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Encoding and decoding mechanisms of pulsatile hormone secretion

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Abstract

Ultradian pulsatile hormone secretion underlies the activity of most neuroendocrine systems, including the hypothalamo-pituitary adrenal (HPA) and gonadal (HPG) axes, and this pulsatile mode of signalling permits the encoding of information through both amplitude and frequency modulation. Thus in the HPA axis, glucocorticoid pulse amplitude increases in anticipation of waking, and in the HPG axis changing gonadotrophin-releasing hormone (GnRH) pulse frequency is the primary means by which the body alters its reproductive status during development. The prevalence of hormone pulsatility raises two crucial questions: how are ultradian pulses *encoded* (or generated) by these systems, and how are these pulses *decoded* (or interpreted) at their target sites? We have looked at mechanisms within the HPA axis responsible for encoding the pulsatile mode of glucocorticoid signalling that we observe *in vivo*. We review evidence regarding the 'hypothalamic pulse generator' hypothesis, and present an alternative model for pulse generation which involves steroid feedback-dependent endogenous rhythmic activity throughout the HPA axis. We also consider the second question concerning the decoding of hormone pulsatility, taking the HPG axis as a model system, and focussing on molecular mechanisms of frequency decoding by pituitary gonadotrophs.

Keywords. Endocrine signalling; ultradian rhythm; pulsatile hormone secretion; glucocorticoid hormones; gonadotrophin-releasing hormone; mathematical modelling

Introduction

Rhythms are fundamental in nature and endocrine rhythms span time frames that have periods ranging from milliseconds to years. Many of these are organised by endogenous mechanisms, including the circadian cycles of many hormones that free-run under constant conditions (1), and even slower circannual endocrine rhythms that are maintained under photoperiodic clamping (2). Hormone secretion is also rhythmic over much shorter time frames, as illustrated by neuroendocrine systems where oscillators controlling neuronal membrane potential, with time frames of milliseconds to seconds, control the coordinated activity of hypothalamic neuronal networks. These characteristically secrete hormone releasing factors into hypophysial portal blood in a pulsatile fashion with ultradian time frames of minutes to hours which in turn, drive the secretion of hormones from the anterior pituitary with similar time frames. The pituitary hormones act on target tissues including the adrenals and gonads which synthesise and secrete glucocorticoid hormones and gonadal steroids in a pulsatile fashion. The magnitude of this pulsatile hypothalamo-pituitary target gland activity is also modulated in a circadian manner by the suprachiasmatic nucleus (SCN) for the hypothalamo-pituitary-adrenal (HPA) axis and in an oestrus/menstrual cycle temporal profile for the hypothalamo-pituitary-gonadal (HPG) axis which also shows circannual cycles for cyclical breeding animals.

Oscillatory signals are well described for both intracellular and cell-to-cell communication in many biological systems (3). When compared to steady state signalling, pulsatile signalling allows greater control, is more robust to degradation, and is generally more energy efficient (4). Moreover, pulsatile signalling provides target sites with a quiescent inter-pulse interval allowing target receptor recovery, and is therefore essential for maintaining tissue responsiveness (5). Pulsatile signals may also be more information rich in the sense that pulse amplitude, pulse duration, pulse shape and inter-pulse interval can all theoretically provide information to the target cell, as compared to amplitude alone with a steady state input. Most importantly, pulsatile signalling permits not only amplitude, but also frequency modulation, and the potential advantage of this is amply illustrated by

comparing the information provided by a black and white TV image (where each pixel has a given signal amplitude) and a colour TV image (where each pixel has a given frequency as well as amplitude).

Ultradian pulsatility underlies the secretion of most hormones (6). Despite this, the physiological implication of these rhythms and the mechanisms underlying their generation (encoding) and interpretation (decoding) at target cells are still unclear. Here, we address both of these questions. Specifically, we have studied mechanisms within the HPA axis responsible for encoding glucocorticoid pulsatility. We review the 'hypothalamic pulse generator' hypothesis, as well as discussing an alternative model for pulse generation which involves steroid feedback-dependent endogenous rhythmic activity throughout the HPA axis. We also consider the second question concerning the decoding of hormone pulsatility, taking the HPG axis as a model system, and focussing on molecular mechanisms of frequency decoding by pituitary gonadotrophs.

Encoding glucocorticoid pulsatility

A pulsatile mode of signalling underlies the activity of the HPA axis, a system crucial for maintaining basal and stress-related homeostasis by regulating the circulating levels of vital glucocorticoid hormones (cortisol in man, corticosterone in rodents). The glucocorticoids govern a broad range of physiological functions, including the regulation of cardiovascular, metabolic, cognitive and immunological activity (7-10). The regulation of HPA activity depends on multiple inputs. In the hypothalamus, the paraventricular nucleus (PVN) receives an indirect input from the SCN which regulates circadian variation in HPA activity, as well as afferent information from brainstem nuclei responding to physical stressors such as hypotension and inflammation, and from limbic areas of the central nervous system that respond to cognitive and emotional stressors (11). The PVN regulates corticotroph activity in the anterior pituitary via two neuropeptides – corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) – released into the pituitary portal circulation and hence the anterior pituitary. Upon stimulation, corticotrophs

secrete corticotrophin (ACTH) into the general circulation, through which it accesses cells of the adrenal cortex initiating the synthesis and secretion of the glucocorticoid hormones.

Glucocorticoids act at target sites via their two cognate receptors – the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) – the expression of which is widespread in areas regulating HPA activity (12-14). The classical effect of glucocorticoids is via activation of their receptors which translocate to the nucleus acting as ligand dependent transcription factors binding to GREs at promoter regions of glucocorticoid sensitive genes (15-18). In addition to these classic genomic effects, there has been considerable recent interest in rapid non-genomic mechanisms through which glucocorticoids can also act (19,20).

In the basal state, glucocorticoids are released in an ultradian pulsatile fashion from the adrenal cortex, which results in rapidly changing levels of hormone concentration observable in blood plasma (21-24), as well as within target tissues such as the brain (25,26). The classic circadian glucocorticoid rhythm, which peaks in the morning in man (27) and evening in the rodent (28), is in fact the result of amplitude (and to a lesser extent frequency) modulation of the underlying ultradian rhythm (22,23,29-33). A particularly striking feature of glucocorticoid pulsatility is the appearance of a more distinct ultradian rhythm during the peak of the circadian cycle, which operates at a frequency of around 1 pulse/h (see shaded region 2 of Figure 1). Variations in amplitude and frequency of the ultradian rhythm not only compose the circadian rhythm, but also characterise changes in HPA activity that occur during early life programming (34), chronic stress (35), lactation and aging (36), and a number of other physiological and pathological conditions (37).

The importance of glucocorticoid pulsatility resides in its ability to provide a digital signalling system which can respond rapidly to stress or changes in environmental conditions. A recent study by Stavreva et al. (38) clearly shows that the pattern of glucocorticoid presented to a tissue is critical for its transcriptional response – an effect that is seen *in vivo* as well as *in vitro*. The details of the mechanisms underlying both genomic and non-genomic signalling of glucocorticoid hormones are beyond the scope of this review, but they not only act directly

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on multiple genes throughout the body, but also have major effects on the 'clock genes' and through them on circadian physiology (39,40).

Despite the significance of glucocorticoid pulsatility (36), surprisingly little is known about the mechanisms that encode this rhythm. Whilst the role of the SCN is well founded in regulating circadian HPA activity (41,42), evidence suggests it is not required for the generation of ultradian rhythmicity. Indeed, lesioning of the SCN completely abolishes the circadian glucocorticoid rhythm in both the adrenals and blood plasma (43-45), but has little effect on the ultradian rhythm, which persists at amplitudes and frequencies comparable to those observed at the peak of the native circadian rhythm (Eleanor Waite personal communication). Furthermore, pulsatility in the HPA axis is regulated independently of the ultradian rhythmicity found in other neuroendocrine systems such as the HPG axis. Thus although glucocorticoids and luteinising hormone (LH) are secreted in pulses with similar frequencies, their release is not concurrent (37).

Given the wealth of evidence showing that the circadian pacemaker resides within the hypothalamus (46), it is perhaps not surprising that it has also been assumed that the ultradian rhythm was the result of a pulse generating neural network within the hypothalamus. We review this hypothesis, covering what we feel to be the most important experimental studies and go on to discuss recent developments from theoretical studies, which have led us to propose a new model for the encoding mechanisms of glucocorticoid pulsatility.

The 'hypothalamic pulse generator' hypothesis
Neural signalling to the anterior pituitary is encoded in the dynamic patterns of hypothalamic neuropeptides released into the portal circulation (47). Both in man and the rat, the major ACTH secretagogue is CRH and to a lesser extent AVP, although oxytocin, norepinephrine, and epinephrine may modulate corticotroph activity (48). Moreover, CRH is the only corticotrophin-releasing factor known to regulate POMC gene expression in the rat (48). In ovine species, CRH is also an important ACTH secretagogue, although there is also evidence to suggest that the predominant corticotroph secretagogue is AVP (49).

Push-pull perfusion studies of the median eminence in free-moving rats show that CRH follows an irregular pulsatile mode of secretion, with a frequency of approximately 3 pulses/h (50). Interestingly, no significant difference is seen in CRH pulse frequency between the morning and evening, indicating a relatively steady frequency over the diurnal period (51). On the other hand, mean CRH concentration levels do vary significantly over the 24 h period, with evening concentration levels nearly twice those of the morning (51). These increased levels result from the fact that mean pulse nadir and peak levels, as well as pulse amplitude, and to a lesser extent pulse duration, are all significantly higher in the evening (51).

An episodic mode of secretion for CRH and AVP has also been demonstrated *in vivo* in a number of other species under basal conditions (48). In the portal blood of unrestrained conscious rams a pulsatile pattern of CRH and AVP secretion is evident (52), and measurement of AVP in the pituitary venous effluent of the unanesthetised horse also demonstrates an episodic secretory pattern (53). In conscious sheep also, both CRH and AVP display a pulsatile secretory pattern in the portal blood (54).

The regulatory oscillator(s) underlying the pulsatile mode of CRH secretion remains to be defined. It is certainly interesting to compare the pulses of CRH with the much more organised pulsatile secretion of gonadotrophin-releasing hormone (GnRH) into portal blood. GnRH and LH release has a relatively consistent frequency which correlates well with organised increases in electrical activity in networks of GnRH-producing neurons (55). In contrast, the irregularity of episodic CRH secretion not only suggests a different underlying mechanism, but may also reflect an inability of CRH-producing neurons to effectively synchronise with one another in a coherent way (51).

ACTH pulsatility and its relationship to CRH
Given this episodic release of CRH, it was not surprising that pulsatile patterns of ACTH measured in blood plasma were also found in many species (4). What has been more surprising, however, is that in most cases the relationship between pulsatile neuropeptides and ACTH rhythmicity is far from straightforward.

In the rat, two ultradian ACTH rhythms are found simultaneously (56). Concentrations of ACTH in blood plasma display fast episodic bursts of variable amplitude that occur at a frequency of approximately 3 pulses/h (so-called 'micropulses'), as well as larger and more prolonged episodes of secretion that appear approximately every 1-2 h (we shall refer to this as 'ultradian'). The two rhythms in ACTH are related in the sense that it is in fact rhythmic variation in micropulse amplitude that makes up the slower ultradian rhythm (56).

Further, in rats blockade of endogenous CRH by passive immunoneutralisation results in a significant reduction in micropulse amplitude without any effect on micropulse frequency (57), which could be attributed to the pulsatile release of other corticotrophin-releasing factors, an intrinsic rhythmicity of the corticotroph, or a combination of these factors (58). Moreover, blockade of endogenous CRH practically completely destroys the slower ultradian rhythm (57), which prompted speculation that the slower ultradian rhythm in ACTH is driven by rhythmic secretion of CRH (57). However, there is currently no solid data that supports this hypothesis, which is actually contradictory to data on the frequency of pulsatile CRH measured in a number of median eminence perfusion studies on rats of the same strain (male Sprague-Dawley), which was 3 pulses/h across the circadian cycle (50,51,59), and which in fact correlates well with the ACTH micropulse frequency.

In larger species, which allow for the simultaneous measurements of portal blood and blood plasma, a straightforward connection between pulsatile CRH/AVP and pulses of ACTH is also absent. In rams, for example, a clear relationship between portal levels of AVP/CRH and temporal patterns of ACTH is not seen (52). In sheep, a large proportion of CRH/AVP pulses are not followed by a significant rise in ACTH, and a significant number of ACTH pulses are not preceded by a pulse in CRH or AVP (54). Moreover, in sheep that have undergone surgical disconnection of the hypothalamus from the pituitary (HPD), pulsatility in ACTH and cortisol is maintained (60).

Bringing together these diverse experimental studies, highlights the lack of any clear causal relationship between changes in portal CRH concentrations and the

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ultradian rhythmicity of ACTH and the glucocorticoid hormones. This suggests that the generation of HPA ultradian activity must involve other factors, presumably at a sub-hypothalamic level.

Network encoding of pulsatility

Some biological systems are endogenously rhythmic and oscillate under the influence of constant stimulation, or even in the absence of stimulation – such systems are often referred to as 'pacemakers' (61). In hepatocytes, for example, continuous stimulation with physiological levels of AVP induces intracellular Ca2+ oscillations, the frequency of which increases with increasing concentrations of agonist (62). The endogenous rhythmicity of these systems typically finds its roots in the underlying regulatory mechanisms that govern the dynamics of that system (3), and these often involve some form of feedback, which may be positive and/or negative, nonlinear in nature, and is often time-delayed (3,61,63). Indeed, circadian time-keeping in neurons of the SCN is achieved through an intricate molecular circuitry involving 'clock genes' which take part in a complex regulatory network consisting of transcriptional and translational feedback loops (64). Feedback also underlies the generation of ultradian rhythmicity at the cellular level. For example, stimulation by serum of cultured murine cell lines induces oscillatory expression of the transcription factor Hes1 at ultradian frequencies (period approximately 2 h), which is due to a negative feedback loop whereby Hes1 binds directly to regulatory sequences in the Hes1 promoter, thereby repressing transcription of its own gene (65). Similarly, it is also now clear that delayed negative feedback underlies rhythmic cytoplasmic-nuclear shuttling of NF-kappaB following TNFalpha stimulation (66). The network comprising the HPA axis also features some of the properties that are common to pacemaker circuitry. In particular, the anterior pituitary, PVN and higher centres are all targets for negative feedback by circulating glucocorticoids (67-70). In addition to feedback, delays are inherent in the HPA network, which arise from transmission times through the blood, as well as delayed response times.

The existence of pacemaker traits in the HPA network has prompted specu-

lation that the generation of ultradian rhythmicity in glucocorticoids is not necessarily due to a neural pulse generator in the hypothalamus, but may actually arise from the complex network of excitatory and inhibitory interactions between CRH, ACTH and the glucocorticoids. This view has mainly come about through a handful of mathematical studies, rather than experimental work, that have developed models characterising the dynamic interactions between HPA hormones (71-73). Most of these studies assume it is feedback at the level of both the pituitary and the hypothalamus that gives rise to the ultradian rhythm. If this were the case then CRH, ACTH and glucocorticoids would all oscillate over the same time frame. However, there is no good evidence for this. Specifically, in the rat the roughly hourly rhythm observed in ACTH (56) and corticosterone (22) is not observable in CRH during either the nadir or peak of the circadian rhythm (51), and if glucocorticoid feedback at the hypothalamic level was important in generating ultradian rhythmicity then removal of this feedback should ablate CRH pulsatility. However, in cultured explants of the macaque hypothalamus, the pulsatile release of CRH persists both in the presence and absence of glucocorticoids (74), and in conscious rats CRH pulsatility in the median eminence is maintained following adrenalectomy (59).

Recently, we have taken a mathematical approach to explore the encoding mechanisms underlying glucocorticoid pulsatility. Our model builds upon one describing dynamic interactions within the HPA axis (75), and focuses on the effects of nonlinear feedback of glucocorticoids mediated by GR at the level of the anterior pituitary. Furthermore, we incorporate the delayed response of glucocorticoid secretion following ACTH stimulation (76), which results from the lack of releasable pools of glucocorticoids and the need to synthesize the hormone prior to secretion.

Our approach does not discount the possibility of a hypothalamic pulse generator, but demonstrates that ultradian pulsatility in ACTH and glucocorticoids can also occur even in the absence of pulsatile input from the hypothalamus, providing the mean levels of hypothalamic drive are sufficiently high (77). These oscillations are born out of the excitatory-inhibitory loop formed by the interactions between the anterior pituitary and the adrenal gland. Implicit in this idea is that there

is a close coupling between ultradian rhythms in ACTH and glucocorticoids, and there is indeed good evidence supporting such a relationship (Figure 1).

It follows from our work that the HPA axis has multiple ways of encoding glucocorticoid pulsatility (Figure 2). During the nadir of the circadian rhythm, when mean CRH levels are low and pulsatile (3 pulses/h), we expect that pulses observed in ACTH and glucocorticoids most likely reflect the activity of pulsatile CRH and other corticotrophin-releasing factors. However, during the peak of the circadian cycle, when CRH release is still pulsatile (3 pulses/h) but mean CRH levels are significantly higher, we predict that these higher mean CRH levels are sufficient to generate the endogenous hourly rhythm in ACTH and glucocorticoids, an intrinsic property of the pituitary-adrenal system (77).

Under both basal and non-basal conditions the HPA axis functions as a closed-loop control system, heavily influenced by negative feedback from circulating glucocorticoids. The regulation of pulsatility in the HPA axis likely involves a number of factors. The complex network of excitatory and inhibitory connections coupled with the pulsatile activity of hypothalamic releasing factors renders the task of unravelling mechanisms regulating glucocorticoid pulsatility extremely difficult. Experimental studies have so far been unsuccessful in identifying both the mechanistic and anatomical origin of the ultradian rhythm. We have been able to demonstrate, using a mathematical approach, that glucocorticoid negative feedback at the level of the anterior pituitary is not just involved in homeostatic regulation of optimal levels of ACTH and glucocorticoids, but is actually involved in generating ultradian HPA activity. The implications of this finding are that the pulsatile patterns of glucocorticoids that we observe in blood plasma may well reflect the integrated activity of pulsatile hypothalamic forcing on an endogenously rhythmic pituitary-adrenal system.

Decoding GnRH pulsatility

In the preceding sections, the HPA axis was used as a model system to consider physiological mechanisms generating pulsatile glucocorticoid signals. Recent work

has demonstrated that target cells are sensitive to the pattern of glucocorticoid to which they are exposed but molecular mechanisms used by cells to decode pulsatile glucocorticoid signals have not yet been extensively explored. Accordingly, we will now focus on the hypothalamo-pituitary-gonadal (HPG) axis as a model system for exploring pulsatile signal decoding.

The neuroendocrine network regulating the HPG axis has many similarities to that which mediates HPA activity. In particular, it too is characterised by an ultradian pattern of hormone secretion. The main role of the HPG axis is in regulating reproductive function. Following its release from the hypothalamus, gonadotrophin-releasing hormone (GnRH) acts via 7 transmembrane region (7TM) receptors to stimulate the synthesis and secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from gonadotrophs in the anterior pituitary. It acts via type I GnRH receptors (GnRHR) to stimulate phospholipase C, activating protein kinases C (PKC) and mobilizing Ca2+. This leads to activation of mitogen-activated protein kinase (MAPK) pathways and Ca2+ effectors such as calmodulin, mediating effects of GnRH on exocytotic gonadotrophin secretion, as well as on expression of many genes including those for the gonadotrophin subunits (78-80). Following their secretion into the general circulation, LH and FSH act to mediate the control of gametogenesis and hormone secretion form the gonads. In a similar manner to the glucocorticoid hormones, the gonadal steroids are self-regulatory via their inhibition of processes at higher centres (81).

GnRH is synthesised in, and secreted from, a relatively small number (hundreds) of hypothalamic neurons and has long been known to be secreted in brief pulses. GnRH pulse frequency varies under different physiological conditions. For example, it varies over the menstrual cycle with pulses on average every 6 h in mid- to late-luteal phases and every 90 min during follicular and early luteal phases (82). GnRH pulse frequencies are higher in rats and mice with physiological pulse intervals of 8-240 min (83). Importantly, GnRH effects on its target cells depend upon pulse frequency as illustrated by early studies showing that constant GnRH suppresses LH and FSH secretion, whereas restoration of GnRH pulses restores gonadotrophin secretion (84). Changing GnRH pulse frequency is also the primary

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means by which the body alters its reproductive status during development, with an increase in GnRH frequency driving the increased gametogenesis and gonadal steroid production at puberty (85). Moreover, stimulation paradigm is crucial for therapeutic manipulation of this system as pulsatile stimulation with GnRH agonists is used to stimulate gonadotrophin secretion in assisted reproduction, whereas sustained treatment ultimately reduces gonadotrophin secretion and this underlies agonist efficacy against steroid hormone-dependent cancers (86).

Although frequency decoding is fundamental to the physiology and pharmacology of the HPG axis, the mechanisms are poorly understood. Most recent work has focused on effects of GnRH on expression of gonadotrophin subunit genes, which (in both gonadotrophs and LbetaT2 cells) are sensitive to GnRH pulse frequency. Increasing GnRH pulse frequency to physiological levels increases its effects on LH-beta, FSHbeta and GnRHR expression, but as frequency is further increased to super-physiological levels transcription is reduced (83,87-93). Computational models suggest that such bell-shaped frequency-response relationships require feedback mechanisms (Figure 3) and these could include GnRHR down-regulation, induction of RGS (regulator of G-protein signalling)-2, inhibition of Ca2+ channels by the calmodulin-dependent G-protein Kir/Gem, or induction of MAPK phosphatases (MKPs). Rapid homologous receptor desensitization can be excluded as a mechanism because type I mammalian GnRHR do not show this behaviour. Uniquely, they lack the C-terminal tails that mediate phosphorylation, arrestin binding and desensitization of numerous other 7TM receptors (79,94-96). Alternative decoding mechanisms involve interplay between Egr-1 and a co-regulator (Nab-2) at the LH-beta promoter. In this model, low GnRH pulse frequency causes transient Egr-1 expression, causing expression of Nab-2 which inhibits LHbeta expression whereas at high pulse frequencies more sustained increases in Egr-1 quench Nab-2 and increase LHbeta transcription (97). For the FSHbeta promoter, similar interplay between c-Fos and the co-regulator TGIF has been proposed to underlie preferential activation at low GnRH pulse frequency (81). Moreover, Ciccone et al. (98) suggest CREB and Inducible cAMP Early Repressor (ICER) may be responsible. Here, high pulse frequencies preferentially induce ICER causing transcriptional

repression by competing for a CRE-site in the FSHbeta promoter (98).

The data above highlight two distinct possibilities; that GnRH frequency decoding reflects feedback effects on signal generation in the cytoplasm, or that frequency decoding occurs at the level of the transcriptome. Using conventional techniques it has been difficult to test the first of these possibilities but we have developed live cell readouts for signalling pathways implicated in frequency decoding, and have used these to explore GnRH signalling, as outlined below.

Nuclear factor of activated T-cells (NFAT) signalling
GnRHR-mediated activation of the Ca2+/calmodulin pathway can influence gonadotrophin subunit gene expression (99-101) and mechanisms by which calmodulins and their effectors interpret frequency-encoded Ca2+ signals are well established (102-105). More recently, NFATs, transcription factors activated by Ca2+/calmodulin-dependent activation of the protein phosphatase calcineurin (which dephosphorylates NFAT), have been implicated in transcriptional regulation by GnRH (106-108). This is of particular interest in light of the role of NFATs as frequency decoders in other systems (109-112). When GnRHR expressing HeLa cells were transduced with NFAT2-EFP (a reporter that translocates to the nucleus after Ca2+/calmodulin/calcineurin activation), GnRH caused dose-dependent NFAT2-EFP translocation although the effect was slower than the underlying Ca2+ response (113). With pulsatile stimulation (5 min pulses, 1 h intervals) responses were reproducible with no measurable desensitisation or reduction in GnRHR expression. Varying GnRHR number influenced response amplitude but not kinetics, and again no desensitisation was seen. At high pulse frequency (30 min) integrative tracking ('saw-tooth' responses) occurred. GnRH also caused dose- and frequency-dependent activation of alphaGSU-, LHbeta- and FSHbeta-luc reporters and these responses were inhibited by cyclosporin A, indicating calcineurin-dependence. Pulsatile GnRH also activated an NFAT-responsive luc reporter but its effect was proportional to cumulative pulse duration (113).

ERK signalling

Like many other 7TM receptors, GnRHR activate the Raf/MEK/ERK cassette (78-80). Activated ERKs can translocate to the nucleus where they phosphorylate transcription factors to control gene expression. GnRH activates ERK1/2, and ERKs can mediate GnRHR-stimulated transcription of the common alphaGSU subunit, as well as the LHbeta and FSHbeta (114-118). ERKs can mediate responses to pulsatile GnRH stimulation (99,119,120), pituitary-targeted ERK knockout can cause infertility (121), and the ERK cascade functions as a frequency decoder in other systems (122-124). To explore ERK signal dynamics we developed a model in which siRNAs are used to prevent expression of endogenous ERK1/2 and recombinant adenovirus (Ad) are used to add back an ERK2-GFP reporter at a physiological expression level (125-127). With this system we found that pulsatile GnRH causes dose- and frequency-dependent ERK2-GFP translocation to the nucleus. The responses were rapid and transient and therefore showed only digital tracking. They also failed to desensitise under any condition tested (dose, frequency and receptor number varied). GnRH also caused dose- and frequency-dependent activation of an Egr1-responsive luc reporter (used as a downstream readout for ERK activation) but the responses were again proportional to cumulative pulse duration. This response, like effects of pulsatile GnRH on FSHbeta-luc, was inhibited by siRNA-mediated knock-down of endogenous ERKs (125). ERK responses are modulated by many ERK interacting proteins including dual-specificity phosphatases (DUSPs), some of which can both scaffold and inactivate ERKs. Using siRNA to target DUSPs, we found that 12 of the 16 DUSPs expressed in HeLa cells influenced ERK responses to sustained stimulation with GnRH or a PKC activator (126,127). Moreover, GnRH can increase expression of nuclear-inducible MKP family DUSPs (117,128-130) and a recent computational model illustrated the potential for pulse frequency decoding by MAPK pathways and inducible phosphatises (131). Accordingly, we tested for effects of cycloheximide (to prevent nuclear-inducible MKPs induction), and used GFP fusions containing ERK mutations (D319N which prevents D-domain-dependent binding of MKPs, and K52R which prevents catalytic activity). These had little or no effect on the

ERK translocation responses arguing against a role for MKPs or ERK-mediated feedback in shaping ERK activation (125).

In interpreting the data above it is important to distinguish between 'frequency-dependence' and 'frequency-decoding'. There are many systems in which increasing pulse frequency increases responses but this could reflect either the increase in cumulative pulse duration or the reduction in inter-pulse interval (132). True frequency decoders sense inter-pulse interval independently of cumulative pulse duration (Figure 3) as illustrated by GnRH effects on expression of genes encoding rodent LHbeta and FSHbeta both of which are increased more effectively at low or intermediate GnRH pulse frequency than at high frequency or with sustained stimulation (83,87-93). Thus our data show that GnRH effects on the Ca2+/calmodulin/calcineurin/NFAT signalling and Ras/Raf/ERK signalling are frequency-dependent, and are consistent with roles for these pathways in mediating pulsatile GnRH effects on gonadotrophin expression. Nevertheless, these pathways appear not to act as genuine frequency-decoders of GnRH signalling in the models used (HeLa and/or LbetaT2 gonadotrophs). We were unable to find evidence for the negative feedback thought to underlie such frequency decoding (i.e. no desensitisation was seen and responses to pulsatile GnRH were not MKP-dependent) in spite of the fact that genuine GnRH frequency-decoding does occur in both models (113,125). These data differ from those obtained by using a mathematical model that predicts cellular responses to GnRH by simultaneous solution of differential equations describing various aspects of the GnRH signalling to LH secretion. Solving these nonlinear equations by machine computation predicts that GnRH effects will desensitise with pulsatile stimulation and that the extent of this desensitisation will increase with GnRH dose, frequency and receptor number (113,133) yet no such desensitisation was seen with either of our imaging assays. However, reduction in cell surface GnRHR number is the primary cause of desensitisation in this model and we have found that 5 min exposure to GnRH does not actually reduce cell surface GnRHR number in the HeLa cell model (113).

Most of the previously developed mathematical models concentrate on a particular aspect of GnRH-signalling rather than on the system as a whole. Earlier

theoretical work on modulation of pre-synthesized gonadotrophins was based on GnRHR kinetics desensitization (134). Furthermore, LH release per se has been the focus of several theoretical studies where frequency dependent modulation of secretion is a consequence of the presumed decrease in GnRHR plasma membrane expression (133,135). Most importantly, it is now clear that type I mammalian Gn-RHR do not undergo rapid desensitisation (95) and although agonists do GnRHR trafficking (136) agonist-induced down-regulation of cell surface GnRHR was not seen with brief GnRH activation (113). Most work on GnRHR signalling has been with sustained stimulation paradigms so signalling with physiological (pulsatile) activation remains relatively poorly understood, as does the mathematical basis for frequency decoding in this system. In this context only one study (132) recapitulates experimentally-determined transcriptional characteristics of GSU synthesis; nonetheless, it reflects a 'top-down' approach by considering the complex GnRH-signalling as a 'black-box', subsuming this process within empirical assumptions regarding translational delays and putative GnRH network modules. There are very few models of transcriptional GSU gene regulation that have been developed from the 'bottom-up' approach, building on the wealth of knowledge for the intracellular pathways activated by GnRH. The Sealfon group (137) used single cell imaging to monitor effects of GnRH on ERK and down-stream transcriptional responses. However their modelling concentrated only on the ERK1/2 pathway in populations of cells and did not take into account pulsatile GnRH stimulation. The effects of GnRH on the Ca2+/calmodulin/calcineurin/NFAT signalling and Ras/Raf/ERK signalling are frequency-dependent can be regarded as sub-modules acting within the overall GnRH signalling pathway. To further explore this mathematically, we have adapted a published model for GnRHR signalling (133) by removal of receptor down-regulation as negative feedback mechanism and have extended it to incorporate two cellular compartments representing the cytoplasm and the nucleus, respectively. We couple both compartments by incorporating into the model the fluxes of activated/inactivated NFAT and ERK1/2 across the nuclear envelope. Thus, the model includes a number of time-dependent evolution equations for all the key players involved in GnRH signalling as outlined above. To this

end we have already calibrated preliminary model parameters using experimental measurements of the nuclear to cytosolic ratios of total NFAT (113) and ERK1/2 concentrations (125) as shown in Figure 4. This model provides an excellent fit for the experimental data but as noted above, digital or integrative tracking is seen with no evidence of adaptation that might underlie genuine frequency decoding so we are currently extending the model to incorporate the action of NFAT and an ERK1/2-dependent transcription factor (e.g. Egr-1) as convergent inputs on GSU promoters.

Concluding remarks

Hypothalamo-pituitary adrenal and gonadal axes are characterised by pulsatile hormone secretion and digital hormonal signalling systems. We have used these two model systems to demonstrate two major facets of neuroendocrine signalling – mechanisms by which they can encode signals, well exemplified in the HPA axis, and mechanisms by which the tissues decode them as described for the regulation of gonadotrophin secretion. Mathematical modelling has been key to elucidation of these signalling systems which exemplify the power of modelling to investigate the organisation of these complex systems.

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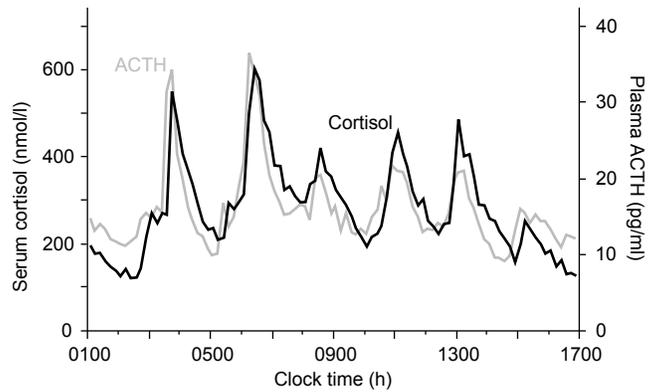
Figures

Figure 1. Human data for ACTH and cortisol from a healthy male sampled at 10 min intervals, adapted from (24). Concentration time series demonstrates strong concordance between rhythmic secretion of ACTH and cortisol, as well as a short time lag in ACTH-induced cortisol secretion.
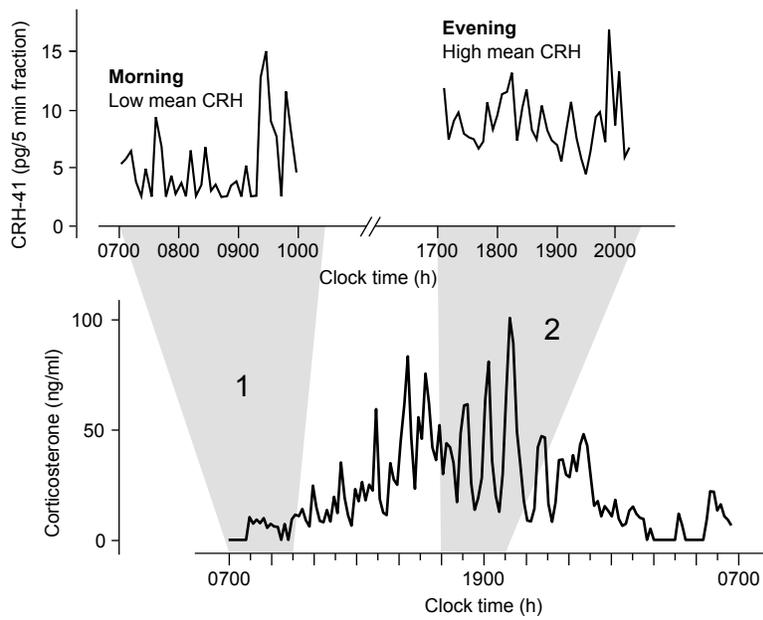
Figure 2. Different ways in which the HPA axis encodes glucocorticoid pulsatility. In region 1, where mean CRH levels are low, glucocorticoid pulsatility may well reflect the irregular high-frequency fluctuations in CRH and other corticotrophin-releasing factors acting on the anterior pituitary. In region 2, higher mean levels of CRH drive on the anterior pituitary are sufficient to excite the intrinsic rhythmicity of the pituitary-adrenal loop, which gives rise to the more distinct roughly hourly rhythm in glucocorticoid secretion. CRH data adapted from (51), glucocorticoid data adapted from (21).

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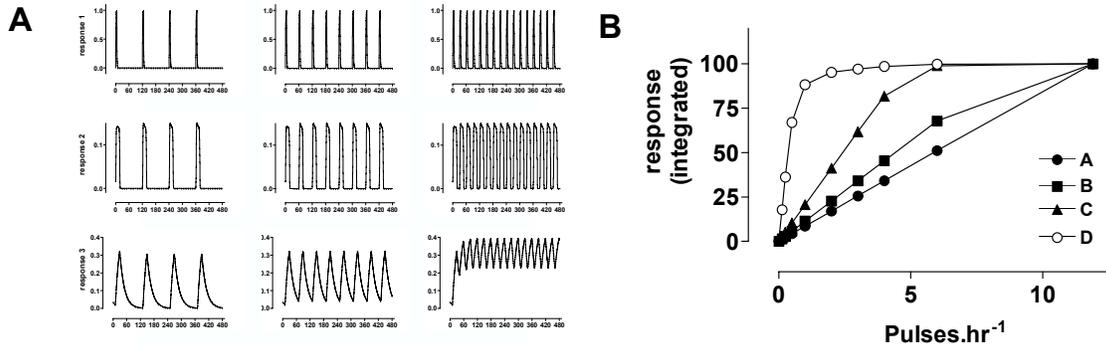
Figure 3. Frequency decoding mechanisms. Panel A illustrates possible responses to a square wave pulsatile stimulation with 5 min pulse duration and 120, 60 or 30 min pulse intervals, eliciting sequential responses (1-3) in a simple vectorial signalling pathway. With such pulsatile inputs upstream responses may have rapid onset and offset, following the input in a process known as digital tracking (rows 1 and 2). However, downstream responses with slower kinetics may not have not returned to baseline before repeat stimulation and this can result in cumulative saw-tooth responses in a process known as integrative tracking (row 3). Integrative tracking can increase the efficiency of signalling and the differential kinetics at different response levels can underlie differences in frequency-dependence (102) as illustrated in panel B. This shows the relationship between pulse frequency and responses calculated as the area under the curve for the input signal (A) or 3 sequential responses (B, C and D) in a vectorial signalling cascade, again using a 5 min stimulus (so that the maximal frequency of 12 pulses.hr-1 is identical to continuous stimulation). Note that maximal activation of response B is only seen with continuous stimulation whereas near maximal activation of response C occurs at 1 pulse.hr-1. Importantly, these processes can not alone explain the genuine frequency decoding (i.e. dependence on inter-pulse interval that is independent of cumulative pulse duration) that is evident in the bell-shaped frequency-response relationships for GnRH effects on gonadotrophin subunit and GnRHR expression (i.e. all the curves in panel B reach the same maximum as pulse frequency increases). Such frequency decoding is thought to require positive or negative feed-back or feed-forward loops (132).
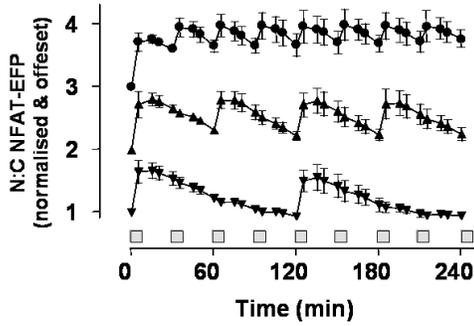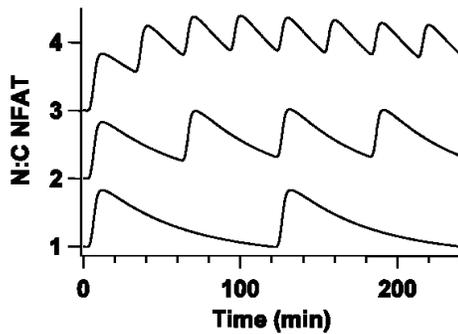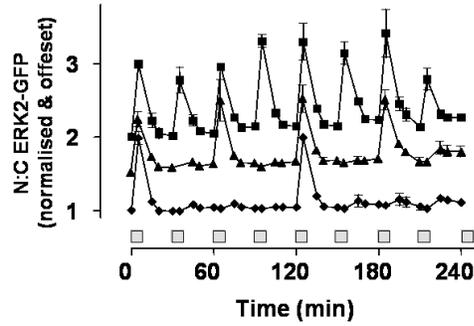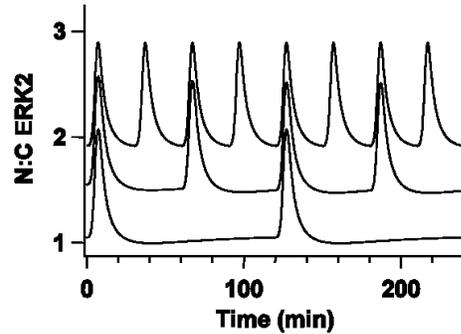
Figure 4. Modelling pulatile GnRHR signalling. Pulsatile stimulation for 5 min with varied GnRH pulse frequency at 30 min intervals, hourly intervals, or every two hours, as indicated. (a) The data shown in the upper panel are the normalised nuclear to cytosolic (N:C) ratio of NFAT2-EFP fluorescence intensity (113). The bottom panel illustrated preliminary model simulations of the whole-cell model NFAT response. (b). The data shown are the N:C ratio of ERK2-GFP fluorescence intensity (unpublished observations). The bottom panel illustrated preliminary model simulations of the whole-cell model ERK1/2 response (125). The data with GnRH at 1hr or 30 min intervals are offset by 1 or 2 units on the vertical axis for clarity.