Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists

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Synchronizing rhythms of behaviour and metabolic processes is important for cardiovascular health and preventing metabolic diseases. The nuclear receptors REV-ERB-α and REV-ERB-β have 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 behaviour 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 dyslipidaemia and hyperglycaemia. These results indicate that synthetic REV-ERB ligands that pharmacologically target the circadian rhythm may be beneficial 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 suprachiasmatic nucleus (SCN) of the hypothalamus functions as the master circadian pacemaker, synchronizing behavioural and physiological rhythms to the environmental light/dark cycle1. 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 temperature2.

The mammalian molecular clock is composed of a transcriptional feedback loop where heterodimers of the transcription factors BMAL1 and CLOCK (circadian locomotor output cycles kaput) or NPAR2 (neuronal PAS domain-containing protein 2) activate the transcription of the period (Per1, Per2 and Per3) and cryptochrome (Cry1 and Cry2) genes. Subsequently the PER and CRY proteins feedback to inhibit BMAL1–CLOCK activity, resulting in a rhythmic, circadian pattern of expression of these genes3. The REV-ERB nuclear receptors have an important role in feedback regulation of the circadian oscillator. Both Bmal1 and Clock are direct REV-ERB target genes4,5 and loss of REV-ERB-α alters circadian behaviour4. The physiological ligand for REV-ERB-α and REV-ERB-β was recently identified as haem6,7, 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 ligands8–11. Here we describe the development of REV-ERB ligands that allowed for characterization of the effects of modulation of this receptor in vivo.

Development of REV-ERB–α/β agonists

We developed two REV-ERB-α/β agonists with sufficient plasma/brain exposure to allow evaluation of their effects in vivo. Both SR9011 and SR9009 (Fig. 1a and Supplementary Fig. 1) dose-dependently increased the REV-ERB-dependent repressor activity assessed in HEK293 cells expressing a chimaeric Gal4 DNA binding domain (DBD): REV-ERB ligand binding domain (LBD) α or β and a Gal4-responsive luciferase reporter (Fig. 1b) (SR9009: REV-ERB-α half-maximum inhibitory concentration (IC50) = 670 nM, REV-ERB-β IC50 = 800 nM; SR9011: REV-ERB-α IC50 = 790 nM, REV-ERB-β IC50 = 560 nM). The REV-ERB ligand GSK4112 (Supplementary Fig. 2), which exhibits no plasma exposure6–11, displays limited activity (Fig. 1b). Both SR9011 and SR9009 potently and efficaciously suppressed transcription in a co-transfection assay using full-length REV-ERB-α along with a luciferase reporter driven by the Bmal1 promoter (Fig. 1c) (SR9009 IC50 = 710 nM; SR9011 IC50 = 620 nM). SR9011 and SR9009 suppressed the expression of Bmal1 messenger RNA in HepG2 cells in a REV-ERB-α/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 co-repressor-nuclear receptor box (CoRNR) peptide fragment of nuclear receptor corepressor 1 (NCoR) using a biochemical assay (Supplementary Fig. 4)6. Direct binding of the SR9009 to REV-ERB-α was also confirmed using circular dichroism analysis (Supplementary Fig. 5) (dissociation (Kd) = 800 nM). Neither compound exhibited activity at other nuclear receptors2,13 (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 mouse14 (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\(^6\) and displayed dose-dependent suppression of expression in response to SR9011 (Fig. 1e). The cholesterol 7α-hydroxylase (*Cyp7a1*) and sterol response element binding protein (*Srebf1*) genes have also been shown to be responsive to REV-ERB\(^1\)\(^\text{16,17}\) and were dose-dependently suppressed with increasing amounts of SR9011 (Fig. 1e). SR9009 displayed a similar effect on these genes (Fig. 1e).

**REV–ERB agonists modulate circadian behaviour**

On the basis of the effects of these compounds on SCN clock activity, we predicted that administration of these compounds would alter circadian behaviour. 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 circadian time 6 (CT6) (6 h after lights on); peak expression of *Rev-erb-α* (Supplementary Fig. 9)). Vehicle injection caused no disruption in circadian locomotor activity (Fig. 2a, top 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, bottom panels). Normal activity returned the next circadian cycle, consistent with clearance of the drugs in less than 24 h. This effect was not due to toxicity as the complete loss of locomotor activity was not observed in an identical experiment using L/D conditions (Fig. 2d). Additionally, mice treated with SR9011 did not display a decrease in strength (Supplementary Fig. 10a) and continued to move as detected in an open field assay (Supplementary Figs 10b, c). Furthermore, we observed no overt toxicity when we examined complete blood counts (Supplementary Fig. 11). We observed that the SR9011-dependent decrease in wheel running behaviour in the mice under constant darkness conditions was dose-dependent (Fig. 2b) and that the potency (half-maximum effective dose (ED\(_{50}\)) = 56 mg kg\(^{-1}\) of SR9009 (i.p., twice per day (b.i.d.)) for 6 days. *P < 0.05; **P < 0.05 versus before SR9011 and during SR9011 treatment. Error bars indicate mean ± s.e.m.; \(n = 6\).

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 whereas *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 (![](https://rdcdn.com/assets/images/prod/100485/2012/05/07/128625/ARTICLE.png)). 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 (12 h/12 h) 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 behaviour, 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
oxygen consumption (VO2) was observed, indicating increased energy consumption. After acclimation, the animals increased fat mass (Fig. 3a); however, food intake was not affected (Fig. 3b). Analysis of wheel running activity during the subject dark period after injection of SR9011 i.p. at CT16 in mice kept under constant darkness indicated that light input into the circadian oscillator has a significant effect on the action of these drugs.

**REV-ERB-α/β agonists modulate metabolism in vivo**

Genetic perturbation of the core clock genes leads to a range of metabolic phenotypes. In addition, REV-ERB-α agonists have been shown to directly regulate genes involved in lipid and glucose metabolism. 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 C57BL/6 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 (VO2) was observed, indicating increased energy expenditure (Fig. 3b). The increase in VO2 was evident in the diurnal and nocturnal phases (Fig. 3c). The increases in VO2 were not due to increased activity, as 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 administration 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 versus 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 1–3-h delay in peak VO2 was observed with administration of SR9011 (Fig. 2i). Given the association between the circadian rhythm and metabolic regulation, and 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. After a single injection of SR9011, we monitored the expression of clock genes in the liver over a 24-h period. When examining the effects of SR9011 treatment on core clock gene expression, the pattern of expression of Per2 was altered, but expression of other genes such as Bmal1 and Npas2 was unaffected (Fig. 4a).

Thus, SR9011 treatment results in alterations in the pattern of circadian expression of clock genes in both the hypothalamus (Fig. 2b, d and 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 expression was shifted (Fig. 4b). Expression of genes involved in cholesterol and bile acid metabolism were also
Drug treatment

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for skeletal muscle revealed a potential mechanism for the increased with SR9011 treatment (Fig. 4b). Examination of gene expression in played a strong circadian pattern of expression that was suppressed time of day differences.

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Glucose oxidation in addition to fatty acid oxidation. The rate-limiting increase in both (Fig. 4c), indicating that treatment resulted in increased oxidation, but the increases in expression were amplified (Fig. 4c).

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Peroxisome proliferator-activated Srebf2 altered. Expression of Srebf2 and Cyp7a1 was decreased (Fig. 4b). 3-Hydroxy-3-methyl-glutaryl-CoA reductase (Hmgcr) was unaffected in this acute model (Fig. 4b). Peroxisome proliferator-activated receptor-γ coactivator 1α and 1β (Pparγ1a and Pparγ1b) both displayed a strong circular 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 levels 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; also called Scl27a1)—were elevated (Fig. 4c). Pparγ1b 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 of key enzymes in the glycolytic pathway (hexokinase (Hk1) and pyruvate kinase (Pkm2)) we noted an increase in both (Fig. 4c), indicating that treatment resulted in 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 (refs 21, 22). We found that SR9011 suppressed the circadian rhythm of Namp1 gene expression in the liver (Supplementary Fig. 15a), indicating that REV-ERB agonist treatment may alter the post-translational acetylation of proteins that may contribute to some of the physiological alterations that we observe.

In contrast to muscle where there was amplification of the circadian expression of genes coupled to fatty acid oxidation and glycolysis, in 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 Dgat2), the genes encoding the enzyme that catalyses the terminal and committed step in triglyceride synthesis, was suppressed with SR9011 treatment (Fig. 4d). Consistent with this pattern, the circadian expression of another gene involved in triglyceride synthesis, monoacylglycerol acyltransferase (Mgat1), is also disturbed (Fig. 4d). Expression of lipid-droplet-associated protein genes including perilipin 1 (Plin1) and hormone sensitive lipase (Hsl; also called Lipe) was also suppressed with SR9011 treatment (Fig. 4d). We also observed suppression of nocturnin (Ccrn4l) expression in both adipose and hepatic tissue (Supplementary Fig. 15b). Similar effects on gene expression were noted in mice 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.
SR9009 improves the metabolic profile in obese mice

On the basis of the alterations in energy metabolism and gene expression that we observed in normal C57BL/6 and BALB/c mice, we sought to examine whether a REV-ERB-α/β agonist would be efficacious in a rodent model of obesity. We initiated the study with 20-week-old C57BL/6 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 high-fat diet and we initiated twice per day dosing (intraperitoneal (i.p.)) of SR9009. Although 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 mice, although handling itself reduced food intake, explaining the weight loss observed in the controls. SR9009-treated mice exhibited a more severe reduction in adiposity compared with vehicle treatment (Fig. 5b). In addition to the decrease in fat mass we also observed a 12% decrease in plasma triglycerides and a 47% decrease in plasma total cholesterol (Fig. 5c).

Plasma non-esterified fatty acids (NEFA) were also reduced (23%) along with plasma glucose (19%) in the SR9009-treated mice (Fig. 5c). There was also a trend towards a decrease in plasma insulin levels (35%). Consistent with the decrease in adiposity we also noted an 80% decrease in plasma leptin and a decrease (72%) in the proinflammatory cytokine interleukin (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, f). Consistent with the decreased plasma triglyceride levels and total cholesterol levels, we observed a significant decrease in the expression of genes encoding lipogenic enzymes (Fasn and Scd1) as well as cholesterol regulatory proteins (Hmgcr and Srebf2) with SR9009 treatment (Fig. 5g). In the WAT, SR9009 treatment resulted in a decrease in the expression of genes encoding enzymes involved in triglyceride synthesis (Fig. 5g) as well as Plin1 (Fig. 5h).

Taken together with the results from the CLAMS experiments (Fig. 3), these data indicate that REV-ERB agonists increase energy expenditure by increasing fatty acid and glucose oxidation in the skeletal muscle. The gene expression data are also consistent with decreased triglyceride synthesis in the liver and WAT as well as a reduction in hepatic cholesterol synthesis.
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 versus vehicle-treated mice. C57BL/6 mice on a high-fat diet were administered SR9009 (100 mg kg\(^{-1}\); i.p., b.i.d., at CT0 and CT12) for 30 days. b, Diet-induced obese mice on SR9009 treatment exhibit lower fat mass versus 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 triglyceride and cholesterol in lean C57BL/6 mice. Normal mice were administered 100 mg kg\(^{-1}\); i.p., b.i.d. (at CT0 and CT12) SR9009 for 10 days. f, Fasting plasma triglyceride and cholesterol are decreased by SR9011 treatment in lean C57BL/6 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 weight 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\(^{-1}\); i.p., b.i.d.). *\(P < 0.05\). Error bars indicate mean ± s.e.m.; \(n = 6–10\) 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 versus vehicle-treated mice. C57BL/6 mice on a high-fat diet were administered SR9009 (100 mg kg\(^{-1}\); i.p., b.i.d., at CT0 and CT12) for 30 days. b, Diet-induced obese mice on SR9009 treatment exhibit lower fat mass versus 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 triglyceride and cholesterol in lean C57BL/6 mice. Normal mice were administered 100 mg kg\(^{-1}\); i.p., b.i.d. (at CT0 and CT12) SR9009 for 10 days. f, Fasting plasma triglyceride and cholesterol are decreased by SR9011 treatment in lean C57BL/6 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 weight 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\(^{-1}\); i.p., b.i.d.). *\(P < 0.05\). Error bars indicate mean ± s.e.m.; \(n = 6–10\) mice.

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, as modulation of the receptors’ activity would be only transient. This is also probably the reason that on the day after administration of the REV-ERB agonist, normal circadian behaviour 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 behaviour. A single injection of either SR9009 or SR9011 resulted in a 1–3-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, indicating 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 examples of genetic alterations to clock genes leading to metabolic disturbances and even metabolic diseases in rodent models. In addition to its role in direct modulation of the positive arm of the mammalian circadian oscillator, REV-ERB has also been demonstrated to have a direct role in the regulation of an array of metabolic genes. Our observations that REV-ERB has also been demonstrated to have a direct role in the modulation of the positive arm of the mammalian circadian oscillator, REV-ERB also regulates an array of metabolic genes. Our observations that REV-ERB has also been demonstrated to have a direct role in the regulation of an array of metabolic genes.

METHODS SUMMARY

Synthesis of SR9009 and SR9011. Compounds were synthesized by reductive amination of 5-nitro-2-thiophencarboxaldehyde with 4-chlorobenzylamine, and sodium triacetoxoborohydride 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 transfections. HEK293 cells were maintained and transfected with vectors previously described.

Mouse studies. For circadian gene expression experiments, male C57BL/6 mice (8–10 weeks of age) were either maintained on a L/D (12 h/12 h) cycle or on constant darkness. At circadian time (CT) 0 mice were administered a single dose (8–10 weeks of age) were either maintained on a L/D (12 h/12 h) cycle or on constant darkness. At circadian time (CT) 0 mice were administered a single dose of 100 mg kg−1 SR9009 or SR9011 (i.p.) and groups of animals were killed at CT0, CT6, CT12 and CT18. Gene expression was determined by real-time QPCR.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions T.P.B. conceived the project. R.N., Y.S. and T.M.K. synthesized the ligands. L.A.S., Y.W., S.B., D.J.K. and T.P.B. designed/analysed and/or performed the CD analysis. T.P.B., L.A.S. and A.A.B. designed, analysed and performed the transfection and biochemical assays. D.J.K. and T.H. designed and analysed the ligands. L.A.S., Y.W., S.B., D.J.K. and T.P.B. designed/analysed and/or performed the studies using the circadian gene expression and behaviour analysis. J.L. performed gene expression analysis. S.-H.Y. and J.S.T. designed and performed the studies using the metabolic studies. Y.W., T.P.B., S.B. and T.L designed/analysed and/or performed the QPCR analysis. S.-H.Y. and J.S.T. performed the studies using the circadian gene expression and behaviour analysis. J.L. performed gene expression analysis. S.-H.Y. and J.S.T. designed and performed the studies using the circadian gene expression and behaviour analysis. J.L. performed gene expression analysis. S.-H.Y. and J.S.T. designed and performed the studies using the circadian gene expression and behaviour analysis.