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

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Introduction

Retinoid-related orphan receptor β (ROR β) 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 synthe-

sized 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 Adelman, 1995). ROR β is such an orphan nuclear receptor, forming a subfamily with the closely related nuclear receptors ROR α (Becker-André *et al.*, 1993; Giguère *et al.*, 1994) and ROR γ (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).

ROR β originally was cloned from rat brain RNA (Carlberg *et al.*, 1994). In rat, the expression of ROR β 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). ROR β '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, ROR β mRNA is expressed exclusively in non-pyramidal neurons of layer IV and V, with the highest levels in primary sensory cortices. In the thalamus, ROR β is expressed in the sensory relay nuclei that project to the respective cortical areas. In the spinal cord, ROR β 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 ROR β expression. ROR β is absent from striatum, hippocampus, cerebellum, the motor nuclei of the cranial nerves and the ventral part of the spinal cord. Additionally, ROR β 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 ROR β 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 ROR β in the context of processing sensory information and circadian rhythm. To understand better the contribution of ROR β to genomic processes that control circadian rhythms, we expanded our study on

the ROR β mRNA distribution in the rat CNS and established the expression pattern of ROR β 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, ROR β mRNA levels oscillate with true circadian rhythmicity, peaking at night-time. To evaluate its overall physiological role, we generated ROR β -deficient mice by gene targeting in embryonic stem (ES) cells and analyzed their phenotypic behavior. We show that ROR β mildly influences circadian rhythmicity: its absence extends the period length τ of the free-running activity rhythm. More importantly, however, the absence of ROR β 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

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

In order to address the question of whether ROR β 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 ROR β 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 ROR β 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 ROR β 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 ROR β expression is truly circadian.

This was substantiated further by establishing a time course of the temporal change of pineal ROR β expression in rats kept in LD or DD conditions (with a documented free-running locomotor activity). ROR β mRNA levels were quantified using a competitive PCR protocol (Becker-André, 1995). Under LD conditions, the ROR β 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 ROR β 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 ROR β expression levels disappears as a consequence of the phase advance-induced collapse of circadian rhythmic behavior. The nocturnal up-regulation of pineal ROR β 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).

ROR β is already expressed during embryogenesis (Schaeren-Wiemers *et al.*, 1997). In order to address the question of at what developmental stage circadian ROR β expression rhythms are established, we analyzed ROR β mRNA levels in pineal gland and retina of rats at various postnatal days. Significant circadian fluctuation of pineal ROR β 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 ROR β 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 ROR β 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 ROR β expression levels.

Generation of transgenic mice carrying ROR β null alleles

To investigate the functional role of ROR β , we generated ROR β -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 ROR β . After homologous recombination, the targeted gene encoded a fusion protein composed of ROR β ’s N-terminal domain and first zinc finger followed by β -galactosidase (Figure 2A). Adult heterozygous ROR β mutant (ROR $\beta^{+/-}$) mice were examined for the distribution of β -galactosidase activity indicative of ROR β 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 ROR β expression in pineal gland and retina. ROR β 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. ROR β -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 ROR β 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 ROR β 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 ROR $\beta^{-/-}$ mice

Cross-breeding of heterozygous ROR $\beta^{+/-}$ mice (F2 and F3 generations) yielded homozygous ROR $\beta^{-/-}$ animals at the expected frequency of 25% (Figure 2B). Reverse transcription–PCR experiments were performed to verify the absence of functional ROR β transcripts in ROR $\beta^{-/-}$ mice. As expected, transcripts from the null mutant gene allele contain the N-terminal ROR β 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 ROR β (Figure 2C, cf. lanes 3 and 4). Young ROR $\beta^{-/-}$

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 ROR $\beta^{-/-}$ mice display: a ‘duck-like’ gait and a hind paw clasp reflex when suspended by the tail (Figure 3). By comparison with ROR $\beta^{+/+}$ littermates, ROR $\beta^{-/-}$ 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. ROR $\beta^{-/-}$ 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, ROR $\beta^{-/-}$

Fig. 2. Targeting of the $ROR\beta$ locus. (A) Scheme depicting the restriction map of the murine $ROR\beta$ locus at the second zinc finger domain, the targeting construct and the resulting targeted $ROR\beta$ locus after homologous recombination. B, *Bam*HI; C, *Cl*aI; 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 $ROR\beta^{+/+}$ and mutant $ROR\beta^{-/-}$ mice. PCR products specific for the N-terminal part of the $ROR\beta$ transcript preceding the disruption site were amplified from both genotypes. In contrast, PCR products derived from the ligand-binding domain (LBD) of the $ROR\beta$ 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.

$ROR\beta^{-/-}$ 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

$ROR\beta$ is expressed appear normally developed. Only in the newborn spinal cord did we find significant differences between $ROR\beta^{+/-}$ and $ROR\beta^{-/-}$ mice: while in $ROR\beta^{+/-}$ 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 $ROR\beta^{-/-}$ mice this staining is dispersed throughout the dorsal horn (Figure 4T and U). The gross anatomical structure of adult $ROR\beta^{-/-}$ spinal cord, however, appears normal when compared with wild-type littermates (not shown).

Histological examination of adult $ROR\beta^{-/-}$ 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 $ROR\beta^{-/-}$ mice is not very different from that of $ROR\beta^{+/-}$ 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). $ROR\beta^{-/-}$ 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 $ROR\beta^{-/-}$ 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 $ROR\beta^{-/-}$ 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 $ROR\beta^{-/-}$ 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 ($\tau = 24.2$ h) than that of their heterozygous and wild-type littermates ($\tau = 23.8$ h) or of the corresponding strains used to breed these animals, C57Bl/6 ($\tau = 23.7$ h) and 129/Sv ($\tau = 23.8$ h; Lopez-Molina *et al.*, 1997).

$ROR\beta^{-/-}$ 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 $ROR\beta^{-/-}$ mice versus older $ROR\beta^{-/-}$ and heterozygous mice. Histological examination revealed strong *lacZ* gene activity in the epithelial cells lining the lumen of the epididymis and vas deferens, but not of the testis and prostate, of both $ROR\beta^{+/-}$ and $ROR\beta^{-/-}$ mice (Figure 7). Intriguingly, no differences between young

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

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

Chromosomal localization of the ROR β gene

We determined the chromosomal localization of the human ROR β 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 ROR β gene to human chromosome 9 (data not shown). To determine its sub-chromosomal position, we performed fluorescence *in situ* hybridization experiments on human metaphase chromosomes using a λ phage clone derived from the human ROR β gene and a chromosome 9-specific gene probe as marker (see Materials and methods). Our results suggest that human ROR β maps to chromosome 9q22 (Figure 8), a region which is syntenic with mouse chromosome 4.

Discussion

The role of ROR β 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 ROR β is expressed in these parts of the photoneuroendocrine system (Schaeren-Wiemers *et al.*, 1997).

Here we show that the ROR β 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 ROR β 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 ROR β is driven by the endogenous pace-maker residing in the SCN. Our results parallel previously reported findings (Baler *et al.*, 1996) showing that the circadian ROR β 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 ROR β , 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 ROR β 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 ROR β 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, ROR $\beta^{+/-}$ 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 $ROR\beta^{+/-}$ and $ROR\beta^{-/-}$ 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 $ROR\beta^{+/-}$ and $ROR\beta^{-/-}$ 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 $ROR\beta^{+/-}$ and $ROR\beta^{-/-}$ 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); $ROR\beta$ 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 $ROR\beta$ 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 $ROR\beta$ and the changed circadian behavior of $ROR\beta^{-/-}$ mice, $ROR\beta$ might be involved in the transcriptional regulation of effectors of the circadian clock. In particular, $ROR\beta$ could participate in the regulation of melatonin synthesis, similarly to ICER (Foulkes *et al.*, 1997), for two reasons: (i) the rhythmic expression of $ROR\beta$ is confined to the two principal melatonin-producing tissues within the CNS, the pineal gland (Axelrod, 1974) and the photoreceptors (Besharse and Ivovone, 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 ROR $\beta^{+/-}$ and ROR $\beta^{-/-}$ mice in response to strong light flashes. Thin lines in the ROR $\beta^{-/-}$ 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 ROR β 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 ROR $\beta^{-/-}$ mice in order to clarify ROR β '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* $\beta^{-/-}$ 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 ROR β .

The phenotypic syndrome of ROR $\beta^{-/-}$ mice suggests a relationship to *vacillans*

ROR β -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, ROR $\beta^{+/+}$, ROR $\beta^{+/-}$ and ROR $\beta^{-/-}$ 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 ROR $\beta^{+/+}$ 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 ROR $\beta^{+/-}$ mice displayed similar free-running periods (median Tau = 23.81, $n = 9$). In contrast, the ROR $\beta^{-/-}$ mice had significantly longer Tau periods than any of the other groups ($P < 0.05$, Kruskal–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 ROR β 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 ROR $\beta^{+/-}$ and ROR $\beta^{-/-}$ 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 ROR $\beta^{-/-}$ mice.

Notwithstanding this, another line of evidence speaks in favor of the assumption that *vacillans* and *rorβ^{-/-}* 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 ROR β 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 ROR $\beta^{-/-}$ 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 ROR β , leading to a phenotype very similar to that of ROR $\beta^{-/-}$ mice.

ROR β plays a role in development

At present, we cannot explain mechanistically the phenotypic abnormalities of ROR $\beta^{-/-}$ mice, and a number of questions remain to be answered. Why do young ROR $\beta^{-/-}$ 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: ROR β -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 ROR $\beta^{-/-}$ 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 ROR $\beta^{-/-}$ 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 ROR $\beta^{-/-}$ mice? This finding is reminiscent of ROR $\alpha^{-/-}$ 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 ROR β 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 ROR β λ clone, and specific signals (arrows) were detected using fluoresceinated anti-digoxigenin antibodies.

Table I. Comparison of ROR $\beta^{-/-}$ and *vacillans* phenotype

Observation	ROR $\beta^{-/-}$	<i>vacillans</i>
Juvenile ataxia	+	+
Duck-like gait in adulthood	+	+
Hind paw clasp 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.

^aThe 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 ROR β affects retinal differentiation and development.

Why do male ROR $\beta^{-/-}$ mice refrain from sexually reproducing until they are 6 months old? ROR β is expressed in parts of the hypothalamus and in the anterior pituitary gland (Schaefer-Wiemers *et al.*, 1997) and, therefore, could play an essential role in the endocrine network of sexual maturation. This would place ROR β 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 ROR β already display in the context of circadian regulation. If ROR β plays a role in the central hormonal regulation of fertility, its role would appear crucial only to male animals, because female ROR $\beta^{-/-}$ mice do not display any reproductive abnormalities. Therefore, another attractive explanation for the male behavior is suggested by considering the expression of ROR β 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 ROR β 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 ROR β as well as the factors that regulate ROR β 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 ROR $\beta^{+/+}$, ROR $\beta^{-/-}$ 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 ROR β 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 (Schaefer-Wiemers and Gerfin-Moser, 1993) on coronal sections of brains, heads and eyes that were cut on a cryostat (14 μm).

ROR β mRNA quantification

RNA quantification was performed using the technique of competitive PCR as described (Becker-André 1995). Briefly, 0.1 μ g 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 ROR β cDNA sequence encoding the putative ligand-binding domain. *In vitro*-transcribed synthetic RNA (103 bases) derived from ROR β 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 ROR β gene locus

A 1.3 kb PCR-generated DNA fragment containing partially the exon which encodes the second zinc finger of ROR β 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 Mouellie et al., 1990). Into this vector was cloned a *Kpn*I-*Not*I 8.0 kb DNA fragment derived from the ROR β locus of a 129/Sv mouse containing intron sequences downstream of the second zinc finger, resulting in the targeting construct pROR β -koc-TK. *Not*I-linearized pROR β -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 ROR β 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 ROR $\beta^{+/-}$ animals used for the various studies were bred by crossing ROR $\beta^{+/-}$ F1 animals with C57Bl/6 mice (resulting in F2 animals) and by crossing ROR $\beta^{+/-}$ F2 animals with C57Bl/6 mice (resulting in F3 animals). Homozygous ROR $\beta^{-/-}$ mice were bred by intercrossing ROR $\beta^{+/-}$ F2 and F3 animals. RT-PCR was used to assay for the absence of functional transcripts from the targeted ROR β locus. RNA was isolated from eyes dissected from ROR $\beta^{+/+}$ and ROR $\beta^{-/-}$ mice (Chomczynski and Sacchi, 1987) and converted to cDNA using either primer rob-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: rob-3, CATGCGAGCACAAATTGAAG, and rob-4, TAGGGAACGTCGGAAGTG, amplifying the N-terminal ROR β cDNA sequence (98 bp) upstream of the disruption site; or rob-3 and rob-5, GAGGGTTTGAAGTGTCTAG, amplifying the ROR β 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 ROR β 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 $\times$ SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated 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 ROR β gene is located at a position corresponding to band 9q22.

Histological analysis

Tissue from *ror* $\beta^{+/-}$ and *ror* $\beta^{-/-}$ mice was frozen in 2-methylbutane at -40°C for 5–10 min and stored at -80°C. Cryosections were cut (15 μ m), 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 MgCl₂, 2 mM K₄Fe(CN)₆, 2 mM K₃Fe(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 ROR $\beta^{+/-}$ and ROR $\beta^{-/-}$ 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/m² 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 M Ω) 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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