HHS Public Access

Author manuscript

Nature. Author manuscript; available in PMC 2012 November 03.

Published in final edited form as:

Nature. ; 485(7396): 62–68. doi:10.1038/nature11030.

Regulation of Circadian Behavior and Metabolism by Synthetic REV-ERB Agonists

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Abstract

Synchronizing rhythms of behavior and metabolic processes is important for cardiovascular health and preventing metabolic diseases. The nuclear receptors REV-ERBα and REV-ERBβ play an integral role in regulating the expression of core clock proteins driving rhythms in activity and metabolism. Here we describe the identification of potent synthetic REV-ERB agonists with *in vivo* activity. Administration of synthetic REV-ERB ligands alters circadian behavior and the circadian pattern of core clock gene expression in the hypothalami of mice. The circadian pattern of expression of an array of metabolic genes in the liver, skeletal muscle, and adipose tissue was also altered resulting in increased energy expenditure. Treatment of diet-induced obese mice with a REV-ERB agonist decreased obesity by reducing fat mass and markedly improving dyslipidemia and hyperglycemia. These results suggest that synthetic REV-ERB ligands that pharmacologically target the circadian rhythm may hold utility in the treatment of sleep disorders as well as metabolic diseases.

In mammals, most if not all tissues display a self-sustaining circadian molecular pacemaker that is responsible for aligning rhythms in various physiological functions. The

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Author Contributions

T.P.B. conceived the project. R.N. and T.M.K. synthesized and analyzed the ligands. L.A.S., Y.W., S.B. D.J.K, and T.P.B. designed/analyzed and/or performed the transfection and biochemical assays. D.J.K. and T.H. designed and performed the CD analysis. T.P.B., L.A.S. and A.A.B. designed, analyzed and performed the metabolic studies. Y.W., T.P.B., S.B. and T.L designed/analyzed and/or performed the circadian gene expression and behavior analysis. J.L. performed gene expression analysis. S-H.Y. and J.S.T. designed and performed the studies using the *Per2-luc* mouse tissues. M.D.C. performed the pharmacokinetic analysis. T.P.B. wrote the manuscript with input from all the authors.

The authors declare no competing financial interests.

suprachiasmatic nucleus (SCN) of the hypothalamus functions as the master circadian pacemaker synchronizing behavioral and physiological rhythms to the environmental light-dark cycle[1]. Optimal coordination of rhythms in metabolic processes with nutrient availability involves signals emanating from the SCN and hypothalamus, as well as autonomous inputs from nutrient-sensors responding to metabolic flux and body temperature[2].

The mammalian molecular clock is composed of a transcriptional feedback loop where heterodimers of the transcription factors BMAL1 (brain and muscle ARNT-like protein 1) and CLOCK (circadian locomotor output cycles kaput) or NPAS2 (Neuronal PAS domain-containing protein 2) activate the transcription of the *Period* (*Per1*, *Per2* and *Per3*) and *Crytochrome* (*Cry1* and *Cry2*) genes. Subsequently the PER/CRY proteins feedback to inhibit BMAL1/CLOCK activity resulting in a rhythmic, circadian pattern of expression of these genes[3]. The REV-ERB nuclear receptors play an important role in feedback regulation of the circadian oscillator. Both *Bmal1* and *Clock* are direct REV-ERB target genes[4,5] and loss of REV-ERBα alters circadian behavior[4]. The physiological ligand for REV-ERBα and β was recently identified as heme[6,7] and based on observations that REV-ERB activity is regulated by a small molecule ligand, we and others have sought to identify and characterize synthetic ligands[8–11]. Here, we describe the development of REV-ERB ligands that allowed for characterization of the effects of modulation of this receptor *in vivo*.

Results

Development of REV-ERBα/β agonists

We developed two REV-ERBα/β agonists with sufficient plasma/brain exposure to allow evaluation of their effects *in vivo*. Both SR9011 and SR9009 (Fig. 1a, Supplementary Fig. 1) dose-dependently increased the REV-ERB-dependent repressor activity assessed in HEK293 cells expressing a chimeric Gal4 DNA Binding Domain (DBD) - REV-ERB ligand binding domain (LBD) α or β and a Gal4-responsive luciferase reporter (Fig. 1b) (SR9009: REV-ERBα IC_{50}=670 nM, REV-ERBβ IC_{50}=800 nM; SR9011: REV-ERBα IC_{50}=790 nM, REV-ERBβ IC_{50}=560 nM). The REV-ERB ligand GSK4112 (Supplementary Fig. 2), which exhibits no plasma exposure[8,10] displays limited activity (Fig. 1b). Both SR9011 and SR9009 potently and efficaciously suppressed transcription in a cotransfection assay using full-length REV-ERBα along with a luciferase reporter driven by the *Bmal1* promoter (Fig. 1c) (SR9009 IC_{50}=710 nM; SR9011 IC_{50}=620 nM). SR9011 and SR9009 suppressed the expression of *BMAL1* mRNA in HepG2 cells in a *REV-ERBα/β*-dependent manner (Supplementary Fig. 3). Consistent with both compounds functioning as direct agonists of REV-ERB, we noted that the compounds increased the recruitment of the CoRNR box peptide fragment of NCoR using a biochemical assay (Supplementary Fig. 4)[6]. Direct binding of the SR9009 to REV-ERBα was also confirmed using circular dichrosim analysis (Supplementary Fig. 5) (K_d=800 nM). Neither compound exhibited activity at other nuclear receptors[12,13] (Supplementary Fig. 6). SR9011 also inhibited the activity of the SCN clock, with reversible inhibition of circadian oscillations in SCN explants cultured from the *Per2:luc* reporter mouse[14] (Fig. 1d). Treatment suppressed the amplitude of the oscillations, but had no effect on the period (Fig. 1d). We observed similar effects in *Per2:luc* fibroblasts

(Supplementary Fig. 7). The compounds displayed reasonable plasma exposure (Supplementary Fig. 8) thus, we examined the expression of REV-ERB responsive genes in the liver of mice treated with various doses of SR9011 for 6-days. The *plasminogen activator inhibitor type 1* gene (*Serpine1*) is a REV-ERB target gene[15] and displayed dose-dependent suppression of expression in response to SR9011 (Fig. 1e). The *cholesterol 7α-hydroxylase* (*Cyp7a1*) and *sterol response element binding protein* (*Srepf1*) genes have also been shown to be responsive to REV-ERB[16,17] and were dose-dependently suppressed with increasing amounts of SR9011 (Fig. 1e). SR9009 displayed a similar effect on these genes (Fig. 1e).

REV-ERBα/β agonists modulate circadian behavior and gene expression in mice

Based on the effects of these compounds on SCN clock activity, we predicted that administration of these compounds would alter circadian behavior. Circadian locomotor activity was examined in mice released into constant dark (D:D) conditions after 1 week of housing in wheel cages in a standard light:dark (L:D) setting. After 12 days in D:D conditions mice were injected with a single dose of SR9011, SR9009 or vehicle at CT6 (peak expression of *Rev-erbα* (Supplementary Fig. 9)). Vehicle injection caused no disruption in circadian locomotor activity (Fig. 2a – *upper panels*). However, administration of a single dose of either REV-ERB agonist resulted in loss of locomotor activity during the subject dark phase (Fig. 2a – *lower panels*). Normal activity returned the next circadian cycle, consistent with clearance of the drugs in less than 24h. This effect was not due to toxicity since the complete loss of locomotor activity was not observed in an identical experiment using L:D (Fig. 2d). Additionally, mice treated with SR9011 did not display a decrease in strength (Supplementary Figure 10a) and continued to move as detected in an open field assay (Supplementary Figures 10b & 10c). Furthermore, we observed no overt toxicity when we examined complete blood counts (Supplementary Figure 11). We observed that the SR9011-dependent decrease in wheel running behavior in the mice under constant darkness conditions was dose-dependent (Fig. 2b) and that the potency (ED_{50}=56 mg/kg) was similar to the potency of SR9011-mediated suppression of a REV-ERB responsive gene, *Srebf1*, *in vivo* (ED_{50}=67mg/kg). *Tau* was not affected by treatment with either drug (data not shown) and the recovery after the drug to resume the normal rhythm is similar to the effect observed after removal of the drug from the SCN explants (Fig. 1d).

We next assessed the expression of core clock genes in hypothalami isolated from mice in D:D conditions. Mice were injected with a single dose of SR9011 or SR9009 at CT0 and hypothalami collected for expression analysis. We observed a range of effects on the pattern of expression of the core clock genes. The amplitude of *Per2* expression was enhanced while *Cry2* was suppressed (Fig. 2c). *Bmal1* expression was affected more subtly with a left shift in the phase of the circadian pattern (Fig. 2c). The circadian pattern of expression of *Npas2* was completely eliminated (Fig. 2c). The pattern of expression of *Clock* was also altered with SR9011 treatment resulting in enhanced amplitude of the oscillation, but also altering the phase so that the *Clock* oscillation was in phase with the *Per2* oscillation (Fig. 2c). SR9009 treatment resulted in similar effects on gene expression (Supplementary Fig. 12). We also examined the effect of both REV-ERB ligands under L:D (12h:12h) conditions. Instead of complete loss of nocturnal locomotor activity, we noted a 1–3 h delay

in the onset of nocturnal locomotor activity (Fig. 2d). Consistent with the more subtle effects on circadian behavior the effects of SR9011 and SR9009 on core clock gene expression in the hypothalamus were less severe than observed under constant darkness (Fig. 2e; Supplementary Fig. 13 and data not shown). Considerable differences were noted in Rev-erbα expression in the D:D vs L:D conditions in terms of their responsiveness to the compounds. In L:D conditions SR9011 had no effect on the circadian pattern of expression whereas under D:D conditions SR9011 completely suppressed the circadian pattern of expression (Supplementary Figure 9). These data suggest that light input into the circadian oscillator has a significant effect on the action of these drugs.

REV-ERBα/β agonists modulate metabolism in vivo

Genetic perturbation of the core clock genes leads to a range of metabolic phenotypes[2,18]. In addition, REV-ERB has been shown to directly regulate genes involved in lipid and glucose metabolism[7,19,20]. We observed clear metabolic effects when SR9011 was chronically administered to Balb/c mice. Mice displayed weight loss due to decreased fat mass (Fig. 3a), however, food intake was not affected (data not shown). Similar results were obtained in SR9009 treatment of C57Bl6 mice (Supplementary Fig. 14). We examined the metabolic effects of SR9011 in more detail using a comprehensive laboratory animal monitoring system (CLAMS). After acclimation, the animals were administered SR9011 twice per day for 10 days. A 5% increase in oxygen consumption (VO_2) was observed suggesting increased energy expenditure (Fig. 3b). The increase in VO_2 was evident in the diurnal and nocturnal phases (Fig. 3c). The increases VO_2 were not due to increased activity since mice displayed a 15% decrease in movement (Fig. 3d). Treatment also had no effect on total daily food intake (Fig. 3e) or the rate of food intake (Fig. 3f) although there was a 10% increase noted in nocturnal food consumption in the SR9011 group. There was also no change in the respiratory exchange ratio (Fig. 3g). Consistent with increased metabolic rate, we observed a decrease in fat mass with SR9011 vs. vehicle treatment (Fig. 3h). Treatment of mice housed in wheel cages in a L:D setting indicated a delayed onset of physical activity (Fig. 2c) and a similar 1–3h delay in peak VO_2 was observed with administration of SR9011 (Fig. 3i). Given the association between the circadian rhythm and metabolic regulation, and in order to understand the potential mechanism underlying the alterations in metabolic rate, we examined the effect of the REV-ERB ligands on the circadian expression of various genes in the liver, muscle and adipose tissue. Following a single injection of SR9011, we monitored the expression of clock genes in the liver over a 24h period. When examining the effects of SR9011 treatment on core clock gene expression the pattern of expression of *Per2* was altered, but others such as *Bmal1* and *Npas2* were unaffected (Fig. 4a). Thus, SR9011 treatment results in alterations in the pattern of circadian expression of clock genes in both the hypothalamus (Fig. 2b, 2d; Supplementary Fig. 9) and liver, but there are clear distinctions in which genes are affected. These data suggest that the REV-ERB ligands differentially affect the central and peripheral clocks.

We also assessed the expression of an array of genes involved in metabolism in the liver in response to SR9011 treatment. The expression of lipogenic genes (*Srebf1*), fatty acid synthase (*Fasn*), and stearoyl-CoA-desaturase 1 (*Scd1*) were clearly altered with SR9011 treatment (Fig. 4b). Both *Srebf1* and *Scd1* expression were suppressed whereas the phase of

Fasn was phase shifted (Fig. 4b). Expression of genes involved in cholesterol and bile acid metabolism were also altered. *Srebf2* and *cholesterol 7α-hydroxylase expression* (*Cyp7a1*) were decreased (Fig. 4b). *3-hydroxy-3-methyl-glutaryl-CoA reductase* (*Hmgcr*) was unaffected in this acute model (Fig. 4b). *Peroxisome proliferator-activated receptor gamma coactivator 1-alpha* and *1-beta* (*Ppargc1a* and *Ppargc1b*) both displayed a strong circadian pattern of expression that was suppressed with SR9011 treatment (Fig. 4b). Examination of gene expression in skeletal muscle revealed a potential mechanism for the increased metabolic rate that we observed in the CLAMS experiments. Expression of the genes that encode the rate limiting enzyme for β-oxidation of fatty acids, *carnitine palmitoyltransferase 1b* (*Cpt1b*), as well as fatty acid transport into the skeletal muscle, *fatty acid transport protein 1* (*Fatp1*), were elevated (Fig. 4c). *Pppargc1b* expression was also elevated along with *uncoupling protein 3* (*Ucp3*) consistent with altered fatty acid metabolism in skeletal muscle (Fig. 4c). The pattern of expression followed the expected diurnal increase in expression of genes involved in fatty acid oxidation, but the increases in expression were amplified (Fig. 4c). When we examined the expression key enzymes in the glycolytic pathway (*hexokinase* (*Hk1*) and *pyruvate kinase* (*Pkm2*)) we noted an increase in both (Fig. 4c) suggesting that treatment resulted increased glucose oxidation in addition to fatty acid oxidation. The rate-limiting enzyme in mammalian nicotinamide adenine dinucleotide (NAD^+) biosynthesis, nicotinamide phosphoribosyltransferase (NAMPT), has recently been demonstrated to be expressed in a circadian manner leading to a circadian pattern of NAD^+ production and thus regulation of the NAD+-dependent deacetylase SIRT1[2122]. We found that SR9011 suppressed the circadian rhythm of *Nampt* gene expression in the liver (Supplementary Fig. 15a) suggesting that REV-ERB agonist treatment may alter post-translational acetylation of proteins that may contribute to some of the physiological alterations that we observe.

In contrast to the muscle where there was amplification of the circadian expression of genes coupled to fatty acid oxidation and glycolysis, in the white adipose tissue (WAT) we observed a suppression of circadian expression of key genes involved in lipid storage. The expression of both *diglyceride acyltransferase 1* and *2* (*Dgat1* and *Dgat 2*), the genes encoding the enzyme that catalyzes the terminal and committed step in triglyceride synthesis, were suppressed with SR9011 treatment (Fig. 4d). Consistent with this pattern, the circadian expression of another gene involved in triglyceride synthesis, *monoacylglycerol acyltransfease* (*Mgat*), is also disturbed (Fig. 4d). Expression of lipid droplet associated protein genes including *perilipin 1* (*Plin1*) and *hormone sensitive lipase* (*Hsl*) were also suppressed with SR9011 treatment (Fig. 4d). We also observe suppression of *Nocturnin* (*Ccrn4l*) expression in both adipose and hepatic tissue (Supplementary Fig. 15b). Similar effects on gene expression were noted in animals treated with SR9009 (data not shown).

Clearly, modulation of REV-ERB activity by a synthetic agonist alters the pattern of expression of many genes involved in metabolism in several tissues including the liver, skeletal muscle and WAT. The alterations that we observed are consistent with decreased lipogenesis and cholesterol/bile acid synthesis in the liver, increased lipid and glucose

oxidation in the skeletal muscle, and decreased triglyceride synthesis and storage in the WAT.

REV-ERBα/β agonists induce weight loss and reduce plasma lipid levels in diet-induced obese mice

Based on the alterations in energy metabolism and gene expression we observed in normal C57BL6 and Balb/c mice, we sought to examine whether a REV-ERBα/β agonist would be efficacious in a rodent model of obesity. We initiated the study with 20-week old C57BL6 mice (average weight = 41g) that had been maintained on a high fat diet for 14 weeks (20% carbohydrate 60% fat). The mice continued on the HF diet and we initiated twice per day dosing (i.p.) of SR9009. While the stress of handling and twice-daily injections caused weight loss in vehicle-treated controls, weight loss of SR9009-treated animals was 60% greater (Fig. 5a). During the treatment period, there was no significant difference in the food intake of SR9009 and vehicle treated animals, although handling itself reduced food intake explaining the weight loss observed in the controls. SR9009 treated mice exhibited a more severe reduction in adiposity (Fig. 5b). In addition to the decrease in fat mass we also observed a 12% decrease in plasma triglycerides (TGs) and a 47% decrease in plasma total cholesterol (Chol) (Fig. 5c). Plasma non-esterified fatty acids (NEFA) were also reduced (23%) along with plasma glucose (19%) in the SR9009 treated animals (Fig. 5c). There was also a trend toward a decrease in plasma insulin levels (35%). Consistent with the decrease in adipocity we also noted an 80% decrease in plasma leptin and a decrease (72%) in the proinflammatory cytokine IL-6 (Fig. 5d). Examination of plasma triglycerides and total cholesterol in lean mice also demonstrated the ability of SR9009 and SR9011 to reduce the levels of these lipids (Figs. 5e, 5f). Consistent with the decreased plasma TGs and total Chol we observed a significant decrease in the expression of genes encoding lipogenic enzymes (*Fasn* and *Scd1*) as well as cholesterologenic regulatory proteins (*Hmgcr* and *Srebf2*) with SR9009 treatment (Fig. 5g). In the WAT, SR9009 treatment resulted in a decrease in expression of genes encoding enzymes involved in TG synthesis (Fig. 5g) as was also observed in lean mice (Fig. 4d). Similar to our observations in lean mice (Fig. 4c), we observed that the REV-ERB agonist induced the expression of genes involved in fatty acid and glucose oxidation (*Cpt1b*, *Ucp3*, *Ppargc1b*, *Pkm2* and *Hk1*) (Fig. 5g). Taken together with the results from the CLAMS experiments (Fig. 3), these data suggest that REV-ERB agonists increase energy expenditure by increasing fatty acid and glucose oxidation in the skeletal muscle. The gene expression data is also consistent with decreased TG synthesis in the liver and WAT as well as a reduction in hepatic cholesterol synthesis. We also examined the effects of SR9009 in a genetic model of obesity (*ob/ob* mice) and after 12-days of dosing we observed that SR9009 suppressed the degree of weight gain normally observed in this leptin deficient mouse with no significant alterations in glucose or insulin tolerance (Fig. 5h and data not shown).

Discussion

We have developed synthetic REV-ERBα/β agonists with sufficient pharmacokinetic properties to examine their activity *in vivo*. These compounds alter the circadian pattern of expression of core clock genes as well as circadian locomotor behavior in mice. A single

injection of either SR9011 or SR9009 results in loss of the subsequent active period in mice maintained under constant dark conditions. Consistent with this major alteration in circadian behavior the circadian pattern of core clock gene expression in the hypothalamus is perturbed. In *Rev-erbα* null mice circadian locomotor activity is also disturbed with the mice displaying a decreased period relative to wild-type mice under constant dark or constant light conditions[4]. We would not necessarily expect to mimic the phenotype of either constant over- or under-expression of REV-ERB with a pharmacological REV-ERB ligand since modulation of the receptors' activity would be only transient. This is also the likely reason that on the day following administration of the REV-ERB agonist, normal circadian behavior is completely restored. We also administered the REV-ERB agonists under L:D conditions to mimic a therapeutic situation as well as the metabolic studies where mice were maintained on this standard L:D cycle. In this case the effects of administration of the REV-ERB ligands were considerably less severe both in terms of alterations in patterns of core clock gene expression in the hypothalamus and in circadian locomotor behavior. A single injection of either SR9009 or SR9011 resulted in a 1 to 3 h delay in initiation of diurnal activity. Consistent with this observation, when we examined oxygen consumption we observed a similar delay in the nocturnal peak in VO_2. Thus, synthetic REV-ERB ligands effectively alter the physiological time of day of mice suggesting that this class of compound may be useful for the treatment of sleep disorders. Additionally, synthetic REV-ERB ligands may hold utility for jet lag where the compounds could be used to realign both the central and peripheral clocks to a new time zone.

The core clock machinery is closely associated with metabolic regulation and there are a myriad of example of genetic alterations to clock genes leading to metabolic disturbances and even metabolic diseases in rodent models[2,18,23–25]. In addition to its role in direct modulation of the positive arm of the mammalian circadian oscillator, REV-ERB has also been demonstrated to play a direct role in regulation of an array of metabolic genes[26–28]. Our observations that the REV-ERB agonists increase energy expenditure decrease fat mass and plasma TGs and cholesterol suggest that these compounds may hold utility in the treatment of metabolic diseases.

Methods Summary

Synthesis of SR9009 and SR9011

Compounds were synthesized by reductive amination of 5-nitro-2-thiophenecarboxaldehyde with 4-chlorobenzylamine and sodium triacetoxyborohydride yielded the secondary amine. A second reductive amination with 1-Boc-pyrrolidine-3-carboxaldehyde yielded the tertiary amine. This compound was treated with trifluoroacetic acid to remove the Boc-protecting group, and then reacted with either ethyl chloroformate (SR9009) or pentyl isocyanate (SR9011) to give the desired products.

Cell Culture and Cotransfections

HEK293 cells were maintained and transfected with vectors previously described[12].

Mouse studies

For circadian gene expression experiments male C57BL6 mice (8–10 weeks of age) were either maintained on a L:D (12h:12h) cycle or on constant darkness. At circadian time (CT) 0 animals were administered a single dose of 100 mg/kg SR9009 or SR9011 (i.p.) and groups of animals (n=6) were sacrificed at CT0, CT6, CT12 and CT18. Gene expression was determined by real time QPCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health Grants (DK080201, MH092769 and DK089984) and the Howard Hughes Medical Institute. L.A.S. is the recipient of an individual National Research Service Award (DK088499).

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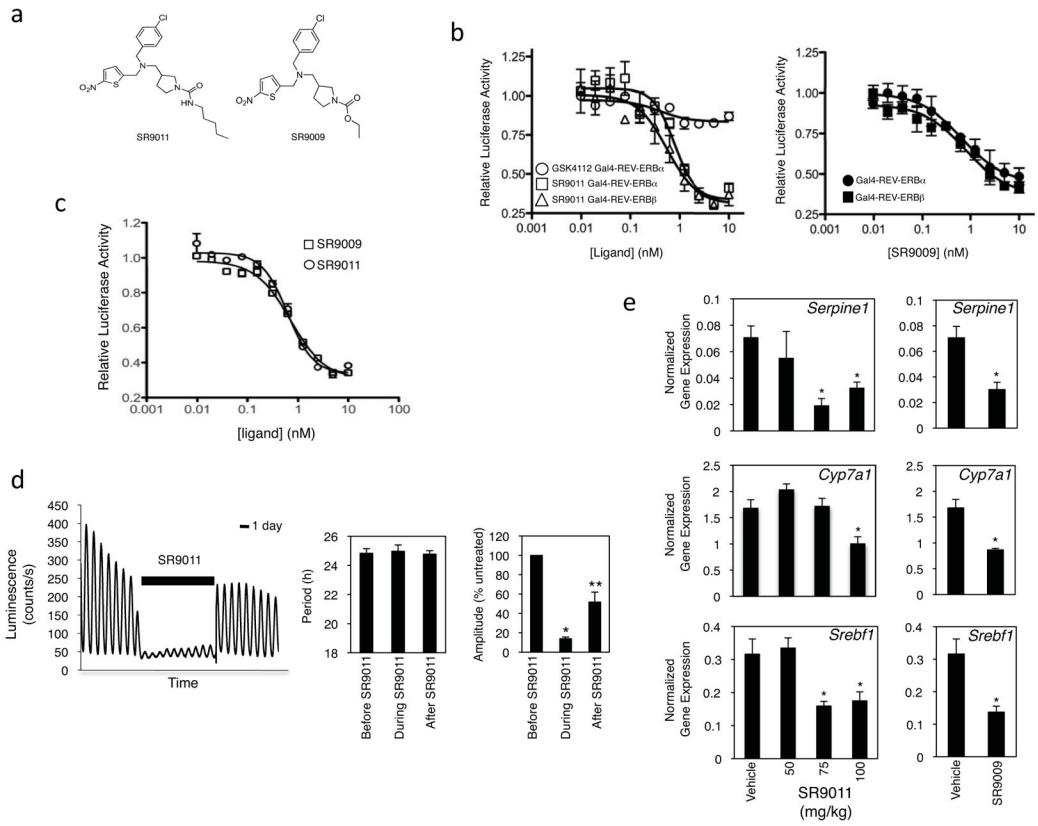

Figure 1. SR9011 and SR9009 are synthetic REV-ERB agonists with activity *in vivo*

a, Chemical structures of SR9011 and SR9009. **b**, GAL4-REV-ERBα and GAL4-REVERBβ cotransfection assays in HEK293 cells illustrating the activity of SR9011 and 9009 and comparing the activity to GSK4112. **c**, Cotransfection assay in HEK293 cells with full-length REV-ERBα and a luciferase reporter driven by the *Bmal1* promoter. **d**, Bioluminescence record from a $Per2^{LUC}$ SCN treated with 5 μM SR9011 as indicated by the bar. The right panels display the period and amplitude of the oscillations prior to, during, and after treatment with SR9011. **e**, Expression of REV-ERB responsive genes after treatment with various doses of SR9011 or 100 mg/kg of SR9009 (i.p., b.i.d.) for 6-days. * indicates $p<0.05$. ** indicates $p<0.05$ vs. before SR9011 and during SR9011 treatment. Error bars indicate mean ± s.e.m. and n=6.

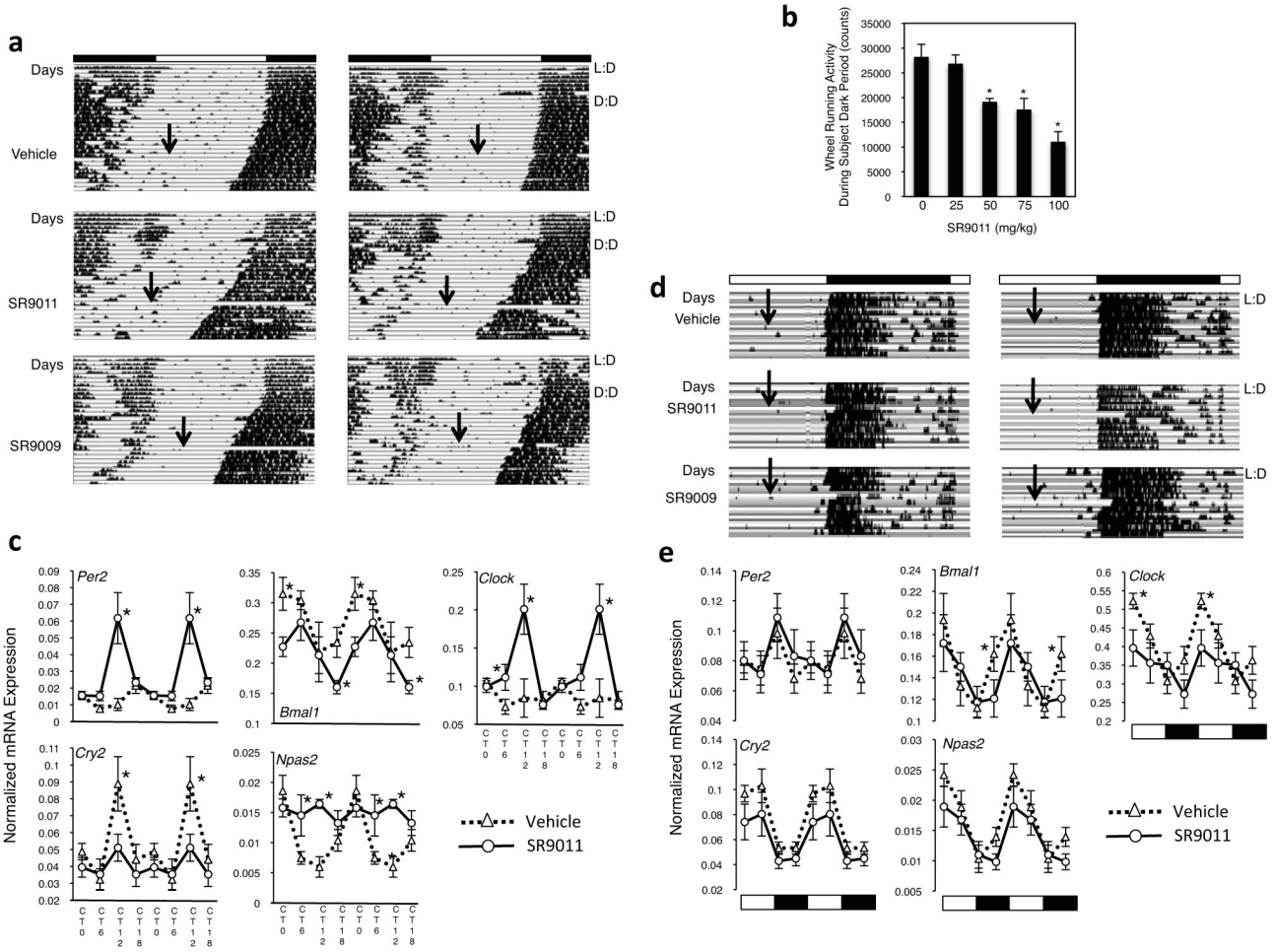

Figure 2. Synthetic REV-ERB ligands alter circadian behavior and the pattern of expression of core clock genes

a, Actograms illustrating the effect of single injections of vehicle, SR9011 (100 mg/kg, i.p.) or SR9011 (100 mg/kg, i.p.) on circadian behavior. C57Bl6 mice were initially maintained on a 12h:12h L:D cycle and altered to constant darkness (D:D) after 7-days. After 12 days on D:D the animals were injected with vehicle or compound at CT6. **b**, Analysis of wheel running activity during the subject dark period following injection of SR9011 i.p. at CT6 in mice kept under constant darkness. **c**, Normalized expression levels of several core clock genes following administration of SR9011 or vehicle under constant dark conditions. C57Bl6 mice were administered SR9011 (100 mg/kg, i.p.) at CT0 on a day of constant darkness. Gene expression was determined and normalized to cyclophilin. Data were double plotted. **d**, Actograms illustrating the effect of single injections of vehicle, SR9011, or SR9009 in mice maintained under 12:12 L:D conditions. **e**, Normalized expression levels of several core clock genes following administration of SR9011 or vehicle under L:D (12:12) conditions. Methods for **e** and **e** were otherwise identical to **a** and **c**. * indicates $p<0.05$. Error bars indicate mean ± s.e.m. and n=6–10 mice.

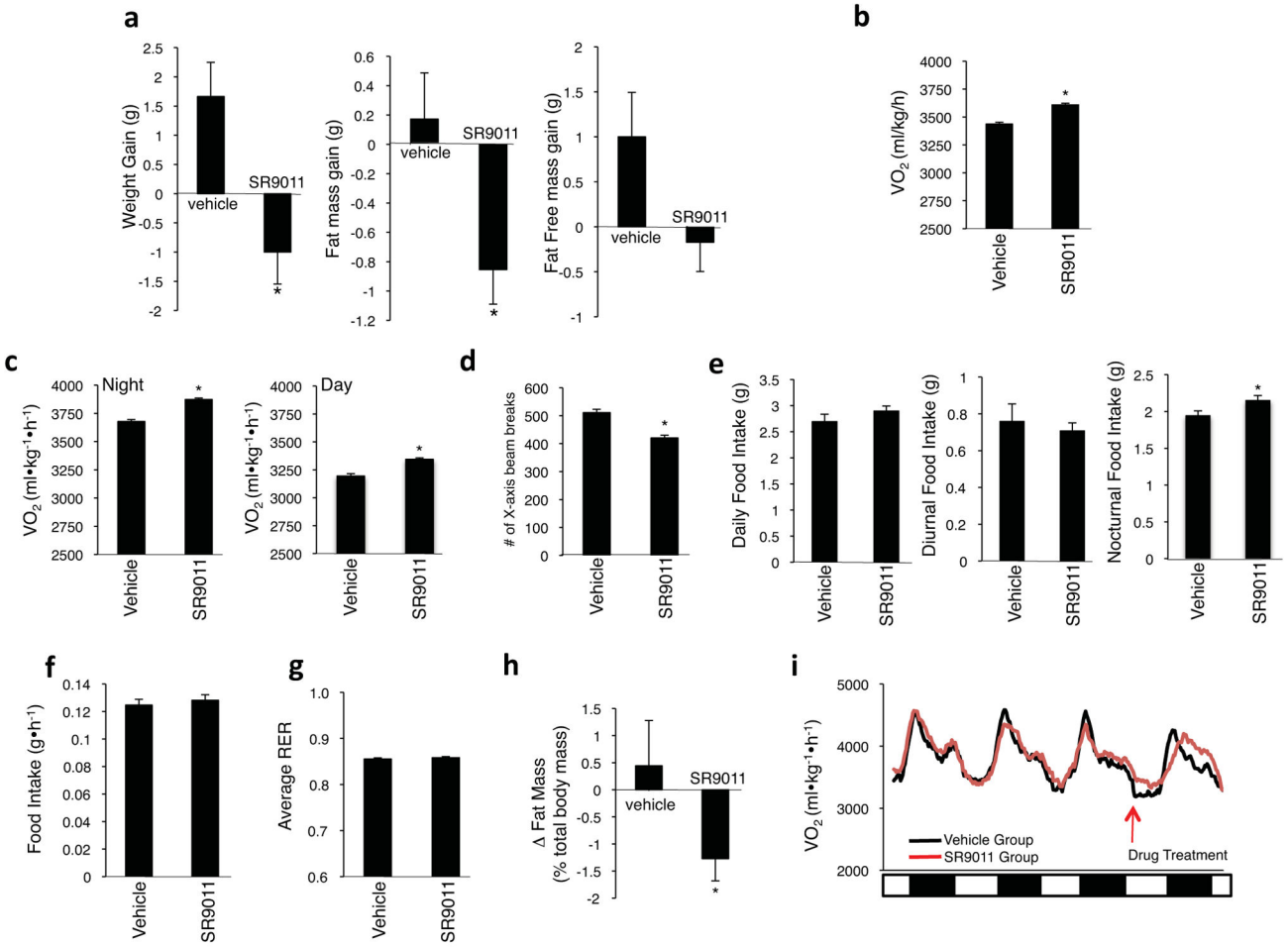

Figure 3. Activation of REV-ERB by SR9011 *in vivo* results in an increase in energy expenditure and weight loss

a, Treatment of mice (Balb/c) with SR9011 results in weight loss and fat mass loss. Animals were dosed with SR9011 (100mg/kg, i.p., b.i.d.) for 12 days. **b**, Oxygen consumption (VO_2) is increased in mice treated with SR9011. Results were obtained in using CLAMS and C57Bl6 mice were dosed as described in **a** except that the duration of treatment was 10 days. **c**, Oxygen consumption (VO_2) is increased during both the diurnal and nocturnal phases of C57Bl6 mice when they are treated with SR9011. Data obtained from the experiment described in **b** was analyzed for time of day differences. **d**, Mice treated with SR9011 are less active in the CLAMS as detected by the number of x-axis beam breaks. **e**, Total daily, diurnal and nocturnal food intake from the animals in the CLAMS study. **f**, The rate of food intake is not altered by SR9011 treatment. **g**, Respiratory exchange ratio (RER) is not altered by SR9011 treatment. **h**, After completion of the 10-day CLAMS experiment animals fat mass was assessed by DEXA. **i**, Results from a CLAMS experiment illustrating the diurnal increase in oxygen consumption prior to and immediately after administration of SR9011. Note the ~3h delay in the diurnal peak in VO_2 following administration of SR9011. For all b.i.d. dosing animals were dosed at CT0 and CT12. * indicates $p<0.05$. Error bars indicate mean ± s.e.m. and n=6–10 mice

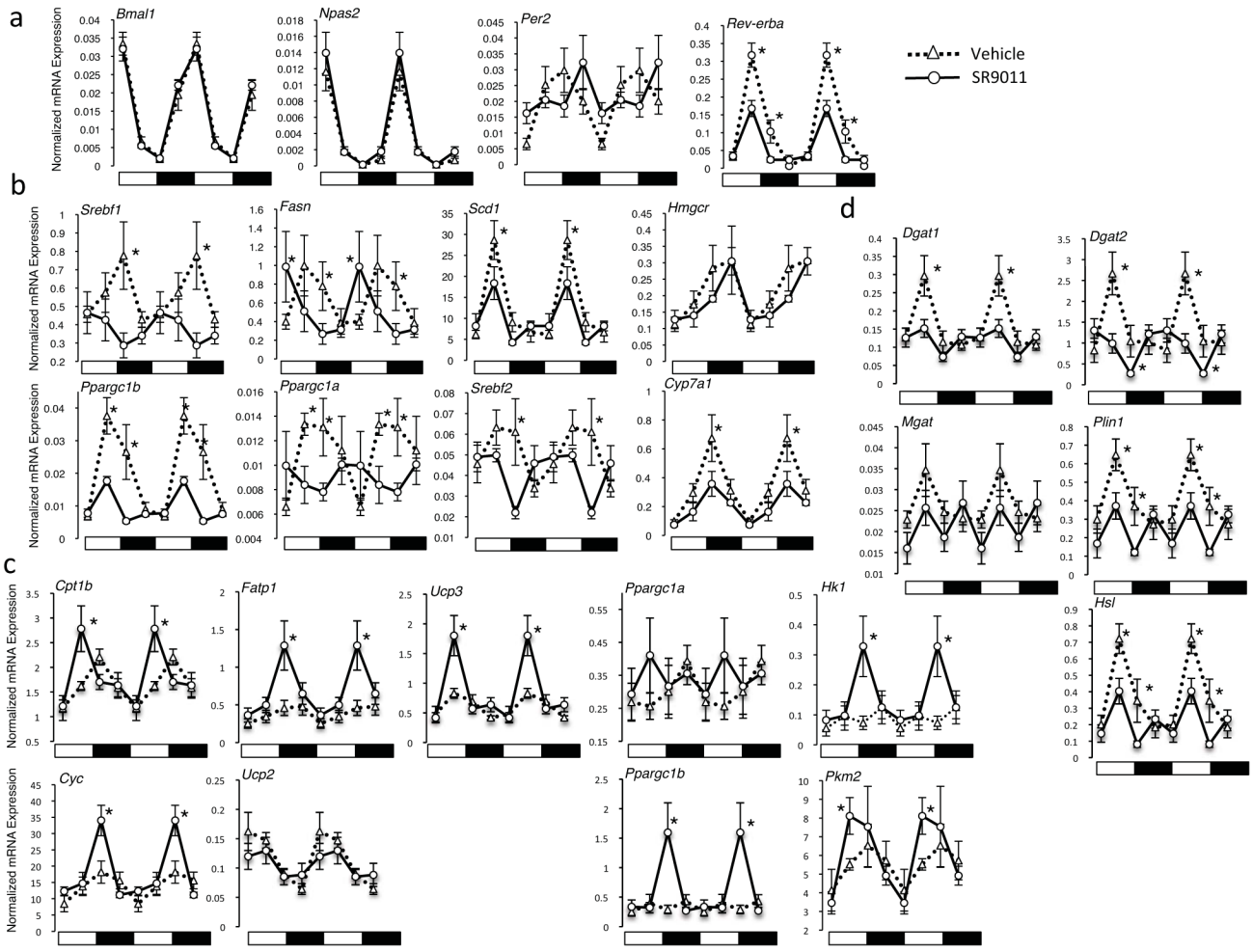

Figure 4. REV-ERB ligands alter the pattern of circadian expression of metabolic genes in the liver, skeletal muscle and adipose tissue

C57Bl6 mice were administered a single dose of SR9011 (100 mg/kg, i.p.) at CT0 and groups of animals (n=6) were sacrificed and gene expression assessed by QPCR. Graphs were double plotted. **a**, Expression of core clock genes from the liver of vehicle treated vs. SR9011 treated mice. **b**, Expression of metabolic genes from the liver of vehicle treated vs. SR9011 treated mice. **c**, Expression of metabolic genes from the skeletal muscle of vehicle treated vs. SR9011 treated mice. **d**, Expression of metabolic genes from the white adipose tissue (WAT) of vehicle treated vs. SR9011 treated mice. * indicates $p<0.05$. Error bars indicate mean ± s.e.m. and n=6–10 mice

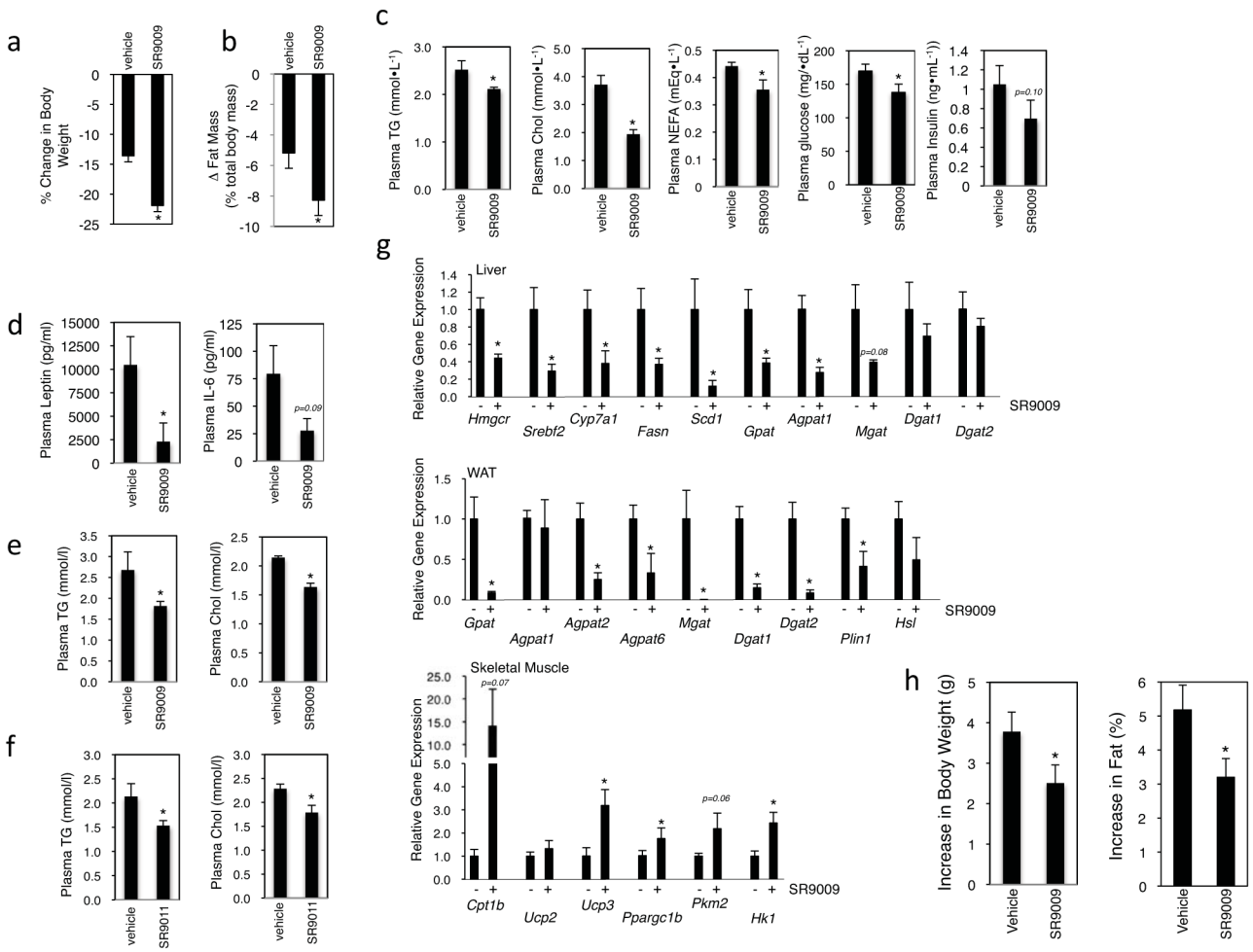

Figure 5. SR9009 treatment results in a decrease in fat mass and in plasma lipids in diet-induced obese mice

a, Diet-induced obese mice on SR9009 treatment lose weight vs. vehicle treated mice. C57Bl6 mice on a high fat diet were administered SR9009 (100mg/kg, i.p., b.i.d, at CT0 and CT12) for 30 days. **b**, Diet-induced obese mice on SR9009 treatment exhibit lower fat mass vs. vehicle treated mice. **c**, Fasting plasma triglycerides (TG), cholesterol (Chol), non-esterified fatty acids (NEFA) and glucose are decreased in SR9009 treated DIO mice. **d**, Plasma leptin and IL-6 levels from DIO mice **e**, Fasting plasma TG and Chol in lean C57Bl6 mice. Normal mice were administered 100 mg/kg, i.p., b.i.d. (at CT0 and CT12) SR9009 for 10 days. **f**, Fasting plasma TG and Chol are decreased by SR9011 treatment in lean C57Bl6 mice. **g**, Expression of metabolic genes in liver, WAT and skeletal muscle of DIO mice treated with SR9009 as described in **a**. Gene expression was measured by QPCR and normalized to *Cyclophilin b* expression. **h**, SR9009 treatment reduces weigh gain in *ob/ob* mice. Body weight and body fat content data are shown from *ob/ob* mice administered SR9009 for 12-days (100 mg/kg, i.p., b.i.d.).* indicates $p<0.05$. Error bars indicate mean ± s.e.m. and n=6–10 mice