MIXED ANALOG/DIGITAL GONADOTROPE BIOSYNTHETIC RESPONSE TO GONADOTROPIN RELEASING HORMONE

Frederique Ruf1, Myung-June Park1,2, Fernand Hayot1,2, Gang Lin3, Badrinath Roysam3, Yongchao Ge1,2 and Stuart C. Sealfon1,2

From the 1Department of Neurology, and 2Center for Translational Systems Biology, Mount Sinai School of Medicine, New York, N.Y. 10029, and from the 3Department of Electrical, Computer, and Systems Engineering at Rensselaer Polytechnic Institute, Troy, N.Y. 12180

Address correspondence to: Stuart C. Sealfon, Neurology Box 1137, One Gustave L. Levy Place, New York, N.Y. 10029, Tel: (212) 241-7075, Fax: (212) 289-4107, Email: stuart.sealfon@mssm.edu

Mammalian reproduction requires gonadotropin releasing hormone (GnRH)-mediated signaling from brain neurons to pituitary gonadotropes. Because the pulses of released GnRH vary greatly in amplitude, we studied the biosynthetic response of the gonadotrope to varying GnRH concentrations, focusing on extracellular-regulated kinase (ERK) phosphorylation and egr1 mRNA and protein production. The overall average level of ERK activation in populations of cells increased non-cooperatively with increasing GnRH, and did not show evidence of either ultrasensitivity or bistability. However, automated image analysis of single cell responses showed that while individual gonadotropes exhibited two response states, inactive and active, both the probability of activation and the average response in activated cells increased with increasing GnRH concentration. These data indicate a hybrid single cell response having both digital (switch-like) and analog (graded) features. Mathematical modeling suggests that the hybrid response can be explained by indirect thresholding of ERK activation resulting from the distributed structure of the GnRH-modulated network. The hybrid response mechanism improves the reliability of noisy reproductive signal transmission from the brain to the pituitary.

Mammalian reproduction and the survival of a species rely on a precise orchestration of temporally and spatially distributed molecular events. The control of reproduction represents a difficult engineering problem because noisy molecular processes within cells that occur on time scales of minutes must regulate brain, pituitary and gonadal activity in a process with an overall periodicity of days to weeks, depending on the species. At the center of this coordinated reproductive activity lies the pituitary gonadotrope, which converts hormone signals secreted by the brain into the biosynthesis and secretion of pituitary hormones controlling gonadal responses.

The hypothalamus secretes discrete pulses of gonadotropin releasing hormone (GnRH, for review see (1-3)). GnRH interacts with high affinity GnRH receptors on the gonadotrope membrane to modulate the biosynthesis and release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (4-6). The function of the reproductive axis depends on appropriate responses of the gonadotrope to GnRH despite the high interpulse variability in the amplitude of GnRH secreted by the brain (3,7-12). Elucidating the mechanisms underlying the response of the gonadotrope to varying concentrations of GnRH is important for understanding the design principles of this key response locus for mammalian physiology.

GnRH directs two distinguishable gonadotrope activities, the biosynthesis of gonadotropins and their secretion. We focus here on the biosynthetic response. The GnRH receptor is a heptahelical G protein coupled receptor that modulates a signaling network
leading to activation of protein kinases and regulation of both transcription and translation (13). The gene network responses include both primary genes, that are activated within the first hour of GnRH receptor activation by preformed transcription factors, secondary genes activated within the first 2 hours and which require new protein synthesis, and tertiary genes, including gonadotropin subunits, that are induced after hours to days (13-16).

The induction of Egr1, a zinc-finger containing transcription factor, represents an early biochemical response to GnRH that is necessary for reproductive competency. Egr-1 is induced rapidly after GnRH stimulation of gonadotropes and is necessary for induction of the tertiary LH subunit gene (14,17-22). The ablation of the egr1 gene in mice prevents LH synthesis and leads to infertility (23,24).

The induction of Egr-1 is mediated by extracellular signal-regulated kinase (ERK) (13,25,26). ERK, which is activated by the addition of two phosphate groups on a Tyr and a Thr, is a GnRH-regulated mitogen activated protein kinase (MAPK). Like all MAPKs, ERK is the final member of a triad of sequential kinases, referred to generically as MAPkinase-kinase (MK2) and MAPkinase-kinase-kinase (MK3). The signaling mediator between the GnRH receptor and the MK3 in the gonadotrope has not been identified.

Biochemical studies of cell signaling and gene regulation assay large populations of cells and quantify the average responses observed. As the response at the level of the single cell may be heterogeneous, there is increasing interest in characterizing signaling and gene regulation within single cells (27-31). In order to facilitate the accurate quantification of signaling and gene responses in a large number of cells and to determine the underlying distributions and mechanisms, we developed and validated an approach based on histological assays and automated image segmentation and feature extraction (32).

The pituitary gonadotrope provides a physiologically important system in which to investigate the mechanisms underlying the response to varying concentrations of GnRH at the level of the MAPK ERK, egr1 gene induction and Egr1 protein synthesis. We found that ERK phosphorylation, egr1 mRNA and Egr1 protein induction followed a mixed analog/digital hybrid pattern of single cell responses. Mathematical simulations undertaken to reconcile the average cell and single cell data obtained suggest a unique mechanism of crosstalk between diverging GnRH-regulated signaling pathways.

**Experimental Procedures**

**Cell culture.** LβT2 cells obtained from Prof. Pamela Mellon (University of California, San Diego, CA) were maintained at 37 degrees C / 5%CO2 in humidified air in phenol-red free DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Gemini, Calabasas, CA) and L-glutamine. For histological assays, 200,000 cells were seeded on poly-D-lysine pretreated glass coverslips (#1.5, 18x18mm, Fisher) in 6-well plates. Cells were synchronized in 0.5% charcoal-treated FBS (CT-FBS), L-glutamine and 25mM Hepes.

**Viability assay.** Live/dead assay (L-3224, Molecular Probes, Eugene OR) was performed according to the manufacturer’s instructions. Permeabilization with 0.1% saponin (Sigma) was used as a positive control. For trypan blue staining (Gibco #15250-061) cells were resuspended in phosphate buffered saline with 10% dye suspension and counted in a hemocytometer.

**Immunohistochemistry.** GnRH diluted in DMEM with 0.5% CT-FBS, L-glutamine and 25mM Hepes was added at the 0, 4, 20 or 100 nM to synchronized cells (5 minutes for MEK and ERK. 60 minutes for Egr1). After 4% formaldehyde (ultrapure EM grade, Polysciences) for 30 minutes at room temperature (RT), permeabilization in 0.2%triton/1X PBS (Triton, Sigma-Aldrich) for 10 minutes at RT, and quenching in 50mM NH4Cl (Sigma-Aldrich) for 5 minutes at RT blocking was performed in phosphate buffered saline/0.1% Tween/5% Bovine Serum Albumin (BSA, Roche Diagnostics, Mannheim, Germany) for 1h at RT. Primary antibody (1:1000) was added and incubated overnight at 4 degrees C. Secondary fluorophore-coupled
antibody was added (1:1000) for 2h, RT. After washing and 4',6'-diamidino-2-phenylindole counterstaining (DAPI, 0.1μg/μL, Sigma-Aldrich), coverslips were mounted in Prolong Gold Antifade reagent (Molecular Probes). The antibodies used were anti-egr1 (sc-110, Santa Cruz), anti-actin (Santa Cruz), anti phospho p42-p44 MAPK (Cell Signaling #9106S), anti-MEK1/2 (Cell signaling #9122), anti-ERK1/2 (Cell signaling #9102), anti-phospho-MEK1/2 (cell signaling #9121S), anti-p21 Waf1/Cip1 (Biosource) and secondary antibodies coupled to Alexa 488 or Alexa 568 (Molecular Probes). Egr-1 blocking peptide was from Santa Cruz Biotechnology (sc-110P). Mitogen-activated protein kinase-ERK kinase (MEK) inhibitor PD98059 was used at 50μM for 30 minutes before GnRH exposure.

In-situ hybridization. Fixation was identical to that for immunohistochemistry except for the use of 0.1% triton/1X PBS. Cells were prehybridized in 4X saline sodium citrate (SSC), 50% formamide, tRNA, unlabeled scrambled oligonucleotide, salmon sperm and DEPC-treated water for 1h at RT then incubated with 60ng of the specific labeled oligonucleotide probe for 2h at 37 degrees C. After washing with 2X SSC at 52 degrees C on a waterbath, cells were counterstained with DAPI before mounting with Prolong Gold Antifade reagent. The in situ hybridization probes were designed, synthesized and labeled as described elsewhere (33,34). Each 50-mer probe contained five amino-modified thymidine residues (Amino-Modifier C6 dT phosphoramidite; Glen Research, Sterling, VA) for the chemical conjugation with activated succinimidyl ester Alexa fluorophores (Molecular probes, Eugene OR). Probe sequences and modification sites are listed in Supplementary Table 2.

Fluorescent microscopy and data analysis. A Zeiss LSM510-META inverted confocal laser-scanning microscope was used for confocal imaging. Imaging was achieved using a 40X/1.3 NA, or 63X/1.3 NA or 100X/1.3 NA oil immersion objective. The blue diode laser 405 nm with bandpass emission filters of 420–480 was used for DAPI visualization. The HeNe laser 543 was used for excitation of Alexa 568 and detected with a 515-540 bandpass filter.

Epifluorescence microscopy used an Olympus BX-60 microscope coupled with a BX-FLA Reflected Light Fluorescence Attachment and a CCD-based image analysis system. Each image field was captured as a digital image using the SPOT Advanced system (Diagnostic Instruments, Sterling Heights, MI). For triple and double-labeled coverslips, images were captured sequentially, then merged in the SPOT Advanced program with Alexa 488 as the green panel, Alexa 568 as the red panel, and DAPI as the blue panel. The image was set to a pixel dimension of 1,520 Å~ 1,080 at a size of 21 Å~ 15 inches in color RGB mode. The digital images were transferred to a Macintosh PC and reduced bicaically in Adobe Photoshop 7.0. The exposure settings were determined empirically for each channel from control background and unchanged within an experiment. At least 10 images of non-overlapping areas with 50-400 cells were assayed for each condition in each experiment.

Automated image quantification and data processing. Digital images were analyzed in a custom automated image analysis suite called 3D-CatFISH (32,35). First, the cell nuclei were segmented using DAPI with the enhanced 3D watershed algorithm (36) followed by model-based object merging (37). Then, a desired region-of-interest was defined based on geometric distance for each segmented nucleus, and specific signals were quantified in nucleus and cytoplasm. The threshold was determined from values obtained for the vehicle-treated slides and maintained without changes for all the other slides of each independent experiment. Each image was visually inspected for possible segmentation errors. Nuclear and cytoplasmic features such as volume, intensity, shape and signal level were output for subsequent analysis. The lowest individual fluorescence levels were used to normalize across coverslips within each experiment and analyzed in Matlab using the Savitzky-Golay function (38,39) and the curve-fitting toolbox.

Quantitative real-time PCR (qPCR). For qPCR experiments, cells were seeded in 12-well plates at 750,000 cells per well. The medium
was replaced 24 h later with DMEM containing 25 mM HEPES (Mediatech), 10% CT-FBS (HyClone Laboratories, Inc., Logan, UT), and glutamine. On the next day, the cells were treated with the indicated concentrations of GnRH or vehicle and were returned to the CO2 incubator for the indicated time of incubation (40 minutes), at which point the medium was replaced with 360μl lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% N-lauroyl-sarcosine, and 0.1 M 2-mercaptoethanol). RNA was isolated according to the method of Chomczynski and Sacchi (40). Total RNA was isolated with the StrataPrep96 kit (Stratagene, La Jolla, CA). After reverse-transcription of 0.5μg of RNA, the samples were diluted 1:20 in dH2O. Later, SYBR green qPCR assays were performed (40 cycles) using 5μl of cDNA template and 5μl of master mix containing the specific primers for the targeted gene and the required qPCR buffers. The results were exported as CT values for subsequent analysis. From the six-replicates of each condition, the mean, standard deviation and fold-changes to vehicle treatment were estimated and normalized to β-actin. The relative copy number of cDNA per assay was determined by running a standard curve with the PCR product for the specific gene.

Immunoblot analysis. 5 million LβT2 cells grown in 10mm dishes were synchronized in low-serum for 24h before GnRH treatment as described. After NP-40 lysis (20mM Tris-HCl, 1% NP-40, NaCl) and centrifugation, 20 μg/well supernatant were loaded onto 10% Tris-HCl ready gel (Biorad) and electrophoresed 1.5h at 100V. After transfer to H-Bond membrane (Hybond-TM ECL (Amersham, Buckinghamshire, UK), blocking for 1h with 5% non-fat dry milk (Bio-Rad) in TBST (Tris-buffered saline and 1% Tween-20) was followed by overnight incubation in primary antibody (1:000) at 4 degrees C. Incubation with the secondary antibody (1:5000) coupled to peroxidase (Santa Cruz Biotechnology) was performed at room temperature for 45 minutes, followed by repeated washings with TBST. Immunoreactive proteins were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. In each case, the blots were stripped and reprobed for the total protein or a control protein to control loading amounts. All immunoblots were quantified by densitometry.

ELISA assay. pERK and total ERK assay kits (Biosource) were used according to manufacturer’s instructions. Briefly, the LβT2 cells were grown in 6-well plates at 2.5 million cells/well and synchronized in low serum for 24h. After treatment cells were washed, lysed in cell extraction buffer (Biosource), boiled and diluted (1:10) into assay buffer. Each sample was divided into two wells for assay of total and phophoERK.

Fluorescent assay sensitivity determination. 1.8μg/μl alexa-568nm (Molecular Probes) was serially diluted 1:2 in DMSO. A GSM-417 microarray printer (Affymetrix) was used to spot dilution replicates on amino-silane coated slides (Corning). The array was imaged using the epifluorescent microscope and analyzed as described above. For testing assay sensitivity in cells, fixed gonadotropes were incubated in serial diluted solutions of DAPI for 5 minutes. The coverslips were imaged and analyzed as described above.

Mathematical modeling. Statistical modeling used parameters obtained by fitting experimental data with two Gaussian curves using the curve-fitting toolbox of Matlab 7.0 (Mathworks) and the simulations were programmed in R. Stochastic modeling was coded in Fortran using Gillespie’s algorithm (41, 42). Network simulations using dynamic modeling was programmed in Matlab. Full descriptions of the modeling assumptions and equations are found in the Supplementary Information.

RESULTS

Average gonadotrope responses to increasing concentrations of GnRH showed graded increases in the activation of ERK and the induction of egr1 mRNA and Egr1 protein. We first characterized the average gonadotrope responses to GnRH in biochemical assays of large populations of LβT2 gonadotrope cells. In order to eliminate response variability arising
from differences in cell cycle, cells were synchronized by low serum. Cell cycle synchronization was confirmed using p21 immunohistochemistry and cell viability by trypan blue exclusion and a two-color live/dead membrane integrity and esterase activity assay (Supplementary Fig. 1). Immunoblot analysis for active, doubly phosphorylated ERK (pERK) in synchronized gonadotropes exposed to GnRH showed that the activated form reached maximal levels within the first few minutes and remained stable for about 40 minutes (Fig. 1A). Egr-1 protein was first detected after 30 minutes and continued to accumulate for the first 60 minutes after GnRH exposure. The induction of Egr1 protein paralleled the time course previously reported for the induction of egr1 mRNA (15). In order to select the optimum time-point to assay ERK, we determined the response to varying concentrations of GnRH over time and found that the level of pERK was stable from 5 to 30 minutes at all concentrations (Supplementary Fig. 2).

GnRH was found to induce a concentration-dependent increase in the average gonadotrope levels of pMEK (MK2), pERK, egr1 mRNA and Egr1 protein (Fig. 1B, C). The average responses determined by biochemical assays of large populations of cells could represent any of three different single-cell mechanisms: a graded (analog) model in which all cells show a similar concentration, a switch-like (digital) mechanism in which individual cells show an all-or-none response, or a hybrid analog/digital mechanism which combines features of both the graded and binary model (Fig. 1D). These mechanisms, which are all consistent with the biochemical results obtained, can only be distinguished by single cell response assays.

The activation of ERK involves two separate phosphorylation steps and the levels of pERK depend on the effects of both kinase and phosphatase. Several mathematical models of the MAPK cascade suggest the theoretical importance of reversibility and product inhibition (43-45). Bistability and hysteresis can occur for some ranges of values of the rate constants (45). Hysteresis has been identified in cell-cycle transitions in Xenopus oocytes (46) and in bacterial metabolism (47). Because determining the presence of hysteresis is important for elucidating the underlying signaling mechanism, we investigated the gonadotrope response for hysteresis, which has not been tested experimentally in mammalian signaling networks.

Hysteresis is dependent on the history of the cells, in that the response of the LβT2 cells to a second concentration of GnRH might depend on the initial concentration of GnRH to which the cells were exposed. If the MAPK cascade showed bistability and hysteresis, then the average levels of pERK would be expected to diverge for cells exposed to increasing versus decreasing concentrations of GnRH. We studied whether hysteresis was observed in the activation of ERK by GnRH. We first determined that the level of pERK following a 10-minute pulse of 100 nM GnRH returned to baseline after 30-45 minutes (Fig. 2A). The levels of pERK were stable for 40 minutes with continuous GnRH (Fig. 1A). Therefore we performed experiments in which LβT2 cells were pre-treated with either 0.1 or 100nM GnRH for 10 minutes before exposure to a different concentration of GnRH. The results of these experiments demonstrated identical concentration-response curves with a Hill-coefficient near unity, whether starting at low or high GnRH concentrations (Fig. 2B-D). Thus we did not observe bistability or hysteresis in this response. Moreover, the observation of a low Hill value indicated that the average response was not ultrasensitive in these cells.

Responses in single cells following GnRH stimulation identified a hybrid activation model- In order to determine the type of single cell response pattern occurring with varying concentration of GnRH, we quantified the activation of ERK and the induction of egr1 mRNA and Egr1 protein in single cells. To facilitate high throughput image analysis and eliminate subjectivity, we automated cell compartment segmentation using an enhanced 3D watershed algorithm and model-based object merging, and subsequent signal quantification (Supplementary Fig. 3; (32)). We validated the automated analysis system by comparing results obtained for the same experimental data using...
manual and automated methods, which showed a high correlation (Supplementary Fig. 4A). Using analysis of slides printed with varying fluorophore concentrations and cells labeled with different concentrations of a nuclear stain, we determined that the protocols utilized gave reliable quantification of signal intensity (Supplementary Fig. 4B, C).

Histological assays for doubly phosphorylated ERK identified two types of cellular responses: an uninduced population with cells having a level of fluorescence comparable to unstimulated cells, and an induced population of cells with increased levels of fluorescence (Fig. 3A, B). The number of pERK positive cells gradually increased as the concentration of GnRH was increased (Fig. 3C).

We also studied the induction of egr1 mRNA and the production of Egr1 protein at the single cell level using fluorescent in situ hybridization (FISH, Fig. 4) and immunohistochemistry (Supplementary Fig. 6). Specificity controls for FISH included competition by unlabeled probe, the use of a scrambled labeled probe, and RNAse pretreatment, all of which eliminated detectable signals (Supplementary Fig. 5). Immunostaining specificity controls included the use of a blocking peptide and inhibition of Egr1 induction by an ERK pathway inhibitor (Supplementary Fig. 5). Our results showed that the single cell pattern of induction of egr1 mRNA (Fig. 4) and Egr1 protein (Supplementary Fig. 6) with varying concentration of GnRH paralleled that observed for pERK. An increasing proportion of cells expressing elevated levels of Egr1 expression were detected with increasing concentrations of GnRH.

Single cell signal quantification showed that in addition to the increasing percentage of cells expressing elevated pERK with increasing concentrations of GnRH, the average response of activated cells also increased (Fig. 5A and Supplementary Table 1). Single cell responses showed large cell-to-cell variation, especially in the induced population (Fig. 5A). Similar hybrid response distributions were obtained for egr1 mRNA and Egr1 protein (Supplementary Fig. 7). We parametrized the pERK response distributions using an empirical statistical model, representing a hybrid analog/digital model in which the cells transition from an inactive to an active state with increasing concentrations and the average level of response in the active state also showed a graded increase with increasing concentrations (See Fig. 1D and Supplementary Information). Simulation with this model showed a reasonable correspondence with both average responses observed (data not shown) and with the single cell response distributions obtained experimentally (Fig. 5B).

**Mathematical modeling of single cell responses to GnRH** - We next developed a dynamic mechanistic model for this signaling pathway. In developing this model, we attempted to understand the basis for the graded average cell response to increasing GnRH, showing a Hill coefficient near unity, the hybrid single cell response distributions, and the highly variable pERK levels found in single activated cells. Stochastic and dynamic models based solely on the ERK cascade were unable to explain both the gradual average single cell responses shown in Fig. 2 and the switching from inactive to active states characteristic of the mixed analog/digital single cell response data shown in Fig. 5 (Supplementary Fig. 8). In view of the known structure of the gonadotrope signaling network in which several parallel kinase cascades are activated downstream of the GnRH receptor (13), we tested the hypothesis that the average and single cell pERK response distributions observed resulted indirectly from the effects of intercellular variation in signaling components and the overall dynamics of the network.

The ERK cascade consists of three sequential kinases, MK3, MEK and ERK and associated phosphatases. MK3 is likely activated by an additional upstream signaling component, represented by MK4 in our model, whose identity in the gonadotrope is not yet known (Fig. 6A). The overall responses to GnRH receptor activation include induction of multiple parallel kinase cascades including ERK, JNK and p38MAPK (13). Consonant with these experimental observations of the signaling network in the gonadotrope, we included the proximal member of a second kinase cascade downstream of MK4, represented by S (Fig. 6A). One potential mechanism for
switch-like behavior is zero-order ultrasensitivity resulting from enzyme saturation, originally described by Goldbeter and Koshland (48). Our experimental data showed that ultrasensitivity was not present in the dynamics of the ERK pathway itself. Therefore, we tested for the possibility that zero-order ultrasensitivity in the S pathway parallel to ERK could lead indirectly to thresholding the ERK pathway response and generate the hybrid pERK response pattern observed with increasing GnRH levels.

The simulation of this network model approximated the experimental results both at the single cell and average cell levels (Fig 6 B, C). Under conditions showing intercellular concentration variations and zero order-kinetics in this parallel pathway, S conversion into Sp showed switch-like behavior, providing an indirect threshold for the ERK pathway (Supplementary Fig. 9). Below the dynamic threshold, MK4* was trapped in an MK4*-S intermediate and only above the threshold concentration did the downstream ERK pathway elements begin to be activated. Even though S had no direct feedback connections with components of the ERK pathway in this model, when the single cell concentrations of all components were selected from Gaussian distributions, S provided an indirect thresholding of the ERK response via the network dynamics. Thus this model can explain the hybrid behavior with a response pattern that approximates results obtained experimentally. This model also suggests a mechanism for crosstalk where other signaling inputs to the gonadotrope can affect thresholding of the GnRH receptor and GnRH response sensitivity (see Discussion).

**DISCUSSION**

As the timing of reproduction is important in the survival of a species, the brain-pituitary-gonadal mechanisms controlling reproductive competency are assumed to be under tight evolutionary constraints. Our study of the single pituitary gonadotrope responses to varying concentrations of the hypothalamic hormone GnRH provides insight into the engineering principles involved in the design of this locus of the regulatory system. We determined the single cell levels of activation of ERK and the induction of egr1 mRNA and Egr1 protein, which is required for reproduction, with increasing concentrations of GnRH. We found that nuclear phosphorylated ERK as well as egr1 mRNA and protein induction by increasing concentrations of GnRH in the gonadotrope showed a hybrid activation pattern, combining a digital response threshold and an analog graded single cell response.

At the population level, we found a gradual response to increasing levels of GnRH with a Hill coefficient close to unity and an absence of ultrasensitivity or hysteresis. The single cell response data showed that the cells distributed among responding cells and cells that remained at baseline, with the probability of activation and the mean response of activated cells increasing as the concentration of GnRH increased. Recent studies have investigated the patterns of single mammalian cell ERK activation and gene induction responses to graded stimuli and the results have been interpreted as consistent with analog, digital and mixed responses, as reported in various experimental systems (28,29,31,49,50). The automated histological approach utilized in the present study improves the accuracy of single cell response experiments by facilitating large sample size studies (Supplementary Fig. 4), which were needed to detect the underlying hybrid activation.

The signaling mechanisms underlying differences in the average cell and single cell responses have not been well studied. The single cell and average cell responses obtained in the gonadotrope could not be simulated using previously reported models of MAPK signaling. Various models of the phosphorylation and dephosphorylation of MAPK signaling cascades have been explored (27,45,51,52). Explicit modeling of the distributed two-step Tyr and Thr phosphorylation that occurs at each kinase in the cascade leads to a steep concentration-response curve that is not applicable to our experimental results (45,51), Supplementary Fig. 8). Ferrell and Machleder modeled experimental results showing switch-like single cell and graded cell population responses (27). In contrast to the gonadotrope responses which occur within
minutes, Ferrell and Machleder studied Xenopus oocyte responses to progesterone that occurred after many hours of exposure and may have involved biosynthetic regulatory loops. The divergence of single cell and population responses in their model depends on postulating a unique single cell distribution of rate constants (27) and does not explain the hybrid responses that we obtained in the gonadotrope.

Our model suggests that a parallel pathway indirectly gates the ERK cascade. The GnRH receptor leads to activation of three MAP kinase cascades in addition to ERK: JNK, p38 MAP kinase and BMK. The model that best simulated the experimental data, which included a second pathway gating the ERK pathway via zero-order ultrasensitivity, provided an interesting mechanism for crosstalk among different extracellular inputs to the gonadotrope. The exact identity of the MK4 substrate in the parallel pathway is not known. However potential candidates include proteins containing MARCKS sites, such as Diacylglycerol kinase zeta, other kinases of the GnRH signaling network, such as kinases of the JNK pathway, or scaffold proteins involved in ERK regulation and regulated by upstream kinase activation, such as Phosphoprotein-Enriched in Astrocytes 15 (PEA-15).

In coordinating reproduction, the gonadotrope integrates a variety of brain, paracrine and endocrine signals that are potentially relevant to optimizing the timing of reproduction. Other extracellular inputs differ in their regulation of various MAPK cascades. For example, activation of insulin or IGF-I receptors in the gonadotrope by insulin does not regulate ERK but does activate the kinase p70S6K, which is also activated by GnRH (M. Fink and S. Sealfon, unpublished data). If p70S6K served as the gating pathway, this would provide a mechanism for other inputs, such as insulin levels as a reflection of nutritional status, to modulate the gating and sensitivity of the GnRH regulated ERK-egr1 pathway driving reproduction. A fundamental question of signal transduction is how specificity is achieved with so many more receptor types than signaling pathways. While the precise network mechanisms involved in ERK gating require further experimentation, the model we have developed suggests a novel network-based signaling crosstalk mechanism that functions in the absence of direct feedback loops.

The use of automated segmentation and signal quantitation facilitated performing many replicated experiments in a large number of cells. This experimental approach was necessary to resolve the hybrid response pattern in the presence of large levels of cell-to-cell variation. Synchronizing the cells and eliminating cell cycle variation did not reduce the levels of extrinsic noise in these experiments. The sources of these high levels of variability most likely resulted from intercellular variations in the levels of rate-determining signaling components. PKC isoform expression varies among primary gonadotropes (53). In transfected cells, GnRH receptor number influences pERK nuclear translocation efficiency (54). However in LβT2 cells, we find much less cell-to-cell variation in GnRH receptor mRNA as determined by FISH (data not shown) in comparison with the downstream responses assayed, which suggests that intercellular variation in receptor number may not be a major contributor to extrinsic response differences. The specific causes of the intercellular response variations have not yet been identified and may have multiple origins including differences in cell geometry and differences in signaling protein concentrations.

It is interesting to speculate why this hybrid response mechanism in the gonadotrope has evolved. The GnRH signaling system is unusual in being largely temporally encoded. GnRH is released by the brain in short ~hourly pulses and the pulse frequency is important in determining the biosynthetic responses (4,5,55-57). Frequency-encoding requires the gonadotrope to process each individual pulse. However, the levels of GnRH secreted by the hypothalamus are highly variable (3,7-12). If a successful signaling event requires activation of a certain number of gonadotropes above a certain threshold level, then a hybrid response mechanism would decrease the rate of signaling failure from the brain to pituitary in the presence of the large variations in GnRH concentration that are observed experimentally. Simulation of the three types of response mechanisms (analog, digital, hybrid) indicated that the hybrid design
showed much lower failure rate with varying GnRH concentrations (see Supplementary Information).

This study addressed the activation of ERK following GnRH stimulation in LβT2 gonadotropes. It is not known if other MAPKs, such as p38 or JNK would also exhibit a mixed analog/digital response to graded GnRH increases, or whether the hybrid response observed for ERK is specific for gonadotropes. Determining the distribution and mechanisms of single-cell responses in complex mammalian systems requires large sample sizes. The use of high-throughput experiments and automated image analysis systems may help resolve the principles underlying mammalian signal transduction.

The signal-decoding systems of the gonadotrope must function reliably despite potentially high levels of cell-to-cell variation. The hybrid digital and analog response mechanism to varying GnRH stimuli appears to be designed to facilitate integration of multiple inputs to the gonadotrope and to optimize the detection of biosynthetic signals at this key reproductive locus.
REFERENCES

1. Moenter, S. M., DeFazio, A. R., Pitts, G. R., and Nunemaker, C. S. (2003) Front Neuroendocrinol 24(2), 79-93
2. Clarke, I. J. (2002) Reprod Suppl 59, 1-13
3. Clarke, I. J., and Pompolo, S. (2005) Anim Reprod Sci 88(1-2), 29-55
4. Haisenleder, D. J., Dalkin, A. C., and Marshall, J. C. (1994) Regulation of gonadotropin gene expression. Raven Press, New York
5. Haisenleder, D. J., Dalkin, A. C., Ortolano, G. A., Marshall, J. C., and Shupnik, M. A. (1991) Endocrinology 128(1), 509-517
6. Pawson, A. J., and McNeilly, A. S. (2005) Anim Reprod Sci 88(1-2), 75-94
7. Clarke, I. J. (1995) J Endocrinol 145(2), 71-272
8. Clarke, I. J., and Cummins, J. T. (1982) Endocrinology 111(5), 1737-1739
9. Moenter, S. M., Caraty, A., Locatelli, A., and Karsch, F. J. (1991) Endocrinology 129(3), 1175-1182
10. Padmanabhan, V., McFadden, K., Mauger, D. T., Karsch, F. J., and Midgley, A. R., Jr. (1997) Endocrinology 138(1), 424-432
11. Skinner, D. C., Caraty, A., Malpaux, B., and Evans, N. P. (1997) Endocrinology 138(11), 4699-4704
12. Tanaka, T., Ohgata, N., Kamomae, H., Takeuchi, Y., Mori, Y., and Karsch, F. J. (1997) Journal of Reproduction and Development 43(1), 101-106
13. Ruf, F., Fink, M. Y., and Sealfon, S. C. (2003) Front Neuroendocrinol 24(3), 181-199
14. Wurmbach, E., Yuen, T., Ebersole, B. J., and Sealfon, S. C. (2001) J Biol Chem 276(50), 47195-47201
15. Yuen, T., Wurmbach, E., Ebersole, B. J., Ruf, F., Pfeffer, R. L., and Sealfon, S. C. (2002) Mol Endocrinol 16(6), 1145-1153
16. Ruf, F., and Sealfon, S. C. (2004) Trends Endocrinol Metab 15(7), 331-338
17. Kaiser, U. B., Halvorson, L. M., and Chen, M. T. (2000) Mol Endocrinol 14(8), 1235-1245
18. Duan, W. R., Ito, M., Park, Y., Maizels, E. T., Hunzicker-Dunn, M., and Jameson, J. L. (2002) Mol Endocrinol 16(2), 221-233
19. Dorn, C., Ou, Q., Swirnoff, J., Crawford, P. A., and Sadowsky, Y. (1999) J Biol Chem 274(20), 13870-13876
20. Wolfe, M. W., and Call, G. B. (1999) Mol Endocrinol 13(5), 752-763
21. Halvorson, L. M., Kaiser, U. B., and Chin, W. W. (1999) Mol Endocrinol 13(1), 106-116
22. Tremblay, J. J., and Drouin, J. (1999) Mol Cell Biol 19(4), 2567-2576
23. Lee, S. L., Sadowsky, Y., Swirnoff, A. H., Polish, J. A., Goda, P., Gavrilina, G., and Milbrandt, J. (1996) Science 273(5279), 1219-1221
24. Topilko, P., Levi, G., Merlo, G., Mantero, S., Desmarquet, C., Mancardi, G., and Charnay, P. (1997) J Neurosci Res 50(5), 702-712
25. Navratil, A. M., Bliss, S. P., Berghorn, K. A., Haughian, J. M., Farmerie, T. A., Graham, J. K., Clay, C. M., and Roberson, M. S. (2003) J Biol Chem 278(34), 31593-31602
26. Fink, M. Y., Wurmbach, E., Ebersole, B. J., Yuen, T., and Sealfon, S. (2003) Connectivity of the GnRH Activated Signal Transduction Pathways and Early Gene Program. In. American Society for Cell Biology, Annual Meeting, San Francisco, CA
27. Ferrell, J. E., Jr., and Machleder, E. M. (1998) Science 280(5365), 895-898
28. Whitehurst, A., Cobb, M. H., and White, M. A. (2004) Mol Cell Biol 24(23), 10145-10150
29. Mackeigan, J. P., Murphy, L. O., Dimitri, C. A., and Blenis, J. (2005) Mol Cell Biol 25(11), 4676-4682
30. Nair, V. D., Yuen, T., Olanow, C. W., and Sealfon, S. C. (2004) J Biol Chem 279(26), 27494-27501
31. French, C. T., Hanneman, W. H., Chubb, L. S., Billings, R. E., and Andersen, M. E. (2004) *Toxicol Sci* **78**(2), 276-286
32. Chawla, M. K., Lin, G., Olson, K., Vazdarjanova, A., Burke, S. N., McNaughton, B. L., Worley, P. F., Guzowski, J. F., Roysam, B., and Barnes, C. A. (2004) *J Neurosci Methods* **139**(1), 13-24
33. Chan, P., Gonzalez-Maeso, J., Ruf, F., Bishop, D. F., Hof, P. R., and Sealfon, S. C. (2005) *J Comp Neurol* **482**(1), 50-73
34. Chan, P., Yuen, T., Ruf, F., Gonzalez-Maeso, J., and Sealfon, S. C. (2005) *Nucleic Acids Res* **33**(18), e161
35. Lin, G., Bjornsson, C. S., Smith, K. L., Abdul-Karim, M. A., Turner, J. N., Shain, W., and Roysam, B. (2005) *Cytometry A* **66**(1), 9-23
36. Lin, G., Adiga, U., Olson, K., Guzowski, J. F., Barnes, C. A., and Roysam, B. (2003) *Cytometry* **56A**(1), 23-36
37. Lin, G., Chawla, M. K., Olson, K., Guzowski, J. F., Barnes, C. A., and Roysam, B. (2004) *Cytometry* **63A**(1), 20-33
38. Savitzky, A. a. G. M. (1964) *Analytical Chemistry* **36**(8), 1627-1639
39. Madden, H. (1978) *Analytical Chemistry* **50**(9), 1383-1386
40. Chomczynski, P., and Sacchi, N. (1987) *Anal Biochem* **162**(1), 156-159
41. Gillespie, D. T. (1977) *Journal of Physical Chemistry* **81**, 2340-2361
42. Gillespie, D. T. (2001) *Journal of Chemical Physics* **115**, 1716-1733
43. Cornish-Bowden, A., and Cardenas, M. L. (2001) *Eur J Biochem* **268**(24), 6616-6624
44. Ortega, F., Acerenza, L., Westerhoff, H. V., Mas, F., and Cascante, M. (2002) *Proc Natl Acad Sci U S A* **99**(3), 1170-1175
45. Markevich, N. I., Hoek, J. B., and Kholodenko, B. N. (2004) *J Cell Biol* **164**(3), 353-359
46. Sha, W., Moore, J., Chen, K., Lassaletta, A. D., Yi, C. S., Tyson, J. J., and Sible, J. C. (2003) *Proc Natl Acad Sci U S A* **100**(3), 975-980
47. Ozbudak, E. M., Thattai, M., Lim, H. N., Shraiman, B. I., and Van Oudenaarden, A. (2004) *Nature* **427**(6976), 737-740
48. Goldbeter, A., and Koshland, D. E., Jr. (1981) *Proc Natl Acad Sci U S A* **78**(11), 6840-6844
49. Broccardo, C. J., Billings, R. E., Chubb, L. S., Andersen, M. E., and Hanneman, W. H. (2004) *Toxicol Sci* **78**(2), 287-294
50. Harding, A., Tian, T., Westbury, E., Frische, E., and Hancock, J. F. (2005) *Curr Biol* **15**(9), 869-873
51. Gunawardena, J. (2005) *Proc Natl Acad Sci U S A* **102**(41), 14617-14622
52. Samoilov, M., Plyasunov, S., and Arkin, A. P. (2005) *Proc Natl Acad Sci U S A* **102**(7), 2310-2315
53. Garcia-Navarro, S., Kalina, M., and Naor, Z. (1991) *Endocrinology* **129**(5), 2780-2786
54. Caunt, C. J., Finch, A. R., Sedgley, K. R., Oakley, L., Luttrell, L. M., and McArdle, C. A. (2006) *J Biol Chem* **281**(5), 2701-2710
55. Krakauer, D. C., Page, K. M., and Sealfon, S. (2002) *J Theor Biol* **218**(4), 457-470
56. Burger, L. L., Dalkin, A. C., Aylor, K. W., Haisenleder, D. J., and Marshall, J. C. (2002) *Endocrinology* **143**(9), 3243-3249
57. Burger, L. L., Haisenleder, D. J., Dalkin, A. C., and Marshall, J. C. (2004) *J Mol Endocrinol* **33**(3), 559-584

**FOOTNOTES**

* We appreciate the help of Tony Yuen for microarray printing, PokMan Chan for microscopy, Ciriyam Jayaprakash and Marc Fink for discussions and critical reading of the manuscript, Pamela Mellon for
providing the LβT2 cells, Tearina Chu for assistance with array printing, and the Mount Sinai Microarray, Real Time PCR, and Microscopy Shared Research Facilities for instrumentation and assistance. This research was supported by NIH Grant DK46943.

FIGURE LEGENDS

**Fig. 1**: Time course and concentration-dependence of average GnRH-induced responses in gonadotrope cells. A. Time course study of ERK phosphorylation (top) and Egr-1 protein (bottom) detection by immunoblot analysis. Right panel shows quantification by densitometry of Egr1 normalized to actin, and pERK normalized to total ERK. B. GnRH concentration-dependence assessed by immunoblots for the activated forms of MEK, ERK and for Egr1. Right panel shows immunoblot quantification. C. egr-1 mRNA levels (normalized to β-actin mRNA) with GnRH stimulation measured by real-time PCR. D. Three possible single cell response models consistent with the signaling and gene induction data shown in panels A-C. Top: graded (analog) single-cell response pattern with similar responses in all cells. Middle: binary (digital, switch-like) single cell response with all-or-none responses. Bottom: hybrid digital/analog model with both switching to an active state and varying levels of activation seen with increasing GnRH levels.

**Fig. 2**: Quantification of average pERK concentration-response and test of hysteresis. A. Determination of the time to return to baseline following a GnRH pulse. LβT2 cells were first exposed to 0.1nM (white) or 100nM (gray) GnRH for 10 minutes followed by 0.1nM GnRH for the time indicated. B. Cells exposed first to 0.1 nM GnRH then to the final concentration. C. Cell exposed first to 100 nM GnRH, then to the final concentration. D. Overlay of panels B and C. Both curves had slopes (Hill coefficients) of 1.0 in this experiment and 1.2 in an independent experiment.

**Fig. 3**: Single-cell ERK phosphorylation with varying GnRH concentrations. A. pERK immunohistochemistry (red) of representative non-overlapping fields of cells exposed to the GnRH concentrations indicated. DAPI for nuclear identification is shown in blue. B. High magnification images. All scale bars = 20 μm. C. Automated quantification of cells showing elevated pERK signal. Error bars represent s.e.m. The results shown were obtained in 10 independent experiments each including at least 100 cells at each GnRH concentration (See Supplementary table 1).

**Fig. 4**: Single-cell GnRH induction of egr-1 mRNA. A. egr-1 mRNA assayed by FISH (red) in cells exposed to the GnRH concentrations indicated assayed for egr-1 mRNA (red) detected by FISH. DAPI nuclear staining is blue. B. β-actin mRNA FISH control assay. All scale bars = 20 μm. C. Automated quantification of cells showing induction of egr1 mRNA. Error bars indicate s.e.m. The results plotted were obtained in 7 independent experiments each including at least 50 cells at each GnRH concentration (See Supplementary table 1).

**Fig. 5**: Distributions of single-cell pERK responses and statistical model simulations. A. Distribution plots of single-cell pERK levels from a representative experiment are shown for each GnRH concentration. B. Simulation of experimental results using an empirical hybrid statistical model. Fitting of both experimental and simulated data with two Gaussian distributions are shown.

**Fig. 6**: Dynamic model for hybrid response single-cell responses. A. Schematic of the network modeled. MK4 is the upstream signaling component activating both the ERK pathway and a parallel kinase pathway, the proximal member of which is represented by S. B. pERK distributions obtained by simulation of individual cells exposed to varying concentrations of GnRH. C. Comparison of the average
pERK responses obtained in two experiments and model simulation. * indicates active form, p indicates phosphorylated form, Z represents specific inactivating enzymes or phosphatases, as indicated.
Figure 1

A. 

B. 

C. 

D. 

Figure 1
Figure 6
Mixed analog/digital gonadotrope biosynthetic response to gonadotropin releasing hormone

Frederique Ruf, Myung-June Park, Fernand Hayot, Gang Lin, Badrinath Roysam, Yongchao Ge and Stuart C. Sealfon

J. Biol. Chem. published online August 17, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M606486200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2006/08/18/M606486200.DC1