Decoding neurohormone pulse frequency by convergent signalling modules

Krasimira Tsaneva-Atanasova*, Christopher J. Caunt†, Stephen P. Armstrong‡, Rebecca M. Perrett§ and Craig A. McArdle†

*Bristol Centre for Applied Nonlinear Mathematics, Department of Engineering Mathematics, University of Bristol, Bristol BS8 1TR, U.K., †Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, U.K., ‡Steroid Hormones Section, Clinical Endocrinology Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892, U.S.A., and §Laboratories for Integrative Neuroscience and Endocrinology, School of Clinical Science, University of Bristol, Whitson Street, Bristol BS1 3NY, U.K.

Abstract
GnRH (gonadotropin-releasing hormone) mediates control of reproduction. It is secreted in pulses and acts via intracellular effectors to activate gene expression. Submaximal GnRH pulse frequency can elicit maximal responses, yielding bell-shaped frequency-response curves characteristic of genuine frequency decoders. GnRH frequency decoding is therapeutically important (pulsatile GnRH can drive ovulation in assisted reproduction, whereas sustained activation can treat breast and prostate cancers), but the mechanisms are unknown. In the present paper, we review recent work in this area, placing emphasis on the regulation of transcription, and showing how mathematical modelling of GnRH effects on two effectors [ERK (extracellular-signal-regulated kinase) and NFAT (nuclear factor of activated T-cells)] reveals the potential for genuine frequency decoding as an emergent feature of the GnRH signalling network, rather than an intrinsic feature of a given protein or pathway within it.

GnRH (gonadotropin-releasing hormone) signalling

Pulsatile signals are used extensively in biological systems for cell–cell and intracellular communication. This enables information to be encoded in pulse frequency, amplitude and shape, and there is considerable interest in the cellular decoding mechanisms. We have explored this in the reproductive endocrine system, where the peptide hormone GnRH is secreted in pulses from neurons in the hypothalamus. It then passes through portal capillaries to the pituitary, where it binds to receptors on gonadotrope cells. Activation of these GnRHRs (GnRH receptors) increases the synthesis and secretion of FSH (follicle-stimulating hormone) and LH (luteinizing hormone) in and from gonadotropes. These gonadotropin hormones have a shared αGSU (α-gonadotropin subunit) as well as distinct LHβ and FSHβ subunits, and GnRH stimulates transcription of all three [1–4]. GnRH pulse frequency varies under different physiological conditions, including the menstrual cycle, where frequency increases in the early follicular phase, causing a surge in gonadotropin secretion that drives ovulation [4,5]. Effects of GnRH on secretion and transcription are dependent upon pulse frequency, and the essential role for pulsatile GnRH in normal physiology is illustrated by conditions in which its perturbation causes infertility [4]. Moreover, it has long been known that pulsatile stimulation of GnRHRs is more effective than sustained stimulation at driving gonadotropin secretion in vitro [6]. Consequently, sustained stimulation can cause chemical contraception, and this is exploited therapeutically in the treatment of hormone-dependent cancers [7].

GnRHRs are G-protein-coupled receptors that act via Gq/11 to activate PLC (phospholipase C), leading to an Ins(1,4,5)P3-mediated mobilization of Ca2+ and consequent activation of calmodulin. This in turn can activate the protein phosphatase calcineurin, causing it to dephosphorylate NFATs (nuclear factors of activated T-cells), exposing a nuclear localization sequence and thereby causing NFAT translocation to the nucleus [8,9]. NFATs are Ca2+-dependent TFs (transcription factors), and there is already considerable evidence that effects of GnRH on transcription of all three GSUs are dependent on Ca2+, calmodulin, calcineurin and/or NFATs [10–12]. Cytoplasmic Ca2+ also acts together with DAG (diacylglycerol) to activate isoenzymes of PKC (protein kinase C) that have many targets, including the MAPK (mitogen-activated protein kinase) cascade. The best characterized of these is the ERK (extracellular-signal-regulated kinase) cascade in which the protein kinase Raf (Raf-1, A-Raf or B-Raf) phosphorylates and activates the protein kinase MEK (MAPK/ERK kinase) (MEK1 or MEK2) which, in turn phosphorylates and activates ERK (ERK1 or ERK2) [2,12,13]. Activated ERK phosphorylates and regulates a large number of target proteins in different cellular compartments. In unstimulated cells, ERK is bound to cytoplasmic proteins such as MEK, but when activated it is released and can translocate to the

Key words: extracellular-signal-regulated kinase (ERK), frequency decoding, gonadotropin-releasing hormone (GnRH), mathematical model, nuclear factor of activated T-cells (NFAT).

Abbreviations used: Ad, adenovirus; EFP, emerald fluorescent protein; Egr-1, early growth response factor-1; ERK, extracellular-signal-regulated kinase; FSH, follicle-stimulating hormone; GFP, green fluorescent protein; GSK3, glycogen synthase kinase 3; GSU, gonadotropin-α subunit; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; Ins(1,4,5)P3, inositol triphosphate; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NFAT, nuclear factor of activated T-cells; PKC, protein kinase C; PLC, phospholipase C; TF, transcription factor.

*To whom correspondence should be addressed (email craig.mcardle@bristol.ac.uk).
nucleus, where it phosphorylates and activates nuclear TFs. It can also be inactivated within the nucleus by nuclear MAPK phosphatases that can either anchor dephosphorylated ERK within the nucleus or release it to be recycled back to the cytoplasm [14]. Many of the transcriptional effects of ERK are mediated by its ability to increase expression of the Egr-1 (early growth response factor-1) [15]. It is already well established that GnRHRs mediate a PKC-dependent activation of ERK and that GnRH effects on GSU expression are dependent on ERK and/or Egr-1 in several models [15–17]. Moreover, ERK and Egr-1 are both required for normal fertility in vivo and are both activated by pulsatile stimulation with GnRH [15,18–20].

Decoding GnRH pulse frequency
GnRH provides a frequency-encoded signal to gonadotropes that is crucial for the physiology and therapeutic manipulation of the reproductive system, yet the mechanisms by which these cells decode GnRH frequency are poorly understood. In many systems, increasing pulse frequency simply increases output until a maximal response is maintained with continuous stimulation. Alternatively, pulsatile stimuli may cause maximal responses at submaximal frequency, generating bell-shaped frequency–response relationships. Such behaviour has been termed genuine frequency decoding (as distinct from simple frequency-dependence). It is thought to require negative-feedback loops [21] and is exemplified by the bell-shaped frequency–response relationships seen for effects of GnRH LHβ or FSHβ expression (as measured using luciferase reporters). However, the nature of the feedback loops remains unclear, and three distinct possibilities are evident in the literature. The first is that negative feedback on upstream GnRHR signalling pathway components reduces information transfer from the cytoplasm to the nucleus at high pulse frequency. Rapid homologous receptor desensitization can be excluded as a potential mechanism, because type I mammalian GnRHRs do not show this behaviour [22]. GnRH does, however, down-regulate cell-surface GnRHRs [23], and a recent mathematical model of GnRH signalling predicts pulse frequency-dependent desensitization of upstream signals as a consequence of GnRH down-regulation [24]. The second possibility is that frequency decoding is due to the interactions of TFs, as exemplified by work on regulation of LHβ expression by Egr-1 and Nab-2. In this scenario, low pulse frequency causes transient Egr-1 expression, causing expression of Nab-2, which inhibits LHβ expression. However, at high GnRH pulse frequency, there is a more sustained increase in Egr1, which increases LHβ expression by quenching Nab-2 [25]. Similarly, GnRH frequency-dependence of effects on FSHβ expression appear to involve interplay of SKIL (SKI-like oncogene) and TGFβ1 (transforming growth factor β-induced homeobox 1) [26] and bZIP (basic leucine zipper) TFs [27]. Indeed, it has been suggested that FSHβ production is modulated by positive TFs and negative co-repressors with different pulse sensitivities [26]. The third possibility is that transcription-dependent negative feedback on upstream inputs occurs at high GnRH pulse frequency. This could include GnRHR-mediated induction of RGS (regulator of G-protein signalling)-2 which could inhibit all responses distal to Gq/11, or induction of MKPs (MAPK phosphatases) which would modulate GnRHR-mediated ERK signalling [28].

Wet laboratory and mathematical modelling of pulsatile GnRH signalling
We have sought to identify sites of negative feedback using fluorescent fusion proteins as live-cell readouts for signalling during pulsatile GnRH stimulation. We focused on NFAT and ERK, both of which decode pulse frequency in other models [29–33]. We used recombinant Ad (adenovirus) to express ERK2 fused to GFP (green fluorescent protein), NFATC1 fused to EFP (emerald fluorescent protein) and GnRHR in HeLa cells. We then stimulated the cells with GnRH pulses (5 min duration, 0.5–4 h interval, varied concentration) and used an automated fluorescence microscopy system for imaging during stimulation. Automated image analysis algorithms were then used to define the outlines of cells and their nuclei. This enabled calculation of N/C (nuclear/cyttoplasmic) ratios of NFAT–EFP and ERK2–GFP as live cell readouts for activation of the Ca2+/calmodulin/calcineurin/NFAT and Raf/MEK/ERK pathways respectively. We found that both reporters translocated rapidly to the nucleus on activation, but that response kinetics differed markedly. With 5 min of GnRH pulses, ERK2–GFP translocated rapidly to and from the nucleus and the N/C ERK2–GFP measure returned to basal values between stimuli (Figure 1), whereas the N/C NFAT–EFP response was slower in onset and offset, so that, at high pulse frequency, the response had not returned to the pre-stimulation value before a subsequent stimulus was added [10,16]. This led to ‘sawtooth’ or cumulative response that are thought to increase signal efficiency with pulsatile stimuli [34]. Irrespective of these differences, we found no evidence for desensitization of responses to pulsatile GnRH with these readouts [6,10] (Figure 1). GnRH pulses caused pulsatile translocation of ERK2–GFP and NFAT–EFP to the nucleus, but neither showed the bell-shaped frequency–response relationship characteristic of genuine frequency decoders.

Most of the data outlined above were obtained in a HeLa cell line engineered to express GnRH. Interestingly, frequency–response relationships for GnRH effects on LHβ and FSHβ transcription were bell-shaped (with luciferase reporters containing LHβ or FSHβ promoters) in this model, demonstrating (i) that genuine GnRH frequency decoding is not restricted to gonadotrope cells, and (ii) that such behaviour can occur in the absence of the negative-feedback loops implicated in GnRH frequency decoding. To explore this further, we have developed a mathematical model for GnRHR signalling based on ordinary differential equations describing GnRHR occupancy and activation.
Figure 1 | Modelling pulsatile GnRH signalling

Left-hand panels: cells transduced with Ad NFAT–EFP (upper panel) or Ad ERK2–GFP (lower panel) received 5 min pulses with GnRH (grey blocks) at 30, 60 or 120 min intervals. Automated imaging was used to follow reporter location (N/C ratio) as readouts for activation, and these values are shown offset on the vertical axes for clarity. Right-hand panels: a mathematical model for GnRH signalling was generated that accurately mirrors the responses of these two pathways to pulsatile GnRH (compare the left- and right-hand panels). Note that ERK responses are more rapidly reversed than NFAT responses, so sawtooth responses occur only with NFAT, and only at high pulse frequency. Based on data taken from [10,16,35].

and downstream effectors [35]. This differs from earlier models [17,21,24,28,36] in that it extends signalling to ERK and NFAT, includes cellular compartmentalization (i.e. nuclear compared with cytoplasm) and importantly, lacks upstream negative feedback. This model accurately predicts wet laboratory data for activation and nuclear translocation of ERK2–GFP and NFAT–EFP (Figure 2) as validated by modelling responses to GnRH pulses at a range of concentrations and frequencies.

Modelling convergent pulsatile signalling

Using the model described above, we considered the situation where two TFs (TF1 and TF2) converge on a single gene promoter that we name GSU (gonadotropin subunit), a generic term used because this is likely to be the case for the αGSU, LHβ and FSHβ gonadotropin subunit genes, as it is for many other ERK and NFAT target genes [8,9,29–33]. We considered the possibility that TF1 is an ERK-activated TF and that TF2 is NFAT (parallel activation network topology) or that TF1 and TF2 are sequentially activated downstream of ERK (sequential activation network topology). We also tested three distinct logic gates for the nature of the action of TF1 and TF2 at the promoter [35]. The first is a CO-OPErATIVE gate that, in biological terms, could reflect the action of one TF to mediate the interaction between the other TF and the cell’s transcriptional machinery, or alternatively, the requirement of physical interaction between the two TFs to orientate distant promoter sites and bring them to close proximity for transcription activation. The second is the AND gate in which both TFs are needed for transcription activation, but there is no functional interaction between them, and the third is the OR gate where either or both TFs can drive transcription, but there is again no functional interaction between the two. The key prediction of our model simulations is that genuine frequency decoding can indeed occur when two TFs are activated either in series or in parallel and converge in a co-operative manner on a gene promoter. Genuine frequency decoding was never seen in simulations with an AND gate or an OR gate, but the CO-OPErATIVE gate predicts this behaviour in the absence of the negative feedback often assumed to underlie it. Clearly, this does not negate the potential importance of negative-feedback loops for shaping ERK and NFAT signals. Indeed, a fundamental feature of the Ras/Raf/MEK/ERK pathway is that it behaves as a negative-feedback amplifier [37]. However, the GnRHR does not undergo rapid homologous desensitization [22] and physiological GnRH pulse duration is low (minutes), features that could minimize the influence of negative feedback (within and between GnRHR signalling modules; Figure 2) and increase reliance on convergent co-operative signalling as a means of frequency decoding.

As our mathematical model was developed using GnRHR-mediated responses, it provides a framework for understanding pulsatile GnRH signalling. It is important to recognize, however, that transcriptional regulation is an extremely
Figure 2 | Model predictions
A simplified view of GnRH signalling is that the GnRHR/Gq/11/PLC signalling module (indirectly) activates the Raf/MEK/ERK and calmodulin (CaM)/calcineurin (Cr)/NFAT modules with no intra- or inter-module negative feedback. With this architecture, modelling of frequency-response relationships predicts that the downstream modules could act independently to drive monotonic transcriptional responses (left- and right-hand panels), precisely as seen with measurement of pulsatile GnRH effects on Egr-1–luc (luc is luciferase) and NFAT-RE–luc [10,16]. Alternatively, they could mediate co-operative and convergent effects, driving non-monotonic responses (centre panel), as seen for pulsatile GnRH effects on FSHβ–luc and LHβ–luc reporters [10,16]. Importantly, the model predicts that frequency-response relationships may be regulatable (i.e. that the amplitude and kinetics of the input to the transcriptome could influence the position of the frequency-response curve as indicated by the horizontal arrows). Also shown is the model detail for the CO-OPERATIVE gate (see [35] for derivation and symbol definition).

complex process involving TF cascades (where immediately-early genes drive expression of late-response genes), and in which multiple TFs form gene-specific combinatorial codes and act in conjunction with epigenetic mechanisms (such as histone modification) at multiple stages of the cascade. We focused on possible convergence of the ERK and NFAT pathways on GSU promoters for four reasons. First, these are the only pathways for which we have experimental measures of the signal passing from the cytoplasm to the nucleus; secondly, ERK and calmodulin effectors, including NFAT, have already been shown to decode pulse frequency in other systems [8,9,31,33]; thirdly, the promoter regions of the αGSU, LHβ and FSHβ all contain response elements likely to mediate effects of ERK (e.g. Egr-1 sites) and NFAT [9]; and fourthly, the Raf/MEK/ERK and Ca2+/calmodulin/calcineurin/NFAT cascades are known to act as co-dependent modules in other systems, notably in the control of cardiac myocyte proliferation where ERK and NFAT converge on composite AP-1 (activator protein 1)–NFAT-response elements in a number of genes controlling cell growth [8,32]. Our modelling illustrates the potential for such convergence as a frequency-decoding mechanism that could well be relevant in the regulation of many target genes by GnRH in gonadotropes or by other stimuli in other cells.

We also performed parameter-sensitivity and numerical continuation analyses to identify the mechanisms and key regulators of these differential responses [35]. This revealed that affinities (dissociation constants) and half-lives of the TF1 and TF2 can have pronounced effects on frequency-dependence of the predicted transcriptional response (see Figures 7 and 8 in [34]). In modelling the CO-OPERATIVE gate, we assumed that Michaelis–Menten kinetics control the formation of the complexes between TF1 and DNA, as well as between TF2 and DNA and between the two TF–DNA complexes [35]. This function can yield non-linear outputs when activation kinetics of the two TFs differ and provides the basis of the predicted non-monotonic responses. However, it clearly represents an oversimplification, as transcription is controlled by the assembly of large protein complexes with multiple TFs and co-regulatory molecules interacting with components of the general transcription unit. Moreover, it is increasingly evident that TFs and other co-regulatory molecules cycle to and from DNA and often do so in synchrony. Thus there is a dynamic assembly and disassembly of transcription-regulating protein complexes at many genes, including the LHβ promoter in GnRH-stimulated cells [15,38]. In this context, one could view the model’s $K_d$ and half-life values for TF1 and TF2 as broad measures of the efficiency of assembly and stability of this multiprotein complex, rather than just the TF–promoter-binding affinity and TF-degradation rate. One intriguing implication of this is that the optimal pulse frequency for any given transcriptional response may itself be regulated by factors such as post-translational modification (i.e. phosphorylation, ubiquitination or SUMOylation of TFs) with the potential to alter the efficiency of assembly and the stability of the multiprotein complex. It has been very well established experimentally that high GnRH pulse frequencies favour activation of the LHβ promoter, whereas slower pulse frequencies favour activation of the FSHβ promoter both in vivo and in vitro [1–3,39], but, to our knowledge, the possibility that these optima themselves vary under different conditions (i.e. through the menstrual cycle or through puberty) has not been explored.

Overview
Although a great deal is yet to be learned about the mechanisms of GnRH frequency decoding, the most important aspect of the analysis described above is the model prediction that two convergent signalling pathways activated by pulsatile stimulation can show genuine frequency decoding behaviour without feedback loops. This
was illustrated for the Ca\(^{2+}\)/calmodulin/calcineurin/NFAT and Raf/MEK/ERK pathways that, under the conditions modelled, do not show frequency decoding as stand-alone modules, but do so when they converge on a COOPERATIVE logic gate. However, the model also predicts that similar behaviour will occur with two pathways in which the convergence is due to genetic and epigenetic regulation (e.g. activation of a TF and an enzyme causing histone modification to permit TF access to the promoter) or in which the pathways converge to influence activity of an effector that is not a TF (e.g. two kinases phosphorylating a target protein on different sites, or an ion channel gated by membrane potential and phosphorylation). The prediction of genuine frequency decoding behaviour in the absence of feedback loops clearly does not negate the importance of such feedback, but merely illustrates an additional possibility. Indeed, the abundance of negative-feedback loops and sites of convergence in signalling networks implies that complex non-monotonic frequency-dependencies will be almost inevitable emergent features of signalling networks, even when the network’s constituent proteins and pathways do not function as stand-alone frequency decoders.

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### References


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