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# Impaired daily glucocorticoid rhythm in *Per1*$^{Brd}$ mice

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**Abstract** Biological clocks have evolved in all kinds of organisms in order to anticipate and adjust to the daily light–dark cycle. Within the last decade, the molecular machinery underlying the circadian system was unraveled. In the present study, the impact of the loss of the *Per1* or *Per2* genes, key components of the core clock oscillator, on body mass, food and water intake, glucose metabolism, and hypothalamic-pituitary-adrenal axis, was investigated in the *Per1*$^{Brd}$ and *Per2*$^{Brd}$ mouse models. The results reveal that the lack of *Per1* but not *Per2* has severe consequences for the regulation of these parameters. Specifically, in *Per1*$^{Brd}$ animals, we found an impaired daily glucocorticoid rhythm, with markedly elevated levels during the day compared to control animals. In addition, *Per1*$^{Brd}$ mice showed significant differences in body mass as well as food and water intake. Although the *Per1*$^{Brd}$ are lighter than wildtype mice, food and water intake per gram body mass is elevated. In addition, the *Per1*$^{Brd}$ mice exhibit an increased glucose metabolism after i.p. injection with glucose. In conclusion, our study presents first evidence for a link between an altered metabolism in *Per1* and *Per2* deficient mice, which in the case of the *Per1*$^{Brd}$ animals might be due to an impaired corticosterone rhythm.

**Keywords** Adrenocortical activity · Circadian rhythm · Clock gene · Fecal glucocorticoid metabolites · *Period*

**Abbreviations** SCN: Suprachiasmatic nucleus · HPA axis: Hypothalamic-pituitary-adrenal axis · *Per*: *Period* · CM: Corticosterone metabolites · LD: Light/dark cycle · WT: Wildtype · GC: Glucocorticoid · AUC: Area under curve

## Introduction

One of the most widespread properties of all organisms is a daily rhythm that evolved as an adaptation to the earth's rotation around its axis, causing day and night (Devlin and Kay 2001). In mammals, this rhythm is controlled by a master clock, which is localized in the suprachiasmatic nuclei (SCN) of the anterior-ventral hypothalamus (Moore and Eichler 1972; Stephan and Zucker 1972). Members of the *Period* gene family, *Per1* and *Per2* are key players in the underlying molecular mechanisms of the master clock (Sun et al. 1997; Tei et al. 1997).

Although we are far from understanding the different roles of *Per1* and *Per2* genes in the mammalian circadian system, some specific functions have already been elucidated by using mice with targeted mutations of *Per1* and *Per2* (Zheng et al. 1999, 2001; Bae et al. 2001; Cermakian et al. 2001). The results confirmed that the *Per* genes together with the *Cry* genes are pivotal elements of the core clock, i.e. of the negative limb of the transcriptional/(post-)translational feedback loop (Albrecht 2004). The *Per1*$^{Brd}$ mice show a shortened free-run in constant darkness (DD) and a longer free-run in constant light (LL). In contrast, *Per2*$^{Brd}$ animals become arrhythmic after a few days in DD but show a short free-run in LL. Furthermore,

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*Per1*$^{Brd}$ and *Per2*$^{Brd}$ animals show deficits in their phase shifting capabilities after a light pulse (Albrecht et al. 1997; Bae et al. 2001; Steinlechner et al. 2002; Spoelstra et al. 2004). Interestingly, there is growing evidence that the *Period* genes are involved in a number of different processes and pathways, i.e. cocaine sensitization (Abarca et al. 2002), sleep (Kopp et al. 2002), and ethanol consumption (Spanagel et al. 2005). Recently, other non-clock functions for *Per* and other elements of the core clock have been described, e.g. in metabolism (*Bmal1*: Rudic et al. 2004; *Clock*: Turek et al. 2005).

Metabolism is largely regulated via several hormonal pathways such as hypothalamic-pituitary-adrenal (HPA) axis. The daily variation in the activity of the HPA axis, which is measured as abundance of plasma glucocorticoids (GCs) is under clock control (Moore and Eichler 1972; Kalsbeek and Buijs 2002). The daily peak of GCs is mainly mediated by a time-of-day dependent sensitivity to adrenocorticotropic hormone (ACTH) of the adrenal cortex (Kalsbeek et al. 1996). In mice, the main GC is corticosterone (Touma et al. 2004) and a practical way to assess the daily rhythm of corticosterone is through sample feces of the animal and measure its fecal corticosterone metabolites (Touma and Palme 2005). This technique has two major advantages over plasma samples: (1) samples can be easily obtained from the cage floor, and (2) there is no feedback of the sampling procedure on subsequent samples (Touma et al. 2004). It has to be taken into account, however, that there is a time lag between the corticosterone peak in plasma and feces. Furthermore, corticosterone is heavily metabolized before excretion. Thus, it is important that the assay to determine corticosterone metabolites in feces is thoroughly validated (Touma and Palme 2005). The assay used in this study has been validated by a radiometabolism study as well as a dexamethasone suppression and an ACTH challenge test (Touma et al. 2003, 2004).

This study is part of a larger project that aims at phenotyping mice with targeted mutations of the *Per1* and *Per2* genes at a behavioural and physiological level. In the present study, we focus on specific physiological aspects of the phenotype of *Per1*$^{Brd}$ and *Per2*$^{Brd}$ mice. As there were some indications from behavioural tests for an altered stress response of the transgenic mice as compared to that of the wildtype (WT) mice (Dallmann 2004), the activity of the HPA axis was examined in more detail. The concentrations of fecal corticosterone metabolites were measured to monitor non-invasively the daily rhythm of GCs. Furthermore, we measured body mass development, and food and water intake because alterations in metabolic functions are likely to be reflected in these parameters. Finally, a glucose tolerance test was carried out in order to test whether an altered glucose metabolism might be responsible for the observed differences in body mass and food consumption.

## Materials and methods

### Animals and housing conditions

We used homozygous adult B6.129S7-*Per1*$^{tm1Brd}$ (*Per1*$^{Brd}$) mice (Zheng et al. 2001) and B6.129S7-*Per2*$^{tm1Brd}$ (*Per2*$^{Brd}$) (Zheng et al. 1999) as well as age matched (B6x129S7)F2 (WT) mice as controls. All animals were derived from a breeding colony at the Max-Planck-Institute of Experimental Endocrinology, Hannover, Germany. We used only male mice with the exception of the glucose tolerance test. Unless otherwise stated, all animals were kept under standard laboratory conditions (12 h of light per day with lights on at 7.00 a.m., 300 lx, 21 ± 1°C). All mice were housed singly in macrolon cages type II (Techniplast, Italy) on standard bedding (Altromin, Germany) and were fed with standard rodent maintenance diet (Altromin 1324, Altromin, Germany) and tap water ad libitum. The *Per1*$^{Brd}$ and *Per2*$^{Brd}$ animals did not show any abnormalities with respect to general health nor could deficiencies in tests for neurological reflexes be found (balance-, eye blink-, ear twitch-, and pupil reflex; Dallmann 2004). We used separate sets of animals for each experiment except for the body mass development.

### Daily rhythm of glucocorticoids

During the sampling period, the eight age-matched males of each genotype were held in special metabolic cages. These consisted of a clear plastic cylinder (17.5 cm diameter × 10 cm height) with a soft plastic mesh (mesh aperture 0.3 cm) floor. In these cages, the urine flows alongside the wall of the catchment funnel and is then led via a tube into a cup, while the feces fall directly through the middle of the funnel and are stored in a small plastic vessel preventing contamination of feces with urine. After an initial habituation period of 4 days in the metabolic cage, feces were collected for 48 h in 3-h intervals. The fecal boli were counted, weighed, and stored at (–20°C until further processing. Briefly, dried fecal samples were homogenized and 0.05 g of each sample was shaken with 1 ml of 80% methanol on a multi-vortex (Buchler Instruments, Austria). Afterwards, the samples were centrifuged and the supernatants were diluted (1:10) with assay buffer (Tris/HCl 20 mM, pH 7.5) before analysis in duplicate in a double-antibody 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) to measure concentrations of corticosterone metabolites. Details regarding the assay used to analyze fecal corticosterone metabolites are described elsewhere (Touma et al. 2003, 2004). The intra- and interassay coefficients of variation were 9.1 and 14.0%, respectively.

### Body mass development, food and water intake

All animals were weighted every other week while maintained in our laboratory, i.e. from the age of 4–20 months. For measurement of food and water intake age-matched (4–6 months old) groups of $Per1^{Brd}$, $Per2^{Brd}$ and WT ($n=10$) mice were selected randomly. While the animals were maintained in their home cages 24-h food and water consumption was measured by weighing the food in the food hopper and the water bottle, every 24 h for five consecutive days. In order to correct for evaporation a water bottle was monitored simultaneously in an empty cage (Karl et al. 2003). As the genotypes exhibited significant body mass differences we corrected absolute food and water intake values for body mass. We calculated the intake per hypothetical 30 g mouse. This hypothetical standard mouse was introduced in order to represent the intake per gram body mass, and at the same time to illustrate the intake in relation to the actual intake per mouse.

### Glucose tolerance test

A glucose tolerance test was carried out following the method described in Röhl et al. (2004) and Cooney et al. (2004). After an overnight fasting (14 h), the blood glucose level of the animals was measured 1 h after lights on using an automatic glucose monitor based on the glucose oxidase method (Glucometer Elite, Bayer, Germany). Directly after the determination of fasting blood glucose level, the animals were injected intraperitoneally with glucose (2 g/kg body mass in distilled water). The blood glucose level was re-determined 30, 60, and 120 min after the injection. Blood samples (each about 5 µl) were collected from small incisions made at the distal part of the tail of the conscious and unrestrained mice. Group sizes were $Per1^{Brd}$ $n=17$ (6 male, 11 female), $Per2^{Brd}$ $n=17$ (6 male, 11 female) and WT = 19 (10 male, 9 female)

### Statistical analysis

Where appropriate, all values measured are given as means ± standard error of means (SEM). To test for statistical differences between the genotypes we used repeated measurement design of the one-way analyses of variance (ANOVA) with the factor "time" as within and "genotype" as between variable and Scheffé's post hoc test where appropriate. All statistical tests were carried out two-tailed using the StatView 5.0 Software (SAS, USA), and the level of significances was set at $P=0.05$. The area under the curve was calculated by numerical integration using the trapezoidal rule, and the built-in algorithm of Origin 6.0 (Microcal Software, USA) was used to compute the results.

## Results

### Daily rhythm of corticosteroids

All genotypes showed a similar daily rhythm regarding the amount of feces excreted over the 48-h sampling period ($F(2,19)=2.493$, $P=0.1068$, $n=8$ per group). Following the pattern of food intake, peak excretion occurred at the end of the dark phase and the trough during the light phase. In the amount of feces produced per 24 h (mean of 2 days sampling) the genotypes were indistinguishable ($Per1^{Brd}$ $1.3\pm0.1$ g; $Per2^{Brd}$ $1.4\pm0.1$ g; WT $1.6\pm0.2$ g; $F(2,21)=1.255$, $P=0.3057$).

The analysis of fecal corticosterone metabolites (CM) revealed a clear daily variation in $Per2^{Brd}$ ($F(7,49)=5.134$, $P=0.0002$) as well as WT ($F(7,49)=4.261$, $P=0.0010$) mice, characterized by high levels in the first half of the night (ZT12–18) and low levels during the first half of the day ($Per2^{-/-}$ ZT6 vs. ZT18 $P=0.0398$; WT ZT3 vs. ZT15 $P=0.0097$). Although there seemed to be a trend towards higher CM concentrations in $Per2^{Brd}$ mice no statistical significant differences between $Per2^{Brd}$ and WT could be detected ($P=0.0871$). In contrast, the $Per1^{Brd}$ animals did not show a daily rhythm ($F(7,49)=0.346$, $P=0.9283$), but excreted constantly high levels of corticosterone metabolites throughout the day (Fig. 1). As a consequence, the CM concentrations of $Per1^{Brd}$ differed significantly from WT ($P=0.044$). They were elevated over those of WT mice during the late night and the day (ZT21 $P=0.032$, ZT0 $P=0.004$, ZT3 $P=0.003$, ZT6 $P=0.009$, ZT9 $P<0.0001$), but not during the early dark phase (ZT12 to ZT18, all $P>0.1$).

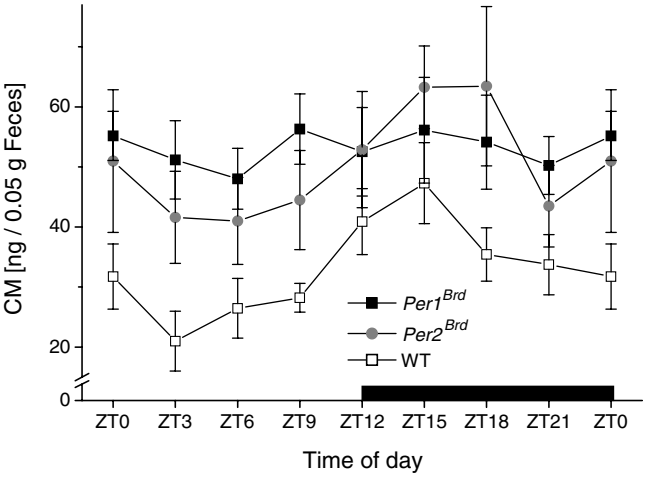


**Fig. 1** Time course of fecal corticosterone metabolite (CM) concentrations in $Per1^{Brd}$, $Per2^{Brd}$ and WT mice. Group size $n=8$ per genotype. The *black bar* indicates the dark phase. Data are given as mean ± SEM. Note that the values for ZT0 are plotted twice. No statistically significant differences were indicated for matters of clarity

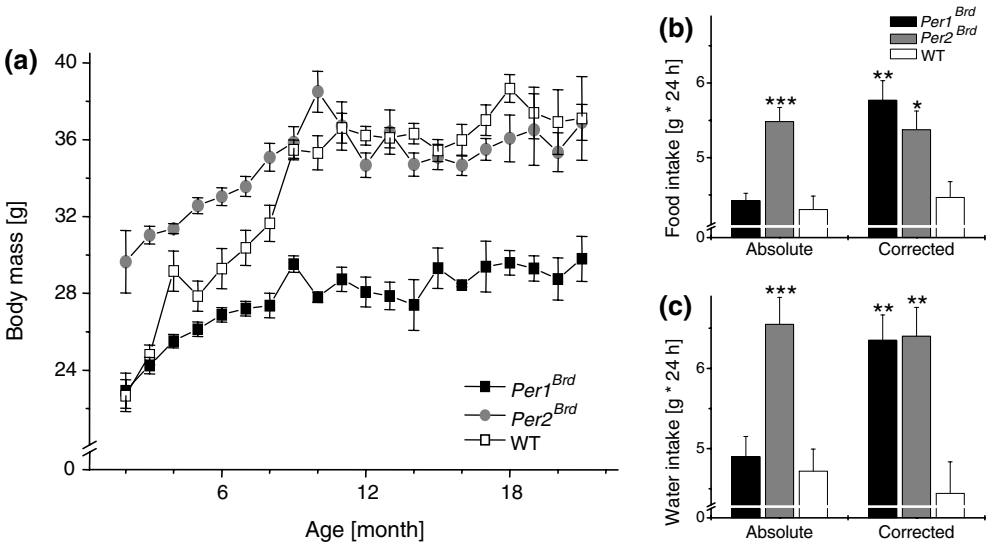


**Fig. 2** Body mass development (**a**) and food and water intake (**b, c**) of all genotypes. Group size was at least 37 and 9 in (**a**) and (**b**, **c**), respectively. Values in (**b**, **c**) are given as absolute values per individual and adjusted for body mass as intake per 30 g body mass. Sig. versus WT given as $*P<0.05$, $**P<0.01$, $***P<0.001$

Body mass development, and food and water intake

The body mass of adult animals (mean body mass for all weightings of each mouse older than 4 months) differed significantly, with *Per1*$^{Brd}$ being smaller than WT animals ($P<0.001$), whereas *Per2*$^{Brd}$ mice could not be distinguished from WT (*Per1*$^{Brd}$: $27.1\pm0.3$ g, $n=56$, *Per2*$^{Brd}$: $34.7\pm0.4$ g, $n=43$, WT: $35.3\pm0.4$ g, $n=37$). This is obviously different for the body mass development during the first 4 months. However, since we did not control for litter size, we cannot exclude effects from the time before weaning.

While absolute food and water intake in *Per2*$^{Brd}$ (food $5.5\pm0.2$; water $6.6\pm0.3$; $n=10$) mice was higher than in the WT (food $4.3\pm0.2$; water $4.7\pm0.2$; $n=9$), the *Per1*$^{Brd}$ (food $4.4\pm0.1$; water $4.9\pm0.3$; $n=12$) mice were indistinguishable from WT in the absolute numbers (*Per2*$^{Brd}$ vs. WT: food $P<0.001$, water $P<0.001$; Fig. 2b). If adjusted for body mass (i.e. calculated as amount consumed per 30 g body mass, Fig. 2c), however, *Per1*$^{Brd}$ (food $5.8\pm0.3$; water $6.3\pm0.3$) and *Per2*$^{Brd}$ (food $5.4\pm0.2$; water $6.4\pm0.4$) mice showed higher food and water consumption than WT (food $4.5\pm0.2$; water $4.4\pm0.4$) animals (*Per1*$^{Brd}$ vs. WT $P<0.01$, *Per2*$^{Brd}$ vs. WT $P<0.05$).

Glucose tolerance test

No significant difference between the sexes could be detected (data not shown). Therefore, the data of males and females were pooled for genotype comparison. In all genotypes, the blood glucose level was elevated following the injection of glucose, but returned to basal levels within 120 min (0 vs. 120 min: *Per1*$^{Brd}$ $P=0.785$; *Per2*$^{Brd}$ $P=0.985$; WT $P=0.714$; Fig. 3). Both, *Per1*$^{Brd}$ and *Per2*$^{Brd}$ mice differed significantly from the WT animals in the time course of the blood glucose levels ($F(2,43)=6.643$, $P=0.003$; WT vs. *Per1*$^{Brd}$ $P=0.015$; WT vs. *Per2*$^{Brd}$ $P=0.001$). Furthermore, the area under the curve (AUC) for blood glucose was significantly different between genotypes ($F(2)=5.539$, $P=0.0068$). While the AUC in the *Per2*$^{Brd}$ ($22.8\pm0.9$) mice was significantly smaller ($P=0.001$) than in WT ($30.2\pm2.1$) the *Per1*$^{Brd}$ ($24.6\pm1.2$) showed only a trend towards a smaller AUC ($P=0.080$).

## Discussion

Compared to WT the *Per1*$^{Brd}$ and *Per2*$^{Brd}$ mice differed markedly regarding physiological parameters associated

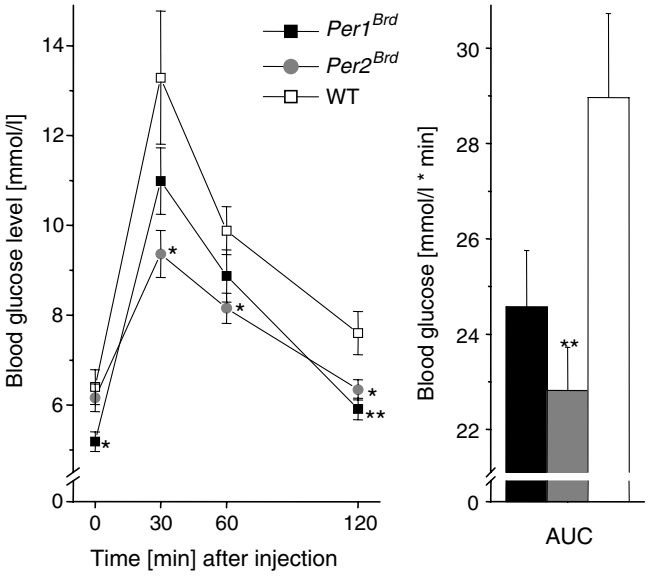


**Fig. 3** Glucose tolerance after overnight fasting. The animals were injected with glucose solution (2 g/kg body mass). Blood was sampled directly before (0), and 30, 60 and 120 min following the injection. AUC area under the curve. Group size: *Per1*$^{Brd}$ $n=17$, *Per2*$^{Brd}$ $n=17$, WT $n=19$. Data are given as means ± SEM. $*P<0.05$, $**P<0.01$

with the activity of the HPA axis. The activity of the HPA axis, which was assessed by measuring CM with a recently established non-invasive technique (Touma et al. 2003, 2004) was altered in the *Per1*$^{Brd}$ mice. As expected for night-active mammals, the results showed a clear daily variation of CM in WT and *Per2*$^{Brd}$ mice, with a peak around the beginning of the dark phase and a trough around the beginning of the light phase. A daily pattern has been shown in various studies investigating plasma (Saba et al. 1963; Verhagen et al. 2004) or fecal samples (mice: Touma et al. 2004; rats: Cavigelli et al. 2005). In contrast, the *Per1*$^{Brd}$ animals exhibited constantly high levels of CM, both during the day and during the night, i.e. the daily rhythm of GC secretion was impaired in these animals. This is surprising because the *Per1*$^{Brd}$ animals do not show an abnormal activity pattern in a light/dark cycle (Zheng et al. 2001). Moreover, the amount of feces did not differ from WT or *Per2*$^{Brd}$ animals. Interestingly, preliminary results suggest that this loss of rhythmicity in GCs can also be found in *Per2*$^{Brd}$/*Cry1*$^{-/-}$ mice (i.e. mice with mutations in both *Per2* and *Cry1* genes and, hence, lack of a major part of the negative feedback loop of the molecular clock work). These mutant mice exhibit constantly low corticosterone levels (H. Oster, personal communication). The same is true for melatonin proficient *Clock* mutant mice, which have low and arrhythmic plasma corticosterone levels in both males and females (D. Kennaway, personal communication). The *Cryptochrome* (*Cry*) and *Clock* genes are proposed to be part of the core clock as well as the *Per* genes (Albrecht 2004), which suggests that the corticosterone rhythm might, to some degree, be dependent on a fully functional clock.

It might be argued that the metabolic cage represents a stressor for the mice as it has been shown to be the case for rats (Eriksson et al. 2004). In order to avoid such an effect, the habituation period of 4 days was chosen even 1 day longer than previously shown as affective (Touma et al. 2004). Indeed, the WT and *Per2*$^{Brd}$ mice showed normal diurnal patterns of fecal CM, indicating habituation. Still, the *Per1*$^{Brd}$ animals might be more prone to stress and might have possibly needed some more time for habituation. If that were the case, however, nighttime levels of CM of the *Per1*$^{Brd}$ mice should have been higher than normal, which they were not. A severely stressed animal, not habituated, should have a much higher CM level as shown in an ACTH challenge test (Touma et al. 2003). By the same argument we can also dismiss the hypothesis that a defect of the *Per1* gene leads to a general hyperactivity of the HPA axis and, hence, to a Cushing syndrome-like status. The level of fecal CM in *Per1*$^{Brd}$ mice is comparable to the basal night levels of CM in WT mice and we describe a loss of rhythmicity that leads to elevated daytime levels, but no extremely high levels as apparent in the Cushing syndrome. One might speculate that in the *Per1*$^{Brd}$ mice signals from the SCN which normally lead to a down-regulation of the HPA axis during the resting period are missing. So far, however, we have no information what the nature of these signals might be.

Furthermore, we showed that the *Per1*$^{Brd}$ are lighter than the WT animals. Elevated levels of corticosterone are associated with reduced body mass (Bartolomucci et al. 2004). Since the *Per1*$^{Brd}$ animals exhibited higher corticosterone metabolite (CM) levels in the feces at least during the day, this seems to provide some explanation for their lower body mass. Their food intake per gram body mass was higher than in the control animals. This suggests that the metabolic rate of the *Per1*$^{Brd}$ mice is higher. In contrast, *Per2*$^{Brd}$ were heavier than WT animals up to 4 months of age and as adults as heavy as the WT controls. We measured food intake only in adult animals, but it seems likely that the higher food intake, both absolute and per gram body mass, contributed to this difference.

It remains unknown whether the differences in the body mass might be a pleiotropic effect of the *Per* genes or whether this is an indirect effect mediated by alterations in the clock as a whole. Recently, however, similar body mass phenotypes have been described for *Clock* mutant mice (Turek et al. 2005).

The glucose tolerance test revealed a significant difference between the genotypes with *Per1*$^{Brd}$ and *Per2*$^{Brd}$ animals having a faster glucose metabolism than the WT mice. Both the basal blood glucose level and glucose tolerance exhibit a daily rhythm which is under the control of the SCN (Yamamoto et al. 1987; La Fleur et al. 2001). In the present study, the initial blood glucose levels after 14 h of fasting were lower in *Per1*$^{Brd}$ than in WT mice, which might be due to a higher metabolic rate in *Per1*$^{Brd}$ mice. In fact, we do have preliminary data showing that at least under constant conditions (DD) *Per1*$^{Brd}$ mice have a slightly higher metabolic rate (Dernbach 2003). However, one cannot exclude a different insulin secretion pattern. Nevertheless, it is possible that the *Per* genes are involved in glucose homeostasis, as it has been recently shown for two other core components of the clock (*Bmal1* and *Clock*), which were found to contribute significantly to the recovery from hypoglycemia after insulin injection (Rudic et al. 2004).

In recent years, the existence of peripheral oscillators has been discovered (Balsalobre et al. 2000). It has been proposed that GCs are one of the pathways by which the SCN synchronizes the peripheral oscillators (Le Minh et al. 2001). This is critically important for organizing the metabolism of the whole body (Penev et al. 1998). The findings of the present study may thus be useful for further investigations on this topic. It would be interesting to know whether peripheral oscillators, especially in the pituitary are still synchronized to the SCN, which has so far only been demonstrated for the kidney (Zheng et al. 2001).

Taken together, we showed that the *Per1*$^{Brd}$ mice exhibit an impaired daily GC rhythm, with markedly elevated CM levels during the day. The *Per2*$^{Brd}$ mice, however, did not differ from WT mice but as well as *Per1*$^{Brd}$ mice showed a tendency towards faster glucose

clearance in the glucose tolerance test. Furthermore, we presented evidence for a higher metabolic rate in *Per1*$^{Brd}$ mice which can be explained by the altered GC rhythm. If investigated on a molecular level, this might yield further insights into the mechanism of the clock and reveal new functions of the *Per* genes.

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