Behavioural food-anticipation in clock genes deficient mice: confirming old phenotypes, describing new phenotypes

Running Title: Food entrainment in clock genes mutant mice

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Abstract

Animals fed daily at the same time exhibit circadian food-anticipatory activity (FAA), which has been suggested to be driven by one or several food-entrainable oscillators (FEOs). FAA is altered in mice lacking some circadian genes essential for timekeeping in the main suprachiasmatic clock (SCN). Here we confirmed that single mutations of clock genes *Per1*$^{-/-}$ and *Per2*^{Brdm1} alter FAA expression in constant darkness (DD) or under a light-dark cycle (LD). Furthermore, we found that *Per1*$^{-/-}$*;Per2*^{Brdm1} and *Per2*^{Brdm1}*;Cry1*$^{-/-}$ double mutant animals did not display a stable and significant FAA either in DD or LD. Interestingly, rescued

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behavioural rhythms in *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mice in DD were totally entrained to feeding time and re-synchronized after phase-shifts of mealtime, indicating a higher SCN sensitivity to feeding cues. However, under an LD cycle and restricted feeding at midday, FAA in double *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutant mice was absent. These results indicate that shutting down one or two clock genes results in altered circadian meal anticipation. Moreover, we demonstrate that in a genetically rescued SCN clock (*Per2*^{Brdm1}*;Cry2*$^{-/-}$), food is a powerful *zeitgeber* to entrain behavioural rhythms, leading the SCN to be more sensitive to feeding cues than in wild-type littermates.

Introduction

In nature food availability is restricted in time and place. Therefore organisms have to develop behavioural and physiological adaptations to anticipate and prepare for regular and predictable food opportunities at any times of the day. Endogenous circadian oscillators, which help organisms to predict and anticipate food availability, are one of the most relevant physiological adaptations (Pittendrigh, 1993). Whereas the main circadian pacemaker in the mammalian brain is contained within the hypothalamic suprachiasmatic nucleus (SCN) (Hastings & Herzog, 2004), one or several food-entrainable oscillators (FEOs) are thought to be responsible for food-anticipatory circadian rhythms (Mistlberger, 1994; Stephan, 2002). The FEO is outside of the SCN (i.e., arrhythmic SCN-lesioned animals show robust FAA) and it has been hypothesized to be elsewhere in the brain (Mendoza, 2007; Davidson, 2009; Mistlberger, 2009). The molecular mechanisms underlying circadian rhythmicity in the SCN depends on interlocking autoregulatory feedback loops involving a set of clock genes, such as the *Period 1* and *2*, *Bmal1* and *Cryptochrome 1* and *2* genes (Takahashi *et al,* 2008). Whereas single disruptions of the genes *Per1* or *Per2* lead to shortening, with a gradual loss, of free-running period of behaviour in constant dark (DD; Zheng *et al,* 1999), double mutations

(*Per1;Per2* or *Cry1;Cry2*) lead to complete and immediate loss of circadian rhythmicity in DD (Bae *et al,* 2001; van der Horst *et al,* 1999). Interestingly, in *Per2* deficient mice ($Per2^{Brdm1}$), a mutation of the gene *Cry2* restores behavioural and molecular rhythmicity in DD (Oster *et al,* 2002). Restoration of behavioural rhythms is also observed in *Per2;Cry1* double mutant mice but only under constant light conditions (LL; Abraham *et al,* 2006).

Even if the anatomical location of FEO remains to be elucidated, it has been shown that mice mutant for the gene $Per2^{Brdm1}$ are unable to anticipate mealtime (Feillet *et al,* 2006), highlighting the role of *PER2* as a critical molecular component for FAA expression. However, it has recently been reported that double $Per1^{ldc};Per2^{ldc}$ mutant animals show FAA similar to wild-type mice (Storch & Weitz, 2009). On the other hand, whereas a study reported absence of FAA in *Bmal1* KO mice (Fuller *et al,* 2008) other studies concluded that *Bmal1* is not necessary for FAA expression (Mistlberger *et al,* 2008; Pendergast *et al,* 2009).

Therefore the aims of this study are to re-evaluate, first, whether mutations in single clock genes such as *Per1* and *Per2* or a double *Per1;Per2* mutation affect FAA. To determine the influence or masking effects of the LD cycle, animals were tested in both DD and LD conditions. Secondly, to test the functionality of the FEO, we assessed the ability of animals to follow both phase-delays and advances of mealtime cycle (Stephan, 1984; 1992). Thirdly, to extend the study on the role of clock genes in food anticipation, since behavioural rhythmicity in $Per2^{Brdm1}$ mutant mice is rescued by the mutation of the clock gene *Cry2*, but not *Cry1* in DD (Oster *et al,* 2002), we evaluated whether FAA is rescued as well in double $Per2^{Brdm1};Cry2^{-/-}$.

Materials and methods

Animals and housing

Adult female F_2 homozygous *Per1* and *Per2* mutant mice and their wild-type littermates were used for the whole experiment. Intercrosses between heterozygous **(C57BL/6× 129S5/SvEvBrd)** F_1 offspring gave rise to F_2 homozygous *Per1* and *Per2* mutant animals. Therefore, mutant and wild-type animals on this mixed background were used in this study. Wild-type (WT) animals were littermates of backcrosses of F_1 *Per1* or F_1 *Per2* heterozygous animals. In previous experiments using $Per1^{-/-}$ and $Per2^{Brdm1}$ mutant mice and their respective WT littermates we did not observe a sex difference in any behavioural (wheel-running amount, entrainment to LD cycle, free-running period in DD or FAA) or physiological parameters (Feillet *et al,* 2006; 2008). The loss-of-function *Per1* mutation ($Per1^{-/-}$) results from deletion of exon 3 to exon 19 of *mPer1*. The deleted region includes the PAS domain, thus precluding protein dimerization. The *Per2* mutation ($Per2^{Brdm1}$) was obtained by deleting part of the PAS domain, thus impairing normal protein dimerization. The $Per1^{-/-}$ and $Per2^{Brdm1}$ mutations are described in detail in Zheng *et al,* (2001) and Zheng *et al,* (1999), respectively.

$Cry1^{-/-}$ and $Cry2^{-/-}$ animals were generated in a hybrid **C57BL/6×129P2/OlaHsd** background (van der Horst *et al,* 1999). For double mutant mice, $Per2^{Brdm1}$ mice were crossed with $Per1^{-/-}$, $Cry1^{-/-}$ or $Cry2^{-/-}$ animals as previously reported (Oster *et al,* 2002). The double-heterozygous offspring was intercrossed to produce wild-type and homozygous mutant animals. Matching wild-type control animals were produced by back-crossing heterozygous animals derived from the $Per1^{-/-}$ and $Cry^{-/-}$ matings to minimize epigenetic effects.

All animals were housed singly in cages equipped with a running wheel (10 cm diameter) in a room at 23 ± 1 °C under a 12/12 light–dark cycle (LD bright light 200 lx; dim red light 5 lx; lights on at 07:00 AM). Under these lighting conditions, times of day were converted to

Zeitgeber times (ZT) in which ZT0 and ZT12 were the onsets of light and darkness, respectively. Food (standard low-fat diet, 105, SAFE, 89290 Augy, France) and water were available *ad libitum* unless otherwise stated. Body mass and food intake were measured weekly during the whole experiment, in DD and LD conditions. During food restriction food intake was measured in the 6h of food access for both DD and LD experiments. All animal manipulations were performed in accordance with the European Committee Council Directive of November 24th 1986 (86/609/EEC), and the French and Swiss national laws.

Experimental design

Food entrainment in DD and mealtime jet-lag test

In a first experiment, after at least two weeks under a LD cycle, animals (n=8 per genotype) were released in constant darkness (DD) for at least two weeks before food entraining conditions. Animals were then entrained to a temporal food restriction with 6h of food access starting at 12h (geographical time) during two weeks. Thereafter, animals were subjected to a 6-h phase advance of mealtime (first *jet-lag* test) and remained in this new feeding schedule for 2 weeks more. Finally, animals were subjected again to a phase change of mealtime but now with a 6-h phase delay of food access. After two weeks animals were put back in *ad libitum* conditions but still in DD conditions before being exposed to an LD cycle.

Food entrainment in an LD cycle

In a second experiment, under LD housing conditions, mice were subjected to 6-h restricted feeding schedules at least for two weeks. Food was provided from *zeitgeber* time 6 (ZT6) to ZT12 (lights off). On the last day of food entrainment, animals were killed at two different time points; ZT4 (time of food anticipation) and ZT16 (n=4-5 per time point and genotype). Brains were removed for immunohistochemistry and trunk blood (1-1.5 ml) was collected in 2-ml Eppendorf tubes containing 10 µl of 4% EDTA for hormonal and metabolic assays.

Circadian Behaviour analysis

Wheel revolutions were recorded in 5 min time bins by CAMS (Circadian Acitivity Motor System, Lyon France) acquisition program and transferred to the ClockLab analysis software (ClockLab; Actimectrics, Evanston, IL, USA) to produce double-plotted actograms and periodograms. χ^2 periodogram analyses were performed using at least 15 days of data in DD. Activity profiles were obtained from each animal, and FAA was determined as the percent of total wheel-running activity during the 24h. Differences in the intensity of FAA between groups were evaluated in activity occurring during the 4h prior to mealtime.

In DD experiments, different to WT animals, in some mutant mice we observed a single behavioural component anticipating mealtime. To dissociate FAA from SCN-dependent activity, we evaluated the duration of FAA over ten days during food restriction (from FAA onset to mealtime; FAA onset was defined as the time bin in which activity is up to 50% of the FAA acrophase per day; Landry *et al,* 2007). To determine whether FAA is stable, we evaluated FAA precision (standard deviation of FAA duration) similarly to the analysis of Landry *et al,* (2007).

Finally, FAA ratios were evaluated in both LD and DD experiments. For LD experiments the FAA ratios were obtained of the FAA counts as a percent of daily wheel-running activity excluding hours 4-12 of lights on (Landry *et al.* 2007). For DD experiments, however, since animals are in free-running conditions and circadian phases are differences between them, FAA ratios were obtained of the FAA (3h prior to mealtime) counts as a percent of the total daily wheel-running activity.

Immunohistochemistry

Brains were fixed overnight in 4% of paraformaldehyde in buffer phosphate (PB, 0.1M), and then allowed to equilibrate in 30% sucrose in 0.1 M PB with 0.02% sodium azide (Sigma, St. Quentin Fallavier, France) at 4°C at least for three days. Brains were sectioned (4 series; 30 µm) on a cryostat and stored in PBS with sodium azide at 4°C. Sections were incubated for 24 hr at 4°C in anti-FOS rabbit polyclonal antiserum (Santa Cruz, Santa Cruz CA, USA; 1:10,000 dilution), 10% donkey serum (Millipore, Road, MA), and PBS with 0.3% Tween-20. Tissue was then rinsed in PBS + 0.05% Tween, incubated in biotinylated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch Laboratories, West Grove PA, USA) for 2 hr at room temperature, and incubated with peroxidase-conjugated avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA) for 1 hr, followed by 0.05% diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.01% H_2O_2.

Cell count

Sections were examined under a light microscope (Leica DMRB; Leica) and images captured using a CCD camera (Olympus DP50; Olympus) on a PC computer. Based on the mouse brain steorotaxic atlas (Paxinos & Franklin, 2001) digital images were taken at the level of the suprachiasmatic nucleus (SCN, -0.46mm from Bregma), the lateral hypothalamus (LH, -1.22mm from Bregma) the ventromedial (VMH) and dorsomedial hypothalamus (DMH) and arcuate nucleus (ARC, -1.70mm from Bregma). Hypothalamic cells immunopositive for c-FOS were counted using the NIH ImageJ software (Rasband, W.S., USA. National Institutes of Health, Bethesda, MD, USA). For analysis, the total number of immunoreactive cells bilaterally per structure was calculated for each animal from the counts of 2-3 images showing the highest number of labeled nuclei.

Hormonal and metabolic assays

Plasma corticosterone was assayed with a commercial ^{125}I RIA kit for mice and rats (ImmuChem Double Antibody, MP Biomedicals, Orangeburg, NY, USA). The limit of sensitivity of corticosterone assay was 7.7 ng/ml. Blood was centrifuged at 5000 rpm for 10 min to obtain blood serum. Serum aliquots of 100 µl were frozen at -80°C for subsequent determination by colorimetric methods of the concentration glucose (method of Trinder, Glucose GOD-PAP Kit BIOLABO, REF: LP80009) and free fatty acids (method ACS-ACOD; Kit NEFA–HR2 Wako).

Statistical analysis

The statistical analysis was performed by *t*-test, one and two-way analyses of variance, (ANOVA) of independent and repeated measures, for all behavioural and physiological experiments, followed by a LSD Fisher *post hoc* test with alpha set at <0.05. Statistical analysis was performed with the statistical package Statistica (version 4.5, 1993; StatSoft, Tulsa, OK, USA). Values are means ± S.E.M.

Results

Food entrainment in clock genes deficient mice under constant darkness conditions

First, we assessed the effects of daily 6-h of food availability in mice mutant for circadian clock genes *Per1*$^{-/-}$, *Per2*^{Brdm1}, *Per1*$^{-/-}$;*Per2*^{Brdm1}, *Per2*^{Brdm1};*Cry1*$^{-/-}$ and *Per2*^{Brdm1};*Cry2*$^{-/-}$ under DD conditions to avoid possible masking effects of the light dark cycle.

All WT, single *Per1*$^{-/-}$ and double *Per2*^{Brdm1};*Cry2*$^{-/-}$ mutant mice exhibit robust free-running rhythms under DD conditions (Fig. 1A). Both *Per1*$^{-/-}$ single and double *Per2*^{Brdm1};*Cry2*$^{-/-}$ mutants had shortened circadian free-running periods (τ) compared to WT animals (Fig. 2;

$F_{2,21}$=10.9, p=0.0005). *Per2*^{Brdm1}, *Per1*$^{-/-}$*;Per2*^{Brdm1}, *Per2*^{Brdm1}*;Cry1*$^{-/-}$ mutant mice, however, were totally arrhythmic immediately after being released in DD conditions (Fig. 1 and Fig. 2). When animals were subjected to daily 6-h period of food availability, WT animals showed FAA independently of a circadian free-running component, indicating the presence of two circadian clocks; the free-running component is likely controlled by the SCN while FAA is thought to be the behavioural output of FEO (Fig. 1A, 1B and Fig. 2).

When we compared FAA intensity from 4h before mealtime, ANOVA revealed significant differences for the factor time (Fig. 1C; $F_{3,126}$=18.5, $p<0.01$) but not for the factor genotype (Fig. 1C; $F_{5,42}$=1.6, p=0.2). However, the genotype X time interaction was statistically different (Fig. 1C; $F_{15,126}$=5.4, $p<0.01$), indicating that FAA was significantly higher in WT animals than in mutant mice 1h before mealtime (Fig. 1C; *post-hoc*, $p<0.01$). Moreover, FAA ratio was significantly different between genotypes, showing an elevated ratio in WT mice (Fig. 1C; $F_{5,42}$=2.6, p=0.04). FAA duration over 10 days of food restriction was significantly different between genotypes, with greater FAA duration in single *Per2*^{Brdm1} and double *Per2*^{Brdm1}*;Cry1*$^{-/-}$ and *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutant mice (Table 1; $F_{5,42}$=2.5, p=0.04). However, because only one behavioural component anticipating mealtime was evident in these mutants, this larger FAA could reflect the entrainment of SCN-dependent activity. Finally, FAA precision showed significant differences between genotypes as well, with a lower standard deviation of FAA duration in WT animals compared to mutant mice (Table 1; $F_{5,42}$=3.9, p=0.005). These results suggest that FAA in WT control animals is much more stable than that in mutant mice.

Although activity profiles of mutant mice showed an increase in activity before mealtime, in double actograms there were not two activity components as in WT mice (Fig. 1A, 1B and Fig. 2). *Per1*$^{-/-}$ mutant mice continued to free-run with an endogenous period that was significantly shortened by restricted feeding (t_{14}=2.24, $p=0.04$). Interestingly daily restricted

feeding in *Per2*^{Brdm1}, but not in double *Per1*$^{-/-}$*;Per2*^{Brdm1} mutant mice, induced a free-running period shorter than in WT animals (Fig. 1A and Fig. 2; $F_{4,28}$=6.5, p=0.0007).

In some double *Per2*^{Brdm1}*;Cry1*$^{-/-}$ mutant mice, there was a significant period of 24h induced by restricted feeding which was not different from the period of WT animals (Fig. 2; $F_{4,28}$=6.5, p=0.0007). Interestingly in rhythmic double *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutant mice, feeding schedules had the ability to entrain the whole circadian behaviour with a significant period of 24-h compared to the free-running period under free-feeding conditions (Fig. 1A and Fig. 2; t_{14}=4.2, $p<0.01$).

Re-synchronization to mealtime phase-shifts

One important characteristic of circadian clocks is their ability to re-synchronize in response to changes of the synchronizer phase, after showing some transient cycles (Pittendrigh & Daan, 1976; Stephan, 2002). Here we observed that WT mice are able to re-synchronize to both 6-h phase advance and delay of feeding time, showing some transient cycles of circadian behaviour (Fig. 3A and B). Short free-running rhythms of wheel-running activity of *Per1*$^{-/-}$ mutant mice continued to free-run without re-synchronization to the new mealtime cycle (Fig. 3A and B). In both *Per2*^{Brdm1} and double *Per1*$^{-/-}$*;Per2*^{Brdm1} mutant mice, food anticipatory activity was absent and no clear transition cycles were evident after shifts of the mealtime.

In double *Per2*^{Brdm1}*;Cry1*$^{-/-}$ mutant mice, but only in those individuals (3/8) that showed a rhythmic behavioural pattern anticipating mealtime, a re-synchronization to the new mealtime was observed after both 6-h phase advance and phase delay (Fig. 3A and B).

Finally, in double *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutant mice, there was a total entrainment of the whole circadian behaviour which was re-synchronized to both phase-advances and phase-delays of the mealtime, showing several transient cycles reminiscent of transitory behaviour observed after a phase-shift of the light-dark cycle (Fig. 3A and B).

Food entrainment in clock gene deficient mice under a LD cycle

Under a 12-12h LD cycle, whereas WT mice exhibited a clear and stable FAA to 6-h of food access, other genotypes such as *Per1*$^{-/-}$ and double *Per1*$^{-/-}$*;Per2*^{Brdm1} and *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutant animals showed FAA significantly lower than that expressed by control WT. *Per2*^{Brdm1} and double *Per2*^{Brdm1}*;Cry1*$^{-/-}$ mutant animals did not exhibit FAA at all (Fig. 4A-C). The ANOVA indicated significant differences for the factor genotype ($F_{5,42}$=6.1, $p=0.0002$) for the factor time ($F_{3,126}$=31.5, $p<0.01$) and the genotype X time interaction (Fig. 1C; $F_{15,126}$=8.4, $p<0.01$). The post-hoc analysis showed that FAA was significant higher 2 and 1h before mealtime in WT animals compared to mutant mice (Fig. 4C; *post-hoc*, $p<0.05$). In addition, FAA ratio in WT mice was also significantly larger than in mutant animals (Fig. 4C; $F_{5,42}$=4.1, $p=0.003$). The absence or reduced FAA in clock gene mutant mice is not the consequence of a hypoactive phenotype in these animals, because wheel-running activity during the night period was similar in most mutants and even significantly higher in double *Per2*^{Brdm1}*;Cry1*$^{-/-}$**,** compared to WT control animals (Fig. 4; $F_{5,42}$=2.9, $p=0.02$).

Hypothalamic activation in clock gene deficient mice entrained to food

c-FOS activation in animals synchronized to a timed 6-h food access and under a LD cycle showed significant differences between genotypes (Fig. 5). In the ARC nuclei c-FOS expression in WT animals was significantly increased at ZT4 (during anticipation) compared to *Per1*$^{-/-}$*;Per2*^{Brdm1} and *Per2*^{Brdm1}*;Cry1*$^{-/-}$ mutant mice (Fig. 5; $F_{5,39}$=4.6 p=0.002). In the SCN nuclei c-FOS immunoreactivity in WT animals was also significantly increased at ZT4 compared to *Per2*^{Brdm1}*, Per1*$^{-/-}$*;Per2*^{Brdm1} and *Per2*^{Brdm1}*;Cry1*$^{-/-}$ mutant mice (Fig. 5; $F_{5,39}$=7.4 p<0.001). In the DMH and VMH nuclei the number of c-FOS stained cells in WT mice at ZT4 was higher than in mutant animals (Fig. 5; DMH, $F_{5,39}$=3.5 p=0.009; VMH, $F_{5,39}$=3.6

p=0.009). Finally c-FOS activity in the LH area was also higher in WT mice during anticipation compared to the other genotypes (Fig. 5; $F_{5,39}$=6.4 p=0.0001). During the night period the number of c-FOS expressing cells was also higher in the LH of WT and *Per1*$^{-/-}$ mutant mice compared to *Per2*^{Brdm1}, *Per1*$^{-/-}$;*Per2*^{Brdm1} and *Per2*^{Brdm1};*Cry1*$^{-/-}$ mutant animals (Fig. 5; *post-hoc* p<0.05).

Physiological changes in clock gene deficient mice synchronized by food

Food restriction in DD leads to an increased body weight only in WT mice. For mutant mice, no significant change was detected during food restriction compared to free-feeding conditions (Table 2; $F_{1,90}$=8.4 p=0.004). Under LD conditions, however, we did not **observe** significant differences between AL and RF conditions in any genotype (Table 2; $F_{1,90}$=0.00 p=0.9), suggesting that body weight changes in response to restricted feeding were similar between mutant and WT mice. 24h food intake during DD and free feeding conditions was not significantly changed between genotypes (Table 3; $F_{5,45}$=2, p=0.1). However, during 6h of food access, double *Per2*^{Brdm1};*Cry1*$^{-/-}$ and *Per2*^{Brdm1}; *Cry2*$^{-/-}$ showed a significant increase in the amount of food intake compared to WT and the other mutants (Table 3; $F_{5,45}$=4.6, p=0.01).

In an LD cycle, food intake was significantly different between genotypes (Table 3; $F_{5,45}$=6.6, p<0.01). Moreover, during the 6-h food access some difference between genotypes in the amount of food eaten was observed (Table 3; $F_{5,45}$=5.0 p=0.0009). Under food restriction and LD conditions, corticosterone concentrations were higher at ZT4 (two hours before mealtime) than at ZT16 in all genotypes (Fig. 6; $F_{1,39}$=38.5 p<0.01). However, this anticipatory peak was significantly higher in WT mice compared to the three other genotypes lacking *Per2* (Fig. 6; $F_{5,39}$=2.7 p=0.03). For glucose concentrations, no differences between genotypes were observed (Fig. 6; $F_{5,40}$=1.5 p=0.2). However, we found a significant difference for the factor

time (Fig. 6; $F_{1,40}$=5.6 p=0.02), indicating a similar entrainment of glucose rhythm between genotypes. No differences between genotypes for free fatty acids concentrations were found (Fig. 6; $F_{5,39}$=0.5 p=0.7), but the ANOVA indicates a significant difference between *zeitgeber* times (Fig. 6; $F_{1,39}$=9.2 p=0.004). These data suggest that whereas the FEO responsible for behavioural food anticipation is altered in mutant mice, entrainment or resetting of peripheral oscillators controlling metabolism may be intact in these animals. Therefore, physiological, but not behavioural, anticipation is still present in these mice.

Discussion

Despite many attempts, the locus of the FEO is still unknown (Davidson, 2009). The study of expression or mutations of mammalian clock genes has provided the opportunity to understand the molecular mechanisms of circadian clocks, including the FEO (Challet *et al,* 2009). Here our results confirm previous data on the lack of FAA in *Per2*^{Brdm1} mutant mice, and give new information on food anticipation in mice bearing mutations in two clock genes.

In DD conditions WT mice show a free-running, SCN-controlled behavioural component as well as a behavioural anticipation of mealtime that is progressively re-entrained after a shift of the mealtime cycle. *Per1*$^{-/-}$ mutant mice only express a free-running component, but do not show a circadian behaviour anticipating mealtime. In some cases *Per1*$^{-/-}$ mutant mice may anticipate mealtime. However, this activity is only present in the free-running behavioural component. Two hypotheses could explain these findings: *1) Per1* mutation leads to a damped or weak FEO, which then would become more strongly coupled to the SCN or *2)* the FEO in *Per1*$^{-/-}$ mutant has also a shortened endogenous period which is unable to entrain to 24 h restricted feeding schedules.

Under LD, LL and DD conditions, FAA is absent in *Per2*^{Brdm1} mutant mice (Feillet *et al,* 2006; present study). While *Per2*^{Brdm1} mutant mice fed *ad libitum* are totally arrhythmic in

DD (Zhen *et al,* 1999; 2001), a free-running behavioural rhythm appears during food restriction, suggesting that a functional SCN clock is restored (but not entrained) by restricted feeding.

Per2^{Brdm1}*;Cry1*$^{-/-}$ mice lose circadian rhythmicity immediately upon release into DD (Oster *et al,* 2002). Here, some of *Per2*^{Brdm1}*;Cry1*$^{-/-}$ mutant mice show a reorganization of wheel-running behaviour and in few cases anticipation of mealtime with re-synchronization of the whole behavioural component. Because two independent behavioural components are not observed, this apparent "FAA" may correspond to SCN behavioural output and not to the FEO-controlled behaviour. Moreover, FAA is very variable each day in these mutants compared to WT animals.

Per2^{Brdm1}*;Cry2*$^{-/-}$ animals in DD maintain a circadian rhythm shorter than WT mice (Oster *et al,* 2002). Surprisingly, this circadian rhythm is fully synchronized by feeding schedules and it follows phase-shifts of mealtime with transitory cycles. Therefore, the restored SCN clock of *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mice is very sensitive to feeding/metabolic cues. *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mice can entrain to LD cycles and show sustained circadian behavioural rhythms. However, the resetting responses of the clock to brief light pulses are absent when light is applied at the activity onset (phase-delay) and potentiated when light is applied during the late night (phase-advances; Oster *et al,* 2002). The alteration of light resetting may lead to a high sensitivity of the main clock to other time cues, like food. It has been proposed that *Per1* gene is involved in the SCN resetting by metabolic cues (Mendoza *et al,* 2005; 2007). In the absence of both PER2 and CRY2, the dimer PER1/CRY1 could compensate the SCN clockwork mechanism in *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutant mice, leading not only to a restoration of the clock (Oster *et al,* 2002), but also to increased responsiveness of the SCN to feeding cues.

Under LD conditions, while WT mice show a strong FAA, only weak FAA is observed in *Per1*$^{-/-}$ and double *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutant mice. Contrary to our original observations

showing comparable FAA in young WT and *Per1*$^{-/-}$ mice under LD (Feillet *et al,* 2006), here we noted a marked reduction of FAA in the same, but older (middle-age) mutants challenged with restricted feeding, suggesting here that *Per1* plays a role in the FEO. The reduced FAA under LD cycle could be due to increased masking response to light that would directly inhibit spontaneous locomotor activity during the light phase. However, the lack of FAA in DD rules out this possibility.

The rescue of circadian rhythmicity in *Per2*^{Brdm1} mutant animals by additional inactivation of the *Cry2* gene is reflected at both behavioural and molecular levels (Oster *et al,* 2002). Here, we examined whether this double mutation could rescue the FAA absent in *Per2*^{Brdm1} mutant animals. Albeit present, FAA in *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutant mice, however, is weaker than in WT animals. In DD, *Per2*^{Brdm1}*;Cry2*$^{-/-}$ mutants mice are synchronized by feeding, with no obvious expression of FAA that could be dissociated from the SCN-controlled rhythm. Indeed there is a rapid and stable entrainment of the total locomotor activity rhythm, rather than two independent behavioural components, thus suggesting either a strong coupling between the SCN and the FEO or an SCN clock highly sensitive to synchronizing effects of mealtime. In LD conditions, it is possible that the FEO remains strongly coupled to the SCN.

In *Per1*$^{-/-}$*;Per2*^{Brdm1}, FAA is weak and unstable. A recent paper has shown apparently normal FAA in double *Per1*^{ldc}*;Per2*^{ldc} mutants (Storch & Weitz, 2009). As suggested by the authors, an important difference between their work and our studies resides in the distinct mutations used. Furthermore, additional experiments in their and our mutants are needed to demonstrate that the apparent FAA is really controlled by a clockwork, as putative regulatory mechanisms of FAA (e.g., associative learning under LD or hourglass processes in DD) may participate more strongly than usual in mice with altered clocks.

Alternatively, in double *Per* mutant animals, other non-circadian alterations could be present. Metabolic and hormonal signals could then be affected by the mutations. Ghrelin has been

reported to be a peripheral signal modulating the intensity of FAA (Blum *et al,* 2009; LeSauter *et al,* 2009). Actually, the number of oxyntic cells expressing ghrelin is reduced in double *Per1;Per2* mutant mice during anticipation (LeSauter *et al,* 2009).

In $Per2^{Brdm1}$ and $Per2^{Brdm1}$;$Cry1^{-/-}$ mutant mice under a LD cycle, FAA is absent, confirming previous results with the same $Per2^{Brdm1}$ mutant animals (Feillet *et al,* 2006). Single mutations of one of these genes, *Per2* (Feillet *et al,* 2006) and/or *Cry* (Iijima *et al,* 2005), abolish and reduce FAA, respectively. However, to consider whether the FEO does not need clock genes to be functional, it is necessary to test the circadian functionality of FAA. Here we gave a first step evaluating this functionality with the mealtime *jet-lag* test. Other tests such as changes in T-cycles (period of feeding cycles different to 24h) in the circadian range (Stephan, 1981), would also be useful in that respect.

c-FOS expression in the all hypothalamic nuclei studied here (ARC, DMH, LH, SCN, VMH) was increased before mealtime in WT animals. c-FOS immunoreactivity increases during anticipation in the DMH of food restricted rats (Angeles-Castellanos *et al,* 2004). The DMH has been hypothesized to be a key structure for the expression of FAA (Gooley *et al,* 2006). However, other studies reported that FAA is still present in rodents with complete DMH ablations (Landry *et al,* 2006). An increased expression of c-FOS in the VMH has also been reported at the time of food expectancy in mice (Ribeiro *et al,* 2007). In mutant mice from the present study, c-FOS activity during anticipation is reduced in both the DMH and VMH nuclei compared to WT animals. Therefore, even if DMH (Moriya *et al,* 2009) and VMH (Mistlberger & Rechtschaffen, 1984) lesions do not abolish FAA, their functional activity is correlated with the manifestation of FAA.

LH neurons, containing hypocretins, become activated in food-restricted animals 1h before food presentation, and orexin mutant mice exhibited reduced FAA (Akiyama *et al,* 2004; Kaur *et al,* 2008). In the LH of WT mice, the number of c-FOS-ir cells during anticipation is

higher compared to mutant mice. Therefore the absence or attenuation of FAA could be dependent in part on a functional arousal system. In line with this hypothesis, is the observation that hypocretin expression is reduced in the hypothalamus of *Per2*^{Brdm1} mutant mice (U. Albrecht, unpublished data).

Taken together, our findings suggest that genetic manipulations of clock genes alter the expression of FAA, showing that the molecular basis of the FEO depends in part on certain known clock genes. Therefore, we confirm our previous data (Feillet *et al,* 2006), highlighting the relevance of the gene *Per2* in FAA. Moreover, since the mutation of *Cry2* does not rescue FAA in *Per2*^{Brdm1} mutant mice, it is possible that some other known (or still unknown) clock genes are implicated in the whole molecular machinery of the FEO. To comprehend that, it will be important to know whether other single, double or triple mutation of clock genes can eliminate FAA. Moreover, more circadian properties of the FEO (re-synchronization, limits of entrainment, persistence, SCN lesions) in all clock genes mutant mice have to be investigated before drawing a final conclusion on the role of classical clock genes in FAA.

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Figure legends

Figure 1. Food synchronization in clock genes deficient mice under constant darkness conditions. *(A)* Representative actograms of WT and mutant mice under constant darkness conditions and 6-h restricted feeding schedules (gray box). After at least 2 weeks under the first restricted feeding schedule, animals were exposed to a 6-h phase-advance of food access, and secondly to a 6-h phase-delay of food access (gray boxes). *(B)* Activity profiles of mice during restricted feeding. Activity waveforms represent the average of 10 days during food restriction. Horizontal bar indicates time of food access. *(C)* Percent of food-anticipatory activity (FAA) over total daily activity from 4 to 1h before mealtime (*left*) and mean (± SEM) of FAA ratios of mice (*right*). * *post-hoc test,* $p<0.05$, WT vs. mutant mice. Means lacking common letters are significant different, *post-hoc test,* $p<0.05$. LD-AL, light-dark cycle and food *ad libitum* conditions; DD-AL, constant darkness and food *ad libitum* conditions; DD-RF, constant darkness and food restriction conditions.

Figure 2. Periods of behavioural rhythms in food synchronized mice. (*Top*) Representative actograms and periodograms of mice under DD conditions during food *ad libitum* conditions and 6-h restricted feeding schedules. (*Bottom*) mean values (± SEM) of activity periods of mice under food *ad libitum* conditions (DD-AL) and food restriction (DD-

RF). Means lacking common letters are significant different, (*post-hoc test, p*<0.05). Numbers in parenthesis indicate the number of animals per group.

Figure 3. Re-synchronization to mealtime phase-shifts. Re-synchronization of behavioural rhythms to 6-h phase advance (A) and 6-h phase delay (B) of the mealtime cycle (shaded gray bars).

Figure 4. Food entrainment in clock gene deficient mice under a LD cycle. Representative actograms (*A*) and activity profiles (*B*; mean ± SEM) of WT and mutant mice under a light-dark cycle and 6-h restricted feeding schedules (shaded gray boxes, actograms; gray bars, activity profiles). (*C*) Percent of food-anticipatory activity (FAA) over total daily activity from 4 to 1h before mealtime (*left*). **post-hoc test, p*<0.05, WT vs. mutant mice. Mean (± SEM) of FAA ratios of mice (*right*). Means lacking common letters are significantly different (*post-hoc test, p*<0.05).

Figure 5. Hypothalamic activation in clock gene deficient mice entrained to food. c-FOS expression in hypothalamic nuclei of mice at ZT4 (during anticipation) and ZT16 (4-h after lights off). Asterisks show significant differences between WT and specific genotypes (*post-hoc test, p<0.05*) at ZT4 or ZT16.

Figure 6. Physiological changes in clock gene deficient mice synchronized by food. (*A*) Corticosterone, (*B*) glucose and (*C*) free fatty acids concentrations, at ZT4 (during anticipation) and ZT16 (4 h after lights off), in animals entrained to 6-h food access (from ZT-6 to ZT12). * *post-hoc test, p*<0.05 day-night differences in each genotype. In A means lacking common letters are significantly different (*post-hoc test, p*<0.05).

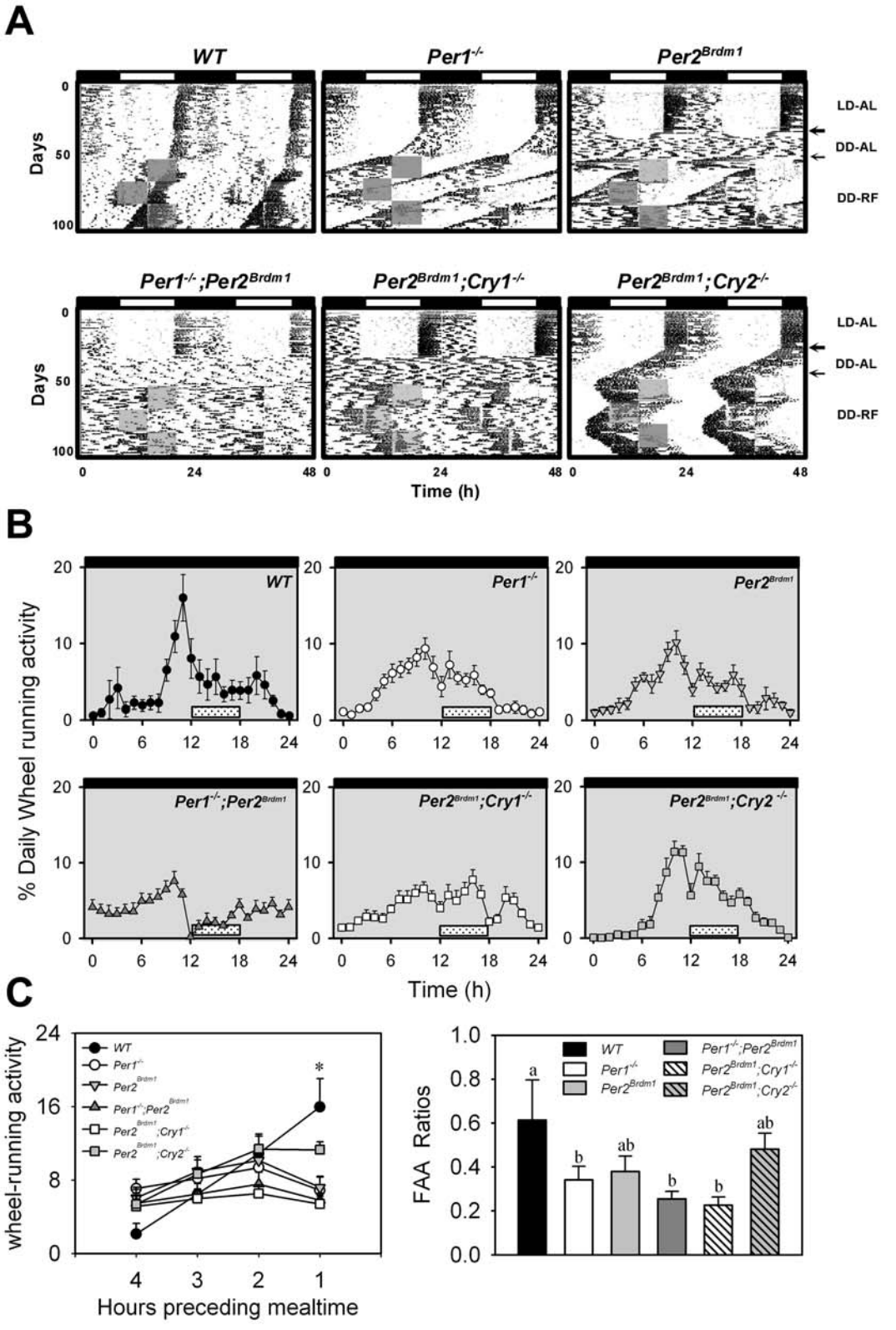
A
WT
Per1-/-
Per2Brdm1
Per1-/-;Per2Brdm1
Per2Brdm1;Cry1-/-
Per2Brdm1;Cry2-/-
Days
LD-AL
DD-AL
DD-RF
Time (h)
B
% Daily Wheel running activity
Time (h)
C
FAA as % of total wheel-running activity
Hours preceding mealtime
FAA Ratios

Figure1

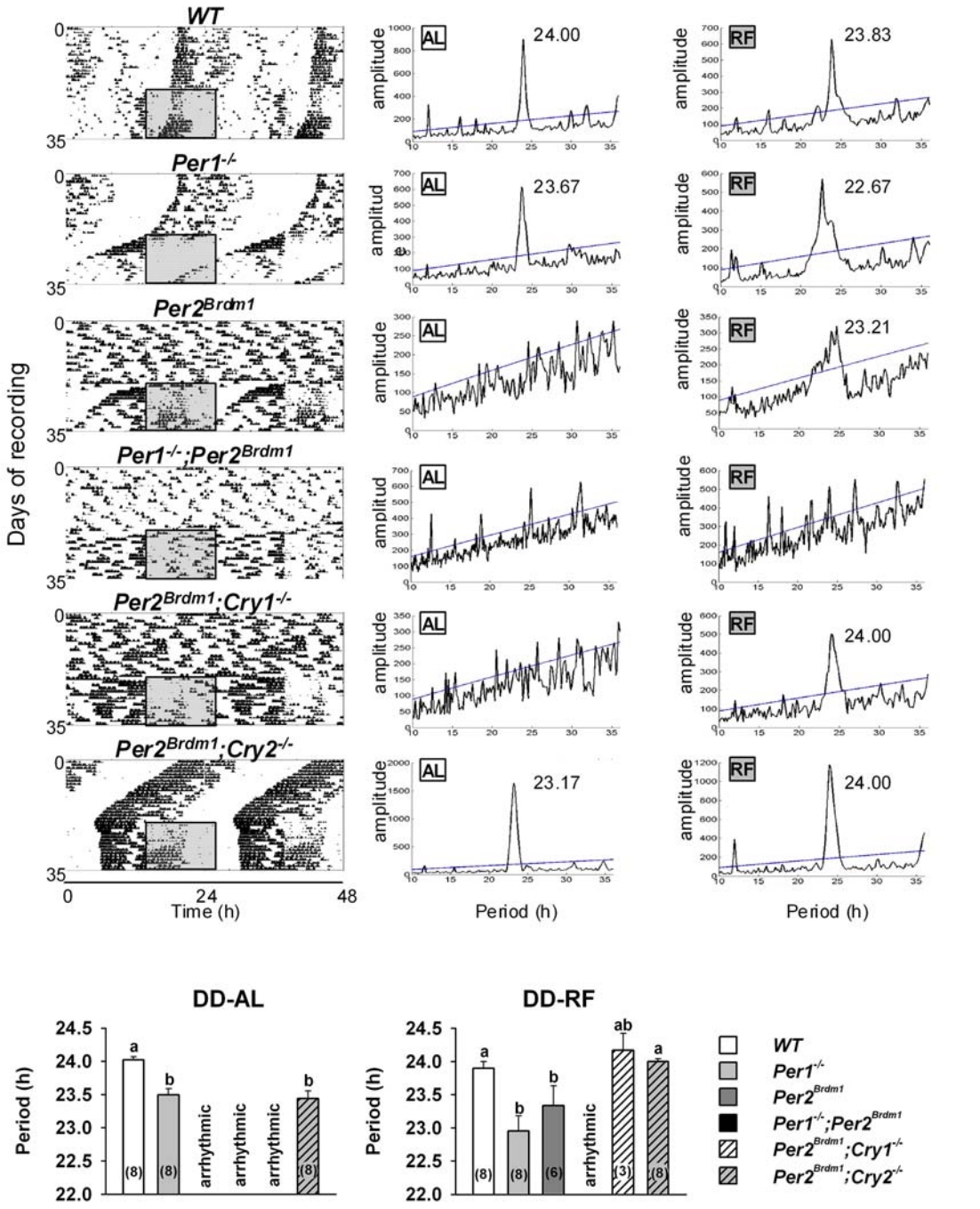
WT
Per1-/-
Per2Brdm1
Per1-/-;Per2Brdm1
Per2Brdm1;Cry1-/-
Per2Brdm1;Cry2-/-
Days of recording
0
35
Time (h)
0
24
48
AL
RF
amplitude
24.00
23.83
23.67
22.67
23.21
23.17
Period (h)
DD-AL
DD-RF
Period (h)
arrhythmic
WT
Per1-/-
Per2Brdm1
Per1-/-;Per2Brdm1
Per2Brdm1;Cry1-/-
Per2Brdm1;Cry2-/-

Figure 2

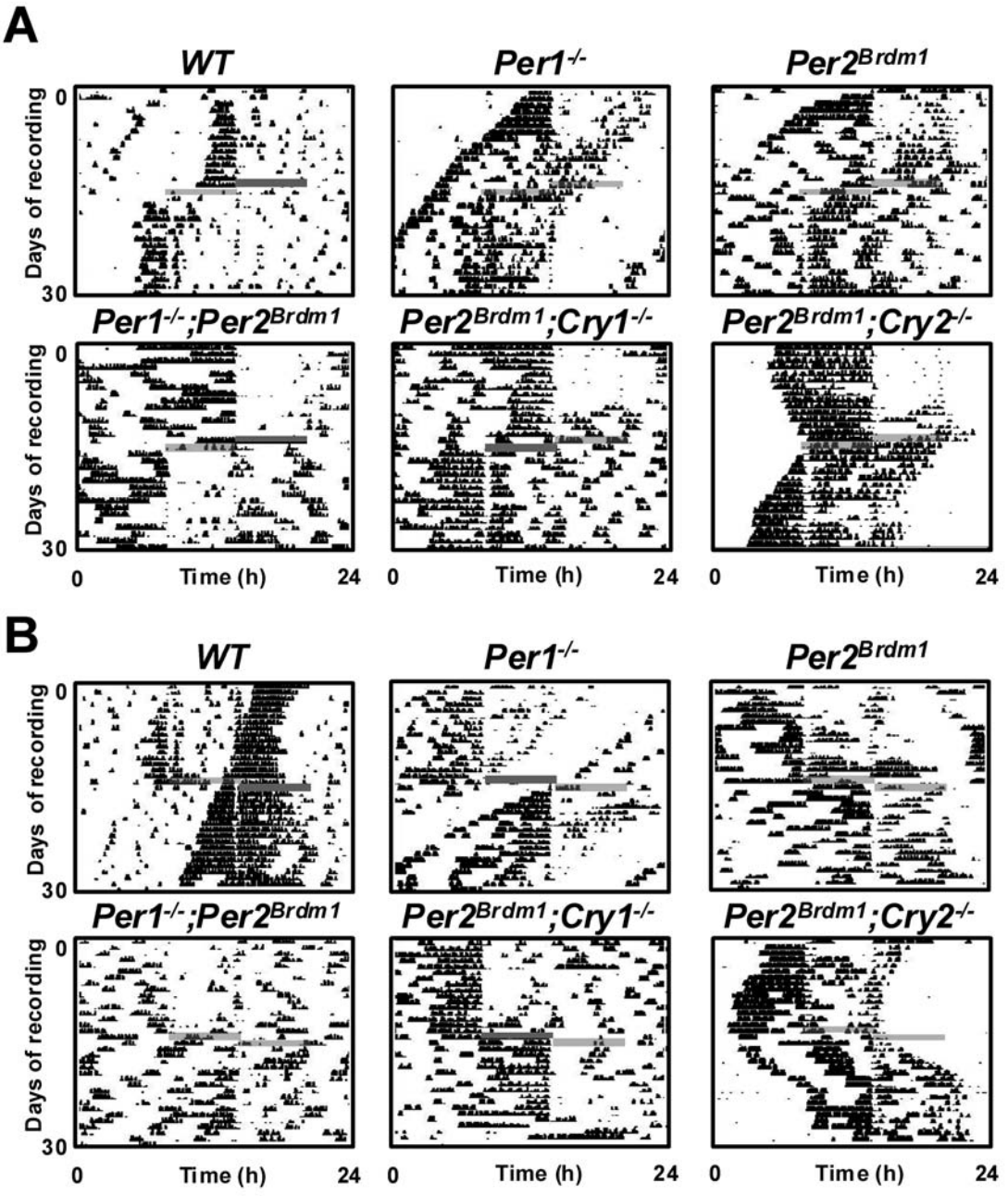
A
WT
Per1-/-
Per2Brdm1
Per1-/-;Per2Brdm1
Per2Brdm1;Cry1-/-
Per2Brdm1;Cry2-/-
Days of recording
0
30
Time (h)
0
24
B
WT
Per1-/-
Per2Brdm1
Per1-/-;Per2Brdm1
Per2Brdm1;Cry1-/-
Per2Brdm1;Cry2-/-
Days of recording
0
30
Time (h)
0
24

Figure 3

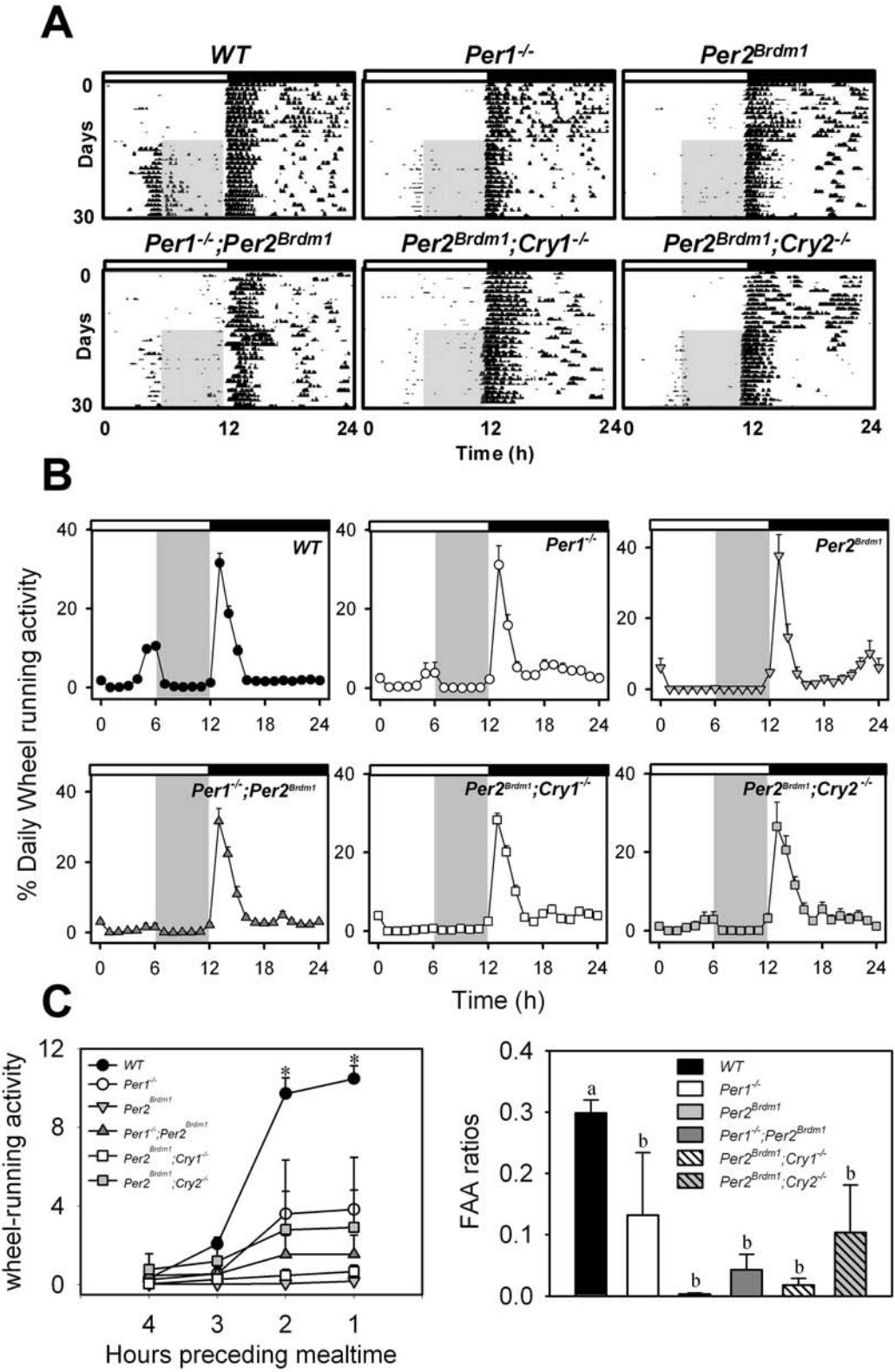
A
WT
Per1-/-
Per2Brdm1
Per1-/-;Per2Brdm1
Per2Brdm1;Cry1-/-
Per2Brdm1;Cry2-/-
Days
0
30
0
12
24
Time (h)
B
% Daily Wheel running activity
Time (h)
C
FAA as % of total wheel-running activity
Hours preceding mealtime
FAA ratios
a
b

Figure 4

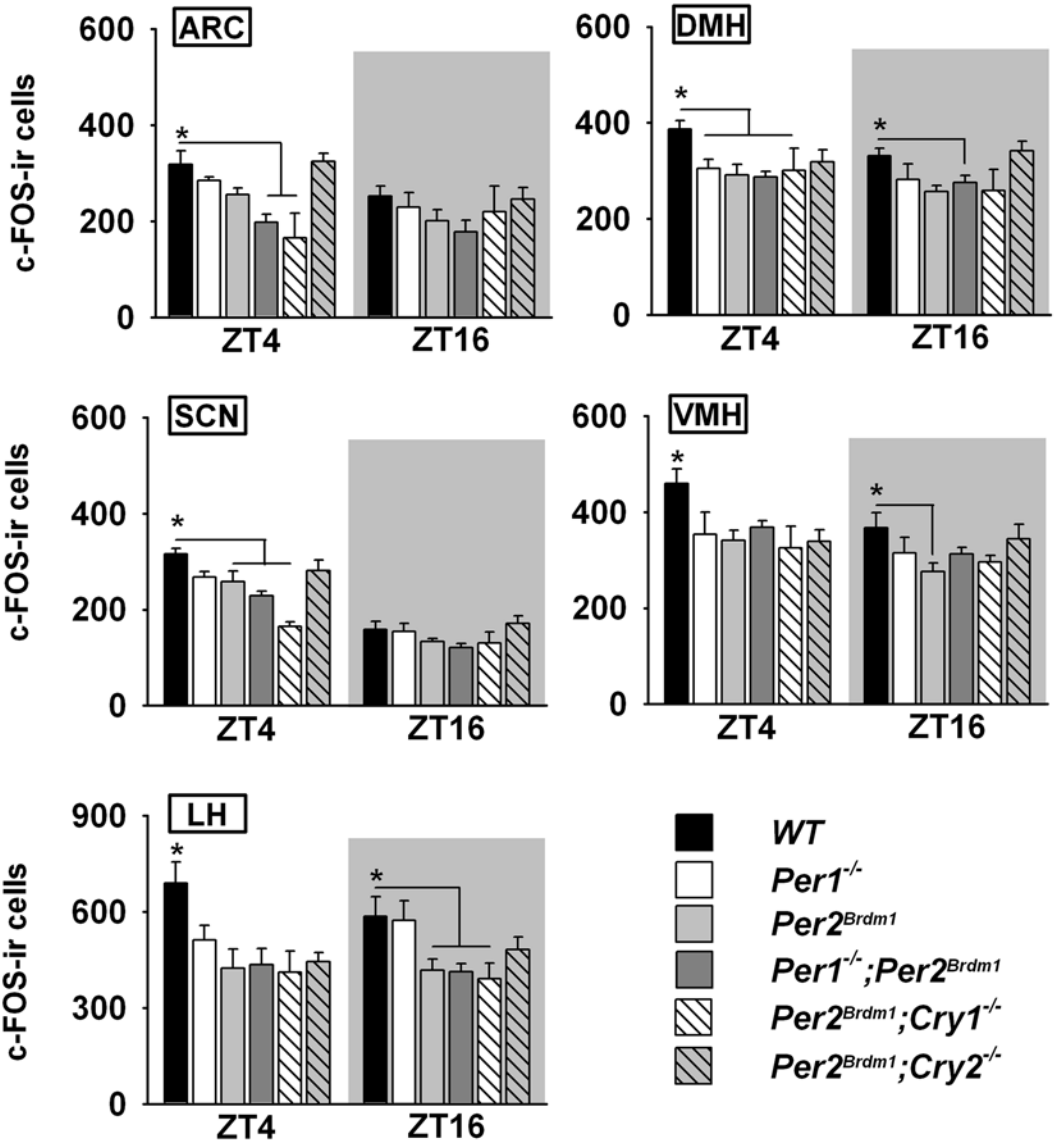
ARC
DMH
SCN
VMH
LH
c-FOS-ir cells
600
400
200
0
900
300
ZT4
ZT16
*
WT
Per1-/-
Per2Brdm1
Per1-/-;Per2Brdm1
Per2Brdm1;Cry1-/-
Per2Brdm1;Cry2-/-

Figure 5

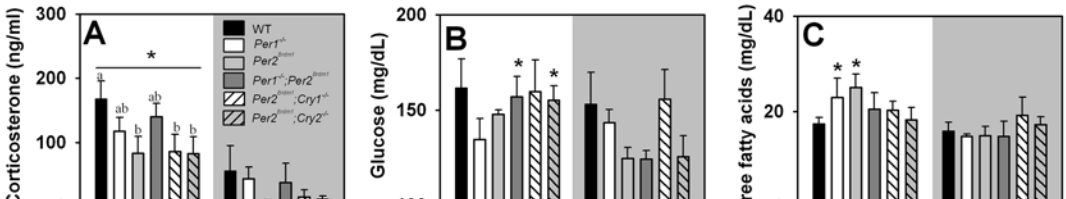
A
B
C
Corticosterone (ng/ml)
Glucose (mg/dL)
Free fatty acids (mg/dL)
300
200
100
0
150
40
20
*
a
ab
b
ab
b
b
WT
Per1-/-
Per2Brdm1
Per1-/-;Per2Brdm1
Per2Brdm1;Cry1-/-
Per2Brdm1;Cry2-/-
ZT-4
ZT-16

Figure 6

Table 1. FAA duration (in minutes) and FAA precision (mean of the standard deviation of FAA duration) in mice under restricted feeding and constant darkness conditions. Values lacking common letters are significant different, *post-hoc test,* $p<0.05$.

Genotype	FAA Duration (min.)	FAA Precision (Std. Dev.)
WT	69.9 ± 4.9[a]	32.6 ± 1.4[a]
***Per1*$^{-/-}$**	85.7 ± 14.3[ab]	73.9 ± 10.4[b]
***Per2*^{Brdm1}**	127.2 ± 25[a]	96.7 ± 14.5[b]
***Per1*$^{-/-}$*;Per2*^{Brdm1}**	65.9 ± 13.5[a]	69.7 ± 13.5[b]
***Per2*^{Brdm1}*;Cry1*$^{-/-}$**	143.7 ± 37[b]	88.0 ± 14.8[b]
***Per2*^{Brdm1}*;Cry2*$^{-/-}$**	131.7 ± 19.4[b]	61.3 ± 7.6[ab]

Table 2. Body weight (BW in grams; g) of mice under food *ad libitum* and restricted feeding (6h food access) in DD and LD conditions. *Different between AL and RF conditions, *post-hoc* $p<0.05$.

Genotype	BW (AL + DD)	BW (RF + DD)	BW (AL + LD)	BW (RF + LD)
WT	26.4±0.4	29.5±0.5*	30.5±0.5	29.1±0.5
***Per1*$^{-/-}$**	24.5±0.4	26.3±0.6	27.1±0.5	27.7±0.5
***Per2*^{Brdm1}**	27.2±0.7	28.5±0.4	30.4±0.4	30.4±0.6
***Per1*$^{-/-}$*;Per2*^{Brdm1}**	23.7±0.8	24.0±0.8	25.2±0.7	27.4±0.5
***Per2*^{Brdm1}*;Cry1*$^{-/-}$**	27.0±0.8	28.3±0.7	30.8±0.7	30.2±0.6
***Per2*^{Brdm1}*;Cry2*$^{-/-}$**	29.4±1.3	29.4±1.2	30.6±1.2	29.9±0.7

Table 3. Food intake (grams; g) of mice under food *ad libitum* (DD and LD) and restricted feeding (RF, 6h food access; DD and LD). Values lacking common letters are significant different, *post-hoc* $p<0.05$.

Genotype	24h Food Intake (*ad-lib*) DD	6h Food Intake (RF) DD	24h Food Intake (*ad-lib*) LD	6h Food Intake (RF) LD
WT	4.5±0.3^{a}	3.6±0.2^{a}	5.6±0.3^{a}	4.2±0.2^{a}
Per1$^{-/-}$	4.6±0.2^{a}	3.8±0.2^{a}	5.5±0.2^{a}	3.2±0.3^{b}
Per2^{Brdm1}	4.7±0.1^{a}	3.7±0.1^{a}	6.8±0.4^{b}	4.5±0.2^{a}
Per1$^{-/-}$;Per2^{Brdm1}	4.3±0.2^{a}	3.5±0.1^{a}	5.8±0.4^{ab}	3.5±0.4^{b}
Per2^{Brdm1};Cry1$^{-/-}$	5.0±0.2^{a}	4.1±0.03^{b}	8.1±0.6^{c}	4.6±0.2^{a}
Per2^{Brdm1};Cry2$^{-/-}$	4.9±0.1^{a}	4.1±0.09^{b}	6.8±0.2^{b}	4.3±0.2^{a}