Gonadotropin-releasing hormone signaling: an information theoretic approach.

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Abstract.
Gonadotropin-releasing hormone (GnRH) is a peptide hormone that mediates central control of reproduction, acting via G-protein coupled receptors that are primarily G_q coupled and mediate GnRH effects on the synthesis and secretion of luteinizing hormone and follicle-stimulating hormone. A great deal is known about the GnRH receptor signaling network but GnRH is secreted in short pulses and much less is known about how gonadotropes decode this pulsatile signal. Similarly, single cell measures reveal considerable cell-cell heterogeneity in responses to GnRH but the impact of this variability on signaling is largely unknown. Ordinary differential equation-based mathematical models have been used to explore the decoding of pulse dynamics and information theory-derived statistical measures are increasingly used to address the influence of cell-cell variability on the amount of information transferred by signaling pathways. Here, we describe both approaches for GnRH signaling, with emphasis on novel insights gained from the information theoretic approach and on the fundamental question of why GnRH is secreted in pulses.

Key words: GnRH, GPCR, NFAT, ERK, mathematical modeling, mutual information.

The Gonadotropin-releasing hormone (GnRH) signaling network

GnRH mediates control of the reproductive system by the CNS. It is a decapeptide hormone that is secreted by hypothalamic neurones into the hypothalamo-hypophyseal portal system and then binds GnRH receptors (GnRHR) on pituitary gonadotropes. It stimulates these cells to synthesise and secrete the gonadotropin hormones, luteinising hormone (LH) and follicle-stimulating hormone (FSH). These pituitary hormones are exocytotically secreted and control gonadal production of gametes and sex steroids. Within seconds of stimulation, GnRH causes fusion of secretory vesicles (containing LH and/or FSH) with the plasma membrane but in the long-term it also increases gonadotropin synthesis, thereby controlling vesicle LH and FSH content. GnRHR are G-protein coupled receptors (GPCRs) that signal primarily via heterotrimeric G-proteins of the G_q, family. Their activation by GnRH drives generation of the second messengers IP_3 (inositol 1,4,5 trisphosphate) and diacylglycerol. IP_3 acts via IP_3 receptors (that are ligand gated Ca^{2+} channels) located on intracellular stores (primarily the endoplasmic reticulum) to mobilize Ca^{2+}. This is followed by Ca^{2+} influx, largely via L-type voltage-gated Ca^{2+} channels, and it is the consequent increase in cytoplasmic Ca^{2+} concentration that is the main drive for the regulated exocytotic LH and FSH secretion (1-3). GnRHR also mediate activation of MAPK (mitogen-activated protein kinase) cascades, causing a largely protein kinase C (PKC)-mediated activation of the MAPK ERK (extracellular signal-regulated kinase) (4-6). GnRH influences expression of many genes in gonadotropes and gonadotrope-derived cell lines (7,8). Notably, it drives transcription of the gonadotrope signature genes (encoding αGSU, LHβ, FSHβ and GnRHR) (3,9,10) all of which can be influenced by activation of PKC and/or ERK (3,10-12). Several Ca^{2+}-regulated proteins are activated by GnRH and can also mediate transcriptional effects of GnRH. These include not only the conventional isoforms of PKC, but also the ubiquitous Ca^{2+} sensor calmodulin (CaM), as well as calmodulin-dependent protein kinases (CaMK), the calmodulin dependent phosphatase calcineurin, and one of its major effectors, the Ca^{2+} dependent transcription factor NFAT (nuclear factor of activated T-cells) (3,13,14). This brief overview omits

many known components of the GnRH signaling network, which are reviewed in more detail elsewhere (1-4,9,10,15-24).

The dynamics of GnRH signaling

Cellular responses to constant stimulation very often desensitize over time as regulatory interactions shape system outputs, and the importance of this is that the dynamics of signaling responses often dictate their effect on downstream responses. This is illustrated by the ERK signaling pathway where multiple negative feedback loops influence ERK response dynamics in different time-frames, and ERK response dynamics have a major influence on cell fate decisions (25-27). For GPCR signaling the best characterised adaptive mechanism is rapid homologous receptor desensitization, a process in which activated GPCRs are phosphorylated by G-protein receptor kinases on Ser and Thr residues that are most often within the receptor's COOH-terminal tail (28,29). This facilitates binding of non-visual arrestins (arrestins 2 and 3) that prevent G protein activation (causing receptor desensitization) and also target the desensitized receptors for internalization, most often via clathrin-coated vesicles (CCVs) (28,29). However, this paradigm cannot be generally applied to GnRHR because type I mammalian GnRHR do not have COOH-terminal tails (16,17,30) and are therefore thought not to undergo agonist-induced phosphorylation or rapid homologous receptor desensitization (31-39). Nevertheless, there are multiple downstream negative feedback mechanisms shaping cellular responses to GnRH, and effects of GnRH on ERK, Ca^{2+} and gonadotropin secretion do characteristically desensitize on constant stimulation (3,5,22,40-43). The situation with GnRH is more complicated however, because physiologically GnRH secretion is pulsatile and this pulsatility is essential for reproduction. GnRH pulses have durations of a few minutes and intervals of ~30 min to several hours in humans, and GnRH effects on its target cells are dependent on pulse frequency (44,45). Moreover, pulse frequency differs under different physiological conditions with changes in frequency driving changes in reproductive status during development and with aging (46-48). In addition to such changes in pulse frequency, the width and shape of GnRH pulses may vary under different physiological conditions. Indeed, recent work suggests that a change from pulsatile to tonic secretion drives the pre-ovulatory gonadotropin surge that is crucial for ovulation and menstrual cyclicity (49), implying that any increase in desensitization to GnRH during the surge is not sufficient to prevent the increased stimulation of gonadotropin secretion in this time-frame. Stimulus dynamics are also crucial for therapeutic targeting of the system as pulses of agonists can increase or maintain circulating gonadotropin levels whereas sustained agonist treatment initially increases, and then reduces, them. This ultimately causes chemical castration and this effect is exploited to treat breast cancer, prostate cancer and other hormone-dependent conditions (9,47,48). The crucial point here is that for many GnRH effects there is a non-monotonic (bell-shaped) frequency-response relationship with maximal GnRH effects on hormone secretion and gene expression being maximal at sub-maximal pulse frequency. In order to explore the interplay between GnRHR stimulus and response dynamics we have combined live-cell imaging (i.e. the nuclear translocation of ERK2-GFP as a readout for ERK activation and the nuclear translocation of NFAT-EFP as a readout for Ca^{2+}/calmodulin/calcineurin activation) with mechanistic mathematical modeling (i.e. ordinary differential equation (ODE)-based modeling of a simplified GnRHR signaling network). These studies are described in detail elsewhere (20,50-52) but the key findings are a) that pulsatile stimulation can increase efficiency and confer specificity on GnRH signaling, and b) that the characteristic bell-shaped frequency-response relationships for GnRH effects on gene expression are unlikely to reflect upstream negative feedback, but could instead be attributable to co-operative convergence of distinct pathways at the transcriptome or to incoherent feed-forward regulatory loops.

Heterogeneity in GnRH signaling

In the mechanistic modeling approach outlined above we seek essentially to model the behavior of an average GnRH responsive cell using a set of ODEs trained on population-averaged wet-lab data. However, this ignores the cell-to-cell response variability (heterogeneity) that is a characteristic feature of biological systems and has been described for effects of GnRH on gonadotropin secretion, cytoplasmic Ca^{2+} concentration, ERK activation and gene expression (8,22,43,50,51,53-57). Such variability is in fact inevitable (even in genetically-identical cloned cell populations) and it is often divided into two parts: intrinsic noise (involving fluctuations due to the discrete and stochastic nature

of the biochemical system at hand) and extrinsic noise (due to interactions of the system with other stochastic systems within and outside the cell, which for example, give rise to differences in amounts of proteins in individual cells or to differences in external environments between individual cells) (58). The key point here is that it is individual cells that have to sense their environment and make appropriate decisions (to express or to suppress specific genes, to die or to survive, to differentiate or to proliferate etc.) in light of it, so this heterogeneity ultimately underpins the health and activity of cell populations.

An information theoretic approach to cell signaling

Understanding of cell-cell variation and its influence on cell fate is relatively poorly developed but one particularly interesting approach has been the application of information theory to quantify information transfer by signaling pathways (59-65). In this context, 'information' is quantified by employing the concept of mutual information (MI) between the signal and the cellular response, I(response; signal), which measures the reduction in the uncertainty about the signal from observing the response. Therefore, estimation of MI can be achieved by exposing cells to a distribution of signals (i.e. hormone concentrations) and measuring responses in individual cells over this distribution (63). Essentially, one treats a signaling pathway as a noisy communication channel and calculates the quality of inference of the signal from the response. MI therefore provides a measure of information transfer that takes cell-cell variation into consideration instead of just averaging it. It is measured in Bits with a system that can unambiguously distinguish between two equally probable states of the environment yielding an MI of 1 Bit.

An important feature here is that MI values are not sensitive to non-linear transformation of the signal or the response, and are therefore uninfluenced by the non-linear and/or undefined input-output relationships often seen in cell signaling pathways. MI values, however, depend on the range and distribution of environmental states considered and, for cell signaling studies, signal probability distributions may be chosen to encompass a full dynamic range (i.e. a full concentration-response curve) or a physiologically-meaningful range. Conversely, MI values can be calculated for different signal probabilities to determine environmental states to which cells are most sensitive (66). A more general feature of this information theoretic approach to cell signaling is that focus is shifted from identification of signaling intermediates to measurement of information transfer, and this shift provides novel perspectives. For example, it is well established that signal amplification occurs through cell signaling cascades with the number of molecules activated increasing from one tier to the next but, information cannot increase through such a cascade because it transfers information about the input but clearly cannot generate it. Accordingly, any increase in signal amplitude any increase in signal amplitude from one tier to the next in the cascade must be coupled to an increase in cell-cell variation. Similar uncoupling between response amplitude and information transfer can occur as cells adapt to stimulation, and we illustrate this schematically in Figure 1 for a simple network where a stimulus (horizontal axis) acts via a single common effector (i.e. a receptor) to activate a network with a bifurcating (inverted Y-shaped) architecture leading to activation of two responses A and B: for example, GnRHR leading to activation of PKC and CaMK. In this scenario the initial population averaged input-output relationships are identical (black lines in A and B) but cell-cell variability is higher for A than for B, as shown by the greater spread of the red dots and the corresponding red frequency-distribution plots. Note that the frequency distribution plots overlap for A so there is a region of uncertainty with individual cells in A being unable to unambiguously distinguish between these two states of the environment. In contrast, the frequency distribution plots for B do not overlap so that the individual cells in B can unambiguously distinguish between these two states or more simply, the MI between response B and the input is greater than that between response A and the input. If the responses measured were activation of downstream effectors A and B, we would conclude that more information (about the concentration of stimulus in the environment) is carried via the receptor to B than via the receptor to A, in spite of the fact that the population-averaged responses are identical.

The influence of negative feedback on information transfer in cell signaling

If we now assume that the system outlined above contains negative feedback loops causing it to desensitize over time (from the initial relationships in Fig1 A and B to the adapted relationships in A' and B'), this adaptation could reduce response amplitude or cell–cell variability (or both). Here we assume that population-averaged response amplitudes are reduced identically for both (i.e. the black line values in A and B are transformed to 20% in A' and B'). However, for the A→A' adaption we assume that cell-to-cell variability and population-averaged responses reduce in parallel. Consequently, the overlap between the frequency distribution plots remains (albeit scaled) so that the quality of sensing is not actually reduced (i.e. in this scenario sensing was equally unreliable before and after adaptation). In contrast, we assume that for the B→B' transition, the population-averaged response reduces without a reduction in cell-to-cell variability so the frequency-distribution plots overlap for the adapted system and the quality of sensing is reduced. Thus, the A→A' adaption illustrates the situation where consideration of population-averaged data shows desensitization, yet the reliability with which the cells sense the concentration of stimulus in the environment has not actually changed. Similarly, consideration of population-averaged data alone suggests that balance of signaling to A and B is unaltered by adaptation yet in terms of information transfer it clearly has, because information transfer via the receptor to A is less than is to B, whereas information transfer to A' and B' is identical. More generally, each of these scenarios illustrates the point that inference quality is dependent on both response magnitude and noise so it is inappropriate to use the size of the system output alone as a measure of information transfer.

We have explored the impact of various sources of noise and the relevance of negative feedback by introducing cell-cell variability in the concentration of protein kinase and also incorporating basal (leaky) activity without stimulus (61). A key finding was that in such a system, negative feedback could increase information transfer by reducing basal activity, and this was confirmed experimentally for the ERK cascade by knock-down of endogenous ERKs and add-back of either wild-type or catalytically-inactive ERK2 (as a GFP fusion for live cell imaging). With either PKC-mediated or EGF receptor-mediated ERK activation, prevention of ERK-mediated negative feedback reduced information transfer because it increased basal activity (61). In a related study we developed a stochastic model for ERK activation and considered two distinct forms of ERK-mediated feedback: rapid negative feedback due to inhibitory phosphorylation of upstream mediators by ERK, and slow negative feedback due to ERK-driven expression of a MAPK phosphatase (57). As expected, simulations revealed that increasing intensity of either feedback loop reduced population averaged ppERK responses (here, the dual phosphorylation of ERK equates to ERK activity). However, this was in stark contrast to information transfer (MI between stimulus and ppERK levels) that was maximal at intermediate feedback levels (Fig.2). Thus, for both loops, high levels of feedback impaired information transfer by reducing the dynamic range of the responses, whereas very low feedback levels also impaired it by permitting noise due to basal (leaky) activity. These findings are summarised in Fig.2, where the difference between the heat maps in Panels A and B is a stark illustration of the fact that population-averaged response amplitude cannot safely be used as a measure of information transfer. In these simulations we also considered the relevance of response trajectory for information transfer as illustrated by panels B and C, and discussed below.

An information theoretic approach to GnRHR signaling

We have used this information theoretic approach to explore information transfer via GnRHR (57). To do so HeLa cells were transduced with recombinant adenovirus (Ad) for GnRHR expression before stimulation. Dual-phosphorylated ERK was measured by fluorescence immunocytochemistry as a readout for ERK activation, and a high content imaging platform was used to obtain single cell measures from large numbers of cells (typically >10,000 cells for each GnRH concentration-response curve). These were used to calculate MI between GnRH concentration and ppERK (I(ppERK;GnRH)), which revealed information transfer between GnRHR and ERK to be <1 Bit (Fig.3). This is comparable to values obtained for cytokine and growth factor signaling in other systems (59,60,64,65,67) and indeed, for EGF-mediated ERK activation in the same model system (61). Here, it should be noted that MI values are calculated for a specific range of inputs, and in these experiments this was typically eight concentrations of GnRH (i.e. 0 or 10^{-12} – 10^{-6} M) so there was a 3

Bit input ($log_2 8$), of which <1 Bit of information was transferred. The sources of the noise impairing information transfer via GnRHR are unknown but work in other models has revealed how extrinsic noise at multiple levels (i.e. variability in the concentrations of multiple effector proteins between individual cells) reduces information transfer (58,68,69). Notably, a recent study revealed that intrinsic noise alone (due to stochasticity in phosphorylation and dephosphorylation of ERK) is insufficient to explain cell-cell variability in ERK responses, and that it is noise extrinsic to the core ERK module that distorts the signal and causes a pronounced loss of information reduction in MI between stimulus and ERK (68). The observed MI values of <1 Bit for GnRH signaling to ERK imply that the system performs worse than a hypothetical one that can unambiguously distinguish between just two equally probable environmental states (e.g., high vs low GnRH concentrations), in spite of the fact that population-averaged measures reveal responses to GnRH that are graded over a broad range of GnRH concentrations. This scenario, which is comparable to that illustrated in Fig.1A, raises two obvious questions; first, whether experimental conditions led to underestimation of information transfer and second, how cells mitigate the loss of information through signaling. For the first we considered the possibility that information transfer might be low because of the use of a heterologous expression system, but this is not the case because I(ppERK;GnRH) values were found to be very similar in HeLa cells (Ad GnRHR transduced for heterologous GnRHR expression) and LβT2 gonadotropes (expressing native GnRHR). It was also not restricted to the ERK pathway because when the nuclear translocation of NFAT-EFP was used as a single cell readout for Ca^{2+}/calmodulin/calcineurin/NFAT activation, the information transfer from GnRHR to NFAT was <0.5 Bits in both HeLa and LβT2 cell models (57). An obvious advantage of the heterologous expression system is that it simplifies comparison of different receptors, so we used it to assess the possible influence of receptor desensitisation on information transfer in Hela cells transduced to express mouse or human GnRHR that lack C-terminal tails and do not show rapid homologous desensitisation, or to express a *Xenopus laevis* (X) GnRHR that has a COOH-terminal tail and rapidly desensitizes (35,41). A chimeric GnRHR consisting of the entire human GnRHR with an added XGnRHR tail was also used, along with a non-signaling mutant (A261K) of the human GnRHR. I(ppERK;GnRH) values were negligible (as expected) for the non-signaling receptor and were comparable (~0.5 Bits) for the other receptors (57). In this model, I(ppERK;GnRH) values were influenced by manipulation of ERK-mediated negative feedback. Notably, the reliability of GnRH sensing was impaired by reducing ERK-mediated negative feedback (by expression of catalytically inactive ERK2) but could also be impaired by increasing ERK-mediated negative feedback (by increasing ERK-driven MAPK phosphatase expression). Thus, consideration of three potential negative feedback mechanisms shaping ERK responses (rapid homologous receptor desensitisation, rapid transcription-independent ERK-mediated negative feedback and slow transcription-dependent ERK-mediated negative feedback) has revealed that both ERK-mediated feedback loops can influence information transfer via GnRHR whereas rapid homologous receptor desensitisation does not (at least for the model and end-points considered).

Turning to the issue of how cells might mitigate loss of information through signaling, an obvious possibility is that they do so by sensing multiple responses. To test this we measured ppERK and the nuclear fraction of NFAT-EFP (NFAT-NF) in the same individual LβT2 cells and calculated not only I(ppERK;GnRH) and I(NFAT-NF;GnRH), but also the joint information obtained by sensing both (57). This revealed MI values of ~0.5 Bits for either response or ~0.7 Bits for joint sensing. We also used transcription reporters (Egr1 driving zsGREEN expression and NFAT driving asRED expression) and this revealed MI values of ~0.8 bits for the ERK reporter, ~0.25 for the NFAT reporter and joint MI values of ~0.8 – 0.9. Qualitatively similar data were obtained with Ad GnRHR-transduced HeLa cells; in every case joint MI values were greater than for either response alone, but the increases were small (typically only 0.1-0.2 Bits).

Another possible explanation for our low MI measurements is that single time-point measures underestimate information transfer. This would be expected for cells that infer inputs (such as GnRH concentrations) from the profiles of outputs (such as ppERK levels) over time (65). We found that I(ppERK;GnRH) values calculated with snap-shot data were higher at 5 than at 360 min (Fig.3), yet the cell clearly has not obtained more information over 5 min than it has over 360 min. Instead, this

simply reveals that MI values are underestimated with the 360 min snapshot. This point is also illustrated by our stochastic stimulations of ERK activation with varied fast and slow feedback intensities, as we actually simulated the evolution of single cell responses over 60 min, enabling calculation not only of MI between stimulus and ppERK at any single time-point, but also calculation of MI between stimulus and single cell response trajectories. The precise gain in information from sensing trajectories was dependent on feedback intensities but in general, MI values were much greater when the response trajectory was taken into account (note the different scales for Fig.2B and C). Although we have not yet explored single cell ERK responses to GnRH in this way, it is well established that sustained ERK activation can have greater effects on gene transcription than transient ERK activation (25). Indeed, we find that the phorbol 12, 13 dibutyrate (PDBu) causes a more sustained increase in ppERK levels, and has a greater effect on Egr1-driven zsGREEN expression, than GnRH does and, importantly, that I(ppERK;PDBu) is greater than I(ppERK;GnRH) in this HeLa cell model (57). This is entirely consistent with GnRHR-mediated information transfer to ERK being sensitive to ERK activation dynamics.

To address the relevance of response trajectory more directly we monitored NFAT-EFP translocation in tracked live cells (70). As shown (Fig.4), continuous stimulation of Ad GnRHR-transduced HeLa cells with GnRH caused a concentration-dependent increase in NFAT-NF. The population-averaged responses increased to maxima at 15-60 min (Fig.4A) and information transfer via GnRHR to NFAT was ~0.5 Bits at all time-points, demonstrating that I(NFAT-NF;GnRH) had not been underestimated by missing a specific time-point. Using the live cell data we could also calculate I(NFAT-NF;GnRH) using the area under the curve for the tracked cell responses or using three time points, and these values were ~0.52 and ~0.55 Bits respectively (as compared to an average of 0.48 for the snap-shot data). Accordingly, although sensing of response trajectory can theoretically increase the MI values, sensing over time provided little or no increase in information transfer via GnRHR to NFAT (70).

Physiologically GnRH is secreted in pulses so we extended these experiments to consider a second pulse, addressing the obvious possibility that cells gain additional information from sensing repeated exposure to GnRH. We initially did so theoretically, by developing a hybrid (deterministic/stochastic) model for GnRH signaling to NFAT. To do so we simplified the deterministic model outlined above by removal of the ERK signaling pathway and transcription regulation steps and used this to simulate NFAT-NF responses to a 15 min pulse of 0, 10^{-11}, 10^{-9} and 10^{-7} M GnRH followed by an interval of 135 minutes without stimulation and then a second GnRH pulse. Although the relative importance of different sources of extrinsic variation is unknown for this cascade, we considered the concentrations of the GnRHR and calmodulin as two obvious possibilities. Here, we can consider two extreme situations. One is that the levels of these effectors in individual cells are constant over time so that although there is cell-cell variability, the signaling network is hardwired in each individual cell. The other is that these levels fluctuate extremely rapidly over time. Since the sources of cell-cell variability in GnRH signaling are unknown, stability of the relevant effectors is inevitably also unknown, so we considered a range of stabilities. By incorporating effector synthesis and degradation into the model we allowed each of these effectors to fluctuate, setting fluctuation lifetime (FL) to 10, 100, 1000 or 10000 min (i.e. from most unstable to most stable, over time). As expected, population-averaged NFAT-NF responses for the stable and unstable systems had comparable means and variance (Fig.5A), in spite of the fact that individual cell response trajectories were more variable with the more unstable effectors (compare Fig.5B left and right hand panels). When effector stability was high, the cells showing greatest responses in pulse 1 also showed large responses in pulse 2, whereas this was not the case when effector stability was low (compare Fig.5B left- and right-hand panels). I(NFAT-NF;GnRH) values calculated using areas under the curve (for the first 15 min of stimulation in either pulse) were ~0.25-0.4 Bits and were comparable for pulse 1 and pulse 2 irrespective of effector stability. Additional information gained by sensing both pulses was negligible with high effector stability but increased to >0.2 Bits at the lowest effector stability (Fig.5E). We also calculated the MI between the pulse 1 and pulse 2 responses (Fig.5F) and this increased from 0 to ~1.8 Bits as FL was increased from 10 to 10000. Thus, these simulations reveal that the additional information from the second pulse is dependent on the nature of the effector lifetime variation. If the heterogeneity reflects a broad distribution of effector concentrations that is constant over time, then

the response in pulse 2 is predictive of that in pulse 1, and there is no additional information from sensing both. This is the situation approached at FL=10000 min where additional information from the second pulse is negligible and the MI between pulse 1 and 2 responses is high. In contrast, if the effector levels (the source of the heterogeneity) are random or change rapidly over time, the response in pulse 2 is less predictive of that in pulse 1 so additional information is obtained by sensing both. This is the scenario with FL= 10, where additional information from the second pulse is relatively high (Fig.5E) and the MI between pulse 1 and 2 responses is low (Fig.5F).

The simulations above illustrate conditions where additional information is, or is not, gained by sensing two consecutive pulses, raising the question of what actually happens in GnRH-stimulated cells. To test this we stimulated Ad GnRHR- and Ad NFAT-EFP-transduced HeLa cells with two separate pulses of GnRH followed by imaging and individual cell tracking as outlined above (Fig.6). I(NFAT-RE;GnRH) values calculated using the areas under the curves for the individual cell response during the first 15 min of each pulse were comparable to one-another (~0.5-0.6 Bits). The additional information due to sensing both pulses was low (~0.1 Bit), and the MI between responses in pulse 2 and 1 was high (~1.0 Bit). Accordingly, the wet-lab data parallel the situation simulated in Fig.5 with high effector stability (FL 10000), implying that the sources of variations are most likely extrinsic to the signaling network and relatively stable over time so that there is little additional information to be gained from sensing trajectories. Thus, the cells can be considered as effectively hard-wired, with undefined but relatively stable differences explaining cell-cell heterogeneity, at least for this readout and time-frame (70).

Summary.

GnRH signaling mechanisms have been the subject of intense scrutiny for decades and this has provided detailed understanding of the GnRHR signaling network componentry and architecture, as exemplified by a web-accessible knowledge-base of GnRHR signaling in LβT2 cells (71). However, stimulus dynamics are of fundamental importance for the physiology and therapeutic manipulation of this system and our understanding of how cells decode such dynamic inputs is still relatively rudimentary. Mathematical modeling using ODE-based mechanistic models has been extremely informative for hypothesis generation and testing. However, such models have typically been trained and validated with data-averaging responses of large cell populations and therefore model the behavior of an “average” cell whilst ignoring the cell-cell heterogeneity. Such heterogeneity is inevitable (even in clonal cell populations), and ultimately underpins the health and activity of cell populations, since it is individual cells that have to sense their environment and make appropriate decisions in light of it. In recent years, information theory-derived statistical measures have been used in cell signaling studies, treating signaling pathways as noisy communication channels and addressing the influence of cell-cell variability on the amount of information they transfer. This approach has provided novel insights into many aspects of signaling, including the finding that negative feedback pathways can both facilitate and impair information transfer. Consequently, information transfer can be maximal at intermediate feedback levels where population-averaged responses are sub-maximal, highlighting the potential risk of equating response amplitude directly to information transfer. Here, it may be useful to recall that information theory was originally developed to explore electronic communication; an engineer working to improve a poor telephone connection would no doubt consider volume and distortion due to noise from different sources but, if we extend this analogy to cell signaling, biologists have focused on volume (i.e. output amplitude) whilst often ignoring the noise that is so prevalent in cell signaling systems. We believe that combined mechanistic and probabilistic modeling are important adjuncts to more conventional molecular and cellular approaches to understand GnRH signaling, and illustrate this here by considering the fundamental question of why GnRH is secreted in pulses. Our mechanistic modeling of pulsatile stimulation with varied pulse width, amplitude and frequency revealed how pulsatility can increase signaling efficiency in the sense that with pulsatile and constant stimuli and identical input integrals, the system output can be much greater with pulsatile stimulation (20). Moreover, this occurs largely because signaling continues in the intervals between the pulses and the extent of this depends on activation and inactivation rates that will differ for different effectors. Consequently, with pulsatile stimuli, input-output relationships for different effectors are not superimposable, and this can give output-specific frequency-response

relationships where no such specificity occurs with concentration-response relationships (20). A third possibility is that repeated pulses of GnRH provide substantial additional information about GnRH concentration, and here the logic is simply that repeating a message can increase the likelihood of it being correctly heard. However, our mixed mechanistic/stochastic modelling and wet-lab experiments argue against this. Instead, we find that single cell responses in one pulse are predictive of those in another, so there is little additional information to be had by sensing both (at least for GnRH effects on NFAT localization with the experimental paradigm considered). Another important general observation here is that, even when joint sensing or trajectory are considered, our I(response;GnRH) values were always <1. It is, of course, possible that joint MI values would be greater for other effector pairs or if more than two effectors/pathways were considered but the data available so far suggests that individual GnRH-responsive cells cannot distinguish between even two GnRH concentrations, and this contrasts to numerous published studies showing dose-dependent effects of GnRH on populations of GnRH-responsive cells. Clearly, cell populations discern GnRH concentrations more reliably than individual cells and this could reflect averaging of responses over multiple cells and/or cell-cell communication providing additional information. The latter possibility is of particular interest as gonadotropes communicate with one-another via gap junctions (37,38), and although it is individual cells that have to sense and respond to GnRH in their environment, these decisions could well be informed by additional information from their neighbours. Again, we suggest that combinations of mechanistic and stochastic modelling with more conventional cellular and molecular experiments, will be needed to address this possibility.

Figure legends

Figure 1. Cell-cell variability and information transfer. The solid sigmoid curves in the upper cartoons illustrate population-averaged responses, with individual dots representing single cell responses from which the population averages are derived. For panels A and B the population-averaged data are identical but there is higher cell-cell variability in A. Consequently, frequency distribution plots shown on the left (for the stimulus concentrations indicated by the dotted lines) overlap for panel A. This creates a region of uncertainty, in that any individual cell in the area of overlap cannot "know" which stimulus concentration it has been exposed to. For panel B, cell-cell variability is much lower so the frequency-distributions do not overlap and there is no area of uncertainty. Mutual information (MI) is a statistical measure of inference quality (how reliably the system input can be inferred from the output). It is measured in Bits (with a system that can unambiguously distinguish two equally probable states of the environment yielding an MI of 1 bit) and would be higher in B than in A. We also illustrate the situation where the cells adapt to their environment such that the population-averaged response is reduced either with a proportional reduction in cell-cell variability (A→A') or with no change in cell-cell variability (B→B'). Note that the frequency-distributions overlap in A' just as they do in A, and in B' whereas they don't in B. Accordingly, the B→B' adaptive response reduces information transfer whereas the A→A' adaptation does not. Note that consideration of population-averaged responses alone can deliver the wrong conclusion; if this were a hormone pre-treatment protocol one could conclude that the system has desensitized from A to A' in spite of the fact that the quality of hormone sensing has not altered.

Figure 2. Stochastic modelling of ERK signaling reveals maximal information transfer at intermediate feedback levels and information gain by sensing response trajectories. Panel A: heat map of population-averaged S-stimulated ppERK responses for single cells with varied fast and slow feedback (FB). The data were generated by simulation as described (57), with fast FB and slow FB at 0.5-3.5 and 0-4 ($\log_{10}$ scale), respectively. The simulations were run for 60 min and 10 min data are shown. Panel B: heat map of predicted I(ppERK;S) for the 10 min snap-shot data corresponding to the left panel. Panel C: heat map of predicted I(ppERK;S) with parameters identical to those for the other panels, except that the simulations were run for 60 min and MI was calculated taking into account response trajectories. Also shown is a cartoon of the system simulated, with stimulus (S) activating an upstream effector (E) which catalyses the activating phosphorylation of ERK. The fast FB loop represents ERK-dependent inhibition of E*→pERK and the slow FB loop mirrors pERK-

driven phosphatase expression and consequent dephosphorylation (inactivation) of pERK. Adapted from (57).

Figure 3. MI as an information theoretic measure of GnRH sensing. Panels A and B show concentration and time-dependent effects of GnRH and PDBu on ERK activity in LβT2 cells, with population-averaged nuclear ppERK values measured by automated fluorescence microscopy and shown in arbitrary fluorescence units (AFU). The single cell measures underlying these plots were also used to calculate MI between ppERK and each of these stimuli, and these are plotted (I(ppERK;stimulus) in Bits) against time in C. The cells were also transduced with recombinant Ad for expression of an ERK-driven transcription reporter (Egr1-zsGREEN). Panel D shows the concentration-dependence of GnRH and PDBu effects on zsGREEN expression (population-averaged responses in AFU) after 360 min stimulation and the MI between zsGREEN and each of these stimuli is also shown for this time. Adapted from Garner et al. 2016 (57).

Figure 4. Live cell imaging reveals little gain in GnRHR-mediated information transfer from sensing NFAT translocation trajectories. HeLa cells transduced with Ad GnRHR and Ad NFAT-EFP were stimulated continuously with 0, 10^{-11}, 10^{-9} or 10^{-7}M GnRH for live cell imaging and calculation of NFAT-NF (nuclear fraction). Population-averaged responses for all tracked cells are shown in panel A and the corresponding I(NFAT-NF;GnRH) values are shown B. I(NFAT-NF;GnRH) values were also calculated from the same tracked cells using the maximum response, the area under the curve for the full 60 min (AUC) or the response trajectories (estimated using 3 time points) as the response. These values are also given in B. Adapted from Garner et al. 2017 (70).

Figure 5. Stochastic modelling reveals the potential for information gain by sensing responses to repeated GnRH pulses. A mixed deterministic/probabilistic model for GnRH signaling was used to simulate NFAT-NF responses to 15 min stimulation with GnRH, followed by a 135 min interval and a second pulse (60 min, GnRH at the same concentration, 0, 10^{-11}, 10^{-9}, or 10^{-7}M, as used in the first pulse). Responses were simulated in 1000 cells at each GnRH concentration/effector stability combination. Panel A shows population-averaged data for simulations with 10^{-7}M GnRH at each of four stabilities (FL 10, 100, 1000 and 10000, means ± SEM, n=1000) and panels B, C and D respectively, show representative traces for 25 individual cells (NFAT-NF and concentrations of GnRHR and calmodulin (CaM) in μM). Panel E shows the additional information gained by sensing both pulses and panel F shows MI between responses in pulse 1 and 2. Panels E and F are plotted against FL (where $\log_{10}$ FL values of 1 and 4 represent the most unstable and stable scenario, respectively). Note that at any given time point, mean values and variance are comparable for NFAT-NF (A) as well as for GnRHR and CaM (C and D), but as effector stability is increased, this increases MI between the pulse 1 and pulse 2 responses (F), and reduces additional information gained from the second pulse (E). Adapted from Garner et al. 2017 (70).

Figure 6. Live cell imaging reveals little gain in information from sensing NFAT translocation responses to repeated GnRH pulses. HeLa cells transduced with Ad GnRHR and Ad NFAT-EFP were stimulated with 0, 10^{-11}, 10^{-9} or 10^{-7} M GnRH for 15 min (first grey bar), then washed (3 times over 5 min) and imaged for a further 2 hr before repeat stimulation (second grey bar) for 60 min using the same concentrations of GnRH. The data shown are population-averaged NFAT-NF values for the tracked cells in one of 3 repeated experiments with control (0 GnRH) values subtracted. MI values calculated using the area under the curve (first 15 min) were 0.58 Bits (pulse 1) and 0.50 Bits (pulse 2). Additional information gained by sensing both and the MI between responses in pulse 1 and pulse 2 were ~0.1 Bits and ~1.0 Bits, respectively. Adapted from Garner et al. 2017 (70).

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