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Egr-1 Binds the GnRH Promoter to Mediate the Increase in Gene Expression by Insulin

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SUMMARY

Insulin increases gonadotropin-releasing hormone (GnRH) gene expression in *in vitro* models of GnRH neurons. Early growth response-1 (Egr-1) is a transcription factor that mediates the effect of insulin on target genes. In the GN11 cell line—an immortalized GnRH-secreting neuronal cell line—insulin maximally increases Egr-1 mRNA after 30 minutes of treatment and Egr-1 protein and GnRH mRNA after 60 minutes of treatment. Egr-1 small interfering RNA blocks the insulin-induced increase in GnRH promoter activity, measured as luciferase expression. Chromatin immunoprecipitation using Egr-1 antibody precipitates DNA in a proximal region of the GnRH promoter but not DNA in a distal region. Mutagenesis of a putative Egr-1 binding site within the proximal region blocks the insulin-induced increase in GnRH promoter activity. Thus, Egr-1 binds the GnRH promoter at a site between –67 and –76 bp from the transcriptional start site to mediate the insulin induced increase in GnRH gene transcription.

Keywords

Egr-1; insulin; GnRH

Introduction

Reproductive maturation and fertility in mammals is made possible by the integration of extracellular signals and coordination of cellular events at the level of the hypothalamus, pituitary, and gonad. Nutritional status is an important determinant of reproductive capacity in mammals; chronic and short-term food restriction impedes reproduction (Schneider and Wade, 2000; Temple and Rissman, 2002). Nutritional signals that inhibit reproductive function during food restriction or are permissive during the fed state are thought to be integrated at the level of the hypothalamus (Bronson, 1988; Cameron and Nosbisch, 1991). Hypothalamic gonadotropin-releasing hormone (GnRH) neurons synthesize and release GnRH, the decapeptide that is the primary signal of the hormonal cascade regulating reproduction. Signals conveying a mammal's nutritional status, therefore act directly or indirectly upon GnRH neurons to affect GnRH synthesis or release.

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Insulin signaling in the central nervous system is necessary for activation of the reproductive axis in rodents. This was illustrated by the development of neuron specific insulin receptor knockout (NIRKO) mice. These mice are obese with elevated insulin levels, decreased fertility and lower luteinizing hormone (LH) levels than wild type mice. The LH levels increase with exogenous GnRH administration, suggesting that the impaired reproductive function is due to GnRH deficiency (Bruning et al., 2000). Our laboratory recently demonstrated that insulin can increase GnRH gene expression in a GnRH-expressing neuronal cell line, suggesting that the observed phenotype in NIRKO mice may be due to insulin acting directly on the GnRH neuron (Kim et al., 2005). These observations were corroborated in a study using a model of conditionally immortalized GnRH-secreting neurons derived from rat hypothalamus (Salvi et al., 2006). In both studies, insulin stimulates a mitogen-activated protein (MAP) kinase pathway but the intermediary transcription factors important to transduce insulin's effects were not elucidated.

Early growth response (Egr-1, also known as NGF-1A, Krox-24, zif268, or TiS8) is a member of the immediate early gene family; gene transcription is induced by stimuli in the absence of *de novo* protein synthesis. A variety of factors can stimulate Egr-1 gene expression, including cellular injury, radiation, and growth factors (Gashler and Sukhatme, 1995). Insulin regulates Egr-1 gene expression in a variety of tissues including hepatic, bone, pancreatic beta cells and vascular endothelial tissue (Barroso and Santisteban, 1999; Tan et al., 2003; Silverman and Collins, 1999; Leung-Theung-Long et al., 2005; Garnett et al., 2005). The induction of Egr-1 gene expression by insulin is primarily mediated by the MAP kinase pathway. PI3 and p38 kinase pathways have a modulating effect on Egr-1 transcription, but are not essential pathways for signal transduction (Keeton et al., 2003). The zinc finger DNA binding domain of Egr-1 binds GC rich regions of its target gene to increase or decrease transcription. Once bound to DNA, Egr-1 can interact with coactivators (Silverman et al., 1998), or corepressors (Swirnoff et al., 1998). Egr-1 target genes include platelet-derived growth factor (PDGF), p53, fibroblast growth factor (FGF), interleukin-2 (IL-2), and LH β (Silverman and Collins, 1999; Theil and Cibelli, 2002).

The importance of Egr-1 to reproduction was demonstrated by the generation of Egr-1 knockout mice (Lee et al., 1996; Topilko et al., 1998). These mice have low LH levels that do not increase after gonadectomy. The gonadotrophs of these mice are atrophic and do not synthesize LH. Egr-1 is involved in GnRH-dependent stimulation of LH β gene expression as was shown by several laboratories (Tremblay and Douin, 1999; Dorn et al., 1999; Russell et al., 2003; Mouillet et al., 2004). Although GnRH neurons were detected in the Egr-1 knockout mice, the function of these neurons was not examined.

In the present study, we describe the regulation of Egr-1 by insulin in the GN11 GnRH-producing neuronal cell line. The regulation of GnRH gene transcription and GnRH neuronal activity is difficult to study *in vivo* because of the scattered distribution and paucity of GnRH neurons. The GN11 cell line is an immortalized, GnRH-expressing neuronal cell line that was generated by targeting expression of the SV-40 Tag to GnRH neurons in transgenic mice with the human GnRH promoter (Radovick et al., 1991). These cells express GnRH mRNA, secrete GnRH decapeptide, express markers specific for differentiated neurons, and display a migratory phenotype (Maggi et al., 2000; Wolfe et al., 2002a). Although *in vitro* mechanisms described in immortalized cell lines may not represent events *in vivo*, the use of immortalized cell lines have allowed significant advances in knowledge of the mechanisms regulating GnRH synthesis and release. The GN11 cell line has been extensively used to investigate the cell-specific regulation of GnRH transcription (Radovick et al., 1994; Zhen et al., 1997b; Wolfe et al., 2002b; Givens et al., 2004; Givens et al., 2005; Kim et al., 2007) and to investigate the mechanisms of GnRH neuronal cell migration (Maggi et al., 2000; Allen et al., 2002; Giampietro et al., 2005). We previously reported that GN11 cells contain insulin receptors, bind insulin,

and that insulin stimulates GnRH promoter activity in these cells (Kim, HH et. al, 2005). Here, we extend these studies and demonstrate that in GN11 cells, Egr-1 binds the GnRH gene promoter to mediate the insulin stimulation of GnRH gene expression.

Materials and Methods

Cell Culture and Transfection

GN11 cells were maintained in DMEM supplemented with 10% FBS and 10 mM glutamine in an atmosphere with 5% CO₂ at 37 C. For the insulin induction experiments, cells were placed in serum free medium and the following morning, insulin was added at the indicated concentration and for the indicated duration.

Transient transfections were performed using the PLUS reagent and Lipofectamine (Invitrogen, Carlsbad, CA) in 6-well plates. The Dual Luciferase Assay System (Promega, Madison, WI) was used to normalize for transfection efficiency and cell number using a tk-renillan luciferase (tk-LUC) reporter plasmid. 0.2 µg of the GnRH reporter constructs was co-transfected with 0.02 µg of the tk-rLUC reporter. For the experiments using RNA interference, 0.2 µg of the siRNA plasmid was also added. If applicable, cells were treated with 10 µM PD98059 or 10 µM Wortmannin for 30 min prior to insulin treatment. After harvesting the cells, luciferase activity was assayed using the Lumat LB 9507 luminometer (Berthold). Experimental luminescence measurement was designated as relative light units (RLU1) and renilla luminescence was designated as relative light units 2 (RLU2). Normalization for efficiency was then calculated as a ratio of RLU1/RLU2. Values were reported as fold increase of relative luminescence when compared to cells treated with vehicle. Samples were run in triplicate and coefficients of variation (CV) between triplicates were lower than 20%.

Plasmid Cloning and Mutagenesis

A small interfering RNA (siRNA) oligonucleotide that successfully targeted Egr-1 mRNA for degradation was published for human microglial cells (Giri et al., 2003). Using software provided by Ambion (Austin, TX), oligonucleotides for the homologous mouse sequence were designed. The Egr-1 siRNA oligonucleotides were: Sense, 5'-GATCCGCATACCAAAATCCAATTATTCAAGAGATAATTGGATTTTGGTATGCCTT TTTTGGAAA-3'; antisense, 5'-AGCTTTTCCAAAAAAGGCATACCAAAATCCAATATCTCTTGAATAATTGGATTTT GGTATGCG-3'. These were inserted into the pSilencer 2.1 vector as suggested by the Ambion. An oligonucleotide that is not homologous to any known mammalian sequence (scrambled siRNA) was purchased from Ambion to use a control.

Mouse GnRH promoter deletion fragments were cloned into the pA3LUC reporter vector (Maxwell et al., 1989) as previously described (Wolfe et al., 2002b). Correct incorporation was confirmed by DNA sequencing. Site-directed mutations were generated with QuikChange kit from Stratagene (La Jolla, CA) and confirmed by DNA sequencing.

Quantitative PCR

Total RNA was harvested from GN11 using Trizol reagent (Invitrogen) following manufacturer's instructions. One microgram of RNA was then reverse transcribed using the iScript cDNA kit (BioRad, Hercules, CA). Real-time quantitative PCR was performed in duplicate using the SyberGreen MasterMix (BioRad) and the ICycler quantitative PCR machine (BioRad). 18S RNA was selected as internal control for cDNA input. The following primers were used: Egr-1 sense 5'ACTACCCCAAACCTGGAGGA GA-3'; antisense 5'-CTTGAGGATTGAAGGCGCTG-3'; 18S sense 5'-CGGCTACCACATCCAAGGAA-3'; antisense 5'-CTGGAATTACCGCGGCT-3'; and GnRH

sense 5' CCCTTTGACTTTCACATCC-3'; antisense 5'GGGTTCTGCCATTTGATCCAC-3'. To determine PCR efficiency, 10 fold serial dilution of cDNA was used as described previously (Wong and Medrano, 2005). PCR conditions were optimized to generate > 95% PCR efficiency and only those reactions with between 95-105% efficiency were included in subsequent analyses. Relative differences in cDNA concentration between baseline and test conditions were then calculated using the comparative threshold cycle (Ct) method (Bustin et al., 2005). Briefly, for each sample, a Δ CT was calculated to normalize for the internal control using the equation: Δ CT= CT(gene)– CT(18S). To obtain differences between experimental and control conditions, $\Delta\Delta$ CT was calculated: Δ CT(sample) – Δ CT(control). Relative mRNA levels were then calculated using the equation fold difference = $2^{\Delta\Delta C_t}$.

Western Blot Analysis

Nuclear extracts of GN11 cells were obtained using a mini-extract procedure (Schreiber et al., 1989). Extracts were size fractionated by SDS-PAGE on an 8% resolving gel and transferred to Protran (Schleicher and Schuell) nitrocellulose membrane by semi-dry transfer. Antibody specific for Egr-1 (Santa Cruz Biotechnologies, Santa Cruz, CA) was used. Antibody binding was visualized using the ECL Plus reagent (Amersham Pharmacia, Piscataway, NJ).

Chromatin Immunoprecipitation

After insulin treatment, cells were cross linked by adding formaldehyde to a final concentration of 1 % directly to media for 10 minutes, harvested, then lysed in SDS buffer for 10 min on ice. Sonication was then performed using the Fisher Model 500 Model Dismembrator (Pittsburgh, PA). Sonication time was optimized to yield DNA fragments between 100 and 500 bp. DNA was precleared with 80 μ L of Protein A agarose beads (Amersham Pharmacia) supplemented with 0.1 mg/mL of salmon sperm DNA and 1 mg/mL of bovine serum albumin. After one hour, the supernatant was collected and incubated overnight with 10 μ g of Egr-1 antibody or without antibody. Protein-DNA complexes were then collected by incubating the sample with 60 μ L of Protein A agarose beads supplemented with 5 μ g of Salmon Sperm DNA and 10 μ g of BSA for two hours. The beads were then rinsed consecutively with high salt, low salt, LiCl, and TE buffers. DNA was eluted from the beads by adding SDS plus 1 mM NaCO₃ solution. After elution, the DNA crosslinks were reversed by incubating the solution in 3M NaCl overnight. Proteinase K was added at 55 c for one hour then DNA was phenol/chloroform extracted and ethanol precipitated using yeast tRNA as a carrier. Quantitative PCR was performed as detailed above using the following primers to amplify a 200 bp region in the proximal GnRH promoter, sense 5'-GACCAGCAGGTGTTGCAAT-3', Antisense, 5'-TCGAGCTTCCGTTGGTAGGT-3' or a 150 bp region of in the distal GnRH promoter, sense 5'-CAACCATCTCTAGTGGGATC-3', antisense 5'-GCCTCGAACTCAGAAATCTG-3'. Relative DNA levels were calculated using the $\Delta\Delta$ Ct method with input DNA as internal control (Chakrabarti et al., 2003).

Statistical Analysis

All results are expressed as mean $\pm$ SEM. Statistical significance was assessed by one-way or two-way ANOVA using the GraphPad Prism 4 program (San Diego, CA), with P<0.05 assigned as significant. Post hoc analysis was conducted using Tukey's multiple comparison test.

RESULTS

Insulin induction of Egr-1 requires activation of a MAP kinase pathway

To determine the effect of insulin on Egr-1 gene expression, relative Egr-1 messenger RNA levels were determined using quantitative real time PCR. As shown in Figure 1A, treatment

of GN11 cells with 30 nM of insulin for 30 minutes resulted in a robust, nearly 20 fold increase in Egr-1 messenger RNA levels whereas treatment of cells with insulin up to concentration of 100 nM resulted in a similar induction of Egr-1. Insulin at a concentration of 30nM was chosen for subsequent studies in order to maximize Egr-1 induction and minimize activation of the IGF-1 receptor as insulin at high concentrations such as 100nM can bind to and activate the IGF-1 receptor (Steele-Perkins et al., 1988). Next, GN11 cells were treated with 30nM of insulin to generate a time course of insulin effect (Figure 1B). Egr-1 mRNA levels increased 20 fold 30 minutes after treatment. Sixty minutes of insulin treatment resulted in an over 5 fold increase in Egr-1 messenger levels indicating that insulin treatment leads to a robust and transient induction of Egr-1 transcription. An increase in Egr-1 protein levels (1.5-fold) occurred 30 minutes after insulin treatment, with a 2.0 fold increase in protein levels 60 minutes after insulin stimulation (Figure 1C).

We previously demonstrated that insulin exerts its effect on GnRH promoter activity via a MAP kinase pathway (Kim et al., 2005); in other cell systems, insulin mediated induction of Egr-1 is dependent upon activation of the Ras/Raf/MAP kinase pathway (Keeton et al., 2003). In order to investigate whether the effect of insulin on Egr-1 is mediated by the MAP kinase pathway in the GN11 cells, cells were pre-treated with PD98059--a specific inhibitor of MEK, the upstream regulator of MAP kinase—then treated with insulin. Similar to previous results, there was a nearly 20 fold increase in Egr-1 mRNA levels 30 minutes after insulin treatment (Figure 2). However, after pre-treatment with PD98029, there was a complete inhibition of insulin-induced Egr-1 transcription. In contrast, the PI3 kinase pathway inhibitor wortmannin did not significantly affect insulin induction of Egr-1 transcription. Insulin thus activates Egr-1 transcription via a Ras/Raf/MAP kinase signaling pathway.

Insulin increases GnRH mRNA levels in GN11 cells

We sought to determine whether insulin can increase GnRH mRNA levels in the GN11 cell line. Because GN11 cells have been shown to produce a GnRH RNA splice variant with exon 2 omitted (Zhen et al., 1997a), PCR primers were located at the very end of exon 1 and beginning of exon 3 such that only an amplicon that included exon 2 would be detectable. Insulin at a dose of 30 nM for one hour resulted in a nearly four fold increase in GnRH mRNA levels compared to untreated cells (Figure 3A). Although treatment with 100 nM of insulin resulted in as nearly a robust increase in GnRH mRNA levels, 30 nM of insulin was used for subsequent experiments in order to maximize response and minimize activation of IGF-1 receptor pathways. GnRH mRNA levels peaked after one hour of 30nM of insulin then declined slightly after two hours of treatment (Figure 3B). GnRH mRNA levels increased slightly after 30 minutes of insulin treatment; results were not statistically different from mRNA levels of untreated cells.

Egr-1 is necessary for the insulin-induced increase in GnRH promoter activity

Small interfering RNA specific to the human Egr-1 gene has been used by others to effectively knockdown Egr-1 mRNA levels (Giri et al., 2003). As shown Figure 4A, insulin treatment results in a 2.6 fold increase in Egr-1 protein that is not affected by the presence of sequence absent in the mouse genome (scrambled siRNA; 3.0 fold induction). In contrast, the mouse Egr-1 siRNA blunted the insulin induced increase in Egr-1 protein levels (1.2 fold increase). Complete blockage of Egr-1 induction is not achieved, possibly because of suboptimal transfection efficiency.

To determine the effect of the Egr-1 siRNA on GnRH promoter activity, we transiently transfected GN11 cells with the Egr-1 siRNA construct and a proximal –1250 bp fragment of the mouse GnRH promoter fused to a luciferase reporter (1250LUC). This construct was previously shown to be sufficient to direct hypothalamic mGnRH gene expression *in vivo*

(Kim et al., 2002) and to be activated by insulin treatment with maximal activity after one hour of treatment (Kim et al., 2005). As shown in figure 4B, the presence of scrambled siRNA did not significantly affect GnRH promoter activity thus indicating that neither the scrambled sequence nor the vector interfered with the insulin stimulation of GN11 cellular pathways. However, in the presence of Egr-1 siRNA, the insulin-induced stimulation of promoter activity was blocked. In data not shown, these results were recapitulated using constructs containing the complete GnRH promoter (3446bp) or the proximal 1005 bp of the promoter.

Egr-1 binds to a proximal region of the GnRH promoter

We postulated that Egr-1 binds the GnRH gene promoter to transduce the insulin stimulation of GnRH promoter activity. Upon inspection of the proximal 1250 bp of the GnRH gene promoter, there are GC rich regions with putative Egr-1 binding sites between –836 bp and –829 bp of the promoter and between –75bp and –67bp of the promoter (Figure 5A). We used chromatin immunoprecipitation to investigate whether Egr-1 binds to the GnRH gene promoter *in vivo* in the presence and absence of insulin. After isolation of immunoprecipitated DNA, PCR was performed using primers that amplified the fragments shown in Figure 5A. In the basal state, there was no significant difference in the amount of DNA recovered after Egr-1 antibody precipitation relative to the no antibody control conditions (Figure 5B). This was true for both the proximal and distal regions of the GnRH promoter. However, after insulin treatment, there was a significant, dramatic increase in amount of DNA recovered after Egr-1 immunoprecipitation relative to the no antibody control (Figure 5B). This effect was seen when the recovered DNA was amplified by the proximal primers, but not the distal primers. These results clearly demonstrate that Egr-1 binds to the proximal region of the GnRH gene promoter after insulin treatment but does not bind this region in the absence of insulin.

The putative Egr-1 binding site in the proximal GnRH promoter is necessary for insulin induction of GnRH promoter activity

To test the role of the putative proximal binding site in the insulin induction of GnRH promoter activity, we generated a mutation of the putative Egr-1 binding site (–67 mutant) previously shown to obliterate Egr-1 binding (Tremblay and Douin, 1999) in the context of the –1250LUC construct. The wild type and mutant sequences of the proximal putative Egr-1 binding site are shown in Figure 6A. A mutation in the GC rich region of the distal promoter region was also generated to investigate the sensitivity of GnRH promoter activity to perturbation of the GC rich sequence (–829 mutant). The wild type and mutant sequences of the distal promoter are shown in Figure 6B. The wild type –1250LUC, –67 mutant, and –829 mutant were transfected into GN11 cells and luciferase activity measured after 60 minutes of insulin stimulation. Figure 6C indicates that cells transfected with the wild type –1250 construct experienced a nearly 2 fold increase in activity after 60 minutes of insulin stimulation consistent with earlier findings. Insulin treatment had no effect on luciferase activity of the proximal, –67 mutant indicating that Egr-1 binding to the proximal site is necessary for the insulin induction of GnRH promoter activity. When GN11 cells were transfected with the –1250LUC construct that contained a mutation of the distal putative binding site (–829 mutant), insulin treatment caused an increase in luciferase activity similar to that seen with the wild type –1250LUC construct. These findings, together with the results of the chromatin immunoprecipitation assay, suggest that Egr-1 binds to the proximal, but not distal putative binding site to increase GnRH promoter activity in response to insulin.

DISCUSSION

In this study, we demonstrate that Egr-1 binds the GnRH promoter in response to insulin to regulate gene expression in a model of immortalized GnRH-expressing neurons. Consistent with its role as an immediate early gene, Egr-1 mRNA levels rise and peak early after

stimulation with insulin then decline quickly, with a corresponding pattern of protein expression (Figure 1). This time course of insulin induced increase in Egr-1 mRNA levels has been observed in many tissues (Barroso and Santisteban, 1999; Tan et al., 2003; Silverman and Collins, 1999; Leung-Theung-Long et al., 2005; Garnett et al., 2005) as has its dependence on the MAP kinase pathway (Leung-Theung-Long et al., 2005; Garnett et al., 2005; Keeton et al., 2003). The GnRH mRNA level peak after one hour of insulin treatment corresponds with the time of maximal Egr-1 protein levels, suggesting that higher protein levels, rather than protein activation, promote binding of Egr-1 to the GnRH promoter. In contrast to our findings, insulin increased Egr-1 mRNA levels to a lesser extent and increased GnRH mRNA levels two hours after insulin treatment in a model of conditionally immortalized and GnRH expressing rat hypothalamic neurons (Salvi et al., 2006). However, this study did not measure GnRH mRNA levels at one hour of treatment and 100 fold greater doses of insulin were used. It is notable, however, that the different models of GnRH neurons respond to insulin by increasing Egr-1 and GnRH gene transcription.

In the model system used, physiologic (30nM) doses of insulin resulted in the greatest activation of Egr-1 transcription and subsequent regulation of GnRH transcription. This is a significant observation because insulin at supraphysiologic concentrations can activate the insulin-like growth factor-1 (IGF-1) receptor and IGF-1 administration has been shown to increase Egr-1 transcription (Criswell et al., 2005) and GnRH transcription (Zhen et al., 1997b). Our results suggest that IGF-1 receptor pathways are not involved in the Egr-1 mediated increase in GnRH transcription since supraphysiologic concentrations of insulin did not result in further increases in Egr-1 or GnRH mRNA levels (Figures 1A and 3A). IGF-1 likely activates GnRH transcription via a different mechanism, as the increase in GnRH transcription occurs four hours after IGF-1 administration (Zhen et al., 1997b), much later than after insulin treatment. Further studies will be required to determine if Egr-1 is important for the regulation of GnRH transcription by IGF-1.

Previous reports found that insulin did not increase GnRH release from the GT1-7 cell line, a GnRH secreting immortalized cell line, after two hours of insulin exposure in static culture (Olson et al., 1995; Anderson et al., 1999). At supraphysiologic concentrations, insulin has been shown to affect GnRH secretion in a model of conditionally immortalized and GnRH expressing rat hypothalamic neurons in perfusion experiments (Salvi et al., 2006). Whether insulin affects GnRH secretion in perfused GT1-7 cells or in GN11 cells is unknown and will be a subject of future study. In addition, a role for Egr-1 in GnRH secretion and whether GnRH gene expression can be coupled to protein secretion is not known.

The finding that Egr-1 is necessary for insulin regulation of GnRH gene expression in our *in vitro* model system is consistent with available *in vivo* data; transgenic mice with knockout of the insulin receptor in neurons (NIRKO) have hypogonadotropic hypogonadism (Bruning et al., 2000) as do Egr-1 null mice (Lee et al., 1996; Topilko et al., 1998). The hypogonadism in NIRKO mice was found to be due to hypothalamic dysfunction (Bruning et al., 2000). The Egr-1 null mice were found to have defects in LH β synthesis but the function of GnRH neurons in these mice was not examined and a potential defect in the regulation of GnRH synthesis or release was not assessed (Lee et al., 1996; Topilko et al., 1998). The significance of the molecular mechanism presented here would be difficult to test *in vivo* in these mouse models because of other factors contributing to the phenotype. In NIRKO mice, insulin may have effects on GnRH afferent neurons complicating analysis of GnRH function. Egr-1 mediates the GnRH receptor-transduced increase in LH β gene expression in pituitary gonadotrophs (Trembley and Douin, 1999; Dorn et al., 1999) thus complicating the analysis of the hypothalamic component to reproduction in Egr-1 null mice.

Egr-1 binds GC rich regions of the promoter of its target genes, binding the consensus sequence GCGG/TGGGCG with the highest affinity (Swirnoff and Milbrandt, 1995; Paillard et al., 2004). Egr-1 has the highest affinity for sequences that preserve guanine at positions 1,3,6,7, and 9 and cytosine at position 2 of the consensus sequence (Swirnoff and Milbrandt, 1995; Paillard et al., 2004). The three zinc fingers of Egr-1 can recognize more than one base of the binding sequence, allowing for variability of the binding sequence. The Egr-1-binding proximal region of the GnRH promoter contains a sequence between -67bp and -76 bp with a GC pattern consistent with the variations that specifically bind Egr-1 (Paillard et al., 2004). The insulin induced increase in GnRH promoter activity was blocked only when the proximal putative Egr-1 site was mutated, (Figure 6C) suggesting that the proximal site is the sole, specific Egr-1 binding site crucial to the insulin-induced increase in GnRH promoter activity. This data, together with the data indicating an increase in Egr-1 mRNA, protein, and binding to the GnRH proximal promoter after insulin treatment, provide evidence that Egr-1 binds the GnRH promoter to mediate the effect of insulin on GnRH mRNA levels. Whether the GnRH mRNA increase is due to post transcriptional modification or an increase in transcription is unknown although the effect of insulin action on the promoter suggests a transcriptional effect. In a previous report (Kim et al., 2005), our laboratory used promoter deletion mapping to identify the region of the mouse GnRH promoter between -1250 and -587 as essential for mediating the stimulatory effects of insulin. Here we report an essential role for Egr-1 sites located at -67 but not -829, for insulin stimulation of promoter activity. These two observations are not mutually exclusive as other *cis* regulatory elements located in the region between -1250 and -587 may also be obligatory for the insulin response. Therefore, *cis* regulatory elements located in the distal promoter, and Egr-1 binding to the proximal promoter might both be required to mediate insulin regulation of the mouse GnRH promoter.

The region of the mouse GnRH gene promoter containing the proximal Egr-1 binding site (Mason et al., 1986) shares approximately 80% homology with the rat (Bond et al., 1989) and 60% homology with the human (Hayflick et al., 1989) GnRH gene promoter. The importance of this region is exemplified by the fact that this region of the rat promoter confers neuron-specific GnRH gene expression *in vitro* and *in vivo* (Eraly and Mellon, 1995; Nelson et al., 2000; Lawson et al., 2002) and this region of the mouse promoter confers neuron-specific GnRH gene expression *in vivo* in mice (Kim et al., 2002). The homologous region of the human promoter, however, is not sufficient to confer cell specific expression *in vivo* in transgenic mice (Wolfe et al., 1996) thus demonstrating species to species differences in GnRH gene regulation. Several transcription factors and cofactors have previously been identified to bind this region (Rave-Harel et al., 2005; Tang et al., 2005). This study demonstrates that this proximal region also mediates growth factor induction of GnRH gene transcription *in vitro*. Whether this region mediates growth factor induction *in vivo* in mice and in other species remains to be determined.

We have demonstrated that insulin, via the MAP kinase pathway, induces Egr-1 transcription, increases protein levels, and induces Egr-1 binding to a proximal site of the GnRH gene promoter to increase GnRH gene transcription in GnRH-secreting GN11 cells. A proposed model of this pathway is shown in figure 7. Briefly, insulin binds to its receptor on GN11 cells to activate the MAP kinase pathway. Activation of the MAP kinase pathway causes an increase in Egr-1 transcription, mRNA levels and protein levels. Finally, Egr-1 binds to the GnRH gene promoter to increase transcription. Our results suggest that GnRH neurons, as represented by our model of GnRH expressing immortalized neuronal cells, are direct targets of insulin action, and may be a locus integrating nutritional cues to the reproductive axis.

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A **B.** **C.**

Fig 1. Dose response and time course of insulin induction of Egr-1 gene and protein expression GN11 cells were serum deprived overnight then treated with insulin A) for 30 minutes at the specified doses or B) with 30 nM at the specified times. Total RNA was extracted, RT-PCR performed and the resultant cDNA amplified using quantitative PCR with primers specific for mouse Egr-1. Relative mRNA levels were calculated using the $\Delta\Delta C_t$ method and graphed as fold increase in mRNA levels in insulin treated cells relative to untreated cells. Each graph represents three (3) separate experiments performed in duplicate. In comparison of insulin treated versus untreated controls, statistical significance of $p < 0.05$ is indicated by the asterisk. C) Nuclear extract was made from GN11 cells after treatment with 30 nM insulin and subjected to western blot analysis using an antibody specific for Egr-1.

Fig 2. Dependency of the insulin-induced increase in Egr-1 expression on the MAP kinase pathway
 GN11 cells were serum deprived overnight, pretreated with 10 μ M of the MAP kinase pathway specific inhibitor PD98059, 10 μ M of the PI3 kinase inhibitor Wortmannin or vehicle for 30 minutes then treated with 30 nM of insulin for 30 minutes. Relative mRNA levels were calculated using the $\Delta\Delta$ Ct method and graphed as fold increase in mRNA levels of insulin treated cells relative to untreated cells. In comparison of treated versus untreated controls, statistical significance of $p < 0.05$ is indicated by the asterisk. Graph represents three (3) separate experiments performed in duplicate.

A.

B.

Fig 3. Dose response and time course of insulin induction of GnRH gene expression in GN11 cells GN11 cells were serum deprived overnight then treated with insulin at the indicated doses for one hour (A) or with 30 nM of insulin for the specified times (B). Relative mRNA levels were calculated using the $\Delta\Delta C_t$ method and graphed as fold increase in mRNA levels in insulin treated cells relative to untreated cells. In comparison of insulin treated versus untreated controls, statistical significance of $p < 0.05$ is indicated by the asterisks. Each graph represents four (4) separate experiments performed in duplicate.

A. **B.**

Fig 4. Egr-1 siRNA interferes with the insulin induced increase in Egr-1 protein and GnRH promoter activity

200 ng of scrambled siRNA or Egr-1 siRNA was transfected into GN11 cells. After 24 hours, media was replaced by serum free media and the next morning, cells were stimulated with 30 nM insulin or vehicle for 60 minutes. A) Nuclear extract was made from GN11 cells and subjected to western blot analysis using an antibody specific for Egr-1. B) Cells were co-transfected with a 1250LUC construct then luciferase activity (RLU1) and renilla luciferase activity (RLU2) were measured. Luciferase activity was corrected for transfection efficiency using the equation $RLU1/RLU2$. The $RLU1/RLU2$ for insulin treated conditions relative to the $RLU1/RLU2$ for untreated conditions is graphed. Upon comparison of the 1250LUC activity alone with 1250LUC co-transfected with siRNA activity, statistical significance of $p < 0.05$ was assigned and is indicated by the asterisk. Graph represents five (5) separate experiments performed in triplicate.

A.

Proximal Promoter

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-176      GACCAGCAGG TGTTCGAATT ACATTACCA TTAAGAGGCT TTTTGTGAGG
GTTTAAACT AACTATTAAG ACTATGGGCT GTGCTGCAAC TGTGCTCACC AGCGGGAAG
ACATCAGTGT CCCAGAAAAA AAAAAATCAT ATAAAAAGGA AGCTAGGCAG ACAGAACTT
CGAAGTACTC AACCTACCAA CGGAAGCTCG A      +28

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Distal Promoter

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-936      CAACCATCTC TAGTGGGATC TGATGCCCTC TTCTGGTGTG TCTGAAGACA
GCGGCAGTGT ACTCATCTTG TATTAAAAAA ATAATTAAGC CAGGTGTGGT GGCACACGCC
TTTAATCCCA GCACTCGGGA GGCAGAGGCA GGCAGATTTC TGAGTTCGAG GC      -775

```

B.

Fig 5. Egr-1 interacts with the GnRH promoter in response to insulin

A) The proximal region of the GnRH gene promoter contains a GC rich region between -67 and -75 bp that has high homology to the Egr-1 consensus sequence (underlined). The distal region of the GnRH gene promoter between -829 and -838 bp also contains a GC rich region.

B) GN11 cells were treated with 30 nM of insulin after serum starvation for 16 hours. Cells were then cross linked using formaldehyde, sonicated, then subjected to immunoprecipitation with no antibody or 10 ug of Egr-1 antibody. DNA was extracted and subjected to quantitative PCR. Data are expressed as fold differences relative to control conditions (in which no antibody is used during the immunoprecipitation). DNA was subjected to PCR using primers that amplified a region of the GnRH promoter between -176 bp and +28 bp ("proximal primers"; *left panel*) or primers that amplified a region between -936 bp and -775 bp ("distal primers"; *right panel*). In comparison between conditions, statistical significance of $p < 0.05$ is assigned and indicated by an asterisk. Each ChIP assay was performed on three different occasions; for each assay, samples were subjected to PCR in triplicate.

A.**WT**

-99 GCTGTGCTGC AACTGTGCTC ACCAG**C**GGGG **A**AGACATCAG TGTCCCAGAA
 CGACACGACG TTGACACGAG TGGT**C**GCCCC **T**TCTGTAGTC ACAGGGTCTT

-67 Mutant

-99 GCTGTGCTGC AACTGTGCTC ACCAT**C**AGGT **A**ACACATCAG TGTCCCAGAA
 CGACACGACG TTGACACGAG TGGT**A**GTCCA **T**TGTGTAGTC ACAGGGTCTT

B.**WT**

-860 TATTAAAAAA ATAATTAAGC CAGGTGTGGT GGCACACGCC TTTAATCCCA
 ATAATTTTTT TATTAATTTC GTCC**A**CACCA CCGTGTGCGG AAATTAGGGT

-829 Mutant

-860 TATTAAAAAA ATAATTAAGC CAGTT**G**CGAT GACACACGCC TTTAATCCCA
 ATAATTTTTT TATTAATTTC GTCA**A**CGCTA CTGTGTGCGG AAATTAGGGT

C.

Fig 6. Mutation of the putative Egr-1 binding site in the proximal region of the GN11RH gene promoter blocks the insulin induced increase in GnRH promoter activity

The putative binding sites of the -1250LUC construct of the GnRH gene promoter were subjected to site directed mutagenesis. A) *Top panel*: sequence of the putative Egr-1 binding site in the proximal mouse GnRH gene promoter. *Bottom panel*: sequence generated by site directed mutagenesis within the -1250LUC construct and used in subsequent studies (-67 mutant). B) *Top panel*: sequence of the putative Egr-1 binding site in the distal proximal mouse GnRH gene promoter. *Bottom panel*: sequence generated by site directed mutagenesis within the -1250LUC construct and used in subsequent studies (-819 mutant). C) GN11 cells were transfected with 200 ng of wild type -1250LUC construct, -67 mutant, or -829 mutant. After

24 hours, media was changed to serum free media and 16 hours later, cells were stimulated with 30 nM insulin for 60 minutes. Luciferase activity (RLU1) and renilla luciferase activity (RLU2) were measured. Luciferase activity was corrected for transfection efficiency using the equation $RLU1/RLU2$. The corrected luciferase activity for the insulin treated samples were normalized to the corresponding untreated sample and this normalized value graphed. The graph represents three (3) separate experiments with triplicates of each condition. In comparison of wildtype and mutant constructs, statistical significance of $p < 0.05$ is assigned and indicated by an asterisk.

Fig 7. Model of the Role of Egr-1 in GnRH Neurons

Insulin binds to its receptor on G11 cells and activates the MAP kinase pathway to increase Egr-1 transcription, mRNA, and protein levels. Egr-1, in turn, binds to the GnRH promoter to increase GnRH transcription and mRNA levels.