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Optimized amplification and single-cell analysis identify GnRH-mediated activation of Rap1b in primary rat gonadotropes

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Abstract

Identifying the early gene program induced by GnRH would help understand how GnRH-activated signaling pathways modulate gonadotrope secretory response. We previously analyzed GnRH-induced early genes in L β T2 cells, however these lack GnRH self-potential, a physiological attribute of gonadotropes. To minimize cellular heterogeneity, rat primary pituitary cultures were enriched for gonadotropes by 40–60% using a sedimentation gradient. Given the limited number of gonadotropes, RNA was amplified prior to microarray analysis. Thirty-three genes were up-regulated 40 minutes after GnRH stimulation. Real-time PCR confirmed regulation of several transcripts including *fosB*, *c-fos*, *egr-2* and *rap1b*, a small GTPase and member of the Ras family. GnRH stimulated *rap1b* gene expression in gonadotropes, measured by a sensitive single cell assay. Immunocytochemistry revealed increased Rap1 protein in GnRH-stimulated gonadotropes. These data establish *rap1b* as a novel gene rapidly induced by GnRH and a candidate to modulate gonadotropin secretion in rat gonadotropes.

Keywords

GnRH; Rap1b; rat primary pituitary cultures; gonadotrope enrichment; early gene; single-cell gene expression

1. Introduction

GnRH plays a key role in the control of mammalian reproductive function. Upon its release from the hypothalamus, the neuropeptide interacts with high-affinity receptors (GnRH receptor) at the surface of the pituitary gonadotrope cells, thereby stimulating the synthesis and secretion of the gonadotropins LH and FSH, which in turn promote gametogenesis and sex steroid production in the ovaries and testes. The biosynthetic and secretory responses to GnRH stimulation are controlled via the modulation of cell signaling pathways and transcription factor activity. A number of genes are induced by GnRH, and their identification would provide insight into the molecular bases of the gonadotrope response to GnRH receptor activation.

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Genomic studies using microarrays and high-throughput quantitative real-time PCR have allowed us and others to study the pattern of gene responses following GnRH receptor activation in the L β T2 gonadotrope cell line [1–5]. In particular, we were interested in the early gene program activated by GnRH in L β T2 cells. Hence, we previously identified 44 genes whose expression was increased within an hour of GnRH exposure, and another 31 up-regulated genes within 3–6 h of exposure ([3] and Ebersole, B. J., T. Yuen, E. Wurmbach, and S.C. Sealfon, unpublished data). Those genes were divided into three main categories: i) genes encoding inducible transcription factors, e.g. *egr2*, *c-fos*, *klf4*, which may be implicated in gonadotropin gene activation; ii) genes encoding modulators of signal transduction, such as *ikb*, *rgs2*, *mkp1*, which might contribute to feedback control of the GnRH-activated signaling cascade; iii) genes encoding cytoskeletal proteins. Furthermore, additional microarray analyses revealed that the amplitude of gene induction was dependent upon GnRH concentration. Most likely, the rates of transcription were influenced by the concentration of GnRH [5]. Another microarray study by Kakar *et al.* identified 149 up-regulated genes and 83 down-regulated genes following one-hour treatment with a GnRH agonist. Consonant with our previous results, the group reported *c-fos*, *egr1*, *Egr2*, *klf4*, and *lrg21* among the up-regulated genes, and observed that most of the genes which exhibited an elevated expression returned approximately to basal levels after 3 h of treatment [1]. More recently, the effects of various GnRH pulse regimens on gene expression were examined in L β T2 cells. Distinct patterns of gene expression were associated with each pulse frequency, with the greatest changes occurring at an hour or less interpulse interval; *Egr1* and *Egr2*, both of which may mediate GnRH induction of the LH β gene promoter, were induced at high pulse frequency, whereas *Egr* corepressors *Nab1* and *Nab2* were induced at low pulse frequency [2]. Importantly, *Egr-1*, in synergy with *SF-1*, was demonstrated to mediate GnRH induction of the LH β gene promoter both *in vitro* and *in vivo* [6–9].

Advances in our understanding of gene responses elicited by GnRH interaction with its receptor have been obtained using L β T2 cells. Consistent with the results obtained in *in vivo* studies, GnRH-stimulated L β T2 cells show a significant induction of LH protein secretion, LH β mRNA, as well as of LH β gene promoter activity [10–14]. However, these immortalized cells do not exhibit self-priming of LH secretory response to GnRH [13], in contrast with primary cultures of female rodent anterior pituitary cells [14–17]. GnRH self-priming or self-potential is a signal amplification device that manifests as enhanced gonadotropin secretion in response to a second, identical GnRH stimulation which is not dependent on changes in LH synthesis or the GnRH receptor [18]. GnRH priming is a critical event in the triggering of ovulation [19] and has been demonstrated *in vivo* in humans and *in vivo* and *in vitro* in rodent models [16,18–22]. Therefore, studying GnRH-activated gene program in primary cultures of anterior pituitary cells is important from a physiological standpoint. Such studies have been impeded due to the heterogeneity of the anterior pituitary cell population, with gonadotropes representing only 5 to 15% of the five secretory cell types contained in the pituitary gland (somatotropes, lactotropes, corticotropes, thyrotropes).

In the present study, we examined early genomic events following GnRH receptor activation in rat primary pituitary cell cultures enriched for gonadotropes. A duration of 40 min post-GnRH stimulation was chosen based on our previous studies establishing a window for the transcription-dependent action of GnRH in self-potential [15]. Because the number of gonadotropes in the anterior pituitary is very restricted, we opted to pre-amplify the RNA prior to conducting high-density oligonucleotide microarray analysis. Expression data were validated by quantitative real-time PCR.

2. Materials and Methods

2.1. Materials

Adult female Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) were maintained in controlled light conditions (12-h light, 12-h dark) for 2 weeks before use. Pituitary glands were removed after CO₂ narcosis and decapitation. The protocols employed in these experiments were reviewed and approved by the University of California Davis Institutional Animal Care and Use Committee. Media and sera for cell culture were purchased from Invitrogen-GIBCO (Carlsbad, CA). Trypsin, kanamycin sulfate, BSA fraction V, 17 β -estradiol (E₂), 8-bromo-cAMP, and GnRH were purchased from Sigma-Aldrich (St. Louis, MO). For immunofluorescence, normal goat serum and mounting medium were obtained from Vector Laboratories (Burlingame, CA), and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG were from Sigma-Aldrich; TOTO-3 iodide was from Invitrogen-GIBCO (Carlsbad, CA). Affinity purified rabbit polyclonal antibody against Rap1 was from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal antibody against Rap1 was from BD Biosciences (San Jose, CA). Monoclonal antibody 518B7 against bovine LH β was provided by Dr. Jan Roser (University California, Davis) [23]. Fura-2/AM and pluronic F-127 were from Invitrogen-Molecular Probes (Carlsbad, CA). Pipettes were pulled from Corning pyrex 7740 glass (Garner Glass, Claremont, CA) and coated with R6010 (K. R. Anderson, Santa Clara, CA).

2.2. Pituitary cell culture

Gonadotrope-enriched cultures—Anterior pituitaries (13–15/experiment) from rats ovariectomized for two weeks were enzymatically dispersed with trypsin and resuspended in Ca²⁺-free Minimum Essential Medium (MEM) containing 0.5% BSA and loaded into a unit gravity sedimentation chamber containing a gradient of 1.0–3.0% BSA in Ca²⁺-free MEM [24]. After equilibration, cells were collected from the bottom of the gradient in 20 \times 50 ml fractions. Fractions 17–20 contained large cells and were combined; this pool represented ~6% of the total cells recovered from the sedimentation chamber. As determined by subsequent immunocytochemistry (Fig. 1), LH-positive cells in this pool ranged from 40–60% compared to < 7% for unfractionated cells. The gonadotrope-enriched pool was pelleted and resuspended in supplemented MEM culture medium [MEM plus 200 μ M kanamycin sulfate, 0.2 nM E₂, and 10% fetal bovine serum (FBS) that had been charcoal-treated to remove endogenous steroids]. For RNA studies, cells from the gonadotrope-enriched pool were plated at 22.5 \times 10⁴ cells per 22-mm well. For immunocytochemical quantification of LH-positive cells, aliquots from the gonadotrope-enriched pool and a pre-fractionation sample were plated at 0.5 \times 10⁴ cells on 12-mm glass coverslips inserted into 22-mm wells. All cells were maintained in a humidified atmosphere (37°C in 5% CO₂). On day 2, medium was replenished (day of plating = day 0).

Heterogeneous pituitary cultures for Rap1 immunocytochemistry and single-cell studies—Each dispersion consisted of one anterior pituitary enzymatically dispersed with trypsin and prepared for culture [25]. For immunocytochemistry, cells were plated at 1.8 \times 10⁴ cells on 12-mm glass coverslips inserted into 22-mm wells. For single cell studies, plating was in 35-mm dishes incorporating a 22-mm glass-bottomed well (Ted Pella, Inc., Redding, CA) at 1.5 \times 10⁴ cells per well. Flooding with supplemented MEM culture medium and maintenance were as above.

2.3. Experimental protocols

RNA studies in gonadotrope-enriched cultures—On day 3 of culture, GnRH was added to experimental wells to achieve a final concentration of 10 nM; control wells

received an equivalent volume of vehicle medium. After 40 min, cells were rinsed with ice-cold PBS, lysis buffer containing guanidinium thiocyanate was added, cells were scraped, triturated, and stored at -70°C . Cell lysates were processed for total RNA using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA) and included an on-column DNase I digestion step.

Heterogeneous pituitary cell immunocytochemistry studies—On day 3 of culture, medium was replaced with control or 1 nM GnRH-containing medium. After 15 min, all media were replaced with control medium and incubation continued for an additional 45 min, following which cells were rinsed with PBS and fixed for immunocytochemistry. Additional GnRH-treated and control wells were rinsed and fixed at that end of the initial 15 min pulse.

Single gonadotrope studies—Cells were loaded with the Ca^{2+} indicator Fura-2 (fura-2/AM, 30 min, 37°C), and culture medium was replaced with an extracellular (EC) medium [26]. Dishes were transferred to an inverted microscope with a temperature regulated stage maintained at 22°C and perfused with EC medium using a peristaltic pump (at 1 ml/min). Once a putative gonadotrope was identified, a tight seal was obtained with a patch pipette; solution flow was switched from control EC medium to challenge EC medium using a valve placed close to the microscope stage. Challenge EC medium contained either 10 nM GnRH, 1 mM 8-bromo-cAMP, or control medium. The rise in $[\text{Ca}^{2+}]_i$, which occurs within a few seconds of GnRH contact, was recorded and used to confirm the cell as a gonadotrope; $[\text{Ca}^{2+}]_i$ was determined by Fura-2 radiometric fluorescence spectroscopy [26] using hardware and software from Photon Technology International (Birmingham, NJ). Groups: a GnRH pulse was administered for 15 min followed by control medium for 25 min, after which cell contents were harvested. In other groups, a pulse of either 8-bromo-cAMP or control medium was administered for 15 min, followed by control medium for 25 min, after which GnRH was administered for ~ 1 min and $[\text{Ca}^{2+}]_i$ increase recorded for gonadotrope confirmation before harvest. Only one cell per dish was harvested. For short term control group a 1 min pulse of GnRH was administered to a putative gonadotrope ~ 5 min after selection and $[\text{Ca}^{2+}]_i$ increase was recorded before harvest. For all groups, cell contents were harvested by aspiration into the patch pipette containing 10 μl reverse transcriptase (AccuRT) cocktail and expelled into a PCR tube. The reaction mixture was immediately incubated for 1 h at 60°C to generate cDNA, and stored at -70°C until determination of Rap1b expression level by quantitative real-time PCR.

2.4. Immunocytochemistry

Single label (LH)—On day 3, cultured cells from the gonadotrope-enriched studies were rinsed with PBS, fixed with pre-cooled methanol:acetone (1:1) for 7 min at -20°C , rinsed in PBS, and blocked in 5% normal goat serum/PBS. Cells were exposed to 1:500 anti-LH for 2 h at room temperature and stained with TRITC-conjugated goat antimouse IgG and 2.4 nM TOTO-3 nuclear DNA stain for 1 h.

Dual label (Rap1, LH)—Following control or GnRH stimulation, pituitary cells were rinsed in PBS, fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, treated with -20°C methanol, and blocked in 5% normal goat serum/PBS. Cells were exposed to 1:100 anti-Rap1 and 1:500 anti-LH overnight at 4°C . Two different Rap1 antibodies, a rabbit polyclonal and a mouse monoclonal antibody, were tested with similar results; the Rap1 polyclonal antibody was used for the results reported here. Both antibodies recognize Rap1b and Rap1a, which are 95% homologous proteins (for review, see [27]). Immunofluorescence staining with FITC- and TRITC-conjugated second antibodies was accomplished in successive incubations. As negative controls, either or both primary antibodies were omitted. To establish absence of overlap between the detection of FITC and

TRITC, single-labeled cells were imaged under similar conditions as dual-labeled cells to confirm proper signal isolation.

2.5. Confocal microscopy and image analysis

A Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) with 40x (gonadotrope-enriched cultures) or 63x (Rap1 immunofluorescence) oil-immersion lenses was used as previously described [28]. For Rap1 protein studies, 2 μm overlapping sections through the entire cell volume of identified gonadotropes were analyzed using the Object Counter3D plugin in ImageJ (<http://rsb.info.nih.gov/ij>). To quantify Rap1-associated punctate areas, threshold criteria for brightness and size for the analysis were established based on preliminary screening of the data: the software identified qualifying number of objects and determined their extent throughout the cell via optical slicing. Object volume and total volume/cell were determined and data expressed as the fold-change (GnRH-treated cells/vehicle-treated cells) for groups terminated at 15 min of treatment or at 60 min. Analysis was carried out on cells from 4 separate pituitary dispersions ($n = 4$); for each group, data represent 1–2 coverslips/dispersion with 2–5 cells analyzed/coverslip. Differences between two groups were analyzed with the *t* test using SigmaStat (Systat Software, San Jose, CA).

2.6. RNA pre-amplification and cDNA microarray analysis

Comparison of Amplification Protocols—The relative performance of different amplification approaches was first determined using RNA from the L β T2 gonadotrope cells in order to select the most reliable protocol to use for amplification of the rat gonadotrope cells.

A) RNA amplification using terminal continuation: L β T2 cells obtained from the Mellon laboratory (University of California, San Diego) were maintained at 37°C in 5% CO₂ in humidified air in DMEM (Mediatech, Herndon, VA) containing 10% fetal bovine serum (Gemini, Calabasas, CA) [13]. Approximately 2×10^7 cells were seeded in triplicate 100-mm dishes and medium was replaced 24 h later with DMEM containing 25 mM HEPES (Mediatech) and glutamine. On the next day, cells were treated with either 100 nM GnRH or vehicle and were re-incubated for 1 h. Total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA). The terminal continuation method [29] relies on the use of a terminal continuation (TC) primer containing a SP6 bacteriophage promoter sequence and a span of CTPs. It has been estimated that over 60% of all human genes are located near CpG islands [30]. While the mechanism of annealing of the TC primer to the 5' regions of mRNA was still under investigation, Che and Ginsberg (2004) proposed that the TC primers preferentially anneal within CpG islands [29]. Briefly, 100 ng RNA from each sample was reverse transcribed in a 20- μl volume containing 10 ng/ μl Oligo(dT) primer, 10 ng/ μl TC primer, 1X first strand buffer (Invitrogen, Carlsbad, CA), 1 mM dNTPs, 5 mM DTT, 20 U RNase inhibitor (Roche), and 5U Superscript II reverse transcriptase (Invitrogen). Reverse transcription was allowed to proceed for 2 h at 42°C. Second strand cDNA synthesis was performed in a 100- μl volume containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.5 U RNase H (Invitrogen), 5 U Platinum Taq DNA polymerase (Invitrogen), and 20 μl first-strand reaction mix. The reaction was allowed to proceed as follows: 10 min at 37°C, 3 min at 95°C, 3 min at 50°C, and 30 min at 72°C. Double-stranded cDNA products were purified using a QIAquick PCR purification kit (Qiagen). Sense RNA was synthesized by *in vitro* transcription using a MegaScript SP6 kit (Ambion, Austin, TX), according to the manufacturer's protocol. The quality of amplified products was assessed by quantitative real-time PCR after reverse transcription of sense RNA using Oligo(dT) primers.

B. RNA isothermal amplification using hybrid DNA-RNA primer: Isothermal amplification of RNA samples for microarray probe labeling was prepared using an Ovation Biotin RNA Amplification and Labeling System (NuGen, San Carlos, CA), following the manufacturer's recommendations. Briefly, 100 ng total RNA was incubated for 60 min at 48°C with 1 µl First Strand Primer, 1.75 µl First Strand Buffer, and 0.25 µl First Strand Enzyme in a 5-µl volume. Second strand cDNA synthesis was performed by incubating the 5 µl first strand cDNA products with 4.5 µl Second Strand Buffer and 0.5 µl Second Strand Enzyme Mix at 37°C for 30 min. RNA amplification and labeling were carried out by incubating the 10 µl second strand cDNA products with 18 µl SPIA Buffer Mix, 1 µl SPIA Primer Mix, 1 µl water, and 10 µl SPIA Enzyme Mix at 50°C for 60 min. Labeled probes were used to hybridize with 4 Murine Genome U74Av2 GeneChip microarrays (Affymetrix, Santa Clara, CA; two vehicle-treated and two GnRH-treated samples). Data analysis was performed using Microarray Suite 5.0 (Affymetrix).

C. RNA amplification using two-cycle T7 RNA polymerase: Two-cycle target labeling was performed according to the manufacturer's protocol (Affymetrix). Briefly, 100 ng total RNA was incubated in a 10-µl volume containing 2 µl T7-Oligo(dT) primer, 2 µl 5X first strand buffer, 1 µl 0.1 M DTT, 0.5 µl RNase inhibitor, 0.5 µl 10 mM dNTP, and 1 µl SuperScript II (Invitrogen) at 42 °C for 1 h. Second strand cDNA synthesis was carried out by incubating 10 µl first strand cDNA products with 4.8 µl water, 4 µl 17.5 mM MgCl₂, 0.4 µl 10 mM dNTP, 0.6 µl *E. coli* DNA Polymerase I, and 0.2 µl RNase H at 16°C for 2 h. *In vitro* transcription was performed using the MegaScript T7 Kit (Ambion). Second strand cDNA products (20 µl) were incubated with 5 µl 10X Reaction buffer, 5 µl each of ATP, CTP, UTP and GTP solutions, and 5 µl Enzyme Mix at 37°C for 16 h. The cRNA generated was purified using an RNeasy kit (Qiagen) and the manufacturer's RNA cleanup protocol. cRNA was subsequently subjected to a second cycle of T7 amplification. First strand cDNA synthesis was performed by incubating 9 µl cRNA with 2 µl 0.2 µg/µl Random Primer, 4 µl 5X first strand buffer, 2 µl 0.1M DTT, 1 µl RNase inhibitor, 1 µl 10 mM dNTP and 1 µl SuperScript II (Invitrogen) at 42°C for 1 h. First strand cDNA products were treated with 1 µl RNase H at 37°C for 20 min. Reaction was stopped by heating at 95°C for 5 min. Second strand cDNA synthesis was carried out by incubating the 21 µl first strand cDNA with 4 µl 5 µM T7-Oligo(dT) Primer, 88 µl RNase-free water, 30 µl 5X second strand buffer, 3 µl 10 mM dNTP, and 4 µl *E. coli* DNA Polymerase I at 16°C for 2 h. The reaction was allowed to proceed for another 10 min at 16°C after the addition of 2 µl T4 DNA Polymerase. Double-stranded cDNA products were then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's recommendations. cRNA generation and labeling were performed using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics). Labeled probes were used to hybridize with 4 Murine Genome U74Av2 GeneChip microarrays (Affymetrix; two vehicle-treated and two GnRH-treated samples). Data analysis was performed using Microarray Suite 5.0 (Affymetrix). Based on these results, the two-cycle amplification was used for the rat gonadotrope RNA samples.

Microarray experiments—A total of 12 Affymetrix Rat Expression Array 230 v2.0 (6 GnRH-treated and 6 vehicle-treated samples), each containing 31,000 gene clusters, were used. Data were analyzed using Affymetrix GeneChip Operating System (GCOS), with candidate regulated genes identified by data concordance across pairwise comparisons.

2.7. Quantitative real-time PCR

One hundred nanograms of total RNA were converted into cDNA and 1/100 utilized in a 40-cycle three-step PCR (95°C/15 sec, 55°C/20 sec, 72°C/30 sec) in 20 mM Tris pH 8.4, 50 mM KCl, 3 mM MgCl₂, 200 µM dNTPs, 0.5X SYBR Green I (Molecular Probes, Eugene, OR), 200 nM each primer, and 0.5 U Platinum Taq DNA polymerase (Invitrogen) using an ABI

Prism 7900 thermal cycler (Applied Biosystems, Foster City, CA). Amplicon size (typically between 100 and 200 bp) and reaction specificity were confirmed by agarose gel electrophoresis. Threshold cycle (Ct) values were determined using SDS software v2.3 (Applied Biosystems). Primer sequences were as follows: r-cjun/S, TGTCTGTATGCTGGGGTGAG; r-cjun/A, TTGCAACACCCTCTTCTTCC; r-cfos/F, CAGCTGCACTACCTATACGTC; r-cfos/R, GTAATGCACCAGCTCAGTCAG; r-fosB/S, CAGGGTCAACATCCGCTAAG; r-fosB/A, GGTGAGGACAAACGAGGAAG; r-egr1/S2, TGTTTCAGGGGAGGCTTTAG; r-egr1/A2, CAGTAGGTAACCGCAGCATT; r-egr2/S, CAGTGGTGTGCAAGTGTCC; r-egr2/A, CAGCTGCAGTGACGTTAAGC; r-egr3/S, CAGCGACCACCTCACTACTC; r-egr3/A, CCCCTTCTCCGACTTCTTC; r-Nr4a3/S, CGTGGTGAAGGTGGATAACACC; r-Nr4a3/A, GCTGCCTTAAAGGTCAGTGC; r-Rap1b/S, GTGGATGTGAATGTGGGAAG; r-Rap1b/A, ATACTGTGGCTCCCTGTTGG; r-GAPDH/S1, CATGGCCTCCAAGGAGTAAG; r-GAPDH/A1, AGATGGTGAAGGTCGGTGTG; r-actin/S1, AGGTGACAGCATTGCTTCTG; r-actin/A1, GCCTCAACACCTCAAACCAC.

2.8. Single-cell quantitative real-time PCR protocol

We adapted a hemi-nested PCR assay for single gonadotrope gene quantitation of Rap1b and Ppia (a reference house-keeping gene), respectively. The assay used two consecutive PCRs, the first with an outer primer set and the second with an inner primer set. Amplification linearity was maintained over a large number of cycles with low non-specific amplification of PCR by-products. Spike oligonucleotides were used to reduce nonspecific amplification by competing with abundant, low affinity DNA sequences for primer binding (random background amplifications), thus improving assay accuracy and detection limit. For the first PCR, single-cell cDNA was split in two aliquots for each gene: sample plus Master Mix 1 (PCR buffer, MgCl₂, dNTP, Platinum Taq DNA polymerase, gene-specific spike oligonucleotide and outer primer set) in a 10- μ l final volume were incubated in a thermocycler (95°C/2 min followed by 8 cycles of 95°C/15 sec, 55°C/20 sec, 72°C/30 sec). The second PCR was performed in a 7900 Prism RT-PCR machine (Applied Biosystems) using 5 μ l of a 1:10 dilution of the first PCR product plus Master Mix 2 (PCR buffer, MgCl₂, dNTP, Platinum Taq DNA polymerase, appropriate TaqMan probe and inner primer set) in a 10- μ l final volume (95°C/2 min and 40 cycles of 95°C/15 sec, 60°C/1 min). Copy numbers were estimated by interpolating Ct values against a standard curve generated using standard oligonucleotides. Assay control wells included non-template controls from PCR1 and 2 Master Mixes. Differences between groups were analyzed by one-way ANOVA followed by Student-Newman-Keuls for multiple comparisons, using SigmaStat. Primer sequences used in the first PCR were as follows: r-Rap1b/S2, TGCTTGAAATCCTGGATACTG; r-Rap1b/A, AGCCTTGTCCTTCTTCATGTAC; r-Ppia/S2, TCTTGTCCATGGCAAATGCT; r-Ppia/A, 5'-GCCATCCAGCCACTCAGTCT; r-Rap1b-spike, TGCTTGAAATCCTGGATACTGTCTAGAGAGTACCGACATTTGACGAGGCAAGGACGTACATGA AGAACGGACAAGGCT; r-Ppia-spike, TCTTGTCCATGGCAAATGCTACCGTCACGTCTATTTTTTGACCCTTGGTAAACACAAACCAGG AGACTGAGTGGCTGGATGGC. Primers used in the second PCR were: r-Rap1b/S, TGCAGGAACGGAGCAGTTT; r-Rap1b/A; r-Ppia/S, GCTGGACCAAACACAAATGGT; r-Ppia/A; r-Rap1b/probe, FAM-CAGCCATGAGAGATC-MGB; r-Ppia/probe, VIC-CCAGTTTTTATCTGCACTGC-MGB. Standard oligonucleotide sequences were as follows: r-Rap1b/std, TGCTTGAAATCCTGGATACTGCAGGAACGGAGCAGTTTACAGCCATGAGAGATCTGTACATGA AGAACGGACAAGGCT; r-Ppia/std, TCTTGTCCATGGCAAATGCTGGACCAAACAAATGGTTCCTCAGTTTTTATCTGCACTGCCA AGACTGAGTGGCTGGATGGC.

3. Results

3.1. Microarray analysis using gonadotrope-enriched primary pituitary cultures requires RNA amplification due to a limited number of cells

Because gonadotropes represent only 5–15% of anterior pituitary cells, a relatively small number of gonadotrope cells were purified from rat primary pituitary cultures. Therefore, it became crucial to reliably amplify the RNA in order to carry out high-throughput gene expression profiling. To this aim, the following three amplification methods were evaluated. While the goal of the present study is the regulation in primary rat gonadotropes, studies to test the protocols used RNA samples from L β T2 gonadotrope cells because they are an abundant and well-characterized source. The rat gonadotrope RNA is limited and therefore is not suitable for protocol development. Using 100 ng of total RNA isolated from the L β T2 gonadotrope cell line, we evaluated: terminal continuation (TC) RNA amplification [29], isothermal amplification using a hybrid DNA-RNA primer, and two-cycle T7 RNA polymerase amplification; these alternative amplification protocols were compared to a standard Affymetrix one-cycle T7 RNA polymerase amplification using 40 μ g of total RNA. The quality of amplified products was assessed by quantitative real-time PCR, by targeting both the 5' and 3' ends of a 1.2 kb mouse gapdh transcript and a 1.9 kb β -actin transcript. The comparison of 5'- and 3'- PCR results was used to determine the degree of 3' biasing of the amplification (the absence of biasing would give a ratio of unity). We compared the amplification approaches to identify which approach gave the maximal amplification yield with minimal 3' biasing. The TC RNA amplification method generated heavily 3' biased products, with 3'/5' ratios of 30 and 50 for the gapdh and β -actin transcripts, respectively (data not shown); therefore it was eliminated. The isothermal amplification method resulted in uneven amplification, as revealed by a skewed scatter plot (Fig. 2B), suggesting sequence dependency.

In the two-cycle amplification scatter plot (Fig. 2C), more low-intensity signals were observed in the lower-left quadrant, as compared to the one-cycle amplification scatter plot (Fig. 2A), and fewer high-intensity signals were spotted in the upper-right quadrant, overall denoting some loss of sensitivity. While the two-cycle T7 amplification method tended to generate 3'-biased amplified products, with a 3'/5' ratio of 7 for β -actin (Fig. 2C) versus <2 using a standard Affymetrix one-cycle T7 RNA polymerase amplification (Fig. 2A), the bias was relatively low, and the expression profile was overall very similar to that of the standard procedure. Thus, the two-cycle T7 amplification was selected as the RNA amplification method for subsequent high-density microarray experiments with rat gonadotropes.

3.2. Microarray analysis of early gene expression in GnRH-stimulated primary gonadotropes identifies 33 up-regulated genes

To investigate the pattern of early gene responses following activation of the GnRH receptor, primary rat pituitary cultures enriched for gonadotropes were treated with 10 nM GnRH or vehicle for 40 min, the RNA amplified by two-cycle T7 amplification and subjected to high-density microarray analysis. Six independent replicate experiments were performed to identify differentially expressed genes. Data revealed the increased expression of 33 genes following GnRH exposure, by at least a 1.5 fold factor (Table 1; the raw data were deposited in the NCBI GEO-Database Accession no. GSE31399). In contrast, no genes were found to be down-regulated. Transcripts induced by GnRH receptor activation included the immediate early genes *c-fos*, *fosB*, and *egr2*, which encode transcription factors. Other GnRH-induced transcripts most notably encoded signal transduction proteins and cell signaling modulators (e.g. *Rap1b*, and *Rgs4*), protein or electron transporters (e.g. *Bet1*, *Rab1*, *Slc38a2*), cell adhesion proteins (e.g. *Cldn4*, *Itga6*), as well as proteins whose molecular functions are still poorly understood (e.g. *Rtn4*, *Dnajb4*).

3.3. Rap1b is confirmed as a novel early gene induced by GnRH in primary gonadotropes

In order to validate the microarray data, we conducted a real-time PCR assay on a subset of up-regulated and mechanistically interesting genes regulated in the primary gonadotropes. With the exception of rap1b and nr4a3, the genes selected for real-time PCR analysis were previously found to be activated in L β T2 cells exposed to GnRH for one hour [[1,3,5,6] and Ebersole, B. J., T. Yuen, E. Wurmbach, and S.C. Sealfon, unpublished data]. Our quantitative assay confirmed the up-regulation of c-fos, fosB, egr2, and rap1b, while others that did not meet our criteria on the microarray (below 1.5-fold change, see Table 1) were found to be up-regulated, e.g. c-jun, egr1, egr3, nr4a3 (Fig. 3). Intriguingly, two novel genes, nr4a3 and rap1b, were established as GnRH-induced. Because previous studies reported a regulatory role for Rap1 in secretion in several exocrine and endocrine cells [31–34], rap1b was further investigated in this work.

3.4. Single-cell studies confirm that GnRH induces rap1b gene expression in primary gonadotropes

Despite a significant enrichment for gonadotrope cells in purified gonadotropes (40–60%) as compared to pituitary cultures, the presence of other pituitary cell populations cannot be completely discounted. Hence, the potential cellular heterogeneity may be responsible for some degree of heterogeneous gene expression. To discriminate the contribution of gonadotropes to the increase in rap1b mRNA expression under GnRH stimulation, we established a single-cell quantitative real-time PCR assay in individual rat gonadotrope cells. As shown in Fig. 4, GnRH induced a 6-fold ($P < 0.001$) increase in the level of rap1b transcripts in single gonadotrope cells, as compared to the unstimulated control. These results confirmed the microarray and real-time PCR data obtained in enriched gonadotrope cultures. As GnRH is known to stimulate an early increase in cAMP in rat pituitary cells [35], we examined the effect of 8-bromo-cAMP on rap1b gene expression: there was a 2-fold increase ($P < 0.02$) in the level of rap1b transcripts. This result suggested that GnRH-induced increase in rap1b mRNA may be partially mediated by cAMP.

3.5. GnRH-induced increase in rap1b gene expression has a similar effect on Rap1b protein levels in primary gonadotropes

Rap1b protein production following GnRH stimulation was evaluated by dual immunofluorescence and confocal microscopy. Pituitary cells were co-exposed to anti-LH and anti-Rap1 antibodies to examine signal co-localization to gonadotropes. As shown in Fig. 5C, at 1 h after initiation of a 15-min GnRH pulse, Rap1 complexes appeared as focal points of intense immunofluorescence staining in LH-positive cells with a distribution that suggested positioning of the complexes at the periphery, in areas compact with LH secretory granules. Although these immunofluorescent objects had a diameter of 500 nm or greater, their roundness was only apparent because they usually extended into multiple optical slices. As the diameter of rat gonadotrope secretory granules is typically 150–250 nm (reviewed in [36]), we could not determine whether the Rap1 complexes were associated with LH secretory granules. Moreover, only a subpopulation of objects was yellow suggesting subcellular co-localization. In contrast, in control cells (Fig. 5A) or in cells fixed after only 15 min of GnRH stimulation (Fig. 5B), Rap1 focal points appeared in smaller aggregates and were more diffusely distributed in the cytoplasm. Object volume quantification (expressed as fold-change, GnRH-treated/vehicle-treated) revealed that Rap1 immunofluorescence was significantly greater 1 h after GnRH stimulation as compared to cells fixed 15 min after GnRH treatment (6-fold; $P < 0.03$; Fig. 5D). Thus, the results on protein expression were consistent with the mRNA data, indicating that the effects observed were specific and mediated by the GnRH receptor.

4. Discussion

Pituitary gonadotropes must decipher instructions received from GnRH receptor activation to respond with the appropriate amount and pattern of gonadotropin secretion. We have investigated the gene network that is activated shortly after GnRH stimulation of gonadotrope-enriched primary cultures of rat pituitary cells, as these early induced genes may encode proteins that notably modulate cellular signaling. We identified 33 up-regulated genes, among which *rap1b*, a widely expressed small GTPase implicated in a number of cellular processes, was novel. We provided evidence for GnRH-induced increase in both *Rap1b* transcript and protein, and confirmed the induction of *rap1b* gene expression at the single gonadotrope level.

Among the up-regulated genes identified in this work, there are genes encoding transcription factors (e.g. *c-Fos*, *FosB*, and *Egr2*), proteins involved in signal transduction or in the regulation of signal transduction (e.g. *Rap1b*, and *Rgs4*), proteins involved in transport (e.g. *Bet1*, *Rab1*, *Slc38a2*), cell adhesion (e.g. *Cldn4*, *Itga6*), and various other processes. By contrast, more than half of the 28 genes whose expression has been found to increase in $L\beta$ T2 cells in response to GnRH are transcription factors (e.g. *Egr2*, *c-Fos*, *Klf4*); the remainder are modulators of signal transduction (e.g. *Rgs2*) and cytoskeletal proteins (e.g. γ -actin and *transgelin*) [3]. Importantly, our microarray data for primary pituitary cells were validated by an independent determination of the expression levels of a subset of genes by real-time PCR. In the present study, most GnRH-regulated genes showed less than a 2-fold induction as determined by microarray analysis (see Table 1). The greatest change detected for primary gonadotropes is 4-fold, as compared to ~50-fold in $L\beta$ T2 cells [3]. Species differences between rat and mouse, primary cells *vs.* transformed cells, and duration of GnRH treatment – 40 minutes in the primary gonadotropes *vs.* 1 hour in the $L\beta$ T2 cell line – may account for these discrepancies. Additionally, despite a marked enrichment in gonadotrope cells, our primary pituitary cultures may still be contaminated by other secretory cell types, thereby resulting in some degree of cell heterogeneity. We conjecture that high basal expression of a given gene in the contaminating cells could potentially mask the detection of gene induction in the gonadotropes. Because differences in the nature of contaminating cells are a potential source of variability between replicated experiments, we performed a total of six replicate experiments in order to minimize the effect of sample variance. More generally, the degree of variability that subsists within any cell population, not to mention noise or random fluctuations among identical cell types, contribute to cell-to-cell variation in gene expression; this can be eliminated by monitoring gene expression at the single-cell level (for review, see [37]). We therefore optimized and implemented a single-cell quantitative real-time PCR assay, which unambiguously substantiated the up-regulation of *rap1b* in single rat gonadotropes. Despite the cellular heterogeneity of primary pituitary cultures, employing them to study the actions of GnRH is physiologically more pertinent than using the $L\beta$ T2 cell line, as primary cultures mimic *in vivo* conditions more closely than transformed cells. Supporting this concept, we previously demonstrated that rat or mouse pituitary cultures responded to estradiol stimulation with an increase in progesterone receptor expression, while $L\beta$ T2 cells did not. Furthermore, GnRH-stimulated LH secretion was augmented by progesterone in rat or mouse pituitary cells, but not in $L\beta$ T2 gonadotropes [14]. This finding is consistent with the absence of GnRH self-priming in the immortalized cell line [13] and the notion that the progesterone receptor plays a role in GnRH self-priming [24]. Pituitary adenylate cyclase-activating polypeptide (PACAP) was previously shown to stimulate the *CGA* and *LH β* genes and reduce *FSH β* mRNA levels in primary pituitary cells [38], but not in $L\beta$ T2 cells [39,40]; thus, based on the data obtained in primary pituitary cells, PACAP was thought to enhance responsiveness to GnRH and regulate the differential secretion of LH and FSH (for review, see [41]). Although PACAP's role was not examined in our study, this peptide has been shown to signal through PKA-

independent and as well as PKA-dependent pathways. For example, select neurotropic actions of PACAP have been shown to operate through cAMP activation of EPAC, a guanine nucleotide exchange factor (GEF) and the GTPase, Rap1 (reviewed in [53]).

In the present work, all of the rat gonadotrope early genes found to be up-regulated by GnRH as determined by real-time PCR were previously shown to be modulated by GnRH in L β T2 cells following a one-hour GnRH treatment [1,3,5,6], except for nr4a3 and rap1b. Nr4a3, also known as neuron-derived orphan nuclear receptor 1 (or NOR-1), is a member of the nuclear hormone receptor superfamily; it is constitutively active, operates in a ligand-independent manner, and is expressed in a variety of energy-dependent tissues such as skeletal muscle, brain, adipose tissue, brain, liver, therefore suggesting a potential role in energy metabolism (for review, see [42]). Rap1, a member of the Ras superfamily of small GTP-binding proteins, is involved in a diverse range of functions, e.g., cell adhesion, polarity, and proliferation [43–45]. Activation of Rap1 involves conversion of the GDP-bound to the GTP-bound form by GEFs, such as Epac1 and Epac2, which are regulated by cAMP, or CalDAG-GEF, which is activated by Ca²⁺ and diacylglycerol [43]. Rap1 has two isoforms, Rap1a and Rap1b, which seem to mediate similar but cell type-specific actions (for review, see [43,46,47]). Rap1b is the predominant isoform and the most abundant Ras family member in platelets [48]. In fact, rap1b knockout mice exhibit 85% lethality, and in surviving rap1b-null mice Rap1b deficiency results in a bleeding defect due to abnormal platelet function [49]. Rap1b was also shown to stimulate mitogenesis in the thyroid both *in vitro* and *in vivo* in a cAMP-dependent manner [44,45].

Of more relevance to gonadotrope function, Rap1 also has a regulatory role in protein secretion, specifically amylase release from parotid and pancreatic acinar cells, cAMP-stimulated neurotensin secretion in an endocrine cell line, and insulin secretion from pancreatic beta cells [31–34]. A parallel potentially exists between the role of Rap1b in insulin secretion and in the priming activity for LH secretion in gonadotropes. Glucagon-like peptide-1 (GLP-1), an intestinally derived incretin, potentiates or primes glucose-stimulated insulin secretion from pancreatic beta cells, and Rap1b has been implicated in this process [34,50]. GLP-1 receptor agonists are important tools in the treatment of type 2 diabetes mellitus, and studies to delineate the signaling pathway have focused on regulation of Rap1b activation and its downstream targets [50,51]. Epac2, the cAMP-regulated activator of Rap1b, is reported to have a primary role in GLP-1 action on insulin secretion [34,50,52]. Unresolved is whether the potentiating action of GLP-1-induced cAMP elevation in pancreatic beta cells operates solely through the Epac2 and Rap1b mechanisms or additionally through a PKA pathway [50,53].

To our knowledge, the effect of GLP-1 or insulin secretagogues on upregulation of rap1b transcripts has not been examined in pancreatic beta cells. In our studies, we observed that cAMP stimulates rap1b gene expression in single gonadotropes. GnRH previously has been shown to stimulate an early increase in cAMP in rat pituitary cells [35], and elevated cAMP, which by itself does not stimulate LH secretion, can substitute for the initial GnRH pulse to potentiate subsequent responses to secretagogues [24,54]. Although our results are consistent with cAMP involvement in GnRH priming, they do not address whether cAMP directly affects Epac/Rap1 activation in addition to increasing Rap1b.

Subcellular compartmentalization is a spatial regulator in signaling pathways, and Epac2 has been found associated with pancreatic beta cell exocytotic machinery (reviewed in [52]) and Rap1 localizes with secretory granule membranes in parotid and pancreatic acini as well as pancreatic beta cells [31,33,34]. Consistent with this, for gonadotropes we observed a distribution of Rap1 immunofluorescent complexes in areas compact with LH secretory granules that was prominent 1 h after GnRH stimulation. Further studies are required to

elucidate its precise role in GnRH-stimulated gonadotropes, but we hypothesize that the small GTPase, Rap1b, is a candidate contributor to GnRH priming.

5. Conclusion

This study demonstrates that GnRH up-regulates Rap1b shortly after stimulation of rat primary gonadotropes. Owing to an optimized amplification of the RNA in gonadotrope-enriched primary pituitary cells, we were able to carry out high-throughput gene expression analysis and identify the early genes that are regulated by GnRH in a highly physiological context. Results were independently validated by real-time PCR. Further, we showed that GnRH induced a significant increase in rap1b transcript levels in single gonadotrope cells, thereby corroborating our findings in cell populations that may exhibit cell-to-cell variation, and to some extent cell heterogeneity. This early increase in rap1b transcript was followed by an increase in the protein detected in the vicinity of LH secretory granules. Rap1b was previously proposed to potentiate protein secretion in other cell types and may be directly implicated in gonadotropin secretion. Further studies are required to elucidate its precise role in GnRH-stimulated gonadotropes, but we hypothesize that the small GTPase, Rap1b, is a candidate contributor to GnRH priming.

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Highlights

- Optimized RNA amplification allowed microarray analysis of GnRH-stimulated gonadotrope-enriched primary pituitary cultures.
- rap1b is a novel early gene induced by GnRH in primary gonadotropes.
- Single-cell real-time PCR demonstrated the rap1b is induced by GnRH in the gonadotropes.
- Rap1b protein was markedly increased in GnRH-stimulated primary gonadotropes.

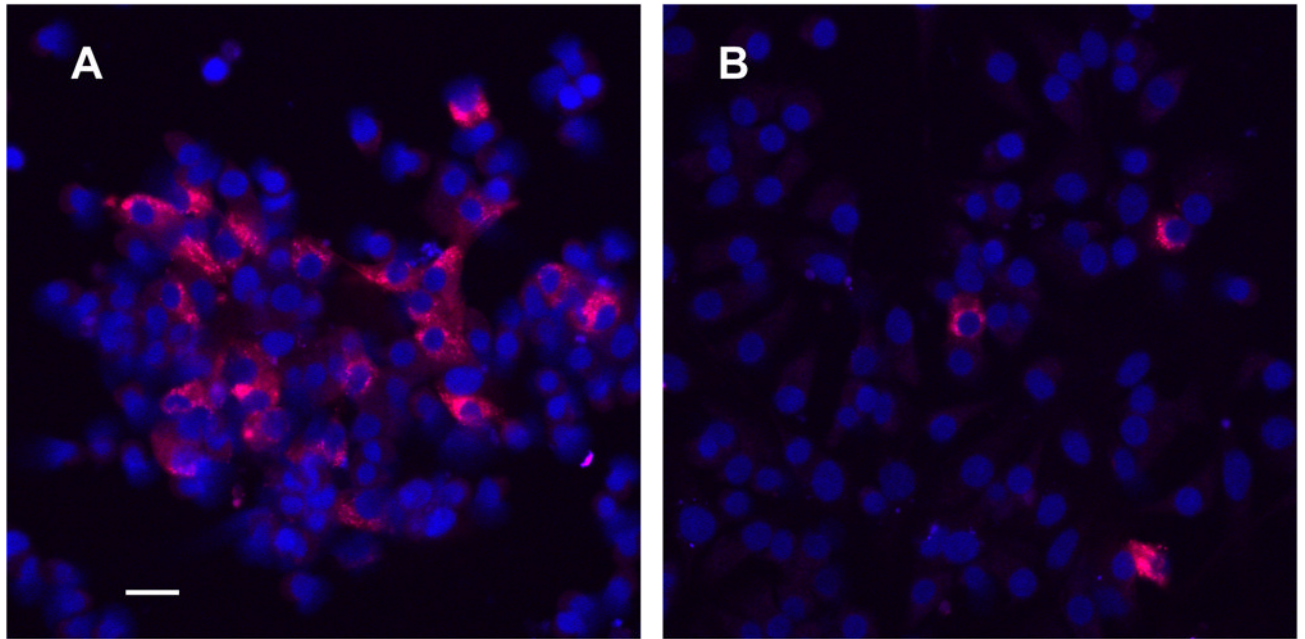


Fig. 1. Localization of LH (red) in gonadotrope-enriched primary pituitary cultures using confocal microscopy

Gonadotrope-enriched (panel A) and unfractionated cells (panel B) from the same pituitary dispersion were cultured for 3 days and fixed and immunostained for confocal microscopy. *Red* identifies LH-positive cells (gonadotropes). *Dark blue* (TOTO-3) identifies cell nuclei. Scale bar (20 μm) in panel A applies to both panels.

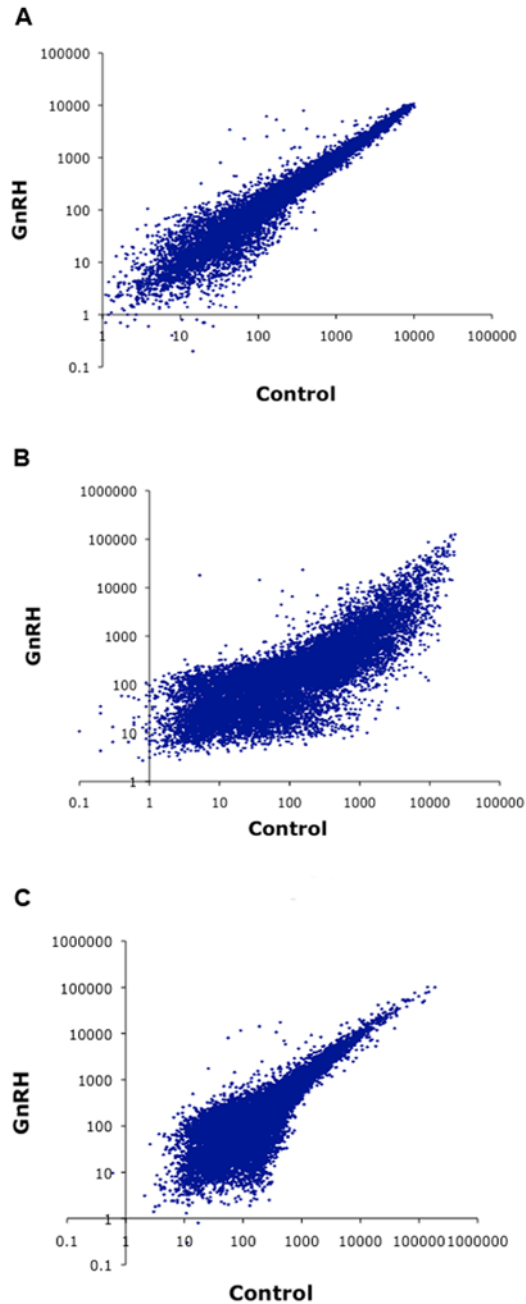


Fig. 2. Comparison of three RNA amplification protocols for microarray analysis
 Scatter plots of microarray data obtained from three different RNA amplification methods. L β T2 cells were treated with 100 nM GnRH or vehicle (Control) for 1 h. RNA was isolated and aliquots were used for amplification procedures. Labeled probes were hybridized to Affymetrix GeneChip U74A microarrays. **A**, Amplification using a standard Affymetrix one-cycle T7 protocol with 40 μ g total RNA. **B**, Hybrid DNA-RNA primer isothermal amplification with 100 ng total RNA. **C**, Amplification using a modified Affymetrix two-cycle T7 protocol with 100 ng total RNA. Copy numbers were estimated by intrapolation of C_T values against a serial dilution of known concentrations of plasmid DNA containing

either mouse GAPDH or mouse β -actin cDNA. Ideally, copy numbers reported for the 3' and 5' assays should yield a ratio of 1.

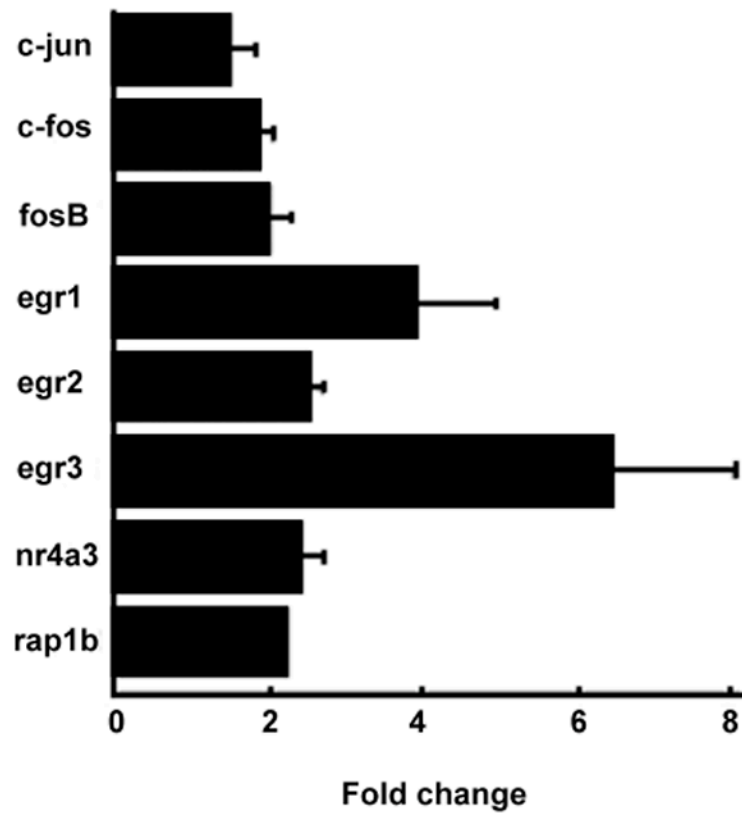


Fig. 3. Validation of the early gene microarray data by real-time PCR

The expression levels of eight candidate genes were evaluated by real-time PCR in vehicle-*vs.* GnRH-treated primary rat gonadotropes. Represented are fold changes relative to the control conditions (i.e. with vehicle). Data from three independent experiments were calculated and plotted. Bars show standard deviations. All genes shown were significant at $p < .05$.

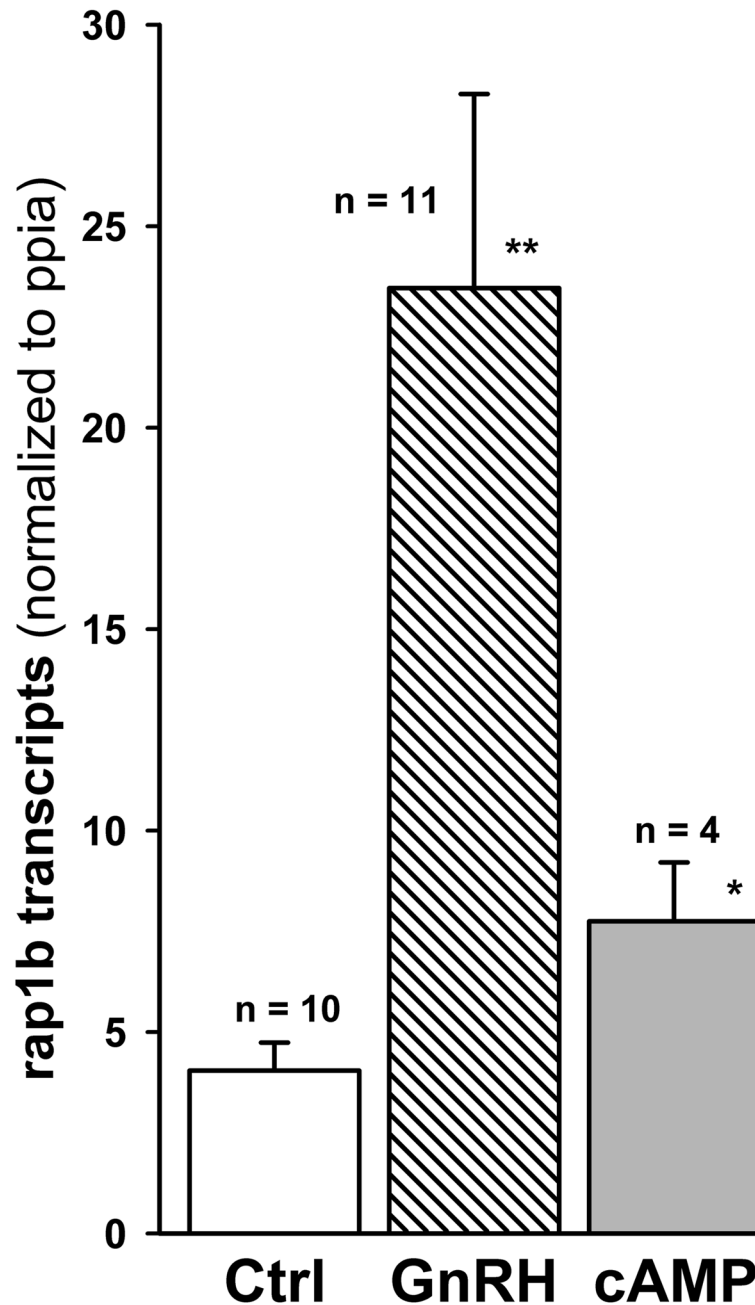


Fig. 4. GnRH activates Rap1b gene expression in single rat gonadotropes

Individual rat gonadotrope cells were perfused with a 15-min pulse of extracellular medium only (Ctrl, control) or with medium containing either 10 nM GnRH or 1 mM 8-bromo-cAMP. Perfusion with control medium only was continued, and 25 min later cells were harvested and immediately processed for reverse transcription, as described in Materials and Methods. Rap1b data were normalized to Ppia transcripts determined in the same gonadotrope. Single cell data for all groups were obtained from two separate pituitary dispersions. The number of individual gonadotropes is in parentheses. Compared to control: ** $P < 0.001$, * $P < 0.02$.

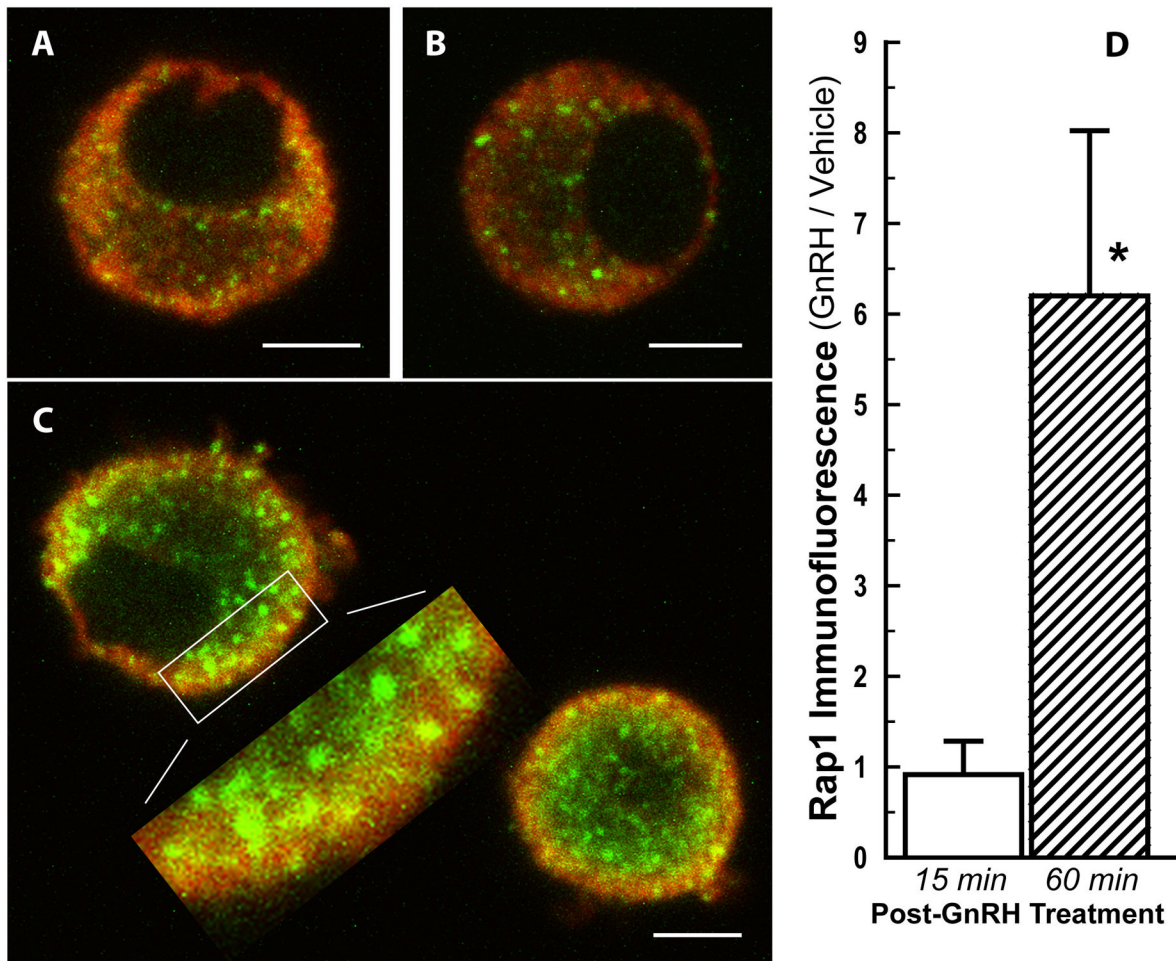


Fig. 5. GnRH activates Rap1 protein expression in primary gonadotropes

Rat pituitary cells were either incubated in the presence of vehicle for 60 min (A), challenged with a 15-min pulse of 1nM GnRH and fixed immediately (B) or 45 min later (C). Fixed cells were subjected to dual immunofluorescence staining for LH (red) and Rap1 (green) and optical sectioning. Panels A–C: Single 2 μ m optical sections at maximum cell diameter (A and B) or at intermediate diameters maximum for the field (C). **Inset of panel C**, an expanded view of immunofluorescent punctuate Rap1-associated objects. Scale bars = 5 μ m; the dimension of the expanded view (C) is approximately 9 x 3 μ m. **D**, Rap1-associated object volume determined from 2 μ m overlapping optical sections through each cell: Fold-change in Rap1 immunofluorescence in gonadotropes (GnRH-treated/vehicle-treated) fixed at either 15 min (open bar) or 60 min (hatched bar). * $P < 0.03$ compared to cells fixed at 15 min.

Table 1

Fold-changes from the early gene microarray experiments

Probe Set	Fold	Gene Symbol	Gene Title
1373759_at	4.17	fosB	FBJ osteosarcoma oncogene B
1387306_a_at	2.85	Egr2	early growth response 2
1398940_at	2.14	Srrm2	serine/arginine repetitive matrix 2 FBJ murine osteosarcoma viral oncogene
1375043_at	2.05	Fos	homolog
1368888_a_at	1.86	Rtn4	reticulon 4
1373093_at	1.85	Errfi1	ERBB receptor feedback inhibitor 1 blocked early in transport 1 homolog
1372710_at	1.80	Bet1	(<i>S.cerevisiae</i>)
1372131_at	1.79	Ubqln2	Ubiquilin 2
1368151_at	1.78	Matr3	matrin 3
1372347_at	1.78	Skil	SKI-like, transcript variant 2
1368044_at	1.76	Scg2	secretogranin 2
1371583_at	1.73	Rbm3	RNA binding motif (RNP1, RRM) protein 3
1387076_at	1.72	Hif1a	hypoxia inducible factor 1, alpha subunit
1398841_at	1.71	Rab1	RAB1, member RAS oncogene family
1371755_at	1.71	EST	Similar to RIKEN cDNA B230219D22
1388547_at	1.69	Cldn4	claudin 4
1369939_at	1.66	Cycs	cytochrome c, somatic
1398797_at	1.65	Hnrpk	heterogeneous nuclear ribonucleoprotein K
1371388_at	1.64	Pdhb	pyruvate dehydrogenase (lipoamide) beta
1387806_at	1.63	Rap1b	RAS related protein 1b
1383401_at	1.61	Tes	similar to Testis derived transcript
1368505_at	1.60	Rgs4	regulator of G-protein signaling 4
1379255_at	1.60	Atp6ap2	ATPase, H ⁺ transporting, lysosomal accessory protein 2
1370286_at	1.60	Slc38a2	solute carrier family 38, member 2
1373156_at	1.59	Alex2	armadillo repeat protein ALEX2
1371642_at	1.59	Eif4a2	eukaryotic translation initiation factor 4A2
1367971_at	1.55	Ptp4a2	protein tyrosine phosphatase 4a2
1376762_at	1.54	Plekha1	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1
1367512_at	1.54	Chmp5	chromatin modifying protein 5
1372722_at	1.53	Dnajb4	DnaJ (Hsp40) homolog, subfamily B, member 4
1383240_at	1.53	Itga6	Integrin, alpha 6
1370238_at	1.51	Usmg5	upregulated during skeletal muscle growth 5
1374529_at	1.50	TSP-2	Thrombospondin 2

Values meeting the criteria for up-regulation are listed in descending order. Primary rat gonadotrope cells were exposed to 10 nM GnRH for 40 min, then harvested and processed for RNA extraction, as described in Materials and Methods. Data analysis was performed by Affymetrix GeneChip Operating System (GCOS). A gene was considered to be up-regulated by GnRH if there is at least 50% concordance across multiple pairwise comparisons of GnRH- vs. vehicle-treated microarrays, and if the fold-change was at least 1.50. Note: using the same selection criteria, no gene was found to be down-regulated after a 40-min GnRH treatment.