Bmal1 function in skeletal muscle regulates sleep

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Abstract: Sleep loss can severely impair the ability to perform, yet the ability to recover from sleep loss is not well understood. Sleep regulatory processes are assumed to lie exclusively within the brain mainly due to the strong behavioral manifestations of sleep. Whole-body knockout of the circadian clock gene Bmal1 in mice affects several aspects of sleep, however, the cells/tissues responsible are unknown. We found that restoring Bmal1 expression in the brains of Bmal1-knockout mice did not rescue Bmal1-dependent sleep phenotypes. Surprisingly, most sleep-amount, but not sleep-timing, phenotypes could be reproduced or rescued by knocking out or restoring BMAL1 exclusively in skeletal muscle, respectively. We also found that overexpression of skeletal-muscle Bmal1 reduced the recovery response to sleep loss. Together, these findings demonstrate that Bmal1 expression in skeletal muscle is both necessary and sufficient to regulate total sleep amount and reveal that critical components of normal sleep regulation occur in muscle.

One Sentence Summary: Bmal1 in skeletal muscle regulates sleep.
The ability to recover from sleep loss is critical for preserving cognitive processes and executive functioning (1–3). The mechanisms that are responsible for the recovery from sleep loss are not well understood. Genetic deletion of the circadian transcription factor Bmal1 (brain and muscle ARNT-like factor; gene symbol Arntl) in mice completely ablates circadian clock function (4) and has effects on sleep that include: increased total sleep amount, increased non-rapid eye movement (NREM) sleep intensity and reduced ability to recover from sleep loss (5). Because Bmal1 whole-body deletion causes a broad range of physical abnormalities (e.g., reduced locomotor activity, joint abnormalities, reduced lifespan; 6,7), we sought to isolate sleep phenotypes from the potential effects of other phenotypes using tissue-specific Bmal1 rescue and knockout models.

We first attempted to rescue electroencephalographic (EEG) sleep-phenotypes in Bmal1 knockout mice by restoring functional Bmal1 expression selectively in brain. To do this we used a transgenic model that rescues Bmal1 expression in the brain of Bmal1 whole-body KO’s, thus restoring circadian behavior (Scg2::tTa; tetO::Bmal1-HA; 8). Surprisingly, transgenic Bmal1 expression in the brain (i.e., brain rescued) did not restore NREM sleep-amount—one of the most prominent sleep changes in whole-body Bmal1 knockouts (Fig. 1A, B). Since circadian rhythms of locomotor activity are restored in this model (8), our finding suggests that the sleep disturbances in Bmal1 knockout mice are not exclusively the result of disrupted circadian behavior. More importantly, the results show that Bmal1 expression in brain does not restore normal sleep amounts in Bmal1 knockout mice, suggesting that expression in other tissues may be important.

To begin this investigation of other tissues we chose mice harboring a transgene that restores Bmal1 specifically in skeletal muscle, but does not restore circadian behavior (i.e., muscle rescued; Acta1::Bmal1-HA). This model rescues several muscle-related phenotypes despite non-cycling BMAL1 levels (8). To our surprise, although REM sleep was unaffected (Fig. 1 B and C), we found that restoring Bmal1 in skeletal muscle completely restored NREM sleep amount to wild-type levels in otherwise Bmal1-deficient mice (Fig. 1C). We also assessed whether Bmal1 function in the skeletal muscle altered sleep intensity by measuring NREM slow wave activity (SWA, 0.5-4 Hz; 9, 10). In our hands, NREM SWA was not altered in Bmal1 knockout mice; similarly, NREM SWA was not significantly altered by rescuing BMAL1 in brain or muscle (Fig. 1B & C). These experiments demonstrate that restoring Bmal1 in the skeletal muscle of otherwise Bmal1-deficient mice is sufficient to restore normal NREM sleep amount, independently of Bmal1 expression in the brain. The diurnal rhythm in sleep amount, however, is not restored (Fig. 1A).

To investigate the effect of Bmal1 function on recovery from sleep loss, we subjected the Bmal1 mouse lines to 6 h of forced wakefulness and monitored sleep EEG’s during recovery (Fig. 2A). The amount and type of recovery sleep observed in response to forced wakefulness is commonly used to assess changes in sleep homeostasis (i.e., sleep drive)—this recovery is typically characterized by increased sleep amount and increased SWA (11). Whole-body KO of Bmal1 did not affect NREM recovery-sleep amount (Fig. 2A, B), but did significantly prevent increased NREM-SWA (Fig. 2C, D). Rescue of Bmal1 in either brain- or muscle-rescued mice reduced NREM recovery-sleep following forced wakefulness (Fig. 2A, B), a finding that indicates Bmal1 restoration in either tissue reduces sleep drive following forced wakefulness. Notably, NREM SWA in whole-body KO mice was rescued by restoring Bmal1 in skeletal muscle, but not brain (Fig. 2A, B). These findings demonstrate that restoring Bmal1 in the
skeletal muscle of Bmal1-deficient mice is sufficient to restore normal SWA following sleep loss.

Our results indicate that the rescue of Bmal1 in skeletal muscle is sufficient to restore both NREM sleep amount and the SWA recovery-responses to lost sleep. We next sought to determine if Bmal1 in skeletal muscle was necessary for the effects we observed on sleep processes by specifically deleting Bmal1 in skeletal muscle (12). Mice lacking BMAL1 in their skeletal muscle had significantly increased baseline NREM sleep amount, a result similar to whole-body Bmal1 knockout mice (Fig. 3A-C). Moreover, when muscle-specific knockouts were examined after 6 h of forced wakefulness, recovery sleep was nearly half that of WT mice (Fig. 3D). Forced wakefulness also increased SWA in these mice when compared to controls (Fig. 3D). This effect on SWA suggests that these mice have higher sleep intensity than WTs during recovery from sleep loss. Together, these data support a role for skeletal muscle and Bmal1 in regulating the ability to recover from sleep loss. Moreover, these data support the conclusion that Bmal1 expression in skeletal muscle is both necessary and sufficient for the regulation of normal NREM sleep amount.

That many of the sleep phenotypes caused by whole-body BMAL1 deficiency are either rescued by muscle-specific Bmal1 expression or recapitulated by muscle-specific Bmal1 deletion suggest that sleep phenotypes in the Bmal1 knockout mice are, in whole or in part, due to loss of BMAL1 in skeletal muscle. Thus, these results suggest an important role for skeletal muscle in sleep regulation, implying that Bmal1-dependent processes in skeletal muscle may be useful therapeutic targets for sleep disorders. In an effort to investigate the therapeutic potential of muscle Bmal1, we examined sleep architecture in wild type mice harboring either the brain or muscle-specific (13) Bmal1 transgene. In these mice, transgene expression is in addition to endogenous Bmal1 expression. Neither baseline sleep nor SWA was significantly altered in brain overexpressed mice (Fig. 4B). Baseline sleep amount was not significantly altered in muscle overexpressed mice, however, baseline SWA was significantly reduced (Fig. 4A), suggesting that overexpressing BMAL1 in the muscle renders mice resistant to sleep loss (14). Furthermore, cholinergic neurons of the basal forebrain, which are important for recovery from sleep loss (15), are more active in Bmal1 muscle-overexpressed mice (Fig. 4 S1).

To further investigate recovery from sleep loss, muscle-overexpressed mice were subjected to 24 h of forced wakefulness by placing mice in a slowly rotating wheel (the 6 hours of forced wakefulness used previously is relatively mild). It is common for mice to exhibit some sleep during prolonged forced-wakefulness paradigms, however, Bmal1 muscle-overexpressed mice were awake more than WT littermates during these 24 h of forced wakefulness (Fig. 4C). Similar to baseline, Bmal1 muscle-overexpressed mice also had less NREM recovery after forced wakefulness (recovery = sleep gained in recovery / sleep lost during forced wakefulness; Fig. 4C) and reduced SWA throughout the 72-h protocol compared to WT mice (Fig. 4D), despite sleeping less. In addition, waking SWA (a measure of accumulating sleep pressure during extensive durations of sleep loss; 16) rose more rapidly and was consistently higher than in WT mice during 24 h of forced wakefulness (Fig. 4D). Combined, these results demonstrate that overexpressing Bmal1 in the skeletal muscle renders mice less sensitive to the effects of sleep loss, portending muscle as a potential therapeutic target for sleep loss.

How might peripheral tissues such as muscle influence sleep? The rapidly emerging area of muscle-derived factors on systemic health provides a potential model for our findings. In particular, there are several examples of muscle-derived factors that alter brain processes. Notably, overexpression of PGC-1α (peroxisome proliferator-activated receptor gamma
coactivator 1α, gene symbol: Ppargc1a) selectively in mouse skeletal muscle reduces the depressive phenotypes induced by stress by preventing plasma kynurenine from reaching the brain (17). Furthermore, PGC-1α activation stimulates release of the muscle-derived peptide irisin into circulation (18). Plasma irisin, in turn, induces BDNF expression in the hippocampus (19). Indeed, PGC-1α expression is rhythmic in skeletal muscle (20) raising the possibility that alterations in Bmal1 expression/function may alter rhythmic PGC1α expression through a change in clock function. However, the studies presented here are not sufficient to determine if the sleep effects of loss/gain of Bmal1 function in skeletal muscle are via core clock or non-clock mechanisms. Furthermore, whole body deletions of other circadian factors such as Per1 and Per2 (21), and Cry1 and Cry2 (22), do not have similar effects on sleep. Other potential contributors could be related to changes in muscle metabolism as Bmal1 metabolic phenotypes have been reported in both muscle mouse-line lines used here (23, 24).

Recent studies have highlighted that sleep disruptions in humans are associated with peripheral circadian desynchrony (25, 26). The current study demonstrates that manipulating levels of the circadian transcription factor Bmal1 specifically in skeletal muscle alters sleep. Moreover, a majority of these effects of Bmal1 on sleep are not dependent on circadian timing in the brain—dependence on circadian timing in the skeletal muscle remains a possibility. Although it has been established that sleep is important for skeletal muscle function (for a review, see 27), these investigations are the first to implicate molecular processes within skeletal muscle in signaling sleep regulatory mechanisms in the brain. Studies in our lab are currently underway to determine the nature of the pathway skeletal muscle uses to signal sleep regulatory mechanisms in the brain.

**Figure 1.** Rescuing Bmal1 in skeletal muscle restores daily non-rapid eye movement (NREM) sleep amount. 24-h electroencephalographic recordings were conducted in undisturbed mice listed in the legend. The 24-h pattern of NREM and REM sleep are shown in A. Whole-body knockout of Bmal1 significantly increased NREM sleep when compared to WT controls (B, ANOVA F_{(2,27)}=11.3, p=0.005; p<0.001, posthoc Tukey’s test). Rescuing Bmal1 in the brain of knockouts did not restore NREM sleep to WT levels (B, p=0.001 vs. WT; Tukey’s test); however, the effect of Bmal1 knockout was reversed when Bmal1 was rescued in the skeletal muscle (C, ANOVA F_{(2,31)}=9.9, p<0.001; p= 0.88 vs. WT, p<.001 vs. KO, Tukey’s test). No differences were found in REM sleep or NREM slow-wave activity. Knockout animals are replotted in B and C to aid in comparison. KO mice were offspring from independent crosses of heterozygous Bmal1 KO’s. Representative electroencephalographic recordings are displayed in D. Grey boxes indicate the active/dark period. Bars and points represent mean ± s.e.m. *, p <0.05. WT (brain) n=11, knockout n=12, muscle rescue n=5, brain rescue n=4, WT (muscle) n=16.

**Figure 2.** Rescuing Bmal1 in skeletal muscle or brain reduces the amount of NREM sleep recovered after forced wakefulness. Continuous sleep recordings during 18-h of recovery sleep were obtained from the nesults were found in REM sleep or NREM slow-wave activity. Knockout animals are replotted in B and C to aid in comparison. KO mice were offspring from independent crosses of heterozygous Bmal1 KO’s. Representative electroencephalographic recordings are displayed in D. Grey boxes indicate the active/dark period. Bars and points represent mean ± s.e.m. *, p <0.05. WT (brain) n=11, knockout n=12, muscle rescue n=5, brain rescue n=4, WT (muscle) n=16.

4
mice following forced wakefulness—compared to WT mice (D, % change over corresponding baseline; p<0.01, Tukey’s test). This reduction in slow wave activity was absent when Bmal1 was rescued in the skeletal muscle (D). Knockout animals in B and D are replicated between graphs to aid in comparison. *, p <0.05.

Figure 3. Knockout of Bmal1 in skeletal muscle increases NREM sleep amount and confers resistance to sleep loss. Selective knockout of Bmal1 in skeletal muscle significantly increased NREM sleep, but not REM sleep, when compared to the same animals prior to tamoxifen treatment (A, B; t(10)=2.52, p=0.036). Treatment of floxed mutant control animals with tamoxifen did not significantly alter NREM sleep or REM sleep (C). Following tamoxifen treatment, mice underwent 6 h of sleep deprivation. Muscle knockout mice also had a significantly altered recovery response to this treatment. Significantly less NREM recovery sleep (t(11)=2.44, p=0.033) and increased NREM slow wave activity (t(11)=2.2, p=0.05) was observed in muscle knockout mice when compared to tamoxifen-treated floxed mutants (D). n=6 per group; *, p <0.05.

Figure 4. Overexpression of Bmal1 in skeletal muscle confers resistance to sleep loss. 24-h electroencephalographic recordings were conducted in undisturbed mice overexpressing Bmal1 in skeletal muscle (A) or brain (B). No differences in baseline sleep amount were found, however, NREM slow wave activity was significantly reduced in mice overexpressing Bmal1 in skeletal muscle (A). Overexpression of Bmal1 in skeletal muscle also significantly decreased the NREM-recovery response to one (24h) day of forced wakefulness by means of a slowly rotating wheel (C, D). It is not unusual for mice to obtain brief amounts of sleep (i.e. micro-sleep) during such an extended regimen of forced wakefulness. This sleep obtained during forced wakefulness was also significantly lower in mice overexpressing Bmal1 (A, ANOVA main effect of genotype F(1, 96)=10.12, p=0.002, main effect of time F(11, 96)=4.1, p<0.001; B, NREM sleep amount t(8)=1.85, p=0.047, NREM recovery sleep t(8)=1.9, p=0.039). NREM slow wave activity (a standard marker of sleep intensity) was consistently lower in Bmal1 muscle-overexpressed mice throughout the 72-h protocol (D, repeated-measures ANOVA F(1,8)=6.57, p=0.04). In contrast, during forced wakefulness waking slow-wave activity was significantly increased in Bmal1 muscle-overexpressed mice (D, repeated-measures ANOVA main effect of genotype, F(1,8)=5.7, p=0.045). Grey bars indicate forced wakefulness, black bars indicate darkness (active period). Data presented as mean ± s.e.m, n=16 muscle overexpression, n=16 littermate controls (muscle), brain overexpression n=8, littermate controls (brain) n=11. *, p <0.05.
Supplementary Materials:

Overexpression of Bmal1 in the skeletal muscle increases Fos-IR in cholinergic neurons.

The finding that Bmal1 muscle overexpression altered brain EEG led us to investigate whether the manipulation had other influences on brain phenotypes that are related to sleep-wake processes. Therefore, we determined the effects of Bmal1 muscle overexpression on neuronal activation in sleep regulatory regions. FOS-immunoreactivity (IR) was measured in the following brain areas: 1) forebrain (nucleus accumbens [NAc/s], basal forebrain [BF], ventral pallidum [VP]); (2) hypothalamus (ventral lateral [VLPO] and medial preoptic [MPOA] areas, suprachiasmatic nucleus [SCN]); (3) thalamus (lateral habenula [LAh]); and (4) hindbrain (ventral [VTA], pedunculopontine [PPT], and laterodorsal [LDT] tegmental areas [S1]). Brains were collected at midday under basal (undisturbed) conditions or immediately after a 6 h of sleep deprivation.

FOS-IR was increased in the forebrain, midbrain, and hindbrain of Bmal1 muscle overexpressed mice relative to WTs (BF: F_{1,16}=26.6; p<0.001, univariate ANOVA; Hb: F_{1,16}=34.5; p<0.001). There were also significant increases in FOS-IR in the forebrain, midbrain and hindbrain (NAc: F_{1,16}=15.5; p=0.001; BF: F_{1,16}=6.8; p=0.01; PPT: F_{1,16}=12.6; p=0.004; Hb: F_{1,16}=26.6; p<0.001; MpOA; F_{1,16}=15.6; p=0.001) of sleep-deprived mice. Most notably, there were no main effects for genotype or treatment in the SCN. This observation indicates that disrupted circadian timing is not responsible for sleep phenotypes in Bmal1 muscle overexpressed mice.

Coincidentally, the brain areas that had increases in FOS-IR in response to Bmal1 muscle overexpression—the basal forebrain, lateral habenula, and pedunculopontine tegmentum—are known to be arousal promoting and have extensive cholinergic tone. We double-labeled with ChAT (enzyme necessary for acetylcholine synthesis) to determine if cholinergic neurons are FOS-positive. Our findings indicate that increases in Fos-IR of Bmal1 muscle overexpressed mice occurred in cholinergic neurons (BF: F_{1,20}=5.6; p=0.02; Hb: F_{1,20}=14.8; p=0.002; PPT: F_{1,20}=13.5; p=0.003; S1).

Overexpression of Bmal1 in the skeletal muscle does not cause circadian impairments in wheel running

The patterns of circadian wheel-running activity have been reported in the majority of mouse models reported here (8.22); however, this wheel-running activity is not available for Bmal1 muscle overexpressed mice. Therefore, we investigated behavioral circadian rhythms in Bmal1 muscle-overexpressed mice and WT littermates.

Bmal1 muscle overexpressed mice entrained to LD and had a nighttime activity onset of 12.2±8.4 min after lights-off. WTs entrained to LD with a nighttime activity onset of 5.4±3.4 min after lights-off (onset: F_{1,14}=0.77; p=0.39, one-way ANOVA). The length of the nighttime activity period averaged 11.0±1.1 h for Bmal1 muscle overexpressed mice and 10.7±0.6 h for WTs (F_{1,14}=0.06; p=0.81, one-way ANOVA). The average duration of a wheel running bout was 176.2±52.3 min for Bmal1 muscle overexpressed mice and 131.6±19.4 min of wheel running for WTs (duration: F_{1,14}=1.3; p=0.27; intensity: F_{1,14}=0.49; p=0.41, one-way ANOVA).
Overexpression of Bmal1 in the skeletal muscle did not change the endogenous rhythm of wheel running. Rhythm period for Bmal1 muscle overexpressed mice averaged 23.7±0.1 h compared with 23.8±0.2 h for WT (F<sub>1,13</sub>=0.90; p=0.36, one-way ANOVA). The duration of the subjective night was also unaffected in Bmal1 muscle overexpression mice compared with WT (10.4±1.1 h vs. 9.1±0.9 h, respectively; F<sub>1,13</sub>=0.18; p=0.68, one-way ANOVA).

Methods

Animals

All mice in the brain-lines and muscle rescued/overexpressed lines were maintained on a 12-h light:12-h dark schedule throughout the study. Food and water were available ad libitum, and animals (10-12 w of age) were individually housed for at least 2 weeks prior to experimental use. All protocols and procedures were approved by the Morehouse School of Medicine Institutional Animal Care and Use Committee.

Bmal1 brain-rescued and brain overexpressed mice used the tetracycline transactivator (tTA) system, which requires two transgenes for expression of the target gene Bmal1 (previously reported in McDearmon et al., 2006, 8; RRID:MGI:3714773). The promoter sequence of the secretogranin II gene (Scg2), which is expressed exclusively in the brain, drives expression of the tetracycline transactivator (tTA). The tTA protein binds to the tetracycline operator (tetO) sequence and drives expression of Bmal1 cDNA. The double transgenic mice were crossed onto a Bmal1 knockout background (RRID:IMSR_JAX:009100) to create brain rescued mice and were crossed onto a Bmal1 WT background to create brain overexpressed mice. Breeding was conducted in-house and genotypes were recorded as they became available from breeding. For rescue lines, animals from approximately 20-25 litters comprised the entire dataset. WT littermates obtained from these litters were kept separate for comparisons in each line. KO mice were offspring from independent crosses of heterozygous Bmal1 KO’s.

Briefly, Scg2::tTA mice express the tTA transcript broadly in the brain and is enriched in the SCN, when assessed by both an oligo to the tTA transcript and by crosses with tetO-promotor-linked reporter lines (29). Furthermore, in situ hybridization in Scg2::tTA X tetO::Bmal1-HA and tetO::Bmal1-HA mice demonstrate that Bmal1 expression is under strict control of the tetracycline transactivator: Hemagglutinin (HA)-tag expression is only detectable in the double transgenic mouse. Both Bmal1 mRNA and protein are constitutively expressed in the brain of brain-rescued mice. (8). Brain specificity has been demonstrated by western blot which shows an absence of HA-staining in both the muscle and liver of double transgenic mice (8).

Bmal1 muscle-rescued and muscle overexpressed mice were generated with the use of a DNA construct consisting of human α-actin-1 promoter sequence positioned upstream of Bmal1 (RRID:MGI:3714769; 8). The transgenic mice were crossed onto a Bmal1 knockout background to create muscle-rescued mice, and also crossed onto a Bmal1 WT background to create brain overexpressed mice.
The skeletal muscle-specific Cre-recombinase mouse (Acta1-cre/Esr1\textsuperscript{\textsection}), RRID:IMSR_JAX:025750) was generated in house (12). Breeding with the floxed Bmal1 mouse (Bmal1\textsuperscript{lox/lox}, The Jackson Laboratory, RRID:IMSR_JAX:007668; 30) generated the inducible muscle knockout mouse. These offspring (Bmal1\textsuperscript{lox/lox}, Acta1-cre/Esr1\textsuperscript{\textsection}) allow selective deletion of the bHLH domain of Bmal1 in skeletal muscle upon tamoxifen administration.

The Acta1 promoter used for both mouse lines (muscle rescued/overexpressed and muscle KO) is a 2.2 kb sequence directly upstream from the human skeletal actin (Acta1) translational start site. Proper developmental and tissue specific expression has been verified previously using an Acta1::CAT mouse line. These findings included a demonstrated lack of expression in the brain (31). Specific Cre-dependent excision of a loxP-flanked gene in mouse striated muscle fiber was also demonstrated in mice expressing Cre recombinase under the control of this same promoter (32). Transgene expression in the inducible muscle knockout mice (Acta1-cre/Esr1\textsuperscript{\textsection}) used here is constitutive and was not detectable in brain by western blot (33).

A lack transgene expression in the brain has also been demonstrated for the Acta1::Bmal1-HA line (8). We verified this finding by western blotting an entire brain hemisphere or gastrocnemius muscle in mice bred at our facility (Figure 1 S1). HA-tag was detected in skeletal muscle, but not brain.

**Surgery**

EEG and EMG electrodes were implanted in anesthetized mice. A prefabricated head mount (Pinnacle Technologies, KS) was used to position three stainless-steel epidural screw electrodes. The first electrode (frontal—located over the frontal cortex) was placed 1.5 mm anterior to bregma and 1.5 mm lateral to the central suture, whereas the second two electrodes (interparietal—located over the visual cortex and common reference) were placed 2.5 mm posterior to bregma and 1.5 mm on either side of the central suture. The resulting two leads (frontal-interparietal and interparietal-interparietal) were referenced contralaterally. A fourth screw served as a ground. Electrical continuity between the screw electrode and head mount was aided by silver epoxy. EMG activity was monitored using stainless-steel Teflon-coated wires that were inserted bilaterally into the nuchal muscle. The head mount (integrated 2 × 3 pin grid array) was secured to the skull with dental acrylic. Mice were allowed to recover for at least 14 days before sleep recording.

**EEG/EMG Recordings**

One week after surgery, mice were moved to the sleep-recording chamber and connected to a lightweight tether attached to a low-resistance commutator mounted over the cage (Pinnacle Technologies). This enabled complete freedom of movement throughout the cage. Except for the recording tether, conditions in the recording chamber were identical to those in the home cage. Breeding was conducted in-house and genotypes were recorded as they became available from breeding. Recordings from approximately 20-25 litters make up the dataset. All wildtype and KO littermates resulting from a litter, up to a maximum of 50%, were recorded with tissue-specific knockout/rescue mice. Mice were allowed a minimum of 7 additional days to acclimate to the tether and recording chamber. Recording of EEG and EMG waveforms began at zeitgeber time (ZT) 0 (light onset). Data acquisition was performed on a personal computer running Sirenia Acquisition software (Pinnacle Technologies), a software system designed specifically for polysomnographic recording in rodents. EEG signals were low-pass filtered with a 40-Hz cutoff and collected continuously at a sampling rate of 400 Hz. After collection, all waveforms were...
classified by a trained observer (using both EEG leads and EMG) as wake (low-voltage, high-frequency EEG; high-amplitude EMG), NREM sleep (high-voltage, mixed-frequency EEG; low-amplitude EMG) or rapid eye movement (REM) sleep (low-voltage EEG with a predominance of theta activity [6–10 Hz]; very low amplitude EMG). EEG epochs determined to have artifact (interference caused by scratching, movement, eating, or drinking) were excluded from analysis. Recordings where artifact comprised more than 5% of total recording time were excluded from analysis. Analysis of NREM delta power and NREM spectral distribution was accomplished by applying a fast Fourier transformation to raw EEG waveforms. Only epochs classified as NREM sleep were included in this analysis. Delta power was measured as spectral power in the 0.5 to 4 Hz frequency range and expressed as a percentage of total spectral power in the EEG signal (0.5-100 Hz) during that time period.

**Forced Wakefulness**

Homeostatic challenge: Six-hour forced wakefulness was conducted in all mouse lines (Figure 2). Following a 24-hr baseline recording, mice were sleep deprived during the first 6 hours of the light phase (ZT 0–6) by gentle handling (introduction of novel objects into the cage, tapping on the cage and when necessary delicate touching) and allowed an 18-hr recovery opportunity (ZT 6–0).

Twenty-four-hour forced wakefulness: Following a 24-h baseline recording, Bmal1 muscle overexpressed lines and WT littermates were moved to a slowly rotating wheel (9 inches in diameter; 1 rpm) adjacent to the recording cage (Figure 3). Mice were confined to this wheel for 24 h beginning at ZT 0 (lights on) during which time they had free access to food and water. Following sleep deprivation, animals were returned to the baseline recording cage and EEG acquisition was continued for a 24-h recovery opportunity.

**Fos- and choline acetyltransferase (ChAT) immunoreactivity (IR)**

Mice were sacrificed by CO₂ inhalation at ZT 6 (ZT0 represented lights-on under LD) after 6 h of forced wakefulness. Brains were immersion-fixed in 4% paraformaldehyde for 24 h then sunk in 30% sucrose (24 h at 4°C). Cryostat sections (40µm-thick) were incubated with a rabbit polyclonal IgG antibody (c-fos; Santa Cruz Biotechnology, Santa Cruz, CA; chicken polyclonal IgY antibody [ChAT]; Novus Biologicals, Littleton, CO) and immunoreactivity was visualized using Vectastain Elite ABC kit with 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromagen (Vector Labs, Burlingame, CA). Sections were mounted with permount, and Fos expression was quantified using ImageJ (National Institutes of Health, Bethesda, MD). Counts of immunostained nuclei were undertaken in the mid-to-posterior region of each brain site.

**Quantitative PCR**

Methods were described previously (33). Briefly, brain (hypothalamic) and skeletal muscle (gastrocnemius) tissues were collected from mice sacrificed at ZT5 and ZT17 (ZT12 was lights-off under a 12:12 light:dark cycle); these are the mid-points of peak and trough Bmal1 gene expression in skeletal muscle (35). Total RNA was extracted from frozen brain and skeletal muscle using Trizol (Invitrogen, Carlsbad, CA) and diluted to 0.1 mg/ml. Samples were converted from RNA to cDNA with Applied Biosystems RT-PCR kit reagents according to the manufacturer’s instruction. Real-time PCR assays were performed using the comparative amplification detection threshold of target gene expression (CT) method. mRNA levels detected
with SYBR Green (Bio-Rad; Hercules, CA) were measured by determining the cycle number at which CT was reached. In each sample, CT was normalized to Gapdh expression (ΔCT) performed on the same plate. Normalized gene expression (ΔΔCT) for each gene with respect to genotype and time point was computed with Bio-Rad CFX Manager.

Western Blotting

Brain hemispheres were homogenized in a microfuge tube using a pellet pestle in 700 ul lysis buffer (20mM Hepes pH 7.6, 400mM NaCl, 1mM EDTA, 5mM NaF, 0.3% Triton-X 100, 5% glycerol, 1mM DTT, 250mM PMSF, and complete protease inhibitor mix (Sigma), and tumbled at 4C overnight prior to quantification and loading on the gel. Muscle proteins were extracted similarly, except 300ul to lysis buffer was used, and the lysis buffer contained 1% Triton X-100 and 10% glycerol. Protein concentrations were determined using a BCA assay kit (Pierce), and separated on an Any-kDa minigel (BioRad). Western blot was performed with anti-HA-HRP conjugated monoclonal antibody (Roche) and anti-Gapdh (Santa Cruz).

Behavioral Phenotyping

Wheel running. Male mice were provided with continuous access to running wheels interfaced to a ClockLab data acquisition system (Coulbourn Instruments, Whitehall, PA) for 2 wk under LD followed by 2 weeks under constant darkness (DD; n=8/genotype). Data were collected in 10 min bins. Nighttime activity onset (designated as zeitgeber time [ZT] 12 under LD and circadian time [CT] 12 under DD) was defined by the initial 10 min period that: 1) exceeded 10% of the maximum activity for the day, 2) was preceded by at least 4 h of inactivity, and 3) was followed by at least 60 min of sustained activity. Nighttime activity offset was defined as the final period of nighttime activity that: 1) was preceded by at least 60 min of sustained activity and 2) was followed by at least 4 h of inactivity. Alpha (measured in h) represented the time between nighttime activity onset and offset. Rhythm period was determined from extrapolation of the least squares line through activity onsets on days 3-14 under LD and DD. The duration (bout) and intensity (count) of wheel running under LD and DD were also measured.

Beam Breaks. A SmartHomeCage data acquisition system (AfaSci, Redwood City, CA) equipped with a multi-layer 3D array of infrared sensors was placed around the home cages of male mice (n=7-8/genotype) to measure gross and fine motor movements under LD (following entrainment) and DD (3rd full 24 h period, CT time derived from each individual animal’s rhythm period). Behavioral parameters included the number of spontaneous movements (counts), % activity in 1 h blocks, distance travelled in home cage, and minutes of gross (ambulatory) and fine (grooming) motor movements.

Statistics

Sleep data were analyzed using one-way analysis of variance (ANOVA), repeated-measures ANOVA or Student’s t. Significance was defined as P ≤ 0.05. Post hoc analysis was conducted using Tukey’s HSD method or student t-test where indicated. Tukey’s HSD method uses the studentized range statistic and maintains family-wise error-rate at 0.05. An appropriate sample size of 5 was predicted with Type I error rate of 0.05 and Type II error rate of 0.2. Standard deviation and mean difference were estimated as 46.4 and 100 min, respectively, based on the existing literature (5) and previous studies in our lab. Sample sizes (biological replicates) for each experiment are indicated in the figure legends.
Figure 1 Supplemental Figure 1. *Bmal1-HA is not detectable in the Brains of Acta1::Bmal1-HA mice.* Fifty micrograms of proteins extracted from either an entire brain hemisphere or skeletal muscle (gastrocnemius) were subjected to western blotting with the indicated antibodies.

Figure 2 Supplemental Figure 1. Rescuing *Bmal1* in skeletal muscle or brain reduces the amount of NREM sleep recovered after forced wakefulness. Continuous sleep recordings during 18-h of recovery sleep were obtained from the mice in Fig. 1 after 6 h of forced wakefulness (squares, dotted line). NREM sleep amount is plotted as a percentage of total sleep for 2-h bins across 24-h days. Each plot includes baseline sleep (circles, solid line) on the preceding day for comparison. (yellow double arrow = forced wakefulness). Points represent mean ± s.e.m. *, p <0.05 Tukey’s. Statistics are not reported for the first 6h (sleep deprivation period).

Figure 3 Supplemental Figure 1. Slow wave activity in inducible muscle knockout mice and controls during undisturbed baseline sleep.

Figure 4 Supplemental Figure 1. Fos-immunoreactivity (IR) in densely cholinergic areas is increased in *Bmal1* muscle-overexpressed mice. Fos-IR in three brain areas known to be involved in sleep—wake regulation was assessed. Male mice were sacrificed at mid-day by CO₂ inhalation in the presence (SD) or absence (Bshn) of 6 h sleep deprivation. **, p <0.05; wild-type n=5; muscle overexpressed n=6. Basal forebrain, $F_{1,16}=26.6$, $p<0.001$, univariate ANOVA; lateral habenula (Hb), $F_{1,16}=75.6$, $p<0.001$; pedunculopontine tegmentum (PPT), $F_{1,16}=31.0$, $p<0.001$.

Figure 4 Supplemental Figure 2. Activity rhythms in *Bmal1* muscle-overexpressed mice are modestly increased when compared to wildtype mice. No differences were found in the circadian rhythm of wheel-running activity between either genotype [Left panel]. Overall locomotor activity measured with infrared sensors revealed increased activity of muscle-overexpressed mice at several time points (n=7-8/genotype; Right panel). *, p<0.05, repeated measures ANOVA [one-way ANOVA, post-hoc]; zeitgeber time 12 = activity onset; LD, light—dark cycle; DD, constant darkness.

Figure 4 Supplemental Figure 3. *Bmal1* muscle-overexpressed mice do not have altered levels of CLOCK:BMAL1-target genes in the brain or muscle. No significant differences in CLOCK:BMAL1-target gene expression between wildtype and muscle-overexpressed mice were found in the hypothalamus at either time point investigated (ZT5 and ZT17). As expected, *Bmal1* expression was significantly elevated in the muscle of muscle-overexpressed mice (32.4-fold increase at ZT5 vs. WT; $F_{1,22}=15.3$, $p=0.001$, two-way ANOVA; 5.1-fold increase at ZT 17, $p=0.003$). **, p <0.05.
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Figure 1

REM

NREM

Wild Type

Knockout

Muscle Rescued

Brain Rescued

REM Sleep (% 2-h interval)

NREM Sleep (% 2-h interval)

Brain overexpressed

Muscle overexpression

Wild type

Brain rescue

Bmal1

Acta1

KO

NREM slow wave activity (relative)

REM Sleep (% 2-h interval)

Awake

NREM

REM

Legend

Wild type (Bmal1<sup>+/+</sup>)

Knockout (Bmal1<sup>-/-</sup>)

Muscle rescued (Bmal1<sup>+/+</sup>; Acta1::Bmal1-HA);

teto::Bmal1-HA)

Brain rescued (Bmal1<sup>+/+</sup>; Scg2::tTA; tetO::Bmal1-HA)
Acta1::Bmal1-HA

| Animal I.D. | Brain   | Muscle  |
|-------------|---------|---------|
|             | 357 369 | 357 369 |

Western blot:

- Anti-HA
- Anti-Gapdh

75kDa

37kDa
Legend

- **Black** Inducible Muscle Knockout 
  \((\text{Bmal1}^{\text{lox/lox}}, \text{Acta1-cre/Esr1}^*)\)

- **Blue** Muscle Knockout

- **Gray** Floxed mutant 
  \((\text{Bmal1}^{\text{lox/lox}})\)

- **Crosshatched** Floxed mutant 
  \((\text{Bmal1}^{\text{lox/lox}})\)
Baseline Sleep

Sleep Deprivation

NREM sleep (minutes)

Wild type
KO
Brain overexpression
Brain rescue

Time (h)

Muscle Overexpressed
Brain Overexpressed
Wild Type

NREM Sleep

24h
Total

Relative NREM Slow Wave Activity

REM sleep (minutes)

Muscle Overexpressed
Brain Overexpressed
Wild Type

24h
Total

NREM sleep gained / sleep lost

Sleep Deprivation

Baseline Sleep

Sleep Deprivation
Littermates

Muscle Overexpressed

Bmal1

LD

DD

Light:Dark Cycle

Zeitgeber Time (h)

Beam Breaks

Constant Darkness

Circadian Time (h)

Beam Breaks
