REVIEW

Fixing the broken clock in adrenal disorders: focus on glucocorticoids and chronotherapy

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Abstract

The circadian rhythm derives from the integration of many signals that shape the expression of clock-related genes in a 24-h cycle. Biological tasks, including cell proliferation, differentiation, energy storage, and immune regulation, are preferentially confined to specific periods. A gating system, supervised by the central and peripheral clocks, coordinates the endogenous and exogenous signals and prepares for transition to activities confined to periods of light or darkness. The fluctuations of cortisol and its receptor are crucial in modulating these signals. Glucocorticoids and the autonomous nervous system act as a bridge between the suprachiasmatic master clock and almost all peripheral clocks. Additional peripheral synchronizing mechanisms including metabolic fluxes and cytokines stabilize the network. The pacemaker is amplified by peaks and troughs in cortisol and their response to food, activity, and inflammation. However, when the glucocorticoid exposure pattern becomes chronically flattened at high- (as in Cushing's syndrome) or low (as in adrenal insufficiency) levels, the system fails. While endocrinologists are well aware of cortisol rhythm, too little attention has been given to interventions aimed at restoring physiological cortisol fluctuations in adrenal disorders. However, acting on glucocorticoid levels may not be the only way to restore clock-related activities. First, a counterregulatory mechanism on the glucocorticoid receptor itself controls signal transduction, and second, melatonin and/or metabolically active drugs and nutrients could also be used to modulate the clock. All these aspects are described herein, providing some insights into the emerging role of chronopharmacology, focusing on glucocorticoid excess and deficiency disorders.

Key Words

- adrenal hormomes
- corticosteroids
- circadian rhythms
- glucose metabolism
- HPA axis (hypothalamus-pituitary-adrenal)

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Introduction

Human physiology and behavior are adapted to daily environmental cycles by means of endogenous circadian clocks. In mammals, the molecular mechanism of these clocks is generated by a transcriptional autoregulatory feedback loop. The 'core' clock genes include the master genes *CLOCK* and *BMAL1* (also named *ARNTL*). Their expression, however, also activates some other proteins that serve as counterbalances and gradually build up in cells over a 12-h period, progressively inhibiting the activity of the master genes. This itself progressively

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reduces the activation of the counterbalances, which then slowly degrade over the next 12 h, causing *CLOCK* and *BMAL1* to bounce back.

However, the mechanism is much more complicated. Clock genes interact with many different signals to produce an integrated output over the 24-h cycle, entraining other cycling activities such as cell division and metabolism, in preparation for the different tasks confined to periods of light or darkness. During the transition hours between activity and rest periods, gene expression increases in a non-linear manner, with a gating system enhancing or softening signal transduction to avoid interferences of misaligned cycles.

Peaks and troughs in adrenal hormones play a pivotal role in mitigating or enhancing the effects of clock genes on their own targets. The exact role of glucocorticoids in this context has yet to be fully elucidated. However, it is generally accepted that their circadian rhythm takes part in the entrainment of peripheral clocks by the master genes and, hence, with the light:darkness cycle. When the endogenous rhythm is disrupted by disease, such as in adrenal insufficiency, a non-physiologic timing of glucocorticoid administration may dysregulate circadian gene expression, as recently described (Venneri *et al*. 2018).

The use of pharmacological intervention on circadian genes has recently gained momentum, as a number of different studies have shown that synchronization of peripheral clocks is achieved not only through classic hierarchical vertical control from the hypothalamic master clock through the peripheral nervous system, which is already relatively independent of glucocorticoids, but also horizontally, through fluxes of nutrients absorbed after food consumption, metabolites produced by the liver and redirected to the peripheral tissues, gastrointestinal peptides, and cytokines derived from the immune system, bone, muscle, and adipose tissue (Fig. 1).

All these different inter-organ signals need to be integrated with the oscillation of the master clock to create a coordinated response. Glucocorticoids act mainly through this horizontal process, influencing the expression of the clock genes directly or the metabolic fluxes indirectly. However, the clock has developed a system of resistance to rapid desynchronization induced by glucocorticoid changes. This counterregulatory mechanism works to avoid rhythm disruption in the event that unexpected acute stress produces sudden changes in glucocorticoid levels. Recent data have also clearly demonstrated the circadian expression of clock

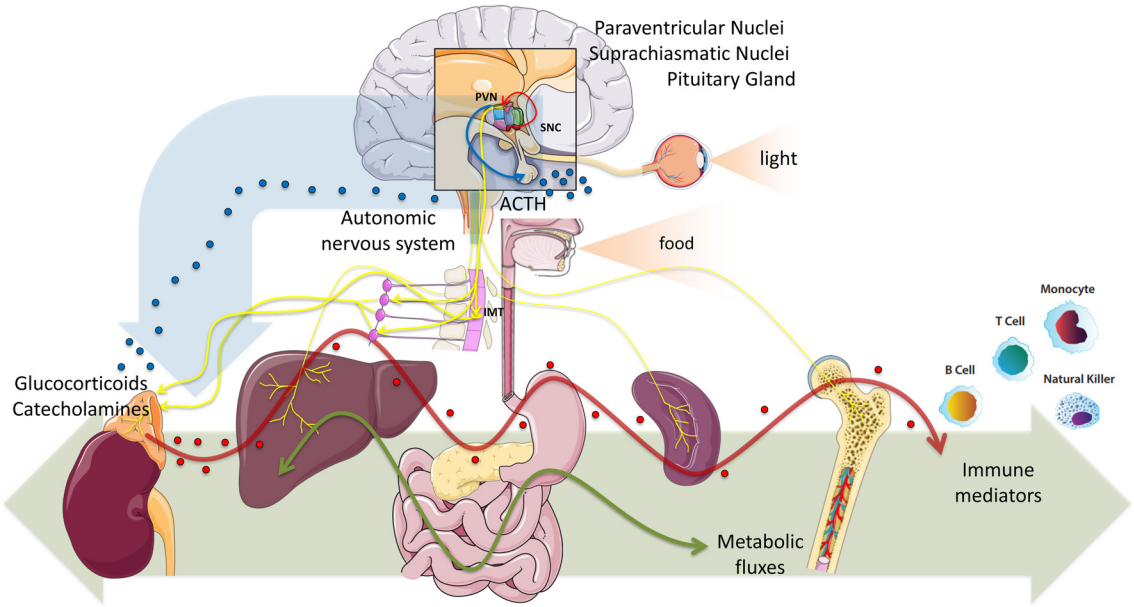

Figure 1
Schematic representation of (1) the traditional vertical-hierarchical control system of the circadian rhythm (thick blue arrow), with control of the suprachiasmatic master clock nuclei (SCN) projecting to the paraventricular nuclei (PVN) and from there to the autonomic nervous system (ANS, yellow lines) and hypothalamic pituitary axis (HPA), blue lines and circles), the ANS provides a direct independent innervation of the adrenal gland by preganglionic fibers from the intermediolateral (IML) column of the spinal cord and postganglionic fibers from the ganglia of the sympathetic chain and (2) the bidirectional horizontal synchronization system (thick green arrow) comprising multiple signals from metabolic fluxes (from food intake and liver processing), cytokines (from immune cells), and peripheral modulation of glucocorticoid modulation (yellow line: ACTH; red line and circles: cortisol and catecholamines).

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genes in the adrenal gland, not only in cortisol-secreting adenomas, but also in aldosterone-producing adenomas and adrenocortical carcinomas (Angelousi *et al.* 2020).

All these aspects are described subsequently, providing some insights into the emerging role of chronopharmacology in hypothalamic–pituitary–adrenal (HPA) axis disorders. The concept of using glucocorticoids, glucocorticoid antagonists, or adrenal steroidogenesis inhibitors to reset the endogenous rhythm in disorders of the HPA axis is briefly presented. Finally, the use of drugs that target metabolism is also reviewed in this context, taking into account the growing awareness of metabolism as a further level of control of the endogenous clock.

The vertical paradigm: hierarchical control of the HPA circadian rhythm

The suprachiasmatic nucleus (SCN) of the hypothalamus receives information about light and darkness and is traditionally considered the 'master clock', coordinating the activities of the 'peripheral' clocks functioning in virtually all other organs (Fig. 1) (Dickmeis *et al.* 2013). The circadian clock is sustained by interlocked transcriptional–translational feedback loops comprised of the master genes *CLOCK* and *BMAL1* (Nader *et al.* 2010, Partch *et al.* 2014, Moreira *et al.* 2018). They heterodimerize in the cytoplasm to form a complex (CLOCK-BMAL1) that binds to E-box elements in the nuclei, thereby enhancing the target genes, including two cryptochrome genes (*CRY1* and *CRY2*) and the core-clock 'period' genes (*PER1*, *PER2*, and *PER3*). Over its 12 h of activity, the CLOCK-BMAL1 complex induces the accumulation of CRY and PER proteins, which peak at the end of daylight. Since these inhibit *CLOCK* and *BMAL1* transcription and hence suppress their own transcription, over the following 12 h of rest, PER is slowly degraded and CLOCK and BMAL1 surge back, resulting in a cycle of about 24 h (Brown *et al.* 2012).

In humans, glucocorticoids peak shortly before activity begins and decline during the remaining 24 h, following a periodic non-linear oscillation (the oscillations do not have a sinusoidal shape). The circadian rhythmicity of cortisol is assumed to play a role in synchronizing the pace of the peripheral clocks with the central master clock (Nader *et al.* 2010, Dickmeis *et al.* 2013). This hypothesis was supported by evidence that dexamethasone injections transiently changed the phase of circadian gene expression in mouse liver, kidney, and heart, but not in the neurons of the SCN (Balsalobre *et al.* 2000). Such behavior was considered to support the presence of 'slave oscillators' that are synchronized by the 'master pacemaker' (via glucocorticoids), as they remain responsive to phase-resetting signals from the SCN, which in turn retains hierarchal independence.

Glucocorticoid fluctuations depend on the intrinsic expression of clock genes at each anatomical site of the HPA axis, classically organized in a hierarchical manner (Moreira *et al.* 2018). The SCN, through the activation of corticotropin-releasing hormone secretion from the paraventricular nuclei of the hypothalamus (PVN), coordinates and controls the rhythmic release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH, in turn, stimulates glucocorticoid production in the adrenal cortex (Dickmeis *et al.* 2013, Leliavski *et al.* 2015) (Fig. 1).

Obviously, the system is much more complicated than this. First, sympathetic innervation is required to maintain the cyclical activity of adrenal steroidogenesis (Ottenweller & Meier 1982). Using viral retrograde trans-synaptic tracer experiments, Buijs demonstrated a neural SCN-adrenal gland network that works in parallel with the HPA axis (Buijs *et al.* 2003). Sympathetic neurons of the SCN project to pre-autonomic neurons of the PVN, which in turn project to the preganglionic sympathetic neurons in the intermediolateral (IML) column of the spinal cord (Buijs *et al.* 2003). The adrenal gland receives both preganglionic and postganglionic sympathetic and parasympathetic innervation (Kesse *et al.* 1988). Most are fibers from the preganglionic sympathetic neurons in the IML, projecting to the medulla and, from there, as postganglionic fibers, to the cortex (Hinson 1990). However, a smaller number of projections, through the splanchnic nerves, are postganglionic sympathetic fibers from the ganglia of the sympathetic chain. Interestingly, vesicle-containing nerve endings have been observed in direct contact with cortical cells in the zona fasciculata of the human adrenal cortex (Dorovini-Zis & Zis 1991). This network controls the circadian output of the adrenal gland in response to classical autonomic activation (e.g. light), but can also regulate sensitivity to ACTH and thus steroidogenesis (Ishida *et al.* 2005, Ulrich-Lai *et al.* 2006).

Second, glucocorticoids can modulate the expression and activity of clock-related genes (see subsequently) (Dickmeis *et al.* 2007). As a consequence, the self-sustained 12-h time-delayed transcriptional/posttranslational negative feedback loop, frequently analyzed using the single-component cosinor linear method, appears over-simplistic (Fig. 2, see Box 1 for cosinor-based-rhythmometry).

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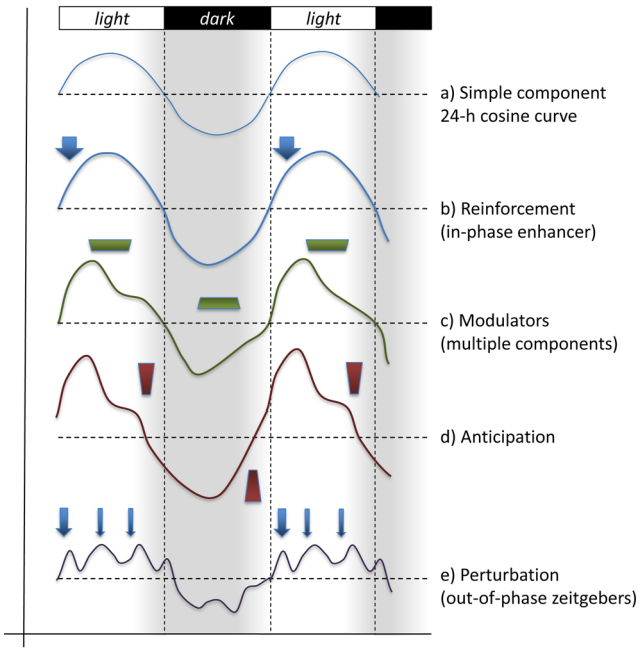

Figure 2
Modelling of the single component 24-h cosinor (A) into a more complex model that better describes human physiology. Glucocorticoid fluctuations act as rhythm enhancers (B), while peripheral modulators (such as metabolic fluxes and cytokines) are responsible for the multiple components (C) shaping the wave of the cosinor. An additional feature of human rhythmometry is the anticipation phenomenon, with a number of genes rushing the transition from active to resting phases (D). When all these components are disrupted by multiple abnormal out-of-phase signals, the overall rhythm is disrupted (E).

Third, pathological or physiological processes such as aging can interfere with this mechanism at either level, leading to circadian disruption. Aging dampens the photoperiodic adaptation, possibly by reducing the amplitude of signal transmission from SCN (Buijink *et al.* 2020), and alters the circadian rhythm of glucocorticoids and melatonin, contributing to the observed circadian derangement (Hood & Amir 2017).

Sex can also influence circadian rhythms. Sex differences have been described at many levels of the circadian system, including in relation to SCN size and HPA axis vulnerability (Bailey & Silver 2014). This leads to a different response and resilience to circadian 'challenges' such as stress: in fact, the adrenal clock seems to have a gating effect on stress response in a sexually dimorphic manner, with ACTH exerting a larger response from female adrenal glands, possibly due to the lower amplitude of the adrenal clock oscillation (Stagl *et al.* 2018). In summary, glucocorticoids and glucocorticoid receptor (GR) are important in re-shaping the cosinor-based rhytmometry, but they are not the only agents involved (Fig. 2B and C). In the next sections, the main endocrine-metabolic contributors to circadian rhythm are briefly reviewed, to provide the background for potential interventions in adrenal disorders.

The horizontal perspective: the importance of peripheral mediators

The classic vertical hierarchic control mechanism is not sufficient to explain how the endogenous self-sustained clocks can remain in alignment despite the various perturbations introduced by cycling human activity or by disease. At least three additional pathways have recently emerged as independent players.

Food and metabolism

It is traditionally believed that eating late at night is related to weight gain and metabolic abnormalities. Furthermore, night and shift work have been considered as risk factors for metabolic and cardiovascular disorders (Biggi *et al.* 2008) and glucose metabolism abnormalities are described in circadian-related metabolic diseases (Stenvers *et al.* 2019). Late sleeping, short sleep duration (<5 h), and late dinnertime or consumption of additional calories later in the evening have been associated with obesity and diabetes (Colles *et al.* 2007, Baron *et al.* 2011, Hsieh *et al.* 2011). Glucocorticoid excess is associated with weight gain, altered food intake, and disrupted metabolism (Pivonello *et al.* 2016).

Eating is included in the non-photic time stimuli synchronizing the endogenous clock (Asher & Sassone-Corsi 2015) (Figs 1 and 2C). It interacts with brain structures projecting to the SCN through mediators such as orexin neurons and ghrelin (Mieda *et al.* 2004, LeSauter *et al.* 2009, Acosta-Galvan *et al.* 2011, Adamovich *et al.* 2017). In turn, leptin receptors in the SCN are time/phase sensitive to leptin modulation (Guan *et al.* 1997). In mice, changing standard alternating light:darkness cycles to a light cycle where dim light replaced the darkness cycle resulted in increased food intake and, even when caloric intake and total motor activity were kept similar, led to excess weight gain (Fonken *et al.* 2010). Consistent with this, *CLOCK*-mutant mice exhibit an attenuated diurnal feeding rhythm and develop obesity and metabolic syndrome (Turek *et al.* 2005). Interestingly, the central SCN oscillator was apparently resistant to transient shifts in feeding time, while peripheral clocks, especially in the liver, were significantly affected (Damiola *et al.* 2000), uncoupling the peripheral oscillators from

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Box 1. Cosinor rhythmometry.

In 1822 Joseph Fourier developed the revolutionary intuition that each time series, regardless of its shape or regularity, can be described by a series of sine waves and cosines of various frequencies, also known as 'spectral analysis' (Fourier & Freeman 1878). If the spectral analysis of a time series identifies a major spectral component of approximately a 24-h period, the investigator can deduce that the study has a circadian rhythmicity. However, most biological studies, assuming that the period is known and of 24 h, use the cosinor method. This method can be used for both non-equidistant data series (which cannot be easily done by the Fourier analysis) and serially independent data. A cosine curve with a given period is fitted to the data by least squares (single-component, Fig. 2A and B). This approach consists of minimizing the sum of squared deviations between the data and the fitted cosine curve. For complex biological rhythms, a multiple-component model helps obtain a better approximation of the signal's waveform when it deviates from sinusoidality. For instance, for blood pressure data, with its typical night drop, a 2-component model consisting of cosine curves with periods of 12 and 24 h is better than a single-component model, as can be easily assessed by the CIs for parameters of rhythmic components derived therefrom. In general, the advantage of cosinor methods is that biological rhythms can be considered as smooth rhythms with some added noise (Fig. 2C, D and E): a model consisting of superimposed cosine curves with known periods adapted from least squares to data (Refinetti *et al.* 2007). The single cosinor approach can be applied clinically by the population mean cosinor method, which provides an estimate of the population from the results of three or more individuals, assuming they were randomly sampled from the same population (Bingham *et al.* 1982). The procedure verifies the similarity of the individual results. Finally, linear regression of cosinor analysis works well if the period is known; when it is not, a non-linear approach can be used. Starting from an initial estimate for the period and applying subsequent iteration sequences aimed at minimizing the residual sum of squares, all parameters can be estimated (Cornelissen 2014).

the central pacemaker. Changes in feeding time reset the phase of rhythmic gene expression in the liver first and only subsequently in other tissues, achieving full synchronization within a week. Studies on time-restricted feeding revealed that insulin-sensitive tissues such as the liver, adipose tissue and pancreas are pivotal in food anticipatory activity, with the liver playing the main role as the communication center (Fig. 2D) (Xie *et al.* 2019). Changes in feeding time are considered to affect body weight, particularly early vs later meals. An extensive study on more than 50,000 adults showed that those eating their largest meal at breakfast experienced a relatively large decrease in BMI compared with those eating their largest meal at lunch or dinner (Kahleova *et al.* 2017). In contrast, eating meals in the evening generally has the opposite effect and patients with night eating syndrome have a higher risk of developing obesity (Colles *et al.* 2007).

However, it is not only the timing but also the quality and quantity of ingredients that control the peripheral oscillators (Vollmers *et al.* 2009, Laermans *et al.* 2014, Pavlovski *et al.* 2018). While the central clock regulates food intake, energy expenditure, and insulin sensitivity, the peripheral tissue clocks are also believed to exert additional control over glucose uptake and secretion, insulin sensitivity, lipid biosynthesis, and catabolism. Eating increases blood glucose levels, which in turn can downregulate the expression of *Per1* and *Per2* in mice fibroblasts (Hirota *et al.* 2002) and indirectly regulate 5' AMP-activated protein kinase (AMPK), which controls the stability of clock component cryptochromes (Lamia *et al.* 2009). The increase in blood sugar and, consequently, insulin seems to lead to an increase in the endogenous synthesis of cholesterol (Jones *et al.* 1993).

Chrono-disruption with a high-fat diet alters the rhythm of both the central and peripheral clocks, attenuating feeding-fasting cycles and acting as a potent zeitgeber for peripheral clocks (Asher & Sassone-Corsi 2015), for example, through the release of additional gastrointestinal peptides or bile (Turek *et al.* 2005, Fonken *et al.* 2010).

Conversely, clock gene expression can be induced by food ingestion in peripheral insulin-sensitive tissues (Oike *et al.* 2014), generating a horizontal feedback loop that is partially independent of the central clock. In obese mice, time-restricted feeding enhanced cAMP response element-binding protein (CREB), mechanistic target of rapamycin (mTOR) and AMPK pathway signaling and increased the oscillations of core and clock-controlled genes, and prevents hyperinsulinemia, hepatic steatosis, and inflammation. These results could be explained through the considerable crosstalk between the cell clock and the triggers induced by feeding (Fuse *et al.* 2012, Hatori *et al.* 2012, Sherman *et al.* 2012, Asher & Sassone-Corsi 2015). Fasting, like the ketogenic diet, induces the phosphorylation of AMPK, which is involved in mitochondrial biogenesis and function. In contrast, a state of satiety stimulates mTOR, which promotes anabolism in response to energy availability in a complex crosstalk with the AMPK pathway (Fuse *et al.* 2012,

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Hatori *et al.* 2012, Sherman *et al.* 2012, Asher & Sassone-Corsi 2015). Finally, mRNA transcript levels of melatonin receptors (MT1, MT2) were significantly higher in type 2 diabetic (T2D) patients than in a normal control group (Peschke *et al.* 2007). Thus, in principle, improving eating habits or using metabolically active drugs could be used to reprogram the clock (Table 1).

The autonomous nervous system

Peripheral clocks are directly controlled by autonomic nervous system (ANS) innervation, as shown in Fig. 1. The ANS can also transmit the SCN's pace to the peripheral clocks, influencing the metabolic processes described previously. Experimental models of sympathetic and parasympathetic denervation of the liver documented a circadian regulation of glucose production by ANS hypothalamic neurons, contributing to lipid metabolism and insulin sensitivity (Kalsbeek *et al.* 2010). Proof of this mechanism is offered by the preservation of the glucometabolic profile (along with normal clock gene oscillation in the liver) under an anti-circadian meal regimen (six meals a day), as opposed to its disruption after sympathetic denervation (Cailotto *et al.* 2005). Interestingly, the persistence of circadian expression of clock genes in hepatic sympathectomy suggests that redundant hormonal feedbacks are involved, including that of glucocorticoids.

The adrenergic system's ability to superimpose a circadian clock gene expression pattern in arrhythmic conditions has been demonstrated in SCN-lesioned mice, in which daily adrenalin injections or sympathetic stimulation produced a robust circadian rhythm of *Per1* gene expression in the liver (Terazono *et al.* 2003). In restricted feeding, both adrenergic stimulation and twice-daily meals exerted a similar entraining effect, suggesting a common pathway. However, feeding every 6 h failed to entrain, suggesting that the adrenergic response to food restriction is hierarchically dominant (Hara *et al.* 2001, Stokkan *et al.* 2001).

In diabetes mellitus, the effects of the ANS on hepatic glucose production are impaired. This has been attributed to a possible lower production of orexin in SCN-controlled hypothalamic neurons, which usually regulate daily variations in sympathetic and parasympathetic tone. In orexin deficiency, such as seen in narcolepsy with cataplexy (Poli *et al.* 2009), the metabolic alterations appear to be independent of body mass, and orexin knockout mice show a significantly altered circadian rhythm of insulin sensitivity and glucose production in the liver (Tsuneki *et al.* 2015).

The dysfunctional ANS circadian rhythm and misalignment of the endogenous cardiac clocks seem to play a role in the development of cardiovascular disease (CVD) (Takeda & Maemura 2015). The loss of the nighttime fall in blood pressure (non-dipper pattern) is the first sign of CVD in Cushing's syndrome (Isidori *et al.* 2015*a*), and patients with mild autonomous cortisol secretion exhibit increased arterial stiffness and cardiac remodeling (Sbardella *et al.* 2018).

Even though an exhaustive discussion of ANS involvement in heart rate variation is beyond the scope of this review, its main influence on circadian heart rate regulation seems to be exerted by ion channel transcription modulation (Tong *et al.* 2013), given the persistence of a robust circadian rhythm in adrenergic blockade models and autonomic denervated heart transplant recipients (Black *et al.* 2019). Interestingly, beta-adrenergic knockout mice models show persistent oscillation of clock-related genes, but with alterations of period and amplitude (Barbagallo F & Isidori AM, unpublished observations). This could possibly lead to inappropriate coupling of oxygen supply

Table 1 Commonly prescribed drugs for diabetes, dyslipidemia, hyperuricemia that target circadian genes.

Drugs	Indication	Circadian gene target
Dipeptidyl peptidase-4 inhibitors	Diabetes mellitus	*DPP4*
Gliclazide	Diabetes mellitus	*ABCC8* and *VEGFA*
Glimepiride	Diabetes mellitus	*KCNJ1* and *ABCC8*
Insulin	Diabetes mellitus	*IGF1R* and *INSR*
Metformin	Diabetes mellitus	*PRKAB1*, *ETFDH* and *GPD1*
Pioglitazone	Diabetes mellitus	*PPARG* and *MAOB*
Alirocumab	Dyslipidemia	*PCSK9*
Atorvastatin	Dyslipidemia	*HMGCR*, *DPP4*, *AHR* and *NR1I3*
Ezetimibe	Dyslipidemia	*SOAT1*
Lovastatin, fluvastatin, pravastatin, rosuvastatin, and simvastatin	Dyslipidemia	*HMGCR*
Fenofibrate	Hypertriglyceridemia	*PPARA*, *NR1I2* and *MMP25*
Allopurinol and febuxostat	Hyperuricemia	*XDH*

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and cardiac requirements. Finally, the renin-angiotensin-aldosterone pathway is also deeply interconnected with the circadian rhythmicity, intrinsic renal and adrenal clock genes, and the HPA axis (see Box 2).

The immune system and inflammation

One of the most important functions of the circadian mechanism is to prevent disease caused by exogenous pathogens. It does this by priming immune function during the active phase while promoting tissue repair during resting hours. Infectious challenges produce a different host response depending on the time of exposure. Circadian peaks and troughs in bone marrow release, peripheral migration, and tissue homing have been demonstrated in almost all immune cell populations (Boivin *et al.* 2003, Silver *et al.* 2012, Adrover *et al.* 2019), resulting in oscillating bloodstream concentrations of inflammatory mediators (Liu *et al.* 2006, Rahman *et al.* 2015).

Many studies have revealed direct interactions between circadian genes and inflammation (Gibbs *et al.* 2012), mostly targeting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Spengler *et al.* 2012, Curtis *et al.* 2015). NF-kB is also one of the key targets mediating the effect of glucocorticoids on inflammation, and glucocorticoids are often given to treat inflammation, without regard to any consequent circadian misalignment (Isidori *et al.* 2020). In fact, circadian rhythm alterations enhance inflammatory response to exogenous pathogens (Castanon-Cervantes *et al.* 2010), but even in the absence of pathogenic challenge, they seem to promote a shift toward a constitutively pro-inflammatory state (Polidarova *et al.* 2017). To add more complexity to this scenario, inflammatory cytokines also affect circadian rhythm: for example, interleukin 1β and tumor necrosis factor alpha can repress the activity of CLOCK/BMAL (Cavadini *et al.* 2007). The typical feature of dysregulated and uncontrolled inflammatory response is sepsis, which is also characterized by altered circadian rhythm. While these two aspects seem tightly intertwined, it is not yet clear whether inflammation disrupts circadian rhythms or pre-existing circadian rhythm disruption enhances inflammatory response in critically ill patients. In trauma patients, alterations in cortisol acrophase and circadian rhythm have been associated with the development of sepsis (Coiffard *et al.* 2019). These findings suggest that the disruption of circadian gene rhythmicity by an external challenge (such as trauma or bacterial infection) increases the susceptibility to sepsis (Coiffard *et al.* 2019). Interestingly, patients with Cushing's are at an increased risk of sepsis (Hasenmajer *et al.* 2020). Glucocorticoids also influence all the components of the immune system, including macrophages, neutrophils, eosinophils, NK cells, T lymphocytes, and B lymphocytes (Kovacs 2014). In critically ill patients, glucocorticoids are often administered continuously or with multiple daily boluses and at supra-physiological dosages: this could be one of the reasons why data on glucocorticoid in sepsis are still controversial. Given its effects on restoration of the circadian rhythm, melatonin has been proposed as an adjunct therapy in sepsis, but data on its effectiveness are not yet available (Colunga Biancatelli *et al.* 2020).

Box 2. Hyperaldosteronism and clock genes.

Circadian rhythm is known to influence blood pressure. The role of clock genes in aldosterone secretion has been recently investigated. First, *Cry*-null mice, lacking the core-clock components *Cry1* and *Cry2*, were shown to exhibit salt-sensitive hypertension due to autonomous aldosterone production, as a consequence of the massive upregulation in the mice counterpart of 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD) (Doi *et al.* 2010). This enzyme is necessary for aldosterone synthesis and also plays a key role in the development of primary aldosteronism (PA) (Konosu-Fukaya *et al.* 2015). PA has two major subtypes, aldosterone-producing adenoma (APA), in which excess aldosterone is secreted by a unilateral adrenal adenoma, and idiopathic hyperaldosteronism (IHA), in which it is secreted due to bilateral adrenal hyperplasia. Doi *et al*. reported that adrenal hyperplasia observed in IHA patients was immunoreactive for type I 3β-HSD (HSD3B1), whereas APA was immunoreactive for type II (HSD3B2), not for HSD3B1. They also reported that HSD3B2 is regulated by ACTH, but HSD3B1 is apparently regulated by other factors, such as clock genes (Doi *et al.* 2010).

In mice, downregulation of *Per1* is associated with lower plasma aldosterone and reduced HSD3B1, reinforcing the idea that *Per1* might be modulating aldosterone levels (Richards *et al.* 2013). Interestingly, ACTH is also reported to stimulate aldosterone secretion, more strongly in APAs, less in IHA (Sonoyama *et al.* 2014); it also stimulates PER1, indicating a potential role for the intrinsic clock gene/ACTH network in the development of APA (Campino *et al.* 2011). Angelousi *et al*. found an increase in *PER1*, *CLOCK*, *BMAL1*, *CRY1*, *REV-ERB*, and *RORA* mRNA expression and protein levels in human APAs compared with the surrounding non-adenomatous tissues, although clock gene expression was not correlated with tumor size, aldosterone level, or plasma renin activity (Angelousi *et al.* 2020). Treatment of human adrenocortical cells with angiotensin caused a significant upregulation of *CRY1* and downregulation of *CRY2* (Tetti *et al.* 2018).

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Outside critical care, the sustained low-grade chronic inflammatory response observed in various models of circadian dysregulation (Isidori *et al.* 2018) suggests that inflammation could contribute to the increased risk of metabolic diseases in shift workers. This may significantly increase both the metabolic consequences of diet-induced obesity (Kim *et al.* 2018) and cardiovascular risk (Schilperoort *et al.* 2020).

Is the glucocorticoid effector system the watchmaker?

The system through which glucocorticoids fine-tune the peripheral clocks while synchronizing the metabolic, inflammatory, and brain responses to acute and chronic stress remains largely underexplored (Fig. 1). Since the discovery of peripheral and central clocks and their phase-shifting (Yamazaki *et al.* 2000), glucocorticoids have been ideal candidates for entraining the periphery with the central pacemaker. Their secretion follows a marked daily rhythm and GR is expressed in almost every peripheral cell except the SCN (Rosenfeld *et al.* 1988, Balsalobre *et al.* 2000). It has also been demonstrated that glucocorticoid treatment could phase-shift circadian clocks in peripheral cells (Balsalobre *et al.* 2000, Cuesta *et al.* 2015) and that this effect did not occur in tissues lacking functional GR. Furthermore, glucocorticoids directly induced clock gene expression in both mouse (Balsalobre *et al.* 2000) and human studies (Yurtsever *et al.* 2016). In fact, glucocorticoid responsive elements have been described in the promoter regions of genes in the *PERIOD* family (Reddy *et al.* 2009), but glucocorticoid-induced transcription seems to depend on more complex mechanisms, such as chromatin accessibility (John *et al.* 2011, Reddy *et al.* 2012) and other mediators such as the circadian gene *Bmal1* (Cheon *et al.* 2013) and its capacity to rhythmically regulate a network of enhancers (Beytebiere *et al.* 2019). Interestingly, glucocorticoid-induced phase-shifting did not occur in PER1 knockout models. This suggests that *Per* plays a major role in mediating the effects of glucocorticoids on other circadian components of the clock mechanism, such as *Bmal1*, *Rev-Erb*, and *Clock* (Koyanagi *et al.* 2006), even though glucocorticoids can directly repress REV-ERB expression (Murayama *et al.* 2019) and CLOCK and CRY have been shown to physically interact with the GR, inhibiting its transcriptional activity (Nader *et al.* 2009, Lamia *et al.* 2011) (Fig. 3). This last observation brought into question the prominent role of endogenous glucocorticoids in regulating peripheral rhythmicity, invoking a hierarchic role for clock-dependent regulation of GR expression in the tissue, rather than daily fluctuations of the circulating hormones.

In the debate over whether the main timekeeper is cortisol, its receptor, or neither of these, it is worth remembering which mechanisms control GR activity.

The GR is located in the cytosol. The presence of chaperone protein HPS90 is fundamental for maintenance of the high-affinity form, while two other binding proteins, the immunophillins FKBP51 and FKBP52, compete to associate with the HPS90-GR complex and can regulate its translocation to the nucleus following ligand binding (Wochnik *et al.* 2005).

The imbalance between FKBP51 and FKBP52 can interfere with GR activation, leading to glucocorticoid resistance. GR is both a target and a transcriptional factor of FKBP52 protein, while FKBP51 interferes with GR activity. Enhanced levels of FKBP5 have been described in several diseases characterized by altered glucocorticoid sensitivity, such as Cushing's syndrome (Resmini *et al.* 2016).

Several studies have recently highlighted the importance of circadian variations in GR activity over glucocorticoid rhythmicity in mediating immune response. A rhythmic inflammatory response to lipopolysaccharide challenge in airway epithelial cells was maintained in the absence of glucocorticoid rhythmic secretion in experimental models that preserved GR ligand availability (Ince *et al.* 2019), while it was lost in adrenalectomized mice (Gibbs *et al.* 2014).

However, the role of the circadian rhythm in modulating glucocorticoid sensitivity in peripheral organs has been best evaluated in 'metabolic' organs (Caratti *et al.* 2018). Glucocorticoids exert different effects on 'metabolic' tissues such as the liver depending on the timing of administration, while other 'non-metabolic' organs such as the lung did not show any difference in target gene expression at different timepoints. Interestingly, the co-binding of GR with circadian nuclear receptor REV-ERBa is necessary for transcription of target genes associated with lipid metabolism in the liver, as shown in *Rev-Erba*-KO mice, which did not present any of the well-known effects of glucocorticoids on lipid metabolism (Caratti *et al.* 2018). Finally, metabolic sensitivity to glucocorticoid is also modulated by hepatocyte nuclear factors (HNFs), such as HFN6 and HFN4A (Reddy *et al.* 2007, Zhang *et al.* 2016, Qu *et al.* 2018).

Specifically, the synchronizing effect of glucocorticoids is attenuated in liver HFN4A knockout mice (Reddy *et al.* 2007). In summary, at physiological

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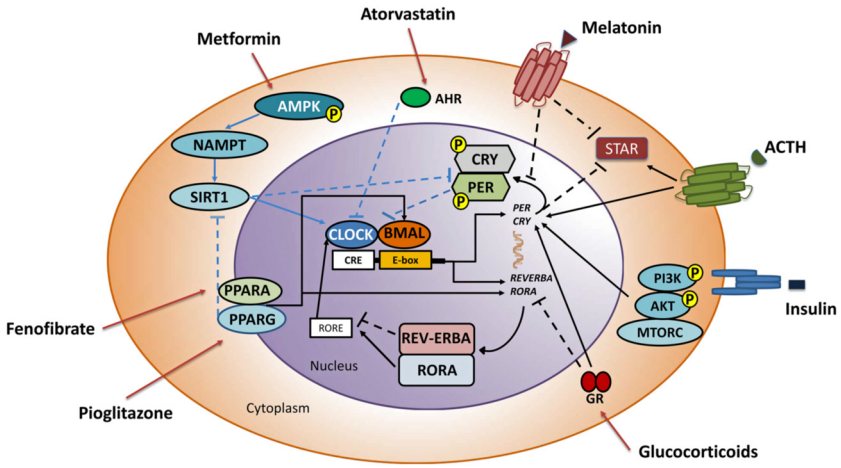

Figure 3

Involvement of the main metabolic drugs and glucocorticoids in the transcriptional feedback loop of the mammalian circadian clock. The CLOCK-BMAL1heterodimer activates transcription of *PER*, *CRY*, *RORA*, and *REV-ERBA* through binding to E-box in their promoters. Upon accumulation, PER and CRY dimerize and translocate into the nucleus to repress CLOCK-BMAL1activity and hence their own transcription. RORA activates and REV-ERB represses RORE-mediated transcription. The Glucocorticoid receptor (GR) interacts with CRY and glucocorticoids can directly repress *REV-ERBA*. ACTH stimulates steroidogenesis (STAR) and *PER*, that in turn blunts ACTH stimulation of STAR that is also inhibited by Melatonin. Metformin activates hepatic Sirtuin 1 (SIRT1) through AMPK-mediated induction of NAMPT. SIRT1 binds CLOCK-BMAL1 in a circadian manner and promotes the deacetylation and degradation of PER. PPARA ligands, such as fibrates, and PPARG ligands, such as pioglitazone, impair SIRT1; *in vitro* PPARA and PPARG regulate the expression of *BMAL1* and *REV-ERBA*. Insulin receptor (INSR) activation increases PI3K, leading to activation of mTOR complex (MTORC), essential for *PER* induction. Atorvastatin is an agonist of aryl hydrocarbon receptor (AHR), which regulates the circadian clock by repressing the CLOCK-BMAL1heterodimer and thereby inhibiting the expression of *PER1*. Solid/dashed blue lines refer to activation/inhibition of signaling steps, respectively. Solid/dashed black lines refer to activation/inhibition of gene transcription, respectively.

concentrations of glucocorticoids, *CRY*-dependent regulation of GR in the liver seems to be the dominant mechanism regulating its time-dependent metabolic sensitivity to glucocorticoids; however, at non-physiological concentrations, such as in adrenal insufficiency or Cushing's syndrome, where *CRY* and *REV-ERBa* are dysregulated (Venneri *et al.* 2018, Isidori 2019), this might not be the case.

Pharmacological targeting of GR used to be considered the ideal way to potentially uncouple the beneficial and detrimental effects of glucocorticoids. However, although in recent decades several compounds selectively modulating GR or partial GR agonists (Koorneef *et al.* 2018) have been tested in metabolic and inflammatory diseases (Lucafo *et al.* 2020), none of them have made it through to clinical trials. The reason probably lies in the tissue-specific complexity, including clock-dependency (Caratti *et al.* 2018), of the cofactors modulating glucocorticoid sensitivity. In the context of circadian rhythm alterations, therapies targeting GR could prevent the development of metabolic alterations induced by altered glucocorticoid rhythm and exposure. In the near future, studies on novel GR antagonists (such as relacorilant) with better pharmacokinetics than mifepristone could provide some insight (see subsequent section), but in the current clinical setting GR targeting does not seem to be a feasible way to 'fix the clock'.

Adrenal disorders as models of circadian rhythm disruption

Hypercortisolism and adrenocortical tumors

Cushing's syndrome involves prolonged exposure to endogenous or exogenous glucocorticoids, resulting in a disturbed circadian rhythm (Alexandraki & Grossman 2010). It is characterized by high morbidity and mortality, especially due to cardiovascular, infectious, metabolic, and psychiatric conditions (Isidori *et al.* 2015*a*,*b*, Pivonello *et al.* 2016, Vitale *et al.* 2017) and largely attributed to the failure of plasma cortisol levels to drop in the late evening and at night. It has been proposed that partial resistance of ACTH-secreting pituitary adenomas to the negative feedback of cortisol alters the secretion of corticotropin-releasing hormone from the hypothalamus, leading to an abnormal circadian rhythm (Moreira *et al.* 2018). This in turn may contribute to dysregulation of the clock system in peripheral tissues, leading to intermediary metabolic alterations and the clinical features of Cushing's syndrome. Conversely, PER2-deficient mice show abnormalities in the HPA axis, elevated corticosterone levels, and a disturbed feeding rhythm (Yang *et al.* 2009). An *ex vivo* study on normal adrenocortical cells suggests that ACTH stimulates *PER1* and *BMAL* expression and that the interplay between ACTH and clock genes is

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crucial in regulating the normal steroidogenic response. Interestingly, melatonin seems to directly inhibit ACTH-stimulated steroidogenesis (Campino *et al.* 2011). In mice with a selective knockout of *Bmal* in the adrenocortical cell, basal steroidogenesis and stress-induced response were maintained (Dumbell *et al.* 2016), albeit with an exaggerated response, especially in females, and the animals were more vulnerable to light-induced time-shift, suggesting that the adrenal clock machinery acts to buffer steroidogenic responses and stabilize circadian glucocorticoid rhythmicity (Engeland *et al.* 2018, 2019).

In relation to cortisol-secreting tumors, only two studies have explored clock gene expression in human adrenal tissue (Campino *et al.* 2011, Angelousi *et al.* 2020). They found that clock genes were expressed but were dysregulated, with an apparent loss of established feedback loops and a distinct pattern between benign (CSA) and malignant adrenal tumors (ACC). Compared to non-adenomatous adjacent adrenal tissue, *PER1*, *CRY1*, and *REV-ERB* were downregulated in CSA, but *REV-ERB* expression was positively correlated and *CLOCK* expression negatively correlated with the severity of hypercortisolism. In contrast, *CLOCK*, *CRY1*, and *PER1* seem to be upregulated and *BMAL1* and *RORA* downregulated in ACC (Angelousi *et al.* 2020). The studies investigating the link between clock genes and hyperaldosteronism are described in BOX 2.

In summary, hypercortisolism is likely to exert direct and indirect effects on non-endocrine peripheral circadian genes, contributing to worsening of comorbidities. The adrenal clock machinery, which normally buffers response to ACTH and stress, appears dysregulated in adrenal tumors, favoring a higher, arrhythmic corticosteroid output. How and when such alterations can be reversible is the outcome of an ongoing multicentric prospective trial on circadian rhythm in the active and remission phases of Cushing's syndrome (NCT03343470).

Adrenal insufficiency

Adrenal insufficiency is characterized by insufficient levels of endogenous glucocorticoids, either due to adrenal dysfunction (the primary form) or to decreased pituitary secretion of ACTH or hypothalamic secretion of corticotropin-releasing hormone (secondary adrenal insufficiency) (Pofi *et al.* 2018). Patients require lifelong glucocorticoid replacement therapy and several studies have demonstrated their increased mortality and morbidity, mostly due to cardiovascular and infectious diseases and malignancies (Bergthorsdottir *et al.* 2006, Quinkler *et al.* 2018). Management of glucocorticoid replacement remains controversial, but most guidelines nowadays advise treating these patients with lower doses of glucocorticoids (usually <30 mg of hydrocortisone or equivalent glucocorticoid daily dosage) in order to match the serum cortisol levels observed in healthy controls (Bornstein *et al.* 2016, Isidori *et al.* 2020). However, several studies failed to demonstrate any clear correlation between glucocorticoid dosage under 35 mg/day and metabolic comorbidities (Bleicken *et al.* 2010, Castinetti *et al.* 2015), leading to the hypothesis that the timing of administration is as important as the dose. In fact, glucocorticoid replacement should mimic endogenous secretion, peaking in the early morning and decreasing during the active phase, with a trough at night. Immediate-release hydrocortisone is therefore administered in two or three doses, with the highest dose in the morning and the lowest at midday and/or in the afternoon, avoiding evening exposure (Bornstein *et al.* 2016). Despite this, peaks and troughs in serum cortisol levels are unavoidable with immediate-release formulations, leading to possible disruption due to the entraining activity of glucocorticoids on peripheral clocks in multiple organs (Balsalobre *et al.* 2000).

According to this hypothesis, patients undergoing multiple daily dose replacement therapy for adrenal insufficiency show significant alterations in circadian gene expression compared to healthy controls (Venneri *et al.* 2018). As observed in other conditions involving a disrupted circadian rhythm, the immune profile of these patients also showed significant alterations, with an increased number of inflammatory monocytes (Isidori *et al.* 2018) and reduced number of $CD16^{+}$ natural killer cells (Bancos *et al.* 2017, Isidori *et al.* 2018), along with an increased incidence of infectious diseases (Isidori *et al.* 2018). This suggests that low-grade inflammation due to circadian disruption could underlie the metabolic comorbidities in adrenal insufficiency, even when patients are treated with adequate tailored daily dosages.

The 'fixing' hypothesis: chrono-pharmacology in glucocorticoid excess and deficiency disorders

Whether the previously described clock alterations are the cause or the consequence of HPA axis dysregulation remains to be fully elucidated. The evidence that normal surrounding tissue or stimulated tumor cells can modulate clock gene expression suggests that clock dysfunction

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is a contributing factor but not the main cause of the disease. This raises the hypothesis that an attempt could be made to realign clock-gene expression. However, this intriguing possibility clashes with evidence that, despite the hierarchical structure of the timing system and its continuous resetting by environmental time cues, the intrinsic activity of both the central and peripheral circadian clocks seem to be largely self-sustained. How can the clocks be fixed, considering that, at the molecular level, they share a similar machinery? An insight comes from the theory that tissue-specific chromatin accessibility dictates clock protein binding (John *et al.* 2011). In other words, the rhythm-specificity of gene expression is conferred by tissue-specific transcription factors – such as HNFs in the liver – that regulate large transcription programs. The second, strongly related concept is that of gating systems, according to which specific, appropriately timed signals can act as gate openers to a different level/phase where the response to identical stimuli is blunted or enhanced. These facilitators or windows could be used to reshape the rhythm and synchronize the otherwise resilient intrinsic clocks. A brief and undoubtedly incomplete list of entrainment mechanisms is provided, starting from basic physiological processes like cell division and moving on to cell nourishment and paracrine-endocrine signaling.

The lesson from the entraining of the clock and cell cycle oscillators

The coordination of the cell cycle with clock genes is one of the best-studied examples of the entrainment mechanism. The transition between the G and S phases of the cell cycle occurs through a gating system that is entrained with clock genes. Using light-responsive zebrafish cell lines, it has been shown that the cell cycle can be synchronized by re-entraining the light:darkness cycles to a different period. The clock uses specific circadian checkpoints to create a window or gate that is either permissive or repressive for cell cycle progression (Laranjeiro *et al.* 2018).

In this context, glucocorticoids exert a double effect: first, directly on the clock gene mechanism, and second, on the genes that are the targets of clock genes. For the latter mechanism, glucocorticoids can delay the expression or degradation of important factors through the gating transition. This could explain why the healing process is dramatically hampered in cases of glucocorticoid excess.

However, these studies have also revealed that not everything has a clock. Embryonic stem cells, which can develop into almost any cell type, do not keep time, and many cancer cells do not keep a regular rhythm: a modified proliferation and differentiation pattern is common to both stem and cancerous cells (Tsuchiya *et al.* 2020).

Glucocorticoids are among the most potent pro-differentiation agents used *in vitro* cell cultures. The glucocorticoid rhythm can promote acceleration or relaxation by moving to or from the gate-point needed to activate specific cell functions, for example, shifting from proliferation into differentiation status.

The clock genes and cell cycle are synchronized to oscillate in coordination around the 24-h period. By experimentally extending the light:darkness period to longer than 24 h, the amplitude of oscillation is lowered and the expression of several clock genes is flattened downstream. This is precisely what is seen in patients receiving glucocorticoids in a non-physiological rhythm (Venneri *et al.* 2018) or in patients with Cushing's syndrome (Isidori 2019). All these observations lead to the hypothesis that medical treatments targeting adrenal disorders could be used to entrain clocks that have become misaligned due to the disorder itself.

Treating Cushing's syndrome and possible autonomous cortisol secretion

Medical treatments for Cushing's syndrome can be classified as pituitary-targeting drugs, steroid synthesis inhibitors, and GR antagonists (Feelders *et al.* 2019). They are generally used when surgery is not indicated or in cases of persistent or recurrent hypercortisolism. Steroidogenesis inhibitors such as ketoconazole or metyrapone effectively lower mean 24-h cortisol values but appear unable to restore the cortisol rhythm (Terzolo *et al.* 1988, Ceccato *et al.* 2018). Conversely, the somatostatin analog pasireotide, alone or in combination therapy with cabergoline or ketoconazole, restored the diurnal cortisol rhythm, albeit, in only half of the patients (van der Pas *et al.* 2013). The activity, kinetics, and toxicity of the drugs used to treat Cushing's syndrome have seldom taken into account the implications of their administration time. Most studies investigate the overall 24-h cortisol output rather than time-of-day-glucocorticoid exposure curves, despite discussions of their value (Alexandraki & Grossman 2011). Taking medications at a specific time (chrono-pharmacology) could help restore physiological cortisol circadian rhythms, thus improving the metabolic and cardiovascular complications associated with hypercortisolism while lowering side effects and toxicity.

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In healthy subjects, the four-hourly administration of eight consecutive doses of metyrapone resulted in a marked suppression of the morning cortisol peak, but afternoon, evening, and nighttime cortisol levels were not significantly different from untreated subjects(Plat *et al.* 1999). Evening administration of metyrapone seems to restore a normal cortisol rhythm in patients with adrenal incidentalomas and autonomous (mild) cortisol secretion (Debono *et al.* 2017). Counterintuitively, to date, none of the trials on Cushing's syndrome treatment have included the normalization of evening/night cortisol levels as a primary or secondary outcome (instead investigating only total 24-h output), even though rhythm alteration is the first hallmark sign of this syndrome.

The MAPEC study showed that taking antihypertensive drugs at bedtime improve the cardiovascular risk of patients with a non-dipping pattern, compared to morning administration (Hermida *et al.* 2010).

Similarly, women who received morning teriparatide treatment showed a lower bone resorption marker (CTX) level and increased lumbar spine bone mineral density compared to those receiving evening teriparatide (Luchavova *et al.* 2011). The chrono-pharmacology of metabolically active drugs will be discussed in the next section.

In short, if the medications used to target the complications of Cushing's syndrome – hypertension, osteoporosis, and diabetes – work better when given with a chrono-pharmacological approach, it follows that the drug used to treat Cushing's syndrome itself should also take cortisol rhythm into account.

Treating adrenal insufficiency

Since the development of modified-release glucocorticoid formulations that better mimic the physiological cortisol daily profile, several studies have described the metabolic advantages of these formulations in treating adrenal insufficiency compared to the multiple daily doses of the conventional regimens. Switching to a once-daily formulation improves BMI and body weight (Johannsson *et al.* 2012, Quinkler *et al.* 2015, Isidori *et al.* 2018), glucose metabolism (Graziadio *et al.* 2018, Isidori *et al.* 2018), and quality of life (Johannsson *et al.* 2012, Isidori *et al.* 2018). The more physiological cortisol rhythm seems to restore the immune alterations observed in these patients by reducing the number of inflammatory monocytes and increasing $CD16^+$ natural killer cells (Isidori *et al.* 2018), ultimately reverting from a low-grade inflammation profile to levels close to healthy controls. These effects have been correlated to an improved circadian gene expression profile (Venneri *et al.* 2018). The effects of once-daily glucocorticoid therapy on metabolism could be due to the reduced overall daily exposure (Johannsson *et al.* 2012), but the link between altered circadian profile and metabolic disruption seems to play a role.

Glucocorticoids are powerful entrainers of peripheral clocks and the immune system is one of the most sensitive to this daily 'reset' (Balsalobre *et al.* 2000). The implications of circadian rhythm disruption were described in more detail in the previous sections. In the total or relative absence of endogenous glucocorticoid secretion, patients with adrenal insufficiency depend on exogenous administration for all the effects of cortisol, including its leading role in transmitting the daily 'central clock' synchronizing impulse to peripheral tissues. The effect is even greater in Addison disease, where adrenal-medullary control of the ANS is also missing and glucocorticoid exposure is the only control mechanism.

To entrain effectively an autonomous oscillatory population such as the clock gene expression loop in peripheral cells, the designed zeitgeber must have an adequate strength and period (Mavroudis *et al.* 2012). In adrenal insufficiency, this suggests that the glucocorticoid peaks should be as close as possible to physiological amplitude and have a period close to the autonomous rhythm of peripheral clocks, which complete a loop in approximately 24 h. That being said, it is easy to speculate that the presence of rapid variations in glucocorticoid levels due to multiple daily dose therapies could desynchronize peripheral clocks, especially if they are not synchronized with other zeitgebers such as ANS activation, meals, or exogenous stress.

Given that circadian disruption has been observed in metabolic diseases and that this is both characterized and enhanced by concomitant low-grade inflammation in the absence of external challenges, the beneficial effects of changing therapy on glucose metabolism, body weight, and immune function should be at least partly due to a more physiological entraining peak of cortisol serum levels.

Melatonin

The hormone melatonin is produced by the pineal gland. It is a robust circadian rhythm marker (Hardeland *et al.* 2011), with production beginning at around 22:00–23:00 h, peaking at 02:00–03:00 h, and reaching its lowest level

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at 09:00–10:00 h (Gooley *et al.* 2011). Production is affected by light and to some extent by body position, but not by activity, sleep, meals, stress, or the menstrual cycle (Skene & Arendt 2006, Marseglia *et al.* 2013). Thus, melatonin seems to be independent of the traditional pathways involving glucocorticoid action. Exogenous administration of melatonin confirms that non-photic stimuli can affect the body clock (Skene & Arendt 2006). Melatonin provides clock time and seasonal information in central or peripheral (adrenal) circadian clocks (Torres-Farfan *et al.* 2003, Mendez *et al.* 2012, Leliavski *et al.* 2015) and also drives darkness-related behaviors, such as sleep propensity. A multi-synaptic pathway controls nocturnal melatonin secretion from the SCN, the paraventricular nucleus, and the upper thoracic spinal cord, culminating in the release of noradrenaline from sympathetic post-synaptic neurons (superior cervical ganglion), which activates β-adrenoceptors in pinealocytes (Perreau-Lenz *et al.* 2003, Ishida *et al.* 2005, Kim *et al.* 2015). The synthesis of melatonin (N-acetyl-5-methoxytryptamine) involves serotonin (5-hydroxytriptamine) acetylation by the rate-limiting enzyme arylalkylamine N-acetyltransferase (AANAT) and N-acetylserotonin methylation by acetyl serotonin O-methyltransferase (da Silveira Cruz-Machado *et al.* 2017). AANAT transcription is regulated by darkness (Torres-Farfan *et al.* 2003); light at night rapidly inhibits AANAT activity (Lewy *et al.* 1980).

The adrenal gland expresses melatonin receptor (Torres-Farfan *et al.* 2003, 2011, Mendez *et al.* 2012, Leliavski *et al.* 2015). A high-amplitude melatonin rhythm imposed on newborn lambs resulted in suppression of the adrenal clock genes *PER1*, *PER2*, *CRY2*, *and CLOCK* (Seron-Ferre *et al.* 2017), whereas *BMAL1* maintained normal clock time–related changes, but with higher values (Torres-Farfan *et al.* 2011, Seron-Ferre *et al.* 2017). Interestingly, the previously described induction of *PER1* by ACTH stimulation of the human adrenal gland can be inhibited, *in vitro*, by simultaneous treatment with melatonin. The ACTH-induced increase in StAR and 3β-HSD protein expression, involved in cortisol and aldosterone production, seems to be blunted by melatonin, in parallel to what occurs for *PER1* suggesting that melatonin can modulate the link between ACTH, clock genes, and adrenal steroidogenesis (Campino *et al.* 2011) (Figs 1 and 3).

Furthermore, the crosstalk between adrenal and pineal glands under inflammatory conditions indicates that glucocorticoids potentiate nocturnal melatonin synthesis by reducing NFκB activity (da Silveira Cruz-Machado *et al.* 2017). Similarly, in stressful conditions, by activating both α and β adrenoceptors, glucocorticoids reduce melatonin synthesis (Fernandes *et al.* 2017) by preventing nuclear translocation of NFκB, which binds to κB elements in the AANAT promoter (Muxel *et al.* 2012). Nevertheless, decreased melatonin levels in parallel with slightly increased insulin levels were documented in both T2D rats and T2D patients (Peschke *et al.* 2015). Finally, a new era of chemoprevention involving the use of melatonin in anticancer therapy is in sight, opening up possible new roles for this neuroendocrine clock mediator (Pinato & Stebbing 2016).

Metabolically active drugs

Metabolically active drugs are often prescribed for patients with adrenal disorders. Even though these drugs could target circadian genes (Zhang *et al.* 2014), the influence of their administration time and their circadian effects have not been extensively studied. One of the most well-known examples is short half-life statins: when taken just before bedtime, they lower cholesterol when the biosynthesis rate is at its highest (Miettinen 1982) (Fig. 3).

The first-line treatment for type 2 diabetes, metformin, modulates molecular clock function in insulin-sensitive tissues in mice (Barnea *et al.* 2012). Table 1 lists the drugs used to treat diabetes, dyslipidemia, hypertriglyceridemia, and hyperuricemia that target at least one circadian gene, according to the database of circadian genes in eukaryotes (Li *et al.* 2017) and DrugBank (Knox *et al.* 2011). For example, metformin targets the circadian gene *PRKAB1*, encoding for a regulatory subunit of AMPK, a master regulator of energy metabolism. Chronic exposure to glucocorticoids can inhibit AMPK activity, exacerbating metabolic impairment (Fig. 3). Metformin counteracts this effect and hence might be beneficial for metabolic complications induced by glucocorticoid excess, especially the accumulation of visceral adiposity (Christ-Crain *et al.* 2008, Seelig *et al.* 2017), even in non-diabetic patients (Pernicova *et al.* 2020). Since most of the drugs described in Table 1 are commonly given to patients with glucocorticoid excess or deficiency, clarifying their impact on clock molecular pathways could expand the available options for chrono-pharmacological treatment.

Conclusions

Glucocorticoids are crucial mediators of the interaction between the central and peripheral clocks. In adrenal disorders, dysregulation of clock synchronization caused

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by disruption of the cortisol circadian rhythm plays a significant role in the development of end organ complications associated with Cushing's syndrome and adrenal insufficiency. Intriguingly, these two conditions, while seemingly opposite in their clinical presentation, share a common pathophysiological pathway in terms of impaired immune function and increased atherosclerotic risk, two systems that are highly sensitive to clock regulation. For these reasons, greater attention should be paid to the medical treatment used to correct glucocorticoid levels. In both replacement and reduction therapies, all attempts should be made to mimic the daily peaks and troughs of cortisol. This means administering medications at an appropriate time that takes account of their pharmacokinetics, to avoid exposure to glucocorticoids late in the evening and at night. In addition, the prescription of metabolic drugs to control glucocorticoid excess should take into account that tissue sensitivity to glucocorticoids can be different throughout the 24 h, hence preference should be given to agents that are more likely to act in phase with the endogenous clock. Endocrinologists, who have a multidisciplinary overview of homeorhesis (as opposed to homeostasis) and the hormonal rhythms that set the pace of so many biological processes, are perfectly positioned to become the new clock repairers: 'Oh dear! Oh dear! I shall be too late!' is Lewis Carroll's reminder to take part in the *Adventures in the Wonderland* of chronopharmacology.

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