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Introduction
The HPA axis
The hypothalamic-pituitary-adrenal (HPA) axis regulates circulating levels of glucocorticoid hormones, and is the major neuroendocrine system in mammals that provides a rapid response and defense against stress. Under basal (i.e., unstressed) conditions, glucocorticoids are released with a pronounced circadian rhythm, characterized by peak levels of glucocorticoids during the active phase, that is daytime in humans and nighttime in nocturnal animals such as mice and rats. When studied in more detail, it becomes clear that the circadian rhythm of the HPA axis is characterized by a pulsatile release of glucocorticoids from the adrenal gland that results in rapid ultradian oscillations of hormone levels both in the blood and within target tissues, including the brain. In this review, we discuss the regulation of these circadian and ultradian HPA rhythms, how these rhythms change in health and disease, and how they affect the physiology and behavior of the organism. © 2014 American Physiological Society. Compr Physiol 4:1273-1298, 2014.

Adrenal glucocorticoid synthesis
Glucocorticoids are synthesized in the zona fasciculata of the adrenal cortex. Glucocorticoid steroidogenesis is activated when ACTH binds to its specific cell surface G-protein-coupled receptor, the melanocortin type-2 receptor (MC2R) (137). Upon ACTH binding, MC2R undergoes conformational changes that activate adenyl cyclase, leading to an increase in intracellular levels of cyclic adenosine monophosphate (cAMP). This intracellular increase in cAMP activates downstream signaling pathways, including the protein kinase A (PKA) pathway. Activation of PKA induces acute synthesis of glucocorticoids via both genomic and nongenomic
mechanisms. The rate-limiting step in glucocorticoid synthesis is the mobilization of cholesterol and its transfer inside the mitochondria, and this process is mediated by the activity of the steroidogenic acute regulatory protein (StAR) (118, 185). Activation of the PKA signaling pathway activates nuclear transcription factors, such as the cAMP response element (CRE) binding protein (CREB), which in turn induce transcriptional activation of a number of steroidogenic genes, including StAR. In parallel to StAR transcription, PKA also induces posttranslational modification of StAR, resulting in phosphorylation at a specific site that activates StAR and increases its ability to transport cholesterol across the mitochondrial membrane (9). Within the mitochondria, cholesterol is subjected to enzymatic modifications by a cascade of steroidogenic regulators, which ultimately lead to glucocorticoid synthesis (21, 36, 80).

Glucocorticoid receptors and genomic responses

Glucocorticoids exert their effects by binding to specific receptors expressed in target tissues. Two receptors for glucocorticoids are known: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These receptors belong to the superfamily of nuclear receptors (48, 123). When inactive, they reside in the cytoplasmic compartment, stabilized by chaperone molecules (140, 147, 215), including the 90-kDa heat-shock protein (HSP-90) (149). Upon glucocorticoid binding, the chaperone-receptor complex dissociates to enable the ligand-receptor complex to translocate to the nucleus, where it functions as transcription factor to modulate genomic events via activation or repression of transcription of glucocorticoid target genes.

GR and MR differ in their affinity for glucocorticoids. The affinity of the endogenous hormones corticosterone and cortisol for MR is approximately 10-fold higher than the affinity for GR. Therefore, variations in circulating levels of glucocorticoids lead to shifts in the balance between MR and GR activity (158). The high-affinity MR is activated at very low concentrations of glucocorticoids, whereas the lower affinity GR is only activated at high concentrations. In basal (non-stressed) physiological states, when glucocorticoid concentrations are low, MR is activated and localized in the nucleus, whereas GR remains latent in the cytoplasm. Once glucocorticoid concentrations increase above a threshold level, for example during the circadian peak or following exposure to stress, GR translocates into the nucleus where it exerts its genomic effects (39, 50, 106, 156, 157, 177).

Differences between MR and GR are also found in their distribution throughout the body. While GR is ubiquitously expressed in the periphery and in the brain, the distribution of MR is more localized to specific organs such as the kidney and...
the heart. In the brain, the distribution of these receptors is also not uniform (156, 157). GR is almost ubiquitously expressed in neurons and glial cells, with particularly high density in the hippocampus and in parvocellular neurons of the PVN. In contrast, MR localization is restricted to the hippocampus, prefrontal cortex, and amygdala (3, 10, 198), structures essential for learning, memory, and emotional behavior. Significantly, both receptors are expressed abundantly in areas regulating the HPA axis, including a number of limbic structures that regulate the activity of the PVN, such as the hippocampus and amygdala, as well as in the anterior pituitary (79).

Nongenomic effects of glucocorticoids

In addition to their genomic effects, glucocorticoids can also modulate cellular activity much more rapidly (i.e., within seconds to minutes) through nongenomic actions; that is, independently of gene expression and de novo synthesis of mRNA and protein.

Rapid nongenomic effects of glucocorticoids occur through a mechanism mediated by G-protein coupled membrane-associated receptors, with a pharmacological profile different from the well known intracellular GR (141, 142). Given the rapidity with which glucocorticoids inhibit the activity of the HPA axis, this effect is likely to be mediated by nongenomic mechanisms at both the level of the pituitary and the brain. For example, corticosterone—administered peripherally—suppresses rat hippocampal cell firing within 20 min (146), while mEPSC frequency increases within minutes in CA1 pyramidal cells from rat hippocampal slices infused with corticosterone. In the rat, a rapid inhibitory effect of glucocorticoids has also been observed in the activity of PVN neurons projecting to the median eminence (102, 115, 164). Furthermore, a fast inhibitory effect of corticosterone on CRF-induced ACTH secretion has been observed in the rat within 15 min of corticosterone administration (75). In addition, a rapid (within 20 min) effect of the synthetic glucocorticoid methylprednisolone on corticosterone secretion has also been observed (178).

Studies from Tasker and colleagues have shown that nongenomic inhibitory effects of glucocorticoids in the PVN are mediated by a mechanism involving glucocorticoid-induced endocannabinoid synthesis. Indeed, Di et al. (51) have shown that rapid glucocorticoid effects on PVN neuronal activity are mediated by the activation of postsynaptic receptors and the dendritic synthesis and release of an endogenous endocannabinoid, which in turn suppresses excitatory synaptic inputs by feeding back onto glutamatergic pre-synaptic terminals.

Evidence for rapid glucocorticoid actions in the brain is also supported by behavioral studies. For example, corticosterone has been shown to inhibit male reproductive behavior as well as medullary neuronal firing in newts (160). Similarly, corticosterone has been shown to have a rapid effect on aggressive behavior, by modulating glucocorticoid-responsive hypothalamic areas (108).

Circadian Rhythms

The suprachiasmatic nucleus and the clock gene network

From the Latin circa diem, circadian means “around a day,” and circadian rhythms refer to biological oscillations with a period of approximately 24 h. Circadian rhythms are highly conserved among living organisms, from cyanobacteria to mammals. Circadian rhythms are characteristic of a number of biochemical, physiological, endocrine, and behavioral functions, and this allows the organism to anticipate and prepare for predictable environmental changes (i.e., the light/dark cycle). The result is an optimization of energy expenditure by timely coordination of physiological processes.

In mammals, circadian rhythms are generated and synchronized by a central clock in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus (126, 135, 188). The activity of the SCN is entrained by the light input received from the retina via the retino-hypothalamic tract, and this allows our physiology to be entrained to solar time (72, 136). The molecular mechanism regulating the circadian activity of each single cell within the SCN consists of an oscillating transcriptional network, including the “clock genes” circadian locomotor output cycles kaput (CLOCK), brain and muscle aryl hydrocarbon receptor nuclear translocator-like (BMAL1), period 1-3 (PER1-3), and cryptochrome 1-2 (CRY1-2) (20, 61, 104). A network of positive and negative transcriptional, translational and posttranslational feedback loops regulates the rhythmic transcription of these genes, with subsequent rhythmic expression of their protein products (155). Signaling between the SCN and the periphery involves both hormonal and neuronal mechanisms (112). Rhythmic activity of the SCN is translated into circadian patterns of gene expression in target tissues, which in turn determine circadian rhythms in the physiological function of that specific organ.

In the SCN, the expression of vasoactive intestinal polypeptide (VIP) is under regulation of the clock machinery. VIP is released with a 24-h cycle, and activates and synchronizes SCN neuronal activity (5, 120). In rodents, lesioning of the SCN results in a loss of physiological circadian rhythmicity, including body temperature, locomotor activity, and hormone secretion (1, 17, 182, 200), even when the 24-h light-dark cycle is maintained, and the circadian rhythm of locomotor activity can be reinstated in arrhythmic animals by transplantation of an intact SCN (153).

In addition to the central master clock in the SCN, clock genes are expressed rhythmically in peripheral tissues, including liver, kidney, skeletal muscle, lung, and adrenal (16, 70, 71, 187). However, although the molecular dynamics of clock genes are the same in the SCN and the peripheral clocks, only the SCN functions as a self-sustaining pacemaker at the whole-tissue level (139, 208, 216); while the SCN can independently generate and maintain its own circadian rhythms, peripheral clocks require SCN-dependent drive to maintain synchronicity with the light-dark cycle (139, 208). Nevertheless, the existence of peripheral clocks suggests that
local clock gene networks can generate and regulate the circadian rhythm of a number of cellular functions, such as daily variations in metabolic and cardiovascular activity, and hormone secretion. Indeed, disruption of clock gene expression in the brain and muscle has been associated with a loss of circadian rhythmicity in locomotor activity (127). Furthermore, ablation of clock genes in the liver leads to a dysfunction in glucose and lipid metabolism (109, 110), and disruption of clock genes in the pancreas is associated with diabetes mellitus in mice (124).

Circadian Rhythm of Glucocorticoid Secretion

Consistent with other circadian physiological functions, the circadian rhythm of the HPA axis is primarily regulated by the central pacemaker located in the SCN (95). The SCN regulates the circadian rhythm of glucocorticoids in part by modulating the release of CRH from neurons in the PVN, and in part via the autonomic nervous system (ANS) through a multisynaptic neural pathway from the SCN to the adrenal gland. Additionally, there is evidence of an intra-adrenal circadian pacemaker that can regulate glucocorticoid synthesis independently from the SCN (144, 175).

SCN regulation of HPA axis circadian rhythms

Neuro-anatomical tracing studies have identified direct axonal projections from the SCN to the PVN, as well as indirect connections via the dorsomedial hypothalamus (DMH) (204, 205). In turn, circadian secretion of CRH regulates circadian release of ACTH from the anterior pituitary (26, 206). Interestingly, the expression of StAR in the adrenal cortex, which is dependent on ACTH signaling, is also characterized by a circadian rhythm, as shown by changes in both mRNA and protein levels throughout the 24-h cycle (63, 145, 175, 195), and this in turn regulates circadian synthesis and release of glucocorticoids (Fig. 2).

A number of studies have demonstrated a role for vasopressin release from the SCN in modulating HPA circadian activity. Vasopressin-containing neurons in the dorsal SCN have been shown to be an important component of the SCN output (94), and experiments using micro-infusions of vasopressin and its antagonists in the PVN and the DMH have shown that vasopressin released from SCN terminals strongly...

Figure 2  Circadian rhythms in the HPA axis. Rat ACTH (A) and corticosterone (B) profiles show a clear circadian variation over the 24-h period with peak levels during the active phase. Steroidogenic acute regulatory protein (StAR), the rate limiting protein in adrenal glucocorticoid synthesis, is also expressed in the adrenal with a circadian pattern that is similar to that observed for ACTH and corticosterone (C). In contrast, the expression pattern of the specific ACTH receptor melanocortin 2 (MC2R) appears to be in antiphase with both StAR and ACTH (D). Reproduced with permission from (145).
inhibits the release of adrenal corticosterone in the rat (93). Furthermore, a decrease in vasopressin release from these SCN terminals in the DMH toward the active dark phase is necessary for the circadian increase in plasma corticosterone (97). The important role of vasopressin in regulating low corticosterone levels during the circadian nadir of HPA axis activity has also been confirmed in a series of experiments in vitro using brain slices in which removal of the SCN from the slice results in a loss of circadian rhythm in the spontaneous firing rate of PVN neurons (194). However, as shown in the same study, this rhythm can be reinstated either by using co-cultures of the slice with SCN tissue, or by a rhythmic (12 h on, 12 h off) perfusion of vasopressin.

In nocturnal animals, where light entrains the SCN during their inactive phase, vasopressin release is important to ensure low circulating levels of corticosterone during the circadian nadir (96). In diurnal species (e.g., man), vasopressin release from the SCN is also induced by light, suggesting that vasopressin has an excitatory effect on HPA axis activity in diurnal organisms. Indeed, this is in accordance with the well-characterized excitatory effect of vasopressin on its neuronal targets (89, 107). These observations clearly suggest that the mechanism through which vasopressin exerts an inhibitory or excitatory effect on CRH release in nocturnal or diurnal species, respectively, does not involve a direct effect of vasopressin on CRH neurons, but depends on vasopressin activation of either an inhibitory or excitatory neuronal pathway regulating CRH release in the PVN. Indeed, although there are direct projections from the SCN to the PVN (204, 205), there is currently no evidence for a direct connection between fibers from SCN vasopressin neurons and CRH neurons in the PVN (18, 199).

A detailed anatomical scheme of the SCN neuronal connections in both nocturnal and diurnal species has been proposed by Kalsbeek and colleagues by comparing the effect of vasopressin on CRH release in the nocturnal rat and in the diurnal Sudanian grass rat (Arvicathanis ansorgei) (95). In accordance with their model, vasopressin is released from the SCN during the day in both nocturnal and diurnal species. In nocturnal species, vasopressin released from the SCN will activate gamma-aminobutyric acid (GABA)ergic interneurons within the PVN and the DMH, which in turn will directly or indirectly (via the DMH) inhibit CRH release during the circadian nadir. In contrast, in the diurnal species, vasopressin release from the SCN will activate glutamatergic interneurons in the PVN and DMH, which in turn will stimulate CRH release. Although the importance of SCN-secreted vasopressin for inhibition of PVN activity has been well described in the rat, a neurotransmitter responsible for SCN stimulatory effect on the HPA axis during the circadian peak has not been clearly identified. A number of studies have also suggested that a stimulatory signal from the SCN via the release of VIP modulates the evening rise in HPA activity (5, 120). Furthermore, a role for neuromedin U in regulating the stimulatory effect of the SCN on HPA axis activity has also been proposed (64).

**ANS regulation of the glucocorticoid circadian rhythm**

Although a circadian rhythm of ACTH has been described in the rat, the circadian variation in corticosterone release throughout the 24-h cycle is more pronounced than the circadian variation in ACTH release. Indeed, the circadian rhythm in ACTH release is often undetectable (4, 44, 97, 98, 145, 195, 210). This dissociation between ACTH and corticosterone during the circadian cycle suggests a diurnal variation in the adrenal sensitivity to ACTH, with higher responsiveness during the peak phase of glucocorticoid secretion. Indeed, it has been proposed that, in addition to the regulatory effect on CRH secretion, the SCN can also regulate adrenal activity independently from hypothalamic-pituitary drive by modulating adrenal sensitivity to ACTH. The adrenal gland receives sympathetic innervation via the thoracic splanchnic nerve. Using transneuronal virus tracing, Buijs and colleagues (19) have identified direct connections between the adrenal gland and neurons located in the intermedio-lateral column of the spinal cord, which receive input from the autonomic division of the PVN, which in turn is innervated by vasopressin neurons from the SCN. This is consistent with studies from Jasper and Engeland (87) showing that transection of the splanchnic nerve in the rat increases corticosterone secretion in the morning but has no effect in the evening, suggesting an inhibitory effect of the splanchnic nerve on corticosterone secretion during the nadir phase of the circadian rhythm. Further studies from the same group have also demonstrated a role for the splanchnic nerve in the regulation of adrenal sensitivity to ACTH in nonstressed rats (88). The mechanism by which the splanchnic nerve regulates adrenal sensitivity to ACTH is not fully understood. A study in which the adrenal responsiveness to different doses of exogenous ACTH was investigated (195) has shown that the decrease in corticosterone levels induced by splanchnic nerve transection during the peak phase of hormone secretion is associated with a decrease in the levels of adrenal cAMP. Since ACTH induces glucocorticoid synthesis via the cAMP/PKA signaling pathway, these data suggest that splanchnic nerve activity regulates adrenal sensitivity to ACTH by affecting intracellular signaling pathways involved in glucocorticoid synthesis. Via a signaling pathway involving cAMP, PKA, and CREB, ACTH induces transcriptional activation of several steroidogenic genes, including StAR. However, in this study, the circadian rhythm of StAR was not affected by lesioning the splanchnic nerve, suggesting that the effects of splanchnic activation at the level of adrenal sensitivity to ACTH may be modulated by a nongenomic mechanism, such as phosphorylation of StAR.

**Circadian clock genes in the adrenal gland regulate rhythmic glucocorticoid synthesis**

In addition to the SCN, key components of the HPA axis, including the PVN, pituitary, and adrenal cortex, express circadian clock genes (63, 81, 95, 143, 144). Indeed, using
Microarray and qPCR techniques to analyze circadian gene expression in the mouse adrenal gland, Oster and colleagues have recently shown that approximately 5% of the whole genome—including several canonical clock genes—demonstrates rhythmic expression (143). Evidence for the involvement of adrenal clock genes in circadian glucocorticoid synthesis has been shown in vivo using mice defective in components of the circadian clock system. In fact, studies in clock gene knockout mice have shown that disruption of the circadian clock in these animals is associated with a disruption of the HPA circadian rhythm. Dallmann and colleagues (46) have shown that, while both Per1 KO mice and Per2 KO mice have markedly elevated levels of circulating glucocorticoids, only Per1 KO mice lack a detectable circadian hormone rhythm. In agreement with this study, a loss of circadian rhythm of corticosterone has been found in Per2/Cry1−/− double mutant mice, whereas circadian rhythmicity of ACTH is maintained in these mice (144). Interestingly, in these animals, a number of clock genes, including Bmal1, Per1, and Per3, which are all rhythmically expressed in the wild-type animals, lack transcriptional circadian rhythmicity. The same authors have shown that transplantation of wild-type adrenal glands restores corticosterone rhythmicity, whereas transplanting adrenal glands from arrhythmic Per2/Cry1 double mutant mice to wild-type adrenalectomized mice dampens the corticosterone circadian rhythm. Furthermore, the circadian rhythmicity of clock genes in the adrenal is independent of circadian rhythmicity of ACTH, as has been shown in a recent study in which circadian rhythms of clock genes such as Per1, Per2, and Bmal1 are maintained in rats after hypophysectomy (58). Taken together, these observations suggest that, although the central pacemaker in the SCN controls peripheral clock gene rhythmicity, clock genes in the adrenal may have an important role in regulating circadian rhythms of glucocorticoid synthesis.

Several rhythmically expressed genes in the adrenal gland encode proteins involved in steroidogenic processes, including proteins involved in cholesterol biosynthesis and transport (e.g., StAR), and proteins implicated in ACTH receptor signaling [e.g., MC2R and the melanocortin 2 receptor accessory protein (MRAP)] (144, 145, 175). Park and colleagues have found that the pattern of StAR expression is consistent with the pattern of ACTH and corticosterone secretion; that is, StAR mRNA and protein levels are maximal during the active phase. Interestingly, the pattern of expression of MC2R mRNA appears to be in antiphase with both StAR expression and ACTH and corticosterone secretion (Fig. 2D). A recent study has shown that, while the ACTH circadian rhythm is unchanged in Bmal1 deficient mice, circadian variations of corticosterone are reduced, with lower corticosterone levels during the peak phase (111). Interestingly, the effect of Bmal1 deficiency on the corticosterone rhythm in these animals is associated with downregulation in the expression of several key genes involved in cholesterol trafficking, including StAR, while the expression of MC2R is elevated.

The mechanisms underlying the regulation of adrenal steroidogenesis by adrenal clock genes have recently been elucidated using Bmal1 adrenal knockdown mice (175). This study has shown that the circadian expression of StAR appears to be directly regulated by the Clock:Bmal1 complex at the level of the StAR promoter in the adrenal gland. Bmal1 adrenal knockdown mice display a flattened circadian rhythm of StAR protein expression, and although the circadian rhythm of corticosterone is maintained in these animals when they are kept under a normal light-dark cycle, corticosterone rhythmicity is lost in mutant mice maintained in constant darkness. This suggests that, while in normal light conditions adrenal circadian rhythmicity is primarily under the control of the SCN via both hypothalamic-pituitary and splanchnic nerve regulation, the adrenal clock pathway may become important for regulation of steroidogenic genes when the central circadian input is disrupted.

A more recent study has shown that in addition to StAR, several of its transcriptional regulators are also rhythmically expressed in the adrenal gland (145), and this includes positive regulators such as the nuclear receptors steroidogenic factor 1 (SF-1) (189) and nerve growth factor IB (NGFIB, also known as Nur77 or NR4A1) (176), as well as the negative regulator nuclear receptor dose-sensitive sex reversal, adrenal hypoplasia congenita determining region on the X-chromosome-1 (DAX1) (217). Consistent with their role in regulating the expression of adrenal StAR, SF-1 and Nur77 mRNA and protein levels are high during the hormonal circadian peak, whereas levels of expression of DAX1 are high during the circadian nadir (145). Whether circadian variation in StAR’s transcriptional regulators is important for the circadian corticosterone rhythm, via regulation of the StAR circadian rhythm, is not known. However, Park and colleagues have shown that disruption of the corticosterone circadian rhythm in rats kept in constant light is associated with a disrupted circadian rhythm of StAR and its transcriptional regulators (145). Whether transcriptional activation of StAR’s regulators is under control of the clock gene machinery, as is the case for StAR, is also not currently known. Taken together, these data suggest that, while the SCN is indispensable for maintaining a circadian rhythm of glucocorticoid secretion, the adrenal clock provides an additional level of control by regulating the steroidogenic pathway.

**Ultradian Rhythm of Glucocorticoid Secretion**

As shown for other neuroendocrine systems that signal through the hypophysial portal system, such as the gonadotropic hormone and growth hormone pathways, the basal activity of the HPA axis is characterized by pulsatile activity. The circadian variation in glucocorticoid levels over the 24-h cycle is not made up of a smooth change in hormone levels, but is in fact characterized by a rapid ultradian,
pulsatile pattern of hormone secretion, with a periodicity of approximately one hour (Fig. 1B). The ultradian glucocorticoid rhythm has been reported in numerous species, including rat (86, 214), rhesus monkey (76, 192), sheep (59), and human (73, 113, 207).

The ultradian rhythm of corticosterone in the rat was first described by Jasper and Engeland using intra-adrenal microdialysis in nonstressed freely behaving animals (86). In addition to the hourly corticosterone pulse frequency, they also observed that both the ultradian frequency and amplitude of the pulses are modulated in a circadian manner (87).

The pattern of corticosterone secretion has also been investigated in blood plasma. An automated blood-sampling (ABS) system developed by Clark et al. (37) enables the remote collection of small blood samples at a high frequency (e.g., every 5-10 min) over extended periods of time (214). This system provides a powerful tool to study glucocorticoid levels in “unstressed” rats over the whole 24-h cycle, and has been used to show that corticosterone levels in the blood are also pulsatile with an ultradian frequency. Analysis of pulse characteristics using the PULSAR algorithm (130) has shown that the pulse amplitude varies in a circadian manner, with low amplitudes in the morning and higher amplitudes in the evening (213).

**Ultradian rhythms of the HPA axis**

There is good evidence that the circadian rhythm of glucocorticoids is regulated by the SCN. However, the mechanisms generating the ultradian pulsatile dynamics of the system remain poorly understood. In rats with SCN lesions, the circadian rhythm of corticosterone is disrupted, but the ultradian pulsatile pattern of corticosterone is maintained (200), which suggests that the origin of the ultradian rhythm of corticosterone is generated independently of the SCN (Fig. 3).

A number of studies have shown that, in addition to endogenous glucocorticoids, an ultradian pattern underlies other components of the HPA axis. Indeed, a small number of studies, both *in vitro* and *in vivo*, have reported episodic release of CRH from macaque hypothalamic explants (131), rapid fluctuations in CRH levels in the median eminence of freely behaving rats (83, 85), and pulsatile patterns of CRH and AVP in the hypophysial-portal circulation of conscious sheep (24, 55). In addition to this, ultradian pulses of ACTH have been observed in the rat (25, 26, 29), sheep (8), and human (73).

Observations of pulsatile CRH have led to a general acceptance that a “hypothalamic “pulse generator”” drives the ACTH and glucocorticoid ultradian rhythm. However, a number of findings are in contrast to this hypothesis. Significantly, there is a mismatch between the frequency of CRH pulses and the frequency of ACTH and corticosterone oscillations. In the rat, CRH pulse frequency is higher (∼3 pulses/h) (83) than the near-hourly oscillation in ACTH (26) and glucocorticoids (214) (Fig. 4). Furthermore, studies in the sheep have shown that pituitary-adrenal ultradian activity is maintained following hypothalamic disconnection from the pituitary (56), suggesting that a supra-pituitary pulsatile drive is not required for pulsatility in ACTH and glucocorticoids.

The pituitary-adrenal system is characterized by both a positive feed-forward pathway whereby pituitary ACTH drives glucocorticoid release from the adrenal cortex, and a negative feedback mechanism through which adrenal glucocorticoids feed back on the anterior pituitary to inhibit ACTH release. A short time delay in the pituitary-adrenal feed-forward pathway has been observed both in humans and in the rat, where each pulse of ACTH is followed by a delayed response in cortisol or corticosterone, respectively (27, 73) (Fig. 5). Furthermore, a rapid inhibitory effect of glucocorticoids on CRH-induced ACTH secretion from the anterior pituitary has been shown (75, 90, 92, 122, 161-163, 209). The observation of this fast inhibitory feedback within the pituitary-adrenal system has led to the hypothesis that this process could provide a potential mechanism within the system for generating oscillatory dynamics.
The origin of the glucocorticoid ultradian rhythm

The interaction between the pituitary corticotrophs and the adrenal fasciculata cells has been modeled mathematically utilizing differential equations that incorporate the rapid glucocorticoid inhibition of ACTH secretion (202). Numerical analysis of the model suggests that the pituitary-adrenal system can support self-sustained ACTH and glucocorticoid oscillations with a physiological ultradian frequency, even under conditions of constant CRH drive to the anterior pituitary. Analysis of this model has provided a number of theoretical predictions (Fig. 6). First, the model predicts that oscillations in ACTH and glucocorticoids can be generated for “intermediate” constant levels of CRH, and that these oscillations occur at the same frequency as endogenous corticosterone oscillations observed in the rat during the peak phase of the circadian cycle (Fig. 6, A and B). Moreover, the model also suggests that these glucocorticoid oscillations become “damped” for higher (or lower) levels of CRH (Fig. 6, A and C). These predictions are consistent with experimental observations that ACTH and corticosterone pulse amplitude increases during the early dark phase are accompanied by increased mean levels of CRH in the median eminence (84), and with the loss of pulsatile corticosterone secretion that occurs both at the circadian nadir of HPA axis activity and following an acute stress response (214), when the levels of CRH are respectively lower and higher than during the circadian peak. The third key prediction of this mathematical model is that glucocorticoid oscillations induced by constant CRH stimulation are driven by oscillations in ACTH, with a phase shift between ACTH and glucocorticoids. This is again consistent with experimental data showing that ultradian ACTH oscillations have been observed in the rat (26,27) and that pulsatile ACTH is a critical factor in regulating pulsatile corticosterone secretion from the adrenal cortex (180).

Consistent with both mathematical and experimental data, high-frequency blood sampling in humans under basal, non-stressed, conditions has revealed a close temporal relationship between pulses of ACTH and cortisol, with a short phase lag between the two hormones (73) (Fig. 5).

The hypothesis that the sub-hypothalamic pituitary-adrenal system can function as an ultradian hormone oscillator has been tested in vivo by studying the dynamics of corticosterone responses to different levels of constant CRH stimulation in conscious freely behaving male rats (201). In
the male Sprague-Dawley rat, the HPA circadian cycle is characterized by a distinct and prolonged nadir phase (7 am–1 pm), when endogenous CRH secretion is minimal (84) and there is no detectable pulsatile secretion of ACTH or corticosterone (28,214). This experimental model allows manipulation of the system by controlling the level of CRH and simultaneously measuring the levels of ACTH and corticosterone, without interference from endogenous HPA hormone release. Constant infusions of CRH (during the circadian nadir) have been shown to induce different temporal patterns of corticosterone secretion that are dependent on the level of CRH. In keeping with the predictions of the mathematical model (202), a constant infusion of CRH induces sustained ultradian corticosterone oscillations that persist throughout the infusion period, with pulse amplitude remaining relatively constant throughout the infusion (Fig. 7A). Furthermore, there is a phase shift between ACTH and corticosterone, with ACTH oscillations preceding corticosterone oscillations, also predicted by the mathematical model (Fig. 7, B and C). This time delay between ACTH and corticosterone pulses reflects the time taken for de novo biosynthesis and release of corticosterone from the adrenal cortex upon stimulation with ACTH (186). Interestingly, this oscillatory response to CRH is dose dependent, and becomes disrupted for higher levels of CRH.

In summary, the hypothesis that a systems-level dynamic balance between positive feed-forward and negative feedback provides a mechanism for generating ultradian oscillations in ACTH and glucocorticoid hormone secretion has been supported by both mathematical modeling and experimental data and these findings provide good evidence for an oscillating mechanism outside the central nervous system.

Adrenal regulation of glucocorticoid pulsatility
Glucocorticoid hormones are lipophilic and therefore cannot be simply packaged in readily releasable vesicles within steroidogenic cells in the adrenal cortex, but need to be newly synthesized in response to ACTH. Given that pulsatile secretion of glucocorticoids is generated by pulses of ACTH, as shown by both mathematical modeling and experimental work (201, 202), it is clear that within the adrenal there must be a remarkably responsive steroid synthesis mechanism that is able to respond rapidly to each pulse of ACTH. Indeed, a recent study in the rat has shown that pulsatile ACTH is necessary for optimal release of corticosterone (180); in rats in which endogenous HPA activity has been suppressed by the addition of a synthetic glucocorticoid (methylprednisolone, MP) to the drinking water, pulsatile infusion of ACTH results in a normal pattern of pulsatile corticosterone secretion (Fig. 8A), whereas an identical dose of ACTH infused at a constant rate has no effect on the adrenal corticosterone secretion.
regulation of StAR in adrenalcortical cells has been shown to be mediated by PKA, playing a role in the immediate initiation of steroidogenesis and glucocorticoid secretion upon stimulation of the adrenal by ACTH (31, 133, 134, 148). Therefore, it is possible that the pulsatile transcription of StAR following each pulse of ACTH may be acting as mechanism for replenishing depleted intracellular stores of StAR (that have been used to generate a pulse of corticosterone) prior to subsequent ACTH pulses. Furthermore, these observations are consistent with previous data showing that only newly synthesized StAR is involved in posttranslational events mediating the rapid synthesis of corticosterone (11). Whether nongenomic events regulating rapid steroidogenesis are also characterized by a pulsatile pattern of activity is currently under investigation.

To better understand the mechanism underlying the in vivo generation of pulsatile corticosterone secretion from adrenal fasciculata cells within the adrenal cortex, the dynamics of steroidogenic gene transcription in relationship to the dynamics of corticosterone secretion has been investigated over a detailed time-course following a single ultradian pulse of ACTH (179). In the rat, pulsatile release of corticosterone, induced by intravenous (IV) administration of a small ACTH pulse, is associated with pulsatile transcription of steroidogenic genes, including StAR (118, 186) (Fig. 8B). This has been shown by measuring rapid changes in the levels of heterogeneous RNA (hnRNA), which is a reliable marker of gene transcription. Specifically, StAR hnRNA levels peak at 15 min after ACTH administration, and return to basal levels within 30 min. A similar dynamic pattern of transcription has been observed for CYP11A, the gene encoding for cholesterol side-chain cleavage cytochrome P450 (P450scc) (36), which catalyses the cleavage of the cholesterol side chain to produce pregnenolone in the inner mitochondrial membrane. In keeping with these findings, Spiga and colleagues (179) have also shown that activation of CREB and its transcriptional regulator TORC2 (transducer of regulated CREB activity 2, also known as CRTC2) also occurs in a highly dynamic pattern following pulsatile ACTH stimulation: PKA-mediated activation of CREB and TORC2, by phosphorylation and dephosphorylation respectively, occurs within 5 min of exposure to a pulse of ACTH, supporting an involvement of these proteins in the rapid (within 15 min) transcription of StAR. An involvement of TORC2 in the CREB-mediated transcriptional regulation of StAR in adrenalcortical cells has been shown in vitro (190, 191). A recent study has shown that a pulse of ACTH also induces pulsatile transcription of the gene encoding for MRAP (119), a protein that is crucial in regulating the level and activity of MC2R at the cell surface, and thus the cell’s responsiveness to ACTH (132) (Fig. 8C). Taken together, these studies clearly suggest that pulsatile secretion of corticosterone is associated with pulsatile transcription of steroidogenic genes in the adrenal gland.

Although corticosterone levels peak within 5 min of an ACTH pulse (given IV), hnRNA StAR levels do not peak until 15 min after the ACTH pulse. The fact that the peak of the secretory event precedes the transcription peak of the steroidogenic enzyme is consistent with evidence that nongenomic posttranscriptional and posttranslational changes in the StAR protein regulate the rapid steroidogenic response. Indeed, rapid activation of proteins involved in steroidogenesis by phosphorylation, such as phosphorylation of StAR on Ser194 mediated by PKA, plays a role in the immediate initiation of steroidogenesis and glucocorticoid secretion upon stimulation of the adrenal by ACTH (31, 133, 134, 148). Therefore, it is possible that the pulsatile transcription of StAR following each pulse of ACTH may be acting as mechanism for replenishing depleted intracellular stores of StAR (that have been used to generate a pulse of corticosterone) prior to subsequent ACTH pulses. Furthermore, these observations are consistent with previous data showing that only newly synthesized StAR is involved in posttranslational events mediating the rapid synthesis of corticosterone (11). Whether nongenomic events regulating rapid steroidogenesis are also characterized by a pulsatile pattern of activity is currently under investigation.

Figure 7 Constant infusion of CRH induces pulsatile secretion of ACTH and corticosterone. (A) Individual corticosterone response to constant CRH infusion. In line with the modeling hypothesis, constant infusion of CRH (0.5 μg/h) induces ultradian corticosterone oscillations that persist throughout the infusion period. This oscillatory response is characterized by an initial rapid increase in corticosterone within approximately 15 min following the onset of the CRH infusion, reaching peak levels by approximately 30 min and returning to low levels by approximately 80 min. Following the initial pulse, the amplitude of the oscillation is relatively constant throughout the infusion. Samples were collected every 5 min from a freely behaving male rat using an automated blood sampling system. Grey bar indicates the period of infusion. (B) ACTH and corticosterone responses to constant CRH infusion. Samples were collected manually at 20 min intervals throughout the infusion via an indwelling cannula implanted in the jugular vein. In agreement with the modeling predictions, CRH induces ACTH and corticosterone oscillations that persist throughout the infusion period. (C) Phase-shifted ACTH and corticosterone response to constant CRH infusion (0.5 μg/h) over the initial activation phase (0-25 min) of the oscillation. This phase shift between ACTH and corticosterone presumably reflects the time taken for de novo biosynthesis and release of corticosterone in the adrenal cortex. Reproduced with permission from (201).
Glucocorticoid Rhythms in Health and Disease

Over the last 20 years, we have used our ABS system to define corticosterone pulsatility in freely behaving rats in their home cages. These studies have revealed that both the circadian and the ultradian pattern shows marked sexual dimorphism and genetic variation. Furthermore, corticosterone rhythms are remarkably plastic, with changes seen both in physiological conditions, such as during lactation and ageing, and in pathological conditions, particularly stress-related disorders.

Physiological changes: Gonadal steroids

A marked difference in the levels of basal corticosterone secretion between male and female rodents is well recognized. Recently, studies using frequent blood sampling to investigate corticosterone pulsatility in the rat have shown a profound difference in the pulsatile profile of corticosterone release over the 24-h cycle between male and female rats (169-172). Compared to male rats, basal corticosterone secretion is enhanced in female rats (169) (Fig. 9, A and B), and this is due to: (i) an increase in the total number of pulses over the 24-h cycle (26 pulses for females vs. 10 pulses for males); (ii) an increase in pulse magnitude (approximately 60 ng of corticosterone/pulse for females vs. 24 ng/pulse for males); and (iii) a greater number of pulses over the 24 h period.

Gonadal steroids have major effects on the pulsatile pattern of corticosterone release in the rat. Following gonadectomy, male rats have increased overall corticosterone secretion similar to that observed in female rats, characterized by an increased number of pulses, pulse amplitude, and...
pulse frequency. On the other hand, ovariectomized females have reduced overall corticosterone levels similar to those observed in male rats, with a reduction in the number of pulses, and decreased pulse amplitude and frequency (169). To better understand whether these changes are due to a direct effect of gonadal hormone withdrawal, further studies have been carried out to investigate the effect of androgen or oestrogen replacement on corticosterone secretion in the gonadectomized male and female, respectively (170): androgen replacement reverses the increase in corticosterone pulse frequency and amplitude induced by castration in male rats; similarly, oestradiol reverses the changes in corticosterone secretory activity in ovariectomized female rats.

Neonatal masculinization or feminization also affects basal corticosterone secretion patterns in adult life in female and male rats, respectively (171,172). Exposure of the female neonate to a single injection of testosterone within 24 h of birth results, in adult animals, in a reduction in the number of corticosterone pulses, as well as pulse amplitude and frequency, over the 24-h period, compared to untreated adult females, and these pulse characteristics are comparable to those observed in normal adult male rats (171). Consistent with this, changes in the adult pulsatile pattern of corticosterone secretion are observed in male rats exposed to neonatal feminization (172): here, rats deprived of pre and postnatal testosterone activity by exposure to the antiandrogen flutamide and the aromatase inhibitor 1,4,6-androstatriene-3,17-dione, respectively, show increased basal corticosterone secretion characterized by an increase in the number, frequency, and amplitude of corticosterone pulses. Interestingly, the amplitude of the corticosterone pulses of prenatal testosterone-deprived rats is similar to those observed in intact females and castrated males (169).

The mechanisms underlying the effects of gonadal steroids on the ultradian rhythm of glucocorticoids are not clear. There is evidence suggesting that androgens and estrogens can affect HPA axis activity at the level of the adrenal gland, anterior pituitary and PVN [reviewed in (67)]. This is supported by an overlap in the expression of gonadal hormone receptor and glucocorticoid hormone receptors in key areas regulating the activity of the HPA axis (67).

Figure 9. Changes in ultradian glucocorticoid dynamics in different physiological and pathological states. (A and B) Ultradian corticosterone oscillations in a female (A) and male (B) Sprague-Dawley rat. Reproduced with permission from (169) (C and D). Ultradian corticosterone oscillations in juvenile (C) and adults (D) rats (unpublished observations). (E and F) Ultradian corticosterone oscillations in a control male PVG rat (E) and a male PVG rat with active immune-mediated adjuvant-induced arthritis (F). Reproduced with permission from (212). In all experiments, corticosterone was measured in blood samples collected at 10 min intervals from freely behaving rats. Shaded regions indicate the dark phase.
Differences in female corticosterone levels across the estrus cycle have been observed. During the diestrous phase, corticosterone levels in female rats are similar to those observed in male rats; in contrast, during the proestrous phase, when the concentrations of progesterone and estradiol are elevated, corticosterone levels are increased (34, 45), and this is paralleled by an increase in CRH mRNA expression in the PVN (82). Consistent with this, inhibition of HPA axis activity by androgens is paralleled by a reduction in CRF-immunoreactivity in the PVN (15). Gonadal steroid hormones can also regulate basal glucocorticoid secretion by affecting GR signaling. Indeed, estrogens regulate the transcriptional activity of a number of proteins involved in GR activity, such as the chaperone protein FKBP51 (78) and the nuclear GR coactivator SRC1 (22). Taken together, these studies suggest that sex steroids can affect the glucocorticoid ultradian rhythm by modulating both hypothalamic CRH drive and GR-mediated glucocorticoid negative feedback.

Physiological changes: Ageing

Studies on ageing rats have revealed marked changes in the pattern of corticosterone pulsatility across their life cycle (117) (Fig. 9, C and D). In juvenile and adult rats, marked changes in pulse amplitude over the 24-h cycle have been observed, with higher amplitude pulses during the peak phase, resulting in the well defined circadian rhythm of corticosterone. In elderly (12-month-old) rats, however, there is a loss of circadian rhythmicity predominantly due to a decrease in pulse amplitude during the peak phase, but also due to an overall loss of ultradian pulsatility throughout the 24 h. These observations are important and suggest a potential link between disrupted corticosterone pulsatility and age-related disorders.

Physiological changes: Reproduction

Changes in corticosterone rhythms have also been observed in female rats over the course of their reproductive cycle (211). Circadian and ultradian rhythms of corticosterone have been studied in virgin rats, rats on day 9 of lactation, weaned dams 2 days after pups removal, and postlactating dams 13 days after pups removal. During lactation, although there is a significant circadian variation in corticosterone release, this is associated with a flattening of the rhythm—due to a decrease in the evening peak levels and an increase in the number of corticosterone pulses throughout the 24 h. Very marked changes in corticosterone release are also observed in rats 2 days after experimental weaning. In this group, corticosterone levels are significantly suppressed throughout the 24-h period, with the number of pulses and pulse amplitude significantly lower than any other group. Interestingly, these effects are reversible, as no differences in either circadian or ultradian rhythms of corticosterone are observed between 13 days postlactating dams and virgin rats.

Physiological changes: Genetic background

Changes in corticosterone pulsatility patterns related to genetic background have also been observed. Ultradian corticosterone rhythms have been investigated in a number of rat strains, including Wistar, Sprague Dawley, Lewis, and Fisher 344. Although they all exhibit a pulsatile pattern of corticosterone release, there are significant differences between strains (213). Lewis rats have a clear circadian rhythm, as seen in female and male Sprague Dawley and Wistar rats, due to changes in pulse amplitude over the 24-h cycle, which is greater in the evening than in the early morning. In contrast, female Fischer rats have higher mean circulating corticosterone concentrations and lack any discernible circadian variation, and this is due to increased pulse amplitude in the morning, which is similar to pulse amplitude in the evening. The mechanism underlying the different pattern of corticosterone secretion between the two strains is not known. Given that female Fisher rats maintain high-pulse amplitudes throughout the morning, it has been proposed that insensitivity to corticosterone feedback, mediated by activation of both GR and MR, might be responsible for the lack of circadian rhythm. Furthermore, in light of the role of splanchnic innervation of the adrenal in the regulation of circadian adrenal responsiveness to ACTH (195), the differential dynamics in pulsatile secretion between the two strains could also be due to differences in sympathetic regulation of adrenal sensitivity. Interestingly, Lewis rats are known to be susceptible to a range of inflammatory conditions, including streptococcal cell wall-induced arthritis (183, 184), whereas the Fisher rat does not develop any of these conditions. This suggests that differences in disease susceptibility may be linked to differences in the dynamics of corticosterone secretion.

Pathological changes: Chronic stress

Chronic inflammation is a model of chronic stress that has high clinical relevance. In particular, a model of chronic inflammatory disease that has been extensively studied in the rat is *Mycobacterium*-adjuvant-induced arthritis. Increased circulating corticosterone and ACTH concentrations, and loss of the normal circadian rhythm of HPA activity, have been observed in Piebald-Viral-Glaxo (PVG) rats infected with the *Mycobacterium*-adjuvant (212) (Fig. 9, E and F). While control PVG rats demonstrate the expected circadian and ultradian patterns of corticosterone release, significant changes in the dynamics of corticosterone secretion occur both prior to the development of arthritis (presymptomatic rats sampled at 6 days after injection of the mycobacterium) and following the development of hind-joint inflammation (13 days after injection). In presymptomatic rats, although a diurnal pattern of corticosterone secretion is still observed, there are changes in the characteristics of the pulses. While no changes in the number of pulses or their amplitude have been observed across the 24-h cycle, in presymptomatic rats the average length of time during which hormone levels rise from baseline to
peak is decreased. Specifically, within each pulse, corticosterone levels rise to peak concentrations more quickly than in control rats. During the symptomatic period, rats show dramatic changes in pulsatility, with an almost doubled number of pulses throughout the 24-h cycle related to continued pulsatility during the normally quiescent lights-on period. Interestingly, neither the amplitude or the duration of the pulses are different from control rats, indicating that the increase in circulating hormone is solely due to this increased number of pulses. It is important to note that in rats exposed to adjuvant-induced arthritis, a decrease in CRH synthesis and secretion has also been observed, together with a very marked increase in pituitary portal levels of vasopressin (2, 34, 68). The lack of changes in corticosterone pulsatility across the 24-h cycle in these animals suggests a consistent level of CRH (and AVP) activation of the pituitary across the day within the range that will generate pulsatile ACTH and corticosterone secretion, as predicted by the mathematical model described by Walker and colleagues (201, 202).

Another model of chronic stress that has been found to affect corticosterone pulsatility in the rat is exposure to constant light. Experimental animals are normally kept under a 12-h light-dark cycle, with light input acting as a zeitgeber processed through the SCN. Disruption of the SCN signal, either by physical lesioning of the SCN or by disruption of the light input, induces a loss of circadian rhythmicity and this is associated with changes in corticosterone pulsatility. Rats maintained under conditions of constant light for a prolonged period of time (5 weeks) lose their circadian corticosterone rhythm, which is due to increased pulsatility during the nadir phase of the circadian cycle, with no difference between the nadir and the peak phase pulse amplitude (200). These same rats have increased levels of CRH mRNA in the morning, which probably accounts for the increased corticosterone secretion during the nadir phase. This suggests that removal of the inhibitory SCN input to the hypothalamic PVN results in sufficient CRH secretion throughout the 24 h to maintain ultradian pituitary-adrenal activity.

The SCN also mediates corticosterone secretion by regulating adrenal sensitivity to ACTH through a mechanism that involves the ANS and splanchnic nerve. It has been shown that light can activate adrenal secretion of corticosterone in a way that is independent of pituitary release of ACTH, but that involves activation of the splanchnic nerve (19, 81). It is therefore possible that the effect of constant light exposure on corticosterone pulsatility may also occur due to a direct effect on the adrenal gland.

Pathological changes: Neonatal programming

Exposure to stress during the neonatal period has profound effects on physiological functions that can be observed in adult life. This phenomenon is known as neonatal programming. Early life stress can also program the development of the HPA axis with changes that persist for the rest of the life of the organism. The effect of neonatal programming on corticosterone pulsatility in the rat has been investigated using a model of early life infection (174). In rats exposed to endotoxin (Salmonella enteritidis) in neonatal age (days 3 and 5 postpartum), basal levels of corticosterone are higher both during the nadir and peak phase of the circadian cycle in adult life. This is due to an increase in both the number and amplitude of corticosterone pulses throughout the 24-h cycle.

The Importance of Pulsatility for Hormonal and Behavioral Response to Stress

The functional interaction between glucocorticoid pulsatility and the response to stress has been investigated in a number of studies where rats are exposed to stress during different phases of their ultradian corticosterone secretory profile (165, 213). Windle and colleagues (213) have shown that the timing of the stressor relative to the phase of the underlying ultradian rhythm is crucial in determining the magnitude of the corticosterone response. Specifically, in rats exposed to a mild stressor such as white noise, the corticosterone response is considerably greater when the stressor is applied during the rising phase of a corticosterone pulse than when the stressor is applied during the falling phase (Fig. 10), which suggests a facilitated stress response during the rising phase and/or an inhibitory effect during the falling phase. This relationship between pulse-phase and the ability of the HPA axis to respond to acute stress has been observed in both male and female rats. These observations indicate the existence of a dynamic interaction between basal glucocorticoid pulsatility and the ability of an animal to mount a hormonal stress response.

Mathematical modeling and in vivo experimental work suggest that ultradian glucocorticoid oscillations can be generated by the feedforward-feedback interaction between the anterior pituitary and adrenal cortex, independent of pulsatile hypothalamic stimulation (201, 202). Therefore, exposure to a stressor, and its associated release of hypothalamic CRH and AVP, can be considered as a transient perturbation to the endogenous oscillatory activity of the pituitary-adrenal system. This mathematical model has also been used to understand in more detail the interaction between pulsatility and stress responsiveness (154). In addition to explaining earlier observations that the magnitude of the stress response depends on the timing of the stress, analysis of this model has also shown that an external stress can act as a resetting mechanism to the phase of the endogenous ultradian rhythm.

The mechanism underlying the differential HPA axis response to stress in relationship to the ultradian corticosterone rhythm has been further investigated in adenalec- tomized rats in which the endogenous hormone is replaced with an IV infusion of hourly pulses of corticosterone (165).
Differential response to stress is associated with changes in the ultradian glucocorticoid rhythm

Both experimental data and mathematical modeling have shown that a facilitated corticosterone response to stress occurs when the stressor is applied during the rising phase of an ultradian pulse (i.e., when the HPA axis is already secreting hormone), or during an interpulse period (when the axis is quiescent). In contrast, the axis is inhibited and unable to respond when the stress is applied during the falling phase of a pulse (154, 214). It is clear therefore that the characteristics of the corticosterone ultradian rhythm are important not only for basal corticosterone secretion but also for maintaining hormonal responsiveness to stress. In light of this, the relationship between the timing of the exposure to stress relative to the phase of the corticosterone pulse has been investigated in a number of experimental models where differences in corticosterone pulsatility have been observed, such as in rats of different strain (213), reproductive stage (173, 211), gender (169-172), and in rats exposed to chronic stress (174, 212).

Differences in the pattern of ultradian rhythmicity have been observed between female Fisher and Lewis rats, and this is reflected in differences in the corticosterone response to stress in these animals (213). The corticosterone response to noise stress is greater in the Fischer than in the Lewis female rat. In the female Lewis rat, stress-induced corticosterone secretion does not occur when the noise is applied during the falling phase of the ultradian pulse. In contrast, female Fisher rats respond equally to a noise stress regardless of when it occurs in relation to the phase of the endogenous corticosterone rhythm. A pronounced difference in the duration of stress-induced corticosterone release has also been observed between the two strains, with Fischer rats showing a more prolonged response.

Differential responses to stress have been observed following manipulation of the gonadal steroid environment of both male and female rats, and changes in the ultradian rhythm are associated with an altered response to both psychological noise stress and LPS-induced immune challenge (169-172). Although there is a significant and prolonged increase in plasma corticosterone in both males and females in response to noise and LPS, the amplitude of the response is higher in females compared to males. Furthermore, gonadectomy reverses this pattern with corticosterone profiles and stress-induced peak corticosterone release in castrated males that are similar to those seen in sham females. In contrast, the effect of ovariectomy results in an ultradian profile and stress-induced peak response similar to those in sham males (169). As seen for basal corticosterone pulsatility, the effects of gonadectomy on the stress response in both male and female are reversed by androgen and oestradiol replacement in male and female, respectively (170). Similarly, neonatal masculinization, which results in reduced pulse amplitude, number, and frequency in adult female rats, is associated with reduced responsiveness to both noise and LPS stress, as seen in normal adult males (171). In contrast, pre and postnatal deprivation of testosterone in

Consistent with Windle et al. (214), exposure of these rats to noise stress results in an increase in ACTH, and this response is more pronounced when the stressor is applied during the rising phase of the corticosterone ultradian pulse than during the falling phase. The differential ACTH response to noise stress, relative to the phase of corticosterone pulse, is also associated with a different behavioral response to the stressor. Indeed, the behavioral response of rats exposed to noise stress is higher in rats that are stressed during the rising phase of the corticosterone pulse, compared to rats exposed to stress during the falling phase of the pulse. Interestingly, in addition to a differential hormonal and behavioral response to noise relative to the phase of the corticosterone pulse, differential neuronal activation in the amygdala occurs when the noise stress is delivered during the rising phase rather than the falling phase of the ultradian pulse (165). These findings are consistent with earlier behavioral studies showing that rats exposed to a male intruder during the rising phase of an endogenous corticosterone pulse are more aggressive than rats exposed to the intruder during the falling phase of the pulse (65, 66).
male rats, which results in increased pulse number, amplitude and frequency, is associated with significantly elevated corticosterone secretion after noise stress or LPS administration (172).

Differential responses to stress have also been observed in female rats at different reproductive stages (173, 211). While in virgin rats noise stress causes a rapid increase in plasma corticosterone concentration, in the lactating group, which has an increased number of pulses across the 24-h cycle, no effects on plasma corticosterone levels are seen following noise stress. Furthermore, in the experimentally weaned group, which show lower basal corticosterone levels and a lower number of pulses, the response to noise is of similar amplitude, but more prolonged, compared to the virgin group (211). A different response to stress has been observed in rats exposed to chronic inflammation (adjuvant-induced rheumatoid arthritis). In these rats, the relationship between the pulse phase and the timing of the stress is maintained throughout the development of the inflammation (212). However, the overall corticosterone response to noise stress is significantly lower in symptomatic rats than in controls. Thus, it is possible that as the number of pulses increases and the interpulse periods reduce in symptomatic rats, there is a greater proportion of time when the rats are unable to respond to stress. These data provide evidence for a direct relationship between increased basal HPA axis activity, associated with chronic disease, and a decreased response to acute stress, and this is indeed consistent with previous data showing a decreased corticosterone response to acute stress in rats with adjuvant-induced arthritis (2, 69), and also in humans with rheumatoid arthritis (33). Interestingly, there is no difference in the corticosterone response to LPS between rats with adjuvant-induced arthritis and control rats.

Changes in basal corticosterone pulsatility in rats neonatally exposed to an inflammatory stress are also associated with a differential corticosterone response to stress (174). Rats that receive endotoxin in early life show an increase in both the amplitude and number of corticosterone pulses during basal conditions in adult life, and are also hyper-reactive to stress (noise and LPS).

**Ultradian Rhythm of Glucocorticoids in Target Tissues**

**CBG regulates the level of glucocorticoids at target tissues**

Once they have been released into the general circulation, approximately 95% of glucocorticoid molecules become bound to carrier proteins, which prevents the steroid from diffusing into cells in target tissues. In the rat, approximately 80% of circulating corticosterone is bound to corticosteroid-binding globulin (CBG), and 10% to 15% is bound to albumin (113). The remaining fraction of circulating corticosterone is unbound and free to diffuse across cell membranes and bind to intracellular GRs and MRs in target tissues. Therefore, steroid hormones bound to carrier proteins are considered biologically inactive and provide a reservoir of inactive circulating hormone, and the levels of CBG regulate the amount of free hormone available for diffusion into tissue. CBG is secreted from hepatocytes (103) with a circadian pattern in the plasma that is in antiphase with the circadian variation in plasma corticosterone levels (77, 114). This results in an increased level of free corticosterone during the circadian peak. In addition, CBG is also released from the liver in response to acute stress in parallel with the adrenal release of corticosterone, and this is thought to be important for maintaining low levels of free corticosterone at target tissues (53, 152).

With respect to the role of CBG in regulating the ultradian rhythm of active free unbound glucocorticoid, CBG acts to accentuate pulse profiles at the tissue level. In fact, it is known that in human, CBG has a relatively low saturation point: 400 to 500 nmol/L cortisol (12). Therefore, when levels of hormone are high, such as during the peak of each ultradian pulse, CBG is already saturated. This results in pulses of free cortisol that are superimposed on pulses of total cortisol in which only about 10% is in the free state. This type of augmented ultradian rhythm of corticosterone has been demonstrated experimentally in the rat (53).

It has also been shown in the rat that free corticosterone levels in the blood show distinct circadian and ultradian rhythms, with a pulse frequency of approximately one pulse per hour (151), consistent with the pulse characteristics of total glucocorticoid hormone in plasma (214). Given that the level of free glucocorticoid depends on the availability of CBG and its affinity for the hormone, a recent study has investigated whether there are dynamic changes in the affinity of cortisol for CBG in human (23). This study has shown that the affinity of CBG for cortisol is remarkably temperature sensitive—the affinity of cortisol for CBG drops approximately 16-fold as the temperature increases from 35 to 42°C. Interestingly, there are circadian and ultradian differences in core temperature that can be between 1 and 4°C in rodents and around 1°C in man. Based on the findings of Cameron et al. (23), these changes in temperature may be sufficient to cause rapid changes in the availability of free glucocorticoid over the course of both the circadian and ultradian rhythms of hormone secretion.

Temperature dependence of glucocorticoids binding to CBG is also an important factor for the free/bound hormone ratio in situations when robust changes in body temperature occur. For example, an increase in body temperature of as little as 1°C in febrile patients may have profound effects, markedly increasing the amplitude of free pulsatile bio-available glucocorticoid at the tissue and cellular level. Indeed, studies in rats have shown changes in the ultradian rhythm of corticosterone secretion in animals exposed to inflammation (174, 212), suggesting that changes in the body temperature of rats exposed to inflammatory stress may have an effect on the corticosterone
binding to CBG, and thus on the pattern of free corticosterone pulsatility.

**Ultradian rhythm of glucocorticoid is maintained in target organs**

Recent studies have shown that the ultradian rhythm of corticosterone secretion is not only observed in the blood but also in tissue targets such as the brain and subcutaneous tissue (52-54, 152). Using the technique of microdialysis in the rat, Droste et al. (53) have shown that the corticosterone ultradian rhythm can be detected in discrete brain regions such as the hippocampus and the striatum, mirroring the pulses detected in the peripheral circulation. In the hippocampus, the frequency of the pulses is approximately one per hour, which fits well with the pulse frequency of corticosterone observed in the blood circulation and in the adrenal gland (Fig. 11). Interestingly, in contrast with plasma corticosterone, the ultradian rhythm of hippocampal corticosterone is not affected by gender (54). Indeed, while both plasma corticosterone levels and pulse amplitude are higher in female rats compared to male rats, there is no difference in either hippocampal corticosterone levels or pulse characteristics between male and female. Although this discrepancy cannot be fully explained, it should be noted that while the plasma corticosterone levels reported by Windle and colleagues (214) and by Seale and colleagues (169) represent both CBG-bound and free hormone, the corticosterone levels in brain measured by Droste and colleagues (53, 54) represent only the free biologically active fraction. Therefore, given that the levels of plasma CBG are higher (by up to 2.5-fold) in female than in male rats (60, 125), the CBG-bound corticosterone fraction can account for most of the differences observed between male and female. In addition to CBG, other factors contribute to the regulation of corticosterone levels in the brain, including the ATP-binding cassette transporter P-glycoprotein (Pgp) (99). However, to date, there are no reported differences in Pgp levels between male and female rats.

The ultradian rhythm of free corticosterone is highly synchronized between the blood and the target tissues both in the rat and in man (14, 152). Dual-probe microdialysis techniques have been used to directly compare, within the same animals, the corticosterone ultradian rhythm between the blood and the subcutaneous tissue, and between the blood and the hippocampus. These studies have shown that corticosterone secretion is highly synchronized between the blood and both the subcutaneous tissue and the brain. However, although there are no differences in the pulse amplitude between the blood, the subcutaneous tissue, and the brain during the nadir phase of the circadian cycle, free corticosterone levels are slightly lower in target tissues, compared with the blood, during the active phase.

As has been seen for plasma corticosterone, changes in the ultradian rhythm have also been observed in the hippocampus. For example, there are changes in the ultradian and circadian rhythms of free corticosterone concentrations in the hippocampus of rats that have been exposed to long-term (4 weeks) voluntary exercise (52). Specifically, exercising rats display increased pulse amplitudes and mean free corticosterone levels during the peak phase of the circadian cycle, compared with control animals during this period, but show no differences in pulse frequency. In contrast, there are no differences in ultradian rhythm characteristics between the two groups during the nadir phase of the circadian cycle.
In man, studies have also been performed showing ultradian and circadian rhythmicity in subcutaneous tissue (14). The same authors have now shown a close correlation between total cortisol levels in the blood and free cortisol levels in the blood and subcutaneous tissue (13).

**The Importance of Glucocorticoid Pulsatility for GR Signaling**

Glucocorticoids act via two distinct intracellular receptors, MR and GR, which upon activation by ligands, translocate into the nucleus where they induce or repress gene transcription. Given that GR is widely expressed throughout the body, the effects of glucocorticoids at target organs are mediated primarily by activation of GR. When glucocorticoid levels are low, GR is retained in the cytoplasm, where it is bound to chaperone molecules, including HSP90 and p23 (149). These molecular chaperones hold GR in a conformation that exposes the ligand binding cleft, and therefore allow GR to be activated by the ligand (150). Once activated, GR undergoes conformational changes that induce dissociation from the chaperone complex, leading to exposure of nuclear localization sequences (NLS) in the GR hinge domain and ligand-binding domain (140). These sequences are important so that importin molecules can recognize and bind at these NLS sequences, and facilitate active transport of GR through the nuclear pore. Once in the nucleus, GR can dimerize and bind to specific regulatory sequences in the DNA, termed glucocorticoid response elements (GREs) (168). This event is followed by the recruitment of cofactors, including members of the p160 family (129) and the histone acetyl transferase (HAT) proteins P300 and CBP (105). Importantly, P300 and CBP are involved in the reorganization of the local chromatin structure to allow access of RNA polymerase and the basal transcriptional machinery at the transcriptional start site (203), ultimately leading to transcription of target genes. It is important to understand how the dynamic ultradian oscillations in glucocorticoid levels we have described above affect the activity of these receptors and their downstream influences on gene transcription.

**Effect of ultradian glucocorticoid rhythm on GR activity: in vitro studies**

The importance of glucocorticoid pulsatility for the physiological regulation of GR activity has been investigated in vitro in a number of cell lines, including mouse corticotroph AtT-20, human HeLa, rat liver HTC, and mouse MCF7(3617) cell lines (38,40,181). Exposure of cells to pulses of ligand (cortisol for human HeLa cells and corticosterone for rat HTC and mouse AtT-20 cells) results in cyclical GR activation. In all three cell lines, each pulse of glucocorticoid initiates a “wave” of GR activation and DNA association. This GR-GRE association decreases rapidly after hormone washout, returning to baseline levels before addition of the next pulse 45 min later.

The pattern of DNA binding has been investigated in more detail in the MCF7(3617) mouse cell line (181). This cell line stably expresses GR protein fused to a green fluorescent protein (GFP) tag, allowing visualization of receptor movement in the living cell. Additionally, these cells also contain an expanded MMTV long terminal repeat giving an array of approximately 1200 GRE sequences. Using live cell imaging and photobleaching techniques, Stavreva et al. have investigated the real-time effect of pulses of corticosterone applied to these cells and visualized the intracellular effect of the treatment on GR activity (Fig. 12A). They have found that each corticosterone pulse is paralleled by cyclic GFP-GR binding to the GREs and that dissociation of GFP-GR from GRE occurs within 10 min from the beginning of the hormone washout period. Further studies in both AtT20 and MCF7 cells (40) have shown that this transient activation, typical of normal ultradian rhythmicity, only occurs in response to the endogenous glucocorticoids corticosterone and hydrocortisone, whereas a single pulse of the synthetic glucocorticoid
analogue dexamethasone results in a prolonged GR activation profile. The dynamics of GR dissociation from, and reassociation with, the ligand, and the mechanism underlying these events, has also been investigated in MCF7 cells (181). When these cells are exposed to pulses of corticosterone, GR rapidly dissociates from the ligand following washout. However, for GR to be able to rebind the ligand when the next pulse of corticosterone arrives, and therefore initiate a second cycle of transcriptional activity, it requires the formation of a complex with chaperone proteins in the nucleus. Indeed, when the chaperone complex formation is blocked using the Hsp90 inhibitor geldanamycin, GR cannot bind corticosterone, and this results in GR failure to associate with GRE, and an accelerated proteasome-mediated degradation of GR.

In other studies, it has been shown that pulsatile exposure to corticosterone also results in dynamic recruitment of Pol II at GRE sites, with periods of reduced Pol II exchange with DNA that coincide with maximal GR-GRE binding. Using chromatin immunoprecipitation (ChIP), it has been shown that in both MCF7 (181) and AtT20 (38) cells, consistent with the pattern of GR binding to DNA, there is also a pulsatile pattern of GR binding to GREs in the promoter sequences of naturally occurring glucocorticoid-responsive genes, including genes encoding metallothionein I and Period 1 (Per1), and the negatively regulated pro-opiomelanocortin (38). This is also reflected in the pattern of transcription of a number of GR-responsive genes, as measured by real time quantitative PCR (RT-qPCR) using primers designed to recognize nascent RNA transcripts (or heteronuclear RNA, hnRNA). Pulsatile corticosterone exposure results in a rapid and transient increase in hnRNA from several GR-regulated genes (38, 181), whereas constant exposure to corticosterone is associated with a continuous increase in hnRNA (181) (Fig. 12B). In addition to this, constant treatment with corticosterone results in accumulation of higher levels of mature RNA (mRNA) and protein, when compared with pulsatile corticosterone (181). Interestingly, when the cells are treated with a synthetic glucocorticoid, such as Dexamethasone, which is known to have a higher affinity for GR—and therefore prolonged GR association with GRE—the duration of the transcriptional response is longer (181).

The mechanism underlying the dynamics of pulsatile gene transcription has been further investigated in AtT20 cells. In this case, GR-mediated cyclical transcription of the Per1 gene, induced by a pulse of corticosterone, is associated with rapid, but transient, generalized protein acetylation at the GRE site within the Per1 promoter, and ChIP assays have revealed the nucleosome core protein H4 as an acetylation target (38). Furthermore, the HAT complex members CBP and p300 have also been found to undergo cyclical recruitment to the GRE region in the Per1 promoter. The pattern of both CBP and p300 recruitment at the Per1 promoter is consistent with the pattern of both GR and RNA Pol II recruitment at the Per1 promoter region. In turn, the pattern of Pol II recruitment is consistent with the pattern of Per1 transcription in this cell line (181). Furthermore, it has been shown that GR-mediated cyclical transcriptional activity at the level of the Per1 promoter involves cyclical intranuclear chaperone protein activity. Indeed, consistent with its effect on corticosterone binding to GR, inhibition of HSP90 with geldanamycin results in complete ablation of ultradian cyclical transcriptional activity at the Per1 gene, as shown by lack of GR, CBP, or p300 recruitment at the GRE and reduced acetylation and transcriptional rate (38).
Consistent with the effect of pulses of corticosterone on cyclic GR nuclear translocation, pulsatile corticosterone infusions induce “waves” of GR activation and DNA binding in the hippocampus of rats (41). Using ChIP, this has been confirmed by showing that the pulses of GR activation result in cyclical recruitment of GR to a GRE in the promoter of the Per1 gene. Furthermore, each pulse of corticosterone induces a pulse of transcription of Per1, as shown by measurements of Per1 hnRNA (Fig 13B). Interestingly, despite a pulsatile pattern of hnRNA, pulsatile corticosterone administration results in an increase in Per1 mRNA that reaches constant levels, suggesting that ultradian activity with pulses at hourly intervals is optimized to maintain Per1 protein level at a steady state.

In addition to the hippocampus, the effect of glucocorticoid pulsatility on GR-induced gene transcription has also been investigated in the liver (181). In a similar manner, pulsatile corticosterone induces cyclic GR-GRE binding, and this is associated with pulsatile binding of GR to regulatory regions in the target clock gene Per1. This in turn is followed by cyclic changes in Per1 hnRNA levels over the duration of the corticosterone pulse. As seen for GR dynamic activity in the hippocampus, these results indicate that in the liver, glucocorticoid signaling is highly dynamic and intrinsically dependent on the secretion pattern of corticosterone released from the adrenal gland. This is very important given that glucocorticoids in the liver regulate numerous vital functions, including glucose metabolism.

**Nongenomic effects of glucocorticoid pulsatility**

A number of studies have shown that the ultradian rhythm of glucocorticoids is important for the transcription of GR-regulated genes in glucocorticoid responsive target organs. In addition to their genomic effects, glucocorticoids also have rapid nongenomic effects on target tissues, such as rapid effects on neuronal activity in the brain (57, 74, 193). Indeed, rapid effects of corticosterone on excitatory and inhibitory inputs to the hippocampus have been described (101). Recently, rapid nongenomic effects of glucocorticoid pulsatility on neuronal activity have been studied using electrophysiology techniques (100). Using a paired-pulse stimulation paradigm, Karst and colleagues have shown that exposure to two pulses of corticosterone increases mEPSC frequency in CA1 hippocampal pyramidal cells, and that this effect is comparable for both pulses. In contrast, when two corticosterone pulses are applied in the basolateral amygdala nucleus,
neurons in this region respond to the first pulse with increased mEPSC frequency—but display a reduced mEPSC frequency in response to the second pulse (100).

Effect of Disrupted Ultradian Rhythm on Hormonal and Behavioral Response to Stress

The existence of an interaction between hormonal and behavioral responsiveness to stress and the ultradian rhythm of corticosterone has been shown in the rat (65,66,165,214). The mechanism underlying the differential HPA axis response to stress in relationship to the ultradian corticosterone rhythm has been further investigated in adrenalectomized rats in which corticosterone is replaced either as a pulsatile or constant infusion (165). In rats infused with constant levels of corticosterone, the ACTH response to stress is suppressed, when compared to rats infused with either vehicle or pulsatile corticosterone. Consistent with these data, disruption of the ultradian pattern of corticosterone secretion results in changes in GR receptor sensitivity with profound effects on the glucocorticoid responsiveness of target tissues (166). In rats implanted with a corticosterone pellet (to abolish corticosterone pulsatility), GR in the hippocampus is downregulated. This downregulation results in decreased GR nuclear translocation and transcription of the GR target gene GILZ in hippocampal CA1 cells in response to a corticosterone injection.

Clinical relevance

It is clear that for optimal transcriptional and nongenomic responses tissues need to be exposed to oscillating concentrations of cortisol. Current hormone replacement regimes fail in two respects. First, they do not provide an anticipatory burst of cortisol release prior to awakening, which is important to ensure that cognitive and metabolic functions are ready for the activities needed first thing in the morning. Second, oral replacement therapy results in smooth levels of steroid throughout the day, which do not provide ultradian regulation for GR signaling mechanisms. It is perhaps not surprising that patients on glucocorticoid replacement have double the age related mortality and, often, severe morbidity in terms of lassitude, tiredness and poor cognitive function. Similarly, little appreciation has gone into the timing of glucocorticoid therapy for inflammatory or malignant disease to maximize effect and minimize side effects.

Conclusions

The HPA axis is a highly dynamic system. Ultradian rhythmicity emerges due to the interaction between the dynamic release of hypothalamic hormones and an intrinsically oscillating pituitary-adrenal network. On a slower time scale, circadian rhythmicity entrained by the SCN modifies these rapidly oscillating levels of glucocorticoid to create a pattern of pulses that vary in amplitude throughout the day. These pulsatile patterns of hormone are vital for maintaining homeostasis, stress responsiveness, and optimal metabolic and cognitive function.

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