a robust circadian rhythm in liver and several other tissues of mouse and rat. Here we report that DBP mRNA levels also oscillate strongly in the suprachiasmatic nucleus (SCN) of the hypothalamus, believed to harbor the central mammalian pacemaker. However, peak and minimum levels of DBP mRNA are reached about 4 h earlier in the SCN than in liver, suggesting that circadian DBP expression is controlled by different mechanisms in SCN and in peripheral tissues. Mice homozygous for a DBP-null allele display less locomotor activity and free-run with a shorter period than otherwise isogenic wild-type animals. The altered locomotor activity in DBP mutant mice and the highly rhythmic expression of the DBP gene in SCN neurons suggest that DBP is involved in controlling circadian behavior. However, since DBP–/– mice are still rhythmic and since DBP protein is not required for the circadian expression of its own gene, *dbp* is more likely to be a component of the circadian output pathway than a master gene of the clock.

Keywords: circadian behavior/DBP/PAR leucine zipper proteins/suprachiasmatic nucleus/transcription factor

Introduction

Circadian rhythms have been observed in a large number of multi- and unicellular organisms, including vertebrates, invertebrates, plants, fungi and bacteria (Takahashi, 1993; Florez and Takahashi, 1995; Kay and Millar, 1995; Rosbash, 1995; Block *et al.*, 1996; Dunlap, 1996). Such oscillations are generated by one or multiple endogenous pacemakers and persist with a period of ~24 h in the absence of external time cues (Takahashi, 1995).

Like any timing device, the circadian system consists of three components: an entrainment pathway, a central oscillator and an output pathway. In most organisms, daily variations in light intensity are the major environmental cues setting the clock (Takahashi, 1993; Rosbash, 1995). In mammals, the central oscillator resides in the suprachiasmatic nucleus (SCN) at the base of the hypothalamus (Rusak and Zucker, 1979). Thus, hamsters display arrhythmic locomotor activity after ablation of the SCN and regain rhythmic behavior after implantation of a fetal SCN (Ralph *et al.*, 1990). Recently, partial rescues of rhythmic running wheel activity have also been obtained with SCN tissue encapsulated in a semi-porous plastic resin. Therefore, diffusible SCN output signals appear to be involved in controlling circadian locomotor activity (Silver *et al.*, 1996).

In two organisms, *Neurospora* and *Drosophila*, genes that are essential for generating circadian oscillations have been isolated (for review see Dunlap, 1996). The best characterized *Neurospora* clock gene, *frq*, fulfills many if not all criteria expected for an intrinsic clock component. Thus, FRQ mRNA and protein levels oscillate with a circadian period, mutations in *frq* change or abolish rhythmicity, *frq* expression is subject to negative feedback regulation, ectopic expression results in phase shift responses and/or cessation of rhythmicity, and *frq* expression is sensitive to light. *Prd-4* and *wc-2* are two additional *Neurospora* clock components (Dunlap, 1996; Crosthwaite *et al.*, 1997). In *Drosophila*, two clock genes, *per* and *tim*, have been identified (Citri *et al.*, 1987; Myers *et al.*, 1995). The products of these two genes form a heterodimer whose accumulation and nuclear translocation follow a circadian rhythm (Gekakis *et al.*, 1995; Sehgal *et al.*, 1995; Saez and Young, 1996; Zeng *et al.*, 1996). Flies with either *per* or *tim* null alleles do not express either of these two proteins in a daily fashion and display arrhythmic locomotor activity (Price *et al.*, 1995; Reppert and Sauman, 1995; Rosbash, 1995). In comparison with *Neurospora* and *Drosophila*, little is known about the genetic basis of circadian oscillation in mammals. Thus far, only two mutations with strong circadian phenotypes and Mendelian inheritance have been described. The first, *tau*, reduces the free-running period of locomotor activity to 22 h and 20 h in hetero- and homozygous mutant hamsters, respectively (Ralph and Menaker, 1988). Remarkably, the SCN implant from a *tau* mutant animal with a shorter free-running period rescued SCN-lesioned wild-type hosts with the period of the donor (Ralph *et al.*, 1990). The second mutation, *clock*, increases the free-running period to 25 h in heterozygous mutant mice and to 27 h in homozygous mutant mice. After some weeks in constant darkness, homozygous *clock* mutant animals become completely arrhythmic (Vitaterna *et al.*, 1994). The *clock* gene has recently been isolated by positional cloning and its protein product has been identified as a PAS helix–loop–helix protein (Antoch *et al.*, 1997; King *et al.*, 1997). Interestingly, the *Drosophila* PER protein also contains a PAS domain, although this protein does not appear to contain a DNA binding domain (for review see Reppert and Weaver, 1997).

The mechanisms by which the central circadian oscillators govern rhythmic behavior and physiology in mammalian organisms are poorly understood. It is believed that the hypothalamus plays a major role in the com-

munication of circadian signals from the SCN to peripheral tissues. Indeed, extensive SCN–intra-hypothalamic connections have been described which lend support to the importance of this output pathway (Moore, 1992).

We have gained access to molecular aspects of circadian regulation in rat and mouse by uncovering a basic leucine zipper transcription factor, albumin site D-Binding Protein (DBP). The expression of the DBP gene oscillates with a very large daily amplitude in liver, kidney, pancreas, heart muscle and lung (Wuarin *et al.*, 1992; Fonjallaz *et al.*, 1996; Lavery *et al.*, 1996), and with a moderate two- to three-fold amplitude in brain. Two proteins, Thyroid Embryonic Factor (TEF) and Hepatocyte Leukemia Factor (HLF), have been shown to share extensive sequence similarity with DBP within their basic leucine zipper (bZip) region and an adjacent peptide segment rich in prolines and acidic amino acids, known as the PAR domain (Drolet *et al.*, 1991; Hunger *et al.*, 1992; Inaba *et al.*, 1992). Similar to the DBP gene, the TEF and HLF genes are expressed according to circadian rhythms in several tissues (Falvey *et al.*, 1995; Fonjallaz *et al.*, 1996).

In order to examine physiological roles of DBP, the transcription factor with the highest abundance and the largest circadian fluctuation of the three PAR proteins, we have established knockout mouse strains homozygous for DBP null alleles. In this paper we show that DBP mutant mice display differences in behavior with regard to length of the period of running wheel activity under constant dark conditions and amplitude of locomotor activity under light-entrained conditions. Moreover, we demonstrate that DBP mRNA accumulation strongly oscillates in the SCN, and that DBP protein is not required for this cyclic mRNA expression.

Results

Generation of transgenic mice carrying DBP null alleles

In order to generate a mouse strain with a DBP null allele, homologous recombination in embryonic stem cells (ES) cells was used (Thomas and Capecchi, 1987). A recombinant bacteriophage containing the mouse (strain 129/Sv) DBP gene was isolated by molecular cloning. This gene is composed of four exons and three introns and spans ~5.8 kb from the cap site to the polyadenylation site. The strategy used for homologous recombination is outlined schematically in Figure 1A. The targeting vector contains a DBP gene fragment encompassing 6.5 kb of 5′-flanking sequences and 120 bp of 5′-non-translated sequences upstream of the *lacZ–neo* cassettes, and a DNA fragment traversing the last 9 bp of the DBP open reading frame, the entire 3′-non-translated region and 900 bp of 3′-flanking sequences downstream of the *lacZ–neo* cassettes. This construct was designed to allow transcription of the bacterial *lacZ* gene from the DBP promoter, once it had inserted into the DBP locus. As the targeting vector contains only 9 bp of the DBP open reading frame 3′ to the *neo* cassette, gene replacement should result in a true null allele of DBP.

ES cells from 129/Ola mice, in which the endogenous *dbp* gene was replaced by the desired homologous recombination event, were used to generate four 129/Ola/C57Bl/6 chimeric males. These were mated with 129/Ola

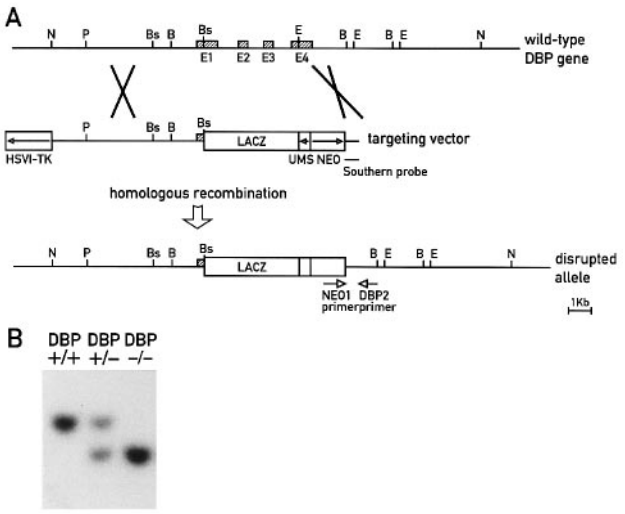

Fig. 1. Generation of a DBP-minus mouse strain. (**A**) Strategy for the replacement of the DBP gene by homologous recombination. A DBP targeting vector, containing a bacterial *lacZ* and a *neo* cassette between DBP upstream sequences (encompassing 6.5 kb of 5′-flanking sequences and 120 bp of 5′-non-translated sequences) and DBP downstream sequences (containing the last three DBP codons, 900 bp of 3′-non-translated sequences and 300 bp of the 3′-flanking region), was electroporated into ES cells. Homologous recombination by a double crossover event lead to the replacement of the entire DBP coding sequence with the *lacZ–neo* sequences. The positions of some restriction sites are depicted. The following abbreviations are used for restriction endonucleases: E (*Eco*RV), B (*Bam*H1), Bs (*Bst*EII), N (*Not*I) and P (*Pvu*I). Note that the *Not*I sites are vector-borne (boundaries of cloned DBP gene region) and do not exist in the genome. LACZ stands for the bacterial β-galactosidase gene; HSVI-TK stands for the thymidine kinase gene of herpes virus I. NEO stands for the neomycin resistance gene, and UMS is a sequence that was inserted to terminate upstream transcripts (but in our hands this sequence was ineffective). The positions of PCR primers and DNA probes used in the identification of recombined alleles are indicated. (**B**) Southern blot analysis of DNA from F_2 mice. Heterozygous DBP± animals were obtained from matings between male mouse chimeras producing 129/Ola DBP mutant gametes with 129/Ola females producing wild-type gametes. The DNA of F_2 animals obtained from isogenic F_1 129/Ola males and females, heterozygous for the DBP mutant allele, was analyzed by Southern blot analysis of DNA digested with the restriction endonuclease *Bam*HI. An example for each of a wild-type mouse (DBP+/+), a heterozygous mouse (DBP+/–) and a homozygous mutant mouse (DBP–/–) is shown. The position of the genomic hybridization probe is indicated in (A).

females to obtain isogenic F_1 offspring in which all alleles other than *dbp* should have been identical. The DNA of tail samples of F_1 and F_2 individuals was examined by Southern blotting to identify hetero- and homozygous DBP mutant animals (Figure 1B). The tail DNA analysis of 152 F_2 animals revealed that mice hetero- or homozygous for the DBP mutant alleles were born in roughly Mendelian ratios (23% DBP+/+, 52% DBP+/–, 25% DBP–/–), suggesting that DBP is not essential for embryonic development. Moreover, homozygous mutant mice are morphologically indistinguishable from wild-type littermates and are fertile. We have not yet systematically monitored the life span of DBP mutant animals, but thus far we have not noticed a high frequency of premature death. Thus, DBP is also dispensable for postnatal life under laboratory housing conditions.

Ribonuclease mapping and immunoblot experiments were performed to verify the absence of DBP gene products in the liver of homozygous mutant mice. As

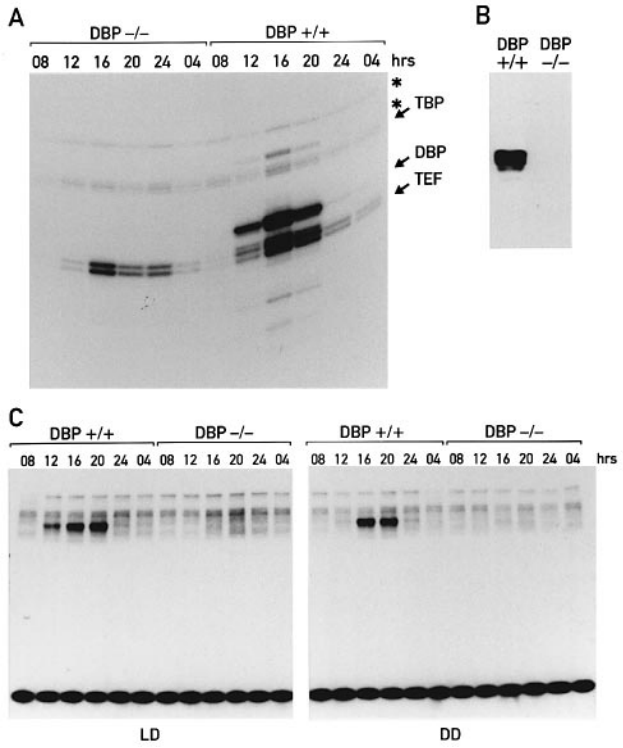

Fig. 2. Expression of DBP mRNA in wild-type and DBP mutant mice. (**A**) Relative levels of DBP, TBP and TEF mRNAs were determined by ribonuclease protection assays in whole cell liver RNA harvested at different times around the clock. ^{32}P-labeled RNA fragments protected by 50 μg whole cell liver RNA were resolved on a 7% urea–polyacrylamide sequencing gel. RNA antisense probes for TBP mRNA and TEF were included in the assays as internal controls for transcripts with constitutive and circadian expression, respectively. Non-digested and partially digested DBP antisense probes are indicated by (*). (**B**) Immunoblot analysis of liver nuclear extracts with DBP antibodies. Purified liver nuclei, harvested at 8 p.m. (pooled from three individuals), were extracted with NUN-buffer (see Materials and methods). Approximately 25 μg of non-histone proteins from DBP+/+ and DBP–/– animals were resolved on a 12 % SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and probed with polyclonal, affinity-purified DBP antibodies. (**C**) Electromobility shift assays with a high-affinity PAR recognition DNA sequence and nuclear extracts from DBP+/+ and DBP–/– mice. NUN extracts (see B) from liver nuclei harvested at different times around the clock were incubated with a radiolabeled double-stranded oligonucleotide encompassing the PAR recognition sequence GTTACGTAAT. Animals were kept either under a 12 h light/dark regimen (LD: lights on 7 a.m., lights off 7 p.m.) or for 2 days in constant darkness (DD) prior to being sacrificed. Note that the major protein–DNA complex displays circadian oscillation and is only detected in DBP+/+ animals. Multiple minor bands with little daily variation can also be discerned. They probably correspond to complexes formed between the DNA probe and various C/EBP isoforms also binding this DNA sequence (see text).

expected, mice homozygous for DBP null alleles express neither DBP mRNA (Figure 2A) nor DBP protein (Figure 2B). In the ribonuclease mapping experiment shown in Figure 2A, Tata box-Binding Protein (TBP) and TEF antisense RNA probes were included as internal controls for constitutively and circadianly expressed mRNAs, respectively. To examine whether the elimination of DBP results in a net decrease of proteins binding to a PAR DNA recognition sequence, electrophoretic mobility shift assays were performed with liver nuclear extracts harvested at different time points from mice kept under either entrained or constant conditions (see legend to Figure 2). The DNA sequence GTTACGTAAT contained in this DNA probe binds all known members of the PAR and most members of the C/EBP bZip protein families (Falvey *et al.*, 1996). As shown in Figure 2C, the most prominent DNA binding activity displays robust circadian accumulation in wild-type animals. Little, if any, DNA binding activity corresponding to this band can be discerned in nuclear extracts from DBP–/– mice, suggesting that DBP is responsible for the major protein–DNA complex observed in wild-type animals.

DBP mutant mice free-run with a shorter circadian period and display less locomotor activity than wild-type mice

Given the rhythmic expression of DBP in several tissues (see Introduction), we were interested in comparing the circadian behavior of wild-type and homozygous DBP mutant mice with regard to period length and amplitude. Such information can be conveniently collected by automated recordings of running wheel revolutions (voluntary locomotor activity) or infrared beam breaks (spontaneous locomotor activity) under entrained (light/dark) and constant (dark/dark) conditions. Since circadian locomotor activity is likely to be affected by many different genes, and since disruption of only one of these genes may result in subtle changes, we considered it important to perform this comparison with isogenic inbred strains, in which the only genetic difference should reside in the presence or absence of the DBP gene (see above).

In a first series of experiments, 32 DBP+/+ and 33 DBP–/– individuals were analyzed for running wheel activity under entrained and free-running conditions. Under both conditions, the locomotor activity of wild-type and mutant mice is clearly rhythmic (Figure 3B). However, determination of the free-running period length revealed a moderate, but statistically highly significant difference ($P < 0.00001$) under constant conditions (dark/dark, DD) (Figure 3A and B and Table I). Thus, the average period length is 23.26 h for DBP–/– mice, compared with 23.76 h for wild-type animals. Visual inspection of the two τ-length distributions (Figure 3A) suggests that DBP–/– mutant mice free-run with more heterogeneous periods than wild-type animals. Therefore, lack of DBP expression may result in not only the shortening of τ, but also in a degeneracy of precision in circadian timing. The analysis of total running wheel activity also revealed significant differences for DBP+/+ and DBP–/– mice under LD conditions (Table I), in that the former generated about 40% more wheel revolutions per day than the latter.

In a second series of experiments, spontaneous locomotor activity of wild-type and DBP–/– mice was determined by infrared beam break recordings (Figure 3C, Table I). Under entrained conditions, DBP+/+ animals are about 64% more active than DBP mutant animals (Table I), similar to what has been observed for running wheel activity. The difference in spontaneous locomotor activity is mainly due to a lower activity of mutant mice during the dark phase (Figure 3D). The residual activity during the light phase does not vary between the two groups. As a consequence, the amplitude of spontaneous locomotor activity between dark and light phases is considerably higher in wild-type as compared with DBP

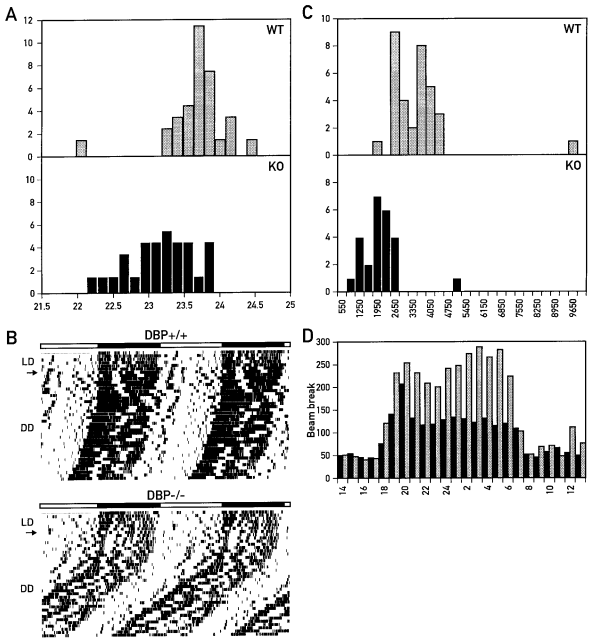

Fig. 3. Circadian behavior of DBP+/+ and DBP–/– mice. (**A**) Period length distributions from 33 DBP–/– and 32 DBP+/+ mice. The average period lengths and the standard errors are given in Table I. The difference between period lengths of DBP+/+ and DBP–/– animals is statistically highly significant ($P < 0.00001$). A single wild-type mouse free-ran with an extremely short period of 22.08 h (see text). (**B**) Actograms of DBP+/+ and DBP–/– animals with free-running periods close to the mean values of the respective groups. Running wheel locomotor activity was recorded for 7 days under LD (lights on 7 a.m., light off 7 p.m.) and 34 days under DD conditions. Forty-eight hour double plots are shown, in which the recordings of each day are replicated and appear under those of the previous day. The arrow indicates the shift from LD to DD. Note that the locomotor activity of the DBP mutant mouse remains rhythmic, even after an extended DD time period. The free-running period lengths of the two presented individuals, measured between DD days 11 and 21, are 23.17 h and 23.68 h for the DBP–/– and DBP+/+ animals, respectively. (**C**) Spontaneous locomotor activities of DBP–/– and DBP+/+ mice. Infrared beam break recordings were performed under LD conditions for 10 days, and the average number of beam breaks/day were determined for each individual. The distributions of these values are presented for 33 DBP+/+ (top panel) and for 25 DBP–/– mice (bottom panel). Note that different individuals from those presented in panel A were used. (**D**) Spontaneous locomotor activities of DBP–/– (black bars) and DBP+/+ mice (grey bars) during the day. The 10 day recordings from mice kept under LD conditions (D) were converted into beam breaks per hour during the day. Each value represents the average from all DBP–/– or DBP+/+ mice and the average of 10 day recordings. Note that the activities during most of the night phase are considerably higher for the wild-type animals, but that these values are very similar for wild-type and mutant animals during the early dark phase and the light phase (see Table I).

mutant mice (Table I). In constant dark (DD), the amplitude of spontaneous locomotor activity of 129/Ola mice is low, irrespective of whether the animals contain the *dbp* gene. This renders it difficult to delineate unambiguously the activity phase, and thus we did not attempt to evaluate the infrared beam break recordings under free-running conditions.

DBP mRNA shows robust circadian expression in the SCN

Circadian locomotor activity is driven by the SCN, the major mammalian pacemaker (see Introduction). The altered circadian behavior of DBP mutant mice prompted us to examine the expression of the *dbp* gene in the SCN. Given the small size of the mouse SCN, this can be most readily accomplished by *in situ* hybridizations with coronal brain sections. The DBP knock-out mouse provided a perfect control for assessing the reliability of such experiments. Figure 4A shows that coronal brain sections yield *in situ* hybridization signals for the DBP antisense probe only for wild-type animals. As expected, hybridization signals for a TEF antisense probe were detected for both wild-type and DBP knockout mice, demonstrating that the quality of tissue sections was comparable for wild-type and knockout animals. The strongest DBP-specific signal was associated with the SCN, when the mice were sacrificed at noon. To examine whether DBP mRNA displays circadian expression in the SCN, serial coronal brain sections containing the SCN were collected at different times from wild-type mice kept under light/dark (LD) conditions (light on 7 h, light off 19 h) or in constant dark (DD) and hybridized to the *dbp* antisense probe. The data presented in Figure 4B illustrate that DBP mRNA accumulates according to a robust circadian rhythm in the SCN, but shows little fluctuation in other brain areas (not shown). Inspection of the hybridization signals at a higher magnification (Figure 4C) suggests that the large majority of SCN cells display rhythmic DBP mRNA accumulation. Moreover, the examination of serial sections across the SCN revealed circadian DBP mRNA accumulation in all parts of the SCN (not shown).

The peak and minimum phases of DBP mRNA expression in the SCN are centered around noon and midnight, respectively, in animals kept under 12 h LD (lights on 7 a.m.) or for two days under DD conditions. This is different from the circadian DBP mRNA accumulation observed in liver (and other peripheral tissues), in which maximal and minimal cellular concentrations were observed at 4 p.m. and 4 a.m., respectively (see Figure 2A). To ascertain the difference in phase angle between the circadian expression of DBP in the SCN and in liver, the accumulation of DBP mRNA was determined by ribonuclease mapping in liver RNA samples from the same individuals (kept in constant darkness) from which the brain tissue sections were collected. Figure 5B shows the results of this analysis. Indeed, maximal DBP mRNA levels are observed in the RNA sample obtained from the mouse sacrificed at 4 p.m. Moreover, at 8 a.m., at which time a strong signal can already be detected in the SCN (Figure 4B), little if any DBP mRNA accumulates in hepatocytes (Figure 5A). The quantification of relative DBP transcript levels in liver and the SCN of the same individuals (Figure 5B) clearly confirms the phase angle difference of DBP mRNA expression in these two tissues.

DBP protein expression is not required for circadian DBP mRNA accumulation in the SCN

In the knockout mice described above, the DBP-encoding DNA sequences were replaced by a bacterial *lacZ* gene. This mutant allele was constructed in the hope that *in situ* staining for β-galactosidase activity would be an expeditious procedure for the determination of the spatial and temporal DBP expression. However, we found that LacZ expression does not faithfully mirror DBP expression in the brain and in other tissues. As shown in Figure 6, only the hippocampus and the dentate gyrus yield a strong

Table I. Volunatary and spontaneous locomotor activity in DBP+/+ versus DBP–/– mice

Parameter	Genotype	
	DBP+/+	DBP–/–
Running wheel activity		
Number of individuals	32	33
Period length[a] (τ, hours)	23.76 ± 0.07	23.26 ± 0.08***
Change in period[b] (hours)	–0.124 ± 0.042	–0.031 ± 0.059
Log power spectral density[c]		
DD (day 10–20)	2.701 ± 0.082	2.468 ± 0.089
Total activity (counts per day)		
LD 12:12	19 120 ± 1546	13 783 ±1088**
DD day 10–20	19 386 ± 1550	15764 ± 1419
Daytime activity (%)	4.1 ± 1.72	4.8 ± 131
Spontaneous locomotor activity (infrared beam breaks = ibb)		
Number of individuals	33	25
LD 12:12 (ibb/day)	3783 ± 245	2299 ± 1688***
Lights on 7 a.m.–7 p.m. (ibb)	833 ± 116	705 ± 74
Lights off 7 p.m.–7 a.m. (ibb)	2950 ± 172	1594 ± 128***
Amplitude (ibb lights off/ibb lights on)	5.42 ± 0.86	2.88 ± 0.46*

Male adult 129/Ola mice (3–5 months of age) with or without functional DBP alleles were analyzed for running wheel or infrared beam break activity as described in Materials and methods. For the evaluation of running wheel activity, the software 'Chronobiology Kit' (Stanford Software Systems) was used, as presented in Vitaterna *et al.* (1994). A homemade piece of software (JERRY2) was used to analyze the infrared beam break data. Different individuals where used for running wheel and beam break recordings. If relevant, mean values ± standard errors are given. Values, for which the difference is statistically significant, are labeled with * ($P < 0.02$), ** ($P < 0.01$) or *** ($P < 0.00001$). Two different statistical methods (Student's *t*-test, assuming normal distributions, and the Mann–Whitney test, assuming non-parametric distributions) have been used to examine the data. Only the maximal *P*-values are given. We noticed a single wild-type individual free-running with an extremely short period of only 22.08 h. At present we do not know whether this individual has suffered a spontaneous mutation in a clock-affecting gene (other than *dbp*), or whether it is genetically identical to the other 31 wild-type mice examined.

[a]The period lengths were determined between DD days 11 and 20.

[b]The changes in period length are given for the periods determined between DD days 1–10 and DD days 11–20.

[c]Log PSD is a measure for the amplitude, based on Fourier analysis of running wheel activity of a 10 day interval (DD days 11–20). For details, see Vitaterna *et al.* (1994).

lacZ hybridization signal, while other areas of the brain of DBP–/– mice, including the SCN, are weakly labeled. Similar results were observed in immunohistochemistry experiments using a polyclonal LacZ antibody with homo- and heterozygous DBP mutant mice (data not shown). These data can be interpreted in two different ways: either DBP is required in a dose-dependent manner for the circadian expression of its own mRNA in the SCN, or the DBP–LacZ mutant allele lacks important intragenic *cis*-acting DNA elements that were deleted in the course of the gene replacement.

To discriminate between these two possibilities, we examined DBP gene expression in a second DBP mutant mouse strain, in which no DBP DNA sequences had been deleted. In this DBP knockout mouse strain, a *neo* cassette was inserted between the genomic DNA sequences encoding the DNA binding and dimerization domains (Figure 7A). As this DBP–*neo* fusion gene still contains all DBP gene sequences, its transcriptional regulation should not deviate from that of the wild-type allele. However, the resulting mRNA should be unable to specify a functional transcription factor, since its putative translation product lacks a dimerization domain.

F_1 and F_2 mice obtained from crossings between 129/Ola/C57Bl/6 chimeric males and C57Bl/6 females were typed by Southern blotting analysis of tail DNA (Figure 7B) and F_2 offspring homozygous for either the DBP mutant or wild-type alleles were used for further analysis. Before examining the expression of the chimeric DBP–neomycin gene by *in situ* hybridization in the brain, we characterized its products in liver using biochemical techniques. While Northern blot and ribonuclease protection experiments revealed the expected fusion mRNA of 3.2 kb (data not shown), Western blot assays failed to detect an immunoreactive protein in nuclear extracts (Figure 7C). Conceivably, dimerization-deficient, and thus DNA binding-deficient, PAR proteins are either unstable, remain cytoplasmic or are lost during the purification of nuclei.

Figure 7D displays the *in situ* hybridization results obtained with mice homozygous for the *dbp–neo* fusion gene that were sacrificed at noon and midnight. Clearly, DBP mRNA still shows circadian expression in the SCN of these DBP-protein-deficient mutant mice. We thus conclude that DBP is not essential for the circadian expression of its own mRNA in the SCN. Rather, the lack of LacZ mRNA accumulation in the SCN of DBP–/– mutant mice (Figure 6) suggests that intragenic *cis*-acting DBP regulatory elements are required for circadian DBP transcription in the SCN.

Discussion

Circadian DBP expression is different in the SCN and in peripheral tissues

Previously published reports (Wuarin and Schibler, 1990; Wuarin *et al.*, 1992; Lavery and Schibler, 1993; Lavery *et al.*, 1996) place PAR bZip transcription factors at an

intermediate hierarchical level in the signal transduction of circadian gene expression. The daily cycle of PAR gene expression in liver and other peripheral organs is likely to be the result of upstream transcriptional regulators, whereas PAR proteins themselves may regulate the circadian expression of downstream genes, such as those encoding cholesterol 7α hydroxylase, coumarin 7-hydroxylase and testosterone 15α hydroxylase (D.Lavery, L.Lopez-Molina, C.Bonfils and U.Schibler, manuscript in preparation). The work presented here opens up the possibility that DBP may also be involved in generating rhythmic signals in the SCN. Indeed, DBP mRNA exhibits a robust circadian expression in SCN neurons. Interestingly, the phase angle of circadian DBP expression is different in the SCN than it is in peripheral tissues, with maximal and minimal levels of mRNA being reached earlier during the day in the former than in the latter. This suggests that circadian DBP expression is driven by different signals in SCN neurons and in cell types of peripheral tissues. Conceivably, rhythmic DBP transcription in the SCN is regulated directly by master clock genes, while in liver and other peripheral tissues it may be controlled by systemic signals whose emission is governed by the SCN. This would explain the phase delay of circadian DBP expression in peripheral cell types as compared with SCN neurons.

Since insertional disruption of the DBP gene did not abolish circadian DBP–Neo mRNA accumulation in the SCN (Figure 7C) or in the liver (data not shown), we can conclude that DBP is not required for the rhythmic expression of its own gene. This is in contrast to the *Drosophila* clock genes *per* and *tim*, and the *Neurospora* clock gene *frequency*, which are all subject to negative feedback regulation (Dunlap, 1996). DBP may thus not be at the top in the hierarchy of clock components, but rather is situated at an intermediate level between the generation and output of circadian signals. The comparison of the expression of the mutant allele obtained by insertion of a *neo* cassette with that of the replacement allele devoid of most *dbp* sequences leads us to conclude that intragenic *cis*-acting regulatory elements are essential for circadian DBP mRNA accumulation in the SCN and the liver. The identification of the regulatory genes upstream of *dbp* in the hierarchy of circadian gene expression will be an enticing endeavor.

DBP mutant mice are altered in their circadian behavior

As shown in this paper, mice homozygous for a DBP null allele show differences in behavior in comparison with otherwise isogenic wild-type mice. First, DBP–/– mice free-run with an about a 30 min shorter period than DBP+/+ mice under constant dark conditions. Although this difference in period length is not as dramatic as that observed for Tau mutant hamsters and for clock mutant mice, it is statistically highly significant (see Table I). Second, under entrained 12 h light/dark conditions, DBP mutant mice exhibit less spontaneous and voluntary locomotor activity than wild-type animals. The difference in spontaneous locomotor activity is limited to the dark

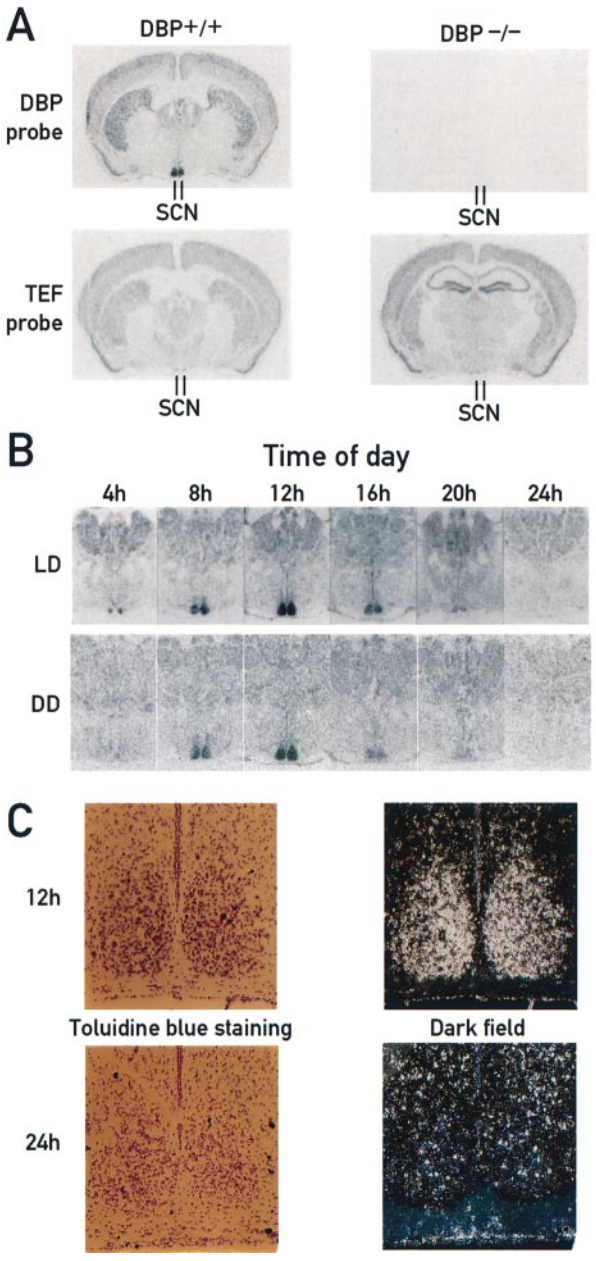

Fig. 4. DBP mRNA accumulates with a circadian rhythm in the SCN. (**A**) Specificity of the *in situ* hybridization procedure. Coronal brain sections from DBP+/+ and DBP–/– mice containing the hypothalamus were hybridized with radiolabeled DBP- or TEF-antisense RNA probes and exposed for film autoradiography. DBP mRNA signals can only be detected in the tissue section from the wild-type animal, while the TEF antisense probe yields signals for both DBP+/+ and DBP–/– mice, as expected. Note the strong DBP mRNA signal in the SCN, at the base of the hypothalamus. The right-hand but not the left-hand panel with the TEF probe contains part of the hippocampus area. This is due solely to a slightly different cutting angle and does not reflect anatomical differences between wild-type and mutant mice. (**B**) Circadian expression of DBP mRNA. Coronal brain sections containing the central part of the SCN were prepared once every 4 h from sacrificed mice (time given below photographs). Animals were kept either under a light/dark regimen (LD, lights on 7 a.m., lights off 7 p.m.) or for 2 days in constant dark (DD). The sections were hybridized to a DBP antisense RNA probe and exposed for film autoradiography, as (A). Only the hypothalamus area is shown. (**C**) Large magnification of *in situ* hybridization to the SCN during peak and minimum phases of DBP mRNA accumulation. The sections shown in (B) were covered with a photographic emulsion and exposed for 8 days before being stained with toluidin blue. Sections prepared at noon and midnight from animals kept for 2 days in constant darkness are shown.

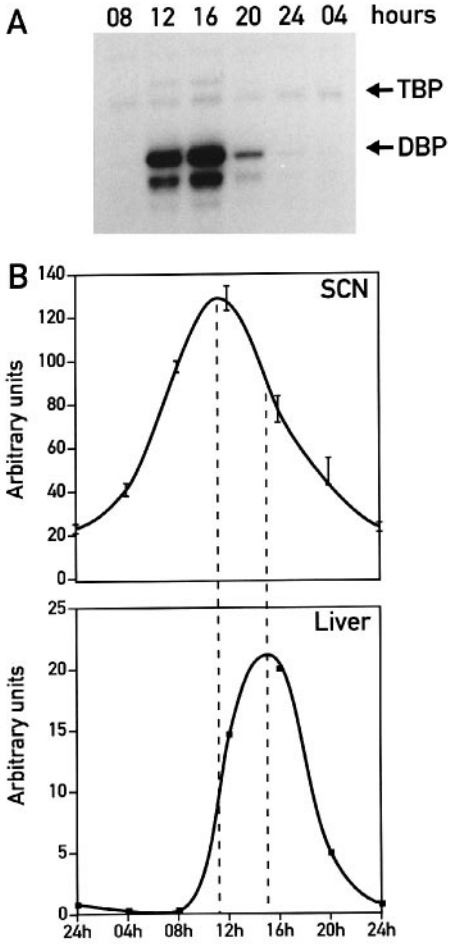

Fig. 5. Circadian expression of DBP mRNA is different in the SCN and in the liver. (**A**) DBP mRNA accumulation in liver. The accumulation of DBP mRNA in liver was determined by ribonuclease protection assays from the same individuals from which the brain sections had been prepared (Figures 4B, panel DD). A TBP antisense probe was included as a control for a mRNA with constant accumulation during the day. (**B**) Comparison of DBP mRNA accumulation in the SCN and in liver. The *in situ* hybridization signals within the SCN (Figure 3B, DD) were quantified by scanning the autoradiograms. Each value is the mean value obtained from the quantification of three serial sections from the same animal. The relative liver DBP mRNA levels from the same individuals were quantified by phosphorimaging analysis of the signals obtained in the ribonuclease protection assays (A). Note the difference in phase angle (~60°) between circadian DBP mRNA accumulation in the SCN and in liver.

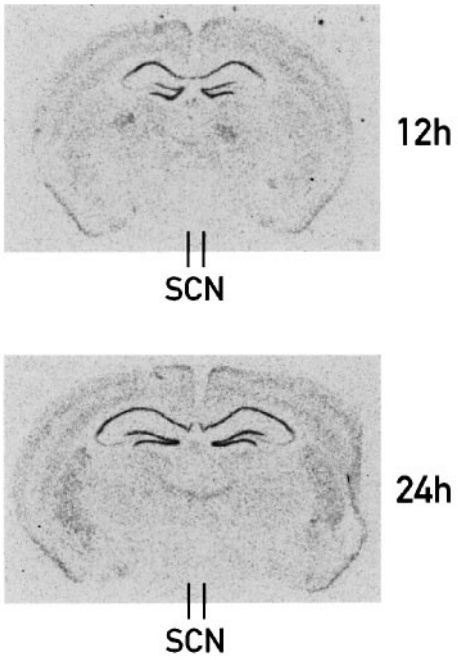

Fig. 6. DBP–LacZ fusion mRNA is not expressed in the SCN. Coronal brain sections containing the central part of the SCN were prepared at noon and midnight from animals kept under a light/dark regimen (LD, lights on 7 a.m., lights off 7 p.m.) The sections were hybridized to a *lacZ* antisense RNA probe and exposed for autoradiography. Note the prominent labeling of the hippocampus and the dentate gyrus, and the absence of signals over the SCN.

phase. As a consequence, the amplitude of spontaneous locomotor activity under entrained conditions is lower in mutant than in wild-type animals. Recently, it has been proposed that higher spontaneous locomotor activity correlates with shorter circadian period length (Sollars *et al.*, 1996). Our findings on DBP mutant mice do not lend support to this conjecture.

The difference in period length between DBP wild-type and mutant animals (0.5 h) is within the range observed for various isogenic mouse strains. However, all attempts to identify genes responsible for inter-strain period length differences by classical outbreeding have failed, probably because such alterations are the result of multiple alleles affecting circadian behavior (Schwartz and Zimmerman, 1990). A moderate lengthening of the free-running period length has been detected in PrP knockout mice (Tobler *et al.*, 1996). However, PrP expression in the SCN has not yet been reported. To our best knowledge, the work presented here is the first report on the knockout of a gene with robust circadian expression in the SCN, showing significant differences in circadian behavior.

The SCN, established as the major pacemaker for circadian locomotor activity, is the only brain region in which we detected robust circadian *dbp* expression. Moreover, the peak DBP mRNA levels are higher in the SCN than in any other examined brain structure. It is thus tempting to speculate that expression of DBP in the SCN is influencing the timing of locomotor activity. Clearly, however, our experiments do not resolve this issue, and it remains entirely possible that DBP expression in other brain areas, or even in peripheral tissues, may account for the behavioral differences observed between wild-type and DBP knockout mice.

Until recently, the feasibility of forward genetics was questionable for mammalian organisms. However, Takahashi and his colleagues (Vitaterna *et al.*, 1994) have identified a mutation in a single Mendelian locus, *clock*, in a screen of several hundred offspring from chemically mutagenized male mice (see Introduction). Recently, the clock gene product has been identified as a PAS helix–loop–helix protein (Antoch *et al.*, 1997; King *et al.*, 1997). The CLOCK mutant protein behaves as dominant-negative over its wild-type counterpart and/or other protein interaction partners. The isolation and characterization of the *clock* gene by Takahashi and his colleagues is groundbreaking, since it demonstrates that forward genetics is applicable even to organisms with very complex genomes. The obvious advantage of forward genetics over

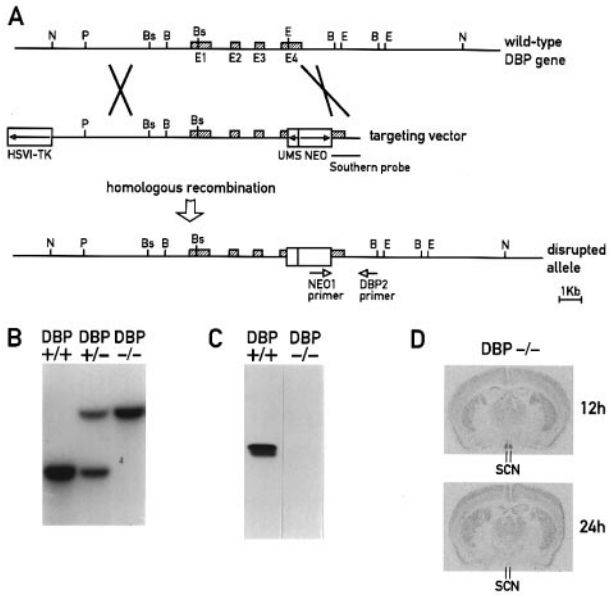

Fig. 7. DBP protein is not required for circadian DBP mRNA expression. (**A**) Strategy for generating a DBP mutant allele with a *neo* cassette insertion between the DNA sequences encoding the DNA-binding and dimerization domains. The targeting vector contains 6.5 kb of 5′-flanking region and the 4.35 kb intragenic region from the cap site to the *Eco*RV site upstream of the *neo* cassette and the intragenic region from the *Eco*RV site to the polyadenylation site, in addition to 1.14 kb 3′-flanking sequences downstream to the *neo* cassette. Abbreviations are as in Figure 1A. (**B**) Southern blot analysis of DNA from DBP+/+, DBP+/– and DBP–/– mice of an F_2 litter. The techniques were the same as those described in Figure 1A. (**C**) Western blot analysis of liver nuclear extracts from DBP+/+ and DBP–/– mice with DBP antibodies. The techniques were the same as those described in Figure 2B. (**D**) Accumulation of DBP–Neo fusion mRNA in the SCN from DBP mutant mice at noon and midnight, detected by *in situ* hybridization. Note the difference in accumulation of DBP–Neo fusion mRNA in the SCN at noon and midnight.

reverse genetics is that the most dramatic phenotypes can be selected for further analysis (Takahashi *et al.*, 1994). However, this approach also has some conceptual limitations. Thus, it can only reveal dominant, semi-dominant or sex-chromosome linked non-lethal mutations, at least in its simplest version. Dominant-negative mutations, such as the one found in the *clock* gene, may interfere with the activity of multiple genes, rendering the interpretation more difficult. Clearly, forward and reverse genetics will have to be used in concert in the genetic dissection of the mechanisms governing circadian entrainment, oscillation and output.

Materials and methods

Cloning of the mouse DBP gene

Phage clones containing the DBP locus were isolated from the 129/Sv library Lambda FixII (No. 946305, Stratagene) kindly provided by Dr Marc Ballivet. A rat DBP cDNA was used as a hybridization probe. The *Not*I inserts of the phage clones were introduced into pBluescript II KS+ (Strategene) and characterized further. For the 'insertion KO', a 11 kb *Not*I–*Eco*RV fragment (*Eco*RV in the fourth exon) as well as a 1.6 kb *Eco*RV–*Rsa*I (*Eco*RV in the fourth exon) fragment were inserted in the *Cla*I and *Not*I sites, respectively, of pTK-NEO-UMS (Bueler *et al.*, 1992), kindly provided by Dr Charles Weissmann. The vector obtained was called pTK-NEO-UMS-DBP1. For the 'replacement KO', a 1.3kb fragment of the DBP gene starting at the last three codons of DBP was first inserted in the *Not*I site of pTK-NEO-UMS. Secondly, the bacterial *lacZ* gene was inserted into the II site. This *lacZ* fragment contained a *Not*I site upstream its translation initiation codon in which a 7 kb *Not*I–*Bst*EII fragment (*Bst*EII in the DBP first exon, upstream of the DBP starting codon) was inserted. The vector obtained was called 'pTK-B.A.-LacZ-NEO-UMS-D'. For the electroporation both recombination vectors were linearized with *Sac*II.

ES cell culturing, generation of germ-line chimeras and genotyping of offspring

Linearized targeting vectors (20 μg) were electroporated (240 V, 500 μF) into 2×10^7 HM-1 ES cells (Magin *et al.*, 1992). Cells were selected and cultured as described (Conquet *et al.*, 1994) except that 0.2 μM 1-(2-deoxy,2-fluoro-beta-D-arabinofuranosyl), 5-iodouracil (FIAU, Bristol-Myers Squibb) was added to the culture medium. The clones that had undergone homologous recombination were detected by PCR with a *neo* cassette-specific oligonucleotide (5′-TCCTCGTGCTTTACGGTATC-3′) and a genomic oligonucleotide (5′-ACTCCACTGCCCCAAGACTG-3′). Recombination events were confirmed further by Southern blotting. ES cells that had undergone the correct recombination event were injected into C57Bl/6 blastocysts according to standard procedures (McMahon and Bradley, 1990). For the 'insertion KO' chimeric males were crossed with inbred C57Bl/6, thus generating non-isogenic lines. For the 'replacement KO' chimeric males were crossed with 129/Ola inbred female mice (Harlan CPB) from which the HM-1 ES cells were derived, thus generating isogenic offspring. Genotyping was performed by Southern blotting tail genomic DNA digested by *Bam*H1 (Biofinex) as described (Talbot *et al.*, 1994). Probes used were the 1.6kb *Eco*RV–*Rsa*I (*Eco*RV in the fourth exon) fragment or the *neo* cDNA. Labeling and probing were performed as described previously (Talbot *et al.*, 1994).

Analysis of liver RNA and nuclear proteins

RNA was purified from mouse livers and analyzed by ribonuclease protection assays as described previously (Schmidt and Schibler, 1995). The following RNA probes were used: TEF, a transcript complementary to rat mRNA sequences between +598 and +693 (Fonjallaz *et al.*, 1996); and TBP, a transcript complementary to mouse mRNA sequences between +36 and +135 (Schmidt and Schibler, 1995). To make a DBP RNA probe encompassing position +1215 (the location of the *neo* cassete insertion) a rat DBP cDNA *Sca*I–*Eco*RII fragment (+1139 and +1233) was subcloned into *Sma*I–pBluescript II KS+. Linearization with *Eco*RI and transcription with T7 RNA polymerase yielded a 159 nucleotide probe which protected a fragment of 94 nucleotides from ribonuclease digestion. Liver nuclei were isolated as described (Lichtsteiner and Schibler, 1989) and proteins were extracted with the NUN (NaCl–Urea–Nonidet P-40) buffer developed by Lavery and Schibler (1993). Immunochemical detection of DBP proteins in nuclear extracts was performed using purified antisera against DBP (Fonjallaz *et al.*, 1996). Approximately 25 μg of proteins in NUN extracts were mixed with 1 vol of 2× Laemmli buffer (Laemmli, 1970) and analyzed in immunoblot experiments, as described previously (Fonjallaz *et al.*, 1996). Electromobility shift assays with NUN extracts were done as described with the following high-affinity PAR recognition DNA sequence (Falvey *et al.*, 1996):

5′-GTTCTTGGTTACGTAATCTCCAATGGTTCTT-3′
3′-CAAGAACCAATGCATTAGAGGTTACCAAGAA-5′

Animal care and handling

Mice between 10 and 14 weeks of age were housed in cages, equipped with running wheels, in ventilated, light-tight cabinets. Inside the cabinet, light/dark regimes and wheel turns per unit of time were controlled and measured by a computer software (Chronobiology Kit, Stanford Software Systems). Free-running period measurements were based on a 10 day interval taken after 3 weeks of a DD regime and were executed with a χ^2 periodogram (Chronobiology Kit, Stanford Software Systems). For the spontaneous activity measurements, mice were kept in cages (without running wheels) equipped with two infrared beam emitters and detectors. Spontaneous activity was defined as the number of infrared beam breaks per mouse per unit of time as assayed by our own computer software (see Table I and Figure 3). Only isogenic animals with a complete deletion of the dbp alleles were examined for locomotor activity. The 129/OLA/C57Bl6 outbred mice containing a *neo* cassette insertion are genetically heterogeneous and thus were not used in behavioral studies.

Mouse tissues were removed within 3 min after decapitation. When expression of DBP was investigated under free-running conditions, the mice were kept in constant darkness for 48 h before being sacrificed, and dissection of tissues was performed under red light.

In situ hybridization on brain sections

Immediately after removal, brains were frozen in isopentane (1 min at −25 and −30°C) and subsequently stored at −70°C until use. Frozen brains were then cut into 14 µm sections in a Microm HM 500M cryostat. The *in situ* hybridization was performed exactly as described by Nef *et al*. (1996), using a riboprobe spanning the entire DBP cDNA. Autoradiograms were digitalized using a Nikon Coolscan Control ver. 1.2E scanner. Relative quantifications were performed directly on the scanning images using NIH Image 1.6 software. Exposure times were 16 h for film autoradiography and 8 days for emulsion autoradiography.

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