

# The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock

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## Summary

In mammals, the master clock of the *suprachiasmatic nuclei* (SCN) and subordinate clocks found throughout the body coordinate circadian rhythms of behavior and physiology. We characterize the clock of the adrenal, an important endocrine gland that synchronizes physiological and metabolic rhythms. Clock gene expression was detected in the outer adrenal cortex prefiguring a role of the clock in regulating gluco- and mineral corticoid biogenesis. In *Per2/Cry1* double mutant mice, which lack a circadian clock, hypothalamus/pituitary/adrenal axis regulation was defective. Organ culture and tissue transplantation suggest that the adrenal pacemaker gates glucocorticoid production in response to adrenocorticotropin (ACTH). In vivo the adrenal circadian clock can be entrained by light. Transcriptome profiling identified rhythmically expressed genes located at diverse nodes of steroid biogenesis that may mediate gating of the ACTH response by the adrenal clock.

## Introduction

The axial rotation of the earth causes extensive yet periodic variations in a wide range of environmental conditions. Most organisms have evolved circadian clocks to optimally adjust their behavior and physiology to such recurring changes (Harmer et al., 2001). In mammals, the master circadian clock resides in the *suprachiasmatic nuclei* (SCN) of the ventral hypothalamus (Ralph et al., 1990; Rusak and Zucker, 1979). To synchronize physiology with external time, the SCN clock emits timing signals to a series of peripheral circadian clocks found in most organs (Schibler et al., 2003). An important goal of contemporary chronobiological research is to understand how central and peripheral circadian pacemakers communicate and how peripheral clocks regulate behavior and physiology.

Signaling between the SCN and the periphery employs both hormonal and neuronal mechanisms. In the case of the adrenal gland, hormonal signaling proceeds along the hypothalamus/pituitary/adrenal (HPA) axis. Specifically, the SCN activates rhythmic release of corticotrophin-releasing hormone (CRH) from the paraventricular nucleus (PVN) that evokes circadian adrenocorticotropin hormone (ACTH) release from hypophysial adrenocorticotrophs (Simpson and Waterman, 1988). ACTH, in turn, regulates circadian corticoid release from the *zona glomerulosa* and the *zona fasciculata* of the adrenal cortex. In addition, neuronal signals generated by the SCN propagate through the autonomic nervous system to the adrenal cortex to contribute to the circadian regulation of glucocorticoid production (Buijs et al., 2003). Transection of the splanchnic nerve renders the

cortex more responsive to ACTH while simultaneously dampening diurnal rhythms of corticoid secretion (Jasper and Engeland, 1994, 1997). The important work of Ishida et al. (2005) shows that photic signals from the SCN are transduced through the autonomic nervous system to the adrenal since denervation of the adrenal abolishes photic induction of the clock gene *Per1* in the adrenal cortex. These neuroendocrinological data, in conjunction with the circadian expression pattern of *Per1* and *Per2* in the adrenal gland of rodents (Bittman et al., 2003; Torres-Farfan et al., 2006; Ishida et al., 2005) and rhythmic expression of clock-controlled genes in the adrenal of primates (Lemos et al., 2006), provide bona fide evidence for the presence of a peripheral circadian clock in the adrenal gland. Among peripheral clocks the one in the adrenal is particularly interesting since adrenal corticoids have been implicated in the synchronization of subordinated oscillators (Balsalobre et al., 2000) and were demonstrated to control metabolic rhythms in many other organs, including liver (Oishi et al., 2005), kidney (Nicholson et al., 1976), and brain (Casanueva and Dieguez, 1999).

The availability of mutant mice lacking a circadian clock (Bae et al., 2001; Bunger et al., 2000; Oster et al., 2002; van der Horst et al., 1999; Zheng et al., 2001), combined with the ease with which adrenal tissue can be cultured (Lindhe et al., 2001) and transplanted (Musholt et al., 2002; Ulrich-Lai and Engeland, 2000), offer new opportunities to capture the physiological function of the adrenal circadian pacemaker. In this study we demonstrate that a light-regulated circadian clock resides in the adrenal cortex and this clock gates the physiological response of this organ to ACTH stimulation via the control of genes encoding

proteins of the complex steroidogenic network. Our work thus identifies a mechanism that bridges the gap between peripheral clock gene oscillations and the control of corticoid-dependent physiological rhythms in the adrenal gland and possibly in many other organs.

## Results

### Localization of a peripheral circadian clock to the outer adrenal cortex

We carried out a circadian microarray profiling with wild-type murine adrenals to identify rhythmically expressed genes. Mice were entrained to a standard light/dark cycle (LD12:12) and transferred to constant darkness (DD; top bar in Figure 1A). On the second day in DD, adrenals were removed at 4 hr intervals for a period of 48 hr and gene expression was assessed with Affymetrix MG430v2.0 microarrays. Using the COSOPT algorithm (Straume, 2004) we identified 1606 annotated genes (<http://www.geneontology.org/>; as of April 2006) with significant circadian expression rhythms (H.O. et al., unpublished data). All microarray data are accessible at the NCBI gene expression omnibus (GEO) database (accession number GSE4253). In keeping with the theme of the present study we focus on genes directly implicated in the circadian clock and those involved in adrenal glucocorticoid metabolism.

The majority of canonical clock genes exhibit a circadian expression pattern in the adrenal. Microarray data revealed circadian rhythms for the expression of *Bmal1* (Figure 1B), *Cry1* (Figure 1D), *Per1* (Figure 1F), *Per2* (Figure 1G), *Per3* (Figure 1H), and *Rev-Erb $\alpha$*  (Figure 1I). *Clock* and *Cry2* transcripts were also detected but did not show an obvious circadian rhythm (Figures 1C and 1E). For the first day of the experiment, clock gene expression was validated by qPCR using a different set of adrenals, with results closely matching those obtained from the microarrays (Figures 1B–1I, open circles).

The adrenal gland consists of a catecholamine-producing medulla enclosed within a three-layered cortex (Figure 1Ba). In situ hybridization was carried out on adrenals collected 46 and 58 hr after “lights off” (Figure 1A) to characterize the expression sites of several clock genes most of which were strongly expressed in the *zona glomerulosa* and the *zona fasciculata* (Figures 1Ba–1Ia). *Bmal1* (Figure 1Ba), *Cry1* (Figure 1Da), *Per1* (Figure 1Fa), *Per3* (Figure 1Ha), and *Rev-Erb $\alpha$*  (Figure 1Ia) were temporally regulated in these layers while *Clock* (Figure 1Ca) and *Cry2* (Figure 1Ea) were not. For *Per2*, we did not detect a difference in expression for the two time points selected (Figure 1Ga), but when adjacent sections were probed for PER2 protein, a clear differential expression was seen in the outer cortical layers (Figure 1Gb). In the *zona reticularis*, only *Bmal1*, *Per3*, and *Rev-Erb $\alpha$*  showed temporally regulated expression (Figures 1Ba, 1Ha, and 1Ia), while in the medulla only *Bmal1*, *Per1* and *Per3* displayed a clear expression difference between both time points examined (Figures 1Ba, 1Fa, and 1Ha).

Taken together, microarray and qPCR in combination with in situ hybridization demonstrate that canonical circadian clock genes are expressed in the *zona glomerulosa* and the *zona fasciculata* and—with the exception of *Clock* and *Cry2*—show a robust circadian rhythm of expression. These findings suggest that the corticoid-producing outer layers of the cortex are chief sites of the adrenal circadian pacemaker.

### The adrenal circadian clock defines ACTH responsiveness

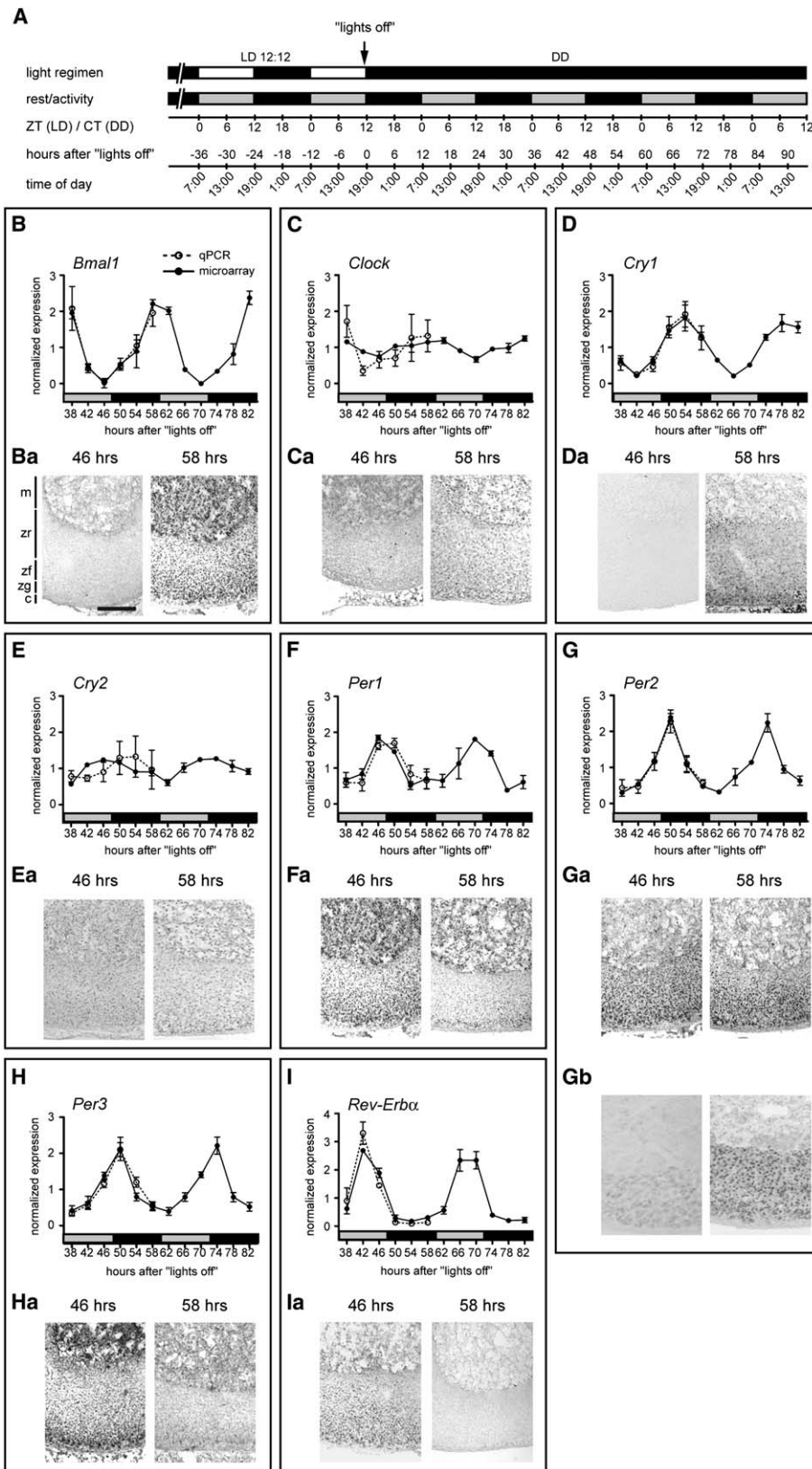
Although the adrenal cortex rhythmically expresses most canonical clock genes, this does not uncover how this pacemaker regulates the circadian properties of this organ. To begin to address this, we analyzed adrenal clock regulation in *Per2/Cry1* double mutant animals that have a disrupted circadian clock (Oster et al., 2002). When we monitored clock gene transcription in the *Per2/Cry1*-deficient adrenal gland, we found that *Bmal1*, *Per1*, *Per3*, and *Rev-Erb $\alpha$* , all rhythmically expressed in the wild-type adrenal (Figures 1B, 1F, 1H, and 1I), are devoid of circadian transcriptional regulation (Figures 2A and 2B).

We next examined how HPA axis hormones were regulated in mutant mice. In wild-type animals kept either under LD or DD conditions, plasma ACTH levels peaked  $\sim 2$  hr prior to the onset of the (subjective) night (Figures 2C and 2D), consistent with previous work (Cheifetz, 1971). ACTH plasma levels of *Per2/Cry1* double mutant animals were steady-state at  $\sim 130$  ng/ml, which is the mean of wild-type zenith and nadir (Figures 2C and 2D). Plasma corticosterone concentration profiles of wild-type mice trailed those of ACTH by  $\sim 2$  hr, peaking at the onset of the (subjective) night (Figures 2E and 2F). Mutant mice lacked not only an overt corticosterone rhythm in serum and feces (Figures 2E–2H), but corticoid levels hovered around nadir concentrations, suggesting that the mutant adrenal cannot properly respond to hypophysical ACTH. Hence, in the absence of a functional clock, input and output signals in the adrenal become to a certain degree uncoupled.

In the intact animal, however, one cannot determine whether the adrenal clock itself or rhythmic serum ACTH causes the rhythmic production of corticosterone. To address this issue, adrenal tissue slices from wild-type or mutant mice were cultured and stimulated with 20 nM ACTH at two opposite time points of the circadian cycle. At 38 hr after “lights off” (Figure 1A), when plasma corticosterone levels were minimal in the wild-type animal (Figure 2F), ACTH evoked a moderate increase of corticosterone in wild-type and *Per2/Cry1* adrenal slices (Figure 3A). ACTH treatment of slices from wild-type adrenals collected at 50 hr (zenith of plasma corticosterone, Figure 2F) evoked a more marked corticosterone release into the medium (Figure 3B). By contrast, mutant adrenal slices treated with ACTH at this time point exhibited basal level corticosterone release reminiscent of that seen at the 38 hr time point. Figure 3C compares the initial rates of corticosterone secretion averaged for two experiments similar to those presented in Figures 3A and 3B. A repeated stimulation after 24 hr of culturing slices in fresh medium gave results similar to those shown in Figure 3C (data not shown), indicating that the differential response observed in the wild-type was not an unspecific effect caused by preparation and culture procedures but most likely reflected an intrinsic rhythm of response. Our organ culture experiments suggest that the adrenal contains a circadian clock that defines—i.e., gates—a time window during which the adrenal most effectively responds to ACTH. Tissue from *Per2/Cry1* mutant mice lacks this clock and, therefore, is unable to differentially respond to ACTH.

### Clock regulation of selective components of the corticosterone biosynthetic pathway

The transcriptional nature of the circadian timing system predicts that the adrenal clock may exert its control on adrenal physiology



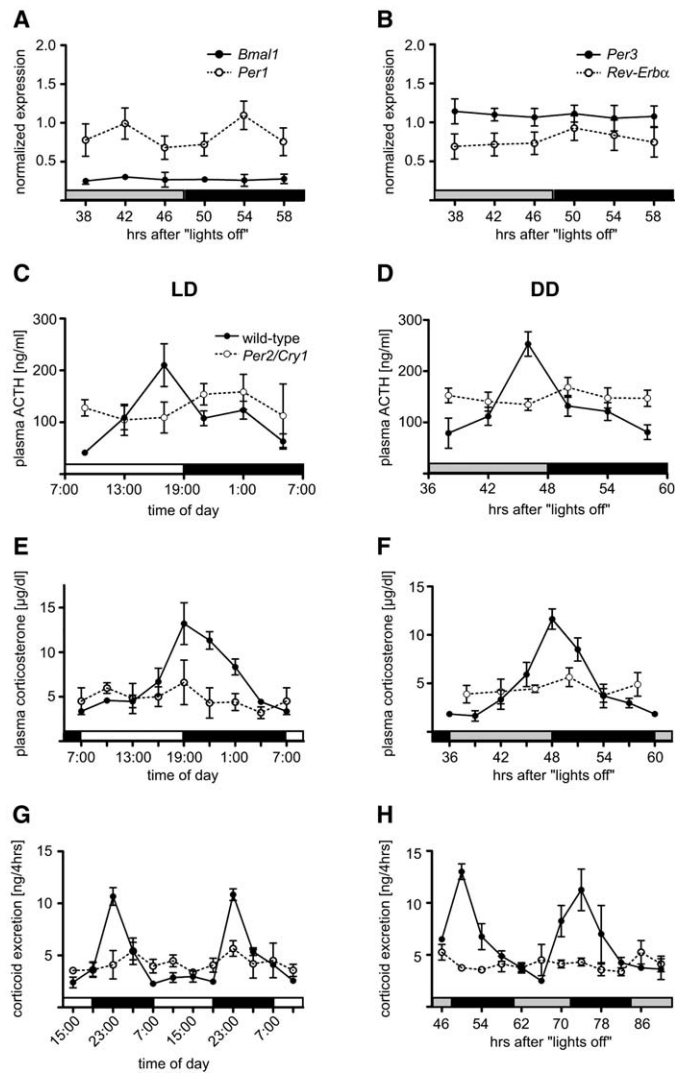
**Figure 1.** Robust circadian expression of clock genes in the murine adrenal cortex

**A)** Diagram of experimental conditions and sampling time points used in this study. Top: zebra bar represents the light/dark schedule to which mice were initially exposed; white segments indicate "lights on" whereas black segments signify darkness. Prior to "lights off" animals were kept for at least 14 days under LD12:12 conditions. Center: wild-type locomotor activity pattern relative to *Zeitgeber* (ZT) and circadian times (CT). Rest/activity patterns echo light conditions in that activity phases (black segments) alternate with resting phases (gray segments). Bottom: Tissue sampling times relative to the "lights off" event and to absolute time.

**B–I)** Circadian expression profiles (mean  $\pm$  SEM) of canonical clock genes in the adrenal gland determined by qPCR (three different adrenals per time point) and microarray analysis (two chips per time point each with a different adrenal). For an explanation of time axis see Figure 1A, top. (**Ba–Fa**, **Ha**, and **Ia**) In situ hybridization micrographs of wild-type adrenals probed for different clock genes at 46 hr (left panel) and 58 hr after "lights off" (right panel). (**Gb**) Differential expression of PER2 protein revealed by immunohistochemistry in the adrenal cortex and medulla. (**Ba**) (left panel) The layered histoarchitecture of the adrenal: adrenal capsule (c); zona glomerulosa (zg); zona fasciculata (zf); zona reticularis (zr), and medulla (m). Bar in (**Ba**) is 0.2 mm.

via rhythmic transcriptional regulation of clock controlled genes. We used our microarray data set to identify those genes implicated in adrenal corticoid metabolism that exhibit a circadian expression pattern. Current gene ontology annotations only partly

reflect the complex regulatory networks underlying physiological processes. We therefore assembled from the literature a more comprehensive representation of the genetic network that controls adrenal corticosterone metabolism (Figure 4). Thirty-three



**Figure 2.** *Per2/Cry1* mutant mice are defective in adrenal clock gene and HPA axis rhythmicity

**A and B** *Bmal1*, *Per1* (A), *Per3*, and *Rev-Erb $\alpha$*  (B) expression rhythms in the adrenal of *Per2/Cry1* double mutant animals in DD as determined by qPCR. Values were normalized to the average wild-type expression level as shown in Figure 1. Absence of rhythmic transcription was also seen for all clock genes of Figure 1 (data not shown). All values are mean  $\pm$  SEM (n = 4 to 6). Gray and black bars delineate rest and active phases in wild-type control animals.

**C** Diurnal rhythm of plasma ACTH in wild-type and *Per2/Cry1* double mutant animals.

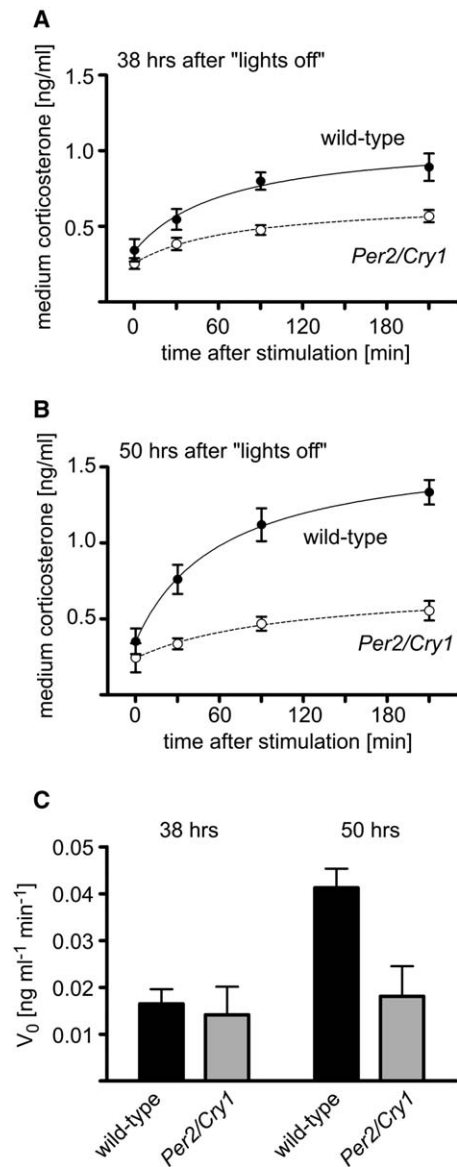
**D** Circadian rhythm of plasma ACTH.

**E** Diurnal rhythm of plasma corticosterone.

**F** Circadian profile of plasma corticosterone.

Diurnal (G) and circadian (H) profiles of fecal corticoid excretion show distinct rhythmicity for wild-type but not for mutant mice. All data presented are mean  $\pm$  SEM (n = 3 to 5). White and black bars indicate light and dark phases; gray and black bars indicate rest and activity phases in wild-type mice.

genes placed at various sites within the network (indicated by gray boxes) are rhythmically expressed (Table S1). The low-density lipoprotein receptor (*Ldlr*) and scavenger receptors (*Scrab2*) regulate the supply of the steroid precursor cholesterol from blood. Other gene products such as P450 oxidoreductase (*Por*) and *trans*-acting transcription factor 1 (*Sp1*) control the activity of steroidogenic enzymes and thereby directly influence steroid biosynthesis. The biggest cohort showing circadian regulations

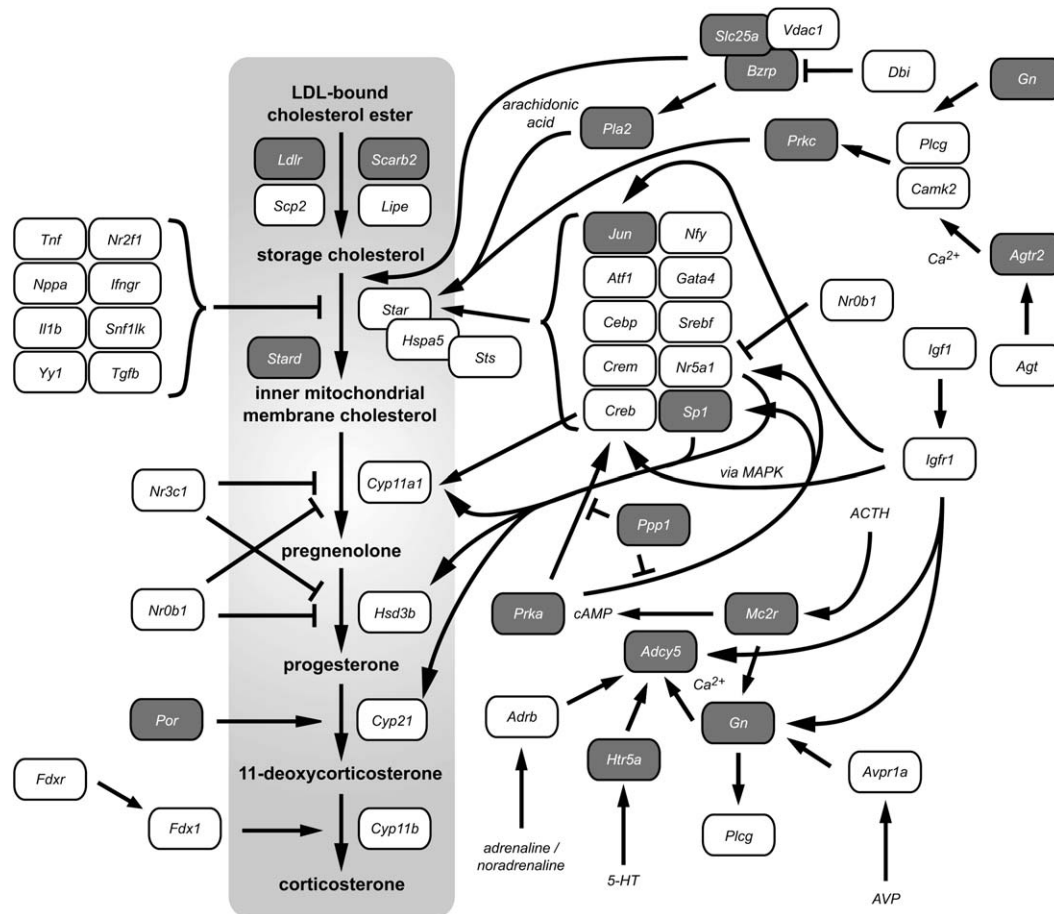


**Figure 3.** A peripheral clockwork residing in the adrenal cortex gates the ability of ACTH to evoke corticosterone release

**A and B** Corticosterone release into culture medium by wild-type and *Per2/Cry1* mutant adrenal tissue culture slices in response to ACTH stimulation at 38 hr (A) and 50 hr (B) after "lights off" (n = 6).

**C** Initial rate of corticosterone release for wild-type and *Per2/Cry1* mutant adrenal slices derived from two experiments, one of which is shown in (A) and (B). All data shown are mean  $\pm$  SEM.

consisted of genes encoding proteins regulating the transport of storage cholesterol to the inner mitochondrial membrane where corticoid production occurs. These include carrier proteins (*Slc25a*) and regulators thereof (*Bzrp*), transcriptional regulators of steroidogenic acute regulatory protein (*Jun*, *Sp1*), and mediators of the ACTH receptor signaling pathway (*Mc2r*, *Adcy5*, several G proteins, protein kinase A, and protein phosphatase 1 subunits). For the 20 genes identified by COSOPT as being most rhythmic, a validation of the microarray data by qPCR was carried out and both methods yielded very similar results (Figure 5). Several of these genes show expression rhythms paralleling glucocorticoid secretion into the blood (compare Figure 2F and Figures



**Figure 4.** Circadian clock-regulated genes involved in the control of adrenal corticosterone biosynthesis

Network of enzymes and regulators of corticosterone production (modified from Delarue et al., 2001; Gallo-Payet and Payet, 2003; Manna and Stocco, 2005; Parker and Schimmer, 1997; Payne and Hales, 2004; Richards, 2001; Sewer and Waterman, 2001; Stocco, 2001; Stocco et al., 2005). Small gray boxes indicate genes identified as rhythmically expressed in our wild-type microarray time series. For gene descriptions see Table S1. The big gray box highlights the corticosterone biosynthesis pathway.

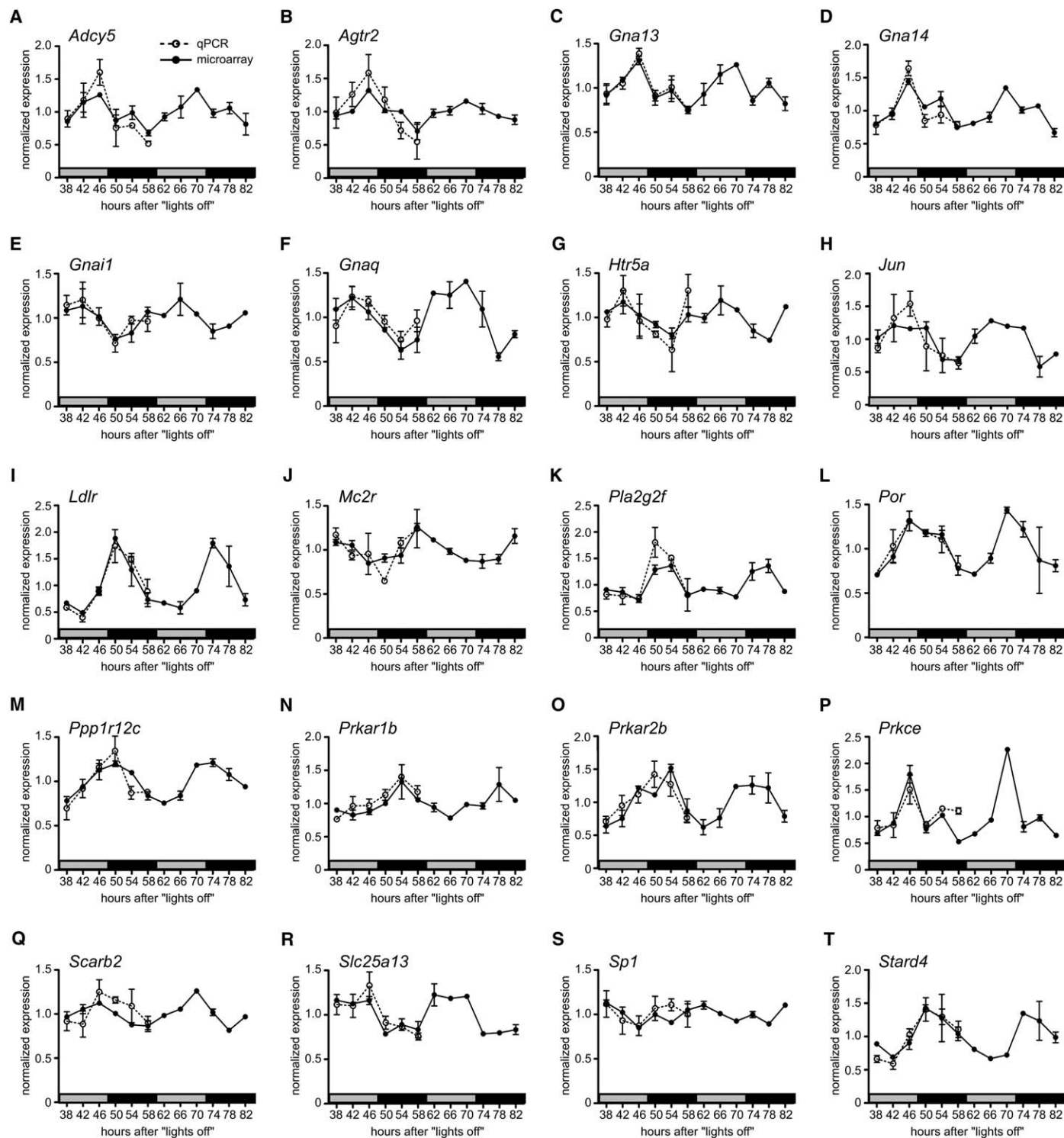
5A–5D, 5H, 5I, 5L–5P, and 5T). However, mRNA production and the availability of a functional protein located in the appropriate cellular compartment may lag behind transcript peaks by several hours (Reppert and Weaver, 2001). Regardless of the caveat, the surprising result of our transcriptome analysis is that within the intricate network of steroid biosynthesis numerous genes distributed over the entire network seem to be candidate targets of the circadian transcriptional machinery.

#### The adrenal clock regulates corticosterone rhythms in vivo

Our comparison of mutant and wild-type in vitro slice cultures revealed a “gating” mechanism in the sense that ACTH stimulation triggers corticosterone release in a temporally controlled fashion. This led us to ask how this gating controls the regulation of circadian corticosterone secretion in vivo. To address this question we transplanted *Per2/Cry1* (arrhythmic) adrenals into wild-type adrenalectomized hosts and vice versa (Figure 6A). We first examined whether the SCN pacemaker was affected in grafted mice by analyzing SCN clock gene expression as well as locomotor activity rhythms. In wild-type hosts carrying wild-type grafts, both *Per1* expression in the SCN and activity rhythms were those seen with unoperated animals (Figure 6B). *Per1* expression peaked during the subjective day and the

free-running period length ( $\tau_{DD}$ ) was  $23.4 \pm 0.1$  hr (mean  $\pm$  SEM,  $n = 5$ ). In both of the crossgenotype recombinations, *Per1* expression and activity rhythms were not significantly affected by the genotype of the graft (Figures 6C and 6D). Wild-type hosts carrying a mutant adrenal showed wild-type LD and DD rhythms ( $\tau_{DD} = 23.6 \pm 0.1$  hr; mean  $\pm$  SEM,  $n = 5$ ) and rhythmic expression of *Per1* in the SCN (Figure 6C). By contrast, mutant hosts carrying wild-type adrenals became immediately arrhythmic when released into DD. Moreover, *Per1* transcription in the SCN was blunted, as was previously reported for unoperated *Per2/Cry1* double-deficient animals (Oster et al., 2002). From these data we conclude that there is no feedback of the adrenal clock on the SCN pacemaker.

Next, grafted mice were examined for HPA axis regulation. We detected rhythmic ACTH levels in wild-type hosts carrying either wild-type or mutant adrenal grafts in both LD (Figure 7A) and DD (Figure 7B). In mutant hosts, a wild-type adrenal was not sufficient to restore circadian ACTH secretion in both lighting conditions (Figures 7A and 7B). Hence, ACTH secretion from the pituitary paralleled the *Per1* expression pattern in the SCN and the behavioral phenotype. By contrast, corticoid rhythms were clearly affected by the genotype of the adrenal transplant. In LD all host/graft combinations showed consistent corticosterone excretion rhythms. However, when either a mutant



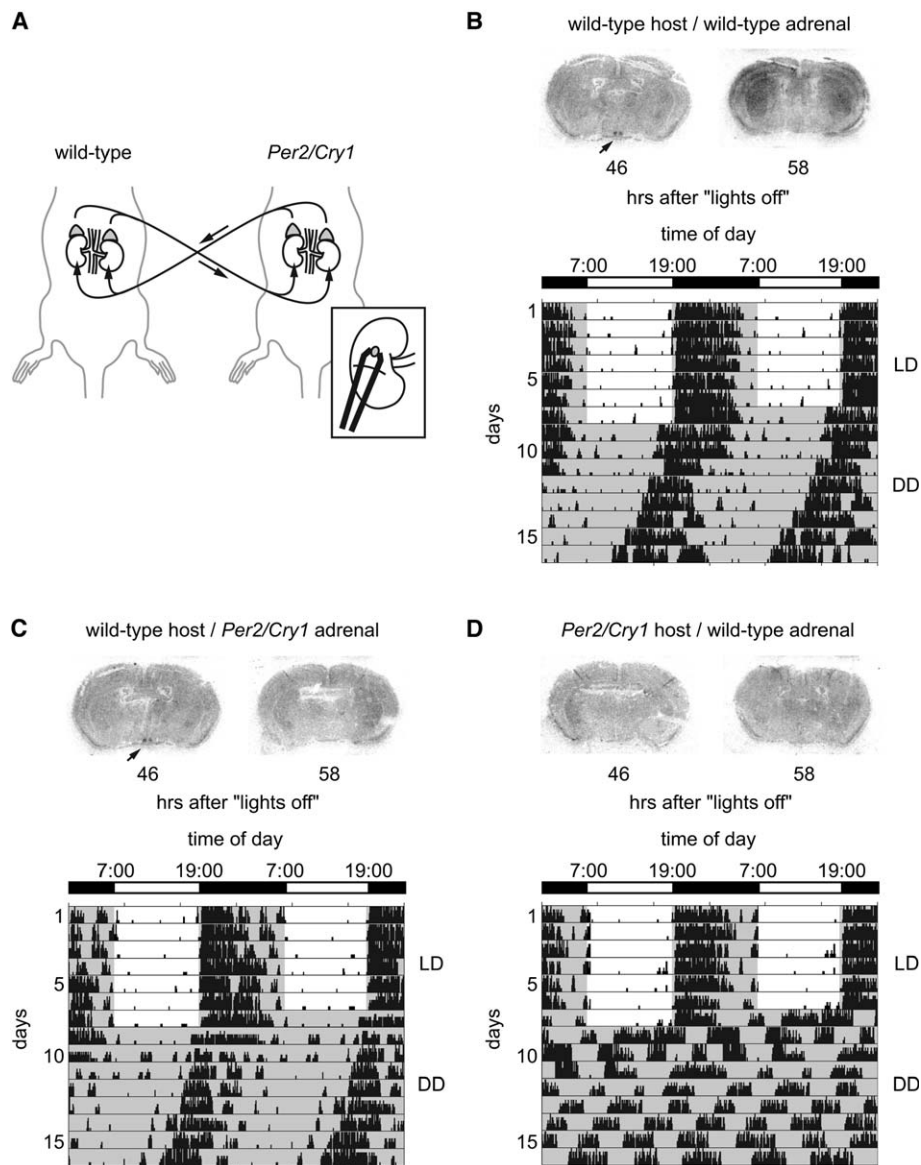
**Figure 5.** Rhythmic expression of genes encoding regulators of steroidogenesis

Microarray (solid lines) and qPCR (dotted lines) time courses for 20 genes identified as rhythmically expressed—by COSOPT—from a wild-type microarray time series. Expression values are normalized to the average mRNA level. All values are mean  $\pm$  SEM ( $n = 3$  for qPCR and 2 for microarrays). Gray and black bars depict subjective day and night.

host or a mutant graft was used, the amplitudes of corticosterone secretion rhythms were dampened by 40%–50% compared to the wild-type host/wild-type graft combination (Figure 7C). These results indicate that the LD cycle can evoke rhythmic corticoid production from a wild-type adrenal in the

absence of a functional SCN clock. In addition, they reinforce our finding that the genotype of the adrenal determines sensitivity of the steroidogenic response to ACTH.

In DD, mutant adrenals transplanted into a wild-type host released corticoids rhythmically, but the amplitude was reduced



**Figure 6.** The functionality of the adrenal clock affects neither *Per1* rhythms in the SCN nor locomotor activity

**A)** Schematic representation of the surgical procedure employed to transplant *Per2/Cry1* double mutant adrenals into wild-type adrenalectomized animals and vice versa. The inset cartoon illustrates how the adrenal grafts are implanted underneath the kidney capsule of the host.

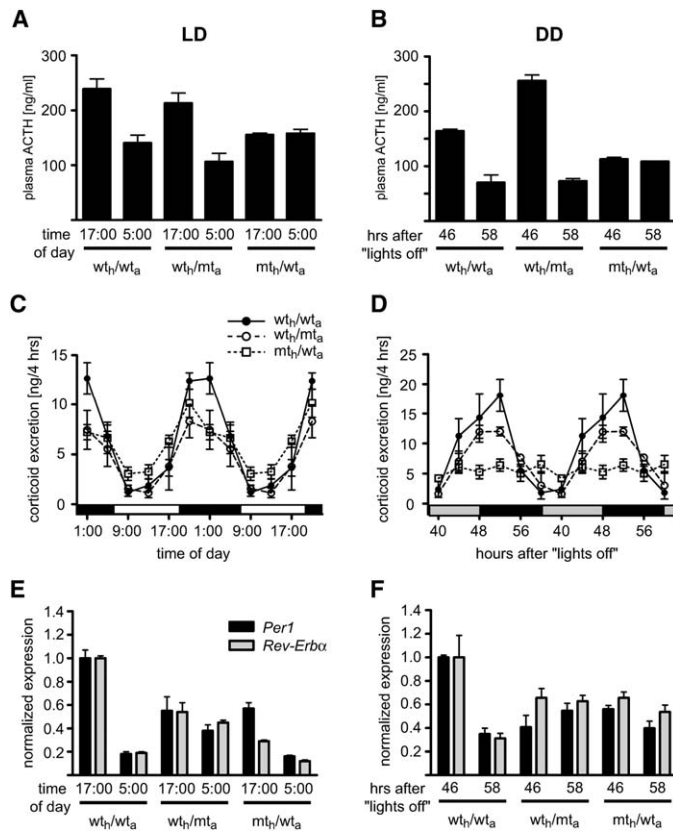
**B–D)** Contact autoradiograph scans of brain sections probed for *Per1* expression 46 and 58 hr after "lights off" (upper panels) and representative double-plotted actograms for the different genotypes in LD and DD conditions (lower panels). **(B)** Auto-grafted wild-type animals, **(C)** wild-type hosts that obtained *Per2/Cry1* mutant adrenal transplants, and **(D)** *Per2/Cry1* double mutants with implanted wild-type adrenals. The arrows in **(B)** and **(C)** point at the SCN.

when compared to that of control operated recipients (Figure 7D). This suggests that the adrenal clockwork influences the amplitude of the corticoid rhythm in response to ACTH, which is rhythmic in the wild-type host (Figures 2D and 7B). Surprisingly, corticoid excretion rhythms were completely abolished in *Per2/Cry1* animals that received wild-type adrenals, indicating that without a properly functioning SCN, a wild-type graft cannot sustain rhythmicity of corticoid production (Figure 7D) in the absence of an entraining light stimulus.

To monitor the molecular status of the clock in the grafts, expression levels of the clock genes *Per1* and *Rev-Erb $\alpha$*  were measured by qPCR. In LD, adrenal clock gene rhythms were paralleling rhythmic hormone secretion (Figure 7E). Of note, this parallelism was also seen with wild-type adrenal grafts transplanted into arrhythmic hosts (Figure 7E, *mt<sub>h</sub>/wt<sub>a</sub>*), which indicates that the LD cycle can entrain a peripheral clock independently of a functional SCN pacemaker. In DD, when combining wild-type hosts and wild-type grafts, both *Per1* and *Rev-Erb $\alpha$*  showed strong differences in expression levels between

the two time points examined (Figure 7F, *wt<sub>h</sub>/wt<sub>a</sub>*). In the case of grafting mutant adrenals into a wild-type host, both clock genes were expressed at similar levels at both times (Figure 7F, *wt<sub>h</sub>/mt<sub>a</sub>*). This suggests that the rhythmic SCN and ACTH signals cannot restore the molecular clock in the grafted mutant adrenal. The observed circadian corticoid rhythm is likely to be driven by the host's SCN pacemaker via rhythmic ACTH release from the pituitary. When a wild-type adrenal was transplanted into a mutant host, rhythmic expression of both clock genes was also largely abolished (Figure 7F, *mt<sub>h</sub>/wt<sub>a</sub>*). Thus, a wild-type adrenal cannot autonomously maintain a circadian rhythm in a clock-deficient host.

It was suggested that adrenal corticoids may serve as a signal that synchronizes peripheral clocks with the SCN pacemaker (Balsalobre et al., 2000). To begin to test this possibility we monitored *Per1* and *Rev-Erb $\alpha$*  expression in kidneys of transplanted animals. In both LD and DD conditions kidney clock gene transcription was not affected by the genotype of the adrenal graft but it fully reflected the genotype of the host. This would suggest



**Figure 7.** HPA axis regulation and adrenal clock gene expression in adrenalectomized mice

**A and B**) Plasma ACTH regulation in grafted animals in LD (**A**) and DD (**B**). Numbers below the x axis depict the time points at which animals were sacrificed ( $n = 3$ ).

**C and D**) Corticoid metabolite excretion rhythms of adrenal-grafted animals in LD (**C**) and DD (**D**). Data are double-plotted for better visualization ( $n = 4$ ).

**E and F**) *Per1* and *Rev-Erb $\alpha$*  expression in adrenal grafts in LD (**E**) and DD (**F**). Relative expression values are normalized to 17:00/46 hr in the control operated animals ( $n = 3$ ). All data are mean  $\pm$  SEM. Abbreviations: w<sub>h</sub>—wild-type host; m<sub>h</sub>—mutant host; w<sub>a</sub>—wild-type adrenal; m<sub>a</sub>—mutant adrenal.

that rhythmic corticosterone per se is not sufficient to re-establish rhythmicity in a mutant kidney clock (Figure S1).

Taken together our transplantation studies show that the adrenal clock can be light-entrained even in the absence of a functional SCN pacemaker. The adrenal clock does not seem to feed back on the SCN; rather it modulates circadian corticosterone rhythms by gating the sensitivity of the adrenal to ACTH.

## Discussion

To thrive in a rhythmically changing environment, organisms have evolved endogenous clocks that synchronize their physiology to the outside world. In mammals, peripheral clocks residing in endocrine glands are prime candidates for transducing rhythmic cues from the SCN to target tissues via a rhythmic release of hormones into the bloodstream. The adrenal, for example, discharges corticoids in a circadian fashion and by this mechanism may control the circadian component of stress response, energy homeostasis, mineral balance, and reproduction (Kawasaki

et al., 1983; Kennaway, 2005; Strubbe and van Dijk, 2002; Zimmermann and Critchlow, 1967). How the peripheral clock of the adrenal carries out this control was the subject of this study.

## The adrenal clockwork

The rhythmic expression of a limited number of clock genes in the adrenal gland was previously reported for rodents (Bittman et al., 2003; Ishida et al., 2005) and monkeys (Lemos et al., 2006), suggesting the existence of an adrenal circadian oscillator. Using qPCR, microarray, and in situ hybridization approaches we have characterized the circadian expression of canonical clock genes in this gland (Figure 1). We show that clock genes are expressed in a rhythmic pattern in the *zona glomerulosa* and *zona fasciculata*, the sites of adrenal corticoid production (Parker and Schimmer, 2001). While some clock genes like *Per1*, *Per3*, and *Bmal1* are also expressed in the adrenal medulla (Bittman et al., 2003; Torres-Farfan et al., 2006), others including *Per2*, the *Cry*s, and *Clock* are—if at all—only weakly expressed in this tissue (Bittman et al., 2003; Torres-Farfan et al., 2006). These and our ISH data thus suggest that a canonical circadian clockwork in the adrenal primarily resides in the outer layers of the cortex, a notion consistent with the rhythmic synthesis of gluco- and mineral corticoids in these layers (Kempainen and Behrend, 1997).

## The role of the peripheral clockwork in regulating ACTH responsiveness of the adrenal

We demonstrate that circadian rhythms of ACTH, corticosterone, and clock gene expression in the adrenal are abolished in *Per2/Cry1* double mutant animals (Figure 2), consistent with the disruption of locomotor activity and clock gene expression rhythms in the SCN and several other tissues in these mice (Oster et al., 2002). Since in the intact *Per2/Cry1*-deficient animal all tissues are mutant, one cannot determine whether the absence of hormonal rhythms is caused by a deficient SCN or by a deficient adrenal clock. We therefore analyzed corticoid production in adrenal slice cultures to characterize the adrenal clock in the absence of an SCN. We found that the adrenal shows a “gated” sensitivity to ACTH that is maintained in the absence of external cues but critically depends on the presence of a functional adrenal clockwork since slices from *Per2/Cry1*-deficient adrenals lack gating (Figure 3).

Using transcriptional profiling we identify circadian expression patterns of multiple genes involved in the regulation of adrenal steroid metabolism (Figures 4 and 5). These genes encompass most facets of the steroidogenic regulatory network including the ACTH signaling cascade. This and the fact that none of the previously identified rate-limiting components of steroid biosynthesis (e.g., steroidogenic acute regulatory protein [*Star*] or cytochrome P450 side-chain cleavage enzyme [*Cyp11a1*]) show circadian transcription rhythms suggest that the adrenal clock exerts its control on corticoid production not through a restricted set of targets but instead uses multiple molecular routes that collectively steer circadian rhythms of adrenal glucocorticoid production. It is presently not clear which adrenal gene products confer gating of the ACTH response. In fact, some of the identified genes (including *Sp1*, *Gna13*, *Mc2r*, and *Scarb2*) show only low-amplitude oscillations. Posttranscriptional regulation and/or the combined effect of synchronized minor transcriptional oscillations, however, may result in robust rhythms at the physiological level. Attractive candidates are



members of the ACTH receptor signaling cascade (like adenylyl cyclase or protein kinase A) that directly transmit the ACTH signal to the steroidogenic machinery. In addition, the supply of the steroid precursor cholesterol from the blood may be controlled via circadian regulation of the corresponding lipoprotein receptor (*Ldlr*) and transporter proteins (*Scarb2*).

### The role of the adrenal clockwork in corticosterone regulation in vivo

We have begun to examine the impact of ACTH gating on the endocrine system in vivo using an adrenal transplantation model. We show that the genotype of the transplanted adrenal affects neither *Per1* expression in the SCN nor locomotor activity rhythms of the animal (Figure 6). A likely explanation of this result is that the SCN does not express glucocorticoid receptors (Rosenfeld et al., 1988).

In arrhythmic *Per2/Cry1* hosts kept in LD, a wild-type adrenal restores corticosterone rhythmicity (Figure 7C). In addition, transplantation of wild-type adrenal grafts into a wild-type animal evokes stronger corticoid rhythms than when mutant grafts are used (Figures 7C and 7D). Both observations illustrate the consequences of a gated adrenal response to ACTH. In the mutant host, a constant ACTH signal is translated into rhythmic corticosterone by the rhythmic wild-type adrenal. In the wild-type host, high-amplitude corticosterone rhythms are achieved because ACTH secretion and adrenal sensitivity rhythms are synchronized, both peaking around the day/night transition (Figures 2 and 3). By contrast, the disrupted clockwork in *Per2/Cry1* adrenal transplants placed into a wild-type mouse hampers synchronization of the two pacemakers. This results in a less efficient reading of the ACTH stimulus and a dampened corticoid rhythm.

The *Per2/Cry1* mouse used in this study is just one animal model with a genetic disruption of the circadian system. Others, like *Per1/2*-, *Cry1/2*-, or *Bmal1*-deficient animals, are likely to give similar results. It is, however, unclear at which level—high, medium, or low—ACTH sensitivity would be arrested in these animals.

A previous report using transplanted *Clock* mutant fibroblasts has shown that genetic disruption of a peripheral clock cannot be rescued by a functional SCN pacemaker (Pando et al., 2002). In accordance with this finding we show that rhythmicity in clock gene expression in mutant adrenal grafts is not restored by the SCN of the wild-type host (Figures 7E and 7F). This result and the dampened amplitude of rhythmic corticoid excretion by these mice indicate that even though the peripheral clock of the adrenal clearly influences corticoid rhythms it is not the rhythm generator.

When wild-type adrenal grafts are placed into a mutant host, corticoid rhythms cannot be rescued under DD (Figure 7B) but can be rescued under LD conditions (Figure 7C). This is also reflected in clock gene expression in the transplants (Figures 7E and 7F). From these data we draw two conclusions: First, the adrenal clock can be made rhythmic by the light/dark cycle in the absence of a functional SCN clock. Second, this rhythm does not persist when mice are transferred to constant darkness. Ishida et al. (2005) have demonstrated that *Per* gene expression in and corticosterone release from the adrenal can be directly activated by a light pulse. This activation requires functional nerve connections between the SCN and the adrenal. It has also been shown that the sensitivity of the adrenal to ACTH

stimulation is regulated via the splanchnic nerve (Jasper and Engelend, 1994, 1997). Together with our finding that the adrenal clock gates the ACTH response, these data suggest that light may directly entrain the adrenal clock via the autonomic nervous system, thereby influencing circadian hormone secretion.

Previous studies using organ cultures have shown that clock gene rhythms can persist for weeks in the absence of an external timing signal (Welsh et al., 2004; Yamazaki et al., 2000; Yoo et al., 2004), a finding that is also reflected in the persistence of rhythmic gating of the ACTH response observed in wild-type adrenal slice cultures (Figure 3). In marked contrast, our transplantation data indicate that in vivo adrenal clock gene expression and corticosterone production rhythms critically depend on the input from the SCN. Circadian clock gene and corticoid excretion rhythms are lost in mutant hosts carrying wild-type adrenals within 48 hr after release into DD (Figure 7). Somewhat contrary to the prevailing view our data suggest that peripheral clocks are not primarily self-sustained generators of rhythms but more likely modulate and stabilize circadian rhythms of physiology. Hence, the principal rhythm generator is the SCN (Davidson et al., 2003; Schibler and Sassone-Corsi, 2002).

The SCN/adrenal/corticosterone regulatory network examined in the present work may serve as a paradigm for the organization of other physiological rhythms, but at the same time our transplantation studies highlight the specificity of some components of this pathway. This may explain why the quest for a singular signaling molecule transmitting time information from the SCN to the body has so far not been successful.

## Experimental procedures

### Animals

All animal experiments were conducted in compliance with the German Law on Animal Welfare (TierSchG §1-11). To identify rhythmically expressed genes by microarray analysis, 4-month-old male C57BL/6J animals were used. Wild-type and *Per2<sup>Brdm1</sup>/Cry1<sup>-/-</sup>* animals used for expression analysis, slice cultures, and transplantation experiments were derived from double heterozygous breeding pairs on a mixed 129Sv/C57BL/6J background (Oster et al., 2002). General mouse handling and behavioral monitoring were performed as described (Jud et al., 2005).

### Microarray hybridization and data analysis

Animals were entrained to a 12 hr light:12 hr dark cycle (LD) for two weeks, released into constant darkness (DD), and sacrificed by cervical dislocation under a 15W safety red light at the indicated time points (see Figure 1A). Eyes were removed prior to tissue dissection and tissue was stored in RNA<sup>later</sup> (Ambion, Huntingdon, United Kingdom) at  $-20^{\circ}\text{C}$  until use. Total RNA samples from whole adrenal preparations were prepared using RNeasy Micro Kit (QIAGEN, Hilden, Germany) and integrity was assessed by analyzing aliquots on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, California). All subsequent experimental procedures, array hybridization (MG430v2.0 chips, Affymetrix, Santa Clara, California), and analytical steps were performed by the RZPD (German Resource Center for Genome Research, Berlin, Germany, <http://www.rzpd.de/protocols>). One adrenal was used per chip. Chips were hybridized in duplicates. Primary data processing and sample comparison were performed using dChip 1.3 software (Li and Wong, 2001). Fluorescence values were normalized according to a MM/PM difference model for each experiment. Only those probe sets qualified as “expressed” in all replicates for at least one time point (including both 24 hr cycles in the wild-type experiment) were used for further analysis. Rhythmic genes were identified using the COSOPT algorithm (Straume, 2004) with a pMMC- $\beta$  cut-off of 0.1 and a period length restriction of 22 to 26 hr.

All microarray data have been submitted to the NCBI gene expression omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/projects/geo/>; series accession number GSE4253).

### Quantitative real-time PCR

Total RNA from whole adrenals was prepared as described for microarrays. cDNA was synthesized using the ThermoScript RT Kit (Invitrogen, Paisley, United Kingdom). Quantitative real-time PCR (qPCR) was performed on an iCycler (Bio-Rad, Hercules, California) with iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's protocol. Primer sequences are found in Table S2. Single well amplification efficiency estimation and relative quantification of expression levels were performed as described (Ramakers et al., 2003).

### In situ hybridization and immunohistochemistry

In situ hybridization using digoxigenin or <sup>35</sup>S-labeled probes on frozen sections was performed as described (Oster et al., 2003; Yaylaoglu et al., 2005). All probe templates were generated by PCR from embryonic day (E) 14.5 mouse embryo and adult brain cDNA. Primer sequences are found in Table S2. Immunohistochemistry on paraffin-embedded sections was performed as described (Oster et al., 2003). The anti-PER2 antibody (Alpha Diagnostic, San Antonio, Texas) was 1000-fold diluted. For signal amplification and visualization Ni-amplified DAB staining with the Vectastain Elite Kit (Vector, Burlingame, California) was used according to the manufacturer's protocol.

### Hormone measurements

Animals were sacrificed by cervical dislocation and decapitated, and blood was collected from the jugular vein. Plasma extracts were prepared using Microvette 300 coated tubes (Sarstedt, Nümbrecht, Germany) and stored at -80°C. To rule out stress-induced effects on hormone levels, animals were put into wheel-running cages equipped with a metal grid floor 2 days prior to sampling. On the following 2 days feces were collected at 4 hr intervals and stored at -80°C until processing. Corticoid extraction was performed as described (Cavigelli et al., 2005). ACTH and corticosterone/corticoid sample concentrations were determined by radioimmunoassay (RIA) using commercial available kits from DiaSorin (Cat.# 27130) and MP Biomedicals (Cat.# 07-120103).

### Adrenal slice cultures

After entrainment to LD mice were released into DD and sacrificed by cervical dislocation. Adrenals were dissected and embedded in 4% low melting agarose on ice. After solidification agarose blocks were trimmed and adrenals sectioned at 200 μm thickness with a vibratome (Model 765, Campden, Lafayette, Indiana). Adrenal slices were placed on top of Millicell-CM culture plate inserts (Millipore, Bedford, Massachusetts) and cultivated in FDMEM/0.1% DMSO/50 μM MeSH/2% FCS/0.1% Gentamycin/0.12 mg/ml Penicillin/0.12 mg/ml Streptomycin at 37°C/5% CO<sub>2</sub>. ACTH stimulation (20 nM) was performed by adding ACTH to the medium. Medium was aspirated and corticosterone levels were evaluated by RIA. For each time point a separate slice was used to circumvent corticoid feedback after medium withdrawal.

The formula used for calculation of the initial release rate ( $v_0$ ) was  $v_0 = -3 \ln(1 - (\text{conc} - \text{conc}_0) / (\text{conc}_{\text{max}} - \text{conc}_0)) \cdot \text{time}^{-1}$  with  $\text{conc}$  = corticosterone concentration,  $\text{conc}_0$  =  $\text{conc}$  at time = 0, and  $\text{conc}_{\text{max}}$  =  $\text{conc}$  at time = ∞. All calculations were done with GraphPad Prism 4 software (GraphPad Software, San Diego, California).

### Adrenal gland transplantations

Transplantations of adrenal gland fragments were performed as described (Musholt et al., 2002). Animals were anaesthetized using Ketamin/Rompun (100/10 mg/kg i.p.), and adrenals were dissected following median laparotomy. After removal of fat, each gland was divided into four pieces. Fragments from wild-type mice or *Per2/Cry1* mutants were transplanted underneath the kidney capsule of adrenalectomized animals of the opposite genotype. Both adrenals from one donor were always transplanted into a single host animal. The capsule of the host kidney was lifted with forceps and incised and the graft was pushed underneath the capsule up to the dorsal pole of the kidney (Figure 6A). The incision was closed by bipolar electro-coagulation. Animals recovered for two weeks prior to transfer to wheel-running cages. Hormone measurements and expression analyses were performed after 2 additional weeks of entrainment.

### Supplemental data

Supplemental data include one figure and two tables and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/4/2/163/DC1/>.

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#### Accession numbers

The microarray data presented in this paper were deposited in the NCBI gene expression omnibus (GEO) database with accession number GSE4253.