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REV-ERBβ is required to maintain normal wakefulness and the wake-inducing effect of dual REV-ERB agonist SR9009

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ABSTRACT

Circadian signaling regulates and synchronizes physiological and behavioral processes, such as feeding, metabolism, and sleep cycles. The endogenous molecular machinery that regulates circadian activities is located in the suprachiasmatic nucleus of the hypothalamus. The REV-ERBs are transcription factors that play key roles in the regulation of the circadian clock and metabolism. Using pharmacological methods, we recently demonstrated the involvement of the REV-ERBs in sleep architecture. Another group reported a delayed response to sleep deprivation and altered sleep cycles in REV-ERBα null mice, indicating a role of REV-ERBα in sleep. Given that REV-ERBβ is structurally and functionally similar to REV-ERBα, we investigated the role of REV-ERBβ in sleep and wakefulness by assessing electroencephalographic recordings in REV-ERBβ deficient mice and the mechanism underlying effects of loss of REV-ERBβ on sleep. Our data suggest that REV-ERBβ is involved in the maintenance of wakefulness during the activity period. In addition, REV-ERBβ-deficient mice administered with dual REV-ERB agonist SR9009, failed to show drug-induced wake increase. Finally, the expression of a number of genes known to mediate sleep and wakefulness were altered in REV-ERBβ null mice.

1. Introduction

Sleep is an essential behavior conserved across species [1]. Long, continuous, and deep sleep is necessary to promote attention, memory and learning [2]. Sleep and wakefulness are regulated by sleep/wake homeostasis and by the circadian clock [3,4]. Sleep/wake homeostasis controls sleep-need accumulation after sleep disruption. Long wake periods during the resting phase generally result in sleep rebounds that compensate for 'lost' sleep hours. Normal sleep homeostasis ensures that daily activity is compensated for by ample sleep during the resting phase. The circadian clock regulates cycles of sleep and wakefulness [4]. The molecular machinery of circadian rhythm consists of a transcriptional translational feedback loop in which heterodimer partners BMAL1 and CLOCK activate the transcription of E-box-containing genes, Period (*PER*), and Cryptochrome (*CRY*). Once PER and CRY accumulate in the cytoplasm, they translocate to the nucleus and inhibit BMAL1 and CLOCK activity. A large body of literature suggests an important role of core circadian clock proteins in sleep/wake behavior in mouse models. *Bmal1* deletion results in increased total sleep and sleep fragmentation [5], and *Npas2* deletion results in decreased non-rapid eye movement (NREM) sleep time during the activity period [6]. Mice lacking both *Cry1* and *2* exhibit increased sleep during the active period [7]. *Per2* mutant mice become active four hours in advance of the dark phase (active phase for mice) [8], whereas *Per1/2* deficient mice become arrhythmic in constant darkness [9] with random sleep/wake behavior distribution throughout the light/dark periods.

Nuclear receptors REV-ERBα and REV-ERBβ are ligand-regulated transcription factors, which repress the transcription of core clock gene *BMAL1*, *CLOCK*, and the *CLOCK* paralog *NPAS2* [10–12]. These genes, together with cryptochrome (CRY) and period (PER) proteins, form the core molecules that compose the circadian clock. Since its original discovery [13–15], REV-ERBα has been extensively studied, with a growing body of literature supporting its crucial role in clock function and metabolism [11,16,17]. REV-ERBα KO mice exhibit shortened circadian period [18], decreased wheel running during the second half of the dark phase [11], and early wakefulness (as early as two hours before the onset of the dark phase), which is followed by a 'sleep rebound' in the middle of the dark phase [19]. Together with the finding that sleep deprivation significantly reduced transcript levels of REV-ERBα in the forebrain [20], these data suggest a role of REV-ERBα

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in maintaining sleep homeostasis and in regulating circadian cycles. In comparison, the role of REV-ERBβ (which is similar in structure and size to REV-ERBα [21]) in the regulating circadian cycles has not been explored, probably due to the traditional view on REV-ERBβ as a compensatory or even redundant protein [18,22,23] [21,23,24]. This view has been challenged by emerging evidence that suggests distinct functions of REV-ERBβ. ChIP-seq experiments using liver tissue revealed varying degrees of overlap in genes regulated by REV-ERBα and REV-ERBβ, indicating there is considerably less redundancy than originally thought [18].

Here, we hypothesize that REV-ERBβ has unique and important roles, contrasting to the traditional view on its being compensatory or redundant. Using EEG recording, we compared REV-ERBβ deficient mice (generated in our lab [25]) and WT littermates on baseline sleep architecture, including wakefulness, SWS, and REM sleep. We also examined the effect of SR9009 – a REV-ERBα/β agonist with a known wake-inducing effect in C57BL/6J mice [25] – in REV-ERBβ-deficient mice and WT. Last, we analyzed expression of a number of potential circadian transcript targets.

2. Materials and methods

2.1. Mice and husbandry

REV-ERBβ KO mice were generated by our group, as previously described [25]. Briefly, REV-ERBβ floxed mice (REV-ERBβ$^{fl/fl}$) were generated by inserting loxP sites flanking exon 3, at the transgenic core in Pennington Biomedical Research Center in Baton Rouge, LA, using standard gene targeting methods. To generate germ-line deletion of REV-ERBβ, REV-ERBβ$^{fl/fl}$ mice were mated with *EIIa-Cre* mice (Jackson Laboratories, Bar Harbor, ME). Mice were further backcrossed to C57BL/6J mice to remove Cre. WT littermates were used as controls. All experiments were conducted at controlled temperature (22–23 °C), humidity ~60%, and 12 h:12 h light:dark cycles (lights on at 6:00AM). Mice had access to regular chow (Harlan 2920X) and water, *ad libitum*. Mice were bred and procedures were conducted in the Scripps vivarium, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and were approved by the Scripps Institutional Animal Care and Use Committee.

2.2. Electroencephalogram (EEG) recording

16-Week old male C57BL/6J mice (n = 8 per genotype recorded for two weeks, two separate 48 h of sleep/wake cycles analyzed) were used for continuous EEG and EMG recording. Stainless steel screw electrodes were implanted under general anesthesia (1–1.5% isoflurane/oxygen vapor mixture). Two electrodes were positioned on the frontal (0.86 mm anterior and each 2.0 mm lateral to bregma) and two on the parietal (2.0 mm posterior and each 2.0 mm lateral to the bregma) bones. The coordinates were based on The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 1977). In order to reduce signal artifacts, one of the electrodes over the frontal cortex was used as ground. Two other electrodes were used for EEG signal detection. In addition, a pair of wire electrodes was inserted in the neck musculature to record active muscle tone through electromyography (EMG) activity. EEG was recorded in a unipolar arrangement (e.g. frontal vs. parietal cortex). Insulated leads from the EEG and EMG electrodes are soldered to male pins (220-P02), cemented to the skull with dental acrylic. After surgery, mice were individually housed in polycarbonate cages, under a 12 h:12 h light:dark cycle at controlled ambient temperature of 25 °C, and humidity of 50–60%. Mice were allowed 14 days to recover from surgery. For EEG recordings, mice were connected to commutators (PlasticOne) with flexible recording cables allowing them unrestricted movements within the cage and habituated to the recording cages for 72 h. The EEG and EMG signals were amplified in a Grass Model Link15 polygraph in a frequency range of 0.3–10 kHz. The EEG and EMG were acquired on a computer monitor using software supplied by DSI (Data Sciences International, St. Paul, MN, USA – Ponemah v5.20) and stored with a resolution of 128 Hz for offline EEG spectral analysis using the software supplied by DSI (Neuroscore v3.0). The polygraphic results are visually scored in 10-s epochs and classified as wake (W), Slow-Wave sleep (SWS), or Paradoxical sleep or Rapid Eye Movement (REM) – sleep. The total time of these vigilance states is calculated and graphed in minutes within 1 h. Neuroscore EEG Delta power of SWS (0.5–4 Hz) spectra was normalized to the total power averaged from all epochs scored as SWS as previously described [26,27].

2.3. Compound administration

SR9009 (PubChem CID: 16020046) was synthesized as previously described [16] and formulated in 15% cremophor (Kolliphor® EL, Sigma-Aldrich, St. Louis, MO, USA) for all animal studies. Mice were administered 100 mg kg^{-1} (i.p.) of the drug at Zietgeber Time 6 (ZT6) during EEG recording. The specifics of administration of SR9009 for sleep studies are indicated in the figures/figure legends.

2.3.1. RT-PCR

Brains from WT or REV-ERBβ KO male mice (N = 12 per group) were collected at ZT6 for transcript processing. Brains were rapidly removed, frozen on dry ice, stored at −80 °C, and sectioned coronally at 20 μm through various regions (cortex, brain stem, and hypothalamus). Total RNA was extracted using RNA STAT-60 reagent (AMS Biotechnology, Cambridge, MA, USA). All RNA concentrations were adjusted in order to load 1 μg of RNA per cDNA reaction. cDNA was synthesized using qScript™ cDNA SuperMix synthesis kit (Quanta Biosciences, Beverly, MA, USA). Quantification of each transcript by quantitative real time-polymerase chain reaction (RT-qPCR) was performed using SYBR Green dye (Quanta Biosciences, Beverly, MA, USA) to detect dsDNA synthesis, and analyzed using cycling threshold (Ct) values. Relative circadian or sleep transcript abundance was normalized to r18s.

Primers for each gene were as follows:

Circadian genes:

Arntl1	forward:	5′-ATAGTCCAGTGGAAGGAATG-3′
	reverse:	5′-CTCCAGGAGGCAAGAAGATTC-3′
Clock	forward:	5′-ACGAAAGTCATCTCACACCG-3′
	reverse:	5′-CATGGCTCCTAACTGAGCTG-3′
Cry1	forward:	5′-CAC TGG TTC CGA AAG GGA CTC-3′
	reverse:	5′-CTG AAG CAA AAA TCG CCA CCT-3′
Cry2	forward:	5′-CAC TGG TTC CGC AAA GGA CTA-3′
	reverse:	5′-CCA CGG GTC GAG GAT GTA GA-3′
Dbp	forward:	5′-GGAACTGAAGCCTCAACCA-3′
	reverse:	5′-CTCCGGCTCCAGTACTTCTCA-3′
Npas2	forward:	5′-ACCTCACAAAGCAACTCCTG-3′
	reverse:	5′-TTGTGACTTGGGTGGATGTG-3′
Nr1d2	forward:	5′-CATGAGGATGAACAGGAACCG-3′
	reverse:	5′-GCATTCTCTGTTTTTCACGCT-3′
Nr1d1	forward:	5′-ATGCCAATCATGCATCAGGT-3′
	reverse:	5′-CCCATTGCTGTTAGGTTGGT-3′
Per1	forward:	5′-CGG ATT GTC TAT ATT TCG GAG CA-3′
	reverse:	5′-TGG GCA GTC GAG ATG GTG TA-3′
Per2	forward:	5′-CCCTGATGATGCCTTCAGAC-3′
	reverse:	5′-TCAGTTCTTTGTGTGCGTCA-3′
Per3	forward:	5′-AAC ACG AAG ACC GAA ACA GAA T-3′
	reverse:	5′-CTC GGC TGG GAA ATA CTT TTT CA-3′