

60 YEARS OF NEUROENDOCRINOLOGY

Glucocorticoid dynamics: insights from mathematical, experimental and clinical studies

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Abstract

A pulsatile pattern of secretion is a characteristic of many hormonal systems, including the glucocorticoid-producing hypothalamic–pituitary–adrenal (HPA) axis. Despite recent evidence supporting its importance for behavioral, neuroendocrine and transcriptional effects of glucocorticoids, there has been a paucity of information regarding the origin of glucocorticoid pulsatility. In this review we discuss the mechanisms regulating pulsatile dynamics of the HPA axis, and how these dynamics become disrupted in disease. Our recent mathematical, experimental and clinical studies show that glucocorticoid pulsatility can be generated and maintained by dynamic processes at the level of the pituitary–adrenal axis, and that an intra-adrenal negative feedback may contribute to these dynamics.

Key Words

- ▶ HPA axis
- ▶ ultradian rhythms
- ▶ glucocorticoids
- ▶ adrenal cortex

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Introduction

Glucocorticoids, the end-product of the hypothalamic–pituitary–adrenal (HPA) axis, are essential hormones that regulate the organism's homeostasis and its response to stress. Glucocorticoids (corticosterone in the rat, cortisol in humans, here referred to as CORT) are synthesized in the adrenal cortex in response to adrenocorticotrophic hormone (ACTH) release from corticotroph cells in the anterior pituitary. ACTH secretion is in turn regulated by the release of the neuropeptides corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus of the hypothalamus (PVN). Upon release from the adrenal glands into the general

circulation, CORT exerts its effects by binding specific receptors, the glucocorticoid and mineralocorticoid receptors (GR and MR, respectively), which are widely expressed in target organs throughout the body. In addition to its metabolic, cardiovascular, immune-suppressive and anti-inflammatory effects, CORT also regulates its own production through negative feedback mechanisms within the HPA axis, which include the inhibition of synthesis and release of ACTH from the anterior pituitary (Jones *et al.* 1977), and inhibition of CRH by direct modulation of neuronal activity in the PVN and other brain structures including the hippocampus,

amygdala and prefrontal cortex, which regulate the activity of the PVN (Jones *et al.* 1977, Dallman *et al.* 1987a,b, Ulrich-Lai & Herman 2009) (Fig. 1a).

Ultradian rhythm of the HPA axis

Under basal (i.e., unstressed) conditions, the secretion of CORT over the course of the day is not constant, but is

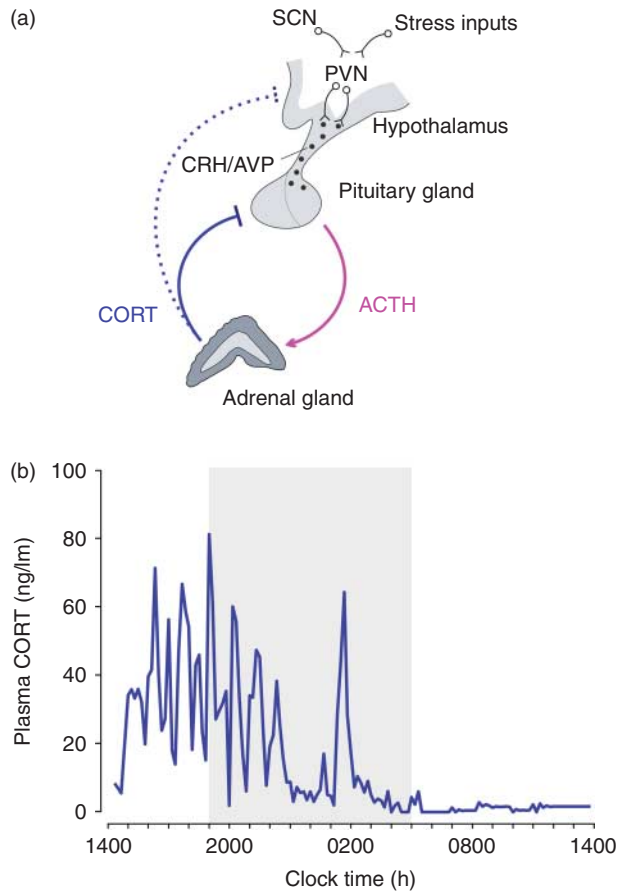


Figure 1

The HPA axis and glucocorticoid ultradian rhythm. (a) Stress and circadian inputs activate the hypothalamic PVN to release CRH and AVP into the hypothalamic–pituitary portal circulation. CRH and AVP activate corticotroph cells in the anterior pituitary, which respond with the rapid release of ACTH into the general blood circulation. In turn, ACTH reaches the adrenal gland where it activates the synthesis of glucocorticoid hormones (CORT). Glucocorticoids regulate the activity of the HPA axis, and thus their own production, through feedback mechanisms acting at the level of the pituitary gland where they inhibit ACTH release, and at the level of the PVN where they inhibit the release of CRH and AVP. (b) Under basal (i.e., unstressed) conditions, CORT levels are characterized by both a circadian and an ultradian rhythm. The data represent an example 24-h profile of plasma CORT from an adult male rat. Shaded region indicates the dark phase. Adapted from Walker JJ, Spiga F, Waite E, Zhao Z, Kershaw Y, Terry JR & Lightman SL (2012) The origin of glucocorticoid hormone oscillations. *PLoS Biology* 10 e1001341. Published under the terms of the Creative Commons Attribution (CC BY) license.

characterized by a circadian pattern, with hormone levels peaking during the active phase of the animal. In addition to this, studies in several species, including the rat and human, have revealed that CORT is released dynamically from the adrenal gland (Jasper & Engeland 1991, 1994), resulting in an ultradian pulsatile rhythm in the blood (Windle *et al.* 1998), as well as in target tissues, such as the brain (Droste *et al.* 2009), and in subcutaneous tissue (Qian *et al.* 2012). In the rat, CORT pulses have a near-hourly frequency, and changes in the amplitude of these pulses throughout the 24-h cycle determine the circadian variation of hormone secretion (Fig. 1b).

The ultradian rhythm of CORT is an important factor in determining the behavioral, neuroendocrine and genomic response to stressors. Furthermore, CORT pulsatility is crucial for physiological activation of GR and MR and for optimal transcriptional responses of glucocorticoid-responsive genes. Because variation in both the amplitude and frequency of CORT pulses occurs in a number of physiological and pathological conditions, including aging and chronic inflammatory disease (reviewed in Spiga *et al.* (2014)), these changes in the pattern of CORT release may be associated with the disrupted physiological functions observed in these conditions. The importance of pulsatility for genomic, behavioral and neuroendocrine responses to CORT has been described in great detail elsewhere (Spiga *et al.* 2014). The purpose of this review article is to discuss how CORT pulsatility is generated, what the mechanisms are that regulate the dynamics of the HPA axis, and how these dynamics become disrupted in disease.

The origin of glucocorticoid pulsatility

The circadian rhythm of the HPA axis is under the control of the suprachiasmatic nucleus (SCN), which directly regulates the pattern of CRH and AVP release from the PVN, and in addition modulates the responsiveness of the adrenal gland to ACTH via the autonomic nervous system and splanchnic nerve (reviewed in Kalsbeek *et al.* (2012)). There has, however, been much less research into the mechanism underlying the ultradian rhythm of CORT, and how this rhythm is maintained at different levels of the HPA axis, despite the significant amount of data highlighting the importance of the ultradian rhythm. A recent study from our group has demonstrated that while the circadian rhythm of ACTH and CORT is lost in rats in which SCN activity has been disrupted, the ultradian CORT pattern is maintained (Waite *et al.* 2012), providing strong evidence for a CORT ultradian pulse

generator functioning independently of the SCN. This is in accordance with data from Jasper & Engeland (1994) showing that lesioning of the splanchnic nerve results in dampened circadian CORT rhythmicity but sustained pulsatility in adrenal corticosterone secretion measured using intra-adrenal microdialysis techniques.

If the SCN does not generate ultradian rhythmicity, could some other area of the hypothalamus be responsible, as has been described for other pulsatile hormones such as secretion of luteinizing hormone and growth hormone (GH)? These pituitary hormones oscillate in response to the pulsatile secretion of their respective hypothalamic secretagogues (REFS) (Belchetz *et al.* 1978, Clarke & Cummins 1982, Plotsky & Vale 1985).

With respect to HPA axis rhythmicity, pulsatility of ACTH has been described in the rat (Carnes *et al.* 1986, 1988*a,b*), sheep (Apostolakis *et al.* 1992) and human (Henley *et al.* 2009*a*). Furthermore, episodic release of CRH has been shown in macaque (Mershon *et al.* 1992), sheep (Caraty *et al.* 1988, Engler *et al.* 1989), and the rat (Ixart *et al.* 1991, 1994). The evidence of pulsatility of CRH led to the acceptance that the ultradian release of CORT was driven by a hypothalamic CRH pulse generator. There were, however, a number of discrepant findings that challenged this concept. For example, studies in the sheep have shown that ultradian rhythms of ACTH and CORT are maintained even after the hypothalamus has been surgically disconnected from the pituitary (Engler *et al.* 1990), suggesting there must be a hypothalamic-independent pulse generator. Further supporting this idea is the observation that in the rat there is a mismatch between the frequency of CRH pulses (~3 pulses/h) (Ixart *et al.* 1991) and the near-hourly frequency of ACTH and CORT oscillations (Carnes *et al.* 1988*a*).

Using a combination of mathematical modeling and *in vivo* experimental approaches, we have proposed that the pituitary–adrenal system possesses an endogenous oscillatory mechanism generating pulses of ACTH and CORT at a physiological ultradian frequency independent of pulsatile release of CRH and/or AVP from the PVN. This hypothesis is based on the knowledge that the activity of the pituitary–adrenal system is governed by an ACTH-mediated positive feedforward pathway regulating adrenal synthesis and secretion of CORT, and by a CORT-mediated negative feedback that regulates ACTH secretion at the pituitary level. An important feature of this feedforward–feedback loop is the slightly delayed secretory response of the adrenal in response to ACTH stimulation that has been observed both in the rat (Carnes *et al.* 1994, Walker *et al.* 2012) and in humans (Henley *et al.* 2009*a*). This time delay

of a few minutes is presumably due to the time needed by the adrenal for *de novo* synthesis CORT in response to ACTH, since in contrast to peptide hormones (e.g., CRH and ACTH), CORT cannot be pre-synthesized and stored within adrenal steroidogenic cells due to its lipophilic nature. Once released into the general circulation, CORT activates a fast (presumably non-genomic) negative feedback mechanism at the level of the pituitary that still remains to be fully characterized (Jones *et al.* 1972, 1974, Rotsztein *et al.* 1975*a,b*, Mahmoud *et al.* 1984, Widmaier & Dallman 1984, Hinz & Hirschelmann 2000, Russell *et al.* 2010).

Mathematical modeling of the pulse generator

To investigate the dynamic interaction between the pituitary and the adrenal gland, we developed a mathematical model of differential equations incorporating CRH acting on the pituitary, the time taken for CORT to be synthesized and secreted by the adrenal gland in response to ACTH stimulus, and the rapid CORT-mediated inhibition of ACTH secretion at the pituitary (Walker *et al.* 2010). In this study, we made the assumption that CORT-driven inhibition at the level of the anterior pituitary was more important for regulating basal HPA dynamics than CORT feedback at the level of the brain.

We used the mathematical techniques of bifurcation analysis and numerical continuation to study the dynamical behavior of this model (i.e., the dynamics of ACTH and CORT) (Walker *et al.* 2010). These techniques allowed us to identify parameter values – in this case, levels of CRH and adrenal time delay – at which there is a qualitative change in the dynamics of the system (i.e., a bifurcation), and then ‘follow’ this bifurcation through parameter space. This results in curves in parameter space that map out regions corresponding to qualitatively different system dynamics.

In accordance with the hypothesis of a sub-hypothalamic pulse generator, we considered the model’s response to constant levels of CRH drive. The model predicted that for certain ‘physiological’ levels of constant CRH drive (e.g., during the circadian peak of HPA axis activity), the pituitary–adrenal system could support self-sustained ACTH and CORT oscillations characterized by a physiological ultradian frequency (~1 pulse/h; Fig. 2*a*). Furthermore, the model predicted that for either higher ‘stress-equivalent’ levels or lower levels of constant CRH, both ACTH and CORT oscillations become dampened, resulting in a steady-state response in hormone secretion;

such responses have been observed *in vivo* following exposure to an acute stressor (high constant CRH), or at the circadian nadir of HPA axis activity (low constant CRH) (Windle *et al.* 1998).

Interestingly, when a ‘physiological level’ of pulsatile CRH (~3 pulses/h) was imposed on the pituitary–adrenal system, the model predicted that the resulting frequency of ACTH and CORT oscillations (~1 pulse/h) would still be predominantly governed by the pituitary–adrenal interaction (Fig. 2b), further supporting an independency of the CORT pulse generator from the dynamics of the hypothalamic drive. Furthermore, when a circadian pattern was imposed on the constant CRH drive, the resultant pattern of ACTH and CORT secretion displayed both circadian and ultradian rhythmicity (Fig. 2c).

To test the validity of our modeling predictions, we then used an experimental approach *in vivo* to investigate the dynamics of ACTH and CORT in response to different levels of constant CRH stimulation in conscious freely behaving male rats (Walker *et al.* 2012). This approach has allowed us to confirm the predictions of our mathematical model, as we have been able to show that ultradian ACTH

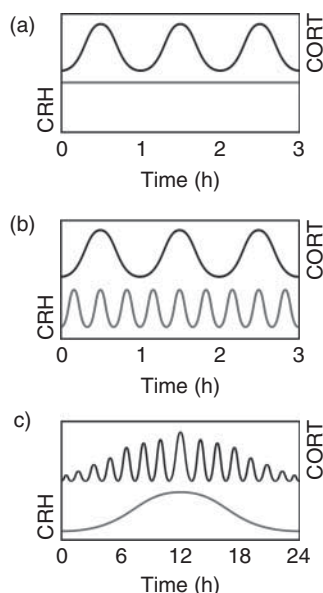


Figure 2

CORT responses (black) to different patterns of CRH drive (grey). Model predictions of CORT pulsatility for constant CRH (a), pulsatile CRH (b) and circadian CRH (c). Response demonstrates a frequency in CORT governed by the pituitary–adrenal system and not by the frequency of the CRH forcing. Data adapted from Walker *et al.* (2010). Adapted from Walker JJ, Terry JR & Lightman SL (2010) Origin of ultradian pulsatility in the hypothalamic–pituitary–adrenal axis. *Proceedings of the Royal Society B: Biological Sciences* 277 1627–1633. Published under the terms of the Creative Commons Attribution (CC BY) license.

and CORT oscillations can be induced by constant infusion of CRH, with resultant CORT pulse amplitude and frequency similar to that of endogenous pulses observed during the circadian peak of hormone release. Consistent with the model, we have also observed the expected time delay between pulses of ACTH and pulses of CORT. Furthermore, constant infusion of a higher dose of CRH resulted in an elevated and sustained level of CORT, similar to the pattern observed in response to severe stressors known to induce a robust increase in hypothalamic CRH secretion.

In summary, both our modeling work and experimental data support the hypothesis of a self-sustained, sub-hypothalamic ACTH and CORT pulse generator that depends on the dynamic interaction between the anterior pituitary and the adrenal cortex.

Our mathematical model also predicts that any disruption in the ACTH feedforward drive (i.e., adrenal delay) or in the CORT feedback mechanism within the pituitary could have an effect on the pulsatile dynamics of ACTH and CORT. Since ACTH is not the only factor that can influence CORT synthesis in the adrenal, it is clearly important to investigate how other factors such as pathogens and inflammatory mediators may interact with the physiological ACTH-mediated adrenal response. Chronic inflammation in the rat is associated with changes in the pulsatile CORT pattern (Windle *et al.* 2001). Furthermore, changes in CORT-mediated signaling at the level of the anterior pituitary can also affect CORT pulsatility, and chronic administration of a GR antagonist leads to changes in the ultradian rhythm of CORT with an increase in the number, height and frequency of CORT pulses, resulting in an overall increase in hormone levels through the 24-h cycle, presumably as a result of an impaired GR-mediated inhibition of ACTH secretion (Spiga *et al.* 2007).

Adrenal regulation of glucocorticoid pulsatility

Studies in human and in the rat have shown that in addition to CORT, ACTH is also released in a pulsatile manner (Fig. 3D), with the ACTH pulse amplitude increasing throughout the day as result of the circadian input from the SCN, via regulation of circadian secretion of CRH (Watts *et al.* 2004). Given that pulsatility of CORT is important for maintaining optimal transcription of glucocorticoid regulated genes (Stavreva *et al.* 2009), we decided to investigate whether pulsatile ACTH is important for optimal transcription of ACTH-responsive

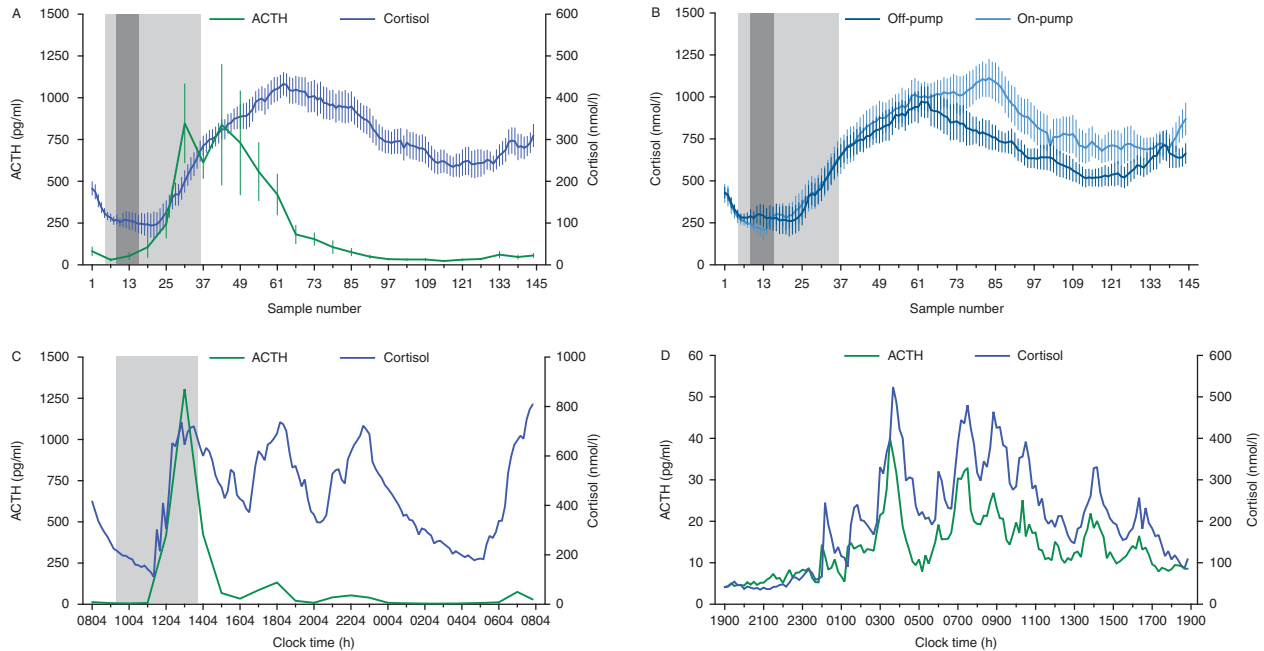


Figure 3

Dynamics of human ACTH and cortisol in health and disease. Changes in cortisol and adrenocorticotrophic hormone (ACTH) levels throughout the 24-h perioperative period of cardiac surgery (A–C), and in a healthy volunteer (D). (A) mean \pm s.e.m. 24-h cortisol and ACTH profile from patients undergoing coronary artery bypass grafting (CABG). (B) specific mean \pm s.e.m. 24-h cortisol profile from patients undergoing CABG using the off-pump or the on-pump technique. In (A) and (B) the light grey area represents the period during which some patients were undergoing surgery. The dark grey area represents the period during which all patients were undergoing surgery. (C) individual 24-h ACTH and cortisol profile of a patient undergoing off-pump CABG; the light grey area represents the period during which the patient was undergoing surgery (09:19–13:49 h). After the initial surge of ACTH and cortisol, both ACTH and cortisol continue to pulse. However, while both the absolute values of ACTH and the pulse amplitude are reduced, the cortisol levels remain elevated. The grey area represents the period of surgery. (D) individual 24-h ACTH and cortisol profile from a healthy volunteer. ACTH and cortisol both display a tightly correlated ultradian rhythm. Reproduced, with permission, from Gibbison B, Spiga F, Walker JJ, Russell GM, Stevenson K, Kershaw Y, Zhao Z, Henley D, Angelini GD & Lightman SL (2015) Dynamic pituitary-adrenal interactions in response to cardiac surgery. *Critical Care Medicine* 43 791–800. Copyright 2015 Society of Critical Care Medicine and Wolters Kluwer Health, Inc. The original source of (D) is Henley DE, Leendertz JA, Russell GM, Wood SA, Taheri S, Woltersdorf WW & Lightman SL, *Journal of Medical Engineering & Technology*, 2009; 33: 199–208, copyright 2009 Informa Healthcare. Reproduced with permission of Informa Healthcare.

steroidogenic genes within the adrenal cortex of the rat. By using a model of suppressed endogenous ACTH secretion, we found that while the adrenal gland responds rapidly to pulses of ACTH, with pulsatile activation of the steroidogenic pathway *in vivo* that parallels pulsatile secretion of CORT, this dynamic response is absent when an identical dose of ACTH is infused at a constant rate (Spiga *et al.* 2011a). Moreover, the responsiveness of the adrenal gland to a pulse of ACTH equivalent to that seen during an acute stress response is also reduced in rats infused with constant ACTH (Spiga & Lightman 2015). These observations suggest that the adrenal is adapted to respond rapidly to individual pulses of ACTH, and that optimal adrenal responsiveness depends on pulsatile ACTH. We have now begun to dissect the mechanisms underlying this phenomenon by investigation of the dynamics of the adrenal steroidogenic pathway.

ACTH induces CORT synthesis by activating its specific cell surface G-protein-coupled receptor, the melanocortin type-2 receptor (MC2R; Mountjoy *et al.* 1994). ACTH binding to MC2R leads to activation of adenylyl cyclase, followed by an increase in intracellular levels of cAMP, which in turn activates downstream signaling pathways including the protein kinase A (PKA) pathway. Since CORT cannot be stored within adrenal cells, the rapid synthesis of CORT that occurs within minutes upon ACTH stimulation *in vivo* must depend on ACTH-induced non-genomic events that include post-translational modification of steroidogenic proteins. This includes phosphorylation of proteins involved in cholesterol metabolism, notably including hormone-sensitive lipase (HSL) and steroidogenic acute regulatory protein (StAR), which regulate the levels of intracellular cholesterol and its transport within the mitochondria matrix,

respectively (Lin *et al.* 1995, Kraemer & Shen 2002). In addition to these rapid non-genomic events, ACTH also regulates the transcription of genes encoding for steroidogenic proteins, including *StAR*, *CYP11A* (the gene encoding for the cholesterol side-chain cleavage cytochrome protein P450_{scc} (Churchill & Kimura 1979), which catalyses the cleavage of the cholesterol side-chain to produce pregnenolone in the mitochondria) and *MRAP* (the gene encoding for the melanocortin receptor accessory protein (MRAP), which regulates the level and activity of MC2R at the cell surface, and thus the cell's responsiveness to ACTH; Metherell *et al.* 2005). We have shown that *StAR*, *CYP11A* and *MRAP* mRNA are normal in rats infused with pulsatile ACTH, but actually decrease in rats infused with constant ACTH (Spiga *et al.* 2011a).

Studies investigating the effects of a single ultradian pulse of ACTH on the dynamics of steroidogenic gene transcription in the rat show that the adrenal steroidogenic pathway is highly dynamic in response to a pulse of ACTH, with rapid changes in the levels of *StAR*, *CYP11A1* and *MRAP* transcription, measured as changes in levels of heteronuclear RNA (hnRNA), peaking 15 min after ACTH administration and returning to basal levels within 30 min (Spiga *et al.* 2011b, Liu *et al.* 2013). It is well known that transcription of *StAR* and other steroidogenic genes is regulated by the phosphorylation of CREB and subsequent CREB binding to the CRE with the target gene promoter. Consistent with this, we found that the dynamic transcriptional activation of *StAR* in response to a pulse of ACTH was associated with rapid and transient phosphorylation of CREB and rapid dephosphorylation and nuclear translocation of the CREB co-activator, transducer of regulated CREB activity 2 (TORC2, also called CRTC2) (Takemori *et al.* 2007), both occurring 5 min after administration of the ACTH pulse.

These findings show that pulsatile events leading to the dynamic transcription of steroidogenic genes in the adrenal gland parallel the pulsatile secretion of CORT. We propose that pulsatile ACTH results in pulsatile expression of *StAR*, and other steroidogenic genes such as *MRAP*, throughout the circadian cycle, and therefore increases in both the amplitude of ACTH pulses and/or responsiveness of the adrenal to ACTH during the circadian peak will lead to increased amplitude in pulsatile transcription of steroidogenic genes, ultimately resulting in the circadian variation in steroidogenic protein levels that we have observed (Fig. 4) (Park *et al.* 2013). In addition to *StAR*, several transcription factors involved in *StAR*'s transcriptional regulation, including the positive regulators steroidogenic factor 1 (SF-1; Caron *et al.* 1997) and Nur77

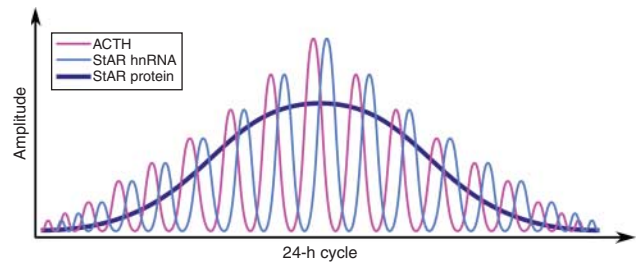


Figure 4

Schematic model of dynamic expression of steroidogenic genes. Pulsatile ACTH induces dynamic transcription of *StAR* (hnRNA); circadian variation in ACTH pulse amplitude, as well as circadian variation in adrenal responsiveness to ACTH, determine changes in the amplitude of *StAR* hnRNA pulses leading to circadian expression of *StAR* protein. Model based on data from Carnes *et al.* (1988a), Spiga *et al.* (2011b) and Park *et al.* (2013).

(NR4A1; Martin *et al.* 2008) and the negative regulator DAX-1 (dosage sensitive sex-reversal (DSS), adrenal hypoplasia congenita (AHC) locus on the X-chromosome; Jo & Stocco 2004), are also rhythmically expressed in the adrenal gland (Park *et al.* 2013). The pattern of expression of SF-1, Nur77 and DAX-1 is consistent with their role in regulating the expression of adrenal *StAR*: while SF-1 and Nur77 mRNA and protein levels are elevated when *StAR* expression reaches its circadian peak, the levels of expression of DAX-1 are high when *StAR* expression is low. Given that ACTH inhibits DAX-1 transcription, the circadian increase in ACTH levels decreases DAX-1 expression, thus reducing its inhibitory effect on *StAR* transcription. The resulting increase in *StAR* expression in turn contributes to increased CORT levels during the circadian peak. It is interesting that in rats in which the CORT circadian rhythm has been disrupted by chronic exposure to constant light, the circadian rhythm of *StAR* and its transcriptional regulators is also disrupted (Park *et al.* 2013), further supporting our hypothesis that the pattern of steroidogenic activity is crucial for maintaining optimal rhythmicity of CORT secretion.

Intra-adrenal glucocorticoid-mediated negative feedback

Our work implicates a key role for rapid CORT feedback of CRH-induced ACTH secretion from the pituitary in regulating the ultradian activity of the system. Since the adrenal cortex expresses GR, as shown by studies both in rodents (Loose *et al.* 1980) and humans (Briassoulis *et al.* 2011), it is plausible to hypothesize that CORT may affect its own synthesis by a local feedback mechanism within the adrenal itself.

There is evidence that CORT can indeed affect steroid synthesis in both the adrenal and other steroidogenic organs including the gonads. With respect to the effect of CORT on the adrenal steroidogenic activity, a number of *in vitro* and *in vivo* studies have shown a decrease in adrenal responsiveness following prior exposure to stressors or ACTH. For example, experiments in cultured adrenal cells have shown that ACTH-induced CORT synthesis is rapidly inhibited (within 1–2 h) when CORT is added to the medium at high concentration (Peron *et al.* 1960, Carsia & Malamed 1979). This is in accordance with studies *in vivo* in the rat showing that previous exposure to a stressor, or to a high concentration of ACTH, inhibits CORT synthesis in response to further stimuli (Langecker & Lurie 1957, Jones & Stockham 1966), suggesting that CORT synthesis is dependent on the prior state of adrenal activity.

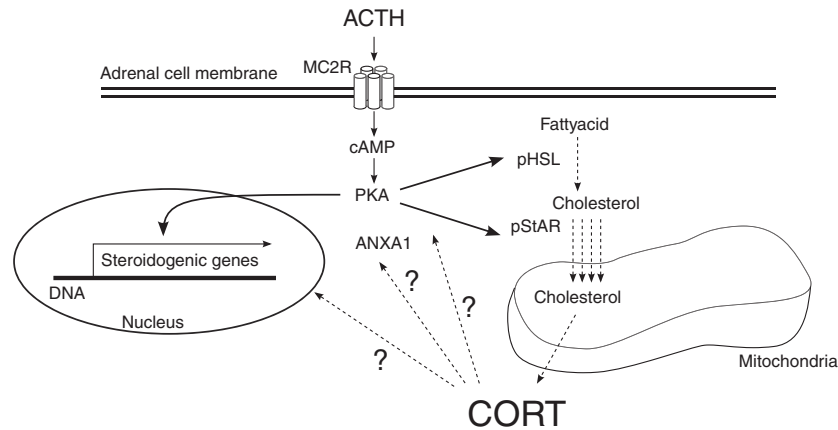
The molecular mechanism underlying CORT-mediated inhibition of steroidogenesis is not clear, but there is evidence that the synthetic glucocorticoid dexamethasone can inhibit the transcription of steroidogenic genes through a mechanism that involves GR (Gummow *et al.* 2006). Following ACTH stimulation and activation of CREB, StAR expression is upregulated by the CREB co-activator SF-1 and inhibited by the co-repressor DAX-1. Interestingly, ACTH can induce the transcription of SF-1 (Ragazzon *et al.* 2006) and Nur77 (Davis & Lau 1994), while inhibiting the transcription of DAX-1 (Ragazzon *et al.* 2006). Gummow *et al.* (2006) have shown that dexamethasone can induce DAX-1 transcription by a mechanism that involves the formation of an SF-1/GR complex, thus leading to inhibition of StAR transcription and ultimately to inhibition of CORT synthesis. In addition to this, CORT can repress the transcription of StAR by inhibiting both the transcription and the activity of Nur77 via a mechanism that involves active GR (Song *et al.* 2004, Martin & Tremblay 2008). In contrast to this, it has been shown that, when overexpressed *in vitro*, DAX-1 can enhance steroidogenic gene expression (Xu *et al.* 2009), and a more recent study has shown that prolonged incubation of the human adrenocortical cell line H295R with dexamethasone can indeed increase both StAR transcription and CORT production (Asser *et al.* 2014). Taken together, these studies motivate the hypothesis that dynamic secretion of CORT may be regulated by a fine balance between ACTH-mediated positive feedforward (involving upregulation and activation of steroidogenic proteins) and CORT-mediated negative feedback within the adrenal (involving GR-mediated inhibition of StAR transcriptional regulators and upregulation of StAR repressors). It is therefore

possible that pulsatile CORT synthesis observed in the adrenal in response to pulsatile ACTH stimulation may in turn be able to dynamically regulate the adrenal response to the steroidogenic stimulus. Furthermore, we have also hypothesized that when CORT secretion is elevated, as seen, for example, during a stress response or in certain conditions of disease or critical illness, this regulatory intra-adrenal feedforward–feedback mechanism may become disrupted, contributing further to abnormal CORT secretion (see the next section in this review).

To investigate this hypothesis further, in Walker *et al.* (2015) we considered the evidence for four candidate mechanisms of adrenal CORT production by studying their ability to reproduce the dynamics of the system in three experimental paradigms: i) administration of an ultradian ACTH pulse; ii) constant infusion of CRH; and iii) exposure to noise stress. Our findings provide evidence for the existence of a mechanism by which CORT can inhibit its own synthesis and/or secretion, and further that this mechanism may be transiently blocked during the early stage of an ACTH pulse. Our hypothesis is that this permits the system to respond rapidly to incoming stressors, whilst preventing uncontrolled release of glucocorticoids. The molecular basis of this ‘systems level’ mechanism remains an open question.

Because our mathematical model predicts that pulsatile CORT can modulate intra-adrenal CORT levels very rapidly, this suggests that, in addition to the genomic effects of CORT on StAR transcription already discussed, CORT can also regulate its own synthesis presumably by interfering with non-genomic mechanisms within the adrenal steroidogenic pathway, for example by regulating the activity (e.g., phosphorylation/dephosphorylation) of steroidogenic proteins including StAR and HSL (Fig. 5).

Rapid non-genomic effects of CORT at the anterior pituitary have been shown to involve annexin 1 (ANXA1), a protein that, upon CORT stimulation, is able to translocate from the cytoplasm to the outer cell surface where it exerts its regulatory effects, including inhibition of ACTH secretion from intracellular vesicles (Taylor *et al.* 1995). Interestingly, ANXA1 is also expressed in the adrenal cortex, and as observed in other tissue, its expression and activity is regulated by CORT (Davies *et al.* 2007). Remarkably, the same study also showed that ACTH stimulation of isolated adrenal gland obtained from ANXA1-null mice exhibited a greater CORT response compared to adrenal obtained from WT mice, suggesting an involvement of ANXA1 in the inhibition of CORT synthesis. Whether ANXA1 is involved in rapid CORT-mediated intra-adrenal negative

**Figure 5**

Model hypothesis of glucocorticoid-mediated regulation of the adrenal steroidogenic network. ACTH binding to its specific receptor MC2R induces rapid glucocorticoid secretion by PKA-mediated non-genomic regulation of steroidogenic proteins involved in cholesterol metabolism. This includes phosphorylation of hormone-sensitive lipase (HSL), a protein that increases the levels of intracellular cholesterol (the precursor of steroid hormones), and phosphorylation of steroidogenic acute regulatory protein (StAR), which promotes the transport of cholesterol into the mitochondria, where cholesterol is converted into pregnenolone by the enzyme side-chain cleavage cytochrome P450. PKA also mediates adrenal genomic activity by inducing the transcription of genes encoding for steroidogenic proteins, including StAR. Our mathematical modeling work suggests that CORT regulates an intra-adrenal inhibition mechanism of CORT synthesis, and that this is an important factor regulating CORT synthesis over the timescales of both the ultradian rhythm and the rapid response to stress. It is therefore our hypothesis that CORT may regulate the steroidogenic response by acting both at a genomic level (e.g., by regulating steroidogenic gene transcription) as well as at a non-genomic level by regulating the phosphorylation of steroidogenic proteins such as StAR and HSL.

feedback within the timescale of an ultradian pulse, however, remains to be elucidated.

In summary, our mathematical modeling work suggests that an intra-adrenal inhibition mechanism of CORT synthesis does indeed exist, and importantly, our model predictions suggest that this rapid intra-adrenal inhibition is an important factor regulating CORT synthesis over the timescales of both the basal ultradian rhythm of the HPA axis and the rapid CORT response to stress. In addition to this, our model points to the existence of a short time delay in this intra-adrenal inhibition, and that upon ACTH stimulation, this local negative feedback mechanism is rapidly antagonized, presumably via ACTH activation of the steroidogenic pathway. Our hypothesis is that these feedforward–feedback mechanisms of intra-adrenal regulation enable rapid glucocorticoid release while at the same time preventing uncontrolled release of glucocorticoids in response to large surges in ACTH associated with stress or diseased states.

Ultradian rhythm of glucocorticoids in human disease and critical illness

The release of ACTH and CORT in humans is also characterized by an ultradian rhythm with a close

temporal relationship between pulses of ACTH and CORT, as has been observed in the rat (Fig. 3D). Furthermore, consistent with changes in the pulsatile pattern observed in a number of disease models in the rat (e.g., chronic inflammation and chronic exposure to constant light), a similar disruption of cortisol pulsatility has been reported in chronic illness in humans. For example, an elevation in CORT secretion due to an increase in the mass of CORT pulses has been observed in patients affected by obstructive sleep apnea, and a complete remission of these hormonal changes was achieved following medical treatment of the condition (Henley *et al.* 2009b).

Given that the ultradian rhythm of CORT secretion is crucial for normal physiological function, it is clear that any disruption in the pulsatile pattern of hormone release could lead to impairment of CORT-regulated functions, including metabolic processes and the immune response. Indeed, maintenance of a normal ultradian rhythm of cortisol may be particularly important in major surgery and critical illness (reviewed in Gibbison *et al.* (2013)). In this regard, a recent study from our group in which the pattern of CORT secretion was investigated in patients undergoing cardiac surgery showed that a disruption in CORT secretion occurs in these patients, with high CORT levels, despite normal ACTH, observed for several hours

after the termination of the surgical procedure through the recovery period (Fig. 3A–C) (Gibbison *et al.* 2015). These data show that dissociation between ACTH and CORT occurs in these patients. It is important to highlight that in these patients both ACTH and CORT still display a pulsatile pattern of secretion, suggesting that although the adrenal sensitivity to ACTH may be altered, the mechanisms regulating pulsatility, including the dynamics of adrenal steroidogenesis, as well as the negative feedback mechanisms at the levels of the pituitary are maintained. It is also noteworthy to point out that, because a delay between the beginning of the surgery and the onset of the ACTH response was observed, it is likely that the HPA axis response in these patients may not be induced by the anesthesia or the surgical procedure itself, but other factors may be involved in the robust hormonal response observed. For example, the time of the ACTH response in these patients is consistent with the time of increase in circulating inflammatory mediators (including TNF, IL1 and IL6) that also remain elevated for about 24 h before returning to normal levels (Lahat *et al.* 1992, Roth-Isigkeit *et al.* 1999, de Mendonca-Filho *et al.* 2006). It is known that circulating cytokines can activate CORT secretion not only via inducing hypothalamic CRH and pituitary ACTH release but also by acting directly at the level of the adrenal gland (Engstrom *et al.* 2008). Indeed, cytokine receptors are expressed in the zona fasciculata of the adrenal cortex, and an increase in cytokines in the adrenal can therefore potentiate the effects of ACTH, as well as exert a direct effect on steroidogenesis, through a mechanism that involves an increase in the expression of steroidogenic genes, including StAR (Tkachenko *et al.* 2011). In addition, because pathogens can induce the expression of a number of cytokines in the adrenal gland itself through activation of toll-like receptors expressed in the adrenal, an immune-adrenal cross-talk regulating CORT synthesis has also been suggested (Judd *et al.* 2000, Zacharowski *et al.* 2006). Therefore, the mechanisms underlying the dissociation between ACTH and CORT secretion and increased adrenal sensitivity that we have observed in our clinical studies may involve circulating and/or intra-adrenal cytokines.

We further investigated the hypothesis of an involvement of inflammatory mediators in the increased adrenal sensitivity to ACTH observed in our studies using a well-established rat model of acute critical illness (administration of lipopolysaccharide (LPS)). The hormonal response to an acute injection of LPS in the rat is similar to what we observe in humans undergoing cardiac surgery. Following a rapid initial HPA axis activation, ACTH levels return to normal within 4 h of LPS administration, whereas

plasma CORT levels remain high for several hours. Since CORT secretion is tightly regulated by the pattern of ACTH under basal conditions, our data suggest that LPS injection increases adrenal sensitivity to ACTH. Interestingly, when rats are injected with a high dose of ACTH that produces plasma ACTH levels (and pattern) similar to those observed after LPS administration, CORT levels return to basal shortly after the decline of ACTH (Gibbison *et al.* 2015), further supporting our hypothesis that factors other than ACTH are responsible for the increased adrenal sensitivity observed both in our clinical and experimental studies. Consistent with the above-discussed role of cytokines in adrenal steroidogenesis, we observed that the sustained increase in CORT levels in rats injected with LPS was paralleled by increased intra-adrenal CORT levels and increased expression of steroidogenic genes including StAR and MRAP, suggesting an LPS-induced increase in adrenal steroidogenic activity. The mechanism by which cytokines affect steroidogenic gene expression has not yet been elucidated, but we found that the increase in steroidogenic gene expression was associated with a dramatic decrease in DAX-1 protein expression. In accordance with a lack of sustained increase in CORT, we did not find such effects on steroidogenic genes in rats injected with a high dose of ACTH (Spiga & Lightman 2015). In summary, both our clinical and experimental studies show that a robust dissociation between ACTH and CORT occurs in conditions of critical illness, and our data from the rat suggest that the changes in the adrenal steroidogenic pathway underlying the increase in adrenal sensitivity may not be induced by the initial peak in ACTH that occurs after surgery (human) or LPS (rat), but rather by inflammatory factors acting within the adrenal cortex to regulate CORT release.

This hypothesis is further supported by recent data from Boonen *et al.* (2014), showing that patients with sustained critical illness also have elevated levels of CORT; interestingly, the same study shows that there is a positive correlation between high levels of cytokines and high levels of CORT in these patients. Furthermore, the same investigators have observed that patients with prolonged critical illness have low levels of ACTH, presumably as a result of increased negative feedback induced by elevated cortisol levels, and this is also associated with alterations in the adrenal steroidogenic pathway including cholesterol-ester depletion as well as reduction in ACTH-regulated steroidogenic gene expression, including MC2R and StAR (Boonen *et al.* 2014). This is contrary to our hypothesis that increased adrenal responsiveness in critical illness is associated with increased steroidogenic activity in the adrenal. However, these effects on adrenal

gene expression observed in sustained critical illness are presumably the consequence of prolonged ACTH depletion observed in these patients, and are consistent with the adrenal alterations observed in mice lacking POMC, the gene that encodes for the precursor of ACTH (Karpac *et al.* 2008). Taken together, these observations suggest that in acute critical illness associated with an inflammatory response, cytokines and other immunomodulators may be responsible for elevated plasma levels of CORT. In contrast, in prolonged critical illness, high levels of plasma CORT may be due to other mechanisms, including reduced CORT metabolism (Boonen *et al.* 2013).

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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