MECHANISMS IN ENDOCRINOLOGY

A sense of time of the glucocorticoid circadian clock: from the ontogeny to the diagnosis of Cushing's syndrome

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Abstract

The circadian rhythm of glucocorticoids has long been recognised within the last 75 years. Since the beginning, researchers have sought to identify basic mechanisms underlying the origin and emergence of the corticosteroid circadian rhythmicity among mammals. Accordingly, Young, Hall and Rosbash, laureates of the 2017 Nobel Prize in Physiology or Medicine, as well as Takahashi's group among others, have characterised the molecular cogwheels of the circadian system, describing interlocking transcription/translation feedback loops essential for normal circadian rhythms. Plasma glucocorticoid circadian variation depends on the expression of intrinsic clock genes within the anatomic components of the hypothalamic–pituitary–adrenal axis, which are organised in a hierarchical manner. This review presents a general overview of the glucocorticoid circadian clock mechanisms, highlighting the ontogeny of the pituitary–adrenal axis diurnal rhythmicity as well as the involvement of circadian rhythm abnormalities in the physiopathology and diagnosis of Cushing's disease.

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Introduction

Circadian rhythms have been widely observed in various organisms spanning from cyanobacteria to humans. Circadian clocks have adopted geophysical cycles as part of predictive homeostasis, thereby

preparing living organisms for environmental fluctuations and regulating sleep patterns, feeding behaviour, body temperature, blood pressure and hormone release (1, 2).

Invited Author's profile

Ayrton Custodio Moreira, MD, PhD is Prof. of Medicine at the Division of Endocrinology and Metabolism, Department of Internal Medicine, Ribeirao Preto Medical School, University of Sao Paulo, Brazil. He has published more than 150 papers in high-quality journals and has held several important administrative positions, including the Dean of the Ribeirao Preto Medical School. In recognition of his outstanding contributions as an educator and researcher, he has received many medical education awards. His main research interest includes the physiology and pathophysiology of the pituitary–adrenal axis, focusing on the glucocorticoid circadian rhythm and the diagnosis of Cushing's syndrome.

The glucocorticoid circadian rhythm in humans had been first described by Pincus in the early 1940s as a diurnal rhythm based on the excretion of urinary ketosteroids (3). Later, the diurnal rhythmicity of the pituitary–adrenal axis had been confirmed in studies involving rats (4) and other mammals, including healthy humans (5). The lack of a diurnal rhythm for plasma and urinary 17-hydroxycorticoid levels in patients with Cushing's syndrome had been first described during the mid-to-late 1950s (6, 7). At that time, Halberg (8) introduced the term 'circadian', which means approximately 24h, to denote daily cycles. The author adopted the terminology from the oscillation theory in physics to describe biological circadian rhythms in terms of their mean levels (Mesor), amplitude, peak, trough (nadir), phase and antiphase. He also coined the concept of environmental synchronisers (*zeitgebers*) and introduced cosinor-based rhythmometry (9, 10).

Studies regarding postnatal emergence of glucocorticoid circadian rhythmicity, both in rats (11, 12) and humans (13, 14), had surfaced during the 1960s. Although Halberg (8) suggested that 24-h rhythms may be related to the transmission of cyclic genetic information from DNA to RNA, the nature of the underlying oscillations remained unknown until 1971, when Konopka and Benzer described the first clock gene *Period (Per)* at the period locus on the X chromosome of *Drosophila melanogaster*. *Per* locus mutations induced alterations in pupal eclosion and adult activity rhythms, including courtship singing (15).

During the 1980s, Young, Hall and Rosbash, recipients of the 2017 Nobel Prize in Physiology or Medicine, began molecular mapping studies on the *Per* locus of *Drosophila* while identifying news transcripts (16, 17). In addition, they observed a feedback loop that enabled *Per*-encoded proteins to cycle their own RNA (18). Searching for genes regulating circadian rhythms in mammals, Takahashi's group identified a new gene named *Clock* (Circadian Locomotor Output Cycles Kaput), which is essential for normal circadian behaviour (19). Since the mid-1990s to the present, several studies have shown cell-autonomous molecular mechanisms for circadian rhythms. These circuits involve at least ten clock genes and their transcript proteins connected by feedback loops, characterising molecular circadian cycles that oscillate at a periodicity of approximately 24 h (20, 21, 22).

The mammalian molecular clock mechanism

Clock genes are expressed in essentially all mammalian cells (22). The cogwheels of the circadian timekeeping system consist of two interlocking transcription/translation feedback loops: a core loop supported by an accessory loop. The circadian molecular clock system in mammals has been extensively reviewed elsewhere (22, 23, 24, 25) with its model being summarised in Fig. 1. Briefly, the core loop comprises circadian genes *Clock* and *Bmal1* (*Arntl*), *Periods* (*Per1*, *Per2* and *Per3*) and cryptochromes (*Cry1* and *Cry2*). The principal mechanism for the circadian timekeeping

Figure 1

Schematic model of the circadian molecular clock in mammals. The cogwheels of the circadian system consist of two interlocking feedback loops. The core loop comprises *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1* and *Cry2* genes. CLOCK-BMAL1 generates an autoregulatory positive feedback loop and also activates the accessory loop consisting of *Rev-Erbα* and *Rorα*. In turn, REV-ERB and ROR regulate the rhythmic transcription of *Bmal1*. Additionally, CLOCK-BMAL1 binds to promoters of clock-controlled genes. The stability of clock proteins is tightly regulated by E3 ubiquitin ligases, kinases and phosphatases.

system depends on two main clock proteins, CLOCK and BMAL1, constituting heterodimeric transcriptional activators of a positive feedback loop. These heterodimers activate their target genes at E-boxes in promoters of core genes *Per1*, *Per2*, *Per3*, *Cry1* and *Cry2*. These genes then interact with CLOCK-BMAL1, inhibiting their own transcription. CLOCK-BMAL1 also activates a second feedback loop that increases the mRNA levels of *Rev-Erba* (*Nr1d1*) and *Rora*. In turn, REV-ERB and ROR proteins inhibit and activate the rhythmic transcription of *Bmal1* respectively. The fine-tuning of the molecular circadian timekeeping system depends on photic and nonphotic environmental synchronisers, such as light–darkness cycles, food ingestion, temperature, rest/activity cycles and social cues (26, 27, 28). Furthermore, light–darkness cycles seem to be a stronger *zeitgeber* than environmental temperature or food availability cycles, at least in mice (29). The primary action of the *zeitgebers* is to synchronise the clock system to a period of exactly 24 h. Additionally, CLOCK, BMAL1 and CRY proteins bind to E-boxes within the promoters of more than a thousand target genes named clock-controlled or output genes (30, 31). Such genes are tissue specific and translate temporal information from clock genes to single cells and whole organs, thereby controlling physiological functions (32, 33).

Circadian rhythm in the hypothalamic–pituitary–adrenal (HPA) axis

The endogenous circadian rhythm in the HPA axis is one of the best-documented cyclic endocrine activities showing daily variations in plasma glucocorticoids: cortisol in humans and corticosterone in rats. Peak concentrations have been reported to occur prior to or at the time of activity onset, daytime in humans and night-time in nocturnal animals and decline over the remaining 24-h period (27, 34, 35). Moreover, molecular mechanisms controlling the HPA circadian rhythm have been widely studied in rodents and non-human primates. Plasma glucocorticoid variations depend on the expression of intrinsic clock genes in the anatomic components of the HPA axis, which are organised in a hierarchical manner (36, 37). Circadian timing signals are propagated downstream from the hypothalamic suprachiasmatic nucleus (SCN) clock genes to other hypothalamic nuclei, pineal gland, pituitary and adrenal clock genes by controlling cyclic hormonal factors and autonomic innervations. Figure 2 provides a schematic representation of the multi-oscillatory system of the HPA axis circadian rhythm in normal subjects.

SCN regulation of the HPA axis circadian rhythm

The mammalian SCN comprises paired bilateral structures of the ventral hypothalamus containing approximately 20 000 and 100 000 neurons in mice and humans respectively (38). Each unilateral SCN has two anatomic subdivisions: a ventral ‘core’ region, which touches the optic chiasm and receives retinal input, and a dorsal ‘shell’ region, which receives input from the core (39). Neurons in the core and shell subregions are distinguished by neurochemical phenotype and vary substantially across species. Vasoactive intestinal polypeptide (VIP), the most abundant neuropeptide in core neurons, is released rhythmically from the core and also strongly involved in SCN coupling. The core also contains calretinin, neurotensin and gastrin-releasing peptide. Shell neurons, on the other hand, contain predominately arginine vasopressin (AVP), angiotensin II and met-enkephalin. These neuropeptides are colocalised with gamma-aminobutyric acid (GABA) in most SCN neurons, while synapses among SCN neurons are mostly GABAergic (39). Cell–cell communication via chemical and electrical synapses may be the primary contributor to normal rhythmicity. Such network properties across the entire SCN provide rhythmic stability to the molecular circadian cogwheels. Although each neuron contains a cell-autonomous circadian oscillator, the SCN network contributes to the synchronisation of cellular oscillations and increases clock precision (39).

The neuroanatomy of the circadian rhythm system, which has been extensively reviewed by Morin (40), is considered complex. The mammalian SCN receives a bilateral input of luminance information via the retinohypothalamic tract (RHT) from photosensitive ganglion cells of the retina (ipRGCs). These cells express the photopigment melanopsin (Opn4), which is not involved in image formation but is essential for SCN light entrainment (41, 42). Moreover, *Per1* and *Per2* genes, which are co-expressed in SCN cells, are induced in response to external light unlike *Per3* (43). This is ascribed to the fact of only *Per1* and *Per2* promoters contain CREs that bind to CRE-binding protein. This light signalling-dependent activation of *Per1* and *Per2* gene expression is distinct from the CLOCK/BMAL1 feedback loop (43). Apart from the RHT, the SCN is also interconnected with many other regions of the brain, such as the median raphe serotonergic pathway, the geniculohypothalamic pathway and the NPY-containing pathway from the thalamic intergeniculate leaflet, which also contribute

Figure 2

Circadian rhythm of the hypothalamic–pituitary–adrenal axis in normal subjects: a multi-oscillatory system. Plasma glucocorticoid variation depends on the expression of intrinsic clock genes in the anatomic components of the HPA axis, which are organised in a hierarchical manner. Circadian timing signals are propagated downstream from the hypothalamic suprachiasmatic nucleus clock genes to other hypothalamic nuclei, pineal gland, pituitary gland and adrenal clock genes by controlling cyclic hormonal factors and autonomic innervations. AVP, arginine vasopressin; GR, glucocorticoid receptor; PVN, paraventricular nucleus; RHT, retinohypothalamic tract.

to circadian rhythm regulation (40). As conventionally accepted, light through the RHT entrains the SCN clock. In addition, recent studies suggest that light may also control peripheral clocks independent of the SCN clock. However, the nature of these additional pathways remains to be elucidated (44, 45).

The SCN output signals are mediated by circadian variation of neuronal firing and humoral output. Neuroanatomical tracing studies have demonstrated a non-uniform distribution of labelled cells across the SCN (46). These studies described three major patterns of SCN efferent projections in rats. The most robust pattern consists of input to the parvocellular portion of the paraventricular nucleus (PVN), periventricular hypothalamic nuclei, preoptic region, subparaventricular zone, dorsomedial nucleus (DMH), retrochiasmatic area and premammillary area (46). The parvocellular portion of the PVN contains neuroendocrine neurons that synthesise corticotrophin-releasing hormone (CRH), as well as AVP in almost half of these SCN neurons. Based on time of death, SCN neurons obtained during the autopsy of young human subjects showed a circadian rhythm among immunoreactive AVP neurons, which peaked during the early morning (47). Moreover, AVP mRNA diurnal variations in rat SCN revealed higher expressions in the morning (48). These data are consistent with those from transgenic rats expressing AVP-enhanced green fluorescent protein fusion gene, which allowed the detection of diurnal AVP mRNA changes in the dorsomedial SCN (49), corroborating the role of AVP as an important output neurotransmitter of the SCN. Notably, AVP is released by the SCN during the

light period in both nocturnal and diurnal species. In nocturnal animals, AVP released during the light period inhibits CRH-containing neurons in the subPVN and DMH nuclei via GABAergic interneurons. In the diurnal species, however, AVP released during the light period stimulates CRH-containing neurons via glutamatergic interneurons in the same nuclei. The existence of different neurotransmitter pathways downstream of the SCN may explain the mechanism behind the 12-h reversal of certain rhythms, including HPA axis circadian activity, in both nocturnal and diurnal species (50).

In addition, endocrine circadian activity relies on a neural connection between the SCN and pineal gland. The SCN controls the circadian rhythm of melatonin synthesis in the mammalian pineal gland via multisynaptic pathways, including preautonomic neurons of the PVN, sympathetic preganglionic neurons in the spinal cord and noradrenergic neurons of the superior cervical ganglion (51). Melatonin blood levels display a circadian variation, with high concentrations during the night, in both diurnal and nocturnal animals, reflecting an internal darkness signal. Melatonin is also an important efferent pathway, distributing temporal cues from mammalian SCN to a large number of tissues expressing melatonin receptors, including the adrenal glands (52). Notably, evidence has shown that melatonin acts on the SCN, in turn directly influencing the circadian clock mechanism. Melatonin could inhibit proteasomal destruction of clock proteins, such as BMAIL and REV-ERB α , and interfere with molecular feedback loops, particularly at night (53).

CRH is the principal neurohumoral signal controlling adrenocorticotrophic hormone (ACTH) secretion and glucocorticoid rhythms (54, 55). CRH is mainly synthesised in the parvocellular and magnocellular neurons of the PVN. Approximately half of the CRH neurons co-express AVP, while their axons project to the median eminence, releasing CRH and AVP to stimulate ACTH release from the anterior pituitary gland (56), which in turn controls the release of glucocorticoids via its stimulatory action on the adrenal cortex (57, 58, 59). Notably, continuous infusion of CRH in healthy males leads to a retention of circadian rhythmicity in ACTH secretion. Thus, other stimulatory factors, such as AVP, may possibly control the circadian rhythm of ACTH and cortisol (60).

Furthermore, evidence has indicated that SCN also regulates adrenal circadian activity independent of CRH–ACTH stimuli. Morphological data support the role of direct sympathetic innervation in modulating adrenal glucocorticoid secretion (61). In addition, physiological studies have shown a circadian rhythm in adrenal responsiveness to exogenous ACTH (27, 62). Notably, plasma corticosterone had a more pronounced (5- to 10-fold) circadian variation amplitude compared to plasma ACTH (27, 62). The hypothesis is that hypothalamic PVN neurons, which receive input from the SCN, project to the sympathetic preganglionic neurons in the spinal cord and provide inhibitory and excitatory inputs to the adrenal cortex, possibly via a cAMP-mediated pathway, thereby increasing free cholesterol transport into the mitochondria for steroidogenesis (63). Therefore, the circadian neuronal control of the adrenal glands amplifies the effects of plasma ACTH rhythmicity on driving the glucocorticoid circadian rhythm (60).

Clock gene expression in the HPA axis

Throughout the last decade, evidence has shown that each anatomic component of the HPA axis possesses a unique circadian expression of clock genes, leading to characteristic circadian signatures for each tissue. Rhythmic clock gene expression had been observed in the adrenal glands of the rhesus monkey and rodents. Like humans, rhesus monkeys are more active during the day. Furthermore, adrenal expression of *Per1* and *Per2* peaked at the beginning of the light phase and was synchronised with the peak of plasma cortisol (37). Conversely, mouse adrenal studies performed under similar photoperiodic conditions reported that *Per1* and *Per2* acrophases occurred closer to the darkness phase in

synchrony with the peak of corticosterone (64). Majority of the canonical clock genes (*Bmal1*, *Cry1*, *Per1*, *Per2*, *Per3* and *Rev-Erba*) exhibited a circadian expression pattern in both the *glomerulosa* and *fasciculata* zones of the mouse adrenal gland (65). However, *Clock* and *Cry2* transcripts did not show an obvious circadian rhythm. Moreover, the circadian rhythmicity of the *Per1*, *Per2* and *Bmal1* genes in the rat adrenal gland remained unaltered after hypophysectomy, indicating that the activity of the adrenal oscillators was independent of SCN signalling via the pituitary gland (66). Additional focused analyses revealed that *STAR* mRNA and protein, a rate-limiting regulator of steroidogenesis, possessed circadian variation with a late-daytime acrophase in accordance with plasma corticosterone profiles in mice (67). Notably, *STAR* gene expression was transcriptionally regulated by the binding of CLOCK/BMAL1 heterodimers to *Star* upstream *cis* elements of the murine promoter, indicating a direct link between adrenocortical clock genes and the regulation of steroidogenesis (67). These studies indicated that the glucocorticoid circadian rhythm was also dependent on a well-organised level of rhythmicity within the adrenal cortex.

Girotti *et al.* (36) examined the 24-h pattern of ACTH and corticosterone secretion, the functional expression of *c-fos*, *Crh*, *Pomc* and *Star* and the expression of *Per1*, *Per2* and *Bmal* clock genes in each component of the HPA axis. They subsequently compared hormone rhythms with circadian rhythms of clock genes in the adrenal gland, pituitary gland and SCN. Among adult rats fed *ad libitum*, the corticosterone rhythm acrophase occurred at the onset of the darkness period and was in phase with *Star*, *Per1* and *Per2* expression rhythms in the adrenal glands. Moreover, *Bmal* temporal expression was in antiphase with *Per1* expression in the adrenal cortex and in phase with *Bmal* circadian expression in the anterior pituitary gland. Interestingly, diurnal rhythms in plasma ACTH and *Pomc* gene expression in the pituitary gland were not as evident as those in adrenal clock genes. However, *Per2* and *Bmal* expressions in the anterior pituitary gland showed a clear rhythm and were in antiphase with each other. In the SCN, *Per1* and *Per2* acrophases occurred during the light period, whereas *Bmal* peaks occurred during the darkness period (68). These genes also showed a clear rhythm in the PVN while being in antiphase with their respective expressions in the SCN. Moreover, *Crh* expression in the PVN displayed an evident circadian rhythm in phase with *Bmal* circadian expression (36).

Our group showed that among rats that had food access *ad libitum* and those that had restricted food

Review	A C Moreira and others	Glucocorticoid circadian clock	179:1	R6
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access, different phases in clock genes expression were observed in the SCN, PVN and ARC. In addition, clock gene expression in the SCN, PVN and ARC responded to feeding schedules in a tissue-specific manner, despite on-going photoperiod *zeitgeber* (69). Recently, Chung *et al.* (70) demonstrated that in mice, adrenal clock-dependent steroidogenesis and the SCN-driven central mechanism regulating corticosterone release cooperate to produce a daily rhythm in circulating glucocorticoids. Taken together, these studies demonstrate that each component of the HPA axis has a distinctive circadian clock gene expression, although the main timing signals are propagated downstream from the SCN to the HPA axis.

Circulating glucocorticoids as a circadian signal

Circulating glucocorticoids, the end-effectors of the HPA axis, regulate a broad spectrum of physiologic functions while also serving as a humoral signal that transduces circadian variation signals from the SCN pacemaker to reset peripheral clock gene expression (71, 72, 59). Thus, glucocorticoids influence brain regions outside the SCN, such as the PVN, pineal gland, central amygdala, stria terminalis, hippocampus and pituitary gland (73), as well as peripheral tissues, such as the liver, gut, muscles, white adipose tissues, lungs, kidneys, bone, skin, blood, cardiovascular and immune/inflammatory systems (59). A recent study suggested that corticosteroid rhythmicity is necessary for maintaining high amplitude with no regulatory impact on clock gene phases in peripheral tissues (74).

At the cellular level, glucocorticoids exert their diverse effects via a specific intracellular receptor, the glucocorticoid receptor (GR), which belongs to the nuclear receptor superfamily and is expressed in almost all human tissues and organs (75, 76). In addition to genomic actions, GRs also exert rapid, non-genomic effects, which are possibly mediated by membrane-localised receptors or by translocation into the mitochondria. The molecular mechanisms of human GR action and variability in glucocorticoid sensitivity have already been reviewed elsewhere (77, 78). Additionally, clock genes and GR expression in peripheral tissues possess a reciprocal interaction. Daily variations in corticosteroids reset circadian rhythms of peripheral clock genes *Per1*, *Per2*, *Bmal1*, *Cry1* and *Cry2*. Meanwhile, CLOCK-BMAL1 proteins modulate the activity and bioavailability of GRs through acetylation (79, 71). In addition, Lamia (80)

showed that both circadian co-regulators, *Cry1* and *Cry2*, interact with GRs in a ligand-dependent manner to oppose the transcriptional response to glucocorticoids. The crosstalk between clock genes and GRs provides a finely tuned molecular feedback loop that modulates the effects of glucocorticoid circadian rhythm in peripheral tissues. Furthermore, the relative activities of both type 1 and type 2 11- β -hydroxysteroid dehydrogenase isoenzymes, which interconvert inactive cortisone into active cortisol, or vice-versa, play an important role in the regulation of tissue cortisol concentrations (81).

Glucocorticoids have also been well known to suppress CRH secretion in the PVN and ACTH secretion in the anterior pituitary gland, establishing a negative feedback loop that resets the activated HPA axis. Glucocorticoids exert rapid negative feedback mediated by GRs in CRH neurons and pituitary corticotrophs through rapid steroid action in the PVN involving the glucocorticoid–endocannabinoid interface via activation of the G-protein-dependent signalling pathway (82). The delayed effects include the inhibition of pro-opiomelanocortin (POMC) and CRH transcription due to mRNA and protein turnover (83). Thus, besides the classical neuroendocrine regulation of the HPA axis, synchronisation can be observed among clock genes in each anatomic component of the HPA axis via feedback loops involving clock genes, proteins and corticosteroids. These reciprocal interactions between molecular and physiological oscillations at multiple levels of the HPA axis result in a robust circadian rhythm of plasma glucocorticoids.

The ontogeny of the glucocorticoid circadian rhythm

The development of the glucocorticoid circadian rhythm follows the gradual maturation of the SCN and HPA axis. Although the ontogeny of the SCN and circadian rhythms in foetal and neonatal rats, hamsters and sheep has been extensively described (84, 85, 86, 87), scanty data are available for primates (87, 88), primarily humans (89, 90). Human foetal SCN neurogenesis is almost completed between the 18th and 30th week of gestation. Moreover, the PVN is already formed at the 25th week of gestation (89), while RHT maturation takes place at the end of gestation. Accordingly, evaluation of the daily ^{14}C -labelled deoxyglucose utilisation patterns in squirrel monkey fetuses during late foetal development confirmed that the SCN was metabolically more active in the morning than at night (91). In addition, light induced

a robust increase in SCN metabolic activity and *Per1* gene expression in baboon infants at ages equivalent to 24 weeks after conception in humans (92). Furthermore, circadian rhythms of several physiological functions, such as heart rate and foetal movements, are present in human foetuses at a gestational age of 29–35 weeks (93). These data provide evidence that the primate SCN circadian clock is already functioning and responsive to light prenatally, while the rat SCN develops gradually with neurogenesis being completed only after birth (84, 85, 86, 87). In addition, ipRGC projection to the SCN via the RHT and maturation of light signalling are completed around P10 in rats, while circadian rhythms of temperature, locomotor activity, drinking and plasma corticosterone also develop after birth (84, 86, 94).

Molecular studies have supported evidence regarding the presence of circadian rhythmicity in SCN clock genes during foetal and neonatal periods in mammals (95, 96, 97). During the foetal stage, *in situ* hybridisation found no detectable *Per1*, *Per2*, *Cry1* and *Bmal1* gene rhythms in the rat SCN at E19, whereas a clear oscillation was observed from P3 to P10 and thereafter (95, 96). A study utilising foetal rat SCN sampling via laser microdissection confirmed the absence of *Per2* and *Bmal1* rhythms at E19, whereas rhythmic expressions of *Rev-Erba* and other genes related to cellular activity (*c-fos*, *Avp* and *Vip*) were observed. However, *Per2* and *Bmal1* expressions exhibited low amplitude but significant rhythmicity at E21 (98). These studies suggested that the development of clock gene rhythms in the foetal SCN could, theoretically, be entrained by *Rev-Erba* oscillations driven by maternal cues rather than the foetal circadian system (98). Another review on the circadian cogwheel development of the

non-primate SCN demonstrated that genes in the negative feedback loop of the core clock become rhythmic earlier than those in the positive feedback loop (99). Unlike that observed in the foetal rat SCN, robust oscillatory expressions of *Bmal-1*, *Per-2*, *Cry-2* and *Clock* in the SCN and adrenal glands, as well as a circadian rhythm of plasma DHEAS, were observed among capuchin monkey foetuses at the end of gestation (100).

The establishment of the HPA axis circadian rhythm in neonatal rats, with a vespertine plasma corticosterone acrophase, has been determined to vary across different studies. Accordingly, such an event had been found to occur on postnatal days P30, P22, P21 or P16 (11, 12, 101, 102). Applying appropriate protocols and using cosinor-based rhythmometry, we had recently conducted a refined study on the emergence of rat postnatal corticosterone rhythm and its relationship with the adrenal rhythmicity of *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2* and *Rora/Rev-Erba* genes and steroidogenesis-related genes (*Star* and *Mc2r*) in rats from the early postnatal period until adulthood (103). In our study, rat pups at postnatal ages P1, P3, P6, P12, P14, P16, P21 and P24 were decapitated at 4-h intervals over a 24-h period. Accordingly, plasma corticosterone circadian rhythm with a morning acrophase (between ZT0 and ZT2, corresponding to 07:00–11:00h) had been observed from P1 until P12 (Fig. 3). Plasma corticosterone rhythmicity, as well as a morning acrophase, at very early postnatal ages had never been previously described. This novel finding was subsequently ascribed to the time of breastfeeding. Conversely, a nocturnal acrophase had been observed at P14, P16, P21 and P24. In our experiments, this rhythm, which was well known in adult rats, had been associated with eye opening in pups at P14. We also demonstrated

Figure 3

Postnatal ontogeny of circadian plasma corticosterone in rats. Plasma corticosterone concentrations at *zeitgeber* time 0, 4, 8, 12, 16 and 20 on postnatal days P1 to P24. Dotted line: mesor; solid line: cosine curve and 95% confidence interval of the acrophase. *Differences between peak and mesor concentrations. Black bars represent the dark phase (Reproduced with permission from: Postnatal Ontogeny of the Circadian Expression of the Adrenal Clock Genes and Corticosterone Rhythm in Male Rats. *Endocrinology*. 2017; 158(5):1339–1346).

circadian variations, as well as a morning acrophase, in the mRNA expression of *Bmal1*, *Per2*, *Per3* and *Cry1* genes from P3, whereas *Rev-Erba* presented a nocturnal acrophase. From P14, *Bmal1*, *Per2*, *Per3* and *Cry1* acrophases advanced by approximately 10h compared to early neonatal periods and became vespertine nocturnal (103), which was consistent with the adult circadian profile as previously described for adult rat adrenal glands (36).

The relationship between plasma corticosterone and clock gene acrophases in adrenal tissues was observed across all postnatal developmental ages. Accordingly, *Per2* and *Cry1* circadian profiles were in phase with the circadian rhythm of plasma corticosterone, whereas *Bmal1* was in antiphase (Fig. 4). Considering that rat SCN maturation and clock gene expression rhythmicity are completed only after P10 (86), we hypothesised that the external rhythmic cues for pups before P10 may have been derived from the maternal environment, entraining the circadian rhythm of adrenal clock genes and corticosterone secretion (103). Although adult rat activity is predominantly nocturnal, mothers approach the litter to feed their pups during the

light phase. The mother's body temperature and tactile stimulation during breastfeeding (104), as well as high milk melatonin levels during night-time (105), may be identified as synchroniser agents. Furthermore, melatonin may synchronise *Per2*, *Bmal1* and *StAR* expression phases in rat foetal adrenal glands in parallel with decreasing corticosterone levels (106, 107). In capuchin monkeys, however, maternal melatonin seems to be a *zeitgeber* for *Bmal1* and *Per 2* expression in the foetal SCN but not in the foetal adrenal glands (100). During the late postnatal phase, the entrainment of rat adrenal circadian variations may shift from maternal and nonphotic to photic cues. In addition to light, daily patterns of food ingestion and motor activity can also act as synchroniser agents in adult rats. Taken together, these data suggest that rats exhibit a progressive postnatal maturation of circadian variations in adrenal clock genes, which is in synchrony with the development of the plasma corticosterone circadian rhythm (103).

Studies in various species have addressed the ontogeny of the postnatal glucocorticoid rhythm and its relationship with circadian expression of clock genes in different components of the HPA axis (87, 108, 103). However, no studies regarding clock gene rhythmicity and its relationship with the ontogeny of the cortisol circadian rhythm have been conducted on human foetal tissues, such as the brain, pituitary gland or adrenal gland. The presence of a 24-h rhythm in umbilical artery plasma cortisol concentrations was demonstrated in human foetuses at term (109), suggesting that the human SCN and adrenal circadian clock systems function prenatally. Another study showed that 2 out of 10 healthy term neonates displayed a cortisol rhythm, which was not coupled to exogenous day/night cycles (110). However, no circadian rhythmicity in cortisol and 17 α -hydroxyprogesterone had been observed in saliva samples from 119 healthy neonates collected 18–24h or 25–55h after birth (111). These data suggest that circadian cortisol secretion may increase later in gestation and may not uniformly occur in all infants of the same age. In addition, given that birth seems to induce re-entrainment, circadian cortisol secretion may be present at birth, despite synchronisation to day/night cycles taking place after the neonatal period (112). Although the aforementioned studies involved human samples collected during the first few hours after birth, the question regarding the onset of the early morning glucocorticoid peak and its maintenance in subsequent postnatal weeks remains unsolved.

Previous data have described the emergence of the plasma cortisol circadian rhythm in children aged between

Figure 4

Acrophases of plasma corticosterone and clock gene expression during postnatal development in rats. Circles indicate the acrophase and 95% confidence interval of *Bmal1*, *Per2*, *Per3*, *Cry1* and *Rev-Erba* expressions and plasma corticosterone concentrations at *zeitgeber* time 0, 4, 8, 12, 16 and 20 on the postnatal days P1 to P24. Black bars represent the dark phase (Reproduced with permission from: Postnatal Ontogeny of the Circadian Expression of the Adrenal Clock Genes and Corticosterone Rhythm in Male Rats. Endocrinology. 2017; 158:1339–1346).

1 and 3 years (13), 6 months (14) and between the second and third month of life (113, 114). Discrepancies in human studies regarding the age wherein plasma cortisol circadian variations appear could be ascribed to the ethical difficulties in obtaining serial blood samples from infants. This limitation had been overcome during the last decades with salivary cortisol measurements, an index of plasma free cortisol that has been used as a tool in longitudinal protocols, given that saliva samples are easier to collect at home many times a day over consecutive days and obtained via a non-invasive procedure that causes no pain or stress for the infant (115, 34).

Four previous longitudinal studies that used salivary cortisol from healthy term infants found that the mean age wherein adult-like rhythm became apparent was between the first and third month of life (116, 117, 118, 119).

All aforementioned studies performed saliva sampling many times a day, including morning, afternoon and night, started collection from 2 weeks until 12 months after birth and had a varying number of healthy term infants. The observed divergences could have been due to the different mathematical definitions of circadian rhythms. One definition of circadian rhythmicity was the amplitude between morning and evening values with a steady decline throughout the day (116). Other definitions of circadian rhythmicity were assumed when night values were less than three times the mean intra-assay variation from the morning value taken as 100% (117), when the median morning cortisol level was significantly higher than the median evening cortisol level (119) or when different methods were compared using multilevel analysis calculations (118). Altogether, these data confirm that salivary cortisol circadian rhythm appears in healthy term infants at least 1 month postnatal, which was earlier than previously thought. In addition, variability had been observed among healthy individuals (117). Indeed, approximately 30% of normal infants presented a circadian cortisol rhythm as early as 2 or 4 weeks postnatal.

Nevertheless, the correlation between plasma and salivary cortisol in preterm infants remains controversial (120, 121). Accordingly, our group was able to observe a correlation between plasma and salivary cortisol levels (120) while also showing the appearance of a salivary cortisol circadian rhythm in preterm infants at a median age of 8 postnatal weeks, similar to term infants. However, individual circadian rhythm in term and preterm infants emerged 2–16 weeks postnatal (122). It is important to point out that daily cortisol variations in our studies should have been maintained for consecutive weeks

in order to be considered an established rhythm. More recently, Ivars *et al.* (123) described the development of a cortisol circadian rhythm in preterm infants on a group level at a corrected age of 1 month. However, they did not specify whether the rhythm had been maintained, as we did in our protocol. We also observed a similar age of salivary cortisol rhythm emergence in monozygotic and dizygotic twin pairs at 7.8 and 7.4 weeks, respectively (124). The heritability index and intraclass correlation coefficients suggest a greater environmental rather than genetic influence, indicating synchrony in the emergence of a cortisol circadian rhythm between each twin pair. This was likely to occur given that the twins shared prenatal and postnatal environmental synchronisers.

Ivars *et al.* found no correlation between the development of salivary cortisol rhythm and behavioural regularity (123). Our study, however, observed a parallelism between the appearance of a salivary cortisol rhythm and the onset of sleep rhythm (122) (Fig. 5). In addition, another study demonstrated a negative correlation between napping and cortisol levels only among older children (125). Taken together, all these data confirm circadian maturation of the HPA axis at a very early stage in human development. Moreover, the association between cortisol circadian rhythm and behavioural development, including sleep patterns, suggest that the complex neuroanatomical pathways, the clock gene system and the HPA axis become fully developed between 31 and 34 weeks post conception and continue to mature thereafter.

Cortisol circadian variation in Cushing's disease

Normal human adult cortisol secretion follows a diurnal variation with plasma levels peaking shortly after morning awakening, termed cortisol awakening response (CAR) (126), and decreasing thereafter, reaching a nadir around midnight. This pattern is maintained by changes in the amplitude of ACTH secretion throughout the day, leading to a mean of approximately 10 cortisol secretory peaks over 24 h (127, 128, 129). The oscillatory pattern had been well described using a waveform-independent deconvolution technique. Analysis of 24-h plasma concentration profiles in healthy subjects revealed that the nyctohemeral pattern of cortisol secretion can be accounted for by an amplitude-modulated, random burstlike mode of cortisol secretion without the need for postulating a tonic mode of cortisol release (130). This

Figure 5

Ontogeny of salivary cortisol circadian rhythm and sleep activity in preterm infants. (A) Mean values of total 24-h sleep time (solid squares), nocturnal sleep time (solid triangles) and diurnal sleep time (open circles). (B) Mean concentration of salivary cortisol in the morning (open circles) and at night (solid triangles) (Adapted with permission from: The emergence of salivary cortisol circadian rhythm and its relationship to sleep activity in preterm infants. *Clinical Endocrinology* 2000 52:423–426).

dynamic concept regarding the physiological mode of cortisol secretion integrates random cortisol pulses with rhythmic circadian variations, eliminating the idea of a tonic glucocorticoid secretion mode (130). Aside from the ACTH-specific amplitude and/or frequency that control secretory burst activity, circadian ACTH and plasma cortisol concentrations in humans probably also depend on the expression of intrinsic clock genes in the HPA axis that oscillate at a periodicity of approximately 24 h, as observed in rodents and non-primate mammals (20, 21, 22). Abnormalities in the HPA axis circadian rhythm were

observed in mice carrying mutated clock genes. Indeed, *Per1*- and *Per2*-null mice showed elevated corticosterone levels with no circadian rhythm (131), whereas *Per1^{Brd}* mice demonstrated elevated glucocorticoid levels with no daily fluctuation. In addition, *Per1^{Brd}* mice were smaller than wild-type mice and showed normal glucose tolerance and no apparent Cushingoid phenotype (132). To the best of our knowledge, no molecular studies on clock genes in Cushing's disease (pituitary ACTH-dependent Cushing's syndrome) have been conducted to date.

Simultaneous with the detailed characterisation of the normal circadian ACTH and cortisol secretion in healthy subjects, abnormalities in the HPA axis were described in patients with Cushing's disease. Accordingly, increased mean pulsatile ACTH amplitude but not frequency had been observed in patients with Cushing's disease (129, 133). This disruption led to the absence of the normal 24-h cortisol secretory variation and the failure of plasma cortisol levels to drop at late evening (127, 134). In addition to these alterations, another remarkable characteristic of corticotropinomas includes the partial resistance to the glucocorticoid inhibitory feedback. This feature is demonstrated by the failure of low-dose dexamethasone to dose-dependently suppress circulating cortisol or its urinary metabolites during a suppression test (135, 136).

Thus far, investigations on the structure and expression of CRH, AVP, ACTH and GR genes have not shown significant abnormalities that could explain the relative glucocorticoid resistance observed in corticotropinomas (137, 138, 139). The loss of cortisol circadian rhythm is a hallmark of patients with Cushing's syndrome of adrenal or ectopic ACTH origin. However, some patients with Cushing's disease may exhibit cortisol circadian variation (140, 141, 142). Indeed, 57% of patients with Cushing's disease completely lost salivary cortisol circadian rhythm, while 43% exhibited persistent diurnal variation albeit with abnormally high cortisol levels (143). HPA activity reflects the circadian release of hypothalamic CRH, which is difficult to measure in plasma and even in the inferior petrosal sinuses (144). Thus, as a substitute for portal vein CRH, published studies have adopted cerebrospinal fluid CRH levels, which was described to have an evening acrophase and an early morning nadir in healthy volunteers (145), similar to the pattern described in rhesus monkeys (146). Notably, the CRH circadian pattern is in opposition to the circadian pattern observed for plasma or cerebrospinal fluid cortisol. On the other hand, patients with Cushing's disease exhibited lower levels of cerebrospinal fluid CRH compared to normal subjects, which returned to normal after pituitary adenectomy

(147, 148). CRH levels in the cerebrospinal fluid were also reduced in patients receiving steroid treatment (148) and dogs with hyperadrenocorticism due to an adrenocortical tumour (149). These data suggest that hypercortisolism causes reduced CRH concentrations.

Based on the aforementioned data, we hypothesised that patients with Cushing's syndrome lose their cortisol circadian rhythm once circadian CRH release in the portal vein is completely abolished. On the other hand, a number of patients with Cushing's disease exhibit only reduced CRH secretion. In such cases, ACTH secretion from the adenoma would be still under the relative control of the SCN circadian clock genes, which would explain the maintenance of the cortisol circadian rhythm in such patients even with abnormally high cortisol levels. However, the potential role of circadian inputs from sympathetic adrenal modulations, as well as the influence of the melatonin rhythm, cannot be ruled out. Indeed, the melatonin circadian rhythm is maintained in majority of the patients with Cushing's syndrome (150). Thus, in corticotropinomas, the glucocorticoid resistance in corticotrophs and the disrupted glucocorticoid circadian rhythm contribute to hypercortisolism and the dysregulation of the clock system in peripheral tissues, leading to intermediary metabolism alterations and clinical features of Cushing's disease (Fig. 6).

Biochemical strategies to establish a diagnosis of Cushing's syndrome have been based on measurements of urinary free cortisol (UFC), low-dose dexamethasone suppression tests (1 mg overnight or longer) and late-night salivary cortisol (LNSC) levels (151, 152, 153).

As will be focused in the present review, the use of LNSC measurements, a simple test that evaluates the presence or absence of cortisol circadian rhythm, in the screening of Cushing's syndrome has increased over time (154).

The importance of salivary steroid analysis for the evaluation of pituitary–adrenal function had been established during the late 1970s (155). However, not until the mid-1980s had its use for diagnosing Cushing's syndrome been suggested (156, 157). Subsequently, two studies clearly showed that salivary cortisol was a highly suitable screening procedure for determining patients with Cushing's syndrome (158, 143). A comparison between normal volunteers, obese patients and patients with Cushing's syndrome revealed a substantial overlap in morning salivary cortisol levels such that patients with Cushing's syndrome exhibited higher cortisol levels that were almost entirely outside the normal range at 23:00h. This difference resulted in a sensitivity and specificity of 93.3% for the diagnosis of Cushing's syndrome (143). Indeed, several studies have confirmed that elevated LNSC levels are an excellent substitute for increased midnight serum cortisol levels in the diagnosis of Cushing's syndrome in adults (143, 157, 158, 159, 160, 161, 162) and children (163, 164). These data have been confirmed using meta-analysis (165, 166). Another strategy to improve the diagnostic accuracy of Cushing's syndrome is the substitution of plasma for salivary cortisol measurement after a 1 mg dexamethasone suppression test (156, 157). Based on previous studies, the combination of saliva collection at 23:00h and after 1 mg dexamethasone overnight improved the diagnostic accuracy of Cushing's

Figure 6

Abnormal cortisol circadian rhythm and dysregulation of clock genes in the pathophysiology of Cushing's disease. Corticotropinomas are partially resistant to cortisol negative feedback (1), leading to increased ACTH secretion (2). In turn, increased cortisol (3a) reduces CRH concentrations (4) and leads to abnormal cortisol rhythm. Disrupted glucocorticoid circadian rhythm and hypercortisolism (3b) deregulate the interaction between the glucocorticoid receptor (GR) and clock system in peripheral tissues (5). PVN, paraventricular nucleus; RHT: retinohypothalamic tract.

syndrome in adults and children (143, 163). It is important to highlight that salivary cortisol diagnostic cut-offs and diagnostic performance vary between reports because of the different assays used (151, 165, 167).

In a more recent prospective study (168), we compared the variability, reproducibility and diagnostic performances of LNSC and UFC using simultaneous samples of 43 patients with Cushing's disease for 3 consecutive days. UFC failed to diagnose 18.6% of patients with Cushing's disease, whereas LNSC failed in only 2.3% (Fig. 7). The receiver-operating characteristic curves confirmed that LNSC was better in diagnosing Cushing's disease than UFC. The superiority of LNSC over UFC for diagnosing either current or early-stage recurrence of Cushing's syndrome had been observed despite high variability for LNSC (168, 169). Considering the variability, at least three LNSC sampling is recommended. Altogether, these data

indicate that loss of salivary cortisol circadian rhythm is an early marker for Cushing's disease, while increased UFC seems to occur later during the natural progression of the disease (168, 169).

Moreover, a distinct phenomenon termed CAR appears to be superimposing the circadian rhythm of cortisol (170, 171). CAR is the rapid increase in cortisol levels within 30–45 min after morning awakening. We showed blunted CAR in patients with active Cushing's disease, which can be related to hypercortisolism upon awakening. This subtle dysfunction of the HPA axis may represent a distinct and additional physiopathological phenomenon overlaying the disrupted cortisol circadian rhythm in patients with Cushing's disease (172).

Concluding remarks

The feedback loops between clock genes and their transcript proteins determine a self-sustained circadian rhythm with a periodicity of approximately 24 h. Moreover, the fine-tuning of the molecular circadian timekeeping system depends on photic and nonphotic environmental synchronisers. Clock genes and their transcript proteins bind to the promoters of more than a thousand clock-controlled target genes. The endogenous circadian rhythm of the HPA axis is propagated downstream from the SCN to other hypothalamic nuclei, pituitary and adrenal circadian clock genes by controlling cyclic hormonal factors and autonomic innervations. Circulating glucocorticoids, which are the end-effectors of the HPA axis, regulate physiologic function while also serving as a transducer of circadian signals from the SCN pacemaker to reset peripheral clock genes, which in turn interact reciprocally with GRs. These interactions between molecular and physiological oscillations result in the plasma glucocorticoid circadian rhythm, the development of which follows the gradual maturation of the SCN and HPA axis. The SCN circadian clock in primates is already functioning and responsive to light prenatally, while salivary cortisol circadian rhythm in infants becomes apparent at around 1 month postnatal. In corticotropinomas, the glucocorticoid resistance in corticotrophs and the disrupted cortisol circadian rhythm contribute to hypercortisolism and clock system dysregulation in target tissues, leading to alterations in intermediary metabolism and clinical features of Cushing's disease. For diagnostic purposes, an abnormal salivary cortisol circadian rhythm seems to be an early marker of Cushing's disease.

Figure 7

Diagnostic performance of late-night salivary cortisol and urinary free cortisol in Cushing's disease. Individual values of three concurrent and consecutive late-night salivary cortisol (LNSC) and urinary free cortisol (UFC) samples obtained from 43 patients with Cushing's disease (CD). The dotted line represents the cut-off values for LNSC (350 ng/dL) and UFC (43 µg/24h). UFC failed to diagnose 18.6% of patients with Cushing's disease, whereas LNSC failed in only 2.3%, confirming the superiority of LNSC. (Reproduced with permission from: Late-night Salivary Cortisol Has a Better Performance than Urinary Free Cortisol in the Diagnosis of Cushing's Syndrome. *Journal of Clinical Endocrinology & Metabolism* 2014; 99 2045–2051.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Review	A C Moreira and others	Glucocorticoid circadian clock	179:1	R14
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Review	A C Moreira and others	Glucocorticoid circadian clock	179:1	R16
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Review	A C Moreira and others	Glucocorticoid circadian clock	179:1	R18
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