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Disruption of retinoid-related orphan receptor β changes circadian behavior, causes retinal degeneration and leads to *vacillans* phenotype in mice

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The orphan nuclear receptor RORβ is expressed in areas of the central nervous system which are involved in the processing of sensory information, including spinal cord, thalamus and sensory cerebellar cortices. Additionally, RORβ localizes to the three principal anatomical components of the mammalian timing system, the suprachiasmatic nuclei, the retina and the pineal gland. RORβ mRNA levels oscillate in retina and pineal gland with a circadian rhythm that persists in constant darkness. RORβ$^{-/-}$ mice display a duck-like gait, transient male incapability to sexually reproduce, and a severely disorganized retina that suffers from postnatal degeneration. Consequently, adult RORβ$^{-/-}$ mice are blind, yet their circadian activity rhythm is still entrained by light–dark cycles. Interestingly, under conditions of constant darkness, RORβ$^{-/-}$ mice display an extended period of free-running rhythmicity. The overall behavioral phenotype of RORβ$^{-/-}$ mice, together with the chromosomal localization of the RORβ gene, suggests a close relationship to the spontaneous mouse mutation *vacillans* described >40 years ago.

Keywords: circadian/fertility/nuclear receptor/retina/spinal cord

Introduction

Retinoid-related orphan receptor β (RORβ) is a transcription factor and belongs to the nuclear receptor family (Carlberg *et al.*, 1994). Members of this superfamily share a common modular structure composed of a transactivation domain, a DNA-binding domain and a ligand-binding domain (Evans, 1988). Typically, their transcriptional transactivation function is regulated by small lipophilic molecules, such as steroid hormones, vitamin D, retinoic acids and thyroid hormone. These molecules are synthesized in the organism and pass readily through the plasma membrane to reach the corresponding receptors inside the cell. In addition to the classical hormone receptors, a growing number of nuclear receptors for which no ligands are known have been identified by homology cloning. These nuclear receptors are referred to as 'orphan' nuclear receptors (O'Malley and Conneely, 1992; Laudet and Adelmant, 1995). RORβ is such an orphan nuclear receptor, forming a subfamily with the closely related nuclear receptors RORα (Becker-André *et al.*, 1993; Giguère *et al.*, 1994) and RORγ (Hirose *et al.*, 1994). Species homologs have been identified in the fly *Drosophila melanogaster* (DHR3; Koelle *et al.*, 1992), in the silk worm *Manduca sexta* (MHR3; Palli *et al.* 1992), in the nematode *Caenorhabditis elegans* (CHR3; Kostrouch *et al.*, 1995) and in the lobster *Homarus americanus* (HHR3; El Haj *et al.*, 1997).

RORβ originally was cloned from rat brain RNA (Carlberg *et al*., 1994). In rat, the expression of RORβ is restricted to the central nervous system (CNS), specifically localizing to areas that are involved in the processing of sensory information (Schaeren-Wiemers *et al.*, 1997). RORβ's distribution pattern matches the chain of hierarchically organized elements represented by the anatomical relay system of the afferent sensory pathways, including receptor organs, receptive areas in spinal cord, nuclei in the mesencephalon and the brainstem, thalamic nuclei and distinct cortical target areas. In particular, in the cerebral cortex, RORβ mRNA is expressed exclusively in non-pyramidal neurons of layer IV and V, with the highest levels in primary sensory cortices. In the thalamus, RORβ is expressed in the sensory relay nuclei that project to the respective cortical areas. In the spinal cord, RORβ expression localizes to layers of the dorsal horn that receive sensory input from the periphery. In contrast, sensory projection neurons in the periphery such as retinal ganglion cells and neurons of the sensory ganglia show only little RORβ expression. RORβ is absent from striatum, hippocampus, cerebellum, the motor nuclei of the cranial nerves and the ventral part of the spinal cord. Additionally, RORβ mRNA-specific hybridization signals are localized to anatomical substrates of the biological clock, the retina, the suprachiasmatic nuclei (SCN) located in the hypothalamus, and the pineal gland, the principle site of melatonin production. The overall distribution pattern of RORβ suggests that this nuclear orphan receptor regulates genes whose products play a role in the context of sensory input integration as well as in the context of the circadian timing system.

The objective of this study was to investigate the functional role of RORβ in the context of processing sensory information and circadian rhythm. To understand better the contribution of RORβ to genomic processes that control circadian rhythms, we expanded our study on

the RORβ mRNA distribution in the rat CNS and established the expression pattern of RORβ in pineal gland, retina and SCN as a function of different environmental light conditions. Here we show that in the pineal gland and in parts of the retina, but not in the SCN, RORβ mRNA levels oscillate with true circadian rhythmicity, peaking at night-time. To evaluate its overall physiological role, we generated RORβ-deficient mice by gene targeting in embryonic stem (ES) cells and analyzed their phenotypic behavior. We show that RORβ mildly influences circadian rhythmicity: its absence extends the period length Tau of the free-running activity rhythm. More importantly, however, the absence of RORβ causes retinal degeneration and leads to a behavioral phenotype which closely resembles that of the natural mouse mutation *vacillans*, including a specific ataxic behavior and the initial inability of male animals to reproduce sexually.

Results

RORβ expression levels oscillate in the rat pineal gland and retina with circadian rhythm

In order to address the question of whether RORβ is a molecular part of the circadian timing system, we investigated its mRNA level in various rat brain areas as a function of circadian time using *in situ* hybridization. Comparing day and night patterns of rats that were kept under light–dark (LD) conditions, we observed a robust nocturnal increase in the amount of RORβ mRNA in pineal gland tissue and in the basal part of the retinal photoreceptor layer (Figure 1A and B 'adult'). In contrast, the level of RORβ mRNA in the pacemaker of the biological clock, the hypothalamic SCN, as well as in the cortex, the various thalamic and hypothalamic nuclei and other places (Schaeren-Wiemers *et al.*, 1997) was found to be unchanged (not shown). Since the primordial criterion for a circadian element is its free-running behavior in the absence of light entrainment, we repeated these experiments using rats that were kept under constant darkness (DD) conditions. The circadian times were determined by monitoring locomotor activity. Again, the pineal gland contains high amounts of RORβ mRNA at subjective midnight and low amounts at subjective midday, as does the retina, displaying a situation identical to that observed under light entrainment conditions (data not shown). Thus, the oscillation of RORβ expression is truly circadian.

This was substantiated further by establishing a time course of the temporal change of pineal RORβ expression in rats kept in LD or DD conditions (with a documented free-running locomotor activity). RORβ mRNA levels were quantified using a competitive PCR protocol (Becker-André, 1995). Under LD conditions, the RORβ mRNA level is very low during the light phase (Figure 1C). About 2 h after the onset of the dark period (CT14), the level rises to reach its maximal value at the beginning of the second half of the night (CT18). At around CT21, the signal starts to collapse rapidly, with a half-life of ~2 h, to reach its minimal value in the early part of the light period (CT2). Similarly, under DD conditions, the profile's minimum is at CT6 and its maximum at CT22. In contrast, the RORβ transcript level starts to rise already in the course of the subjective day, although with a much flatter gradient than under LD conditions.

Circadian rhythmicity of behavioral and biochemical processes can be suspended for a couple of days by a phase advance shift such that melatonin production ceases until the new entrainment cycle is adapted. We achieved this by reducing the light period of a given day to 4 h and starting a new LD 12:12 regimen 12 h later. Rats were kept for 2 days after this phase shift and the arrhythmicity of their circadian cycle was documented by monitoring their locomotor activity. The data in Figure 1C ('PS') demonstrate that the oscillation of pineal RORβ expression levels disappears as a consequence of the phase advance-induced collapse of circadian rhythmic behavior. The nocturnal up-regulation of pineal RORβ gene expression is suppressed until the new circadian rhythm is installed. A similar situation was observed in the retina, as monitored using *in situ* hybridization (not shown).

RORβ is already expressed during embryogenesis (Schaeren-Wiemers *et al.*, 1997). In order to address the question of at what developmental stage circadian RORβ expression rhythms are established, we analyzed RORβ mRNA levels in pineal gland and retina of rats at various postnatal days. Significant circadian fluctuation of pineal RORβ mRNA levels starts during the first postnatal week (Figure 1A), coinciding with the completion of the sympathetic innervation and the onset of melatonin synthesis (Klein *et al.*, 1981), two hallmarks of the developmental maturation of the pineal gland. The retinal RORβ expression apparently is not subject to circadian changes until the end of the second postnatal week (Figure 1B), when the retina has matured and the eyes open, coinciding with the functional completion of the retino-hypothalamic tract (RHT) which forms the anatomical basis for the visual entrainment of the SCN. Although we observed circadian changes in the inner cellular layer at adult stages (Figure 1B), these changes were contradictory in a different rat strain (PVG/Lister hooded) and, therefore, were not considered of further interest for this study.

In conclusion, two major components of the circadian timing system display circadian changes in RORβ mRNA levels that persist under free-running conditions: the retina as the major part of the visual entrainment pathway; and the pineal gland as an important target organ and effector of the output pathway. In contrast, the central clock SCN is not affected by RORβ expression levels.

Generation of transgenic mice carrying RORβ null alleles

To investigate the functional role of RORβ, we generated RORβ-deficient ($ROR\beta^{-/-}$) mice by gene targeting in ES cells. We have used a targeting vector (Le Mouellic *et al.*, 1990) in which the β-galactosidase gene replaced the second zinc finger of the DNA-binding domain of RORβ. After homologous recombination, the targeted gene encoded a fusion protein composed of RORβ's N-terminal domain and first zinc finger followed by β-galactosidase (Figure 2A). Adult heterozygous RORβ mutant ($ROR\beta^{+/-}$) mice were examined for the distribution of β-galactosidase activity indicative of RORβ protein expression in various tissues. As expected, enzyme activity was found in retina, pineal gland, SCN, spinal cord (Figure 4C, G, L, P and T) and other brain areas (not shown), matching the recently

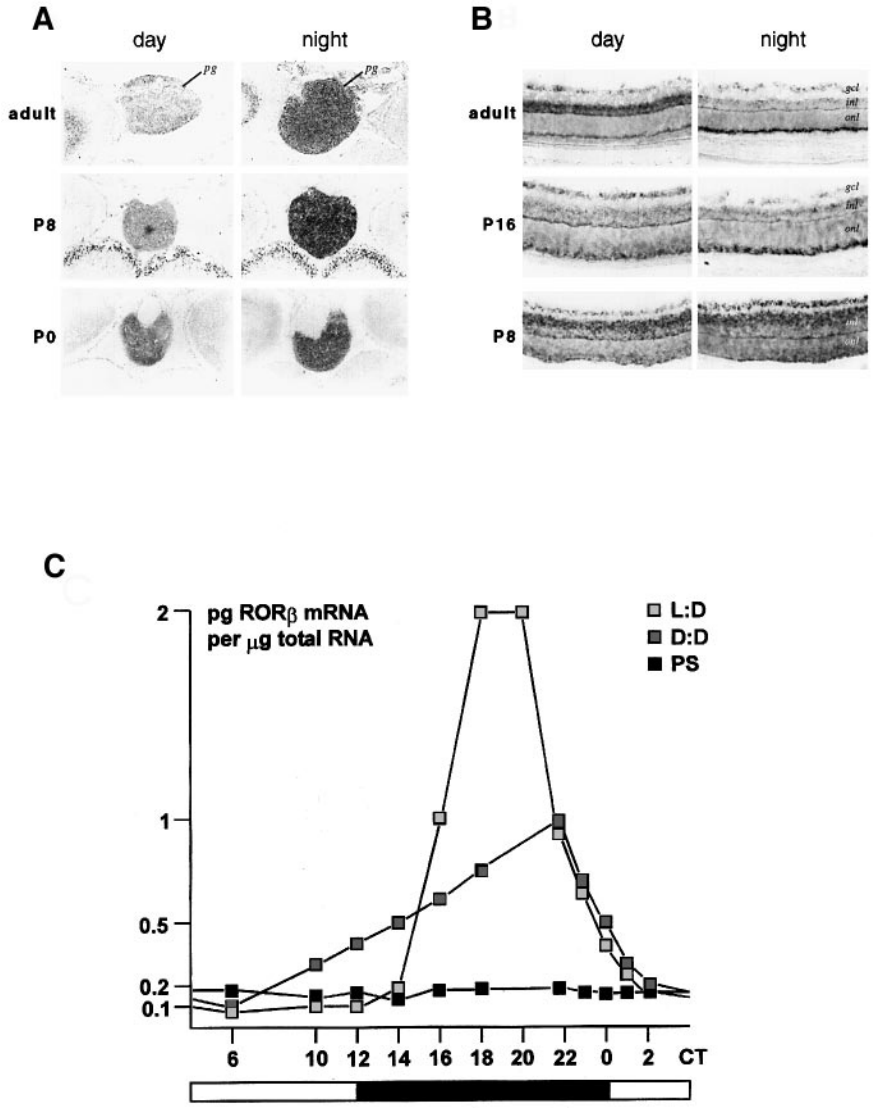

Fig. 1. Circadian rhythm of RORβ expression in pineal gland and retina. RORβ distribution in rat pineal gland and retina in the daytime and nighttime was analyzed by *in situ* hybridization. (**A**) Coronal sections of brains dissected from adult, newborn (P0) and 8-day-old (P8) rats at the level of the pineal gland. Note the increase in signal intensity in the pineal gland (*pg*) shifting from day to night. (**B**) Cross-sections of retina dissected from adult, 8-day-old (P8) and 16-day-old (P16) rats. RORβ-specific hybridization occurs in the ganglion cell layer (*gcl*), the inner nuclear layer (*inl*) and the photoreceptor layer (or outer nuclear layer, *onl*). Note the massive staining localizing to the basal part of the photoreceptor layer in nocturnal retina. (**C**) Time course of RORβ mRNA levels in rat pineal gland. Animals were kept under illumination conditions mimicking natural day–night switches with equal lengths of both phases (L:D) or under constant darkness conditions (D:D), or were subjected to a phase advance shift (PS). The free-running locomotor activity of animals kept under DD conditions was recorded to determine their subjective day and night phases; CT12 equals onset of activity. At the indicated times, the animals were sacrificed, the pineal glands were dissected and RNA was extracted. Levels of RORβ mRNA were determined by competitive RT–PCR and normalized to total RNA.

published *in situ* hybridization data (Schaeren-Wiemers *et al*., 1997).

Phenotypic examination of RORβ$^{-/-}$ mice

Cross-breeding of heterozygous RORβ$^{+/-}$ mice (F2 and F3 generations) yielded homozygous RORβ$^{-/-}$ animals at the expected frequency of 25% (Figure 2B). Reverse transcription–PCR experiments were performed to verify the absence of functional RORβ transcripts in RORβ$^{-/-}$ mice. As expected, transcripts from the null mutant gene allele contain the N-terminal RORβ sequence preceding the disruption site (Figure 2C, lanes 1 and 2) but they lack all of the sequence information downstream of the disruption site, including the entire ligand-binding domain of RORβ (Figure 2C, cf. lanes 3 and 4). Young RORβ$^{-/-}$ mice are undersized as compared with wild-type siblings and initially display reduced muscular strength. When they begin to walk, they sometimes fall sideways and roll over. With increasing age, they gain normal muscular strength and acquire the two most conspicuous phenotypic traits which adult RORβ$^{-/-}$ mice display: a 'duck-like' gait and a hind paw clasping reflex when suspended by the tail (Figure 3). By comparison with RORβ$^{+/+}$ littermates, RORβ$^{-/-}$ mice have normal grasping, limb flexion and righting reflexes, respond to auditory stimulation, show normal rearing, balancing and climbing responses, and display normal temperature-related pain reflexes. RORβ$^{-/-}$ mice have a normal posture and appear to be alert, although they are more docile than their wild-type littermates. Placed in a new environment, RORβ$^{-/-}$

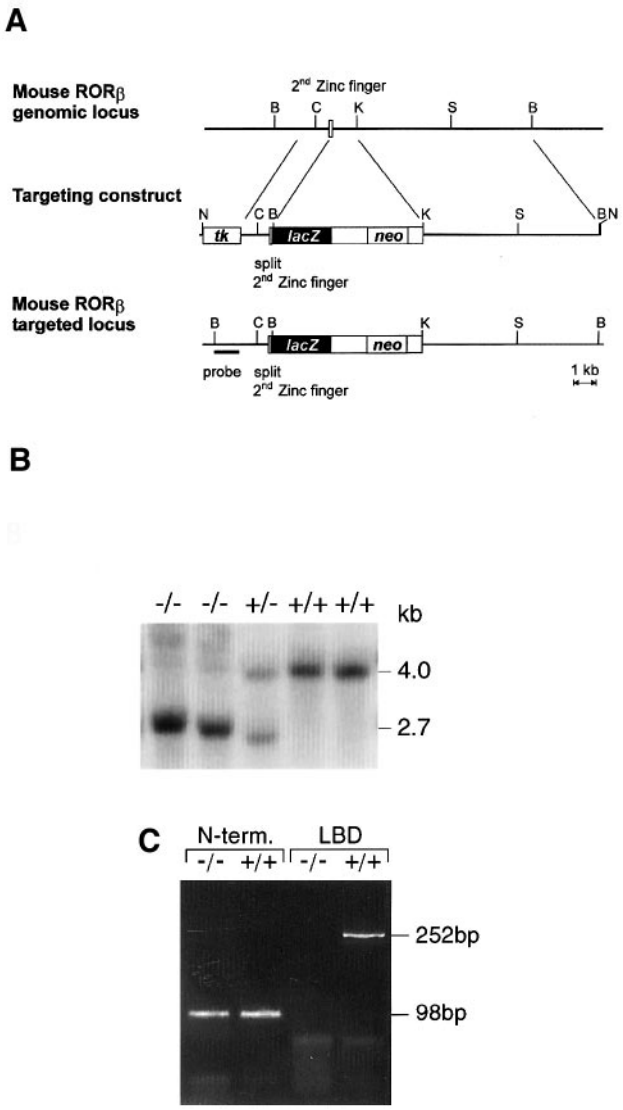

Fig. 2. Targeting of the RORβ locus. (**A**) Scheme depicting the restriction map of the murine RORβ locus at the second zinc finger domain, the targeting construct and the resulting targeted RORβ locus after homologous recombination. B, *Bam*HI; C, *Cla*I; K, *Kpn*I; N, *Not*I, S, *Sma*I; *tk*, thymidine kinase gene; *neo*, neomycin resistance gene; *lacZ*, β-galactosidase gene. (**B**) Southern hybridization analysis of genomic DNA purified from tail biopsies of offspring. The DNA was digested with *Bam*HI and *Kpn*I and hybridized with the DNA probe indicated in (A). A 2.7 kb band was indicative of the targeted allele, a 4.0 kb band was indicative of the wild-type allele. (**C**) RT–PCR analysis of RNA extracted from the retina of wild-type RORβ$^{+/+}$ and mutant RORβ$^{-/-}$ mice. PCR products specific for the N-terminal part of the RORβ transcript preceding the disruption site were amplified from both genotypes. In contrast, PCR products derived from the ligand-binding domain (LBD) of the RORβ transcript downstream of the disruption site were only obtained from wild-type RNA.

mice initially are reluctant to explore, i.e. they do not move ahead but display erratic movements, for ~1 min.

RORβ$^{-/-}$ mice show no gross anatomical changes in the brain and display a *lacZ* expression pattern very similar to that of the heterozygous animals, although generally a stronger staining was observed possibly due to the presence of two *lacZ* alleles per genome (compare Figure 4M, Q and U with L, P and T). In particular, the pineal gland, the SCN, the cortex and all other brain areas in which RORβ is expressed appear normally developed. Only in the newborn spinal cord did we find significant differences between RORβ$^{+/-}$ and RORβ$^{-/-}$ mice: while in RORβ$^{+/-}$ mice the staining is confined to the second and third layer of the dorsal horn, matching the *in situ* hybridization pattern (Schaeren-Wiemers *et al*., 1997), in RORβ$^{-/-}$ mice this staining is dispersed throughout the dorsal horn (Figure 4T and U). The gross anatomical structure of adult RORβ$^{-/-}$ spinal cord, however, appears normal when compared with wild-type littermates (not shown).

Histological examination of adult RORβ$^{-/-}$ eyes, in contrast, revealed a severely malformed retina (compare Figure 4B and D with A and C). The tissue appears disorganized, lacking the layer structure typical of normal retina. The retina also seems to be collapsed, being composed of significantly fewer cells. Interestingly, until shortly after birth, the developing retina of RORβ$^{-/-}$ mice is not very different from that of RORβ$^{+/-}$ mice with respect to cell number (Figure 4E–H), yet the first abnormalities could be seen at the level of the presumptive inner nuclear layer (arrows in Figure 4E and F). RORβ$^{-/-}$ retina, therefore, is likely to suffer from impaired cellular differentiation and degenerative cell loss during the first weeks after birth. Functional tests of the visual capabilities of RORβ$^{-/-}$ mice confirmed our histological observations. Electroretinograms (ERGs) and intracortical visual evoked potentials (VEPs) showed that little, if any, visual activity is measurable in the retino-cortical pathway of adult RORβ$^{-/-}$ mice (Figure 5). Their retinal and cortical responses to strong light flashes are close to background activity, in agreement with the anatomical finding of grossly abnormal retinas.

Studies on circadian photoreception in mice with genetically abnormal retinas [e.g. *rd/rd* mice (Argamaso *et al.*, 1995)] have led to the hypothesis that there are specialized non-visual photoreceptors within the mammalian retina which detect LD cycles and provide circadian entrainment (Foster *et al.*, 1991; Czeisler *et al.*, 1995). Despite grossly abnormal retinas, the mammalian visual subsystem that mediates light-induced circadian responses remains functionally intact. Therefore, we were not surprised that the locomotor activity and temperature rhythms of RORβ$^{-/-}$ mice were entrained by LD conditions (Figure 6). However, when the mice were housed in constant darkness for up to 8 weeks, we observed robust free-running circadian rhythms of both locomotion and body temperature, with a Tau period 0.4 h longer (τ = 24.2 h) than that of their heterozygous and wild-type litter mates (τ = 23.8 h) or of the corresponding strains used to breed these animals, C57Bl/6 (τ = 23.7 h) and 129/Sv (τ = 23.8 h; Lopez-Molina *et al*., 1997).

RORβ$^{-/-}$ mice are generally fertile, with one puzzling exception: male animals consistently fail to sexually reproduce during the first 6 months of their life; thereafter, they assume normal reproductive behavior until the onset of senescence. Given this male-specific behavior, we looked for potential abnormalities in the genital organs of young RORβ$^{-/-}$ mice versus older RORβ$^{-/-}$ and heterozygous mice. Histological examination revealed strong *lac*Z gene activity in the epithelial cells lining the lumen of the epididymis and vas deferens, but not of the testis and prostate, of both RORβ$^{+/-}$ and RORβ$^{-/-}$ mice (Figure 7). Intriguingly, no differences between young

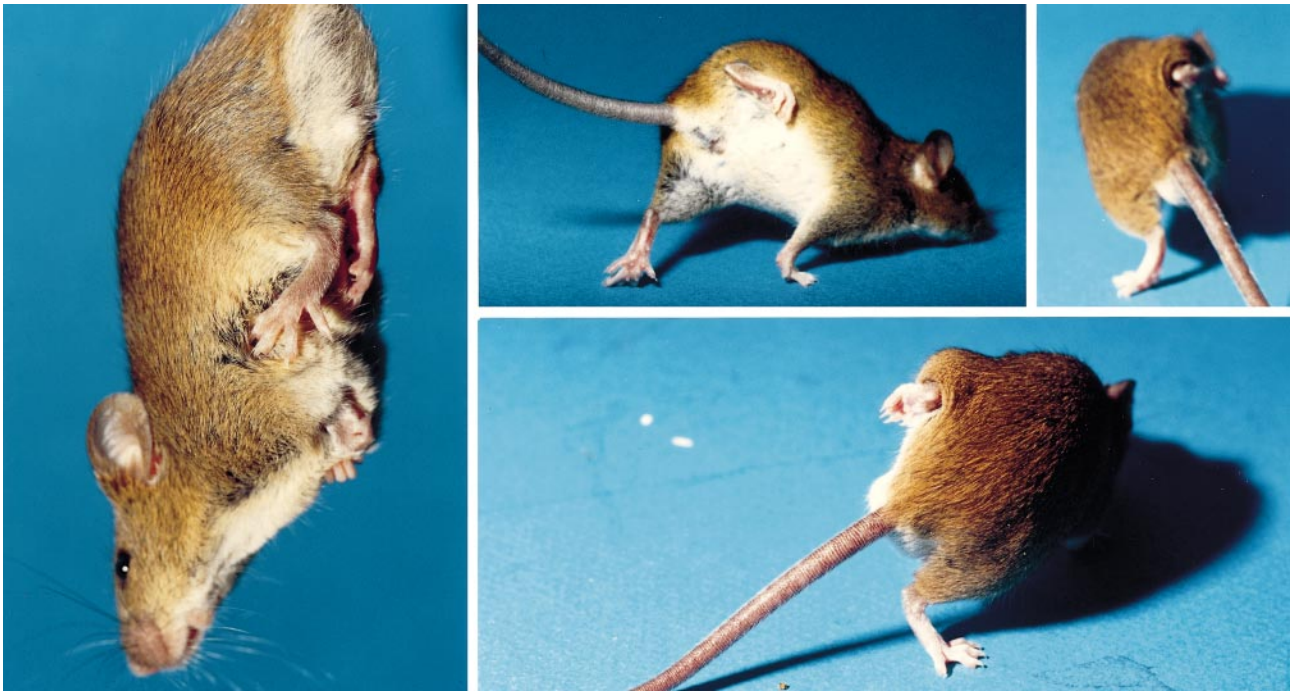

Fig. 3. Phenotypic appearance of a RORβ$^{-/-}$ mouse when suspended at the tail and when walking, respectively.

and old homozygous and heterozygous male animals were apparent in the distribution pattern of RORβ-specific staining, the anatomical presentation of the tissue and the sperm load of the conducting vessels.

Chromosomal localization of the RORβ gene

We determined the chromosomal localization of the human RORβ gene as part of the basic characterization of this gene, contributing to the knowledge about the human genome and providing data which might be of potential therapeutic interest. Based on PCR experiments using a chromosomal human–hamster cell hybrid panel (Bios Laboratories, CT), we localized the RORβ gene to human chromosome 9 (data not shown). To determine its subchromosomal position, we performed fluorescence *in situ* hybridization experiments on human metaphase chromosomes using a λ phage clone derived from the human RORβ gene and a chromosome 9-specific gene probe as marker (see Materials and methods). Our results suggest that human RORβ maps to chromosome 9q22 (Figure 8), a region which is syntenic with mouse chromosome 4.

Discussion

The role of RORβ in the circadian timing system

The mammalian photoneuroendocrine system converts changes of ambient light conditions into rhythmic endocrine signals. The principle anatomical components of this system include the retina, the SCN and the pineal gland (Klein *et al.*, 1991; Moore, 1995; Takahashi, 1995). We have shown previously that the orphan nuclear receptor RORβ is expressed in these parts of the photoneuroendocrine system (Schaeren-Wiemers *et al.*, 1997).

Here we show that the RORβ mRNA levels exhibit striking circadian changes in the pineal gland and in retina during the day–night cycle that persist in constant darkness. Furthermore, we show that the nocturnal increase in RORβ mRNA levels can be abolished by a change in the light entrainment condition (phase advance shift), dissociating the coordination of the clock output (Meijer, 1991) and disrupting for several days the behavioral and biochemical circadian rhythms (Tamarkin *et al.*, 1979; Perlow *et al.*, 1980). It is, therefore, very likely that the rhythmic expression of RORβ is driven by the endogenous pacemaker residing in the SCN. Our results parallel previously reported findings (Baler *et al.*, 1996) showing that the circadian RORβ mRNA expression in pineal gland is under photoneural regulation involving β-adrenergic and cAMP-dependent mechanisms. This is reminiscent of studies reported on the inducible cAMP early repressor (ICER; Molina *et al.*, 1993), an isoform of cAMP-responsive element-binding modulators (CREM; Stehle *et al.*, 1993). Similarly to RORβ, ICER transcript levels in pineal gland are regulated through β-adrenergic stimulation of the cAMP signaling pathway, cycle with circadian rhythm under the control of the SCN and are down-regulated rapidly by light pulses during the night. In contrast, ICER and the other CREM isoforms are only expressed weakly in retina, and their expression levels do not cycle in this tissue (Stehle *et al.*, 1995). Thus, while the circadian expression pattern of ICER is restricted to the pineal tissue, the circadian expression oscillation of RORβ extends to both principal SCN-distal parts of the biological clock, the pineal and the retina.

In the present study, we have shown that mice lacking RORβ show a significant difference in their circadian behavior: under constant darkness conditions, they free-run with a Tau period ~0.4 h longer than wild-type littermate controls, RORβ$^{+/-}$ or C57Bl/6 mice. Several mutations have been described so far that change the free-running circadian period in mammals. Some of these mutations shorten the circadian period: *tau* mutation

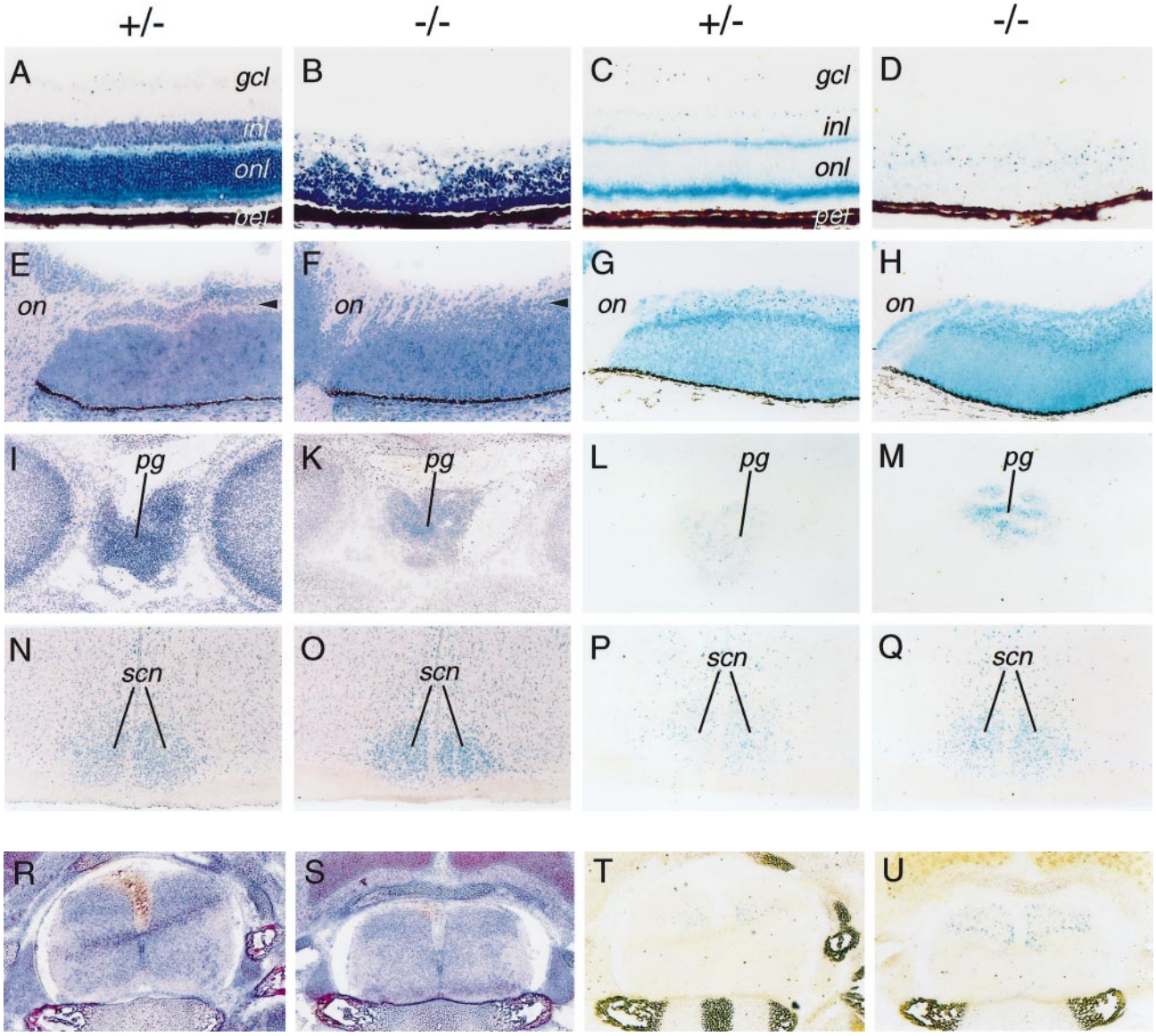

Fig. 4. Distribution of β-galactosidase activity in various tissues of RORβ$^{+/-}$ and RORβ$^{-/-}$ mice. Adult retina (**A**–**D**), postnatal (P3) retina (**E**–**H**), postnatal (P3) pineal gland (**I**–**M**), adult SCN (**N**–**Q**) and postnatal (P3) spinal cord (**R**–**U**) of RORβ$^{+/-}$ and RORβ$^{-/-}$ mice. Tissue sections were either stained with the β-galactosidase substrate X-gal alone (C, D, G, H, L, M, P, Q, T and U), additionally counter-stained with hematoxylin and eosin (A, B, I, K, N and O) or stained with hematoxylin and eosin alone (E, F, R and S). The arrowheads in (E) and (F) point to differences at the level of the presumptive inner nuclear layer of developing RORβ$^{+/-}$ and RORβ$^{-/-}$ retinas. Abbreviations: *on*, optic nerve; *pg*, pineal gland; *scn*, suprachiasmatic nucleus.

in hamster (Ralph and Menaker, 1988); mutant, non-polysialylated neural cell adhesion molecule (NCAM) (Shen *et al.*, 1997); and null mutation of the transcription factor DBP gene (Lopez-Molina *et al.*, 1997). Other mutations extend the circadian period: prion protein PrP gene null mutation (Tobler *et al.*, 1996); RORβ gene null mutation (this study); and mutant CLOCK gene (*Clock*; Vitaterna *et al.*, 1994). *Clock* is the only mutation known in mammalian organism which affects the two fundamental properties of circadian systems: the period length and the persistence of rhythmicity. The corresponding gene recently has been identified by positional cloning and transgenic rescue experiments (Antoch *et al.*, 1997; King *et al.*, 1997) and encodes a novel member of the basic helix–loop–helix-PAS family of transcription factors. Both, CLOCK and RORβ are expressed in the SCN and the retina, but there are, to our knowledge, no reports of CLOCK expression in the pineal gland or of a circadian expression pattern of CLOCK transcript levels in these organs.

Given the spatio-temporal expression of RORβ and the changed circadian behavior of RORβ$^{-/-}$ mice, RORβ might be involved in the transcriptional regulation of effectors of the circadian clock. In particular, RORβ could participate in the regulation of melatonin synthesis, similarly to ICER (Foulkes *et al.*, 1997), for two reasons: (i) the rhythmic expression of RORβ is confined to the two principal melatonin-producing tissues within the CNS, the pineal gland (Axelrod, 1974) and the photoreceptors (Besharse and Iuvone, 1983; Blazynski and Dubocovich, 1991; Tosini and Menaker, 1996), and parallels that of melatonin biosynthesis on a circadian basis; and (ii) the

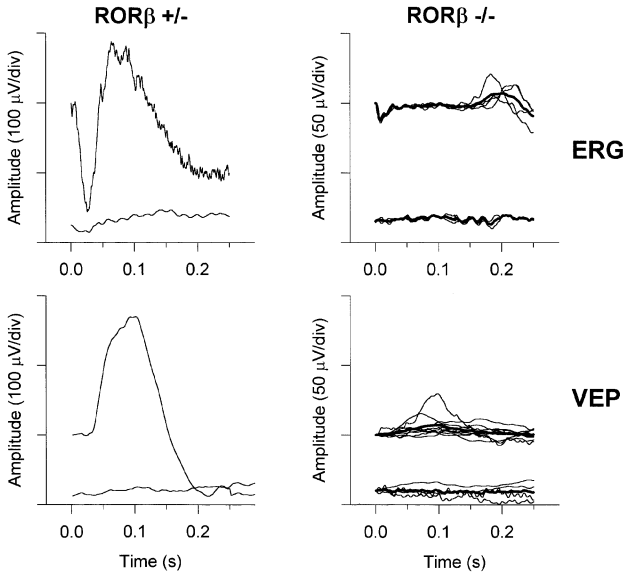

Fig. 5. Electroretinograms (ERGs) and cortical visual evoked potentials (VEPs) recorded in RORβ$^{+/-}$ and RORβ$^{-/-}$ mice in response to strong light flashes. Thin lines in the RORβ$^{-/-}$ recordings represent individual responses; the thick lines represent the overall average. The lower records in each panel represent the 'noise' responses to occluded stimuli of the same intensity. Note the different scales in the records from the homozygous and the heterozygous animals.

onset of rhythmic expression of RORβ in retina and pineal gland coincides with the start of melatonin production in these tissues. It would be very interesting, therefore, to measure melatonin levels in RORβ$^{-/-}$ mice in order to clarify RORβ's role in this context. Unfortunately, most of the laboratory mouse strains, including strain 129/Sv used for the present gene disruption study, do not produce measurable amounts of melatonin. Amongst the rare exceptions is strain C3H (Goto *et al.*, 1989). Backcrossing the *ror*β$^{-/-}$ allele into the genetic background of the C3H strain should, therefore, allow analysis of the potential deviations in pineal function at the level of melatonin synthesis in the absence of RORβ.

The phenotypic syndrome of RORβ$^{-/-}$ mice suggests a relationship to vacillans

RORβ-deficient mice display a syndrome of abnormalities that comprise juvenile ataxia and adult gait peculiarities, circadian activity deviations, retinal malformation and degeneration, and developmentally delayed onset of male fertility. Overall, this phenotype is conspicuously similar to that described >40 years ago for a spontaneous mouse mutation for which the name *vacillans* was proposed (Sirlin, 1956), now believed to be extinct (Doolittle *et al.*, 1996). The two phenotypes are summarized and compared in Table I. There are two discrepancies, however:

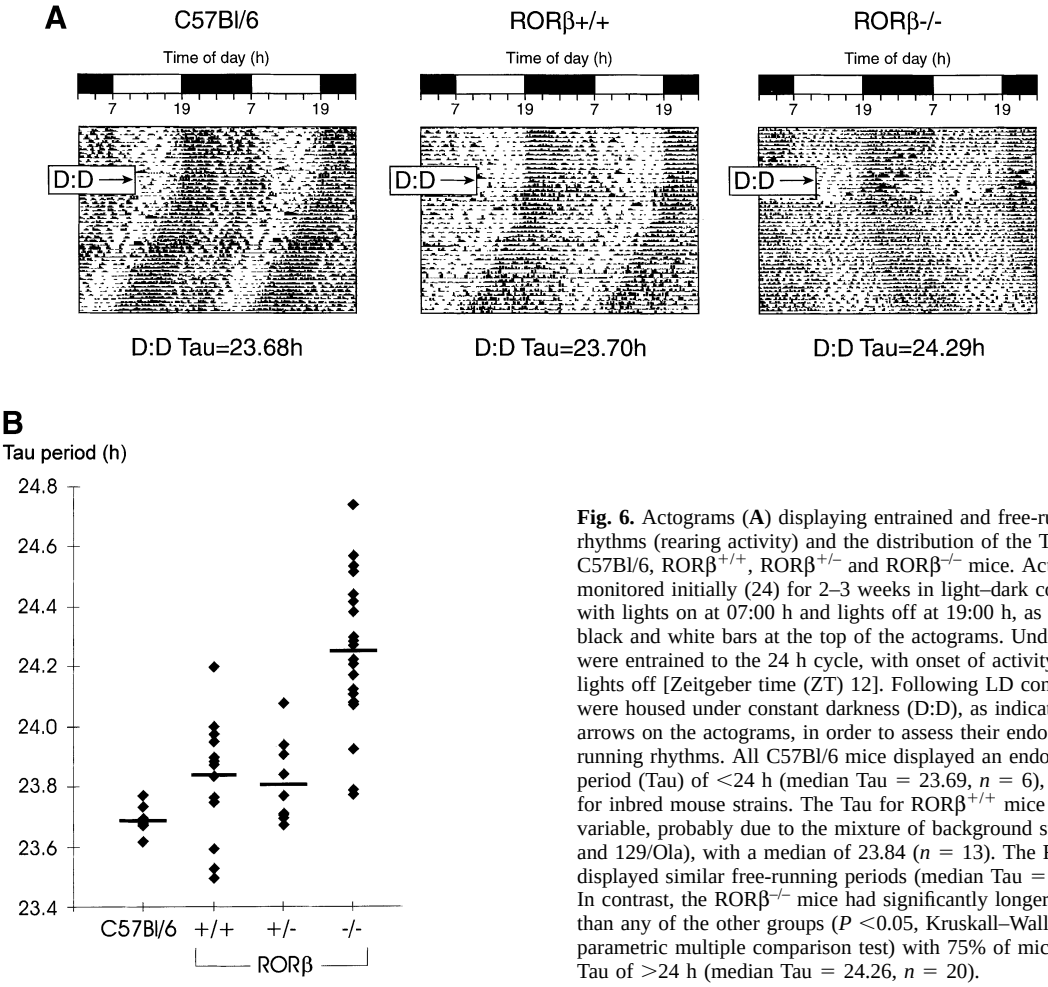

Fig. 6. Actograms (**A**) displaying entrained and free-running activity rhythms (rearing activity) and the distribution of the Tau period (**B**) of C57Bl/6, RORβ$^{+/+}$, RORβ$^{+/-}$ and RORβ$^{-/-}$ mice. Activity was monitored initially (24) for 2–3 weeks in light–dark conditions (LD) with lights on at 07:00 h and lights off at 19:00 h, as indicated by the black and white bars at the top of the actograms. Under LD, all mice were entrained to the 24 h cycle, with onset of activity occurring at lights off [Zeitgeber time (ZT) 12]. Following LD conditions, mice were housed under constant darkness (D:D), as indicated by the arrows on the actograms, in order to assess their endogenous free-running rhythms. All C57Bl/6 mice displayed an endogenous rhythm period (Tau) of <24 h (median Tau = 23.69, n = 6), which is typical for inbred mouse strains. The Tau for RORβ$^{+/+}$ mice was more variable, probably due to the mixture of background strains (C57Bl/6 and 129/Ola), with a median of 23.84 (n = 13). The RORβ$^{+/-}$ mice displayed similar free-running periods (median Tau = 23.81, n = 9). In contrast, the RORβ$^{-/-}$ mice had significantly longer Tau periods than any of the other groups (P <0.05, Kruskall–Wallis non-parametric multiple comparison test) with 75% of mice displaying a Tau of >24 h (median Tau = 24.26, n = 20).

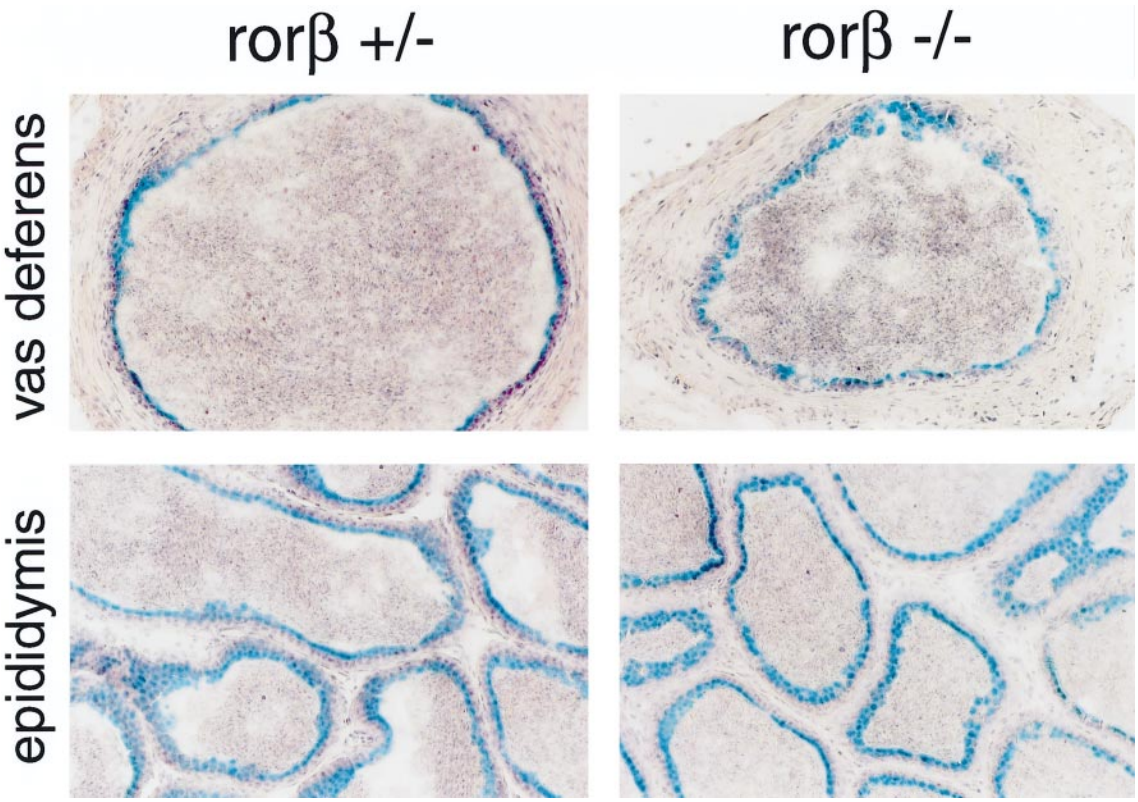

Fig. 7. Expression of RORβ in epididymis and vas deferens as shown by β-galactosidase activity staining in tissue cryosections. Note the strong staining in the epithelial cell lining, in both RORβ$^{+/-}$ and RORβ$^{-/-}$ tissue.

(i) deviations in circadian behavior were not described for *vacillans*, probably because they were not tested; and (ii) retinal abnormalities in *vacillans* were not reported. Vision is not an important sensory quality in rodents' strategy to perceive their environment. There are a number of natural mutant mouse strains which suffer from retinal degeneration (e.g. rd/rd, C3H; Bowes *et al.*, 1990) which show no conspicuous behavior as to orientation and locomotion in a well-known environment. It is conceivable, therefore, that blindness passed unnoticed when the phenotype of *vacillans* mice was studied, although it was noted that 'on coming near the edge of a table *vacillans* are apt to fall off it' (Sirlin, 1956), which is also a typical behavior of RORβ$^{-/-}$ mice.

Notwithstanding this, another line of evidence speaks in favor of the assumption that *vacillans* and *ror*β$^{-/-}$ are mutual phenocopies. *Vacillans* was shown to be genetically linked with *brown* (Sirlin, 1956). Recently, *brown* was mapped to mouse chromosome 4 in a region which is syntenic to human chromosome 9p23 (Abbott *et al.*, 1991; Bell *et al.*, 1995). We have mapped the RORβ locus to human chromosome 9q22 which is within the only other region of the human chromosome 9 (9q22–33) syntenic to mouse chromosome 4. It is tempting, therefore, to conclude that the respective mutations in *vacillans* and RORβ$^{-/-}$ mice are genetically related. However, since *vacillans* mice are apparently no longer available, this hypothesis cannot be proven. It is conceivable that the *vacillans* mutation affected another gene, which could nevertheless cross-talk or even functionally interact with RORβ, leading to a phenotype very similar to that of RORβ$^{-/-}$ mice.

RORβ plays a role in development

At present, we cannot explain mechanistically the phenotypic abnormalities of RORβ$^{-/-}$ mice, and a number of questions remain to be answered. Why do young RORβ$^{-/-}$ mice suffer from ataxic movement and display the typical duck-like gait later in adulthood? We think this could reflect a reduced fine-tuning of motor activities as a result of impaired integration of sensory input information. A potential contribution by abnormal development of the dorsal horn of the spinal cord is conceivable: RORβ-positive neurons localize normally to lamina III of the spinal cord, relaying sensory input from the periphery; dissipation of these neurons to all dorsal layers of RORβ$^{-/-}$ spinal cord could reflect changes in the wiring architecture of afferent input pathways resulting in the overlapping of sensory and other, e.g. nociceptive, modalities. Sensory perception (touch, pressure) at the level of the foot pad elicited at each step the RORβ$^{-/-}$ mouse takes consequently could provoke a withdrawal reflex as if it was elicited by a noxious stimulus (pain). Interestingly, DHR3, the homolog of the RORs in *Drosophila*, is required to complete embryogenesis, and mutant DHR3 can cause defects in the pattern formation of the peripheral nervous system (Carney *et al.*, 1997), suggesting common principles of physiological action of this evolutionarily old nuclear receptor in both insects and mammals.

Why does the retina degenerate in RORβ$^{-/-}$ mice? This finding is reminiscent of RORα$^{-/-}$ and *staggerer* mice (Hamilton *et al.*, 1996; Steinmayr *et al.*, 1998) in which cerebellar Purkinje cells do not terminally differentiate and consequently degenerate, leading to a massive overall cerebellar malformation and atrophy. Detailed histological

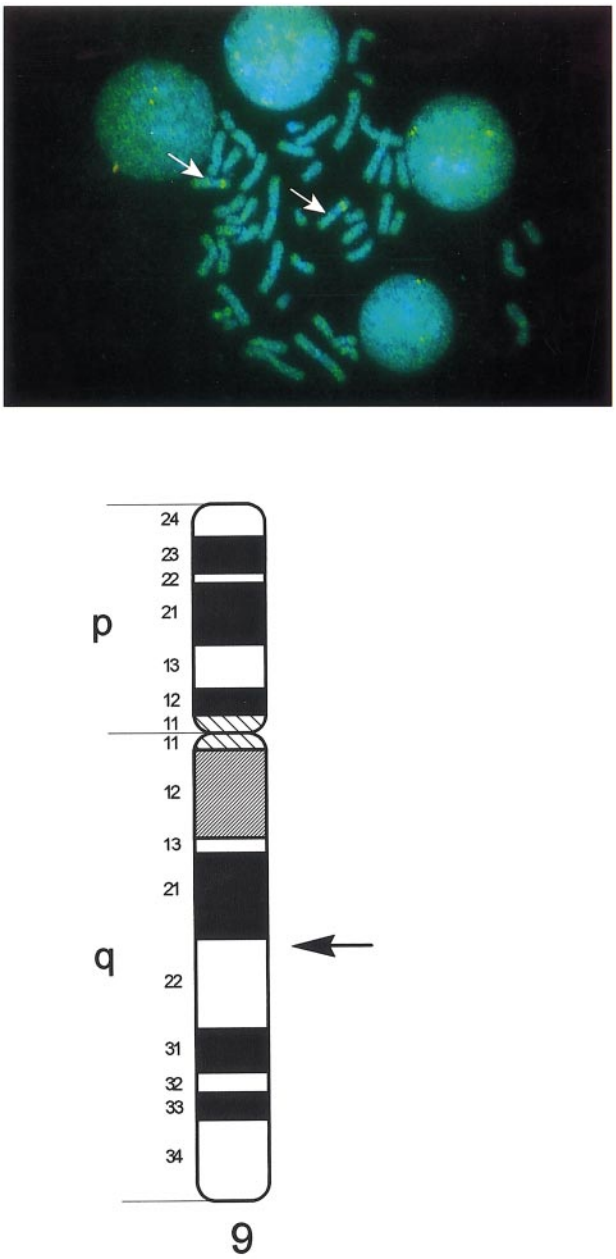

Fig. 8. The human RORβ gene localizes to chromosome 9q22. Fluorescence *in situ* hybridization was carried out using the digoxigenin dUTP-labeled 12 kb DNA insert of a genomic human RORβ λ clone, and specific signals (arrows) were detected using fluoresceinated anti-digoxigenin antibodies.

Table I. Comparison of RORβ$^{-/-}$ and *vacillans* phenotype

Observation	RORβ$^{-/-}$	*vacillans*
Juvenile ataxia	+	+
Duck-like gait in adulthood	+	+
Hind paw clasping reflex	+	+
Docile	+	+
Males do not breed before 6 months of age	+	+
Retinal degeneration	+	n.s.
Extended Tau period	+	n.s.
Mutation linked to mouse chromosome 4	a	+

n.s.: not specified.
[a]The human gene localizes to a region on chromosome 9 syntenic with mouse chromosome 4.

studies will be necessary to identify the retinal cell types that are principally involved and to understand how the lack of RORβ affects retinal differentiation and development.

Why do male RORβ$^{-/-}$ mice refrain from sexually reproducing until they are 6 months old? RORβ is expressed in parts of the hypothalamus and in the anterior pituitary gland (Schaeren-Wiemers *et al.*, 1997) and, therefore, could play an essential role in the endocrine network of sexual maturation. This would place RORβ into the context of the transcription factor ICER, which plays an important regulatory role in the physiology and development of the hypothalamic–pituitary–gonadal axis (Foulkes *et al.*, 1992, 1993), further adding to the similarity which ICER and RORβ already display in the context of circadian regulation. If RORβ plays a role in the central hormonal regulation of fertility, its role would appear crucial only to male animals, because female RORβ$^{-/-}$ mice do not display any reproductive abnormalities. Therefore, another attractive explanation for the male behavior is suggested by considering the expression of RORβ in the epididymis and the vas deferens. Potentially impaired terminal maturation and/or inappropriate storage of the sperm cells could constitute the underlying cause of the male abnormality. Whatever the shortcomings are, however, they have to be mild enough to be overcome and compensated for later in life.

A final answer to the question about the biological function of RORβ will have to await the identification of target genes regulated by this receptor. To this end, the corresponding null mutant animals will be of great utility. Knowledge of the genes which are regulated by RORβ as well as the factors that regulate RORβ will be prerequisite to a sound understanding of the molecular function of this orphan nuclear receptor.

Materials and methods

Experimental animals

Male Sprague–Dawley rats were maintained under light–dark conditions (12h:12h; LD) and constant darkness (DD), or subjected to an 8 h phase advance shift (PS). Activity rhythms were monitored using infra-red beam devices. Rats were deeply anesthetized using pentobarbital and killed at different time points under bright light or dim red light, respectively. Eyes and pineal glands were frozen to –50°C in 2-methylbutane (Fluka, Switzerland) for 5–10 min and stored at –80°C.

Male RORβ$^{+/-}$, RORβ$^{-/-}$ and wild-type littermate mice were housed singly under controlled lighting conditions (lights on 07:00 h, 30–50 lux; lights off 19:00 h), with food and water available *ad libitum*, in cages equipped with two levels of light beam grids. The upper grid monitored rearing activity and the lower grid monitored locomotor activity in the horizontal plane. In addition, under general anesthesia, some of the mice had undergone intraperitoneal implantation of PDT-4000 E-mitters (physiological data transponder, Mini-Mitter Co., Inc.) for the monitoring of core body temperature. Both the number of beam breaks and E-mitter data were recorded on-line and stored in 15 min time bins (Benwick Electronics, AM1051 system). The consequent actograms were displayed using TAU software (Mini-Mitter Co., Inc.).

In situ hybridization

Synthetic cRNA probes were derived from a recombinant plasmid (pBS^{-}, Stratagene, La Jolla, CA) containing the cDNA of RORβ spanning its coding sequence (1.6 kb; Carlberg *et al.*, 1994). Antisense and sense probes were synthesized by *in vitro* transcription using T3 or T7 RNA polymerase and digoxigenin-labeled UTP (Boehringer Mannheim, Germany). *In situ* hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993) on coronal sections of brains, heads and eyes that were cut on a cryostat (14 μm).

RORβ mRNA quantification

RNA quantification was performed using the technique of competitive PCR as described (Becker-André 1995). Briefly, 0.1 μg of total RNA extracted from rat pineal gland was subjected to a single-tube RT–PCR using M-MuLV reverse transcriptase (Life Technologies, Gaithersburg, MD) and *Taq* polymerase (Roche, Nutley, NJ). Oligonucleotide primers (20mer) were designed using the RORβ cDNA sequence encoding the putative ligand-binding domain. *In vitro*-transcribed synthetic RNA (103 bases) derived from RORβ cDNA and containing an internal 4 bp deletion served as internal standard in the co-amplification process. Target- and standard-specific PCR amplification products were separated on 3.5% metaphor agarose (FMC Bioproducts, Rockland, ME).

Targeting the RORβ gene locus

A 1.3 kb PCR-generated DNA fragment containing partially the exon which encodes the second zinc finger of RORβ and its 5′-flanking sequences was cloned into the vector pBluescript (Stratagene) via *Xba*I and *Sac*II. After sequencing of the insert, a 1.8 kb thymidine kinase gene (*tk*) cassette was cloned into the *Xba*I site of this plasmid. The resulting 3.1 kb insert was released from the plasmid using *Not*I and *Sac*II and cloned into the pGN vector (Le Mouellic *et al*., 1990). Into this vector was cloned a *Kpn*I–*Not*I 8.0 kb DNA fragment derived from the RORβ locus of a 129/Sv mouse containing intron sequences downstream of the second zinc finger, resulting in the targeting construct pRORb-koc-TK. *Not*I-linearized pRORb-koc-TK was electroporated into 129/Ola ES HM1 cells as described (Magin *et al*., 1992). Chimeric mice were mated with C57Bl/6 mice, and offspring (F1 generation) were genotyped by Southern hybridization of tail DNA using as a probe a DNA fragment derived from the RORβ locus upstream of the 1.3 kb *Xba*I–*Sac*II DNA fragment (Figure 3A). Genomic DNA was digested using *Bam*HI and *Kpn*I and size separated in 0.8% agarose gel. Heterozygous RORβ$^{+/-}$ animals used for the various studies were bred by crossing RORβ$^{+/-}$ F1 animals with C57Bl/6 mice (resulting in F2 animals) and by crossing RORβ$^{+/-}$ F2 animals with C57Bl/6 mice (resulting in F3 animals). Homozygous RORβ$^{-/-}$ mice were bred by intercrossing RORβ$^{+/-}$ F2 and F3 animals. RT–PCR was used to assay for the absence of functional transcripts from the targeted RORβ locus. RNA was isolated from eyes dissected from RORβ$^{+/+}$ and RORβ$^{-/-}$ mice (Chomczynski and Sacchi, 1987) and converted to cDNA using either primer rorb-1, GTAACAAGACGACCGAGGAG (derived from the DNA-binding domain upstream of the disruption site), or rob-2, GTGGAGTCGTATGTCCGACA (derived from the ligand-binding domain). PCR was carried out on each of the cDNA pools using the primer pairs: rorb-3, CATGCGAGCACAAATTGAAG, and rorb-4, TAGGGAACGTCGGAAGTG, amplifying the N-terminal RORβ cDNA sequence (98 bp) upstream of the disruption site; or rorb-3 and rorb-5, GAGGGTTTGAAGTGTCGTAG, amplifying the RORβ cDNA sequence (252 bp) flanking the disruption site including part of the ligand-binding domain. PCR products were separated on a 3% MetaPhor agarose gel (FMC BioProducts).

Chromosomal localization of the human RORβ gene

Digoxigenin-labeled probe, derived from a genomic λ clone insert 15 kb in size, was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluorescein-ated anti-digoxigenin antibodies (Bios Laboratories, New Haven, CT) followed by counter-staining with 4,6′-diamidino-2-phenylindole (DAPI). In the same experiment, a probe was co-hybridized which is specific for the p16 INK4A locus previously mapped to 9p21 (Kamb *et al*., 1994). Measurements of 10 specifically hybridized chromosomes 9 demonstrated that the RORβ gene is located at a position corresponding to band 9q22.

Histological analysis

Tissue from *ror*β$^{+/-}$ and *ror*β$^{-/-}$ mice was frozen in 2-methylbutane at –40°C for 5–10 min and stored at –80°C. Cryosections were cut (15 μm), air-dried on glass slides (Menzel, Marburg, Germany), fixed in 0.05% glutaraldehyde for 5 min at room temperature, washed with phosphate-buffered saline (PBS) and incubated overnight at 37°C in PBS containing 4 mM $MgCl_2$, 2 mM $K_4Fe(CN)_6$, 2 mM $K_3Fe(CN)_6$ and 0.4 mg/ml XGal. After washing with PBS, the sections were counter-stained (optional) with hematoxylin and eosin and mounted.

Recording electroretinograms (ERGs) and visual evoked potentials (VEPs)

Anesthetized RORβ$^{+/-}$ and RORβ$^{-/-}$ mice (urethane 20%, 0.8 ml/100g, administered intraperitoneally) were mounted in a stereotaxical device and dark adapted for 30 min before recording. Light flashes of rod-saturating intensity (10 cd·s/m^2 at the cornea) were delivered, and ERGs were recorded from a corneal silver–silver chloride electrode. The VEPs were recorded from a tungsten electrode (0.5 MΩ) positioned in layer 4 (0.4 mm below the cortical surface) of the binocular cortex (3 mm lateral of the lambda) contralateral to the stimulated eye. Signals were amplified (10 000-fold), band-pass filtered (ERG: 0.1–300 Hz; VEP: 0.1–100 Hz) and averaged (ER: five sweeps; ERG: 100 sweeps).

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References

Abbott,C., Jackson,I.J., Carritt,B. and Povey,S. (1991) The human homolog of the mouse *brown* gene maps to the short arm of chromosome 9 and extends the known region of homology with mouse chromosome 4. *Genomics*, **11**, 471–473.

Antoch,M.P. *et al*. (1997) Functional identification of the mouse circadian *Clock* gene by transgenic BAC rescue. *Cell*, **89**, 655–667.

Argamaso,S.M., Froehlich,A.C., MacCall,M.A., Nevo,E., Provencio,I. and Foster,R.G. (1995) Photopigments and circadian systems of vertebrates. *Biophys. Chem*., **56**, 3–11.

Axelrod,J. (1974) The pineal gland: a neurochemical transducer. *Science*, **184**, 1341–1348.

Baler,R., Coon,S. and Klein,D.C. (1996) Orphan nuclear receptor RZRβ:cyclic AMP regulates expression in the pineal gland. *Biochem. Biophys. Res. Commun*., **220**, 975–978.

Becker-André,M. (1995) PCR-aided transcript titration assay: an improved version of competitive PCR for the evaluation of absolute levels of rare mRNA species. In Sarkar,G. (ed.), *PCR in Neuroscience*. Academic Press, San Diego, CA, pp. 129–146.

Becker-André,M., André,E. and DeLamarter,J.F. (1993) Identification of nuclear receptor mRNAs by RT–PCR amplification of conserved zinc-finger motif sequences. *Biochem. Biophys. Res. Commun.*, **194**, 1371–1379.

Bell,J.A., Rinchik,E.M., Raymond,S., Suffolk,R. and Jackson,I.J. (1995) A high-resolution map of the brown (b, Tyrp1) deletion complex of mouse chromosome 4. *Mamm. Genome*, **6**, 389–395.

Besharse,J.C. and Iuvone,P.M. (1983) Circadian clock in *Xenopus* eye controlling retinal serotonin *N*-acetyltransferase. *Nature*, **305**, 133–135.

Blazynski,C. and Dubocovich,M.L. (1991) Localization of 2-[^{125}I]iodo-melatonin binding sites in mammalian retina. *J. Neurochem*., **56**, 1873–1880.

Bowes,C., Li T., Danciger,M., Baxter,L.C., Applebury,M.L. and Farber,D.B. (1990) Retinal degeneration in the rd mouse is caused by a defect in the β subunit of rod cGMP-phosphodiesterase. *Nature*, **347**, 677–680.

Carlberg,C., Hooft van Hujisdujinen,R., Staple,J.K., DeLamarter,J.F. and Becker-André,M. (1994) RZRs, a new family of retinoid-related orphan receptors that function as both monomers and homodimers. *Mol. Endocrinol.*, **8**, 757–770.

Carney,G.E., Wade,A.A., Sapra,R., Goldstein,E.S. and Bender,M. (1997) DHR3, an ecdysone-inducible early–late gene encoding a *Drosophila* nuclear receptor, is required for embryogenesis. *Proc. Natl Acad. Sci. USA*, **94**, 12024–12029.

Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem*., **162**, 156–159.

Czeisler,C.A., Shanahan,T.L., Klerman,E.B., Martens,H., Brotman,D.J., Emens,J.S., Klein,T. and Rizzo,J.F. (1995) Suppression of melatonin secretion in some blind patients by exposure to bright light. *N. Engl. J. Med*., **332**, 6–11.

Doolittle,D.P., Davisson,M.T., Guidi,J.N. and Green,M.C. (eds) (1996) *Genetic Variants and Strains of the Laboratory Mouse*. Oxford University Press, Oxford, UK, p. 807.

El Haj,A.J., Tamone,S.L., Peake,M., Sreenivasula Reddy,P. and Chang,E.S. (1997) An ecdysteroid-responsive gene in lobster—a potential crustacean member of the steroid hormone receptor superfamily. *Gene*, **201**, 127–135.

Evans,R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science*, **240**, 889–895.

Foster,R.G., Provencio,I., Hudson,D., Fiske,S., De Grip,W. and Menaker,M. (1991) Circadian photoreception in the retinally degenerate mouse (*rd/rd*). *J. Comp. Physiol. A*, **169**, 39.

Foulkes,N.S., Mellström,B., Benusiglio,E. and Sassone-Corsi,P. (1992) Developmental switch of CREM function during spermatogenesis: from antagonist to activator. *Nature*, **355**, 80–84.

Foulkes,N.S., Schlotter,F., Pévet,P. and Sassone-Corsi,P. (1993) Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. *Nature*, **362**, 264–267.

Foulkes,N.S., Borjigin,J., Snyder,S.H. and Sassone-Corsi,P. (1997) Rhythmic transcription: the molecular basis of circadian melatonin synthesis. *Trends Neurosci.*, **20**, 487–492.

Giguère,V., Tini,M., Flock,G., Ong,E., Evans,R.M. and Otulakowski,G. (1994) Isoform-specific amino-terminal domains dictate DNA-binding properties of RORα, a novel family of orphan hormone nuclear receptors. *Genes Dev.*, **8**, 538–553.

Goto,M., Oshima,I., Tomita,T. and Ebihara,S. (1989) Melatonin content of the pineal gland in different mouse strains. *J. Pineal Res.*, **7**, 195–204.

Hamilton,B.A. *et al*. (1996) Disruption of the nuclear hormone receptor RORα in *staggerer* mice. *Nature*, **379**, 736–739.

Hirose,T., Smith,R.J. and Jetten,A.M. (1994) RORγ, the third member of ROR/RZR orphan receptor subfamily that is highly expressed in skeletal muscle. *Biochem. Biophys. Res. Commun.*, **205**, 1976–1983.

Kamb,A. *et al*. (1994) A cell cycle regulator potentially involved in genesis of many tumor types. *Science*, **264**, 436–440.

King,D.P. *et al*. (1997) Positional cloning of the mouse circadian *Clock* gene. *Cell*, **89**, 641–653.

Klein,D.C., Namboodiri,M.A.A. and Auerbach,D.A. (1981) The melatonin rhythm generating system: developmental aspects. *Life Sci.*, **28**, 1975–1986.

Klein,D.C., Moore,R.Y. and Reppert,S.M., eds (1991) *Suprachiasmatic Nucleus: The Mind's Clock*. Oxford University Press, Oxford.

Koelle,M.R., Segraves,W.A. and Hogness,D.S. (1992) DHR3: a *Drosophila* steroid receptor homolog. *Proc. Natl Acad. Sci. USA*, **89**, 6167–6171.

Kostrouch,Z., Kostroucha,M. and Rall,J.E. (1995) Steroid/thyroid hormone receptor genes in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA*, **92**, 156–159.

Laudet,V. and Adelmant,G. (1995) Lonesome orphans. *Curr. Biol.*, **5**, 124–127.

Le Mouellic,H., Lallemand,Y. and Brulet,P. (1990) Targeted replacement of the homeobox gene *Hox-3.1* by the *Escherichia coli lacZ* in mouse chimeric embryos. *Proc. Natl Acad. Sci. USA*, **87**, 4712–4716.

Lopez-Molina,L., Conquet,F., Dubois-Dauphin,M. and Schibler,U. (1997) The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. *EMBO J.*, **16**, 6762–6771.

Magin,T., McWhir,J. and Melton,D. (1992) A new mouse embryonic stem cell line with good germ line contribution and gene targeting frequency. *Nucleic Acids Res.*, **20**, 3795–3796.

Meijer,J.H. (1991) Integration of visual information by the suprachiasmatic nucleus. In Klein,D.C., Moore,R.Y. and Reppert,S.M. (eds), *Suprachiasmatic Nucleus: The Mind's Clock*. Oxford University Press, Oxford, pp. 107–119.

Molina,C.A., Foulkes,N.S., Lalli,E. and Sassone-Corsi,P. (1993) Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell*, **75**, 875–886.

Moore,R.Y. (1995) Organization of the mammalian circadian system. In Chadwick,D.J. and Ackwill,K. (eds), *Circadian Clocks and their Adjustment*. *Ciba Foundation Symposium 183*. Wiley, Chichester pp. 88–106.

O'Malley,B.W. and Conneely,O.M. (1992) Orphan receptors: in search of a unifying hypothesis for activation. *Mol. Endocrinol.*, **6**, 1359–1361.

Palli,S.R., Hiruma,K. and Riddifird,L.M. (1992) An ecdysteroid-inducible *Manduca* gene similar to the *Drosophila* DHR3 gene, a member of the steroid hormone receptor superfamily. *Dev. Biol.*, **150**, 306–318.

Perlow,M.J., Reppert,S.M., Tamarkin,L., Wyatt,R.J. and Klein,D.C. (1980) Photic regulation of the melatonin rhythm: monkey and man are not the same. *Brain Res.*, **182**, 211–216.

Ralph,M.R. and Menaker,M. (1988) A mutation of the circadian system in golden hamsters. *Science*, **241**, 1225–1227.

Schaeren-Wiemers,N. and Gerfin-Moser,A. (1993) A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: *in situ* hybridization using digoxigenin-labelled cRNA probes. *Histochemistry*, **100**, 431–440.

Schaeren-Wiemers,N., André,E., Kapfhammer,J.P. and Becker-André,M. (1997) The expression pattern of the orphan nuclear receptor RORβ in the developing and adult rat brain suggests a role in the processing of sensory information and in circadian rhythm. *Eur. J. Neurosci.*, **9**, 2687–2701.

Shen,H., Watanabe,M., Tomasiewicz,H., Rutishauser,U., Magnuson,T. and Glass,J.D. (1997) Role of neural cell adhesion molecule and polysialic acid in mouse circadian clock function. *J. Neurosci.*, **17**, 5221–5229.

Sirlin,J.L. (1956) *Vacillans*, a neurological mutant in the house mouse linked with *brown*. *J. Genet.*, **54**, 42–48.

Stehle,J.H., Foulkes,N.S., Molina,C.A., Simmoneaux,V., Pévet,P. and Sassone-Corsi,P. (1993) Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. *Nature*, **365**, 314–320.

Stehle,J.H., Foulkes,N.S., Pévet,P. and Sassone-Corsi,P. (1995) Developmental maturation of pineal gland function: synchronized CREM inducibility and adrenergic stimulation. *Mol. Endocrinol.*, **9**, 706–716.

Steinmayr,M. *et al*. (1998) *Staggerer* phenotype in retinoid-related orphan receptor α-deficient mice. *Proc. Natl Acad. Sci. USA*, **95**, 3960–3965.

Takahashi,J.S. (1995) Molecular neurobiology and genetics of circadian rhythms in mammals. *Annu. Rev. Neurosci.*, **18**, 531–553

Tamarkin,L., Reppert,S.M. and Klein,D.C. (1979) Regulation of pineal melatonin in the Syrian hamster. *Endocrinology*, **104**, 385–389.

Tobler,I. *et al*. (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature*, **380**, 639–642.

Tosini,G. and Menaker,M. (1996) Circadian rhythms in cultured mammalian retina. *Science*, **272**, 419–421.

Vitaterna,M.H. *et al*. (1994) Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. *Science*, **264**, 719–725.

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