Behavioral Characterization and Modulation of Circadian Rhythms By Light and Melatonin in C3H/HeN Mice Homozygous for the RORβ Knockout#

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Running Head: Modulation of Circadian Rhythms in C3H RORβ Knockout Mice

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Abstract

This study reports for the first time the effects of RORβ receptor gene deletion (RORβ(C3H)−/−) in C3H/HeN mice on behavioral and circadian phenotypes. Pineal melatonin levels showed a robust diurnal rhythm with high levels at night in wild type (+/+) and heterozygous (+/-) mice. The RORβ(C3H)−/− mice displayed motor (“duck gait,” hind paw clasping reflex) and olfactory deficits, and reduced anxiety and learned helplessness related behaviors. Circadian rhythms of wheel-running activity in all genotypes showed entrainment to the light/dark (LD) cycle, and free running in constant dark, with RORβ(C3H)−/− mice showing a significant increase in circadian period (τ). Melatonin administration (90 µg/mouse, s.c. for 3 days) at CT 10 induced phase advances while exposure to a light pulse (300 lux) at CT14 induced phase delays of circadian activity rhythms of the same magnitude in all genotypes. In RORβ(C3H)−/− mice a light pulse at CT 22 elicited a larger phase advance in activity rhythms and a slower rate of re-entrainment after a 6 hour advance in the L/D cycle compared to (+/+). Yet, the rate of re-entrainment was significantly advanced by melatonin administration at the new dark onset in both (+/+ and −/−) mice. We conclude that the RORβ nuclear receptor is not involved in either the rhythmic production of pineal melatonin or in mediating phase shifts of circadian rhythms by melatonin, but it may regulate clock responses to photic stimuli at certain time domains.

**Keywords:** C3H/HeN mice; RORβ gene; circadian activity rhythms; knockout mice; melatonin receptors; suprachiasmatic nucleus
Introduction

Retinoid-related orphan receptors (ROR) belong to a superfamily of nuclear hormone receptors comprised of over forty transcription factors (7, 24). Members of this superfamily share a common modular structure consisting of a transactivation domain, a DNA-binding domain, and a ligand-binding domain (24). The family of ROR receptors is a subfamily of orphan receptors comprising RORα, RORβ and RORγ (21). RORβ is a nuclear receptor expressed in areas related to processing of sensory information as well as circadian rhythmicity (27, 33). Recently, structural data and structure-function analysis identified all-trans retinoic acid (ATRA) as a bona fide ligand for the RORβ nuclear receptor (37).

Circadian oscillations of the mammalian biological clock within the suprachiasmatic nucleus (SCN) are driven by a main transcriptional/translational feedback loop of clock gene products. The negative arm of the loop involves the circadian oscillation of three Period (Per) genes and two Cryptochrome (Cry) genes, potent repressors of CLOCK/BMAL1-induced transcription. The positive loop is driven by the transcription factors CLOCK and BMAL1 positively influencing Per and Cry rhythmic transcription (16). Recently, Hogenesch and colleagues (32) demonstrated a second, stabilizing feedback loop, where the nuclear receptors Rev-Erbα and RORα are repressors and activators, respectively, on the same RORE element of the *Bmal1* promoter, generating the rhythms of *Bmal1* transcription. This is an elegant demonstration that RORα, a member of the family of ROR orphan receptors is a necessary component of the core circadian function.
RORβ receptor mRNA oscillates with a circadian rhythm in the retina (1, 33). In the pineal gland, RORβ receptor expression exhibits a robust daily rhythm under photoneural regulation involving an adrenergic/cAMP mechanism (1, 3) which suggests its involvement in the daily rhythm of melatonin production (3). Disruption of the RORβ receptor leads to behavioral phenotypes (i.e. duck-like gait, delayed onset of male fertility, blindness due to retinal degeneration) (1) resembling the spontaneous mouse mutation vacillans described in 1956 (35). C57BL/6 mice lacking the RORβ receptor entrain to a light/dark cycle, in spite of the retinal degeneration that causes blindness (1). When running in constant dark, however, the period of activity \( (\tau) \) is longer than in wild type mice (1). In assessing involvement of the RORβ gene product as a component of the biological clock, the response to any agent that phase shifts the clock may be altered in mice lacking the RORβ receptor. Here we studied the response of RORβ knockout mice with a C3H/HeN background to the phase shifting effect of light during the night.

Melatonin, a hormone synthesized primarily in the retina and the pineal gland following a circadian rhythm with high levels at night, modulates neuronal activity and phase shifts circadian rhythms (10, 20, 23). In the suprachiasmatic nucleus, inhibition of neuronal activity is mediated through activation of \( \text{MT}_1 \) melatonin receptors (23), while phase shifts of neuronal firing rhythms \textit{in vitro} require activation of the \( \text{MT}_2 \) melatonin receptors (9, 19, 20). However, paradoxically, phase shift of overt circadian rhythms of activity \textit{in vivo} requires activation of \( \text{MT}_1 \) receptors, as this effect is absent in \( \text{MT}_1 \) knockout mice (9, 19). Initially, melatonin was suggested as the natural ligand of retinoid orphan receptor β (RORβ), however, further research did not confirm this finding (4). The \( \text{MT}_1 \) melatonin receptor and RORβ gene are both localized on arginine vasopressin (AVP) containing neurons in the Siberian Hamster suprachiasmatic
nucleus (36). However, it is not known whether RORβ receptor activation participates in melatonin-mediated phase shifts of circadian rhythms or affects photic phase shifts. The goal of this study was to investigate the role of the RORβ nuclear receptor in various behavioral domains, in the diurnal rhythm of pineal melatonin levels, or in melatonin- and light-mediated circadian phase shifts and acceleration of re-entrainment.
Methods

Experimental Animals

The ROR\(\beta\) gene knockout was initially generated in the 129/Sv mouse and was made congenic with the C57Bl/6J mice (1). The ROR\(\beta\) gene was disrupted downstream of the exon encoding the DNA-binding domain via insertion of and in-frame fusion with the lacZ gene. ROR\(\beta\) (C57Bl/6J) knockout mice were then backcrossed with C3H/HeN (rd/rd) mice at Glaxo-Wellcome (Geneva, Switzerland). ROR\(\beta\)(C3H) mice used for experiments reported here were at least of the F6-F7 generation. All animals were genotyped at the time of weaning and at the time of sacrifice by PCR using DNA obtained from a tail sample. Genomic DNA (0.1-0.5 \(\mu\)g) was subjected to PCR using the following primer pair: the upstream primer derived from the ROR\(\beta\) gene GGAGCCAGCAGAACAATG, and the downstream primer derived from the lacZ gene CCTCTTCGCTATTACGCCAG. All mice were bred under a 12:12 light:dark cycle (250 lux at the level of the cage). Mice were group housed under controlled lighting conditions with food and water available \textit{ad libitum}. All animal care and procedures were performed according to institutional guidelines and to the National Institute of Health standards, and were approved by the Institutional Animal Care and Use Committee of Northwestern University.

Behavioral phenotyping of ROR\(\beta\)(C3H) mice

Basic behavioral screening: All behavioral screening was performed according to the methods described by Crawley (2000) (8). ROR\(\beta\)(C3H)\(^{+/+}\) mice (male: n = 14, female: n = 2, 6.3±1.0 mo old) and ROR\(\beta\)(C3H)\(^{+/}\) mice (male: n = 8, female: n = 10, 6.5 ± 0.8 mo old) were kept in a 14:10 L:D cycle. All testing was performed between ZT 3 and ZT 7 (ZT0 lights on). All mice
underwent an examination of basic physical characteristics [weight, piloerection (%), whiskers (%), bald patches (%) and palpebral closure (%)], and general behaviors (sniffing, licking, rearing, jumping, moving around the cage and wild running). Mouse behaviors were observed and quantified individually in a clean cage. Results were expressed as % of animals displaying each behavior during a 1 min period.

**Neurological reflexes, muscle strength & coordination:** Reflexes observed included, righting, eye blink, ear twitch, whisker orienting, and smell. Muscle strength was assessed using the hanging wire test and motor coordination using the vertical pole test (8).

**Emotional Behavior:** Behaviors in the emotional domain used methods previously developed (8, 28) and included a 5 minute open field test (anxiety) (48 x 48 x 10 cm with a total of 64 squares each 6 x 6 cm), a 5 minute elevated plus maze (anxiety) (two open arms 30 x 6 cm crossed with two closed arms 30 x 5 x 15 cm elevated to a height at 60 cm) and a 6 min forced swimming test (learned helplessness) (11.5 cm in diameter x 14 cm in height). For the tests in the emotional domains, the mice were exposed to the three tests in the order of less stressful to most stressful: the Open Field, then the Elevated Plus Maze, and lastly the Forced Swimming Test.

**Quantification of Pineal Melatonin Levels**

Melatonin levels in pineal glands from male and female mice were determined by radioimmunoassay using the Buhlmann-saliva direct kit (ALPCO, Windham, NH), based on the Kennaway G280 melatonin antibody (40). Briefly, pineal glands were homogenized in assay buffer and spin at 12,000 rpm. Aliquots of the supernatant were incubated with the anti-melatonin antibody and 2-[¹²⁵I]-iodomelatonin for 20 hours. The antibody bound fraction was
precipitated by solid phase second antibody and counted. The concentration of melatonin in the samples was interpolated from a standard curve. The intrassay precision at 3.56 expressed as the coefficient of variance was 4.1 %, while the interassay precision at 3.39 pg/ml expressed as the CV was 7.5 %. The assay limit of analytical sensitivity was 0.2 pg/ml and the functional sensitivity was 0.9 pg/ml.

**Circadian Activity Rhythms**

Circadian activity rhythms were determined in mice housed in individual cages (18 x 30 x 12 cm) equipped with activity wheels. Circadian activity rhythms were measured with a magnetic microswitch that detected wheel rotations on line with a PC. Data were collected and analyzed using CLOCK LAB (Actimetrics, Evanston, IL). Circadian time (CT) 12 was defined as the onset of running wheel activity.

*Circadian Phase Shifts:* Male and female RORβ(C3H)−/−, RORβ(C3H)+/−, and RORβ(C3H)+/+ wild-type mice (2-15 month) were maintained in a 12:12 L:D cycle (300 lux at the level of the cage) for 2 weeks and then transferred to constant and complete darkness. Once a stable free running activity pattern was established, mice received either vehicle (3% ethanol/saline) or melatonin (90 µg in vehicle, s.c.) at CT 10 for three consecutive days. Each mouse was treated with vehicle or melatonin using a crossover design three weeks apart. Light pulses (300 lux, 15 min) were given at either CT14 or at CT 22 to animals free running in constant dark. All treatments were performed under dim red light (15 watt, GE soft light with red 1A Safe Light Kodak filter). The time of the treatment was predicted using Clock Lab software. Steady state onsets of free running activity were determined over a period of 7-10 days, both prior to and after
the treatment. Transient shifts in activity during the three days of melatonin treatment or during the three days that followed the light pulse were not included in the steady state onsets. The magnitude of phase shifts was calculated as the difference between the pre- and post-pulse activity onset.

Re-entrainment to a 6 hour Advance to the L:D Cycle: Mice were kept in a 12:12 L:D cycle (40-60 lux at the level of the cage) for at least 3 weeks. Following this entrainment the onset of darkness was advanced by 6 hours. Vehicle (3% ethanol/saline, s.c.) or melatonin (90 µg/mouse in vehicle, s.c.) or were injected at dark onset for three consecutive days. Daily advances (hours) in running wheel activity onsets were recorded everyday for 10 days.

Statistical Analysis
Statistical analysis was conducted using SPSS (Windows, release 10.0, Chicago, IL) or Graph Pad Prism 4.3 for Windows (GraphPad Software, San Diego, California, USA) software packages. Student’s t test for comparisons between two groups or one or two-way analysis of variance (ANOVA) with the Bonferroni post hoc test for multiple comparison were applied. All analysis was considered statistical significant with P values ≤ 0.05.
Results

Behavioral phenotypic characterization of ROR(C3H)$^{+/+}$ and ROR(C3H)$^{-/-}$ mice

Table 1 shows the physical characteristics, general behavior, and neurological reflexes of RORβ(C3H)$^{+/+}$ mice and mice with genetic deletion of the RORβ gene. No differences were observed in basic physical characteristics between RORβ(C3H)$^{+/+}$ and RORβ(C3H)$^{-/-}$ mice with the exception of palpebral closure. Over 60% of the RORβ(C3H)$^{-/-}$ group showed at least one nonfunctional eyelid (Table 1). RORβ(C3H)$^{+/+}$ and RORβ(C3H)$^{-/-}$ mice showed identical general behaviors while in their home environment with the exception of grooming which was greater in the RORβ(C3H)$^{-/-}$ group (Table 1).

RORβ(C3H)$^{-/-}$ mice showed several impaired neurological reflexes (Table 1 and 2). Neural reflexes that appear to be impaired in RORβ(C3H)$^{-/-}$ mice were the ear twitch, the eyeblink, and the whisker orienting reflexes (Table 1). RORβ(C3H)$^{-/-}$ mice took almost 4 times longer to advance to the smell target as compared to their RORβ(C3H)$^{+/+}$ counterparts (Table 2).

All RORβ(C3H)$^{-/-}$ mice exhibited the prominent and obvious phenotypic “duck-like gait” and hind paw clasping reflex when suspended by the tail (1). Motor coordination was similar in the RORβ(C3H)$^{-/-}$ mouse as compared to the RORβ(C3H)$^{+/+}$ as shown in the vertical pole test (Table 2). However, the RORβ(C3H)$^{-/-}$ mouse exhibited less muscle strength as compared to the RORβ(C3H)$^{+/+}$ group in the hanging wire test (Table 2).
In the emotional domain, RORβ(C3H)^−/− mice showed a high level of approach behavior in the Open Field paradigm, as they transversed over 3 times more square grids as compared to the RORβ(C3H)^+/+ group (Fig. 1A). They also showed more exploratory behavior as determined by their number of rearing and assisted rearing which was almost five times the level of the wild type group (Fig. 1A). No differences in transfer latency or number of defecations were shown (not shown).

In the Elevated Plus Maze paradigm, RORβ(C3H)^−/− mice exhibited similar behavior to that of the RORβ(C3H)^+/+ in the total number of arm entries, number of entries into open and closed arms; RORβ(C3H)^−/− mice, however, spent considerably more time in the open arms (Table 3) representing less anxiety related behavior. Additionally, RORβ(C3H)^−/− mice spent less time with risk related behaviors meaning less apprehension as compared to their RORβ(C3H)^+/+ counterparts.

In the Forced Swimming Test, we assessed whether the “duck gait” abnormality affected the ability to of the RORβ(C3H)^−/− mice to swim. Interestingly, RORβ(C3H)^−/− mice exhibited a more pronounced swim behavior and hence spent significantly less time in the state of immobility as compared to the RORβ(C3H)^+/+ group (Fig. 1B).

*Circadian Phenotypic Characteristics*: Figure 2 A and B shows actograms from male and female mice maintained in a 12/12 L/D cycle showing circadian rhythms of wheel-running activity with higher levels at night. Although the circadian rhythm is maintained across genotypes, the RORβ(C3H)^−/− mice showed significantly reduced wheel revolutions/min [total: 6,384 ± 2,195, p
< 0.01; day (Rho): 790 ± 239, p < 0.01; night (alpha): 5,593 ± 1,986, p < 0.01; n=12] when compared with the RORβ (C3H)+/+ mice [total: 36,092 ± 3,032; day (Rho): 5,115 ± 597; night (alpha): 30,977 ± 2721, n=15], respectively, or RORβ(C3H)+/+ mice (not shown). The amplitude of the rhythm determined by χ² periodogram shows a main effect for genotype (F(2,32) = 5.11, p< 0.05) being more robust in the RORβ(C3H)+/+ (1,926 ± 127, n=15; p < 0.05) and RORβ(C3H)+/+ (2,189 ± 64, n=4, p < 0.05) mice than in the RORβ(C3H)-/- (806 ± 104, n=12) mice (Fig. 2A and B). No main effect for gender or group interaction was observed.

Mice of either genotype or gender entrained to the 12:12 L:D cycle (Figs. 2 and 3). Although the RORβ(C3H)-/- mice are visually blind (1), they stably entrain to the light/dark cycle in a similar fashion as the RORβ(C3H)+/+ counterpart (Fig. 2A and 2C, Fig. 3A-C), suggesting that the RORβ(C3H)-/- mutation did not affect light perception by the circadian system. When released in constant dark, RORβ(C3H)-/- mice free run (Fig. 3A-C) with a period significantly longer than RORβ(C3H)+/+ or RORβ(C3H)+/+ mice [tau: RORβ(C3H)+/+: 23.59 ± 0.04 h, n = 29; RORβ(C3H)+/+: 23.58 ± 0.03 h, n = 36; RORβ(C3H)-/-: 23.98 ± 0.07 h, n = 21] (p < 0.001) (Fig. 3D).

Pineal melatonin levels during day and night

Pineal melatonin levels were determined in pineals collected from the three genotypes, RORβ(C3H)+/+, RORβ(C3H)+/+ and RORβ(C3H)-/- during the day (ZT 9-11) and during the night (ZT 20-22) (Fig. 4). A robust and significant diurnal rhythm of pineal melatonin with low levels during the day and high levels during the night was observed (F_{1,103}=30.08, p < 0.001). For all
genotypes pineal melatonin levels were significantly higher during the night [RORβ(C3H)+/+ (p<0.05), RORβ(C3H)+/− (p<0.001), and RORβ(C3H)−/− (p<0.05)].

Light-induced phase shifts of circadian activity rhythms

Figure 5 shows representative wheel-running activity records from RORβ(C3H)+/+, RORβ(C3H)+/− and RORβ(C3H)−/− mice kept in constant dark and exposed to a light pulse (300 lux, 15 min) at CT14 (Fig. 5A, B, C) or CT 22 (Fig. 5D, E, F). Light pulses administered during either the early or the late subjective night produced phase shifts of locomotor activity of similar direction and magnitude in all three genotypes (Fig. 6B). When administered at the beginning of the subjective night phase (CT 14-18) light induced phase delays [-2.10 ± 0.08 h, n = 14 for RORβ(C3H)+/+, -1.87 ± 0.07 h, n=14, for RORβ(C3H)+/− and -2.01 ± 0.13 h, n=14, for RORβ(C3H)−/−]. Following exposure to light during the late subjective night (CT 20-24), all three groups phase advanced. However, the magnitude of the shifts was significant larger than for mice lacking RORβ gene (F2,43 = 73.09, p < 0.0001) [0.72 ± 0.11 h, n = 10 for RORβ(C3H)+/+, 0.86 ± 0.05 h, n = 17, for RORβ(C3H)+/− and 1.80 ± 0.07 h, n = 17, for RORβ(C3H)−/−].

Melatonin-mediated phase shifts

Mice free-running in constant dark were injected with vehicle or melatonin (90 µg/mouse) for three consecutive days, at the melatonin window of sensitivity: CT 9-11. Figure 7 shows representative actograms of circadian rhythms of wheel-running activity obtained in all three genotypes free-running in constant dark and their response to vehicle (Fig. 7A, C, E) or melatonin (Fig. 7B, D, F) at CT 10. Mice lacking the RORβ receptor responded to melatonin with a phase advance of similar magnitude (1.00 ± 0.22 h, n = 8) as the heterozygous (0.90 ±
0.11 h, n = 15) or the wild type (1.26 ± 0.26 h, n = 11) (Fig. 7). Melatonin did not affect the tau of circadian activity in either genotype (tau change: ROR(C3H)\(^{+/+}\): -0.02 ± 0.03, n = 11; ROR\(\beta(C3H)^{+/+}\): -0.01 ± 0.02, n = 15; ROR\(\beta(C3H)^{-/-}\): -0.02 ± 0.04, n = 8).

**Rate of re-entrainment after phase advance of the light/dark cycle**

The rate of re-entrainment of ROR\(\beta(C3H)^{+/+}\) mice after a 6 hours phase advance was slower than the rate of wild type mice (\(F_{1,260} = 42.31, p < 0.0001\)) (compare Fig. 8A and B). ROR(C3H)\(^{+/+}\) mice needed ~ 7 days to complete the 6 hours advance in the wheel-running activity onset, while ROR\(\beta(C3H)^{-/-}\) mice needed more than 10 days to complete the advance. Melatonin administration facilitated the rate of re-entrainment in both the wild type and ROR\(\beta(C3H)^{-/-}\) mice (Fig. 8), indicating that ROR\(\beta(C3H)^{-/-}\) mice respond to melatonin with the same efficacy as their wild type counterpart.
Discussion

Results from this study show that the RORβ nuclear receptor is involved in sensory processing and that its absence leads to specific behavioral changes. The prolonged circadian period (\textit{tau}) and the reduced rate of re-entrainment to a phase advance of the light/dark cycle in the RORβ(C3H) knockout mice confirm the involvement of the RORβ nuclear receptor in circadian rhythm regulation. In response to melatonin administration RORβ(C3H) knockout mice phase advance the onset of circadian activity rhythms as the wild type counterparts. These results suggest that the RORβ nuclear orphan receptor is not involved in melatonin-mediated phase shifts of running wheel activity. Moreover, in the pineal gland of the C3H mouse the transcription factor RORβ appears not necessary for the circadian production of melatonin, despite the cyclic nature of this gene’s activity.

RORβ(C3H) knockout mice have the same behavioral phenotype as previously reported for RORβ(C57) knockout mice (1) in that they display a duck like gait, male inability to reproduce until 6-9 months of age, retinal degeneration, and an increased free running period in constant darkness. As with RORβ(C57) knockout mice, the phenotype of RORβ(C3H)\textsuperscript{−/−} mice are very similar to that reported for the \textit{vacillans} mutant mouse 50 years ago (35). The pattern of expression of the RORβ mRNA in the central nervous system suggests a role for RORβ in the processing of sensory information as well as in circadian rhythm (27, 33). Our results show a deficit in the RORβ(C3H)\textsuperscript{−/−} mice’s ability to perceive and process sensory information including smell and touch (ear and whisker reflex). Furthermore, the eyeblink response was also altered in the RORβ(C3H)\textsuperscript{−/−} mice, which may represent an additional sensory deficient. However, because
the RORβ(C3H)/− mice showed a high incidence of palpebral closure, motor dysfunction cannot be ruled out as a contributing factor for the lack of responsiveness to the eyeblink test. Interestingly, RORβ(C3H)/− mice showed reduced muscular strength contrary to a previous report (1), demonstrating normal muscular strength in adult C57 mice lacking the RORβ receptor.

Our results show a deficit in the ability to perceive and process sensory stimuli including smell and touch (ear and eye blink twitch reflex) in the RORβ(C3H)/− mouse and provides corroborating behavioral data to the already reported expression of the RORβ mRNA in such areas as the anterior olfactory nucleus posterior (AOP) (33) and sensory cortices (thalamus & cerebellum) (1, 26, 33, 38).

We report for the first time that RORβ(C3H)/− mice are less prone to anxiety related behaviors as evidenced in their performance in the Open Field Test and the Elevated Plus Maze. In the Open Field paradigm the animals were placed in a novel and unfamiliar environment to create conflict by evoking both approach and avoidant behaviors. As such, this test has been cited as inducing moderate anxiety in the animal (11). Our data suggest the RORβ(C3H)/− mouse to be less inhibited, more curious and more responsive to the novel environment thus showing less anxiety-related behavior in comparison to the wild type. Similarly, in the Elevated Plus Maze, RORβ(C3H)/− mice appear to exhibit less anxiety related behaviors thus they are more receptive to novel situations and environments despite their visual deficits.
C3H RORβ⁻/⁻ mice exhibited less depressive-like behavior as appear to show a resistance to learned helplessness induced by the forced swimming test as compared to the C3H RORβ⁺/⁺. Taken together, these animals appear to have above average emotional behavioral characteristics. Numerous mutant animal models show altered anxiety and depression-related behaviors: for example, the mouse knockout for the μ opioid receptor (41) or for the tac1 gene encoding substance P and neurokinin A (6) and the most remarkable, the Clock mutant mouse, carrying a mutation of the circadian gene clock (12). Behavioral changes in clock mice are very similar to the ones reported here, including increased exploratory behavior and less depressive-like behavior. These results suggest a potential interaction of RORβ with clock or other clock genes that may be involved in the modulation of behaviors in the emotional domain.

Disruption of the RORβ orphan receptor does not affect rhythmic synthesis of pineal melatonin in mice with a C3H/HeN background, one of the few melatonin-producing mouse strains. The rhythmic expression of RORβ mRNA coincides with melatonin production in both the retina and pineal gland (1-3), which originally suggested that this nuclear receptor may somehow be involved in the regulation of melatonin production. However, the absence of ROR response elements in the promoter of the AANAT gene suggests that the RORβ receptor may not influence melatonin synthesis. Indeed, our results showed a robust rhythm of melatonin synthesis for the wild type, heterozygous, as well as the RORβ(C3H) knockout mice, arguing against the involvement of the RORβ receptor in the production of pineal melatonin.

RORβ(C3H)⁻/⁻ mice perceive light signals to entrain physiological rhythms to the 24 h light/dark cycle, and phase shift circadian activity rhythms in response to a light pulse when running in
constant dark. The running wheel activity patterns of RORβ(C3H)$^{-/-}$ mice, however, is significantly less robust than with RORβ(C3H)$^{+/+}$ mice showing only small bursts of activity during the subjective night. RORβ(C3H)$^{-/-}$ mice have genetically abnormal retinas due to the lack of the RORβ receptor gene as well as the rd/rd mutation of the C3H background (1, 13). As demonstrated for the RORβ knockout mouse in a C57 background (1) or for the rd/rd C3H mouse (13), the massive loss of cones and rods does not impair the circadian responses, suggesting that in RORβ(C3H)$^{-/-}$ mice, the elements in the visual system mediating circadian responses are intact. In a similar way, RORβ(C3H)$^{-/-}$ mice respond to light with phase shifts into the same direction as the wild type and the heterozygous mice, supporting the concept that in spite of the abnormality of the retinal visual system C3H mice lacking the RORβ receptor still conserve the elements of the retinal circadian system. These elements may include the melanopsin-containing retinal ganglion cells (17) that may account for the non-visual retinal functions as pupil reflex and photoentrainment of circadian rhythms in rod and cone deficient mice (18).

The expression of RORβ mRNA in areas related to circadian physiology (pineal gland, SCN, retina) (1, 33) and the longer free-running period (1) and present results), point towards a role of this nuclear receptor in circadian biology. The nature of that involvement and the targets of RORβ, however, remain to be elucidated. It is conceivable that the endogenous rhythm of activation of RORβ regulates transcriptional activation of clock genes in the SCN. In fact, all-trans retinoic acid activation of RAR or RXR nuclear receptors phase shifts the rhythmic expression of the per2 gene in smooth muscle cells in vitro and in the vasculature in vivo (25). Both the staggerer mutant mouse, lacking a functional RORα receptors, as well as the Rev-erb α
knockout line, have a shorter free running period (32). These transcription factors seem to compete for the same response element on the Bmal1 gene promoter forming part of a positive stabilizing loop (32). On the contrary, RORβ knockout mice show a longer tau, suggesting that this gene product may bind to a different response element or be part of another stabilizing loop. One interesting finding is the larger phase advances observed in ROR(C3H)−/− mice after light pulses administered during late subjective night. This finding suggests that the RORβ nuclear receptor negatively modulates the light-induced phase advance. Whether the potentiation in light-mediated phase shifts in the C3H mouse strain with genetic deletion of the RORβ gene is related to the expression of a circadian period larger than 24 h is not known. Indeed, several studies reported that light-mediated phase advances are positively correlated with the length of the circadian period (29, 34), while others did not observe this correlation (30).

The ability of melatonin to phase advance wheel-running activity rhythms in mice lacking the RORβ gene suggests that this nuclear receptor does not affect the melatonin-mediated phase shifts. In mice, activation of MT1 or MT2 melatonin receptors mediate two distinct responses: inhibition of neuronal firing (MT1) and phase shifts of neuronal firing rhythms in the SCN in vitro (MT2) (9, 20, 23). However, phase shift of overt activity rhythms in vivo appears to require activation of MT1 receptors (9). Here, we demonstrated that the orphan receptor RORβ does not contribute to the phase shift of circadian rhythm of activity by melatonin. Activation of the RORβ receptor was postulated to be the mechanism by which melatonin may transmit photic information from the circadian pacemaker to peripheral tissues, i.e. GnRH mRNA rhythm in the GT1-7 neurons depends on the circadian effect of melatonin on an enhancer region of the GnRH gene containing consensus sequences for the RORβ response elements (31). Recently, however,
the transcription factor involved in melatonin-mediated repression of GnRH gene expression was shown to be COUP-TFI and not RORβ (14).

Taken together these results show that RORβ does not seem to be involved in the relay of photic information by melatonin and may not be a nuclear melatonin receptor (4, 15, 39). However, AVP neurons in the Siberian hamster SCN co-express MT₁ melatonin receptor and the RORβ nuclear receptor mRNAs (36). Similarly, TSH cells of the rat pars tuberalis co-express the MT₁ melatonin receptor and the RORβ orphan receptor (22). While it is unlikely that RORβ nuclear receptor is involved in melatonin-mediated phase shifts, we cannot exclude the possibility that other MT₁ melatonin receptor-mediated responses may require the presence of a functional RORβ receptor.

The rate of re-entrainment following a phase advance of the light/dark cycle is slower in the RORβ(C3H)−/− mouse. This phenotypical characteristic of the RORβ(C3H)−/− correlates with a longer free-running period of the RORβ(C3H)−/− compared to wild type mouse (1; this paper). RORβ(C3H)−/− mice showed the longest free running period in comparison with both wild type and heterozygous mice and the slowest rate of re-entrainment. A longer period in the RORβ knockout mice and therefore the natural tendency to delay counteracts the advance imposed by the light/dark cycle and therefore slows down the re-entrainment process in comparison to wild type mice possessing a period shorter than 24 h. Melatonin accelerates the rate of re-entrainment in the RORβ(C3H)−/− mouse, demonstrating again that the RORβ receptor does not mediate melatonin’s effects on circadian rhythms.
Here we demonstrate that melatonin-mediated effects on circadian activity rhythms are not mediated by activation of the nuclear receptor RORβ. Similarly the synthesis of melatonin is not affected in mice lacking the RORβ receptor. The role of the nuclear RORβ receptor in circadian rhythms is demonstrated by extended tau and reduced rate of re-entrainment in mice lacking this receptor, underpinning RORβ as one of the numerous molecules that may be part of the fine-tuned structure of the circadian biological clock.
Acknowledgments

We thank Dr. Stephanie Hauge for her participation in early experiments and Mr. Kenneth Yun, Ms. Elizabeth Manna, Ms. Iwona Stepien and Ms. Paula Ginter for expert technical assistance. RORβ(C3H)^/- mice were created by Dr. Michael Becker-Andre (Glaxo-Wellcome, Geneva, Switzerland) and kindly provided to us by Glaxo-Smith-Kline (Stevenage, UK). Our thanks to Jeremy M. Davies for editorial assistance.
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References


Legends for Figures

Figure 1. Gross locomotor, anxiety and learned helplessness behaviors in RORβ(C3H)+/+ and RORβ(C3H)^/- mice.

A. Exploratory behavior in the open field test. Each test session was for 5 min. The ordinate represents the number of behaviors expressed as mean and SEM in RORβ(C3H)+/+ mice (n=16; white columns) or in RORβ(C3H)^/- mice (n=18; black columns).

* p<0.05 when compared with RORβ(C3H)+/+ mice (t-test).

B. Time of immobility in the forced swim test. The ordinate represents time of immobility (sec) during the last 4 min of a 6-min forced swimming for RORβ(C3H)+/+ mice (n=16; white column) and RORβ(C3H)^/- mice (n=18; black column).

* p<0.05 when compared with RORβ(C3H)^/- mice (t-test).

Figure 2. Circadian rhythm of wheel-running activity in the ROR(C3H)^+/+, RORβ(C3H)^+ and RORβ(C3H)^/- maintained in a light/dark cycle.

Panels A and C: Single-plotted actograms showing representative wheel-running activity patterns of male (A) and female (C) ROR(C3H)^+/+ (males: n=7; females: n=8), RORβ(C3H)^+ (males: n=3; females: n=1) and RORβ(C3H)^/- (males: n=8; females: n=4) mice. The bar on the top represents the 12/12 light/dark cycle. The ROR(C3H)^+/+ and RORβ(C3H)^+ mice showed robust wheel-running activity rhythms, while activity was reduced in RORβ(C3H)^/- mice.

Panels B and D: Peaks in the corresponding χ² periodogram for each mouse show the dominant and secondary peaks during a 16 days block, for males (B) and females (D). Peaks above the diagonal line (99.9% confidence) were considered significant.
Figure 3. Representative actograms of running wheel activity rhythms in RORβ(C3H)\(^{+/+}\), RORβ(C3H)\(^{+/-}\), and RORβ(C3H)\(^{-/-}\). (A-C): Representative double plotted actograms for RORβ(C3H)\(^{+/-}\), RORβ(C3H)\(^{+/-}\), and RORβ(C3H)\(^{-/-}\) mice maintained in a 12 h light/12 h dark cycle (LD) for 2 weeks and then released into constant dark (DD) on the day denoted by the arrow to assess their endogenous free running rhythms. (D) \(\tau\) period (h) of RORβ(C3H)\(^{+/+}\) (n=29), RORβ(C3H)\(^{+/-}\) (n=36), and RORβ(C3H)\(^{-/-}\) (n=21) male and female mice. RORβ(C3H)\(^{-/-}\) mice showed a significant (F\(_{2,85}=21.69\), p<0.0001) increase in the free-running period in dark/dark compared to the other genotypes.

* p< 0.001 when compared with \(\tau\) in either RORβ(C3H)\(^{+/+}\) or RORβ(C3H)\(^{+/-}\) (Bonferroni post-hoc test).

Figure 4. Pineal melatonin levels during the light and dark cycle in RORβ(C3H)\(^{+/+}\), RORβ(C3H)\(^{+/-}\) and RORβ(C3H)\(^{-/-}\) mice. Ordinate represents melatonin levels expressed as pg/pineal at ZT 9-11 (white columns) and ZT 20-22 (black columns). Melatonin levels where determined using the ALPCO radioimmunoassay kit as described in Methods. A robust rhythm of pineal melatonin content with low levels during the day and high levels during the night was observed for each RORβ(C3H) genotypes (+/+: males: n=12, females: n=16; +/-: males: n=12, females: n=12; -/-: males: n=7, females: n=10) (F\(_{1,103}=30.08\), p < 0.001). *p<0.05, ** p<0.001 when compared to day time melatonin level (Bonferroni post-hoc test).

Figure 5. Representative actograms of running wheel activity rhythms showing the effect of a light pulse during the early and late subjective night in RORβ(C3H)\(^{+/+}\), RORβ(C3H)\(^{+/-}\) and
RORβ(C3H)\textsuperscript{+/−} mice. RORβ(C3H)\textsuperscript{++} (A, B), RORβ(C3H)\textsuperscript{+−} (C, D), and RORβ(C3H)\textsuperscript{−−} (E, F) mice were maintained in constant dark for two weeks. Once a stable free running activity pattern was established mice received a light pulse (300 lux, 15 min) either at CT 14 (A, C and E) or at CT 22 (B, D and F) on the day denoted by the circle.

Figure 6. Phase response curve to light pulses on the circadian rhythm of wheel-running activity in RORβ(C3H)\textsuperscript{++}, RORβ(C3H)\textsuperscript{+−}, and RORβ(C3H)\textsuperscript{−−} mice. Mice were maintained in constant dark for two weeks. (A, B, C). Once a stable free running activity pattern was established mice received a light pulse (300 lux, 15 min) at different circadian times between CT 12 and CT 2. (D). A light pulse (300 lux) at CT 14-18 phase delayed circadian rhythm of wheel-running activity for all genotypes (+/+: n=14; +/−+: n=29; −−: n=14). (E). A light pulse (300 lux) at CT 20-24 phase delayed circadian rhythm of wheel-running activity for all genotypes (+/+: n=10; +/−+: n=17; −−: n=17) with the advance being significantly more pronounced for RORβ(C3H)\textsuperscript{−−} (F\textsubscript{2,43} = 73.26, p < 0.0001). *p<0.001 when compared with RORβ(C3H)\textsuperscript{++} (Bonferroni post-hoc test).

Figure 7. Melatonin phase advanced wheel-running activity rhythms in RORβ(C3H)\textsuperscript{++}, RORβ(C3H)\textsuperscript{+−} and RORβ(C3H)\textsuperscript{−−} mice (A-F) Representative double-plotted actograms. Mice were maintained in constant dark for two weeks. Once a stable free running activity pattern was established RORβ(C3H)\textsuperscript{++} (A: n=8, B: n=11), RORβ(C3H)\textsuperscript{+−} (C: n=10, D: n=15), and RORβ(C3H)\textsuperscript{−−} (E: n=9, F: n=8) male and female mice were treated with either vehicle (3% ethanol/saline, sc) or melatonin (90 µg/mouse in vehicle, sc) at CT 10 for three consecutive days. Arrows point to CT 10 on the first day of treatment. (G) Melatonin-mediated phase advanced of
circadian activity rhythms. Mice free-running in constant dark were treated with either vehicle (white columns) or melatonin (black columns) at CT 10. Melatonin administration resulted in significant phase advances for all three genotypes ($F_{1,55}=44.39$, $p<0.0001$). * $p<0.01$; **$p<0.001$ in comparison with vehicle (Bonferroni post-hoc test).

Figure 8. Effect of melatonin on the rate of re-entrainment to a 6 hours advance in dark onset. After running wheel pattern stabilized in a light/dark cycle, dark onset was advanced 6 hours. Vehicle (3% ethanol/saline, sc) or melatonin (90 µg/kg) were administered at the new dark onset. Melatonin administration facilitated the rate of re-entrainment to the new dark onset in both the RORβ(C3H)$^{+/+}$ mice (vehicle: $n=16$; melatonin: $n=14$) ($F_{1,225}=5.21$; $p < 0.05$) and the RORβ(C3H)$^{-/-}$ mice (vehicle: $n=12$; melatonin: $n=12$) ($F_{1,135}=4.57$; $p < 0.05$).
Table 1. Physical characteristics, general behavioral and neurological reflexes of RORβ(C3H)\(^{+/+}\) mice and RORβ(C3H)\(^{-/-}\).

<table>
<thead>
<tr>
<th></th>
<th>Number of animals exhibiting the indicated behavior</th>
<th>(p) value ((\chi^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piloerection</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 0 (0%) 0 (0%)</td>
<td>ns</td>
</tr>
<tr>
<td>Bald patches</td>
<td>ROR(^{(C3H)}^{-/-}) (n=18) 1 (0.06%)</td>
<td>ns</td>
</tr>
<tr>
<td>Whiskers</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 16 (100%) 18 (100%)</td>
<td>ns</td>
</tr>
<tr>
<td>Palpebral closure</td>
<td>ROR(^{(C3H)}^{-/-}) (n=18) 1 (0.06%) 11 (61%)</td>
<td>(p&lt;0.01)</td>
</tr>
<tr>
<td><strong>General behavior</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sniffing</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 16 (100%) 18 (100%)</td>
<td>ns</td>
</tr>
<tr>
<td>Licking</td>
<td>ROR(^{(C3H)}^{-/-}) (n=18) 0 (0%) 0 (0%)</td>
<td>ns</td>
</tr>
<tr>
<td>Rearing</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 16 (100%) 18 (100%)</td>
<td>ns</td>
</tr>
<tr>
<td>Wild running</td>
<td>ROR(^{(C3H)}^{-/-}) (n=18) 0 (0%) 1 (0.06%)</td>
<td>ns</td>
</tr>
<tr>
<td>Jumping</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 0 (0%) 0 (0%)</td>
<td>ns</td>
</tr>
<tr>
<td>Grooming</td>
<td>ROR(^{(C3H)}^{-/-}) (n=18) 2 (19%) 11 (61%)</td>
<td>(p&lt;0.01)</td>
</tr>
<tr>
<td>Moving around cage</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 16 (100%) 18 (100%)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Neurological reflexes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Righting reflex</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 16 (100%) 18 (100%)</td>
<td>ns</td>
</tr>
<tr>
<td>Ear twitch reflex</td>
<td>ROR(^{(C3H)}^{-/-}) (n=18) 13 (81%) 4 (22%)</td>
<td>(p&lt;0.01)</td>
</tr>
<tr>
<td>Eyeblink reflex</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 16 (100%) 13 (72%)</td>
<td>(p&lt;0.05)</td>
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<tr>
<td>Whisker orienting reflex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jumping</td>
<td>ROR(^{(C3H)}^{+/+}) (n=16) 0 (0%) 0 (0%)</td>
<td>ns</td>
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</tbody>
</table>
Table 2. Olfactory, muscle strength and motor coordination in RORβ(C3H)+/+ mice and RORβ(C3H)+/−.

<table>
<thead>
<tr>
<th></th>
<th>RORβ(C3H)+/+ (n=16)</th>
<th>RORβ(C3H)+/− (n=18)</th>
<th>p value (t-test)</th>
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<tbody>
<tr>
<td>Smell test (sec)</td>
<td>35.6 ± 5.7</td>
<td>128.3 ± 20.5</td>
<td>p &lt; 0.001</td>
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<tr>
<td>Vertical pole test (sec)</td>
<td>38.5 ± 5.9</td>
<td>32.3 ± 4.7</td>
<td>ns</td>
</tr>
<tr>
<td>Hanging wire test (sec)</td>
<td>58.7 ± 1.3</td>
<td>42.9 ± 4.1</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Weight(g)</td>
<td>28.3 ± 7.8</td>
<td>17.0 ± 1.3</td>
<td>ns</td>
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</tbody>
</table>

Table 3. Anxiety-related behaviors in RORβ(C3H)+/+ and RORβ(C3H)+/− mice.

<table>
<thead>
<tr>
<th>ELEVATED PLUS MAZE</th>
<th>RORβ(C3H)+/+ (n=16)</th>
<th>RORβ(C3H)+/− (n=18)</th>
<th>p value (t-test)</th>
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<tr>
<td>Total arm entries</td>
<td>3.81 ± 2.88</td>
<td>2.83 ± 0.60</td>
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<tr>
<td>Entries into open arm</td>
<td>0.31 ± 0.19</td>
<td>0.83 ± 0.25</td>
<td>ns</td>
</tr>
<tr>
<td>Time spent in open arms (sec)</td>
<td>0.97 ± 0.64</td>
<td>57.9 ± 19.3</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Entries into closed arms</td>
<td>3.50 ± 0.68</td>
<td>2.00 ± 0.42</td>
<td>ns</td>
</tr>
<tr>
<td>Time spent in closed arms (sec)</td>
<td>270.1 ± 4.3</td>
<td>233.1 ± 20.8</td>
<td>ns</td>
</tr>
<tr>
<td>Time spent in center platform (sec)</td>
<td>17.2 ± 4.4</td>
<td>10.5 ± 2.5</td>
<td>ns</td>
</tr>
<tr>
<td>Number of risk behavior</td>
<td>6.87 ± 1.06</td>
<td>2.33 ± 0.63</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM. Mice lacking the RORβ gene exhibit less anxiety-like behavior than wild type controls.
Figure 1
Masana et al., 2007
Figure 2
Masana et al., 2007
Figure 3
Masana et al., 2007

A. ROR (C3H)+/+  

B. ROR (C3H)+/-  

C. ROR (C3H)-/-  

D. 

![Graph showing tau (h) for different ROR (C3H) genotypes]

Figure 4
Masana et al., 2007

![Graph showing pineal melatonin levels in different ROR (C3H) genotypes.](image)
Figure 5
Masana et al., 2007

<table>
<thead>
<tr>
<th>CT 14</th>
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<tr>
<td>ROR (C3H)**</td>
<td>ROR (C3H)**</td>
</tr>
<tr>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>B</td>
<td>E</td>
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<td>C</td>
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Figure 6
Masana et al., 2007
Figure 7
Masana et al., 2007

<table>
<thead>
<tr>
<th>Vehicle</th>
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<tbody>
<tr>
<td>ROR (C3H)+/+</td>
<td>A</td>
</tr>
<tr>
<td>ROR (C3H)+/−</td>
<td>C</td>
</tr>
<tr>
<td>ROR (C3H)−/−</td>
<td>E</td>
</tr>
</tbody>
</table>

![Graph showing phase shift (h) for different genotypes with error bars and asterisks indicating significance.](image-url)
Figure 8
Masana et al., 2007