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

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Keywords: Circadian, clock genes, food-anticipatory activity, hypothalamus, suprachiasmatic

Received 5 November 2009, revised 16 January 2010, 27 January 2010, 29 January 2010, accepted for publication 1 February 2010

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). Although 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 (Davidson 2009; Mendoza 2007; Mistlberger 2009). The molecular mechanisms underlying circadian rhythmicity in the SCN depend 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). Although 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−/−), 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 Per2Brdm1 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 Per1d/c−;Per2d/c− mutant animals show FAA similar to wild-type (WT) mice (Storch & Weitz 2009). On the other hand, although a study reported the 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 light–dark (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, because behavioural rhythmicity in Per2Brdm1 mutant mice is rescued by the mutation of the clock gene.
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Cry2, but not Cry1 in DD (Oster et al. 2002), we evaluated whether FAA is rescued as well in double Per2\textsuperscript{Brdm1},Cry2\textsuperscript{−/−}.

Materials and methods

Animals and housing

Adult female F\textsubscript{1} homozygous Per1 and Per2 mutant mice and their WT littermates were used for the whole experiment. Intercrosses between heterozygous (C57BL/6\times 129S5/SvEvBrd) F\textsubscript{1} offspring gave rise to F\textsubscript{2} homozygous Per1 and Per2 mutant animals. Therefore, mutant and WT animals on this mixed background were used in this study. WT animals were littermates of backcrosses of F\textsubscript{1}, Per1 or F\textsubscript{1}, Per2 heterozygous animals. In previous experiments using Per1\textsuperscript{−/−} and Per2\textsuperscript{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\textsuperscript{−/−}) results from the deletion of exon 3 to exon 19 of mPer1. The deleted region includes the PAS domain, thus precluding protein dimerization. The Per2 mutation (Per2\textsuperscript{Brdm1}) was generated by deleting part of the PAS domain, thus impairing normal protein dimerization. The Per1\textsuperscript{−/−} and Per2\textsuperscript{Brdm1} mutations are described in detail in Zheng et al. (2001) and Zheng et al. (1999), respectively.

Cry1\textsuperscript{−/−} and Cry2\textsuperscript{−/−} animals were generated in a hybrid C57BL/6\times 129S2/OlaHsd background (van der Horst et al. 1999). For double mutant mice, Per2\textsuperscript{Brdm1} mice were crossed with Per1\textsuperscript{−/−}, Cry1\textsuperscript{−/−} or Cry2\textsuperscript{−/−} animals as previously reported (Oster et al. 2002). The double-heterozygous offspring was intercrossed to produce WT and homozygous mutant animals. Matching WT control animals thus precluding protein dimerization. The Per2 mutation (Per2\textsuperscript{Brdm1}) was generated by deleting part of the PAS domain, thus impairing normal protein dimerization. The Per1\textsuperscript{−/−} and Per2\textsuperscript{Brdm1} mutations are described in detail in Zheng et al. (2001) and Zheng et al. (1999), respectively.

Food entrainment in DD and mealtime jet-lag test

In a first experiment, after at least 2 weeks under a LD cycle, animals (n = 8 per genotype) were released in constant darkness (DD) for at least 2 weeks before food entraining conditions. Animals were then entrained to a temporal food restriction with 6 h of food access starting at 12 h (geographical time) during 2 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 2 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 (RF) schedules at least for 2 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% ethylene diamine tetraacetic acid (EDTA) for hormonal and metabolic assays.

Circadian behaviour analysis

Wheel revolutions were recorded in 5 min time bins by CAMS (Circadian Activity Motor System, Lyon, France) acquisition program and transferred to the ClockLab analysis software (ClockLab Version 2.61; Actimetrics, Evanston, IL, USA) to produce double-plotted actograms and periodograms. χ\textsuperscript{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 24 h. Differences in the intensity of FAA between groups were evaluated in activity occurring during the 4 h 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 10 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 baseline; Figure 2A). To determine whether FAA is stable, we evaluated FAA precision [standard deviation (SD) 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 from 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, because animals are in free-running conditions and circadian phases are differences between them, FAA ratios were obtained from the FAA (3 h prior to mealtime) counts as a percent of the total daily wheel-running activity.

Immunohistochemistry

Brains were fixed overnight in 4% paraformaldehyde in buffer phosphate (PB, 0.1 M), 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 3 days. Brains were sectioned (four series; 30 μm) on a cryostat and stored in phosphate buffer saline with sodium azide at 4°C. Sections were incubated for 24 h at 4°C in anti-FOS rabbit polyclonal antisera (Santa Cruz, CA, USA; 1:10 000 dilution), 10% donkey serum (Millipore, Road, MA, USA), 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 h at room temperature, and incubated with peroxidase-conjugated avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA, USA) for 1 h, followed by 0.05% diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.01% H\textsubscript{2}O\textsubscript{2}.

Cell count

Sections were examined under a light microscope (Leica DMRB; Leica Microsystems, Rueil-Malmaison, France) and images captured using a digital camera (Olympus DP50; Olympus France, Rungis, France) on a PC computer. Based on the mouse brain stereotaxic atlas (Paxinos & Franklin 2001), digital images were taken at the level of the SCN (−0.46 mm from Bregma), the lateral hypothalamus (LH, −1.22 mm from Bregma), the ventromedial (VMH) and dorsomedial (DMH) hypothalamic nuclei. The Hoechst stain was used to label nuclei. Images showing the highest number of labelled nuclei.

Hormonal and metabolic assays

Plasma corticosterone was assayed with a commercial 125I RIA kit for mice and rats (ImmuChem Double Antibody, MP Biomedicals,
Food entrainment in clock genes mutant mice

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 LD 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 (\(\tau\)) compared with WT animals (Fig. 2; statistical analysis).

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 least significant difference 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 ± SEM.

Figure 1: Food synchronisation in clock genes deficient mice under constant darkness conditions. (a) Representative actograms of WT and mutant mice under constant darkness conditions and 6-h RF schedules (grey box). After at least 2 weeks under the first RF schedule, animals were exposed to a 6-h phase-advance of food access, and secondly to a 6-h phase-delay of food access (grey boxes). (b) Activity profiles of mice during RF. Activity waveforms represent the average of 10 days during food restriction. Horizontal bar indicates the time of food access. (c) Percent of FAA over total daily activity from 4 to 1 h 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 significantly different, post hoc test, \(P < 0.05\). LD-AL, LD cycle and food ad libitum conditions; DD-AL, constant darkness and food ad libitum conditions; DD-RF, constant darkness and food restriction conditions.

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Orangeburg, NY, USA). The limit of sensitivity of corticosterone assay was 7.7 ng/ml. Blood was centrifuged at 2404 \(g\) 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 BIOLOABO, REF: LP80009) and free fatty acids (method ACS-ACOD; Kit NEFA–HR2 Wako).
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 RF 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 significantly different (post hoc test, $P < 0.05$). Numbers in parenthesis indicate the number of animals per group.
Food entrainment in clock genes mutant mice

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,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,b). In both Per2Brdm1 and double Per1−/−;Per2Brdm1 mutant mice, food-anticipatory activity (FAA) was absent and no clear transition cycles were evident after shifts of the mealtime.

In double Per2Brdm1;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,b).

Finally, in double Per2Brdm1;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 LD cycle (Fig. 3a,b).

Food entrainment in clock gene deficient mice under a LD cycle

Under a 12–12 h LD cycle, although WT mice exhibited a clear and stable FAA to 6-h of food access, other genotypes such as Per1−/− and double Per1−/−;Per2Brdm1 and Per2Brdm1;Cry2−/− mutant animals showed FAA significantly lower than that expressed by control WT. Per2Brdm1 and double Per2Brdm1;Cry1−/− mutant animals did not exhibit FAA at all (Fig. 4a–c). The ANOVA indicated significant differences for the factor genotype (F3,42 = 6.1, P = 0.0002) for the factor time (F3,126 = 31.5, P < 0.01) and the genotype × time interaction (Fig. 1c; F15,126 = 8.4, P < 0.01). The post hoc analysis showed that FAA was significantly higher 2 and 1 h before mealtime in WT animals compared with 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; F3,42 = 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 Per2Brdm1;Cry1−/−, compared with WT control animals (Fig. 4; F3,42 = 2.9, P = 0.02).

Table 1: FAA duration (min) and FAA precision (mean of the SD of FAA duration) in mice under RF and constant darkness conditions

<table>
<thead>
<tr>
<th>Genotype</th>
<th>FAA duration (min)</th>
<th>FAA precision (SD)</th>
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<tbody>
<tr>
<td>WT</td>
<td>69.9 ± 4.9a</td>
<td>32.6 ± 1.4a</td>
</tr>
<tr>
<td>Per1−/−</td>
<td>85.7 ± 14.3ab</td>
<td>73.9 ± 10.4b</td>
</tr>
<tr>
<td>Per2Brdm1</td>
<td>127.2 ± 25a</td>
<td>96.7 ± 14.5b</td>
</tr>
<tr>
<td>Per1−/−;Per2Brdm1</td>
<td>65.9 ± 13.5a</td>
<td>69.7 ± 13.5b</td>
</tr>
<tr>
<td>Per2Brdm1;Cry1−/−</td>
<td>143.7 ± 37b</td>
<td>88.0 ± 14.8b</td>
</tr>
<tr>
<td>Per2Brdm1;Cry2−/−</td>
<td>131.7 ± 19.4ab</td>
<td>61.3 ± 7.6ab</td>
</tr>
</tbody>
</table>

Values lacking common letters are significantly different, post hoc test, P < 0.05. SD, standard deviation.

F2,21 = 10.9, P = 0.0005. Per2Brdm1. Per1−/−;Per2Brdm1, Per2Brdm1;Cry1−/− mutant mice, however, were totally arrhythmic immediately after being released in DD conditions (Figs 1 and 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 (Figs 1a,b and 2).

When we compared FAA intensity from 4 h before mealtime, ANOVA showed significant differences for the factor genotype (Fig. 1c; F3,126 = 18.5, P < 0.01) but not for the factor genotype (Fig. 1c; F3,42 = 1.6, P = 0.2). However, the genotype × time interaction was statistically different (Fig. 1c; F15,126 = 5.4, P < 0.01), indicating that FAA was significantly higher in WT animals than in mutant mice 1 h 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; F5,42 = 2.6, P = 0.04). FAA duration over 10 days of food restriction was significantly different between genotypes, with greater FAA duration in single Per2Brdm1 and double Per2Brdm1;Cry1−/− and Per2Brdm1;Cry2−/− mutant mice (Table 1; F5,42 = 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 SD of FAA duration in WT animals compared with mutant mice (Table 1; F5,42 = 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 no two activity components as in WT mice (Figs 1a,b and 2). Per1−/− mutant mice continued to free-run with an endogenous period that was significantly shortened by RF (t14 = 2.24, P = 0.04). Interestingly, daily RF in Per2Brdm1, but not in double Per1−/−;Per2Brdm1 mutant mice, induced a free-running period shorter than in WT animals (Figs 1a and 2; F4,28 = 6.5, P = 0.0007).

In some double Per2Brdm1;Cry1−/− mutant mice, there was a significant period of 24 h induced by RF which was not different from the period of WT animals (Fig. 2; F4,28 = 6.5, P = 0.0007). Interestingly, in rhythmic double Per2Brdm1;Cry2−/− mutant mice, feeding schedules had the ability to entrain the whole circadian behaviour with a significant period of 24 h compared with the free-running period under free-feeding conditions (Figs 1a and 2; t14 = 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,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,b). In both Per2Brdm1 and double Per1−/−;Per2Brdm1 mutant mice, food-anticipatory activity (FAA) was absent and no clear transition cycles were evident after shifts of the mealtime.

In double Per2Brdm1;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,b).

Finally, in double Per2Brdm1;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 LD cycle (Fig. 3a,b).
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 with 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 with 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 with 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 with 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 (BW) only in WT mice. For mutant mice, no significant change was detected during food restriction compared with 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 BW changes in response to RF were similar between mutant and WT mice. Twenty-four-hour 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 6 h of food access, double Per2\(^{Brdm1}\);Cry1\(^{-/-}\) and Per2\(^{Brdm1}\);Cry2\(^{-/-}\) showed a significant increase in the amount of food intake compared with 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
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 LD cycle and 6-h RF schedules (shaded grey boxes, actograms; grey bars, activity profiles). (c) Percent of food-anticipatory activity (FAA) over total daily activity from 4 to 1 h 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, \( P < 0.05 \)).

were higher at ZT4 (2 h 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 with 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 ZT (Fig. 6; \( F_{1,39} = 9.2, P = 0.004 \)). These data suggest that although 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 \( \text{Per2}^{Brdm1} \) 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 RF schedules.

Under LD, LL and DD conditions, FAA is absent in Per2Brdm1 mutant mice (Feillet et al. 2006; present study). Although Per2Brdm1 mutant mice fed ad libitum are totally arrhythmic in DD (Zheng 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 RF.

Per2Brdm1;Cry1−/− mice lose circadian rhythmicity immediately upon release into DD (Oster et al. 2002). Here, some of Per2Brdm1;Cry1−/− mutant mice show a reorganization of
Food entrainment in clock genes mutant mice

Table 2: BW (g) of mice under food ad libitum (AL) and RF (6 h food access) in DD and LD conditions

<table>
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<tr>
<th>Genotype</th>
<th>BW (AL + DD)</th>
<th>BW (RF + DD)</th>
<th>BW (AL + LD)</th>
<th>BW (RF + LD)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>26.4 ± 0.4</td>
<td>29.5 ± 0.5*</td>
<td>30.5 ± 0.5</td>
<td>29.1 ± 0.5</td>
</tr>
<tr>
<td>Per1−/−</td>
<td>24.5 ± 0.4</td>
<td>26.3 ± 0.6</td>
<td>27.1 ± 0.5</td>
<td>27.7 ± 0.5</td>
</tr>
<tr>
<td>Per2Brdm1</td>
<td>27.2 ± 0.7</td>
<td>28.5 ± 0.4</td>
<td>30.4 ± 0.4</td>
<td>30.4 ± 0.6</td>
</tr>
<tr>
<td>Per1−/−,Per2Brdm1</td>
<td>23.7 ± 0.8</td>
<td>24.0 ± 0.8</td>
<td>25.2 ± 0.7</td>
<td>27.4 ± 0.5</td>
</tr>
<tr>
<td>Per2Brdm1,Cry1−/−</td>
<td>27.0 ± 0.8</td>
<td>28.3 ± 0.7</td>
<td>30.8 ± 0.7</td>
<td>30.2 ± 0.6</td>
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<tr>
<td>Per2Brdm1,Cry2−/−</td>
<td>29.4 ± 1.3</td>
<td>29.4 ± 1.2</td>
<td>30.6 ± 1.2</td>
<td>29.9 ± 0.7</td>
</tr>
</tbody>
</table>

*Different between AL and RF conditions, post hoc P < 0.05.

Table 3: Food intake (g) of mice under food ad libitum (DD and LD) and RF (6 h food access; DD and LD)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>24 h Food intake (ad lib) DD</th>
<th>6 h Food intake (RF) DD</th>
<th>24 h Food intake (ad lib) LD</th>
<th>6 h Food intake (RF) LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.5 ± 0.3*</td>
<td>3.6 ± 0.2a</td>
<td>5.6 ± 0.3a</td>
<td>4.2 ± 0.2a</td>
</tr>
<tr>
<td>Per1−/−</td>
<td>4.6 ± 0.2a</td>
<td>3.8 ± 0.2a</td>
<td>5.5 ± 0.2a</td>
<td>3.2 ± 0.3b</td>
</tr>
<tr>
<td>Per2Brdm1</td>
<td>4.7 ± 0.1a</td>
<td>3.7 ± 0.1a</td>
<td>6.8 ± 0.4b</td>
<td>4.5 ± 0.2a</td>
</tr>
<tr>
<td>Per1−/−,Per2Brdm1</td>
<td>4.3 ± 0.2a</td>
<td>3.5 ± 0.1a</td>
<td>5.8 ± 0.4ab</td>
<td>3.5 ± 0.4b</td>
</tr>
<tr>
<td>Per2Brdm1,Cry1−/−</td>
<td>5.0 ± 0.2a</td>
<td>4.1 ± 0.03b</td>
<td>8.1 ± 0.6c</td>
<td>4.6 ± 0.2a</td>
</tr>
<tr>
<td>Per2Brdm1,Cry2−/−</td>
<td>4.9 ± 0.1a</td>
<td>4.1 ± 0.09b</td>
<td>6.8 ± 0.2b</td>
<td>4.3 ± 0.2a</td>
</tr>
</tbody>
</table>

Values lacking common letters are significantly different, post hoc P < 0.05.

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 variable each day in these mutants compared with WT animals.

Per2Brdm1,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 Per2Brdm1,Cry2−/− mice is very sensitive to feeding/metabolic cues. Per2Brdm1,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 Per2Brdm1,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, although WT mice show a strong FAA, only weak FAA is observed in Per1−/− and double Per2Brdm1,Cry2−/− mutant mice. Contrary to our original observations showing comparable FAA in young WT and Per1−/− mice under LD (Feillet et al. 2008), here we noted a marked reduction of FAA in the same, but older (middle-age)
mutants challenged with RF, 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 Per2Brdm1 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 Per2Brdm1 mutant animals. Albeit present, FAA in Per2Brdm1;Cry2−/− mutant mice, however, is weaker than in WT animals. In DD, Per2Brdm1;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−/−;Per2Brdm1, FAA is weak and unstable. A recent paper has shown apparently normal FAA in double Per1−/−;Per2Brdm1 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 show 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 Per2Brdm1 and Per2Brdm1;Cry1−/− mutant mice under a LD cycle, FAA is absent, confirming previous results with the same Per2Brdm1 mutant animals (Feillet et al. 2006). Single mutations of one of these genes, Per2 (Feillet et al. 2006) and/or Cry1 (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 24 h) in the circadian range (Stephan 1981) would also be useful in that respect.

c-FOS expression in 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 with 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.

LN hypocretins, containing hypocretins, become activated in food-restricted animals 1 h before food presentation, and hypocretin 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 with 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 Per2Brdm1 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, because the mutation of Cry2 does not rescue FAA in Per2Brdm1 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.

References


**Acknowledgments**

The authors thank Stéphanie Dumont for her excellent help for hormonal and metabolic assays and Dr Dominique Sage for her support and help with the behavioural setup recording. This work was supported by ANR-07-JCJC-0111 (J.M. and E.C.), CNRS (J.M. and E.C.) and SNF grants (U.A.).