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Publication Date

2017

DOI

Peer reviewed

Homeostatic effects of exercise and sleep on metabolic processes in mice with an overexpressed skeletal muscle clock

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Abstract

Brain and muscle-ARNT-like factor (Bmal1/BMAL1) is an essential transcriptional/translational factor of circadian clocks. Loss of function of Bmal1/BMAL1 is highly disruptive to physiological and behavioral processes. In light of these previous findings, we examined if transgenic overexpression of Bmal1/BMAL1 in skeletal muscle could alter metabolic processes. First, we characterized *in vivo* and *ex vivo* metabolic phenotypes of muscle overexpressed mice (male and female) compared to wild-type littermates (WT). Second, we examined *in vivo* and *ex vivo* metabolic processes in the presence of positive and negative homeostatic challenges: high-intensity treadmill running (positive) and acute sleep deprivation (negative). *In vivo* measures of metabolic processes included body composition, respiratory exchange ratio (RER; VCO_2/VO_2), energy expenditure, total activity counts, and food intake collected from small animal indirect calorimetry. An *ex vivo* measure of insulin sensitivity in skeletal muscle was determined from radioassays. RER was lower for muscle overexpressed females compared to WT females. There were no genotype-dependent differences in metabolic phenotypes for males. With homeostatic challenges, muscle overexpressed mice had lower energy expenditure after high-intensity treadmill running. Acute sleep deprivation reduced insulin sensitivity in skeletal muscle in overexpressed males, but not WT males. The present study contributes to a body of evidence showing pleiotropic, non-circadian, and homeostatic effects of altered Bmal1/BMAL1 expression on metabolic

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processes, demonstrating a critical need to further investigate the broad and complex actions of Bmal1/BMAL1 on physiology and behavior.

Keywords

Insulin sensitivity; Resting energy expenditure; VO2 max; Restricted sleep; Treadmill running; Molecular circadian clock

1. Introduction

Brain and muscle-ARNT-like factor (Bmal1/BMAL1) is most commonly known for its function as a positive transcriptional-translational factor of the molecular clock in the brain and periphery [1]. Its expression is essential for maintaining daily rhythms of physiology and rest/activity. The necessity of Bmal1/BMAL1 for maintaining circadian timekeeping has been identified in studies with whole-body and tissue-specific knockout (KO) mice [2, 3, 4]. Studies with whole-body and tissue-specific knockout (KO) mice have also demonstrated the essentialness of Bmal1/BMAL1 for maintaining metabolic homeostasis; compromised mitochondrial respiration [5] and reduced sensitivities to glucose and insulin [6, 7, 8, 9] in skeletal muscle, pancreas, and liver have been reported *in vivo*.

The present study is unique in that we used a transgenic overexpressed model instead of whole-body or tissue-specific knockout (KO) models. This allowed us to study the dynamics of metabolic processes in the absence of compromised circadian timekeeping. We selected an overexpression model of Bmal1/BMAL1 specific to the skeletal muscle for several important reasons. First, muscle overexpressed Bmal1/BMAL1 micehave robust behavioral and molecular rhythms; overexpression does not shift the timing or amplitude of other clock (*Per2*) and clock-controlled (*Dbp*) genes [see 2.2 of Materials and Methods and ref. 3]. Second, transgenic overexpression of other gene products driven by the *Acta1* promoter has yielded several advantageous metabolic and behavioral phenotypes as reported previously in *Biochimie* [10]. Thus, this is one of few studies to examine *in vivo* and *ex vivo* (metabolic) phenotypes of males and females in an overexpressed line driven by the *Acta1* promoter in comparison to wild-type littermates (WT).

We also aimed to determine if the dynamics of metabolic processes were amplified by positive and negative homeostatic challenges of high-intensity treadmill running (positive) and acute sleep deprivation (negative). It is well documented in rodents and humans that metabolic and endocrine function are altered in opposing directions by high-intensity exercise [11, 12] and restricted and fragmented sleep [13, 14, 15]. It is also known that sleep deprivation alters DNA-binding of BMAL1 [16]. Given previous findings, we hypothesize that overexpression of Bmal1/BMAL1 would alter the dynamics of metabolic processes *in vivo* and *ex vivo*. We also hypothesize that genotype-dependent differences in metabolic processes would be amplified in the presence of both positive and negative homeostatic challenges.

2. Materials and Methods

2.1 Transgenic muscle overexpressed mice

Adult (10–12 weeks) mice were used in all experiments. Animals were housed in a temperature-controlled vivarium (23°C) under an entrained 12 h-12 h light-dark cycle. Food and water were provided *ad libitum*. This transgenic muscle overexpressedline (*Acta:Bmal1*) has been used by McDearmon *et al.* 2006 [3] to examine the differential effects of *Bmal1* expression in brain versus skeletal muscle on circadian and skeletal muscle physiology. Mice were bred on a CD1 strain background and were back-crossed to C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) for ten generations. Overexpressed *Bmal1* in skeletal muscle was derived from a DNA construct consisting of a human alpha-skeletal actin (*Acta1*) promoter sequence positioned upstream of *Bmal1* tagged to a hemagglutinin complex.

2.2 Clock and Clock-controlled gene expression profiles of transgenic overexpressed mice

Real-time PCR assays have previously been performed using the comparative amplification threshold of target gene expression (C_T) method. mRNA levels of clock regulatory genes (*Bmal1, Per2, Per3, Dbp, Rev-Erb-alpha*) were detected with SYBR Green (Bio-Rad; Hercules, CA). In each sample, C_T was normalized to *Gadph* expression (C_T) performed on the same plate and normalized gene expression (C_T) was computed with Bio-Rad CFX Manager (Bio-Rad; Hercules, CA). Brain (hypothalamic) and skeletal muscle (gastrocnemius) were collected from *Acta:Bmal1* mice and wild-type littermates (WTs). Mice were sacrificed at the daily mid-points of maximal peak (~ZT 23.5, with ZT 12 representing lights-off under a 12 h light: 12 h dark photocycle) and minimal trough (~ZT 11.5) of *Bmal1* gene expression in skeletal muscle (ZT 5 [run]; ZT 17 [rise]; n=5–6 per genotype and time point). Results from our laboratory corroborate with results from PCR assays of *Per2* and *Dbp* derived from brain and skeletal muscle and collected near the daily maximal peak of *Bmal1* gene expression from the transgenic (*Bmal1*) muscle overexpressed mice reported in McDearmon et al. [ref. 3]. To no surprise, *Acta:Bmal1* mice had 32.4-fold more *Bmal1* expression at ZT 5 (post-hoc t-test; p=0.001) and 5.1-fold more *Bmal1* expression at ZT 17 (post-hoc t-test; p=0.003) compared to WTs. There were no main effects of genotype for expression of *Per2, Per3, Dbp*, and *Rev-Erb-Alpha* in skeletal muscle. Also, there were no main effects for expression of any clock regulatory gene, including *Bmal1*, in the brain of *Acta:Bmal1* mice compared to WTs.

2.3 Energy expenditure and body composition

24-hr energy expenditure (EE) was measured using a 4-chamber Oxymax FAST system (Columbus Instruments; Columbus, OH) at thermoneutral housing conditions (25.9 degrees C). Body composition was measured using an EchoMRI within 1 week of measurement of gas exchange, and mice were acclimated to the calorimetry room and chamber for at least two days prior to calorimetry. Mice underwent measurement for 24-hr EE as previously described in Zhang *et al.* 2012 [17]. Briefly, mice were enclosed in the chamber with food and water, and fresh air was provided at 0.39–0.40 LPM. Chamber air was sampled at 0.3 LPM, with 120-sec reference and cage settle times, and 30-sec reference and sample measurement times, with reference air sampled every 30 min. Data from the first 1 hour of measurement were not analyzed to allow for acclimation and air equalization.

2.4 VO$_{2max}$

VO$_{2max}$ measurement was also performed as previously described in Zhang *et al.* 2012 [17]. Briefly, mice were placed in the treadmill, which was then sealed, with fresh air provided at 0.75 LPM, and air was sampled every 10 sec at 0.3 LPM. After 20 min, the treadmill was started and VO$_{2max}$ was assessed using the following protocol: 10m/min at 0° incline for 5 min and at 15° for 5 min; at 25° for 2 min each at 10m/min, 15m/min, 17m/min. 19m/min, 21m/min, 23m/min, 25m/min, 27m/min, and 29m/min, or until exhaustion.

2.5 Food intake

Food intake was measured and averaged over four days. Mice were acclimated to wire cage bottoms for two days prior to being provided with pre-weighed food between 0830 and 1000. Intact and scattered food was measured, along with body weight, at the same time of day.

2.6 Insulin Sensitivity

Mice were undisturbed or had been sleep-deprived in their home cages for 6 h at the time of skeletal muscle extraction (midday). For acute sleep deprivation, mice were subjected to non-stressful gentle handling described previously in Longordo *et al.* 2011 [see 2.7 of Materials and Methods and ref. 18]. A separate group of *Acta:Bmal1* mice were fasted during the 6 h of sleep deprivation. Left and right solei were incubated in Krebs-Ringer bicarbonate buffer (in mM: 117 NaCl, 4.7 KCl, 24.6 NaHCO$_3$, 1.2 KH$_2$PO$_4$, 1.2 CaCl$_2$, and 2.5 MgSO$_4$) gassed with 95% O$_2$-5% CO$_2$. The first incubation was in the presence of 2 mM pyruvate for 30 min at 37°C. Following wash, the solei were incubated with Krebs-Ringer bicarbonate buffer containing 1 mM 2-deoxyd-[1,2-^{3}H]glucose (1.5 mCi/ml) and 7 mM d-[^{14}C]mannitol (0.45 mCi/ml). One soleus was stimulated with insulin for 10 min. The other soleus was unstimulated. Dosing for sub-optimal insulin-stimulated glucose uptake in soleus muscle was determined from the development of a dose-response curve with male WTs derived from *Acta:Bmal1* breeding. Insulin doses ranged from 0.25, 0.50, 1.00, 1.50, and 2.50 μunits/μl (n=3/concentration). The sub-optimal dose for experimentation was found to be 0.66 μunits/μl (Fig. 1). After insulin stimulation, the muscles were digested with 250 μl of 1 N NaOH at 80°C for 10 min, and were then neutralized with 250 μl of 1 N HCl. 350 μl of scintillation liquid was added for dual-label radioactivity counting of 1 mM 2-deoxy-d-[1,2-^{3}H]glucose (1.5 mCi/ml) and 7 mM d-[^{14}C]mannitol (0.45 mCi/ml).

2.7 Sleep Deprivation

Six hours of gentle handling is common for a majority of animal studies that examine homeostatic, physiological effects (recovery) of short-term sleep deprivation [ref. 18]. Under this paradigm, non-stressful gentle handling begins at lights-on when the daily physiological drive to sleep is maximal [ref. 18]. Following 6 h of gentle handling, animals are given an 18 h opportunity to recover lost sleep. Typically, mice bred on a C57 background in our laboratory recover ~30–40% of sleep lost (~1.8 to 2.4 h of additional sleep relative to baseline levels) and have normal sleep/wake rhythms and amounts the following night/day.

Results

3.1 Metabolic phenotyping in Bmal1/BMAL1 muscle overexpressed mice reveal sex-specific differences

In these experiments, we aimed to determine the influence of Bmal1 overexpression in skeletal muscle(*Acta:Bmal1*) on metabolic processes *in vivo*. To examine this, we measured VO_2, respiratory exchange ratio (RER, VCO_2/VO_2), energy expenditure, total activity counts, and food intake in male and female mice (n=8–12/sex and genotype).

At the time of calorimetry in male mice, means ± SEMs for body weight averaged 29.14±0.52 g for *Acta:Bmal1* mice versus 29.42±0.45 g for wild-type littermates (WTs; p=0.69; Student's t-test). There was no main effect of genotype for any metabolic process in male mice (Table 1). VO_2 levels averaged 2320.43±33.20 ml/kg/hr for *Acta:Bmal1* male mice versus 2367.07±32.73 ml/kg/hr for male WTs (p=0.53; Student's t-test). RER averaged 0.963±0.01 for *Acta:Bmal1* male mice versus 0.965±0.005 for male WTs (p=0.86; Student's t-test). Energy expenditure averaged 0.3382±0.01 kcal/hr for *Acta:Bmal1* male mice versus 0.3485±0.006 kcal/hr for male WTs (p=0.24; Student's t-test). Total activity counts/min averaged 9.51±0.49 for *Acta:Bmal1* male mice versus 11.16±0.77 for male WTs (p=0.13; Student's t-test). Measures of food intake across four days averaged 4.20±0.15 g/day for *Acta:Bmal1* male mice versus for 4.17±0.11 g/day for male WTs (p=0.88; Student's t-test). Weight gain was calculated by comparing the body weight at the time of calorimetry with the time of food-intake assessment (42–70 days later). There was similar weight gain (g/day) between male *Acta:Bmal1* mice (0.034±0.010 g/day) compared to male WTs (0.036±0.005 g/day; p=0.64).

At the time of calorimetry in female mice, means ± SEMs for body weight averaged 22.12+0.26 g for *Acta:Bmal1* female mice versus 22.82±0.36 g for female WTs (p=0.13; Student's t-test). There was a main effect of genotype for RER and total activity counts in female mice (Table 1). Muscle overexpressed female mice were: 1) more active (15.77±0.91 counts for *Acta:Bmal1* female mice vs. 12.52±0.83 for female WTs; p=0.02; Student's t-test); and 2) had lower RERs (0.940±0.01 for *Acta:Bmal1* female mice vs. 0.960±0.005 for female WTs; p=0.03; Student's t-test). Otherwise, 24 h VO_2 levels averaged 2812.65±61.81 ml/kg/hr for *Acta:Bmal1* female mice versus 2798.01±32.49 ml/kg/hr for female WTs (p=0.84; Student's t-test). Measures of food intake across four days averaged 4.12±0.66 g for *Acta:Bmal1* female mice versus 4.18±0.06 g for female WTs (p=0.66; Student's t-test). EE averaged 0.31±0.01 kcal/hr for *Acta:Bmal1* female mice versus 0.32±0.01 kcal/hr for female WTs (p=0.15; Student's t-test).

3.2. Bmal1/BMAL1 muscle overexpressed mice have unique metabolic profiles in response to high-intensity treadmill running

Next, we wanted to determine if a positive homeostatic challenge amplified or unmasked metabolic phenotypes. It is well documented that acute exposure to high-intensity exercise can have immediate (and sex-specific) effects on metabolic pathways [19]. At the time of the treadmill test, body weights differed slightly but significantly between *Acta:Bmal1* female (21.7±0.2 g) and WT female mice (23.2±0.3 g; p=0.003, Student's t-test). Body weights did

not significantly differ between *Acta:Bmal1* males (28.5±0.8 g) male WTs(29.5±0.5 g; p=0.340, Student's t-test). In regards to exercise capacities, no differences were found between the number of 10-sec intervals of treadmill running that the mice were able to complete (female, p=0.715; male, p=0.458; Table 2). There were also no differences in the top treadmill speed reached (female, p=0.840; male, p=0.448; Table 2).

Once body weights were considered, no differences were found in VO_{2max} (female, p=0.100; male, p=0.654; Table 2). This lack of genotype- and sex-dependent differences in VO_{2max} when adjusting for body weight was recapitulated by a regression analysis (p=0.394; ANCOVA). There were also no differences in maximal RER (female, p=0.977; male, p=0.632; Table 2). The maximal energy expenditure (kcal/h) during the VO_{2max} treadmill test was analyzed using its two major determinants—body weight and number of running intervals completed—as covariates. Peak running energy expenditure (EE) was significantly higher in WT mice compared to *Acta:Bmal1* mice when co-varied against body weight (p=0.004), running intervals completed (p=0.001), or both covariates in the same analysis (p=0.008; Table 2). There was no effect of sex (p=0.177) and no interaction (p=0.610). In general, female mice weighted less than male mice (p=0.001).

3.3. Acute sleep deprivation leads to insulin insensitivity in Bmal1/BMAL1 muscle overexpressed mice

We also wanted to determine if a negative homeostatic challenge amplified or unmasked metabolic phenotypes. Several studies have found that whole-body and tissue-specific loss of Bmal1/BMAL1 function alters the ability to recover from acute sleep deprivation [4, 20]. Only male mice were selected for this experiment because metabolic phenotyping revealed no genotype-dependent differences in body weight, RER, total activity, and food intake.

Weight of soleus muscle did not differ between genotype and treatment (p=0.840 [genotype]; p=0.752 [treatment]; two-way ANOVA). For mice left undisturbed in their home cages, there was no difference in the extent of insulin-stimulated glucose uptake (*Acta:Bmal1* mice: 8621.7±925.1 nmol/ml; WT: 7649.21±893.12 nmol/ml; p=0.63; two-way ANOVA [genotype]). After acute sleep deprivation, however, the extent of glucose uptake (relative to unstimulated muscle) was significantly lower in *Acta:Bmal1* mice compared to WTs; 13.9±5.8% increase from basal levels in *Acta:Bmal1* mice versus 62.8±9.5% increase from basal levels in WTs (p=0.04; two-way ANOVA [time]; Fig. 2). Fasting across sleep deprivation (versus non-fasting) did not further change the extent of insulin insensitivity in *Acta:Bmal1* mice (12.3±6.2% increase from basal levels [fasted] vs. 13.9±5.8% increase from basal levels [non-fasted]; p>0.05; Student's t-test).

Discussion

In the present study, we phenotyped a unique mouse line with tissue-specific transgenic overexpression. We aimed to identify possible homeostatic, non-circadian effects of circadian clock genes on physiological and behavioral processes. We examined the dynamics of metabolic processes in *vivo* and *ex vivo*, including energy expenditure, food intake, and insulin sensitivity in the absence and presence of homeostatic challenges: high-intensity exercise and acute sleep deprivation. The circadian clock gene of focus was Bmal1/

BMAL1. Systemic Bmal1/BMAL1 regulates circadian timekeeping, sleep, and metabolic processes. Loss of function of Bmal1/BMAL1 causes arrhythmia, hypoactivity/ hypersomnia, inflammation, insulin insensitivity, and reduced mortality [2, 3, 4, 20, 21]. Therefore, one benefit of studying the dynamics of metabolic processes in this particular transgenic overexpressed line (*Acta: Bmal1*) is that we were able to study metabolic processes in the absence of compromised morbidity (e.g. circadian timekeeping) and mortality.

Our analyses revealed sex-specific differences of Bmal1/BMAL1 overexpression (in skeletal muscle) on metabolic processes *in vivo*; *Acta: Bmal1* female mice had lower respiratory exchange ratios (RER) coupled with more general locomotor activity compared to wild-type littermates (WTs). These findings complement each other because it suggests a shift towards greater activation and recruitment of fat as an energy source; although total fat mass did not differ between genotypes Most notably, there were positive and negative alterations in metabolic processes with positive (high-intensity wheel running) and negative (acute sleep deprivation) homeostatic challenges, respectively; *Acta: Bmal1* mice had lower energy expenditures with exercise, but became insulin insensitive (in skeletal muscle) with sleep deprivation.

4.1 Differences energy balance driven by Bmal1 overexpression

Our hypothesis was based on recent studies that have identified changes in mitochondrial and fat signaling cascades driven by changes in systemic Bmal1/BMAL1 expression; For example, changes in systemic Bmal1/BMAL1 expression alter AMPK signaling, glucose synthesis, breakdown, and transport, fatty acid and protein metabolism, and mitochrondrial respiration [5, 6, 7, 8, 9]. However, a majority of these studies were in Bmal1/BMAL1 knockout lines. Whole-body loss of Bmal1/BMAL1 is linked to numerous morbidities and mortality related to circadian timekeeping, sleep, and metabolism [2, 20, 21].

Despite this lack of a main effect for genotype, there was a within-sex effect of *Bmal1* overexpression on physical activity and RER. Compared to their female WT littermates in the present study, female *Acta:Bmal1* mice were significantly more physically active in every dimension measured, and also had lower RER, indicating higher net fat oxidation. Further, although there was no difference in body weight at the time of calorimetry, *Acta:Bmal1* females were significantly lighter than female WTs when food intake was assessed. The unmasking of different metabolic profiles with sex is not a surprise. In recent years, several studies have used the sex chromosome complement model to unmask differences in rhythms of activity [22], food intake [22, 23], and lipoprotein levels [24].

4.2 Bmal1/BMAL1 muscle overexpressed mice sensitive to positive (exercise) and negative (sleep deprivation) homeostatic challenges

Although we did not discover genotype-dependent differences in metabolic profiles prior to treadmill running, we were not surprised to find a main effect of Bmal1 overexpression (in skeletal muscle) on metabolic profiles after treadmill running. This is largely because transgenic overexpression driven by the *Acta1* promoter has yielded several advantageous metabolic and behavioral phenotypes: more physically active, better exercise and fitness

capacities, enhanced clearance of lactic acid and reactive oxygen species, healthier metabolic profiles, higher fecundity, and longer lifespan as reported previously in *Biochimie* [10]. In the present study, we found that *Acta1: Bmal1* mice have lower energy expenditures after high-intensity treadmill running. This means that *Acta1: Bmal1* mice can work harder on less fuel, and can recover quicker and have better work capacities with repeated training [25].

We were surprised to find a main effect of *Bmal1* overexpression (in skeletal muscle) on insulin sensitivity of the skeletal muscle; loss of Bmal1/BMAL1 function and sleep deprivation (NREM and REM) for an extensive duration (96 hours) both compromise insulin sensitivity [6, 7, 8, 27, 28]. No study in rodent models has yet to report a compromise in insulin sensitivity with short durations of sleep deprivation (6 hours), but 12 hours of sleep fragmentation can also compromise insulin sensitivity [29]. Plus, few studies have actually examined insulin sensitivity of skeletal muscle *ex vivo*. Six hours of sleep deprivation is also known to alter *Per2* clock gene expression but not *Bmal1* clock gene expression in the lateral habenula: an arousal-promoting area of the brain [30]. Thus, it is interesting that a metabolic deficiency in transgenic overexpressed mice did not arise until an acutely negative homeostaticsleep challenge. This suggests that Bmal1/BMAL1 and downstream metabolic cascades in skeletal muscle is sensitive to sleep deprivation.

In fact, it is also known that sleep deprivation alters DNA-binding of BMAL1 [16], and that disruption to *Bmal1*/BMAL1 in skeletal muscle significantly alters metabolic genes [26]. However, the system is clearly more complicated and suggests that while *Bmal1* overexpression in skeletal muscle does not change expression profiles of clock regulatory genes in skeletal muscle and brain [see 2.7 of Materials and Methods and ref. 3], the works of Peek et al. 2013 [ref. 31] and Bass and Takahashi, 2010 [ref. 32] suggest there still may be direct, downstream changes in metabolic regulatory genes and fluxes including systemic and tissue-specific levels of glucose, ATP/AMP, glucocorticoids, catecholamines, and lactate.

4.3 Putative Mechanisms of Actions

Our present phenotypic findings warrant further mechanistic investigation. This is one of few studies to transgenically overexpress clock genes in order to delineate tissue-specific effects on physiology and behavior [see ref. 3]. Otherwise, a calorie-restrictive diet has been shown to promote the induction of *Bmal1* expression in skeletal muscle [33]. Further, it is notable to mention previous studies that have either developmentally or directly altered *Bmal1*/BMAL1 expression in order to identify effects on mitochondrial and insulin signaling processes.

In regards to mitochondrial oxidation, silencing *Bmal1* expression *in vivo* and *ex vivo* silences mitochondrial oxidation [5, 34]. Arresting mitochrondrial oxidation can also downregulate *Bmal1* expression [34]. In regards to insulin signaling, developmentally knocking-out or pharmacologically-arresting *Bmal1* expression alters blood glucose levels, insulin sensitivity in skeletal muscle, and downregulates GLUT4 and glucose regulatory genes in skeletal muscle. First, example, Dyar et al. [ref. 9] discovered that developmental knock-out of *Bmal1* in skeletal muscle corresponds with: 1) a shift in the amplitude and

timing of clock, clock-controlled, and glucose regulatory genes in skeletal muscle; 2) insulin insensitivity in skeletal muscle; and 3) downregulation of GLUT4. Similarly, Schroeder et al. 2015 [ref. 35] discovered that pharmacologically-arresting *Bmal1* expression immediately corresponds with: 1) insulin insensitivity in skeletal muscle; 2) downregulation of GLUT4 and glucose regulatory genes (*Hk2, Pfkm*); and 3) a change in body composition after three months. Mice lacking *Bmal1* in skeletal muscle weighed less and had less fat mass. Although we did not investigate molecular signaling cascades in the present study, our metabolic phenotypes suggest that there is a possible change in the distribution of glucose and fat oxidation regulatory genes.

4.4 Conclusions

We found that altering the dynamics of the intrinsic biological clock in skeletal muscle alters the dynamics of metabolic processes *in vivo* and *ex vivo*. A major benefit of our model is that we were able to alter the expression of a critical positive transcriptional/translational factor in the absence of a compromise in circadian timekeeping [3]. We found an influence of Bmal1 overexpression on physical activity and RER, implicating differential glucose vs. fat oxidation *in vivo*. We also found a positive influence of Bmal1 overexpression on exercise capacity and a negative influence of Bmal1 overexpression on insulin sensitivity. To conclude, the present study contributes to a body of evidence showing pleiotropic, non-circadian effects of Bmal1 on physiological processes. These pleiotropic effects appear to be driven by auxiliary biological factors such as sex as well as environmental factors such as homeostatic challenges. Overall, these findings demonstrate the critical need to further investigate the complexity of Bmal1/BMAL1 actions on physiology and behavior.

Acknowledgments

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Highlights

1. Mice with overexpressed muscle clocks work harder on less fuel and recover quicker from exercise

2. Overexpressed mice are more sensitive to the positive metabolic effects of maximal exercise

3. Overexpressed mice are more sensitive to negative metabolic effects of acute sleep loss
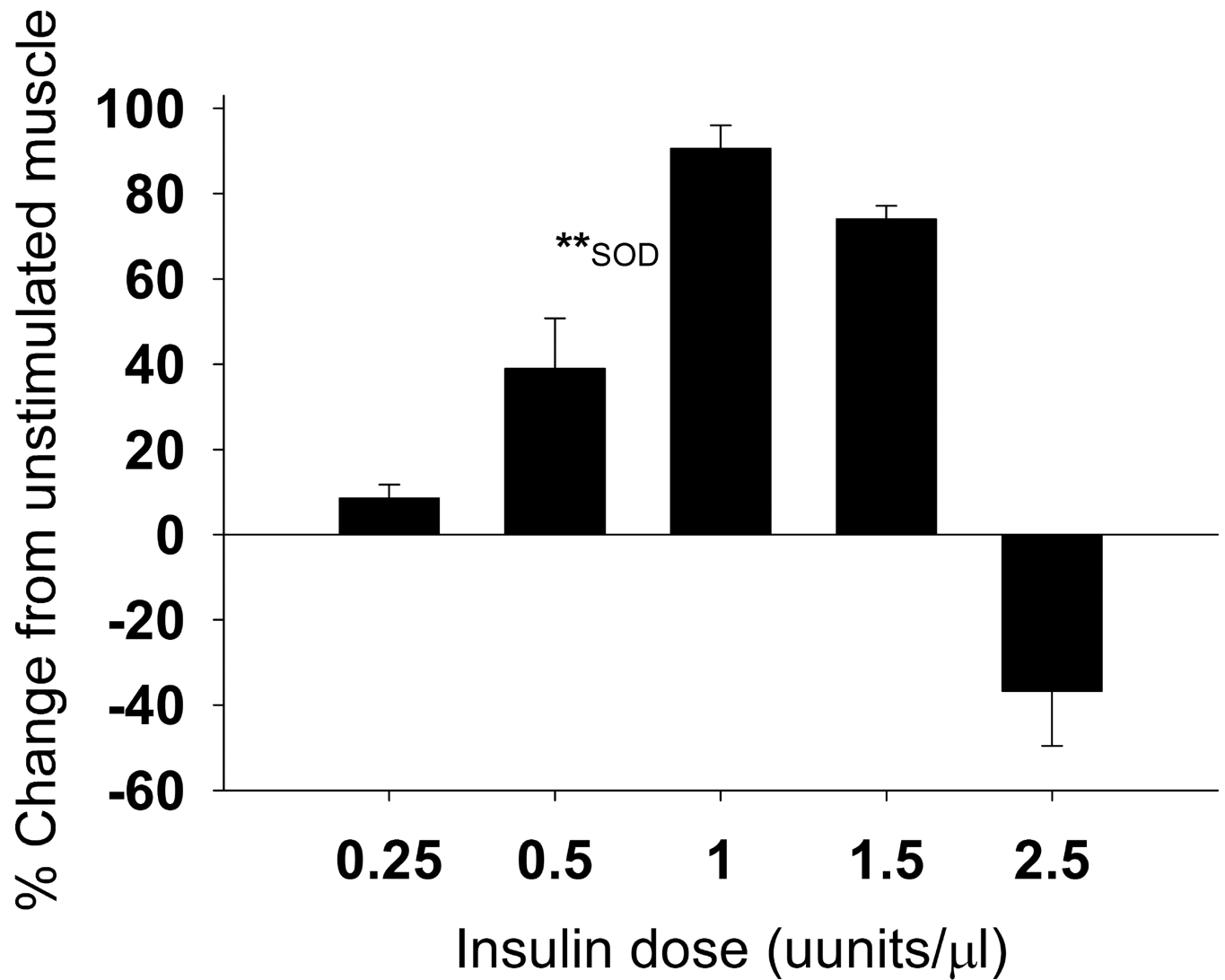
Figure 1. Dose response curve of insulin-stimulated glucose uptake in soleus muscle
Soleus muscle extracted at midday was stimulated with insulin and subjected to radioassay analyses with 2-deoxy-d-[1,2-^{3}H]glucose (1.5 mCi/ml) and 7 mM d-[^{14}C]mannitol. Means ±SEM % change in the extent of glucose uptake (nmol/ml) in stimulated versus unstimulated muscle extracted from the same mouse. *SOD, sub-optimal dose.
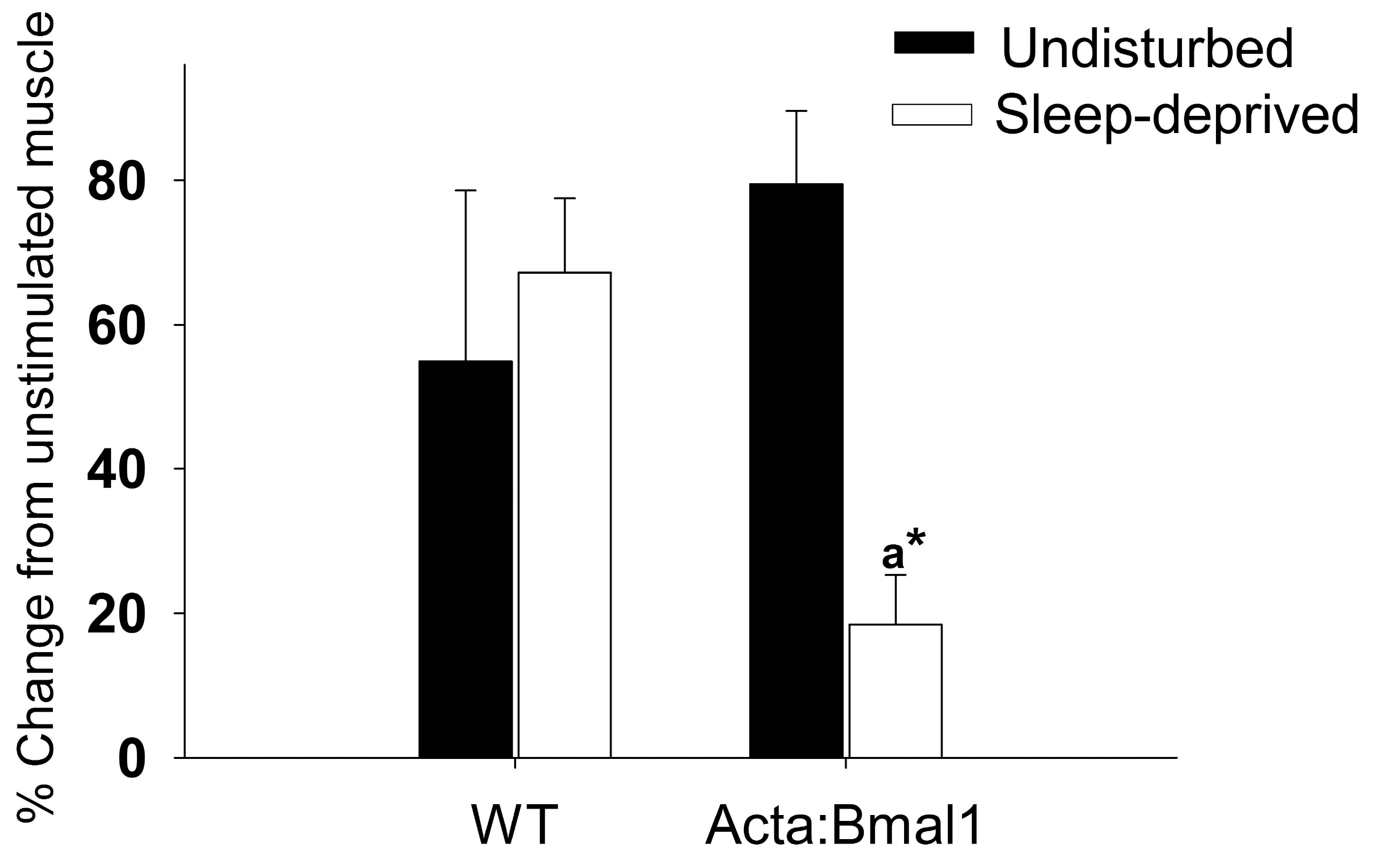
Figure 2. Negative homeostatic (sleep) challenge reduces insulin sensitivity in skeletal muscle of mice with muscle-specific clock gene overexpression

Mice were left undisturbed or had been gently handled for 6 h in their home cages immediately prior to muscle extraction at midday. Means±SEM % change in the extent of glucose uptake (nmol/ml) in stimulated versus unstimulated muscle extracted from the same mouse. a*, p<0.05; two-way ANOVA.

Table 1

Female mice with muscle-specific clock gene overexpression are more metabolically and physically active.

	Male		Female	
	Wild-type	*Acta:Bmal1*	**Wild-type**	*Acta:Bmal1*
BW (g)	29.42±0.45	29.14±0.52	22.82±0.36	22.12±0.26
Fat (g)	2.41±0.22	2.88±0.48	1.76±0.25	1.43±0.12
Lean (g)	22.31±0.57	21.82±0.53	18.40±0.63	17.62±0.39
Activity	11.16±0.77	9.51±0.49	12.52±0.83	15.77±0.91 [**]
VO2 (ml/kg/h)	2367.07±32.73	2320.43±33.20	2798.01±32.49	2812.65±61.81
VCO2 (ml/kg/h)	2291.85±33.15	2246.12±47.31	2706.04±34.03	2637.33±44.96
RER (VCO2/VO2)	0.9650±0.0048	0.9633±0.0100	0.9600±0.0001	0.9400±0.0100 [**]
Heat (kcal/h)	0.3485±0.0060	0.3382±0.0100	0.3200±0.0100	0.3100±0.0100

BW, body weight; RER, respiratory exchange ratio; EE, energy expenditure;

[**] $p < 0.05$, Student's t-test.

Table 2

Mice with muscle-specific clock gene overexpression have lower energy expenditures during positive homeostatic (exercise) challenges.

	Male		Female	
	Wild-type	*Acta:Bmal1*	**Wild-type**	*Acta:Bmal1*
BW (g)	29.49±0.48	28.50±0.80	23.22±0.34	21.70±0.24 [**]
Fat (g)	2.41±0.22	2.74±0.45	1.88±0.25	1.40±0.14
Lean (g)	22.31±0.57	21.82±0.53	18.74±0.62	17.35±0.32
10-s run intervals	118.2±4.8	112.2±6.5	123.2±7.3	119.0±8.14
Max speed (m/min)	21.7±0.8	20.6±1.2	22.3±1.3	21.9±1.3
VO2max	7693.92±139.85	7575.22±237.01	8672.67±132.69	8236.43±192.98
RER (VCO2/VO2)	1.12±0.02	1.14±0.04	1.08±0.01	1.08±0.02
EE (kcal/h)	1.17±0.03	1.09±0.02 [**]	1.02±0.03	0.9±0.02 [**]

BW, body weight; RER, respiratory exchange ratio; EE, energy expenditure;

[**] $p<0.05$, Student's t-test.