Circadian phenotyping of obese and diabetic db/db mice

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Highlights:

- Most *db/db* mice display behavioral arrhythmicity, probably due to polydipsia.
- Their endogenous period of temperature rhythm is lengthened.
- *Db/db* mice show increased molecular responses to light in the master clock.
- Genetic obesity and type 2 diabetes affect speed of the master clock and photic resetting.

ABSTRACT:
Growing evidence links metabolic disorders to circadian alterations. Genetically obese *db/db* mice, lacking the long isoform of leptin receptor, are a recognized model of type 2 diabetes. In this study, we aimed at characterizing the potential circadian alterations of *db/db* mice in comparison to *db/+* control mice. By using telemetry devices, we first reported arrhythmicity in general activity of most *db/db* mice under both light-dark cycle and constant darkness, while their rhythm of body temperature is less dramatically disrupted. Water access restricted to nighttime restores significant rhythmicity in behaviorally arrhythmic *db/db* mice, indicating a masking effect of polydipsia when water is available a*d libitum*. Endogenous period of temperature rhythm under constant dark conditions is significantly increased (+ 30 min) in *db/db* compared with *db/+* mice. Next, we studied the oscillations of clock proteins (PER1, PER2 and BMAL1) in the suprachiasmatic nuclei (SCN), the site of the master clock, and detected no difference according to the genotype. Furthermore, c-FOS and P-ERK1/2 expression in response to a light pulse in late night was significantly increased (+80 and +55%, respectively) in the SCN of these diabetic mice. We previously showed that, in addition to altered activity rhythms, *db/db* mice exhibit altered feeding rhythm. Therefore, we investigated daily patterns of clock protein expression in medial hypothalamic oscillators involved in feeding behavior (arcuate nucleus, ventro- and dorso-medial hypothalamic nuclei). Compared with *db/+* mice, very subtle or no difference in oscillations of PER1 and BMAL1 is found in the medial hypothalamus. Although we did not find a clear link between altered hypothalamic clockwork and behavioral rhythms in *db/db* mice, our results highlight a lengthened endogenous period and altered photic integration in these genetically obese and diabetic mice.

Keywords:
Diabetes, Obesity, Circadian rhythm, Hypothalamus

Abbreviations:
ANOVA, analysis of variance;
ARC, arcuate nucleus;
CRY, cryptochrome;
DMH, dorso-medial hypothalamic nucleus;
NS, non-significant;
PB, phosphate buffer;
PBS, phosphate buffer saline;
PER2, Period 2;
P-ERK1/2, Phosphorylated extracellular signal-regulated kinase1/2;
SCN, suprachiasmatic nucleus;
VMH, ventro-medial hypothalamic nucleus;
ZT, Zeitgeber time.

1. Introduction

In mammals, most metabolic parameters are under the control of the circadian timing system. The master circadian clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, is reset by the environmental light-dark cycle and synchronizes secondary oscillators present in many regions of the brain (e.g., nuclei of the medial hypothalamus, involved in feeding-fasting cycle) and in most peripheral organs (e.g., liver and adipose tissue) [1, 2]. The circadian control of metabolism is reflected, for instance, by daily variations of metabolic hormones, such as leptin, and plasma glucose [3, 4]. The molecular clockwork relies on transcriptional and translational feedback loops involving clock genes and proteins and generating a rhythmic transcriptional activity with a ~24-h period. The main feedback loop involves CLOCK-BMAL1 heterodimer, which stimulates the transcription of *Period* (e.g., *Per1-2*) and *Cryptochrome* (*Cry 1-2*) genes. PER-CRY heterodimers inhibit in turn the transcriptional activity of CLOCK-BMAL1. CLOCK-BMAL1 also stimulates the transcription of clock-controlled genes, many of them being also involved in metabolism control, thus providing a molecular mechanistic basis for a circadian control of metabolism [1].

In turn, metabolic cues can affect circadian rhythmicity. Feeding time and concomitant changes in nutritional and hormonal signals are potent synchronizers of peripheral oscillators, such as the liver and white adipose tissue [5-8]. Under certain conditions of feeding (hypo- or hyper-caloric diets), metabolic cues affect the master clock and modify its circadian responses to light [9-13]. This reciprocal relationship is potentially of great importance for human health since metabolic disorders like obesity or diabetes are concomitant to circadian disturbances [1, 4]. Various experimental models of obesity or diabetes were previously used to study the impact of metabolic disorders on circadian rhythmicity. For example, diet-induced obesity in rodents lengthens the endogenous period of wheel-running behavior and body temperature under constant darkness, and reduces photic resetting of the master clock, as shown by smaller light-induced phase-advances and slower rate of re-entrainment after a jet-lag (phase-advance) [11, 12]. Genetically obese *ob/ob* mice, lacking functional leptin, display no change in the endogenous period, but display altered photic resetting, as shown by larger light-induced phase-delays and faster rate of re-entrainment after a delayed light-dark cycle [14, 15], and disturbances of peripheral clocks [16]. Moreover, insulino-dependent diabetes, induced in rodents by the chemical destruction of β-pancreatic cells, leads to circadian disturbances in peripheral clocks [17, 18] and alterations of photic resetting of the master clock [19, 20].

Db/db mice, lacking functional leptin receptor, are a recognized model of obesity and type 2 diabetes [21]. Therefore, *db/db* mice offer a model of choice to study the circadian alterations

resulting from obesity combined with severe diabetes. Slight alterations in transcriptional levels of *Per2* in SCN, as well as impairments of hepatic molecular clockwork have previously been shown in *db/db* mice [22]. The aim of this study was first to further investigate circadian alterations in the master clock of *db/db* mice, by studying free-running rhythms, circadian oscillations of clock proteins (PER1, PER2 and BMAL1), as well as behavioral and molecular responses to light. Wild-type mice fed with high fat and *ob/ob* mice display slower rate of re-entrainment after an advanced light-dark cycle, suggesting that obesity in both cases alters light-induced phase-advances [12, 15]. The study of circadian responses to light in late night was expected to highlight the additional effect of severe diabetes in *db/db* mice. Second, because *db/db* mice display alterations in feeding behavior, including daytime hyperphagia [23], we wondered whether alterations in feeding rhythm could be related to alterations of molecular clockwork in the SCN or in downstream structures, such as arcuate (ARC), ventro- (VMH) and dorso-medial hypothalamic (DMH) nuclei, possibly involved in feeding rhythm.

2. Material and Methods

2.1. Ethics statement

All experiments were performed in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996), the French National Law (implementing the European Union Directive 2010/63/EU) and approved by the Regional Ethical Committee of Strasbourg for Animal Experimentation (CREMEAS AL/01/06/03/12**).**

2.2. Animals, housing and diet

Eight-week-old male BKS(D)-*Lepr*^{db}/JOrlRj (*db/db)* mice and control littermates (*db/+*) were purchased from Janvier Labs breeding centre (Le Genest-Saint-Isle, France). Unless otherwise stated, they were housed in individual cages, kept at 23 ± 1°C under a 12:12 h light-dark cycle with lights on at 07:00 AM and lights off at 07:00 PM, defining Zeitgeber time (ZT) 0 and ZT12, respectively. Food (standard chow pellets, 105, SAFE, Augy, France) and tap water were available *ad libitum*.

2.3. Circadian phenotyping of db/+ and db/db mice

In a first series, 32 *db/+* mice and 32 *db/db* mice were studied. Among them, 15 *db/+* and 16 *db/db* mice were implanted intraperitoneally under isoflurane anesthesia, with Vitalview telemetry devices (Mini-Mitter Co., Sunriver, OR, USA) to record general activity and body temperature in 5 min bins during all the experimental procedure. After surgery, animals were

maintained at 23°C under a 12 h light/12 h dark cycle (lights on at 7:00 AM for 2 weeks before experiments began. Other mice were exposed to the same conditions, without telemetry. Mice were first exposed to a light-dark cycle (lights on, 7 AM; lights off, 7 PM) for two weeks. The quantity of water consumed in 24 h was estimated by weighing water bottles. Then, mice were transferred under constant darkness (DD) for 10 days to determine their endogenous period. Afterwards, mice were resynchronized to a light-dark cycle (lights on 7:00 AM; lights off 7:00 PM) during 3 weeks. On the day of sacrifice, mice were transferred in constant darkness and sacrificed every 6 h at projected ZT2, 8, 14 and 20 (projected ZT12 corresponding to the time of light offset the day before). A dim red light (TL-D 18W Red SLV, Philips, <3 lux at the level of animals) was used to euthanize mice. Brains were collected to determine oscillations of clock proteins by immunohistochemistry in suprachiasmatic nucleus (SCN) and medial hypothalamus.

A second series of 8 mice (4 *db/+* and 4 *db/db*) was implanted with telemetry devices, as reported above. Mice were kept under a 12:12 h light-dark cycle. During baseline, water was available *ad libitum*. Then, daily access to water was restricted to 8 h per day (from ZT12 to ZT20) during two weeks. Thereafter, water was again available *ad libitum* for two weeks.

2.4. Photic resetting of SCN clock

In a third series, 28 mice (n=14 per genotype) were transferred under dark conditions after two weeks of habituation under a light-dark cycle (12 h light; 12 h dark) cycle, mice were exposed to a 6-h phase advance of the light-dark cycle (jet-lag test, advance shift). After two weeks under the new light-dark cycle, mice were transferred to constant darkness. On the first night, half animals (7 *db/+* and 7 *db/db* mice) were exposed to a 30-min white light pulse (200 lux at the level of the animals) at projected ZT22 (*i.e.* the late period of the subjective night). Mice were euthanized under dim red light (see above) one hour after the beginning of the light pulse. Dark control animals (n=7 per genotype) did not receive the light pulse and were sacrificed at the same time and in the same conditions as light-exposed mice.

2.5. Immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital (i.p. 150 mg/kg) and intracardially perfused with saline (NaCl 0.9%) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB). Each brain was rapidly removed and post-fixed in same fixative for 24 h, then cryoprotected in 30% sucrose for 48-72 h. Brains were quickly frozen in isopentane cooled at -

40°C and stored at -80°C. Coronal cryosections of 30 µm through the SCN and the medial hypothalamus (ARC, VMH and DMH) were prepared on a cryostat at -20°C.

Free-floating sections were washed in 0.01M Phosphate buffer saline (PBS) and incubated in a solution of 3% of H_2O_2 (Sigma-Aldrich, St Louis, MO, USA) in PBS for 30 min. Brain sections were then rinsed in PBS for 10 min three times. For PER2, BMAL1, c-FOS and P-ERK1/2 staining, sections were incubated for 2 h in a blocking solution in 10% normal goat serum in PBS with 0.3% Tween-20, followed by an incubation with a rabbit polyclonal anti-PER2 (1:3000, Cat # PER-21-A, Alpha Diagnostic International, San Antonio, TX, USA), anti-BMAL1 (1:3000, ab3350, Abcam, Cambridge, MA, USA), anti-c-FOS antibody (1:10,000, SC-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or a rabbit monoclonal anti-Phosphorylated extracellular signal-regulated kinase1/2 (P-ERK1/2) antibody (1:40,000; #4370, Cell Signaling Technologies, Danvers, MA, USA) for 18 h at 4°C. For PER1 staining, sections were blocked in 10% normal horse serum in PBS with 0.3% Tween-20, followed by incubation with a goat polyclonal anti-PER1 (1:3000; SC-7724; Santa Cruz Biotechnology).

Sections were then rinsed three times for 10 min in PBS-T20 and, for PER2, BMAL1, c-FOS and P-ERK1/2, incubated for 2 h at 4°C with a biotinylated anti-rabbit IgG made in goat (Vectastain Standard Elite ABC Kit PK6101, Vector Laboratories, Inc., Burlingame, CA, USA) diluted 1:500 in in PBS with 0.3% Tween-20. Secondary antibody used for PER1 was a biotinylated anti-goat IgG, made in horse (BA-9500, Vector Laboratories Inc.), diluted 1:500 in in PBS with 0.3% Tween-20. Sections were rinsed in in PBS with 0.3% Tween-20 and incubated for 1 h at room temperature with an avidin–biotin–peroxidase complex (Vectastain Standard Elite ABC Kit; Vector Laboratories Inc.). Sections were rinsed three times for 10 min in PBS, and incubated with 0.05% 3,3'-diaminobenzidine (Sigma-Aldrich) with 0.015% H_2O_2 in tap water. Then, sections were rinsed with PBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in toluene and coverslipped with Eukitt (Chem Lab, Zedelgem, Belgium).

2.6. Quantification of immunostaining

Images were taken on a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) connected to an Olympus DP50 digital camera (Olympus France, Rungis, France). For staining intensity, all lighting parameters on the microscope and the camera software (Viewfinder Lite; Olympus) were standardized to ensure stable lighting throughout the image capture procedure. Using the atlas of Paxinos and Franklin [24]), one brain section per structure per animal was carefully selected to compare the same level of structures between animals,

corresponding to bregma levels -0.46mm for SCN (Coronal plate 35 of the atlas), -1.58mm for ARC and VMH (Plate 44), -1.82mm for DMH (Plate 46). Boundaries of structures were chosen by comparison with sections stained with Cresyl Violet and by using anatomical landmarks (e.g., third ventricle, fornix and mammillothalamic tract for DMH). Quantifications were performed with ImageJ software (W. S. Rasband, U.S. National Institutes of Health, Bethesda, MD, USA) by delimiting so-called "Regions of Interest" covering each side of the nuclei. For each animal, 3 pictures were taken: one of the target structure, one part of the section without specific staining (internal capsule) and one of the slide without section. The latter was substracted from the two other images to compensate for inhomogeneities in the illumination of the image field. Then, the mean intensity of gray pixel value was calculated and averaged for both sides of target structures, and for unstained tissue, which corresponds to tissue background, varying between animals. By substracting the mean intensity of background tissue to the mean intensity of selected structure, we obtain the final value of intensity, proportional to the quantity of targeted protein.

2.7. Statistical analysis

Data are presented as mean ± SEM. Normality was assessed with a Shapiro-Wilk test. Student's t test was used to compare two groups. Two-way analysis of variance (ANOVA) was performed to assess the effects of [genotype and light conditions] or [genotypes and time] and the interaction between these factors (Statistica 10.0, StatSoft, Tulsa, OK, USA). p values > 0.05 were considered non-significant (NS).

The period of entrainment (T) under a light-dark cycle and the endogenous period (t) under constant darkness were measured for general cage activity and body temperature by using the χ^2 Periodogram function of Clocklab software (Actimetrics, Evanston, IL, USA). For assessing daily rhythmicity, we also used a cosinor analysis to determine mean level, amplitude and acrophase of the considered parameter with SigmaPlot software (Systat software Inc., San Jose, CA, USA). Data for body temperature under a light-dark cycle were fitted to the following regression: $[y=A+B\cdot\cos(2\cdot\pi\cdot(x-C)/24)]$ where A is the mean level, B the amplitude, and C the acrophase of the rhythm. For assessing circadian rhythmicity of body temperature in constant darkness, we used a cosinor analysis to determine mean level, amplitude, acrophase, and endogenous period of the considered parameter with SigmaPlot. Data were fitted to the following regression: $[y=A+B\cdot\cos(2\cdot\pi\cdot(x-C)/D)]$ where A, B and C were defined as above, while D is the endogenous period of the free-running rhythm. Cosinor analyses of clock proteins (PER1, PER2 and BMAL1) were performed on data at points ZT2, 8, 14 and 20. To clarify the

graphic representations, ZT2 point was double-plotted in Figures 2 and 4, but the repeated point was excluded for statistical analysis.

3. Results

3.1. Locomotor activity and body temperature rhythms

General activity and body temperature rhythms of *db/+* mice were synchronized on the light-dark cycle (Figure 1A and B) and free-ran under constant darkness conditions with an endogenous period close to 23.7 h for both parameters (Table 1). By contrast, under both light-dark cycle and DD conditions, most *db/db* mice (12/16 and 10/16, respectively) displayed arrhythmicity of general activity (Figure 1C and D; Table 1). Despite much disruption of body temperature rhythm in *db/db* compared to *db/+* mice, significant rhythmicity can be detected under both light-dark cycle and constant darkness conditions, albeit with lower amplitude (Table 1). When measurements were possible, the endogenous period in constant darkness was significantly longer in *db/db* mice for both activity and temperature rhythms (+30 and +18 min, respectively; Table 1). Moreover, the mean temperature was significantly lower in *db/db* mice (36.3 ± 0.05 vs 34.6 ± 0.1°C in *db/+* and *db/db* mice, respectively; $p<0.01$). We also observed that *db/db* mice consumed six-fold more water than *db/+* mice (6.3 ± 0.2 *vs* 39.1 ± 3.0 ml/day, for *db/+* and *db/db* mice, respectively; $p<0.01$), revealing a polydipsia characteristic of severe diabetes.

Next, we challenged *db/db* and *db/+* mice with a daily restricted access to water to investigate its impact on daily rhythmicity. Scheduled access to water restores significant behavioral rhythmicity in non-rhythmic *db/db* mice, while not altering rest/activity rhythm in *db/+* mice (Figure 2; Table 2).

3.2. Circadian oscillations of clock proteins in SCN

PER1, PER2 and BMAL1 oscillations were not affected in the SCN of *db/db* mice compared with *db/+* mice (2-way ANOVA, no effect of genotype, $p>0.05$ for each protein) (Figure 3). An effect of time was found for the three proteins ($p \leq 0.01$). A cosinor analysis revealed a significant circadian rhythm for PER2 in both genotypes, with a peak close to ZT 15-16 (Table 3). The acrophase of PER1 oscillation appeared to be close to ZT12.5 in both genotypes. The circadian rhythmicity, however, was not significant in *db/db* mice since the amplitude did not reach the threshold of significance ($p= 0.077$). BMAL1 peaked around ZT18-19 in both

genotypes, while the amplitude of the rhythm did not reach the threshold of significance for *db/+* mice (p=0.068).

3.3. Photic resetting in SCN of db/db mice

Given that rhythms of body temperature of *db/db* mice were less disrupted than general activity, we tried to quantify the rate of reentrainment of *db/db* mice after a 6-h advance of the light-dark cycle (i.e. the numbers of days necessary for resynchronization). Unfortunately, the weaker rhythmicity of body temperature rhythm in *db/db* mice as compared to *db/+* controls, precluded a precise quantification of rate of reentrainment (Figure 4).

As expected, a light pulse at the end of the subjective night increased the levels of the markers of cellular activation c-FOS and P-ERK1/2 in the SCN of control mice [25, 26] (Figure 5). Interestingly, the light-induction of c-FOS and P-ERK1/2 was respectively twice and 1.5 times higher in *db/db* mice, suggesting a higher responsiveness to light-induced phase-advances in the diabetic and obese mice.

3.4. Clock protein oscillations in medial hypothalamus

Since *db/db* mice show an altered pattern of feeding, with increased food intake during the light period [23], we wondered whether molecular clockwork was altered in oscillators of medial hypothalamus involved in feeding rhythm, like arcuate nucleus (ARC), ventro- and dorso-medial hypothalamic nuclei (VMH and DMH, respectively). In VMH and DMH, the levels of PER1 and BMAL1 were similar between *db/+* and *db/db* mice and no circadian oscillation was detected (Figure 6). Similarly, BMAL1 levels did not oscillate and nor they differ between *db/+* and *db/db* mice in ARC. We found a significant rhythmicity of PER1 in ARC of *db/+* mice (p=0.0076), which was lost in *db/db* mice (NS). However, no effect of genotype was found (effect of genotype: NS), although the interaction [genotype x time] was very close to significance threshold (p=0.055).

4. Discussion

This study provides new information on the circadian alterations of obese and diabetic *db/db* mice. General activity of *db/db* mice is arrhythmic under both light-dark cycle and constant dark conditions, while body temperature, although disrupted, is still rhythmic under both conditions of lighting. The free-running period of body temperature is lengthened in *db/db* compared to *db/+* mice. After a light pulse in late night, the photic induction of c-FOS and P-ERK1/2 is increased in SCN of *db/db* compared with *db/+* mice while their rate of

resynchronization is quicker, suggesting alterations in photic responses of the master clock. Almost no alteration is detected in the daily oscillations of clock proteins in the SCN, VMH, DMH and ARC. Therefore, our results do not support a direct relationship between altered patterns of activity and feeding and alterations in SCN and medial hypothalamic oscillators.

4.1. Activity and temperature rhythms in db/db mice

Given that our *db/db* mice were 8 weeks of age at the beginning of the experiment, they exhibit severe diabetes [21]. The observed behavioral arrhythmicity in most *db/db* mice (Figure 1, Table 1) is consistent with a previous study [22], while another work showed only a decreased amplitude of rest/activity rhythm [27]. Because *ob/ob* mice do not show similar alterations of rest/activity rhythm [14, 15], the prominence of arrhythmicity of motor behavior, but not body temperature, in *db/db* mice suggests functional links with diabetes. Here we report an important polydipsia in *db/db* mice, characteristic of severe diabetic state [21]. Restriction of water during the dark phase was able to restore daily variations in rest/activity in behaviorally arrhythmic animals. Therefore, we propose that behavioral arrhythmicity of *db/db* mice is due in most part to a masking effect of polydipsia. In *db/db* mice under a light-dark cycle, there may be also a decreased masking effect of light that normally inhibits motor activity of nocturnal mice during the light phase, including in mice with defective circadian clocks [28, 29]. This hypothesis of masking effects is in accordance with the fact that rhythms of general activity and temperature are not correlated in *db/db* mice, while both rhythms totally fit in *db/+* mice. *Db/db* mice also display hypothermia, which is consistent with defects in thermogenesis and cold tolerance previously reported [30]. Of interest, a previous study showed that feeding restricted to nighttime also increases the robustness of behavioral rhythmicity in *db/db* mice [22].

4.2. Free-running period and oscillations of clock proteins in the SCN of db/db mice

Furthermore, the endogenous period is increased in *db/db* mice, which highlights a faster master clock. Of note, diet-induced obese mice also display lengthened free-running period [11, 12], whereas the endogenous period is not changed in obese *ob/ob* mice [14, 15]. These differential effects between *ob/ob* and *db/db* mice suggest that the lengthening of the endogenous period in *db/db* mice is not due to obesity or defective leptin signaling, but could result from severe diabetes. In that view, lengthening of the endogenous period in high-fat fed mice would be due to processes not directly related to diabetic symptoms. To try to explain the functional defects of the SCN clock in *db/db* mice, we investigated clock protein oscillations in their SCN (Figure 2). We did not detect any significant difference between *db/+* and *db/db* mice for the three

proteins studied, PER1, PER2 and BMAL1. The circadian rhythmicity did not reach, but was close to, the threshold of significance for BMAL1 in *db/+* mice and PER1 in *db/db* mice. The difference between *db/+* and *db/db* mice is thus very subtle. These results are consistent with those of Kudo and colleagues, who reported no difference in transcriptional levels of *Per1* and found a slight increase of *Per2* mRNA in the SCN of *db/db* mice [22]. Here PER1 and PER2 levels also tend to be slightly increased in *db/db* mice. Further investigations are needed to assess whether this modest increased amplitude could strengthen the negative feedback loop and somehow lengthen the endogenous period of the SCN clockwork in *db/db* mice.

4.3. Photic responses of SCN in db/db mice

Classical light-induced phase-shifts of rest/activity rhythm cannot be determined in *db/db* mice because of behavioral arrhythmicity or noisy circadian rhythmicity. At least at molecular levels, a light pulse in late night induces a higher increase of c-FOS and P-ERK1/2 in the SCN of *db/db* mice compared with *db/+* mice (Figure 3). Of note, *db/db* mice are known to develop retinopathy due to hyperglycemia-induced microvascular damage, and even loss of ganglion cells [31]. In view of the higher sensitivity to light of the SCN cells in *db/db* mice, it is unlikely this effect is due to altered photo-reception or transduction in their retina and favors the idea of intra-SCN effects. The higher responsiveness of the *db/db* SCN to light may partly be due to their longer free-running period (i.e., close to 24 h) that would increase light-induced phase-advances in late night. This possibility, however, needs to be tested further because high-fat fed mice that also have a longer period (also close to 24 h) display a reduction in light-induced phase-advances [12], suggesting additional metabolic modulation of photic resetting (see below).

Db/db mice are hyperglycemic and their rhythm of glucose is reversed (peak during the night). Their insulinemia is also elevated, with higher values during the day, opposite to the rhythm of insulin in *db/+* mice [23]. Glucose availability has been shown to modulate SCN responses to light. Blocking glucose utilization with 2-deoxy-D-glucose reduces light-induced phase-shifts in mice [32]. By contrast, streptozotocin-induced hyperglycemia in mice increase the shifting effect of light during early night [20]. Furthermore, glucose has been shown to alter the circadian firing rate of SCN slice *in vitro* [33]. Glucose uptake of neurons being independent of insulin [34], the increase of glucose availability in *db/db* mice may affect the metabolic activity of SCN cells. Molecular metabolic actors being closely connected with molecular clockwork, this could impact the circadian responses to light of SCN cells.

We investigated the effects of light exposure in early night (i.e., inducing phase-delays) in *db/db* mice and found no difference in light-induced expression of c-FOS and P-ERK1/2 between *db/db* and *db/+* mice (see Supplemental Fig. S1). This result is reminiscent of the lack of molecular changes observed in the SCN of *ob/ob* mice exposed to light in early night [15] and different from the decrease in c-FOS observed in streptozotocin-induced diabetic rats [35]. This indicates that in *db/db* mice, molecular responses to light are not changed in early night, and are specifically altered in late night. The increased molecular responses in the SCN of diabetic *db/db* mice after a light pulse in late night are actually opposed to those induced by similar light in high-fat fed mice which display weaker induction of c-FOS and P-ERK1/2 in the SCN, correlated with smaller phase-advances of behavioral rhythm, in spite of longer free-running period as compared to chow-fed control mice [12]. This suggests that alterations in photic resetting of *db/db* mice are not a passive consequence of obesity, but could be due to two causes, namely the lack of leptinergic activity and/or severe diabetes. The lack of leptin has already been associated with altered circadian phenotype in *ob/ob* mice [15, 16]. Photic resetting associated with phase-advances is decreased in *ob/ob* mice, because a slower resynchronization was found after a phase-advance of the light-dark cycle [15]. Together, these comparative data suggest that severe diabetes, rather than obesity, could explain the potentiated photic responses during late night in *db/db* mice.

4.4. Alterations in medial hypothalamic oscillators of db/db mice

Db/db mice also show altered feeding behavior, with higher food consumption during the light phase [23]. Phase-advance of feeding rhythm has also been observed in diabetic Zucker (*fa/fa*) rats, bearing a missense mutation in long-form leptin receptor [36]. Since nuclei of the medial hypothalamus have been involved in feeding rhythms [2], we investigated the clock protein oscillations in ARC, VMH and DMH. Contrary to our previous study [37], here we did not detect oscillations of PER1 in DMH of control mice. Due to the sharp and transient increase of PER1 in the mouse DMH, the chosen time of sampling in the present study might not be adequate to detect this rhythm. As previously shown [37], we found a significant rhythmicity of PER1 in ARC. ARC being a crucial structure involved in the generation of feeding rhythm [38], it would be tempting to think that alteration of PER1 rhythmicity in ARC could be involved in altered feeding behavior of *db/db* mice. However, our data do not support such a hypothesis.

5. Conclusions

These results show that *db/db* mice display circadian alterations. At behavioral level, *db/db* mice are arrhythmic, most likely due to a masking effect of polydipsia. The rhythm of body temperature is less disrupted and free-ran under constant darkness with a longer endogenous period compared with *db/+* mice. Moreover, altered photic response of the SCN clock in *db/db* mice, characterized by higher induction of c-FOS and P-ERK1/2 after a light pulse in late night, could be due to severe diabetes. Therefore, the present study provides new experimental information showing that genetic obesity and type 2 diabetes impacts the circadian timing system by affecting the master clock.

Authors' contributions

E.G., C.S.K., B.G.L., P.P., F.C. and E.C. designed the experiment, S.D. and E.G. performed immunohistochesmitry and analyzed the data, E.G. made the figures and wrote the first version of the manuscript that was corrected by all co-authors.

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Legends to figures

Fig. 1. Representative double-plotted actograms of general activity (**A**, **B**) and thermograms of body temperature (**C D**) of *db/+* (left) and *db/db* mice (right). After 2 weeks in 12:12 h light-dark (LD) cycle, mice were transferred under constant darkness (DD) for 10 days (indicated with arrows), before being resynchronized to new a regular light-dark cycle for 3 weeks (LD pZT, projected Zeitgeber time. Projected nighttime is indicated with a shaded area.

Fig. 2. Double-plotted actograms of general activity of *db/+* (left) and *db/db* mice (right) challenged with daily restricted access to water. After 2 weeks in 12:12 h light- dark cycle with water *ad libitum*, water access was limited to 8-h per day, from ZT (Zeitgeber time) 12 to ZT20 (shaded rectangle) during two weeks (indicated with arrows). ZT, Zeitgeber time.

Fig. 3. Circadian profiles of PER1, PER2 and BMAL1 expression (means ± SEM) in the suprachiasmatic nucleus (SCN) of *db/+* (black circles) and *db/db* (white circles) mice. Data for ZT2 are double-plotted. pZT, projected Zeitgeber time. Projected nighttime is indicated with a shaded area. Scale bar: 100 μm. Significant and non-significant cosinor regressions are shown with solid and dashed curves, respectively.

Fig. 4. Double-plotted thermograms of *db/+* (left) and *db/db* mice (right) before and after a 6-h phase advance of the light-dark cycle. Arrows on day 11 indicate the first day of the 6-h advance.

Fig. 5. c-FOS and P-ERK1/2 responses in the suprachiasmatic nucleus (SCN) of *db/+* and *db/db* mice exposed (Light pulse) or not (Dark control) to a 30-min light pulse at projected ZT 22 (means ± SEM). Groups with different letters are significantly different ($p<0.05$). Representative photomicrographs of c-FOS and P-ERK1/2 staining in SCN of *db/+* and *db/db* mice. Scale bar: 100μm.

Fig. 6. Circadian profiles of PER1 and BMAL1 expression (means ± SEM) in the arcuate nucleus (ARC), ventromedial and dorsomedial hypothalamus (VMH and DMH, respectively) of *db/+* (black circles) and *db/db* (white circles) mice. Data for ZT2 are double-plotted. pZT, projected Zeitgeber time. Projected nighttime is indicated with a shaded area.

Legends to supplemental figures

Suppl Fig. S1. c-FOS and P-ERK1/2 responses in the suprachiasmatic nucleus (SCN) of *db/+* and *db/db* mice exposed to a 30-min light pulse at projected ZT 13 (means ± SEM). Groups with different letters are significantly different ($p<0.05$).

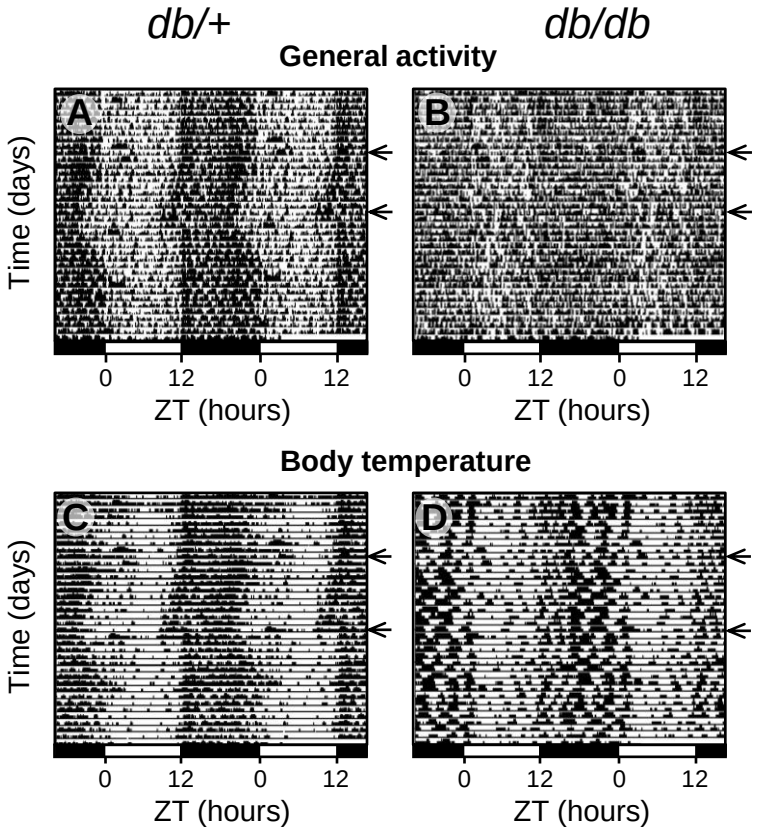
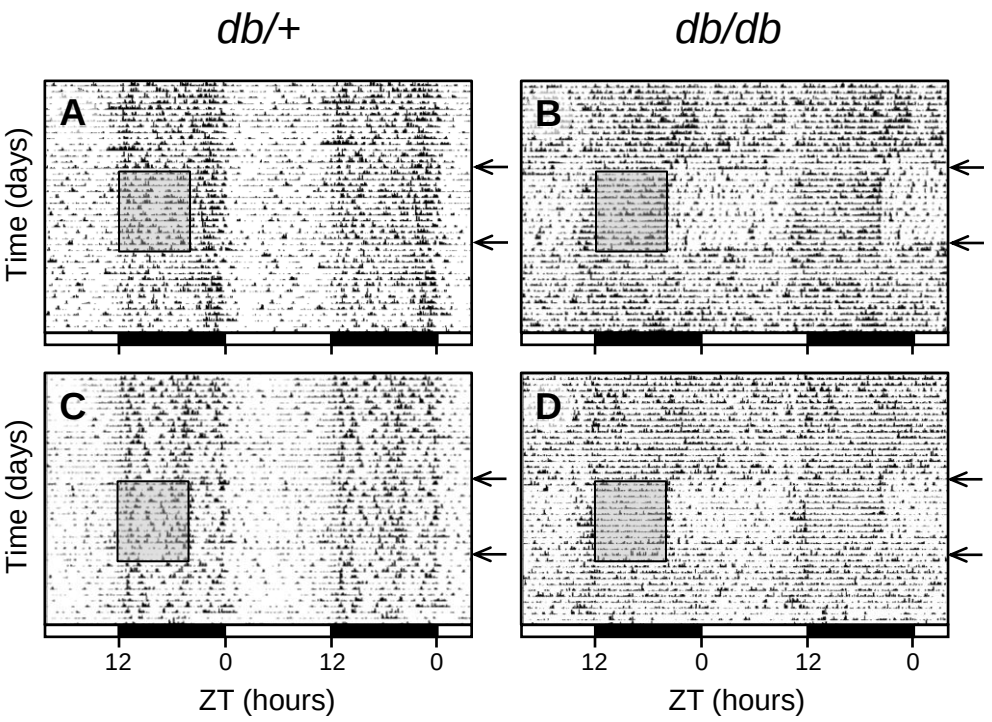

db/+
db/db
General activity
A
B
Time (days)
0
12
0
12
ZT (hours)
0
12
0
12
ZT (hours)
Body temperature
C
D
Time (days)
0
12
0
12
ZT (hours)
0
12
0
12
ZT (hours)

Figure 1


db/+
db/db
A
B
C
D
Time (days)
Time (days)
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12
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ZT (hours)
ZT (hours)

Figure 2

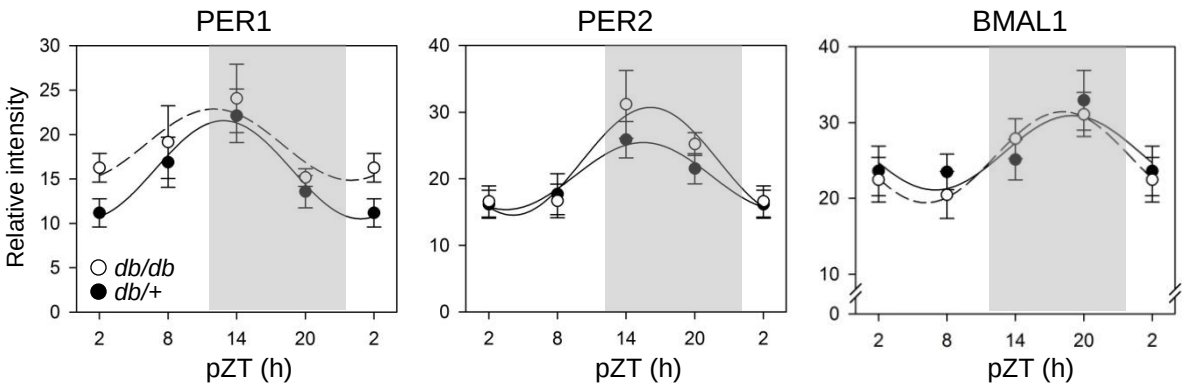


Figure 3

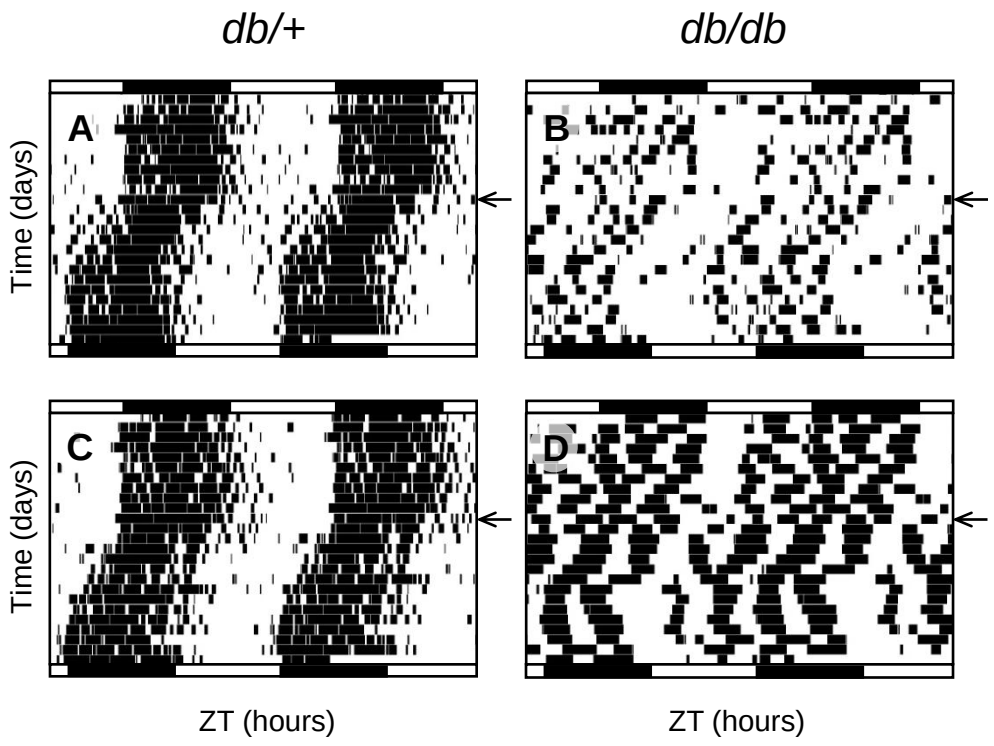
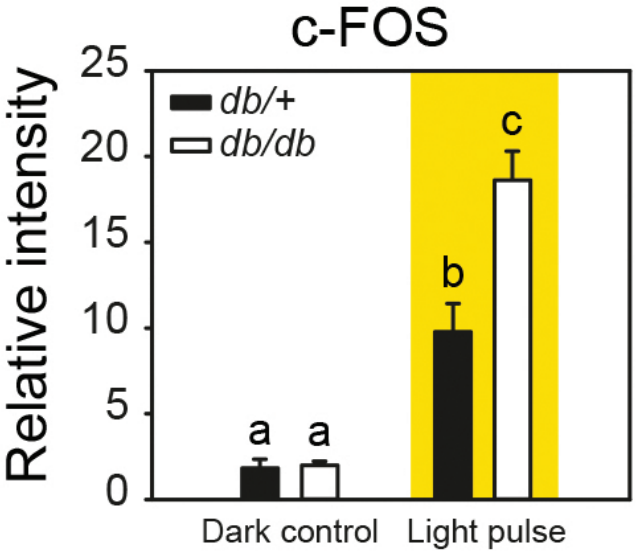
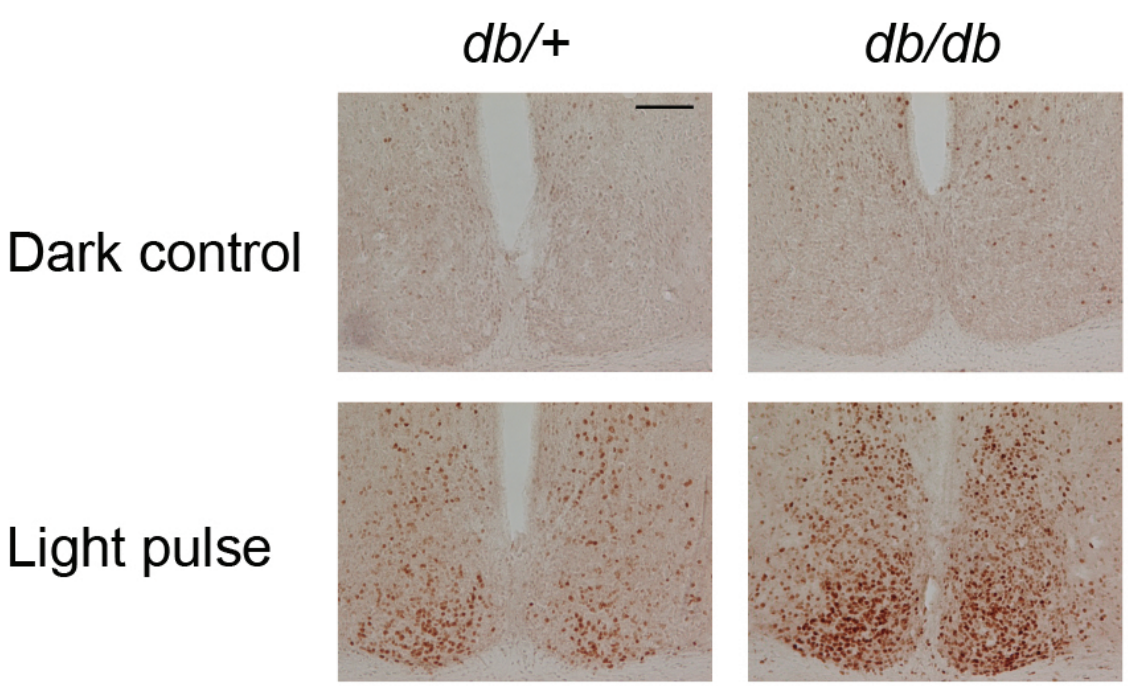
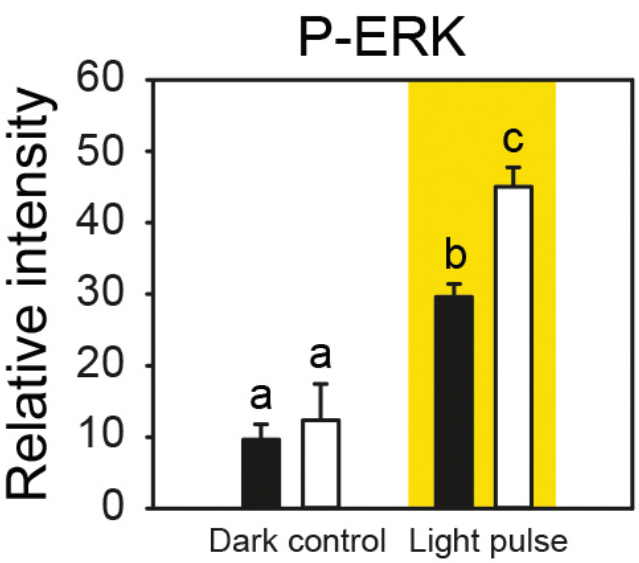


Figure 4


c-FOS
Relative intensity
25
20
15
10
5
0
db/+
db/db
a
a
b
c
Dark control
Light pulse


db/+
db/db
Dark control
Light pulse


P-ERK
Relative intensity
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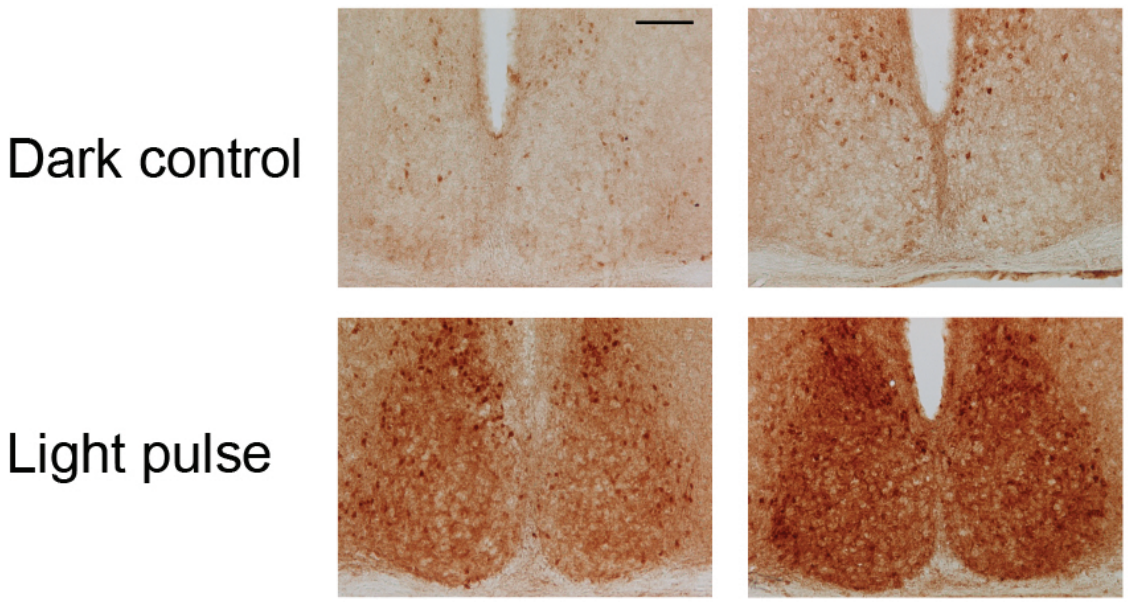
50
40
30
20
10
0
a
a
b
c
Dark control
Light pulse


Dark control
Light pulse

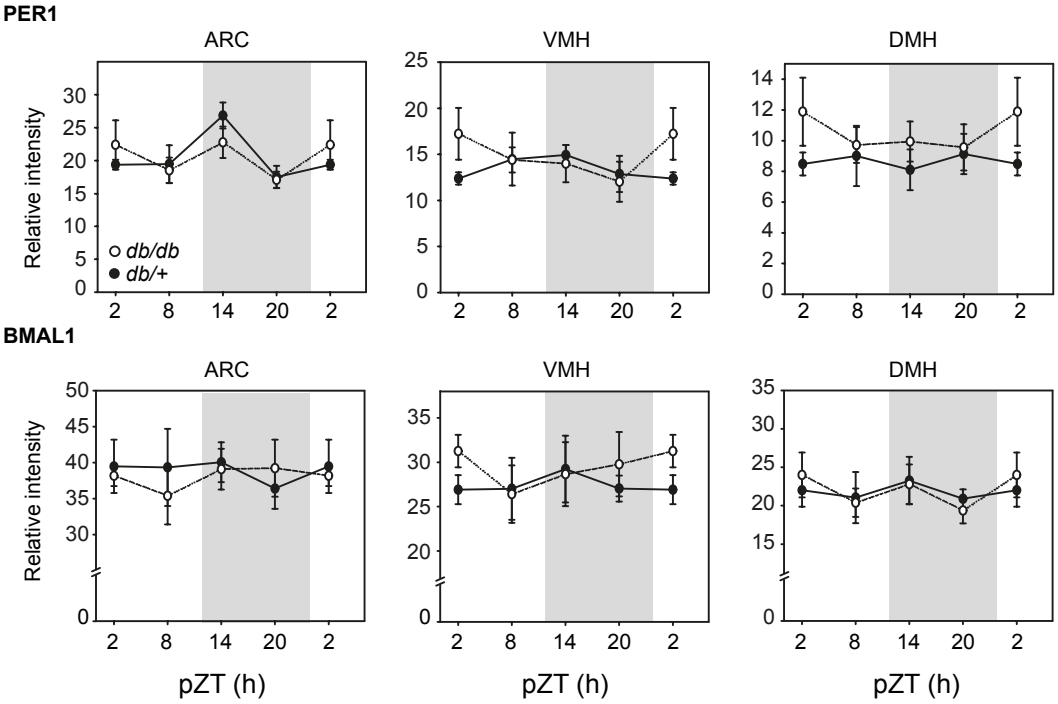
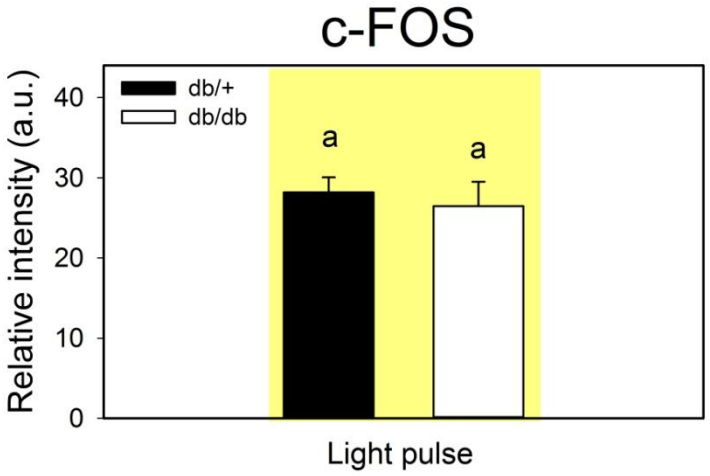
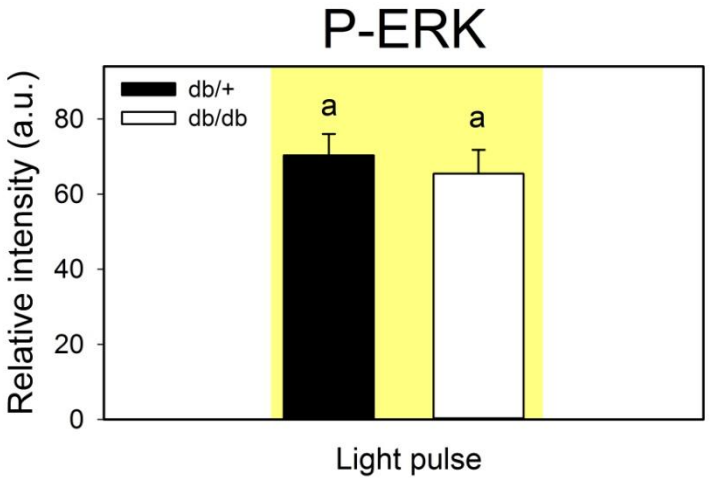

PER1
ARC
VMH
DMH
Relative intensity
db/db
db/+
BMAL1
ARC
VMH
DMH
Relative intensity
pZT (h)
pZT (h)
pZT (h)

Figure 6





Supplemental Fig. S1