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Research paper

Circadian phenotyping of obese and diabetic *db/db* mice

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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 *ad 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.

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

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.

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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) 1 h 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 0.1 M 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.