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.

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...
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 genes.
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).
Buijink, C (2018). CLOCK mutant mice exhibit an uncoupling of the peripheral oscillators from the central oscillator. It interacts with brain leptin receptors in the SCN are time-sensitive to leptin modulation. Late sleeping, short sleep duration 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).

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 (Beebig 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-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

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.
Using hormones and drugs as entrainers, for example, through the release of additional knockdown of Per1 and Per2 in mice fibroblasts (Hirota et al. 2002) and indirectly regulate S' 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 AMPK 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, Asher & Sassone-Corsi 2015).
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 sympathectomized 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. 2015a), 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>
<thead>
<tr>
<th>Drugs</th>
<th>Indication</th>
<th>Circadian gene target</th>
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<tbody>
<tr>
<td>Dipeptidyl peptidase-4 inhibitors</td>
<td>Diabetes mellitus</td>
<td>DPP4</td>
</tr>
<tr>
<td>Gliclazide</td>
<td>Diabetes mellitus</td>
<td>ABCB8 and VEGFA</td>
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<tr>
<td>Glimepiride</td>
<td>Diabetes mellitus</td>
<td>KCNJ1 and ABCC8</td>
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<tr>
<td>Insulin</td>
<td>Diabetes mellitus</td>
<td>IGFR1 and INSR</td>
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<tr>
<td>Meftrormin</td>
<td>Diabetes mellitus</td>
<td>PRKAB1, ETFDH and GPD1</td>
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<tr>
<td>Pioglitazone</td>
<td>Diabetes mellitus</td>
<td>PPARG and MAOB</td>
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<tr>
<td>Alirocumab</td>
<td>Dyslipidemia</td>
<td>PCSK9</td>
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<tr>
<td>Atorvastatin</td>
<td>Dyslipidemia</td>
<td>HMGCR, DPP4, AHR and NR1I3</td>
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<td>Ezetimibe</td>
<td>Dyslipidemia</td>
<td>SOAT1</td>
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<tr>
<td>Lovastatin, fluvastatin, pravastatin, rosuvastatin, and simvastatin</td>
<td>Dyslipidemia</td>
<td>HMGCR</td>
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<td>Fenofibrate</td>
<td>Hypertriglyceridemia</td>
<td>PPARA, NR1I2 and MMP25</td>
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<tr>
<td>Allopurinol and febuxostat</td>
<td>Hyperuricemia</td>
<td>XDH</td>
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Table 1 Commonly prescribed drugs for diabetes, dyslipidemia, hyperuricemia that target circadian genes.
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-κB 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 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 (Sonomaya 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).
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 (Schliperoort 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
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. 2015a,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
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 Bmal1 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 (Berghorsdottir 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...
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 (chronopharmacology) could help restore physiological cortisol circadian rhythms, thus improving the metabolic and cardiovascular complications associated with hypercortisolism while lowering side effects and toxicity.
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.
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-hydroxytryptamine) acetylation by the rate-limiting enzyme aryalkylamine N-acetyltransferase (AANAT) and N-acetylsertotonin 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...
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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