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Regulation of *Lhb* and *Egr1* Gene Expression by GNRH Pulses in Rat Pituitaries Is Both c-Jun N-Terminal Kinase (JNK)- and Extracellular Signal-Regulated Kinase (ERK)-Dependent[1]

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ABSTRACT

Pulsatile GNRH regulates the gonadotropin subunit genes in a differential manner, with faster frequencies favoring *Lhb* gene expression and slower frequencies favoring *Fshb*. Early growth response 1 (EGR1) is critical for *Lhb* gene transcription. We examined GNRH regulation of EGR1 and its two corepressors, Ngfi-A-binding proteins 1 and 2 (NAB1 and NAB2), both in vivo and in cultured rat pituitary cells. In rats, fast GNRH pulses (every 30 min) stably induced *Egr1* primary transcript (PT) and mRNA 2-fold ($P < 0.05$) for 1–24 h. In contrast, slow GNRH pulses (every 240 min) increased *Egr1* PT at 24 h (6-fold; $P < 0.05$) but increased *Egr1* mRNA 4- to 5-fold between 4 and 24 h. Both GNRH pulse frequencies increased EGR1 protein 3- to 4-fold. In cultured rat pituitary cells, GNRH pulses (every 60 min) increased *Egr1* (PT, 2.5- to 3-fold; mRNA, 1.5- to 2-fold; $P < 0.05$). GNRH pulses had little effect on *Nab1/2* PT/mRNAs either in vivo or in vitro. We also examined specific intracellular signaling cascades activated by GNRH. Inhibitors of mitogen-activated protein kinase 8/9 (MAPK8/9 [also known as JNK]; SP600125) and MAP Kinase Kinase 1 (MAP2K1 [also known as MEK1]; PD98059) either blunted or totally suppressed the GNRH induction of *Lhb* PT and *Egr1* PT/mRNA, whereas the MAPK14 (also known as p38) inhibitor SB203580 did not. In summary, pulsatile GNRH stimulates *Egr1* gene expression and protein in vivo but not in a frequency-dependent manner. Additionally, GNRH-induced *Egr1* gene expression is mediated by MAPK8/9 and MAPK1/3, and both are critical for *Lhb* gene transcription.

Egr1, *follicle-stimulating hormone, gonadotropin-releasing hormone, gonadotropins, luteinizing hormone, MAPK1/3(ERK), MAPK8/9(JNK), neuroendocrinology, pituitary hormones*

INTRODUCTION

The gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are dimeric protein hormones composed of a common α subunit (CGA) and a unique β subunit [1]. The subunit genes are regulated by hypothalamic GNRH in both a coordinate and a differential manner. GNRH differentially regulates subunit mRNA synthesis via changes in pulse frequency, with faster-intermediate GNRH pulse frequencies (8- to 60-min pulse intervals) favoring *Cga* and *Lhb*, and slower-frequency pulses (≥120-min pulse intervals) favoring *Fshb* [2].

The signal transduction mechanisms responsible for interpreting GNRH pulse frequency and differentially regulating β-subunit gene expression are not well understood. The GNRH receptor (GNRHR) is a member of the G protein-coupled receptor family [3, 4]. Ligand-bound GNRHR activates several members of the G protein family, including G_q and G_{11}. Activated G_q stimulates phospholipase C, resulting in increased inositol 1,4,5,-trisphosphate (IP_3), elevated diacylglycerol levels, and activation of protein kinase C (PKC) [5, 6]. GNRHR activation also stimulates a transient increase in intracellular calcium (Ca^{2+}) derived from IP_3-induced release of Ca^{2+} from intracellular storage pools and from influx via L-type voltage-gated calcium channels, which can stimulate other Ca^{2+}-sensitive protein kinases [5, 7]. Additionally, we and others have shown that GNRH stimulates activation of mitogen-activated protein kinase (MAPK) signaling cascades (MAPK1/3 [extracellular signal-regulated kinase, or ERK], MAPK8/9 [c-Jun N-terminal kinase, or JNK], and MAPK14 [p38]), and that members of this family are important in transducing GNRH pulse information in gonadotrophs [2].

GNRH-induced MAPK1/3 activation is via both PKC-dependent and independent mechanisms [2, 8]. We reported that GNRH pulses are more effective than continuous GNRH to stimulate sustained pituitary MAPK1/3 phosphorylation in rats, that MAPK1/3 phosphorylation is maximal after slow-frequency GNRH pulses [9, 10], and that inhibition of the pathway using a MAP Kinase Kinase 1 (MAP2K1, also known as MEK1) inhibitor blocked the GNRH-induced increase in *Cga* and *Fshb* mRNAs, but not *Lhb* mRNA, in primary pituitary cells [9]. GNRH also induces MAPK8/9 activation via a PKC-independent mechanism [11, 12]. Recently, we reported that MAPK8/9 blockade completely suppressed the GNRH-induced increase in *Lhb* transcription in perifused rat pituitary cells [13]. GNRH also increases MAPK14 activation via a PKC-dependent mechanism [14], but inhibition of MAPK14 activation had no effect on *Lhb* or *Fshb* transcriptional or gonadotropin secretory responses to pulsatile GNRH in rat pituitary cells [13].

The mechanism(s) by which MAPK1/3 and MAPK8/9 regulate β-subunit transcription have not been explored fully. MAPK1/3 and MAPK8/9 activation stimulates a number of transcription factors that are important in the regulation of the *Lhb* and *Fshb* subunit genes, including cFOS (FOS), cJUN (JUN), the ETS protein ELK1, and EGR1 [15]. The rodent

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Fshb proximal promoter contains a low-affinity activator protein-1 (AP1) half-site that binds JUN/FOS heterodimers and is important for maximal GNRH induction of the murine *Fshb* promoter in LβT2 cells [16]. This AP1 half-site is involved in MAPK1/3 activation of *Fshb* transcription, because treatment of LβT2 cells with a MAP2K1 inhibitor or cotransfection of a dominant/negative FOS expression vector reduced GNRH-stimulated *Fshb* promoter activity [16]. GNRH also regulates *Fshb* gene expression indirectly via changes in pituitary activin and follistatin (FST). Fast-frequency GNRH pulses selectively stimulate FST expression, reducing activin bioavailability and suppressing *Fshb* gene expression [2, 10, 17–20].

The rat *Lhb* promoter also contains a region that is highly homologous with a consensus AP1 site (−159/−153 bp [21, 22]), and mutation of this site diminishes *Lhb* promoter activity [23]. However, *Lhb* transcriptional responses to GNRH are primarily through actions on EGR1 and other transcription factors that bind to the proximal and distal GNRH-responsive regions [15]. EGR1 (also known as NGFI-A, Krox24, and zif268) is an immediate early gene of the zinc-finger subfamily and is expressed in many cell types during development and in differentiated cells in response to numerous types of signals and stress stimuli (for a review, see Thiel and Cibelli [24] and Knapska and Kaczmarek [25]). In the reproductive axis, EGR1 plays an essential role based on findings that *Egr1* knockout mice are either completely infertile or subfertile, reflecting a lack of LHB synthesis in the gonadotroph (CGA and FSHB were unaffected [26, 27]). Two EGR1-binding sites have been identified in the proximal GNRH-responsive region of the *Lhb* promoter that are highly conserved across species [26, 28–31], and mutations within these EGR1-binding sites abrogate the GNRH induction of *Lhb* promoter reporter constructs in gonadotroph-derived cell lines [32–34]. Also, it has been observed that rat pituitary *Egr1* mRNA expression is greatest during proestrus and is increased after ovariectomy (OVX), and the post-OVX increase can be blocked by estrogen [35], suggesting that GNRH plays a physiological role in regulating pituitary *Egr1* expression.

Recent reports demonstrated that expression of *Egr1* mRNA and protein and the two EGR1 corepressors Ngfi-A binding proteins (NAB1 and NAB2) are differentially regulated by GNRH pulse frequency in gonadotroph-derived cell lines. Kanasaki et al. [36] showed that EGR1 protein levels are 3-fold greater in LβT2 cells receiving GNRH pulses every 30 min vs. every 120 min. Similarly, Lawson et al. [37] reported that *Egr1* mRNA increased rapidly and transiently in response to GNRH and was maximally stimulated by faster-frequency GNRH pulses (<60-min interpulse interval). In contrast, maximal *Nab1* and *Nab2* mRNA expressions were seen after slower-frequency GNRH pulses (≥60-min interpulse interval [37]). These findings suggest that regulating EGR1 synthesis and bioavailability plays a role in GNRH pulse frequency actions on the *Lhb* gene.

The aims of the present study were to investigate whether GNRH pulse frequency differentially regulates *Egr1*, *Nab1*, and *Nab2* transcription in normal rat pituitary cells, and to assess the effects of MAPK8/9, MAPK1/3, and MAPK14 blockade on *Egr1* and *Nab* gene expression.

MATERIALS AND METHODS

In Vivo Studies

Adult (225–250 g) Sprague Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN) were used for all experiments. For in vivo studies, rats were housed in a light-controlled (lights on 0500–1700 h) and temperature-controlled (25°C) room and allowed access to food and water ad libitum. All surgeries were performed under isoflurane (2.5% isoflurane, balance O_2; ISOTHESIA; Vetus Animal Heath, Burns Veterinary Supply Inc., Westbury, NY) anesthesia. At the completion of experiments, rats were euthanized by decapitation under anesthesia. The University of Virginia Animal Care and Use Committee approved all animal experimentation procedures described within this report.

To study the effects of GNRH pulse frequency, we used a GNRH-deficient (castrate and testosterone-replaced) rat model. Rats were castrated, and two 20-mm testosterone-containing silastic implants were inserted subcutaneously (serum testosterone levels were 4–5 ng/ml, 24 h after implant insertion) as described previously [20]. An indwelling right jugular cannula was also inserted at the time of castration for i.v. GNRH administration, and experiments began 24 h after castration. Rats (n = 5–7 per group) received 25-ng GNRH pulses (in 0.25 ml of 0.9% saline/0.1% bovine serum albumin) either every 30 or 240 min for 1–24 h. Controls were pulsed with vehicle only. Animals were killed 5 min after the last pulse, based on previous data in rats that β-subunit primary transcript (PT) responses to GNRH are maximal 5 min after a pulse [38]. Pituitaries were collected, snap frozen in liquid nitrogen, and stored at −70°C until RNA was extracted. In studies to determine the effects of GNRH pulse frequency on EGR1 protein, four to six rats per group received GNRH pulses (25 ng; i.v.) every 30 min or every 240 min for 8 h (controls were pulsed with vehicle only [10]).

In Vitro Studies

Pituitaries from adult female rats were pooled and dissociated in medium containing 0.35% collagenase, 0.1% hylauronidase, and 0.01% DNase. After dissociation, the cell suspension was aliquoted into culture wells (2×10^6 to 3×10^6 cells per well) containing 22-mm plastic coverslips coated with Matrigel (BD Biosciences, Bedford, MA). The cells were cultured for 48 h before beginning each experiment, and the media were augmented with testosterone (500 pg/ml) to allow *Lhb* mRNA expression in response to pulsatile GNRH [39, 40]. For perifusion studies, 48 h after plating cells, coverslips were inserted into custom-made chambers and allowed to equilibrate for 1 h before initiating treatment. The perifusion flow rate was 200 μl/min, and 100-μl pulses were administered during a 10-sec duration via autosyringe pumps. Studies were conducted as four separate experiments (12 chambers per experiment), with all treatment groups represented in each experiment (three chambers per treatment per experiment [39]).

Time course of Egr1 and Nab transcriptional responses to GNRH. Cells received two prepulses of GNRH (1 nM; 5-min duration; or media pulses to controls) 2 h and 1 h before beginning each experiment. GNRH (1 nM) was administered to groups of cells for durations of 5, 10, 45, or 120 min. After GNRH treatment, cells were lysed and RNA extracted (n = 5–6 per group).

The role of MAPK8/9 (JNK) and MAPK14 (p38) in the regulation of Egr1 and Nab transcripts by pulsatile GNRH. Perifused rat pituitary cells were given pulses of GNRH (peak chamber concentration, 200 pM; medium pulses to controls) every 60 min for 24 h [13]. GNRH pulses every 60 min were chosen because this intermediate pulse frequency stimulates both *Lhb* and *Fshb* expression in vitro [9]. For MAPK8/9 blockade studies, cells were perifused with medium containing the MAPK8/9-specific inhibitor SP600125 (SP; 20 μM; EMB Biosciences, San Diego, CA) or vehicle (inactive SP isoform; 20 μM; EMB Biosciences). For MAPK14 inhibitor studies, cells were treated with medium containing the MAPK14-specific blocker SB203580 (SB; 20 μM; EMB Biosciences) or vehicle (0.1% dimethyl sulfoxide [DMSO]). The SP and SB doses selected were based on previously published reports showing effective suppression of GNRH-induced activation of the MAPK8/9 or MAPK14 pathways within gonadotroph-derived cell lines [14, 41, 42]. Cells were recovered 10 min after the last pulse, and RNA was extracted.

The role of MAPK8/9 (JNK) and MAPK1/3 (ERK) in the regulation of Egr1 and Nab transcripts by pulsatile GNRH. Rat pituitary cells were cultured as described above. Forty-eight hours after dissociation, cells were treated with the MAP2K-specific inhibitor PD98059 (PD; 50 μM; EMB Biosciences), the MAPK8/9-specific inhibitor SP600125 (20 μM), or vehicle (0.25% DMSO). All cells were also treated with 1 μM bromocriptine (EMB Biosciences) to suppress MAPK1/3 activational responses to the removal of dopamine in lactotropes [9]. The selected dose for PD was based on previous studies showing that 50 μM PD blocks the MAPK1/3 activational response to GNRH in rat pituitary cells in vitro [9, 43]. One hour later, cells received two prepulses of GNRH (1 nM; 5-min duration; or media pulses to controls) 2 h and 1 h before the final GNRH pulse. The final 1-nM GNRH pulse was administered for 10 min, which was optimal for both *Lhb* and *Egr1* transcriptional responses. Cells were then lysed, and RNA and protein were extracted (n = 4 per group).

αT3 cells: time course of Egr1 and Nab mRNA expression in response to GNRH. After initial results showed minimal *Nab1/2* transcript responses to GNRH, which contrasts with earlier data in gonadotroph-derived cell lines [37], we used αT3 cells to assess the time course of *Egr1* and *Nab* transcript expression in response to GNRH. αT3 cells were plated at a density of 1.0×10^6 cells onto 22-mm diameter coverslips coated with Matrigel diluted 1:3. Cells were incubated in Dulbecco modified Eagle medium augmented with 10% fetal bovine serum. Twenty-four hours later, the cells were transferred to serum-free medium for 16 h and then treated with 10 nM GNRH (or vehicle; 0-h time) for 10, 45, or 120 min. After GNRH, coverslips were rinsed twice in PBS, and cells were recovered and processed for RNA and protein.

RNA Preparation and Measurement of Egr1 *and* Nab *Transcripts*

Total pituitary RNA was extracted using the acid guanidinium method [44]. Residual genomic DNA was removed by treatment with 1 unit of RNase Free DNaseI per microgram of RNA (Roche Molecular Biochemicals, Indianapolis, IN) at 37°C for 1 h. RNA preparations were confirmed to be DNA free by PCR in the absence of a preceding RT reaction. Primary transcripts and mRNAs were measured by real-time PCR as described previously [10]. The PCR assay primers were: *Lhb* PT forward, 5′-AGAGGCTCCAGGTAAGATGGTA-3′; *Lhb* PT reverse, 5′-CTTTTGCATTCCAGGTCCTGGA-3′; *Fshb* PT forward, 5′-TTTCCCCAGGAGAGATAGCCAA-3′; *Fshb* PT reverse, 5′-GCAA ACTGCTCTGTAAGTCAGA-3′; *Egr1* PT/mRNA forward, 5′-AACAACCC TACGAGCACCTG-3′; *Egr1*-PT reverse, 5′-CCCCAGACATCCCTCTAA CA-3′; *Egr1* mRNA reverse, 5′-AGCGGCCAGTATAGGTGATG-3′; *Nab1* mRNA forward, 5′-AAGGACAATGCCCTGCTG-3′; *Nab1* PT forward, 5′-TCTGAGGGAATGTTACAGACTGA-3′; *Nab1* PT/mRNA reverse, 5′-GGA GACAATTCATCTCTTTCACC-3′; *Nab2* mRNA forward, 5′-CGTGAGGG CAAACAGCTTAG-3′; *Nab2* PT forward, 5′-AAGCAGGCATTCTTT GGATG-3′; *Nab2* PT/mRNA reverse, 5′-GTGCTCTCTCGGGCTACTTG-3′; *Gnrhr* mRNA forward, 5′-ATGCTGGAGAGTTCCTTTGC3′; and *Gnrhr* mRNA reverse, 3′-CCGTCGGCTAGGTAGATCAT-5′. Primers for *Fst* mRNA have been reported previously [19, 38]. To confirm amplification of a single product, the PCR reaction was followed by melt-curve analysis. To create a standard for each subscript, RNA from a pooled rat pituitary cDNA sample was amplified by PCR using the aforementioned primers and then subcloned into PGEM T-EZ (Promega, Madison, WI). The identity of all PCR products was confirmed by DNA sequencing. Each PCR reaction was optimized for annealing temperature and Mg^{2+} concentration to obtain a PCR efficiency of 90%–105%. Each sample was measured against a standard curve of 2.0E1 to 1.28E–4 pg of plasmid in 1:5 dilutions. All samples from within an experiment were measured in the same assay in triplicate. Mean intraassay CVs are *Lhb* PT (9.0%), *Fshb* PT (7.1%), *Egr1* PT (9.3%), *Egr1* mRNA (6.8%), *Nab1* PT (7.9%), *Nab1* mRNA (9.5%), *Nab2* PT (12.8%), *Nab2* mRNA (8.0%), *Gnrhr* mRNA (6.7%), and *Fst* mRNA (6.7%).

Protein Preparation and Western Blot Immunostaining

For the in vivo experiment, pituitary protein was prepared as described previously [10]. For some in vitro experiments, protein was precipitated from the phenol:isoamyl:chlorofom extract after lysis in acid guanidinium by 3 volumes of cold acetone. Precipitates were washed three times with 95% ethanol, 0.3 M guanidine hydrochloride, and 2.5% glycerol and then were solubilized directly into Laemmli sample buffer (2% SDS; 62.5 mM Tris, pH 6.8; 8.5% glycerol; and 1.5% β-mercaptoethanol). Pituitary protein lysates (20 μg for in vivo samples or 10% of total for in vitro samples) were resolved by 8% SDS-PAGE and then transferred to nitrocellulose filters. Blots were then immunostained for EGR1, phosphorylated p44/p42 ERK (MAPKs 3 and 1, respectively), or phosphorylated cJUN, with primary antibodies (rabbit) obtained from Cell Signaling Technologies (Beverly, MA). Protein loading was determined by immunostaining for GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit (Millipore, Billerica, MA). Immunoactivity was detected using SuperSignal West Pico chemiluminescent system (Pierce, Rockville, IL), followed by autoradiography. Protein bands were quantified by densitometry using TotalLab Software (Amersham Biosciences, Piscataway, NJ).

Analysis

All data were examined by ANOVA. Significant differences ($P < 0.05$) were determined posthoc by Duncan multiple range test. Prior to analyses, all measurements were transformed to the logarithmic scale to attain equal variation among treatments.

RESULTS

We have shown previously that *Lhb* transcription is maximally stimulated by faster-frequency (30 min) and *Fshb* by slower (240 min) GNRH pulses [20]. To examine potential links between frequency-dependent regulation of the *Lhb* gene and specific downstream transcriptional mediators, the effects of GNRH pulse frequency in vivo on *Egr1*, *Nab1*, and *Nab2* PTs and mRNAs were determined (Fig. 1). GNRH pulses every 30 min increased *Egr1* PT and mRNA 2- to 3-fold between 1 and 24 h ($P < 0.05$ vs. 0-h time point). In contrast, GNRH pulses every 240 min only increased *Egr1* PT (6-fold; $P < 0.05$) after 24 h of pulses but increased *Egr1* mRNA 4-fold between 4 and 24 h of treatment, and *Egr1* mRNA levels were significantly greater at 24 h in rats receiving GNRH pulses every 240 min vs. 30 min ($P < 0.05$). Neither GNRH pulse frequency had much effect on *Nab1* or *Nab2* gene expression, although 30-min pulses induced a small increase in *Nab2* mRNA at 1 h. The GNRH-induced increases in *Egr1* gene expression had similar effects on EGR1 protein levels (Fig. 2). Although there was variability among samples, 8 h of GNRH pulses every 30 min or 240 min increased EGR1 protein levels to a similar degree (3- to 4-fold vs. controls; $P < 0.05$).

Figure 3 shows the time course of β-subunit, *Egr1*, and *Nab* gene expression responses to GNRH in cultured rat pituitary cells. GNRH rapidly increased *Lhb* PT (1.8-fold 5 min after GNRH pulse), peaked at 10 min (3-fold), then declined but remained elevated vs. controls at 45 min and 120 min ($P < 0.05$ vs. 0-h time controls). *Fshb* PT levels also increased rapidly after GNRH (1.6-fold at 5 min), with maximal (3-fold; $P < 0.05$) increases seen at 10 min before declining to <50% of controls between 45 min and 120 min ($P < 0.05$ vs. 0 min). *Egr1* PT increased to a peak (3.5-fold; $P < 0.05$) 10 min after GNRH, returned to control levels at 45 min, and was suppressed by 40% after 120 min ($P < 0.05$). *Egr1* mRNA increased approximately 2-fold between 5 min and 45 min after GNRH ($P < 0.05$), then returned to basal levels at 120 min. *Nab1* PT and mRNA changed little during the GNRH treatment time course, although *Nab1* PT was transiently lower (30% vs. control; $P < 0.05$) 45 min after GNRH. *Nab2* PT increased 1.5-fold 5 min after GNRH before returning to basal after 10 min of treatment ($P < 0.05$), whereas *Nab2* mRNA showed little change.

In gonadotroph-derived cell lines, GNRH has been reported to induce large increases in *Egr1* mRNA [36, 37], which contrasts with our observations either in vivo or in cultured primary pituitary cells. To confirm that this difference reflects cell type, we investigated *Egr1*, *Nab1*, and *Nab2* gene responses to GNRH in αT3 cells (Fig. 4A). GNRH increased both *Egr1* PT and mRNA, with maximal responses seen at 10 min (45-fold) for PT and 45 min (150-fold) for mRNA ($P < 0.05$ vs. 0-h time). Changes in *Nab1* PT and mRNA after GNRH were modest and only significant at 120 min (1.9- and 1.7-fold, respectively; $P < 0.05$), whereas *Nab2* PT increased 2- to 3.5-fold 45–120 min after GNRH, and mRNA increased 5-fold at 120 min ($P < 0.05$). The large changes in *Egr1* gene expression reflected similar increases in EGR1 protein levels; GNRH increased EGR1 protein 13- and 4.7-fold at 45 and 120 min, respectively ($P < 0.05$; Fig. 4C).

We have reported that the MAPK8/9 inhibitor SP600125, but not the MAPK14 inhibitor SB203580, abolished the *Lhb* transcriptional response to pulsatile GNRH in perifused rat pituitary cells [13]. To determine whether blockade of *Lhb* PT reflects altered pituitary *Egr1* gene expression, GNRH pulses (every 60 min for 24 h) with or without SP or SB were given to perifused rat pituitary cells, and *Egr1*, *Nab1*, and *Nab2* PTs and

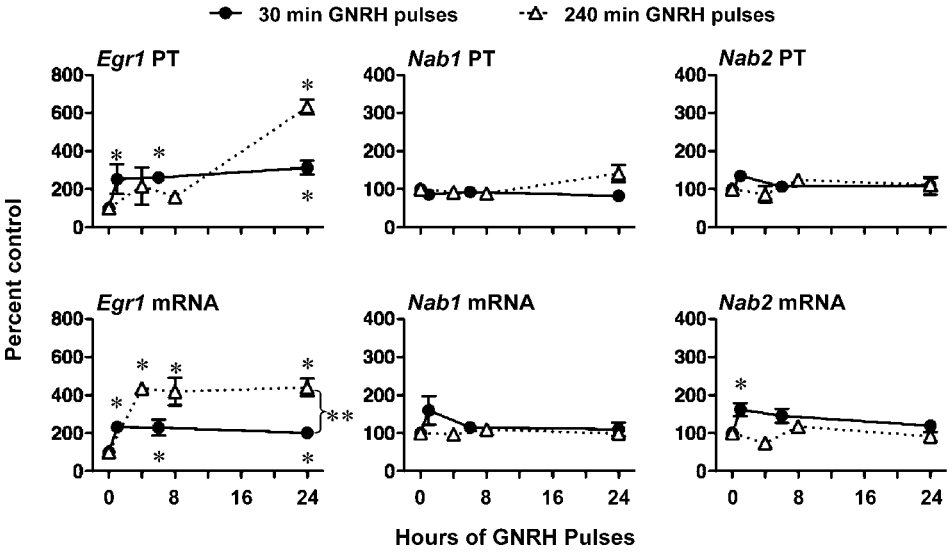

FIG. 1. The effect of GNRH pulse frequency in vivo on *Egr1*, *Nab1*, and *Nab2* PTs and mRNAs. Rats received 25-ng GNRH pulses either every 30 min or 240 min for 1–24 h (vehicle pulses to controls; n = 4–6 per group). Pituitaries were collected 5 min after the final pulse, and RNA was extracted. Primary transcripts or steady-state mRNAs were measured by quantitative real-time PCR. Data are expressed as percent 0-h controls ± SEM. Data were analyzed by two-way ANOVA with frequency and time as main effects. Basal levels (femtograms of plasmid/nanograms of RNA) for *Egr1* PT and mRNA are 0.35 ± 0.06 and 34.0 ± 1.4; *Nab1* PT and mRNA are 0.07 ± 0.01 and 0.8 ± 0.1; and *Nab2* PT and mRNA are 0.70 ± 0.07 and 14.8 ± 1.01. *Significant difference ($P < 0.05$) vs. controls (0 h). **Significant difference between GNRH pulse-frequency paradigms at 24 h.

mRNAs were measured (Fig. 5). *Egr1* PT and mRNA increased 2.5-fold and 1.5-fold, respectively, in GNRH-treated cells ($P < 0.05$ vs. controls). The SP blocked the *Egr1* PT and mRNA responses to pulsatile GNRH, but SB had no effect. *Nab1* and *Nab2* PTs and mRNAs were not different from vehicle controls after either GNRH or either inhibitor treatment, but *Nab2* mRNA was greater in SP plus GNRH-treated cells vs. SP-only cells ($P < 0.05$). GNRH regulation of the *Gnrhr* gene has been reported to be MAPK8/9 dependent, because GNRH-induced *Gnrhr* promoter activity was blocked in αT3 cells stably expressing a dominant-negative MAP2K4 (also known as MKK4/JNKK [45]). To determine whether the effects of MAPK8/9 blockade on *Egr1* gene expression are indirect (i.e., via downregulation of Gnrhr), we measured *Gnrhr* mRNA. Twenty-four hours of GNRH pulses increased Gnrhr mRNA 2.5-fold ($P < 0.05$ vs. vehicle controls), but neither MAPK8/9 nor MAPK14 blockade altered basal or GNRH-induced increases in *Gnrhr* mRNA (data not shown).

Evidence in gonadotroph-derived cell lines [36, 46–49], and recently in pituitary-specific MAPK1/3 (ERK2/1) knockout mice [50], suggests that MAPK1/3 regulation of EGR1 is fundamental for *Lhb* gene expression. To determine the importance of MAPK1/3 in the regulation of *Egr1* gene expression, and hence *Lhb*, we examined the effects of GNRH pulses with or without the MAP2K1 inhibitor PD in primary rat pituitary cells. For comparison, we also measured the effects of GNRH with or without the MAPK8/9 inhibitor SP. GNRH (three pulses, every 60 min, cells recovered 10 min after the last pulse) increased *Lhb* PT 2-fold and *Egr1* PT 1.5-fold ($P < 0.05$; Fig. 6). MAP2K1 blockade by PD reduced both basal *Egr1* PT and mRNA and completely abrogated the GNRH-induced increases in *Lhb* and *Egr1* PT. MAPK8/9 blockade by SP also suppressed both basal *Egr1* PT and mRNAs but, in contrast to our data in perifused rat pituitary cells after 24 h of treatment (Fig. 5), short-term SP only partially suppressed the GNRH induction of *Lhb* and *Egr1* PTs. As seen earlier, GNRH had little effect on *Nab* gene expression in rat pituitary cells, although GNRH did increase *Nab2* mRNA 1.5-fold ($P < 0.05$). Nonetheless, both inhibitors suppressed basal *Nab1* PT and GNRH-induced increases in *Nab2* mRNA. PD also suppressed basal *Nab2* PT and mRNA ($P < 0.05$).

Previously, we found that both MAPK8/9 and MAP2K1 blockades influence *Fshb* gene expression after 24 h in perifused rat pituitary cells [9, 13], so we also examined the effects of SP and PD on *Fshb* PT in this short-term treatment model. GNRH pulses increased *Fshb* PT 2.3-fold. Both SP and PD suppressed basal *Fshb* PT ($P < 0.05$ vs. controls), but GNRH induction was not impaired. The suppression of basal *Fshb* PT by MAP2K1 blockade may be indirect, at least in part, because PD increased basal *Fst* mRNA 1.5-fold vs. vehicle controls ($P < 0.05$), whereas SP had no effect on either basal or GNRH-induced *Fst* mRNA (data not shown).

Because MAPK1/3 appeared to play a major role in *Egr1* gene expression, and we have previously shown that androgens increase pituitary MAPK1/3 activation [43], we determined whether the levels of testosterone (500 pg/ml) added to cultured rat pituitary cells (required to allow *Lhb* gene responses to pulsatile GNRH [40]) affect *Egr1* expression. After 24 h of androgen (testosterone or dihydrotestosterone)

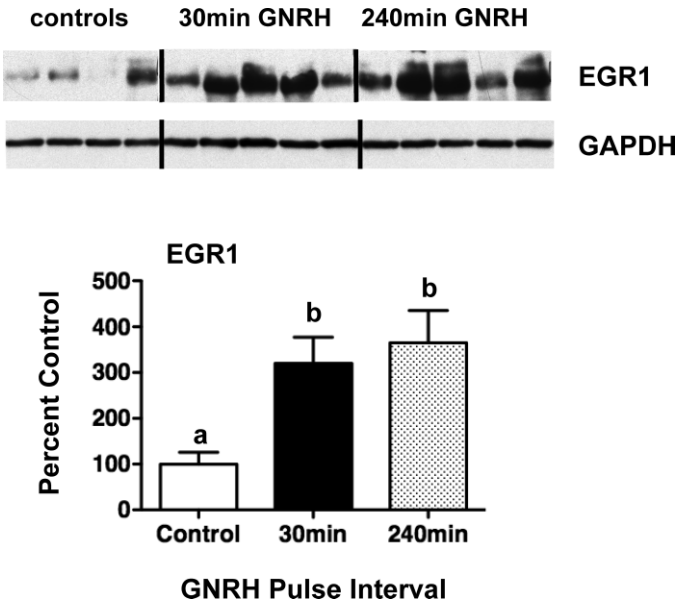

FIG. 2. The effects of GNRH pulse frequency on EGR1 protein in vivo. Top: Representative Western blots of pituitary protein from rats pulsed with GNRH either every 30 min or 240 min for 8 h (n = 4–5 per group). Blots were immunostained for EGR1 and GAPDH. Protein amounts are 20 μg per lane. Bottom: Changes in EGR1 densitometry normalized to GAPDH for protein loading and expressed as percent 0-h (±SEM) controls. Points with different letters are significantly different ($P < 0.05$).

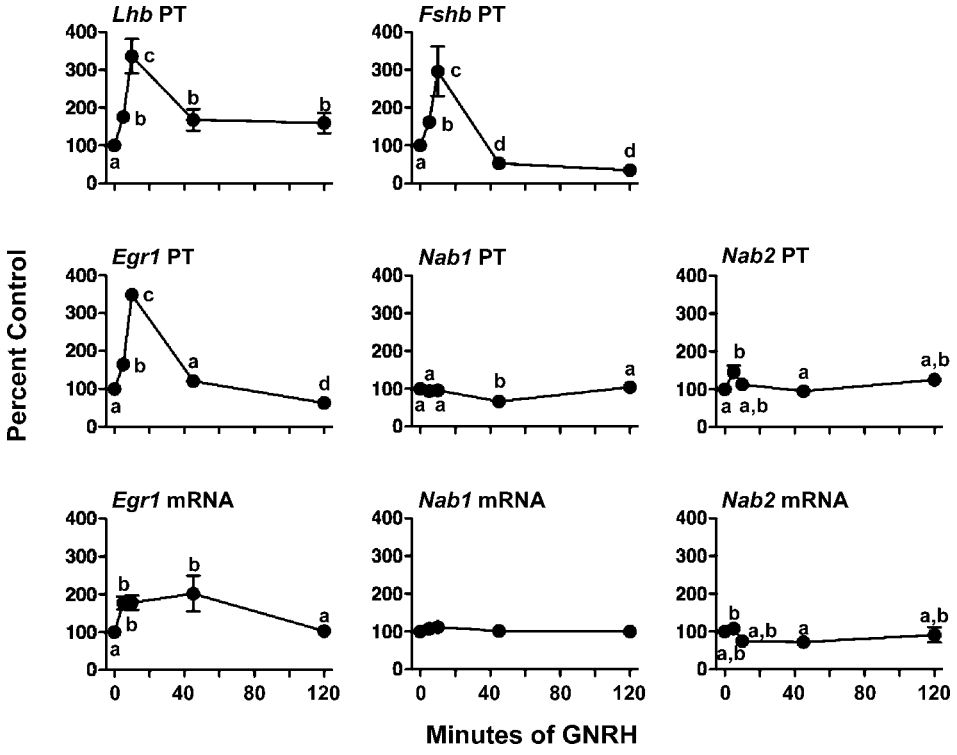

FIG. 3. The effects of GNRH on *Lhb* and *Fshb* PTs, and *Egr1*, *Nab1*, and *Nab2* PTs and mRNAs in cultured rat pituitary cells. Rat pituitary cells received two prepulses of GNRH (1 nM; media pulses to controls; 60-min interval). One hour later, cells were treated with 1 nM GNRH for 5–120 min (media to 0-h time point). Data are presented as percent (±SEM) of 0-h control (n = 4–5 per group). Groups marked with different letters are statistically different ($P < 0.05$). Basal levels (femtograms of plasmid/nanograms of RNA) for *Lhb* PT and *Fshb* PT are 0.59 ± 0.02 and 57.6 ± 2.9; *Egr1* PT and mRNA are 4.7 ± 0.9 and 230.2 ± 22.6; *Nab1* PT and mRNA are 0.15 ± 0.01 and 2.4 ± 0.2; and *Nab2* PT and mRNA are 0.93 ± 0.06 and 24.2 ± 2.6.

treatment, *Egr1* PT and mRNA levels were no different compared with cells in steroid-free media (data not shown).

Finally, to determine both efficacy and specificity of the inhibitors, we examined the effects of MAPK8/9 and MAP2K1 blockade on basal and responses to GNRH (cells recovered 10 min after GNRH pulse; Fig. 7) for EGR1, phosphorylated p42/p44 MAPK1/3, and phosphorylated JUN protein levels. Significant stimulatory responses to GNRH were not seen (Fig. 7), which was anticipated based on previous time course studies showing that 10 min after GNRH is a little early to observe effective increases in EGR1 expression or MAPK1/3 and JUN phosphorylation [37, 41]. The SP tended to decrease basal EGR1 protein, but it did not reach significance. In contrast, PD reduced EGR1 protein to nearly undetectable levels ($P < 0.05$). The PD suppressed MAPK1/3 phosphorylation 65%–75% ($P < 0.05$). Of note, SP also partially suppressed MAPK1/3 phosphorylation (approximately 25%–30%; $P < 0.05$). The cross-reactivity of SP to inhibit MAPK1/

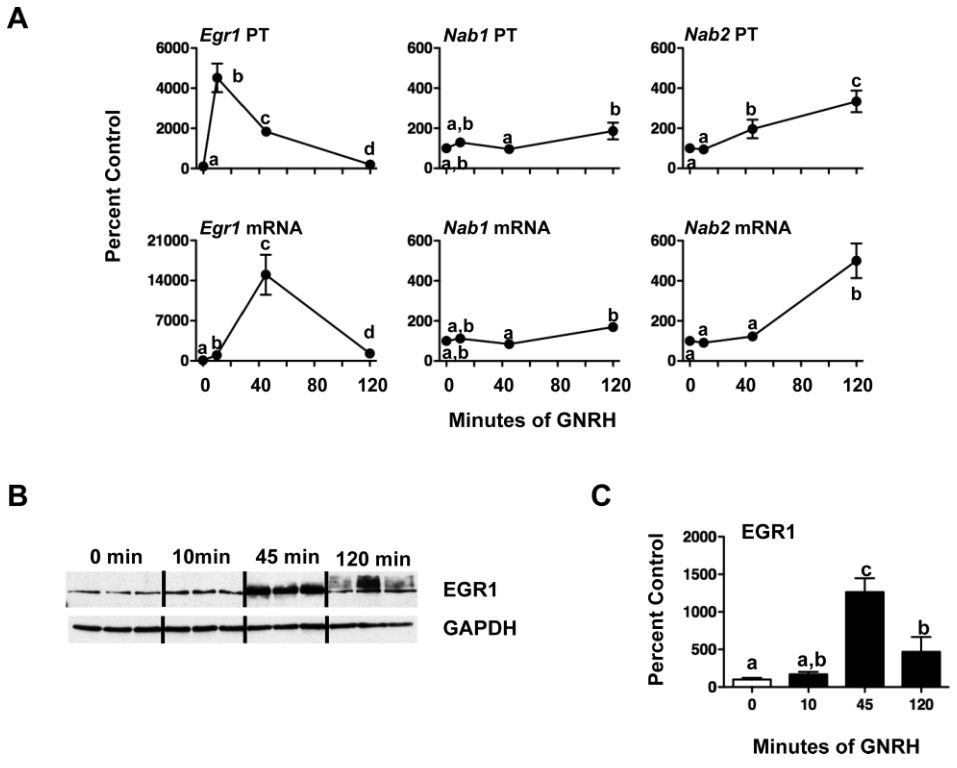

FIG. 4. The regulation of *Egr1*, *Nab1*, and *Nab2* transcripts and EGR1 protein in αT3 cells. **A**) Gonadotroph-derived αT3 cells were treated with 10 nM GNRH for 10–120 min. After completing the study, cells were recovered, and PT/mRNA levels were determined. Data are presented as percent (±SEM) control (n = 6 per group). Groups marked with different letters are statistically different ($P < 0.05$). Basal levels (femtograms of plasmid/nanograms of RNA) for *Egr1* PT and mRNA are 0.06 ± 0.01 and 3.4 ± 0.3; *Nab1* PT and mRNA are 0.021 ± 0.002 and 7.4 ± 0.7; and *Nab2* PT and mRNA are 0.004 ± 0.001 and 1.2 ± 0.1. **B**) Representative Western blots of protein from gonadotroph-derived αT3 cells treated as in **A** (n = 3 of 6 per group). Blots were immunostained for EGR1 and GAPDH. Protein amounts are 10% of culture lysate. **C**) Changes in EGR1 densitometry normalized to GAPDH for protein loading and expressed as percent 0-h (±SEM) controls. Points with different letters are significantly different ($P < 0.05$).

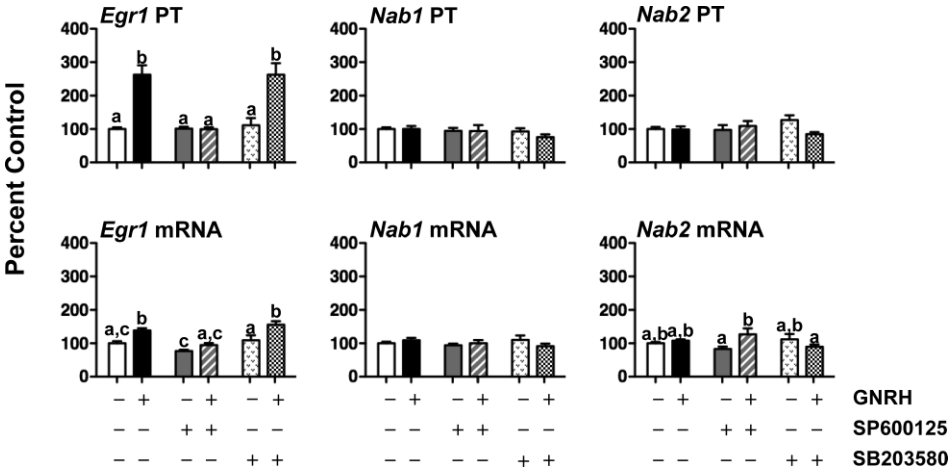

FIG. 5. The effects of MAPK8/9 and MAPK14 blockade on *Egr1*, *Nab1*, and *Nab2* PTs and mRNAs. Cultured rat pituitary cells received pulses of GNRH (200 pM; media pulses to controls; 60-min interval) in the presence of the MAPK8/9 blocker (SP600125; 20 μM), MAPK14 blocker (SB203580; 20 μM), or vehicle for 24 h. After completing the study, pituitary cells were recovered, and *Egr1*, *Nab1*, and *Nab2* PTs and mRNAs were measured (n = 6 per group). Data are presented as percent (±SEM) control. Groups with different letters are statistically different ($P < 0.05$). Basal levels (femtograms of plasmid/nanograms of RNA) for *Egr1* PT and mRNA are 2.7 ± 0.2 and 82.0 ± 5.8; *Nab1* PT and mRNA are 0.09 ± 0.01 and 1.1 ± 0.1; and *Nab2* PT and mRNA are 0.8 ± 0.1 and 29.0 ± 1.2.

3 has been noted previously [51]. Of note, SP specifically reduced cJUN phosphorylation by approximately 60% ($P < 0.05$), whereas PD had no effect.

DISCUSSION

These results expand our previous investigations into the mechanisms responsible for differential regulation of *Lhb* and *Fshb* transcription by GNRH pulses. We reported previously that in vivo GNRH pulses every 30 min preferentially increased *Lhb* PT levels and also stimulated a transient increase in FSHB transcription, which declined to basal levels coincidentally with an increase in *Fst* mRNA. In contrast, GNRH pulses every 240 min maximally increased *Fshb* PT, which was correlated with the suppression of *Fst* mRNA but had either a modest or no effect on *Lhb* PT [10, 20]. These and other data suggest that pulsatile GNRH acts on the gonadotroph both directly and indirectly to regulate *Fshb* transcription, but its actions on *Lhb* remain unclear. Because EGR1 has been shown to be critical for *Lhb* transcriptional responses to GNRH, we examined whether GNRH pulse frequency regulates EGR1 and its corepressors NAB1 and NAB2.

In rat pituitary cells, we found that *Egr1* responded to GNRH with a rapid but short-duration burst of transcription, and *Egr1* mRNA increased during 45 min; in perifused rat pituitary cells, *Egr1* expression continued to respond to pulsatile GNRH (every 60 min) for at least 24 h. In vivo, fast-frequency GNRH pulses (every 30 min) maintained a rise in both *Egr1* PT and steady-state mRNA levels during 24 h, whereas slow-frequency GNRH pulses (every 240 min) only increased *Egr1* transcription after 24 h. Surprisingly, a GNRH-induced increase in *Egr1* mRNA was seen earlier (beginning at 4 h), suggesting that slow-frequency GNRH pulses increase *Egr1* mRNA via a nontranscriptional mechanism, such as increased mRNA stability. The regulation of *Egr1* mRNA by RNA stability has been reported in several cell types [52–56]. The half-life of the *Egr1* mRNA is approximately 30 min, but it increased to 70 min when protein synthesis was inhibited with cyclohexamide, indicating posttranscriptional regulation of *Egr1* mRNA by a labile protein [52]. The *Egr1* mRNA has a lengthy 3′ untranslated region (3′ UTR; >1000 bp) containing

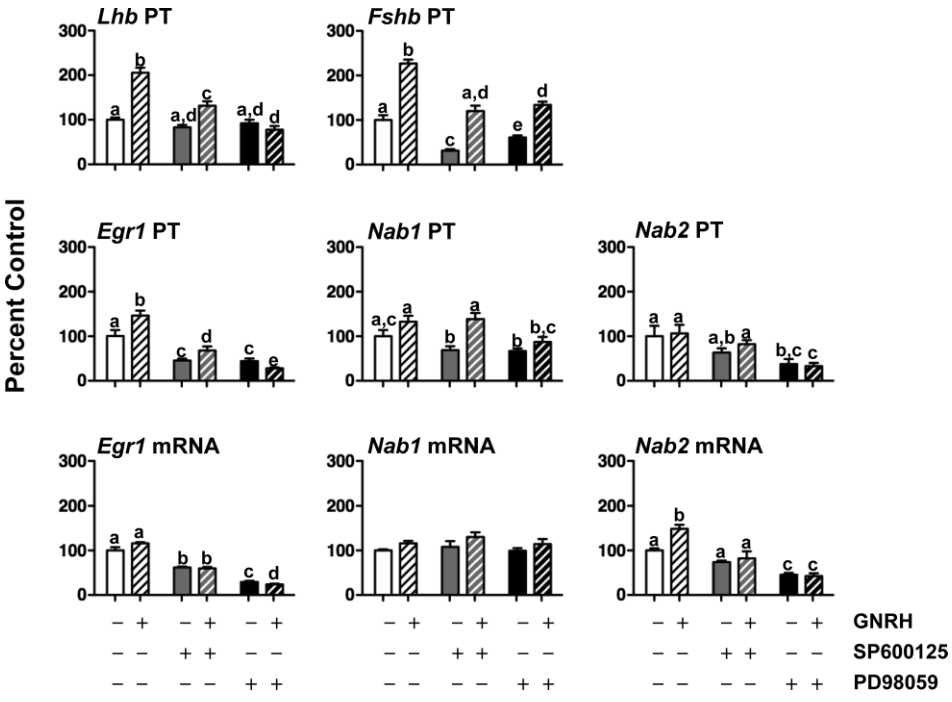

FIG. 6. The effects of MAPK8/9 and MAPK1/3 blockade on *Egr1*, *Nab1*, and *Nab2* PTs and mRNAs. Rat pituitary cells were pretreated with: MAPK8/9 blocker (SP600125; 20 μM), MAP2K1 blocker (PD98059; 50 μM), or vehicle for 1 h. Then, cells received two prepulses of GNRH (1 nM; media pulses to controls; 60-min interval). One hour later, cells were treated with 1 nM GNRH for 10 min (media to controls). After completing the study, pituitary cells were recovered, and *Lhb* PT, *Fshb* PT, and *Egr1*, *Nab1*, and *Nab2* PTs and mRNAs were measured (n = 4 per group). Data are presented as percent (±SEM) control. Groups marked with different letters are statistically different ($P < 0.05$). Basal levels (femtograms of plasmid/nanograms of RNA) for *Lhb* and *Fshb* PTs are 0.25 ± 0.01 and 63.2 ± 7.0; *Egr1* PT and mRNA are 3.3 ± 0.5 and 153.1 ± 11.2; *Nab1* PT and mRNA are 0.13 ± 0.02 and 3.5 ± 0.1; and *Nab2* PT and mRNA are 0.60 ± 0.14 and 25.4 ± 1.2.

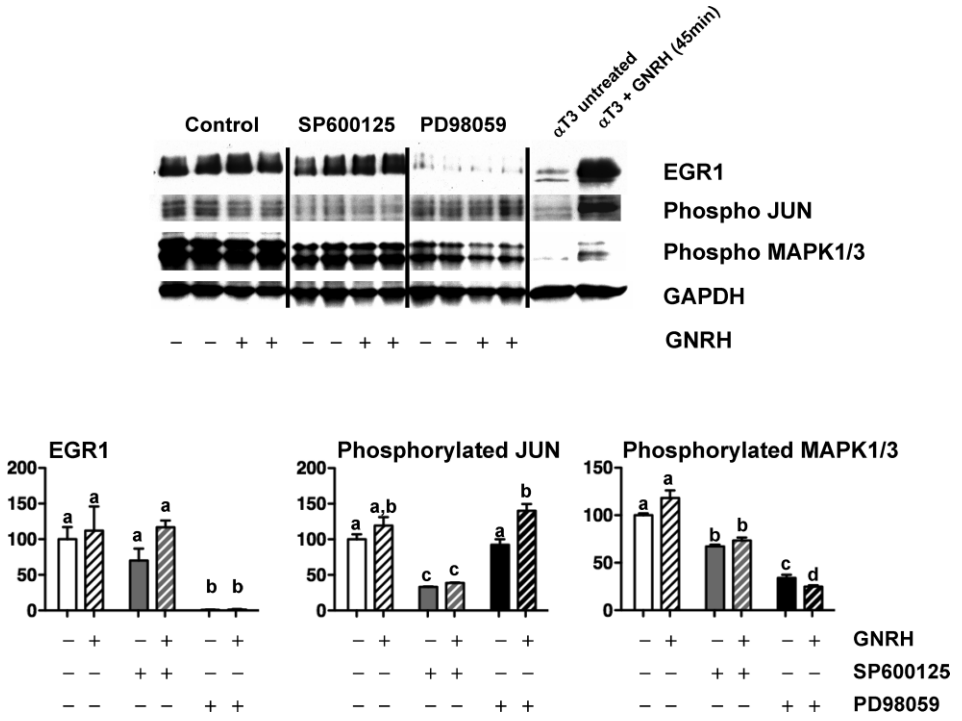

FIG. 7. The effects of MAPK8/9 and MAPK1/3 blockade on EGR1, phosphorylated cJUN, and phosphorylated MAPK1/3. Top: Representative Western blots of protein from rat pituitary cells that were treated as in Figure 6 (n = 4 per group). Blots were immunostained for EGR1, phosphorylated cJUN, phosphorylated MAPK1/3, and GAPDH. Untreated αT3 protein lysates or αT3 cells plus 50 nM GNRH for 45 min were included as negative and positive controls, respectively. Protein amounts are 10% of culture lysate. Bottom: Changes in EGR1, phosphorylated cJUN, and phosphorylated MAPK1/3 densitometry normalized to GAPDH for protein loading and expressed as percent (±SEM) control. Points with different letters are significantly different ($P < 0.05$).

several elements that are known to regulate mRNA stability, such as adenosine/uracil (AU)-rich elements, polypyrimidine tracts, cold shock domain sequences (CDSs), and alternative polyadenlyaltion sites [57, 58]. Chauvin and Nilson [58] have reported that although GNRH does not affect *Egr1* mRNA stability per se in LβT2 cells, the 3′ UTR-enhanced GNRH induction of a luciferase reporter and treatment with a CDS siRNA attenuated GNRH induction of *Lhb* mRNA.

GNRH pulses stimulated an increase in EGR1 protein, but an effect of pulse frequency was not seen. However, we cannot exclude the possibility that posttranslational effects of GNRH pulse frequency on EGR1 may play a role in *Lhb* gene regulation. EGR1 is regulated by a number of mechanisms that can alter transactivational activity and/or protein turnover. EGR1 is Ser/Thr phosphorylated by a number of kinases, most notably casein kinase II [59, 60], PKC [60], and AKT1 [61]. Inhibitors of protein phosphatases 1 and 2A also increase EGR1 phosphorylation [62]. The function of phosphorylated EGR1 is uncertain and has been reported to either enhance [62, 63] or inhibit [59, 60] EGR1′s ability to bind and transactivate DNA directly or via other transcription factors. Lysine residues on EGR1 are also posttranslationally modified by acetylation, sumoylation, and/or ubiquitination. EGR1 acetylation increases EGR1 protein stability [64], and sumoylated EGR1 induces the tumor suppressor phosphatase and tensin homologue [61]. Multiubiquitinated EGR1 associates with proteosome complex 8 and is targeted for degradation via the ubiquitin-dependent proteosome pathway, and proteosome blockade results in increased EGR1 accumulation [65]. Recently, Walsh and Shupnik [66] have suggested that ubiquitination and degradation of EGR1 by the proteasome are critical for *Lhb* transcription. When proteasome activity was inhibited in LβT2 cells, GNRH induction of an *Lhb* promoter reporter was suppressed, and both *Egr1* mRNA and protein levels accumulated to high levels. They hypothesized that proteasome activity in the gonadotroph allows for degradation and clearing of transcription factors—in this case EGR1—from the *Lhb* promoter to initiate the next round of transcription. Perhaps slow-frequency GNRH pulses do not stimulate ubiquitination and/or proteasome-directed degradation of EGR1, resulting in high levels of *Egr1* mRNA and protein and relatively low levels (vs. fast frequency) of *Lhb* transcription.

The mechanism(s) by which GNRH regulates *Egr1* expression are not fully known. The rodent *Egr1* promoter has five serum response elements (SREs; a distal group of three and a proximal group of two), a cAMP response element (CRE), and an AP1 site [46, 67]. The distal group of SREs contributes the majority of the *Egr1* promoter responsiveness to GNRH [46], but mutation of the CRE or expression of a dominant-negative CRE-binding protein (CREB) also reduced GNRH induction of *Egr1* expression [46, 49]. The role of the AP1 site in GNRH regulation of the *Egr1* promoter has not been examined. The SREs bind serum response factor and recruit binding of ternary complex factors, such as the Ets protein, ELK1 [24]. Expression of a dominant-negative ELK1 blocks induction of EGR1 protein by a GNRH agonist in αT3 cells [49]. Also of note, ELK1 is a substrate for all three MAPK pathways [68], and as such connects MAPK signaling to SRE-mediated transcription of the *Egr1* gene.

Several laboratories have reported that the *Egr1* transcriptional response to GNRH is PKC dependent [32, 34, 69]. Further, *Egr1* promoter activity or EGR1 protein levels in αT3 and LβT2 cells can be suppressed by MAPK1/3 blockade [36, 46, 49]. Recently, Bliss et al. [50] reported that pituitary-specific *Mapk1/3* (ERK2/ERK1) double-knockout mice result in female, but not male, infertility, with reduced basal and GNRH-stimulated *Egr1* gene expression and a loss of LHB biosynthesis. Prior to the development of this mouse model, a regulatory link between *Lhb* and the MAPK1/3 pathway was not well established in primary pituitary cells. In fact, we and others found that MAP2K1 blockade did not suppress *Lhb* mRNA expression or promoter activity in response to GNRH [9, 70]. However, in the present study we report that PD98059 completely blocked GNRH induction of *Lhb* PT, *Egr1* PT and mRNA, and EGR1 protein in primary rat pituitary cells. The explanations for the differences between this and our former studies are uncertain but may be related to the efficacy of blockade during the treatment duration (3 h vs. 24 h) or *Lhb*

gene expression markers measured (PT vs. steady-state mRNA). In previous studies, we measured the effects of MAP2K1 blockade on *Lhb* mRNA expression in perifused rat pituitary cells given GNRH pulses every 60 min for 24 h. It is possible that the actions of PD on *Lhb* expression are transient, and/or that MAP2K1 blockade can be compensated for by another signaling pathway(s), after an extended duration of GNRH pulse treatment. In previous experiments, we determined the effects of PD plus GNRH on steady-state *Lhb* mRNA by Northern dot blotting vs. measuring *Lhb* PT using quantitative real-time PCR. Thus, PD may suppress *Lhb* transcription but not significantly deplete the pool of steady-state mRNA, which has a much longer half-life [71].

We have also reported that MAPK8/9 blockade by either the inhibitor SP600125 or the dominant negatives of MAP2K4 and/or 7 (also known as MKK4/7) inhibited the *Lhb* transcriptional response to GNRH in both perifused rat pituitary cells (24-h treatment duration) and LβT2 cells (6-h treatment duration [13]), whereas MAPK14 blockade had no effect. Here, we report that GNRH induction of *Egr1* PT and mRNA in perifused rat pituitary cells is also MAPK8/9, but not MAPK14, dependent; however, in shorter-duration (3 h) studies, SP was only partially effective in suppressing GNRH-induced increases in *Lhb* and *Egr1* PTs. We acknowledge that blocking MAPK8/9 activity with chemical inhibitors is not completely specific, and kinase inhibition by SP is not limited to MAPK8/9 [51]. Indeed, we observed that 3 h of SP treatment also partially suppressed MAPK1/3 activation (Fig. 7), although it was quite specific for inhibiting cJUN phosphorylation. However, the present results and our earlier data in LβT2 cells using dominant-negative MAP2K4 and MAP2K7 proteins [13] support a role for GNRH-induced MAPK8/9 activation in *Egr1*-dependent *Lhb* transcription. Furthermore, MAPK8/9 has been implicated in the regulation EGR1 in several other cell types [72–76].

The ability of EGR1 to transactivate target genes may be altered by expression of its corepressors NAB1 or NAB2. NAB1 is constitutively expressed in most cell types [24, 77], whereas NAB2 is induced by the same stimuli as EGR1, but responses are often delayed [78]. The human *NAB2* promoter contains 11 EGR1-binding sites and is strongly stimulated by EGR1, suggesting that EGR1 induction of *NAB2* is a negative-feedback circuit to regulate EGR1 activity [79]. In LβT2 cells, a single GNRH pulse increased *Nab1* and *Nab2* mRNAs 2- to 4-fold 1–4 h after pulse [37]. We saw similar responses to GNRH for *Nab2* gene expression in αT3 cells, whereas *Nab1* was largely unaffected. In contrast to LβT2 or αT3 cells, *Nab1* and *Nab2* transcripts showed little change after GNRH either in vivo or in cultured rat pituitary cells, indicating that GNRH does not significantly regulate EGR1 bioavailability via the NAB proteins in normal rat pituitary cells.

In conclusion, these findings reveal that pulsatile GNRH increases *Egr1* transcription both in vivo and in cultured rat pituitary cells, but not in a frequency-dependent manner and not to the degree seen in gonadotroph-derived αT3 cells. Additionally, GNRH induction of *Egr1* transcription and mRNA expression is dependent on MAPK8/9 and MAPK1/3 but not on MAPK14. These results suggest that both MAPK8/9 and MAPK1/3 are critical regulators of *Lhb* gene expression, in part by mediating the *Egr1* transcriptional response to pulsatile GNRH.

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