High-fat diet-induced hyperinsulinemia and tissue-specific insulin resistance in Cry-deficient mice

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1Circadian Rhythms Group, Max Planck Institute of Biophysical Chemistry, Göttingen, Germany; 2Institute of Experimental and Clinical Pharmacology and Toxicology, University of Lübeck, Lübeck, Germany; 3Department of Genetics, Cancer Genomics Center, Erasmus University Medical Center, Rotterdam, Netherlands; and 4Department of Internal Medicine I, University of Lübeck, Lübeck, Germany

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Barclay J.L., Shostak A., Leliavski A., Tsang AH., Jöhren O., Müller-Fielitz H., Landgraf D., Naujokat N., van der Horst GT., Oster H. High-fat diet-induced hyperinsulinemia and tissue-specific insulin resistance in Cry-deficient mice. Am J Physiol Endocrinol Metab 304: E1053–E1063, 2013. First published March 26, 2013; doi:10.1152/ajpendo.00512.2012.—Perturbation of circadian rhythm in mammals, either by environmental influences such as shiftwork or by genetic manipulation, has been associated with metabolic disturbance and the development of obesity and diabetes. Circadian clocks are based on transcriptional/translational feedback loops, comprising positive and negative components. Whereas the metabolic effects of deletion of the positive arm of the clock gene machinery, as in Clock- or Bmal1-deficient mice, have been well characterized, inactivation of Period genes (Per1–3) as components of the negative arm have more complex, sometimes contradictory effects on energy homeostasis. The CRYPTOCHROMEs are critical interaction partners of PERs, and simultaneous deletion of Cry1 and -2 results in behavioral and molecular circadian arrhythmicity. We show that, when challenged with a high-fat diet, Cry1/2−/− mice rapidly gain weight and surpass that of wild-type mice, despite displaying hypophagia. Transcript analysis of white adipose tissue reveals up-regulated expression of lipogenic genes, many of which are insulin targets. High-fat diet-induced hyperinsulinemia, as a result of potenti-tiated insulin secretion, coupled with selective insulin sensitivity in adipose tissue of Cry1/2−/− mice, correlates with increased lipid uptake. Collectively, these data indicate that Cry deficiency results in an increased vulnerability to high-fat diet-induced obesity that might be mediated by increased insulin secretion and lipid storage in adipose tissues.

circadian clock; lipid uptake; insulin; cryptochrome; diet-induced obesity

GENETICALLY ENCODED CIRCADIAN clocks are present in most organisms. They respond to external timing signals such as light and food availability and coordinate internal physiological processes throughout the course of the day for maximal fitness. At the molecular level, the mammalian circadian clock is comprised of interlocking transcriptional/translational feedback loops (TTLs) of positive and negative components. The positive arm of the mammalian core TTL consists of the transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1 or ARNTL), which dimerize and bind to E-box cis-elements on target promoters. The CLOCK-BMAL1 complex drives the transcription of three period (Per1–3) and two cryptochrome genes (Cry1 and -2). TheCRY and PER proteins, in turn, form the negative arm of the clock, completing a time-delayed feedback and impinging on their own transcription by inhibiting CLOCK and BMAL1 (6, 38), resulting in a rhythm approximating 24 h. Clock gene rhythms are translated into physiological functions via rhythmic regulation of clock output genes that make up to 10% of the transcriptome of each cell (40). At the systemic level the mammalian circadian timing system shows a hierarchical organization, with a master pacemaker residing in the suprachiasmatic nuclei (SCN) of the hypothalamus. SCN clocks are synchronized to external time by the daily light-dark cycle, perceived through photoreceptors in the eye. From the SCN, synchronizing neurohumoral signals are sent to subsidiary oscillators in the periphery and other regions of the brain (44). Unlike the core clock genes, which are ubiquitously expressed, clock-controlled output genes are tissue specific, reflecting the physiological function of the respective organ or cell type (49).

Perturbation of the mammalian circadian clock has been linked to various pathophysiologies such as cancer (18), mood disorders (8), sleep disturbances (30), and metabolic disrup-tion (12, 14). In humans, the correlation between external circadian perturbation and adverse metabolic consequences has been discussed (5, 16). For example, human shift workers have an increased propensity for developing higher body mass index (BMI) and metabolic syndrome (2, 4). The role of various core clock genes in metabolic regulation has been best demonstrated using mutant mouse models. Young adult Bmal1−/− mice exhibit impaired glucose tolerance and gluconeogenic potential (28, 42, 48). The role of various core clock genes in metabolic regulation has been best demonstrated using mutant mouse models. Young adult Bmal1−/− mice exhibit impaired glucose tolerance and gluconeogenic potential (28, 42, 48). The role of Bmal1 in the regulation of adipogenesis is disputed. On one hand, Bmal1 is promoting adipogenesis (47), since in Bmal1−/− mice expression levels of adipogenesis markers and regulators of triglyceride and fatty acid synthesis are reduced in adipose tissues. Concurrently, circulating lipids are increased in these mice, correlating with increased lipoprotein lipase (Lpl) expression in the liver and ectopic lipid accumulation in liver and muscle (48). On the other hand, Bmal1 has been shown inhibit adipocyte differentiation via transcriptional regulation of components of the Wnt signaling pathway (21). ClockΔ19 mice show impaired gluco-neogenesis in addition to hyperphagia and obesity (42, 50), although the metabolic effects of this mutation appear to strongly depend on the genetic background (26). At the level of the pancreas, both ClockΔ19 and Bmal1−/− mice display defective insulin secretion (33, 43).
In regard to the negative arm of the TTL, the metabolic consequences of clock gene disruption become less clear. On one hand, several studies suggest that, whereas Per2−/− and Per2Brd mice are already heavier under normal chow (NC) conditions, Per2−/−, Per1/2/3m1Drw, and Per3m1Drw mice are all susceptible to diet-induced obesity (10, 20, 56). On the other hand, Per1Brd and young Per2−/− mice show reduced body weight (10, 20). Per2−/− mice also show increased absolute food intake when kept on a high-fat diet (HFD) (56), whereas food intake is reduced in Per1/2/3m1Drw and normal in Per3m1Drw mice (11). Under NC conditions, targeted disruption of Per2 has been shown to result in increased food intake (10), whereas another report shows no effect (56). Per1Brd mice show normal food intake but are still hyperphagic when food intake is corrected for body weight (10). Finally, Per2−/− mice have been recently reported to have increased glucose-stimulated insulin secretion (GSIS) (59), contrary to what has been reported for ClockΔts and Bmal1−/− mice (33, 43). One could argue that the PER proteins are not ideal representatives of the negative arm, since they appear to predominately modulate the timing of the negative feedback rather than directly inhibiting CLOCK and BMAL1 (25, 35), whereas CRY proteins are much stronger inhibitors of E-box-mediated transcription (19, 46). The effect of Cry deficiency on metabolism has not yet been investigated. This is of particular interest, since these mice are reported to display low body weight, similar to that seen in the Per1Brd mice (7, 10). To close this gap, the current study characterizes the response of Cry1/2−/− mice to diet-induced obesity, providing mechanistic insight into the physiological anomalies seen under these conditions.

MATERIALS AND METHODS

Animals. Male and Cry1/2−/− mice (52) on a C57BL/6J background and wild-type (WT) controls, 8–10 wk old, were entrained to a 12:12-h light-dark (LD) schedule with lights on [Zeitgeber time (ZT) 0] at 7:00 A.M. Mice were housed individually and kept under constant temperature (20.0 ± 0.5°C) and humidity (50–60%) conditions with ad libitum access to NC (3.4% kJ fat, Ssniff V1536) or high-fat chow (45% kJ fat, Ssniff EF D12451) and water. Activity was measured using custom-made infrared detectors mounted on top of each cage and analyzed using ClockLab software (Actimetrics, Evanston, IL). After 10 wk, mice were killed by cervical dislocation at the indicated time points. In the dark phase, a 5-W safety red light was used to avoid acute light effects. Gonadal, subcutaneous, and perirenal fat pads were isolated and weighed. Serum and tissues were collected and frozen. Gonadal fat was collected in 4% paraformaldehyde for paraffin embedding and sectioning. Hepatic lipid content was determined by Oil Red O staining on frozen sections followed by densitometric analysis using Image J software (NIH, Bethesda, MD). All animal experiments underwent ethical assessment and were licensed and approved by the Office for Consumer Protection and Food Safety of the State of Lower Saxony and executed in accordance with the German Law on Animal Welfare.

Serum analysis. Serum insulin levels were determined using the Ultrasensitive Mouse Insulin ELISA (Mercodia, Uppsala, Sweden).
Serum glucose was measured using an Accu-Chek Aviva glucose monitor and test strips (Mannheim, Germany). Circulating triglycerides were determined using the Serum Triglyceride Determination Kit (Sigma, St. Louis, MO), and free fatty acids were measured using the Serum Fatty Acid Kit (Zen-Bio). Cholesterol was measured using the Cholesterol Assay Kit (Cayman Chemical, Ann Arbor, MI). Leptin was measured using the Mouse Leptin ELISA Kit (Crystal Chem, Downers Grove, IL) and corticosterone using the ImmuChem Double Antibody125I-Radioimmunoassay Kit (MP Biomedicals, Solon, OH).

**Insulin and glucose tolerance tests.** Metabolic tests were performed on male mice after 10 wk of HFD. Mice were starved from ZT0 to ZT4 and injected intraperitoneally with 1.0 IU/kg insulin (ipITT) or 2 g/kg glucose (ipGTT). Blood was sampled from the tail vein at indicated time points between ZT4 and ZT6, and glucose levels were determined as described above.

**Calorimetric measurements.** Mice were placed in metabolic cages in LD with lights on at 7:00 A.M. Food intake, water intake, locomotor activity, O₂ consumption, and CO₂ production were continuously monitored using an open-circuit indirect calorimetry system (TSE Phenomaster Systems, Bad Homburg, Germany). After an initial phase of acclimatization for at least 48 h, data were sampled every 15 min for three to four additional days. The respiratory exchange ratio (RER) was estimated as the ratio of V˙CO₂ produced (ml/h) to V˙O₂ consumed (ml/h). Energy expenditure (EE, kcal·h⁻¹·kg⁻¹) was calculated using the formula (3.941 + 1.106 × RER) × 0.001 × V˙O₂ and normalized to body weight (54).

**Fig. 2.** Adipocyte hypertrophy, hepatic lipid accumulation, and arrhythmic metabolic profiles in Cry1/2⁻/⁻ mice. Fat pad weights (A), gonadal adipocyte size (B), and hepatic lipid accumulation (C) after 10 wk of HFD (n = 7–10). *P < 0.05 relative to WT determined by t-test. B: representative images of gonadal adipocytes from WT and Cry1/2⁻/⁻ mice (scale bar represents 100 µM). D and E: 24-h profiles of respiratory exchange rates (RER, D) and energy expenditure (EE, E) in WT and Cry1/2⁻/⁻ mice after 10 wk of NC (top) or HFD (middle) (n = 4–8). Dotted lines indicate SE. Bottom: diurnal variations in RER (D) and EE (E). *P < 0.05, dark vs. light phase within genotype and condition by 2-way ANOVA.
Quantitative real-time PCR. Relative quantification of mRNA levels by quantitative real-time PCR (qPCR) was performed as previously described (39). Total RNA was extracted using TRIzol reagent (Life Technologies, Darmstadt, Germany), cDNA synthesis was performed by reverse transcription (Life Technologies) using random hexamer primers. qPCR was performed using iTQ SYBR Green Supermix (Bio-Rad, Hercules, CA) on an Bio-Rad C1000 Thermal Cycler and a CFX96 Real-Time system (95°C for 3 min, 40 cycles at 94°C for 15 s, 60°C for 15 s and 72°C for 20 s, 95°C 10 s, and then 95°C for 10 s followed by a melt curve from 65°C to 95°C at 0.5°C increments for 5 s). Primer sequences are available on request. Eef1a was used as a reference gene, and data were expressed as relative quantitation (ΔΔCT method).

Adipocyte histology. Freshly isolated gonadal fat pad samples were fixed with 4% paraformaldehyde and embedded in paraffin. Tissue sections were sectioned at 8 μm and stained with hematoxylin-eosin. Adipocyte size was determined with Image J software (NIH).

Insulin-stimulated protein kinase B phosphorylation. Mice were injected with 1.0 IU/kg insulin or saline intraperitoneally at ZT6, and tissues were collected in liquid nitrogen 10 min later. Tissues were homogenized and immunoprecipitated using protein kinase B (Akt) antibody (no. 9272; Cell Signaling) and 500 μg protein lysate. Membranes were probed using phospho-Akt (Ser473) antibody (no. 9271; Cell Signaling), stripped, and reprobed with Akt antibody. Quantitation of densitometry was performed, and data were expressed as the ratio of phospho-Akt to Akt, relative to saline-injected controls within each genotype. For comparison of Akt levels between genotypes, tissues lysates were blotted and probed using Akt antibody; membranes were then stripped and reprobed with GAPDH antibody (ab9485; Abcam).

Ex vivo GSIS. Pancreata were dissected from HFD mice at ZT3 into Krebs-Ringer buffer, cut lengthways in half, weighed, and incubated in Krebs-Ringer buffer with 2 mM glucose at 37°C with 5% CO2 for 30 min. Tissues were transferred into fresh Krebs-Ringer buffer with 2 mM glucose for 10 min, and buffer was assayed for insulin as described above and expressed relative to tissue weight.

Ex vivo lipid uptake. Epididymal fat pads were collected at ZT3 into DMEM (low glucose) with 0.1% fatty acid free-BSA. Pieces were cut and weighed. One group was incubated directly for 1 h ≥ 30 nM insulin at 37°C with 5% CO2. A second group was preincubated for 24 h with 10 nM insulin in DMEM/BSA, briefly washed with DMEM/BSA, and stimulated as described. Tissue was transferred into Krebs-Ringer buffer with 0.3 μM oleic acid for 1 min, washed rapidly three times in cold PBS, and briefly homogenized in 0.5 ml PBS. Scintillation fluid (4.5 ml) was added, and samples were counted. Counts were normalized to tissue weight.

Statistical analysis. All data are shown as means ± SE. Statistical comparisons were performed using GraphPad Prism software (GraphPad, La Jolla, CA). t-Tests or two-way ANOVA with Bonferroni post hoc test were used. P values <0.05 were considered significant.

RESULTS

Cry1/2−/− mice are more susceptible to diet-induced obesity. As has previously been reported (7), Cry1/2−/− mice showed slightly decreased body weight under NC conditions (Fig. 1A), whereas overall food intake was not significantly altered (Fig. 1B). However, under a HFD, male Cry1/2−/− mice responded more rapidly than WT males and gained much more weight than WT mice over the course of 10 wk (Fig. 1A). Surprisingly, and despite their increased weight gain, Cry1/2−/− mice ate less than WT controls under HFD (Fig. 1B), which translated into an increase in energy-to-body mass conversion rate of threefold compared with WT mice and specific for the HFD condition (Fig. 1C). Under HFD conditions, WT mice showed increased food consumption during the light phase (27) but continued to eat most of their food during the night, whereas Cry1/2−/− mice displayed arrhythmic feeding behavior regardless of diet condition (Fig. 1D) and mirrored by total home cage activity distribution (Fig. 1E).

Cry1/2−/− have increased fat pad weight and adipocyte hypertrophy in the absence of overt calorimetric disruption. Congruent with their increased body weight, HFD-fed Cry1/2−/− mice displayed enlarged gonadal, subcutaneous, and perirenal fat deposits (Fig. 2A) and adipocyte hypertrophy, as evident from the increased gonadal adipocyte size (Fig. 2B), suggesting that the effect on fat mass was, at least in part, a result of enhanced lipid uptake/lipogenesis as opposed to adipogenesis. Lipid metabolism was similarly altered in liver tissue with increased lipid accumulation evidenced by Oil Red O staining on liver sections (Fig. 2C). In contrast, circulating lipids were not significantly altered in Cry1/2−/− mice. A trend toward up-regulated corticosterone was seen (P = 0.08 at both time points), whereas leptin blood levels were not changed in Cry mutant mice (Table 1). To determine whether this obesity phenotype was a result of altered energy substrate utilization or EE, mice were placed in calorimetric cages after 10 wk of NC or HFD (Fig. 2, D and E). WT mice showed dampened metabolic rhythms under HFD, with reduced diurnal variation in RER (Fig. 2D) and no significant difference between light- and dark-phase averages for EE (Fig. 2E). In contrast, Cry mutant RER and EE profiles were arrhythmic both under NC and HFD conditions, mirroring food intake and activity profiles (Fig. 1). Both genotypes used mostly lipids as an energy source during HFD, as indicated by a lowered RER compared with NC conditions (Fig. 2D), but no significant differences in overall RER/EE were recorded between genotypes.

Table 1. Serum lipid and hormone profiles in Cry1/2−/− and WT mice under HFD

<table>
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<tr>
<th>Serum Parameters (concentration)</th>
<th>WT</th>
<th>ZT6</th>
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Values are means ± SE, WT, wild type; HFD, high-fat diet; ZT, Zeitgeber time. Shown are circulating lipids, leptin, and corticosterone at ZT6 and ZT18 in WT and Cry1/2−/− mice (n = 5–6) and serum lipid and hormone profiles of WT and Cry1/2−/− mice under HFD.
Cry1/2−/− mice have increased insulin-targeted gene expression in white adipose tissue, despite overall insulin resistance. Focusing on gonadal adipose under HFD conditions, transcriptional analysis was performed targeting genes involved in lipid handling at two opposite diurnal time points, ZT6 (noon) and ZT18 (midnight). We observed upregulation of fatty acid synthase (Fas), Lpl, acetyl-CoA carboxylase (Acc1), acyl-CoA synthetase 4 (Acsl4), diacylglycerol acyltransferase (Dgat1 and -2), and leptin and downregulation of phosphoenolpyruvate carboxykinase (Pepck) expression in Cry1/2−/− mice at either one or both time points (Fig. 3, A–H). Strikingly, many of the affected genes are known direct targets of insulin signaling, indicating increased insulin stimulation. Circulating insulin levels were determined at ZT6 and ZT18, and ipGTT and ipITT were performed between ZT4 and ZT6 (Fig. 4, A–C). Under NC conditions, Cry mutants had similar insulin levels as WT controls. However, under HFD, Cry1/2−/− mice displayed hyperinsulinemia at both time points (Fig. 4A). Moreover, Cry1/2−/− mice showed signs of insulin resistance, with impaired glucose uptake in response to both glucose and insulin stimulation (Fig. 4, B and C).

Cry1/2−/− mice show tissue-specific insulin resistance. Elevated insulin levels correlated well with the observed upregu-

![Graphs showing mRNA expression of lipid-handling genes](http://ajpendo.physiology.org/)

**Fig. 3.** mRNA expression of lipid-handling genes in white adipose tissue (WAT). A–H: mRNA expression of lipid handling and insulin target genes in gonadal WAT under HFD conditions (n = 4–6). Fas, fatty acid synthase; Lpl, lipoprotein lipase; Acc1, acetyl-CoA carboxylase; Acsl4, acyl-CoA synthetase 4; Dgat1 and -2, diacylglycerol acyltransferase-1 and -2; Pepck, phosphoenolpyruvate carboxykinase. *Significant effect of genotype (P < 0.05). #Significant effect of time (P < 0.05) determined by 2-way ANOVA.
lation of insulin target genes in the adipose tissue. At the same time, however, impaired insulin-mediated glucose uptake was suggestive of insulin resistance. Given that white adipose tissue (WAT), the major site of insulin-stimulated lipogenesis, is responsible for only 3–5% of the glucose uptake after insulin stimulation (24), we hypothesized that in Cry1/2−/− mice insulin resistance might be restricted to other glucose-consuming tissues such as muscle and liver, whereas WAT would remain insulin sensitive. Indeed, we found that insulin injection at ZT6 resulted in increased Akt phosphorylation in the adipose tissue of Cry1/2−/− mice and not in WT mice (Fig. 5A). Conversely, insulin injection had no effect on Akt phosphorylation in the muscle or liver of either WT or Cry1/2−/− mice (Fig. 5, B and C), indicating insulin resistance in these tissues. Two-way ANOVA revealed a significant interaction (P < 0.0001) for the factors tissue and genotype, indicating that there is genotype and tissue specificity in the response to insulin stimulation. These differences cannot be explained by altered Akt baseline levels in Cry mutant mice, as shown by Western blotting using anti-GAPDH as loading controls (Fig. 5, A–C, insets on right). Of note, our paradigm yielded only moderate insulin stimulation effects under NC conditions (Fig. 5, A–C, left), probably reflecting the fact that some of the animals might have fed, and thus produced insulin, before treatment.

Insulin has a strong stimulatory effect on WAT lipogenesis (13). Consistent with increased insulin signaling in Cry1/2−/− mice, we observed elevated expression of genes indicative of increased lipid storage. To test if increased lipogenesis may contribute to the observed adipocyte hypertrophy in the mutants, lipid uptake by WAT was examined. Insulin-stimulated gonadal fat pads from Cry1/2−/− mice displayed enhanced lipid uptake compared with fat pads from WT mice (Fig. 5D). Under nonstimulated conditions, Cry1/2−/− fat pads also showed increased basal lipid uptake; however, this only reached significance by t-test (P = 0.0127). When fat pads were preincubated with insulin for 24 h, reflecting the hyperinsulinemic state in HFD-fed Cry mutant mice, this effect became even clearer. Whereas subsequent insulin stimulation did not alter lipid uptake responses in Cry1/2−/− explants, insulin responses were blunted in WT fat pads, indicating insulin resistance (Fig. 5E). We also tested for effects of insulin on lipid mobilization in vivo by measuring free fatty acids at two time points during ipGTTs. However, no significant effects of glucose administration were observed in either genotype (data not shown).

*Insulin secretion is potentiated in Cry1/2−/− mice. It has been recently shown that deficiency in Clock, Bmal1, or Rev-erba results in impaired insulin secretion, whereas Per2 deficiency has the opposite effect (33, 43, 53, 59). Ideally, insulin secretion would be measured in vivo under fasted conditions to reduce endogenous glucose and insulin levels. However, under fasted conditions, Cry1/2−/− mice displayed elevated circulating glucose and elevated circulating insulin (Fig. 6A), confounding an in vivo approach. Therefore, pancreatic insulin secretion was examined ex vivo and was found to be enhanced under basal (nonstimulated) glucose concentrations (2 mM) (Fig. 6B). Given that insulin secretion appears to be clock-regulated (33, 43, 53, 59), clock gene expression was examined in pancreas. In WT pancreas, rhythmic expression of Per2, Rev-erba, and Bmal1 was observed, with Per2 and Rev-erba peaking toward the end of the light phase and Bmal1 showing an inverted rhythm (Fig. 6C). In Cry1/2−/− pancreas, elevated, but nonrhythmic expression of Per2 and Rev-erba was observed, whereas Bmal1 expression was downregulated (Fig. 6C). Retarded insulin secretion in Rev-erba−deficient islets was attributed to reduced insulin exocytosis due to the downregulation of key exocytotic genes (53). At ZT18, these same genes were found to be upregulated (Munc18) or unchanged (Vamp3 and Syntaxin1a) in Cry1/2−/− mice (Fig. 6D).

**DISCUSSION**

We and others (23) have shown that Cry mutant mice are lean under NC conditions. However, during a HFD challenge,
and in spite of overall decreased food intake, Cry1/2−/− mice gained more weight than age-matched WT controls, resulting in a profound increase in energy efficiency (Fig. 1). Despite being attributable to increased fat depots and adipocyte hyper trophy, the diet-induced obesity phenotype in the Cry1/2−/− mice could not be correlated with alterations in EE or substrate utilization (Fig. 2). However, transcriptional analysis of gonadal WAT revealed that the expression levels of a number of genes involved in lipid uptake, many of which are direct transcriptional targets of insulin signaling such as Leptin, Fas, Lpl, and Pepck, were altered in Cry1/2−/− mice relative to WT controls (Fig. 3). Insulin rises after food intake and signals via a plethora of pathways to directly or indirectly induce the transcription of lipogenic regulators (such as Fas, Acc1, and Lpl) and inhibit glycerol production (via the suppression of Pepck) in WAT (15, 45, 55, 58). Importantly, insulin has also been shown to increase the enzymatic activity of both ACC1 and LPL, thereby further driving lipogenesis (17, 58).

**Fig. 5.** Tissue-specific insulin sensitivity in Cry1/2−/− mice. Insulin-stimulated protein kinase B (AKT) serine-473 phosphorylation (p) at ZT6 from WT and Cry1/2−/− mice as determined by ip (IP) WB and densitometric quantitation (n = 3) in gonadal WAT (A), muscle (B), and liver (C). *P < 0.05 relative to saline injection in the same genotype determined by t-test. Insets: Western blot (WB) for AKT (−60 kDa) under nonstimulated conditions with anti-GAPDH (GDH; −40 kDa) as loading control. D and E: ex vivo uptake of [14C]oleic acid in gonadal WAT (n = 5) before (D) and after (E) incubation with insulin (10 nM) for 24 h. *P < 0.05 relative to WT within the same insulin conditions determined by 2-way ANOVA.
5), suggesting that increased insulin-regulated gene expression in the adipose tissue is physiologically meaningful.

Adipose gene expression has previously been evaluated in Bmal1−/− mice (48). Contrary to what we observed in the Cry1/2−/− mice, Bmal1−/− mice display a transcriptional profile suggesting retarded adipogenesis, with concurrent reduced adipocyte size and increased ectopic lipid accumulation. ClockΔ19 animals of a C57BL/6 background as used in this study show adipocyte hypertrophy similar to that seen in Cry1/2−/− mice (51), although the lack of a thorough transcriptional profile or adipocyte physiology studies makes it difficult to determine the mechanism behind this phenotype. Despite their increased body weight, HFD-fed Cry1/2−/− mice showed none of the classical obesity-related changes to circulating lipids (51), with cholesterol, triglyceride, and free fatty acid levels remaining on par with those of WT controls (Table 1). This contrasts with ClockΔ19 and Bmal1−/− mice, which both display hypercholesterolemia and hypertriglyceridemia (48, 51). Perhaps hypophagia in the Cry1/2−/− mice contributes to this difference, whereas ClockΔ19 mice are hyperphagic (51).

Strikingly, at ZT18, circulating insulin was increased to approximately threefold the concentrations seen in WT mice, again contrasting with ClockΔ19 mice, which are hypoinsulinemic (51). This effect depends on an interaction of genotype and diet, since under NC conditions insulin is normal in Cry mutant mice (our data) or even reduced (23). Poor glucose tolerance (Fig. 4), together with decreased central appetite regulation (Fig. 1), is suggestive of a selective state of insulin resistance most prominently affecting the regulation of glucose uptake. However, given that adipose tissue is a major target of insulin, but responsible for only 3–5% of glucose uptake after insulin stimulation (24), it seems feasible that increased insulin signaling in adipose tissue may contribute to the HFD-induced obesity phenotype in Cry1/2−/− mice, despite insulin resistance in other tissues. We demonstrate tissue-specific insulin resistance in Cry1/2−/− mice after HFD, with WAT remaining insulin sensitive while muscle and liver are no longer responsive (Fig. 5). Interestingly, although Cry mutant livers do not display increased lipid uptake in response to insulin stimulation, elevated lipid accumulation was observed after HFD challenge. One explanation would be that the increased lipid load during HFD promotes hepatic lipid uptake independent from insulin, e.g., via leptin (34) or glucocorticoids (36). Alternatively, local effects of Cry function on hepatic lipid

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metabolism may be involved (29, 57). The differential regulation of insulin in WT mice compared with mutants made it impossible to assess directly whether the Cry1/2−/− mice actually display WAT insulin hypersensitivity. To accurately determine this, mice need to be fasted to achieve equally low basal insulin levels. This was not possible using the current model, since Cry1/2−/− mice develop fasting hyperglycemia and concurrently maintain hyperinsulinemia relative to WT controls (Fig. 6).

Recent studies targeting the positive arm of the clock have shown that Clock or Bmal1 deficiency leads to hypoinsulinemia due to impaired insulin secretion from islets (33, 43). A similar phenotype has been reported in a model of Rev-erba deficiency, although Zhao et al. recently showed that Per2−/− mice display increased GSIS, perhaps suggesting that, by affecting the negative arm of the clock machinery, the opposite phenotype is achieved (53, 59). Indeed, Cry1/2−/− mice display increased insulin secretion under basal glucose levels (Fig. 6). We show that pancreatic expression of Rev-erba is arrhythmic and regulated at a relatively high level, similar to Per2, contrasting with models of both Rev-erba and Clock deficiency (33, 53). Vieira et al. suggested that insulin secretion was impaired because of the downregulation of genes encoding exocytosis components, and these were either up-regulated in the case of Munc18 or unchanged (in the case of Vamp3 and Syntaxin1a) in Cry1/2−/− mice (53). It is unclear whether upregulation of Munc18 alone can explain the observed hyperinsulinemia, and we cannot rule out the possibility that other factors are involved. Rev-erba deficiency also results in decreased β-cell proliferation (53), making it interesting to speculate whether increased Rev-erba, as seen in our model, may lead to exacerbated proliferation and contribute to the hyperinsulinemic phenotype. However, insulin mRNA levels per se were not increased in pancreata of Cry1/2−/− mice (Fig. 6).

Several lines of evidence suggest that the effects of Cry deficiency are mediated via the deregulation of the circadian timing system. In humans, circadian misalignment occurs in shift-working populations, who show a propensity for increased BMI and metabolic syndrome (2). Shift workers tend not to overeat per se, with total caloric intake remaining normal, but the macronutrient content and the timing of their food consumption is altered (31). Food timing is known to have profound influences on obesity and metabolic dysfunction (3, 22). However, although Cry1/2−/− mice exhibit hyperinsulinemia and selective insulin resistance in the liver and muscle but show high insulin sensitivity in adipose tissue under HFD conditions, promote lipid accumulation and expedite the development of obesity and metabolic dysfunction.

Collectively, studies show that most mouse models of clock gene deficiency display alterations in energy homeostasis; however, the nature of that phenotype differs dramatically between different models. Cry1/2−/− mice are more susceptible to diet-induced obesity, in spite of hypophagia. Under HFD, Cry1/2−/− mice exhibit hyperinsulinemia and selective insulin resistance in the liver and muscle but show high insulin sensitivity in adipose tissue and consequent increased lipid uptake. Similar symptoms have been observed in shift workers (31), suggesting that Cry mutant mice might become a valuable tool for studying the mechanisms of diet-induced obesity in the presence of circadian metabolic disruption in humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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