

Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators

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The circadian timing system in mammals is composed of a master pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus and slave clocks in most peripheral cell types. The phase of peripheral clocks can be completely uncoupled from the SCN pacemaker by restricted feeding. Thus, feeding time, while not affecting the phase of the SCN pacemaker, is a dominant *Zeitgeber* for peripheral circadian oscillators. Here we show that the phase resetting in peripheral clocks of nocturnal mice is slow when feeding time is changed from night to day and rapid when switched back from day to night. Unexpectedly, the inertia in daytime feeding-induced phase resetting of circadian gene expression in liver and kidney is not an intrinsic property of peripheral oscillators, but is caused by glucocorticoid signaling. Thus, glucocorticoid hormones inhibit the uncoupling of peripheral and central circadian oscillators by altered feeding time.

Keywords: adrenalectomy/circadian gene expression/clock/corticosterone/GR knockout

Introduction

In mammals, most physiology and behavior is subject to daily oscillations that are driven by an endogenous circadian timing system. Current evidence suggests that this system has a hierarchical structure: a master pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus synchronizes slave oscillators in most tissues (for reviews see Brown and Schibler, 1999; Reppert and Weaver, 2001; Ripperger and Schibler, 2001). As indicated by its name, the circadian clock can measure time only approximately and therefore has to be reset every day by external time cues (*Zeitgebers*). Light,

the major *Zeitgeber* for the central pacemaker, entrains the phase of oscillations in SCN neurons via the retinohypothalamic tract, and glutamate appears to be the major neurotransmitter in this process (Ding *et al.*, 1994; Shirakawa and Moore, 1994; Ebling, 1996). Light pulses delivered to animals kept in constant darkness during the subjective night not only elicit phase shifts, but also induce a surge of immediate early gene expression in SCN neurons. The products of these immediate early genes include the circadian clock components PER1 and PER2, and several transcription factors, such as c-Fos, Jun-B and EGR3 (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997; Morris *et al.*, 1998). It is likely that some of these regulatory proteins participate in the light-induced phase shifting.

Seven genes essential for mammalian circadian clock function have been identified. These include the two period isoforms *mPer1* and *mPer2* (Shearman *et al.*, 1997; Sun *et al.*, 1997; Tei *et al.*, 1997; Zheng *et al.*, 1999), the two cryptochrome isoforms *mCry1* and *mCry2* (Kume *et al.*, 1999; van der Horst *et al.*, 1999), two genes, *Clock* and *Bmal1*, encoding PAS helix–loop–helix transcription factors (King *et al.*, 1997; Bunger *et al.*, 2000) and *Tau*, encoding casein kinase 1 ϵ (Lowrey *et al.*, 2000). The products of these genes have been assembled into a model of a molecular oscillator, which is based on interlocked feedback loops in gene expression (Shearman *et al.*, 2000). In this model, the two cryptochromes repress transcription of *mCry* and *mPer* genes via interference with the two PAS helix–loop–helix transcription factors *Clock* and *Bmal1*. The cyclic accumulation of *mPER2* then acts to generate a rhythmic expression of *Bmal1*, whose oscillation may reinforce the negative feedback loop of *mCry* expression. Post-transcriptional regulation also appears to play an important role in rhythm generation (Keesler *et al.*, 2000; Lowrey *et al.*, 2000; Toh *et al.*, 2001). Clock-controlled genes such as *Arginine Vasopressin* or *Dbp*, whose products display cyclic accumulation but are not required for clock function, can be regulated by the same interlocked feedback loops of gene expression that drive the rhythmic expression of bona fide pacemaker genes (Jin *et al.*, 1999; Ripperger *et al.*, 2000).

Circadian gene expression of clock and clock-controlled genes is not only observed in the SCN, but also in most peripheral tissues. Moreover, circadian gene expression persists in organ explants for several consecutive days and can be induced in immortalized rat and mouse tissue culture cells (Balsalobre *et al.*, 1998; Yamazaki *et al.*, 2000; Yagita *et al.*, 2001). Hence, it appears that peripheral cells contain bona fide circadian timekeepers that can generate circadian oscillations during several consecutive days. The phase angle relationship between different mRNA accumulation cycles is very similar in SCN neurons and peripheral cell types, and mutations in

essential clock genes affect these oscillators in a similar way. Therefore, central and peripheral oscillators are likely to function according to similar molecular principles (Balsalobre *et al.*, 1998; Yagita *et al.*, 2001).

In spite of their resemblance in molecular make-up, central and peripheral circadian clocks differ from each other in that only the former are self-sustained. Hence, it appears that the SCN must periodically re-entrain peripheral oscillators in order to prevent the dampening of circadian gene expression in organs such as liver, kidney, heart and pancreas (Yamazaki *et al.*, 2000). The mechanisms by which the SCN accomplishes this task are not yet understood, but they are likely to be complex. In fact, ligands of the glucocorticoid receptor (Balsalobre *et al.*, 2000a) and two other nuclear receptors, RAR α and RXR α (McNamara *et al.*, 2001), calcium ionophores and chemicals that activate either protein kinase A, protein kinase C and/or MAP kinases (Akashi and Nishida, 2000; Balsalobre *et al.*, 2000b; Yagita and Okamura, 2000) can all trigger circadian gene expression in tissue culture cells. Moreover, some of these signals also provoke phase shifts in peripheral tissues of intact animals (e.g. Balsalobre *et al.*, 2000a; McNamara *et al.*, 2001). It is thus likely that multiple signaling pathways are used for phase resetting of circadian peripheral oscillators. Balsalobre *et al.* (2000a) have recently demonstrated that dexamethasone, a glucocorticoid receptor agonist, acts as a strong phase-shifting agent for peripheral oscillators in the mouse. However, since the phase of oscillators is the same in hepatocytes that do or do not express glucocorticoid receptors, glucocorticoid hormones cannot be the only *Zeitgeber* signal for peripheral clocks.

Feeding time has recently been shown to be a dominant *Zeitgeber* for rhythmic gene expression in tissues such as liver, pancreas, kidney and heart (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). Thus, if nocturnal animals like mice or rats are fed exclusively during the day for extended periods, the phase of circadian gene expression in several examined organs becomes completely inverted. Interestingly, however, restricted feeding has no influence on the phase of rhythmic gene expression in the SCN (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). These observations raise the possibility that the SCN entrains peripheral clocks indirectly by imposing activity and resting phases, and thus feeding behavior. According to this model, food metabolites or signals elicited by feeding behavior or food processing would be the principal timing cues for peripheral oscillators.

Upon switching nocturnal animals to daytime feeding, it takes several days until the reversed phase is established (Damiola *et al.*, 2000). This relatively slow phase adaptation is reminiscent of the time-consuming photic entrainment of the SCN master clock, which manifests itself in jet lag after long-distance east- or westbound trips. Here we demonstrate that the sluggish phase resetting of peripheral clocks by altered feeding time is not caused by an intrinsic inertia of circadian oscillators to adapt to the new *Zeitgeber* time, but by active signaling pathways that counteract rapid phase resetting. Thus, in the absence of glucocorticoid hormones or the glucocorticoid hormone receptor, feeding time can induce large phase shifts in peripheral tissues such as the liver, thereby inverting the phase of their oscillators within a few days.

Results

Daytime feeding phase-shifts peripheral oscillators faster in adrenalectomized compared with sham-operated mice

As shown previously, food is a dominant *Zeitgeber* for circadian oscillators in several mouse tissues, including liver, pancreas, kidney and heart. Mice, which are nocturnal animals, ingest ~80% of their daily food intake during the night (N.Preitner, unpublished results). As a consequence, restricting feeding time to the dark period does not significantly alter the phase angle of cyclic gene expression (Damiola *et al.*, 2000). However, when food is offered only during the light phase during several consecutive days, the phase of circadian gene expression of clock and clock-controlled genes becomes completely inverted after 7–10 days (Damiola *et al.*, 2000). Among several tissues examined, this phase resetting occurred most rapidly in the liver. We wished to determine whether glucocorticoid hormones, which efficiently induce circadian gene expression in cultured cells and elicit potent phase shifts in peripheral tissues of intact animals, participate in the food-induced resetting of peripheral oscillators. To this end, the kinetics of phase changes in the rhythmic expression of the clock genes *mPer1*, *mPer2* and *mCry1*, and the clock-controlled gene *Dbp*, were compared in livers and kidneys of normal (sham-operated) and adrenalectomized mice. As shown in Figure 1A and B, the accumulation profiles of the mRNA encoded by these genes are strikingly different during the second day after initiation of daytime feeding. In sham-operated animals (right panels), the peak and trough levels of some mRNAs are offset by only ~4 h when compared with mice fed *ad libitum* or during the night (Figure 2A and C). For example, in control animals, *Dbp* mRNA, whose circadian transcription is regulated directly by components of the molecular oscillator (Ripperger *et al.*, 2000), reaches peak and trough levels in liver and kidney at about *Zeitgeber* times ZT14 and ZT2, respectively, during the second day of daytime feeding (ZT0 = time when the lights are switched on). However, much larger phase shifts are observed in tissues of adrenalectomized mice. Thus, during the second day after restricted feeding, *Dbp* mRNA peak levels are observed in liver between ZT0 and ZT4, which is significantly closer to the time at which daytime feeding-induced phase resetting is completed (Figure 2B and Damiola *et al.*, 2000).

In kidney, phase reversal by daytime feeding takes somewhat longer than in liver (Damiola *et al.*, 2000). Thus, as we have observed previously for the transition from *ad libitum* to daytime feeding, the expression of some genes (e.g. *Dbp*, *mPer2* and *mCry1*) is not yet rhythmic in kidney during the second day of daytime feeding. Nevertheless, *mPer1* mRNA, which in kidney is the sole examined transcript with a clearly cyclic accumulation during the second day of restricted feeding, already follows a profile close to that observed after complete phase reversal.

In contrast to the observations made in liver and kidney, daytime feeding does not affect circadian gene expression in the SCN (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). As shown by the *in situ* hybridization experiments presented in Figure 1C, this holds true for rhythmic *mPer1* and

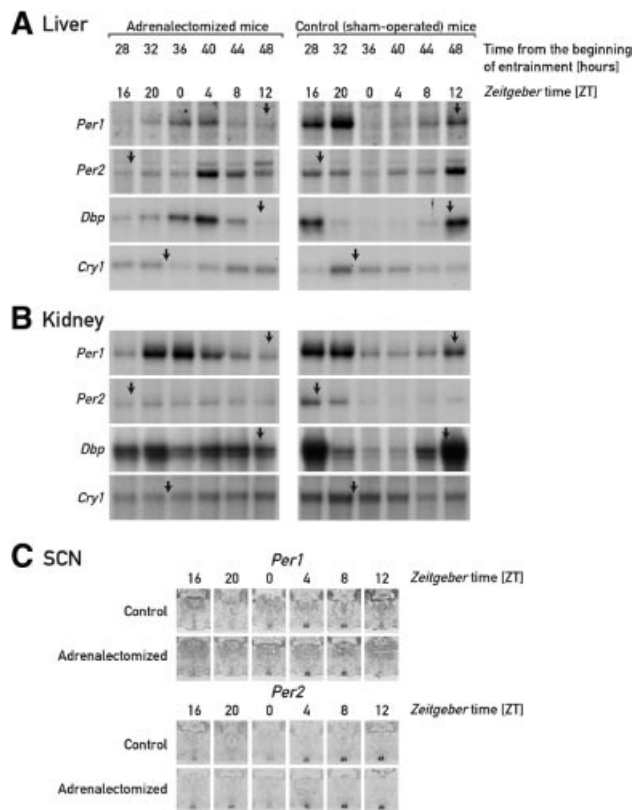


Fig. 1. Accumulation of circadian transcripts in adrenalized and sham-operated mice at the onset of daytime feeding. Adrenalized mice and sham-operated mice, kept under a 12 h light–dark regimen (lights on ZT0), were switched from *ad libitum* to daytime feeding. On day D, food was removed at ZT12 and provided exclusively during the light phase of the following days. During the second day (D+1) of restricted feeding, animals were killed at 4 h intervals and the livers, kidneys and brains were collected for monitoring the accumulation of mRNAs specified by the three clock genes *mPer1*, *mPer2* and *mCry1*, and the clock output gene *Dbp*. Relative transcript levels in liver (A) and kidney (B) were determined by RNase protection assays using radiolabeled antisense RNA probes. *Tbp* (Tatobox-binding protein) mRNAs served as a control for a transcript whose accumulation does not oscillate during the day (not shown). The approximate times at which peak levels of mRNA accumulation are observed in animals fed *ad libitum* or during the night are ZT12 (*mPer1*), ZT17 (*mPer2*), ZT11 (*Dbp*) and ZT22 (*mCry1*) (see Damiola *et al.*, 2000 and Figure 2C). These times are indicated by arrows on top of the respective panels. The accumulation of *mPer1* and *mPer2* mRNA in the SCN was assessed by *in situ* hybridization to coronal brain sections, using ³⁵S-labeled antisense RNA probes (C). Only the hypothalamus regions containing the SCN (small pairwise structures at the base of the hypothalamus) are depicted. At ZT20, the signal obtained for *mPer2* mRNA is somewhat higher in the adrenalized animal compared with sham-operated animals, but this difference does not significantly change the phase of *mPer2* mRNA accumulation.

mPer2 mRNA accumulation in both sham-operated and adrenalized mice. Hence, as reported in previous studies (Balsalobre *et al.*, 2000a; Damiola *et al.*, 2000; Stokkan *et al.*, 2001), neither feeding time nor glucocorticoid signaling appear to influence the phase of the central molecular pacemaker.

Similar experiments to those presented in Figure 1 were also performed with mice fed exclusively during the night, or for extended time periods during the day. The experiments presented in Figure 2 indicate that glucocorticoid signaling does not influence the phase of circadian

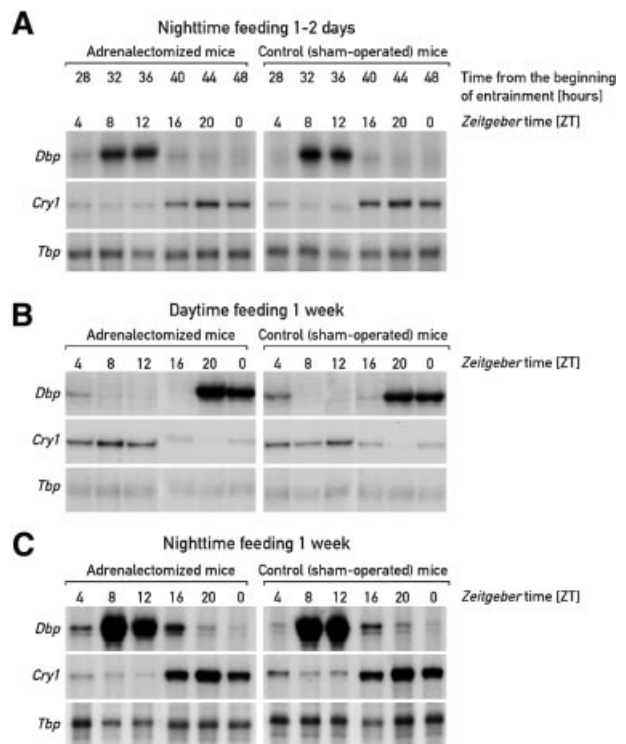


Fig. 2. Hepatic accumulation of *mCry1* and *Dbp* transcripts in night-time- and daytime-fed adrenalized and sham-operated mice. Adrenalized mice and sham-operated mice fed *ad libitum* were kept under a 12 h light–dark regimen (lights on ZT0) before they were switched to night-time feeding [(A) and (C)] or daytime feeding (B). One day (A) or 1 week [(B) and (C)] after restricted feeding had been initiated, animals were killed at 4 h intervals and the livers were collected for monitoring the accumulation of *Dbp* and *mCry1* transcripts. *Tbp* mRNA was included as a transcript whose accumulation is neither circadian- nor feeding time-dependent.

gene expression in animals subjected to night-time feeding for either 1–2 days (Figure 2A) or 1 week (Figure 2C). This is in keeping with the previously published finding that the phases of circadian gene expression are indistinguishable in animals fed *ad libitum* or exclusively during the night (Damiola *et al.*, 2000). We also noticed that after extended periods of daytime feeding, the steady-state phase of cyclic mRNA accumulation is the same in the presence or absence of glucocorticoid hormone signaling (Figure 2B).

We draw two major conclusions from the results presented thus far. First, glucocorticoid signaling strongly slows down—but does not prevent—the adaptation of circadian gene expression to a feeding schedule that is in conflict with the normal activity phase of the animal. Secondly, glucocorticoids are not required for the phase setting of circadian oscillators in liver and kidney under conditions in which food is available throughout the 24 h day (see also Damiola *et al.*, 2000) or during the normal activity phase.

Daytime feeding changes the profile of daily glucocorticoid secretion

In animals fed *ad libitum*, glucocorticoid hormones are secreted by the adrenal gland in a circadian and episodic fashion (Carrillo *et al.*, 1980; Tronche *et al.*, 1998). The

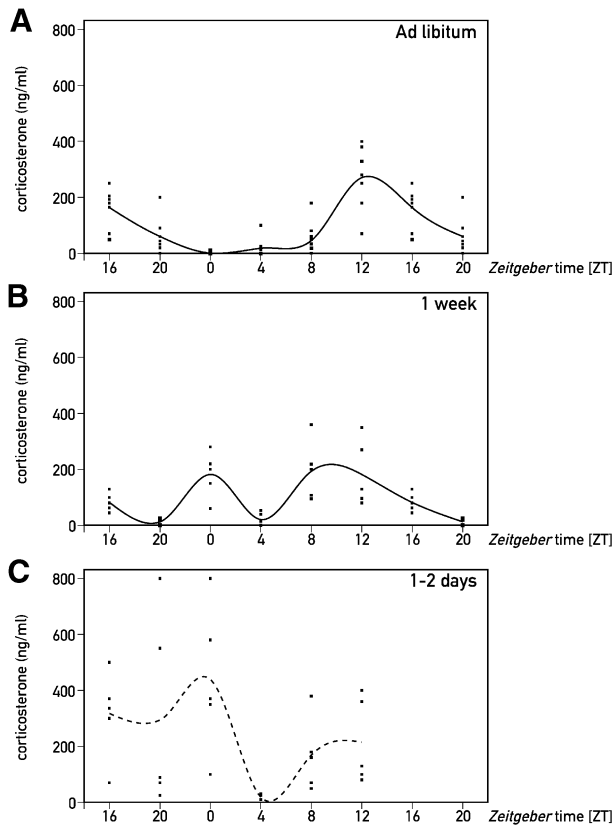


Fig. 3. Diurnal corticosterone levels in animals fed *ad libitum* or during the day. Daytime-dependent corticosterone serum levels were determined in mice fed *ad libitum* (A), or in mice fed exclusively during the day for 7 days (B) or 1–2 days (C). For each time point, the blood was collected from five [(B) and (C)] to seven (A) individuals. As indicated in the text, due to the episodic nature of glucocorticoid secretion, the individual variability is small during nadir times but large during zenith times. In the steady-state profiles shown in (A) and (B), the values obtained for ZT16 and ZT20 are repeated to visualize the bimodal distributions.

episodes, consisting of short bouts of hormone secretion (Shiraishi *et al.*, 1984), are not synchronized between animals. The SCN pacemaker is believed to control the daily fluctuations of corticosterone levels via the hypothalamus–pituitary gland–adrenal axis and additional routes (Lejeune-Lenain *et al.*, 1987). In both diurnal and nocturnal mammals, serum glucocorticoids reach peak levels around or just before the onset of the activity phase. Given the strong inhibition of food-induced phase resetting by glucocorticoid signaling, we wished to examine whether daytime feeding affects the temporal pattern of serum glucocorticoids. To this end we monitored the serum concentrations of corticosterones at 4 h intervals around the clock in mice fed *ad libitum* (Figure 3A), in mice at the onset of daytime feeding (Figure 3C) and in mice exposed for 1 week to daytime feeding (Figure 3B). After 1 week, the inverted phase of circadian gene expression has been fully established in the peripheral organs examined. Because of the episodic nature of corticosterone secretion (Carrillo *et al.*, 1980), the values obtained for the 5–7 individuals killed at a given time point vary greatly at times when these hormones reach high circadian levels, but are low in all animals at nadir times of glucocorticoid secretion. As a consequence, the

circadian phase of glucocorticoid secretion is characterized best by the trough levels, which are highly reproducible. In accordance with published data (Cheifetz, 1971), corticosterone plasma levels are lowest at the dark–light transition (ZT0) and highest at the light–dark transition (ZT12) in animals kept under a 12 h light–dark regimen (Figure 3A). Interestingly the temporal glucocorticoid profile is bimodal in mice stably entrained to daytime feeding, with a peak of short duration between ZT20 and ZT4 (zenith at ZT0) and a broader peak between ZT4 and ZT20 (zenith between ZT8 and ZT12) (Figure 3B). The average levels of both peaks are in the range of 200 ng/ml, which resembles the average peak level measured for animals fed *ad libitum*.

Based on these results, we conclude that daytime feeding establishes a bimodal temporal pattern of corticosterone secretion, of which only the first peak appears to depend upon feeding time. The second oscillation is akin to the glucocorticoid profile recorded in animals fed *ad libitum* in that it reaches maximal values just before the light–dark transition. This second wave of corticosterone secretion may be controlled primarily by the SCN pacemaker, which does not change its phase in daytime-fed animals. At the onset of daytime feeding, corticosterone levels can attain particularly high levels during the dark phase (Figure 3C). Conceivably, these elevated glucocorticoid concentrations could be responsible for slowing down daytime feeding-induced resetting of peripheral oscillators (see Discussion).

The inhibition of food-entrained phase resetting by glucocorticoids requires the glucocorticoid receptor and is tissue autonomous

The observed inhibitory effect of glucocorticoid signaling on feeding time-induced phase resetting could be accomplished via direct cell-autonomous mechanisms or indirect mechanisms involving systemic signals whose production and/or release into the blood is controlled by glucocorticoid hormones. To discriminate between these two scenarios, we examined phase resetting of circadian gene expression in livers and kidneys of wild-type mice and GR^{Alfp}Cre mice, carrying glucocorticoid receptor null alleles exclusively in the hepatocytes (Kellendonk *et al.*, 2000). As shown in Figure 4A (left panel), on the second day of daytime feeding the phases of circadian expression of *Dbp*, *mPer1*, *mPer2* and *mPer3* in the livers of GR^{Alfp}Cre mice are already shifted to values close to those observed 1 week after the animals had been subjected to the new feeding regimen. In contrast, the cyclic gene expression of these genes is only moderately advanced in the kidneys of GR^{Alfp}Cre mice (Figure 4B, left panel) and in kidneys or livers of wild-type mice (Figure 4A and B, right panels).

The expression of *mPer1* behaves somewhat differently when compared with that of the other circadian genes examined. Under normal circumstances, it is high during the dark phase of the first day and low throughout the period of daytime feeding examined in the livers of GR^{Alfp}Cre mice. Relatively low levels of *mPer1* mRNA have also been observed in the livers of adrenalectomized animals (Figure 1A). As outlined in the previous section, corticosterone levels are particularly high during the dark phase at the onset of daytime feeding, when *mPer1* mRNA

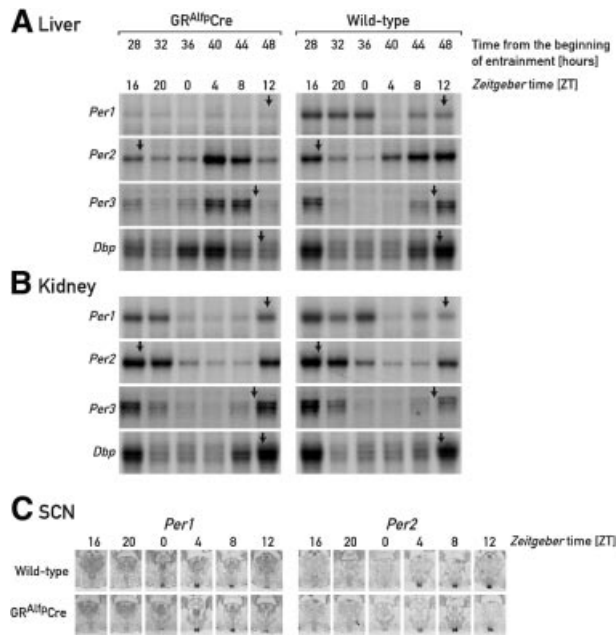


Fig. 4. Accumulation of circadian transcripts in GR^{AlfpCre} and wild-type mice at the onset of daytime feeding. GR^{AlfpCre} and wild-type mice were switched to daytime feeding as explained in the legend to Figure 1. The hepatocytes of GR^{AlfpCre} mice harbor a glucocorticoid receptor null allele and thus cannot respond to corticosterone signaling. The relative mRNA levels were determined by RNase protection assays for liver (A) and kidney (B), and by *in situ* hybridization for the SCN (C). For comparison, the approximate peak times of mRNA accumulation in animals fed *ad libitum* or during the night are indicated by arrows (see Figure 1).

accumulates to high cellular concentrations. This correlation could indicate that *mPer1* is directly regulated by glucocorticoid hormones (see Balsalobre *et al.*, 2000a and Discussion). We noticed, however, that in kidneys of adrenalectomized animals, *mPer1* expression is not significantly diminished when compared with sham-operated animals (Figure 1B). Thus, in contrast to the observations made with liver, in kidney glucocorticoid signaling does not appear to be required for high *mPer1* mRNA expression.

We also compared the circadian *mPer1* and *mPer2* mRNA accumulation profiles in the SCN of GR^{AlfpCre} and wild-type mice on the second day of daytime feeding (Figure 4C). As expected from the data presented in Figure 1, rhythmic gene expression is not significantly altered in the SCNs of mice subjected to restricted feeding, in spite of the high corticosterone levels measured during the dark phase at the onset of daytime feeding. In fact, neither glucocorticoid signaling nor restricted feeding causes phase shifts in circadian gene expression in SCN neurons (Balsalobre *et al.*, 2000a; Damiola *et al.*, 2000; Stokkan *et al.*, 2001).

The switch from daytime to night-time feeding results in a rapid phase resetting of circadian gene expression in liver and kidney

For nocturnal animals, daytime feeding is in conflict with their activity phase, and although feeding time is a dominant *Zeitgeber* for circadian clocks in several peripheral tissues, it takes several days to adapt the

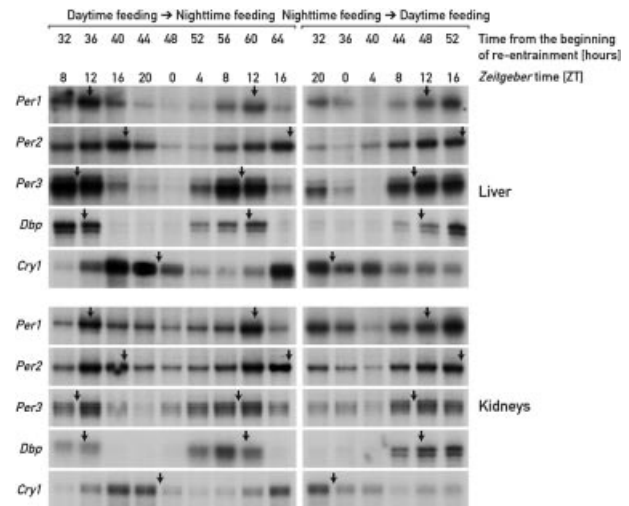


Fig. 5. Accumulation of circadian transcripts in mice switched from daytime to night-time feeding or vice versa. Mice kept under a 12 h LD regimen were entrained to daytime feeding or night-time feeding for 8 days, and then switched to night-time or daytime feeding, respectively. At the times after feeding time reversal (re-entrainment) indicated, liver and kidney RNAs were subjected to RNase protection assays with the probes indicated on the left-hand side of the panels. *Tbp* mRNA was included as a transcript whose accumulation is neither circadian- nor feeding time-dependent (not shown). For comparison, the approximate peak times of mRNA accumulation in animals fed *ad libitum* or during the night are indicated by arrows.

phase of these oscillators to the unusual feeding schedule. In the results above, we demonstrated that the inertia in this phase resetting is not an intrinsic property of peripheral oscillators, but is caused principally by glucocorticoid signaling. We wished to determine whether the phase resetting in peripheral organs proceeds with different kinetics when mice are switched from an abnormal to a normal feeding schedule than when they are switched in the reverse direction. To this end, we subjected mice to 8 days of daytime feeding and then switched them to night-time feeding. Circadian gene expression was recorded at 4 h intervals between 32 and 64 h after the switch from daytime to night-time feeding. The data depicted in Figure 5 show that, in liver, the night-time entrained phase of circadian gene expression is already approached 2–3 days after changing the feeding regimen. For example, on the second day after switching from daytime to night-time feeding, *Dbp* mRNA reaches zenith levels between ZT8 and ZT12, similar to what had been observed in animals fed *ad libitum* or during the night. The phase changes proceed with slightly slower kinetics in kidney, in which the phases of several mRNA accumulation profiles lag behind those monitored in liver by ~2 h. We have previously noticed that circadian kidney gene expression also adapts somewhat more slowly to daytime feeding, at least for certain examined genes.

In control experiments, we have monitored circadian gene expression in mice switched from restricted night-time feeding to daytime feeding 2–3 days after reversal of the feeding regimen. The data presented in Figure 5 (right panels) are comparable to those reported for animals switched from unrestricted to daytime feeding (see Figure 1A and B and Damiola *et al.*, 2000). They confirm that in animals with an intact hypothalamus–pituitary

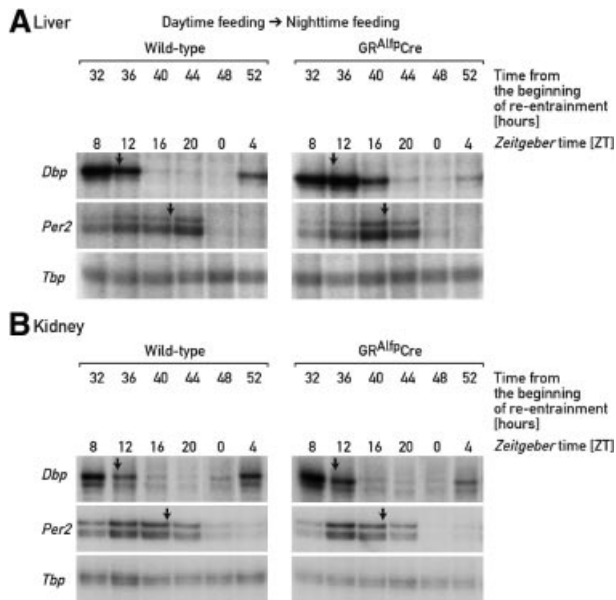


Fig. 6. Accumulation of *Dbp* and *mPer2* mRNA in $GR^{AlfpCre}$ and wild-type mice switched from daytime to night-time feeding. Mice kept under a 12 h LD regimen were entrained to daytime feeding during 8 days, and then switched to night-time feeding. At the times after the feeding time reversal (re-entrainment) indicated, liver (A) and kidney (B) RNAs were subjected to RNase protection assays with *Dbp*, *mPer2* and *Tbp* probes. *Tbp* mRNA was included as a transcript whose accumulation is neither circadian- nor feeding time-dependent. For comparison, the approximate peak times of mRNA accumulation in animals fed *ad libitum* or during the night are indicated by arrows. Note that glucocorticoid signaling [compare left and right autoradiographs in (A)] has little if any effect on the rapid phase adjustments in mRNA accumulation cycles observed in mice switched from daytime to night-time feeding.

gland–adrenal axis, phase adaptations are reached slowly when the feeding schedule is set in conflict with the activity phase.

In order to test whether glucocorticoid signaling also affects the phase adaptation in circadian gene expression accompanying the switch back from daytime to night-time feeding, we compared the *Dbp* mRNA accumulation profile 2–3 days after changing the feeding regimen in livers and kidneys of wild-type and $GR^{AlfpCre}$ mice. Figure 6A demonstrates that the kinetics of phase resetting of *Dbp* and *mPer2* mRNA accumulation are similar in wild-type and $GR^{AlfpCre}$ mice on the second day after swapping feeding time. In a control experiment we re-examined *Dbp* mRNA accumulation 2–3 days after swapping mice of the two genotypes from night-time to daytime feeding, and observed that the phase of circadian *Dbp* mRNA accumulation shifted considerably faster in the liver of $GR^{AlfpCre}$ mice (data not shown), as expected from the data presented in Figure 4. Moreover, no significant differences between the *Dbp* mRNA accumulation patterns could be noticed in the kidneys of wild-type and $GR^{AlfpCre}$ mice, which both express the glucocorticoid receptor (Figure 6B).

Discussion

The circadian timing system in mammals is constructed in a hierarchical fashion: a master pacemaker localized in

SCN neurons synchronizes slave clocks in most cell types of peripheral tissues (Yamazaki *et al.*, 2000). While light is clearly the major *Zeitgeber* for the central clock, recent reports suggest that feeding time is the dominant timing cue for at least some peripheral clocks (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). Thus, in nocturnal rodents, such as rats and mice, daytime feeding completely uncouples the phase of circadian gene expression in liver, kidney, heart and pancreas from that of the SCN pacemaker. These observations opened the possibility that the SCN may entrain peripheral clocks indirectly by setting the activity phase, and thus feeding time, if food availability is not in conflict with the normal activity phase. Similar to the phase resetting of the SCN pacemaker by large changes in the photoperiod, the phase adjustment of peripheral oscillators by feeding time is gradual rather than instantaneous. Even in the liver, in which food-induced phase shifting proceeds somewhat faster than in other tissues examined, several consecutive days of daytime feeding are required to reach the new and inverted steady-state phase (Damiola *et al.*, 2000). The inertia in phase resetting could reflect the possibility that the limited daily phase-shifting capacity is an intrinsic property of peripheral clocks. However, the results presented in this study offer an alternative interpretation: namely, that a rapid phase adjustment in response to altered feeding time is inhibited by glucocorticoid hormone signaling. In fact, daytime feeding can induce large phase shifts in mice in which glucocorticoid signaling has been interrupted either by adrenalectomy or ablation of the glucocorticoid receptor gene.

In previous work, the glucocorticoid analog dexamethasone has been demonstrated to be a potent phase-shifting agent for circadian gene expression in peripheral mouse tissues (Balsalobre *et al.*, 2000a). Moreover, dexamethasone efficiently stimulates circadian gene expression in rat-1 fibroblasts grown in tissue culture (Balsalobre *et al.*, 2000b). For three reasons, glucocorticoids have been considered to be particularly well suited as *Zeitgebers* for peripheral oscillators used by the SCN to synchronize peripheral clocks. First, their cyclic secretion is under the control of the hypothalamus–pituitary gland–adrenal axis, which in turn is controlled by the SCN. Secondly, with the noteworthy exception of the SCN, the glucocorticoid receptor is expressed in all cell types examined (Rosenfeld *et al.*, 1988; Balsalobre *et al.*, 2000a), a pattern that is a prerequisite for an input component of the ubiquitous peripheral clocks. Thirdly, in the SCN of newborn animals, the glucocorticoid receptor gene is expressed, but it becomes silenced within the first weeks after birth (Rosenfeld *et al.*, 1988), just before the animals display robust circadian gene expression in peripheral organs (S.A. Brown and U. Schibler, unpublished observations). In view of the different phase angles determined for circadian gene expression in the SCN and in peripheral tissues, at least some signaling pathways affecting the phase in the latter should not be operative in the former. However, although glucocorticoid hormones fulfill all of these prerequisites of *Zeitgebers* for peripheral clocks, the glucocorticoid receptor is dispensable for the synchronization of circadian liver gene expression under steady-state conditions. Thus, the steady-state phase of circadian liver gene expression is identical in $GR^{AlfpCre}$ mice, whose

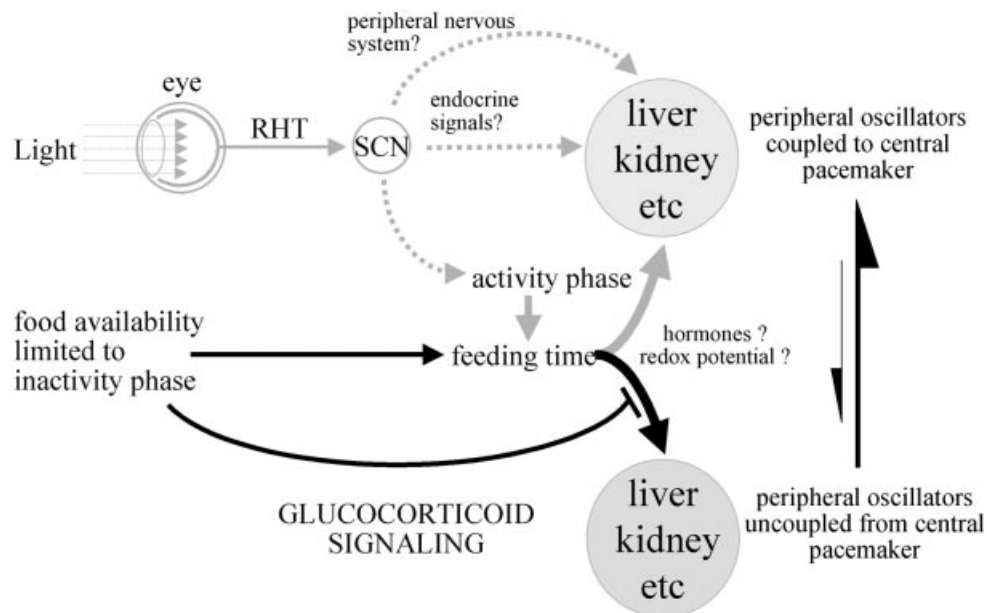


Fig. 7. The phase entrainment of circadian oscillators in peripheral organs. The scheme displays a hypothetical model of the synchronization of peripheral oscillators by the central SCN pacemaker and feeding time. The SCN is entrained by the photoperiod via synaptic connections with the retina (retino–hypothalamic tract, RHT). In turn, the SCN master clock entrains circadian gene expression in peripheral tissues (e.g. liver, kidney) through direct neuronal and humoral pathways, or indirectly by determining the activity phase and thus feeding time. When food, the dominant *Zeitgeber* for (at least some) peripheral oscillators, is only available during the resting phase (light phase in nocturnal rodents), the phases of these clocks are inverted. This process is slowed down by glucocorticoid signaling, probably through the abundant secretion of corticosterones during the dark phase in animals switched to daytime feeding. In contrast to the slow-phase adaptation of peripheral gene expression accompanying the switch from night-time to daytime feeding, during which peripheral oscillators become uncoupled from the central pacemaker, the switch back from daytime to night-time feeding causes an almost instantaneous phase inversion. The molecular mechanisms by which food resets the phase of peripheral oscillators are not known, but they may involve changes in the redox potential [NAD(P)H/NAD(P)⁺ ratio] or in hormones whose secretion is provoked or suppressed by food metabolites.

hepatocytes are homozygous for a glucocorticoid receptor null allele, and wild-type mice (Balsalobre *et al.*, 2000a). Hence, glucocorticoid hormones cannot be the sole *Zeitgeber* for peripheral tissues.

Given the known connection between glucocorticoid signaling and fasting (Dallman *et al.*, 1999), one might have anticipated that glucocorticoid signaling may play a role in the phase resetting of circadian gene expression by feeding time. However, Stokkan *et al.* (2001) have demonstrated that the rephasing of cyclic gene expression in rat liver cannot be mimicked by repeated daily injections of corticosterones at times when these hormones peak in animals subjected to restricted feeding. As shown here, glucocorticoids inhibit rather than promote the phase adjustments of peripheral oscillators to daytime feeding, and circadian gene expression can experience large phase shifts in response to restricted feeding, once the counteracting glucocorticoid signaling pathway is inactivated. That peripheral oscillators are capable of conducting very large phase shifts is also demonstrated by an experiment in which mice are first entrained to daytime feeding and then switched back to night-time feeding. This switch is accompanied by a very rapid phase reversal in circadian liver gene expression, both in the presence and absence of glucocorticoid signaling. Hence, it appears that glucocorticoid signaling slows down exclusively phase changes provoked by a feeding regimen that is in conflict with the daily activity phase, but has little effect when the phase is switched from an abnormal to a normal feeding schedule.

Glucocorticoid signaling could be used by the SCN master clock to prevent a rapid uncoupling of peripheral

oscillators. However, our results demonstrate that the temporal pattern of serum glucocorticoid hormones changes significantly during daytime feeding, in that it follows a bimodal distribution in daytime-fed animals. In particular, at the onset of daytime feeding, corticosterone levels are unusually high, probably as a consequence of decreased glucose levels. It is, thus, difficult to decide whether the circadian glucocorticoid secretion controlled by the SCN or that provoked by restricted feeding is responsible for the observed phenotype. A further complication is introduced by the episodic nature of corticosterone secretion. As glucocorticoids are secreted in bouts, their levels vary dramatically between sera harvested from different individuals, and this makes the comparison of individuals difficult. Real-time monitoring of blood samples in individual animals would be a more reliable way to follow glucocorticoid secretion profiles, but unfortunately this method is difficult to apply to mice, given their small blood volume.

We noticed that in livers and kidneys of animals with intact glucocorticoid signaling, daytime feeding affects the expression of *mPer1* more dramatically than that of other circadian genes. Thus, while the steady-state phases of *mPer1* and *Dbp* mRNA accumulation are nearly identical (see Damiola *et al.*, 2000), they are transiently different at the onset of daytime feeding (Figures 1A and B, 4A and B). Previously published experiments already suggested that *mPer1* is a direct target of glucocorticoid signaling (Balsalobre *et al.*, 2000a,b). In keeping with this conjecture, *mPer1* contains two almost perfect GRE consensus sequences of the type AGAACAN₃TGTTCT in the

5'-flanking region (AGAACACGATGTTCC centered around -1088) and in the first intron (gGAACATCC-TGTTCT centered around +490), respectively, with respect to the transcription start site determined by Hida *et al.* (2000). Moreover, dexamethasone rapidly induces a surge of *Per1* mRNA accumulation in rat-1 tissue culture cells or peripheral cell types of intact mice (Balsalobre *et al.*, 2000a,b). We thus consider it likely that the altered expression of *mPer1* after switching mice to daytime feeding participates in the glucocorticoid-dependent kinetics of food-induced phase resets in liver and kidney. We also wish to emphasize, however, that after 1 week of daytime feeding, corticosterone accumulates in the blood in a biphasic temporal pattern, the phase of the second peak of which resembles the circadian pattern of glucocorticoid secretion in animals fed *ad libitum*. Thus, once the new steady-state phase is reached after an extended duration of daytime feeding, glucocorticoid signaling does not appear to interfere with the food-imposed reversed phase of circadian gene expression in peripheral tissues.

Restricted feeding not only affects the phasing of circadian gene expression, but it can also entrain food-anticipatory activity (see Davidson and Stephan, 1999 and references therein). Thus, shortly before the daily meal is offered to laboratory rodents, they display increased locomotor activity and body temperature. Interestingly, food-anticipatory behavior does not depend on either a functional SCN pacemaker or an intact pituitary gland. Whether circadian gene expression in other brain regions or in peripheral tissues is involved in driving the feeding-entrainable oscillator remains to be examined.

How does feeding time reset the phase of oscillators in organs such as liver and kidney? As mentioned in the Introduction, a plethora of chemical signals can induce circadian gene expression in tissue culture cells, and at least some of them may be involved in food-induced phase resetting. Additional bloodborne signals (e.g. insulin, glucagon and leptin), whose secretion is known to depend on food metabolites, may also play a role. However, a recent report by Rutter *et al.* (2001) opens up the possibility that feeding may also influence circadian gene expression more directly. At least *in vitro*, the DNA binding of the Clock-BMAL1 or NPAS2-BMAL1 heterodimers, which constitute the positive limbs of the central circadian feedback loop, is highly sensitive to the ratio of NAD cofactors. The reduced electron carriers NADH or NADPH strongly stimulate the occupancy of E-box motifs by Clock-Bmal1 heterodimers, while the oxidized versions NAD⁺ or NADP⁺ inhibit this protein-DNA interaction. As the ratio of reduced to oxidized NAD cofactors is likely to depend on the availability of fuels such as glucose, the metabolic state of cells may directly influence molecular oscillators in liver and other peripheral tissues through changes in redox potential. If applicable, this mechanism would have to be specific to peripheral oscillators, since feeding time does not impinge on the phase entrainment of the central SCN pacemaker (Damiola *et al.*, 2000; Stokkan *et al.*, 2001).

The scheme depicted in Figure 7 summarizes how the SCN pacemaker, feeding time and glucocorticoid signaling may control the phase entrainment of peripheral oscillators. The elucidation of the signals involved in the

complex interactions between central and peripheral clocks, and of the mechanisms that govern the interplay between metabolism and circadian oscillators, has just begun. We hope that further studies addressing these issues will be greatly facilitated by the possibility of investigating circadian gene expression in tissue culture cells that are readily amenable to genetic and biochemical manipulation.

Materials and methods

Animal care and handling

All experiments were performed with mice between 10 and 16 weeks of age. Adrenalectomized and sham-operated C57BL/6J mice were purchased from IFFA CREDO. GR^{Alfp}Cre and their respective wild-type control mice were established at the Deutsches Krebsforschungszentrum (Heidelberg, Germany) (Kellendonk *et al.*, 2000). GR^{Alfp}Cre mice have a mixed genetic background (129-C57BL/6-FVB/N). The animals were kept under a 12 h light-dark regimen (lights on at ZT0). Mice fed during the day received food from ZT0 to ZT12, whereas those fed during the night received food from ZT12 to ZT0.

RNase protection experiments

Mouse tissues were removed within 4 min after decapitation, frozen in liquid N₂ and stored at -70°C until use. The extraction of whole-cell RNA and its analysis by RNase protection assays were performed as described (Schmidt and Schibler, 1995). The *Dbp* antisense RNA probe is complementary to rat *Dbp* mRNA (+1126 to +1221) (Fonjallaz *et al.*, 1996). The *Tbp* probe is complementary to mouse *Tbp* mRNA (+36 to +135) (Schmidt and Schibler, 1995). The *Per3* and *Cry1* probes (kindly provided by A.Balsalobre) are complementary to mouse *Per3* mRNA (+367 to +572) and mouse *Cry1* mRNA (+83 to +312), respectively (Balsalobre *et al.*, 2000a). The *Per2* probe is complementary to mouse *Per2* mRNA (+165 to +287) (Balsalobre *et al.*, 1998). The *Per1* probe is complementary to mouse *Per1* mRNA (+2397 to +2523) (a generous gift from J.Ripperger).

In all cases, the plasmids were linearized with a suitable restriction enzyme, and the antisense RNA probes were prepared by *in vitro* transcription of the linearized templates with T7 or T3 RNA polymerase using [α -³²P]UTP. Autoradiography was performed with an intensifying screen (Fuji) at -70°C for 1-5 days.

In situ hybridization to coronal brain sections

Immediately after removal, brains were frozen in isopentane (4 min at -20°C) and stored at -70°C until use. Serial coronal brain cryosections of 12 μ m above the optical chiasma were prepared using standard procedures. *In situ* hybridizations with sections through the central SCN were performed as described previously (Nef *et al.*, 1996). The *mPer1* and *mPer2* riboprobes used in these experiments were prepared from the templates pKS-mPer1-F1 and pKS-mPer2-nqF1 (a generous gift from S.Brown), using RNA polymerases T3 and T7 for the antisense and sense strands, respectively. pKS-mPer1-F1 and pKS-mPer2-nqF1 were obtained by cloning the inserts of the plasmids pcDNA3.1-P1 and pcDNA3.1-P2, respectively, into the plasmid vector pBS-KS. To this end, the inserts of pcDNA3.1-P1 and pcDNA3.1-P2 were excised with *Xba*I and *Cla*I-*Not*I, respectively. The plasmids pcDNA3.1-P1 and pcDNA3.1-P2 (Jin *et al.*, 1999) were kindly provided by Dr Steven Reppert.

Determination of corticosterone levels

Mice were killed within 10 s after entering the animal facility (to avoid interference of stress with corticosterone levels) and, after decapitation, their blood was collected. After coagulation (10 min at room temperature and 10 min on ice), samples were centrifuged at 3000 r.p.m. (920 g) for 20 min and the sera were stored at -70°C until use. Corticosterone measurements were carried out using the corticosterone ¹²⁵I RIA kit from ICN Biomedicals, Inc. (Costa Mesa, CA) according to the manufacturer's instructions.

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