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The molecular mechanism underlying the gonadotropin releasing hormone pulse sensitivity of the follicle stimulating hormone beta subunit gene

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The Molecular Mechanism Underlying the Gonadotropin Releasing Hormone Pulse Sensitivity of the Follicle Stimulating Hormone Beta Subunit Gene

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Devendra S. Mistry

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2011

The Dissertation of Devendra S. Mistry is approved, and it is acceptable in quality

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University of California, San Diego

2011

Epigraph

"An experiment is a question which science poses to Nature, and a measurement
is the recording of Nature's answer."

Max Planck

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List of Abbreviations

αGSU, Alpha Gonadotropin Subunit

AC, Adenylate Cyclase

ACN, Arcuate nucleus

AP-1, Activator Protein 1

ARE, Activin Responsive Element

AVPV, Anteroventral Periventricular nucleus

Ca^{2+}, Calcium ion

cAMP, Cyclic Adenosine Monophosphate

CBP, cAMP response element-binding protein

CC: Coiled Coil Region

CR: Conserved Repression domain

ChIP, Chromatin Immuno-Precipitation

CMV, cytomegalovirus

CO_2, Carbon Dioxide

CRE, cAMP response element

CREB, cAMP response element-binding protein

CREM, cAMP response element-modulator

Ct, Cycle threshold

D box: Degradation box

DAG, diacylglycerol

DAX1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region

on the X chromosome, gene 1

DBD: DNA binding domain

DCREB, DIEDML CREB mutant

DHD: Dachshund homology domain

DIEDML, (amino-acids) Asp-Ile-Glu-Asp-Met-Leu

DMEM, Dulbecco's Modified Eagle Medium

DNA, Deoxyribonucleic Acid

EDTA, Ethylenediaminetetraacetic acid

Egr1, early growth response 1

EMSA, Electrophoresis Mobility Shift Assay

ER, Estrogen Receptor

ERα, Estrogen Receptor α

ERE, estrogen response element

ERK1/2, extracellular signal regulated kinase 1/2

FBS, fetal bovine serum

FMA, functional hypothalamic amenorrhea

FRET, Fluroscense Resonence Energy Transfer

FSH, follicle stimulating hormone

FSHβ, follicle stimulating hormone beta subunit

FSHR, FSH receptor

GnRH, gonadotropin releasing hormone

GnRHR, GnRH receptor

Gmx, Glutamax

GPCR, G-Protein Coupled Receptor

HA, hemagglutinin

hCG, Human Chorionic Gonadotropin

HD, homeodomain

HDAC, histone deacetylase complex

hpg, hypogonadal

HPG axis, hypothalamic pituitary gonadal axis

ICER, inducible cAMP early repressor

IP, immunoprecipitation

IP3, inositol triphosphate

JNK, c-jun N-terminal kinase

KCREB, mutant form of CREB

KNDy (neurons), Kisspeptin-Neurokinin and Dynorphin (expressing neurons)

LDS, lithium dodecyl sulfate

LH, luteinizing hormone

LHβ, luteinizing hormone beta subunit gene

LHR, LH Receptor

LiCl, Lithium Chloride

MAPK, mitogen activated protein kinase

Mg^{2+}, Magnesium Ion

Mek, MAPK kinase

MCREB, mutant form of CREB

NaCl, Sodium Chloride

Nab, Ngfi-A binding protein

NCOR, nuclear receptor corepressor

NF-Y, nuclear factor-Y

NLS: nuclear localization sequence

PBS, phosphate buffered saline

PCOS, polycystic ovarian syndrome

PCR, polymerase chain reaction

PIP2, phosphatidylinositol 4,5-bisphosphate

PKA, protein kinase A

PKC, protein kinase C

PLC, phospholipase C

PLL, poly-L-lysine

PR, progesterone receptor

PS, penicillin-streptomyocine

PVDF, polyvinylidene difluoride

QPCR, quantitative PCR

SDS, Sodium Dodecyl Sulfate

SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SF1, steroidogenic factor 1

RIPA buffer, radioimmunoprecipitation assay buffer

RNA, Ribonucleic Acid

Rsk, ribosomal kinase

RT, reverse transcription

SAND: Sp100, AIRE1, NucP41/75 and DEAF1

siRNA, small interfering RNA

Ski, Sloan Kettering Institute (protein)

SKIL, ski-oncogene like protein

SBE, SMAD binding element

SMAD, phosphorylated mothers against decapentaplegic

SnoN, Ski like Novel Protein N (Also known as SKIL)

SUMO, Small Ubiquitin-like Modifier.

TE, Tris/EDTA

TEM, Transmission Electron Microscopy

TGF, Transforming Growth Factor

TGIF, 5'-Thymine Guanine-'3 interacting factor

Tris, tris(hydroxymethyl)aminomethane

USF, upstream stimulating factors

YFP, Yellow Flurescent Protein

Acknowledgements

I would like to thank my committee members, colleagues, friends and family members who helped in many ways while working on this dissertation.

First and foremost, I am grateful to my supervisor, Prof. Nicholas JG Webster, for his supervision, umpteen discussions on research protocols and explication of the data. He kept a perfect balance between supervision and encouragement for independence.

I would also like to thank my other thesis committee members, Prof. Mark Lawson, Prof. Pamela Mellon, Prof. Alexandra Newton, Prof. Gina Sosinsky and Prof. Wylie Vale, for scientific and career guidance and advice.

Thanks are also due to my co-workers, especially, Dr. Rie Tsutsumi for initiating me into the wonderful world of gonadotropin biology. Dr. Shweta Sharma for the camaraderie in the lab, for all the academic inputs during the lab meetings and teaching me yin and yang of qPCR. Dr. Marina Fernandez for collaboration and contribution on primary pituitary culture studies. Dr. Supriya Sen, Ms Jing Li, Dr. Emily Rickert and all the erstwhile and current colleagues are also thanked for all their help and support. Special thanks to Prof. Mark Lawson and his lab member Steven Cardenas for collaboration on the pulse perifusion studies. Also, many thanks to Prof. Gina Sosinsky, Mason Mackey,

Chapter 4-9, in part, is a reprint of the material as it appears in Gonadotropin-Releasing Hormone Pulse Sensitivity of Follicle Stimulating Hormone-Beta Gene Is Mediated by Differential Expression of Positive Regulatory Activator Protein 1 Factors and Corepressors SKIL and TGIF1 2011. Mistry, Devendra et al. Molecular Endocrinology, 2011. The dissertation author was the primary investigator and author of this paper.

Last but not the least, I would like to thank my parents for their blessings, constant support and encouragement. I would also like to thank my dear wife for being so considerate and supportive right from my formative stages as a graduate student till now. Special thanks to my soon to be born baby for providing me with a boost in motivation and encouragement to graduate on time.

Gratitude is the fairest blossom which springs from the soul. ~Henry Ward Beecher

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Tsutsumi R, **Mistry DS,** Webster NJ. (2010) Signaling responses to pulsatile GnRH in LβT2 gonadotrope cells. J Biol Chem. 285: 20262-72.

Sharma S, Sharma P, **Mistry DS**, Mellon P, Chang RJ, Olefsky JM, Webster NJ. (2010) PPARg agonist Rosiglitazone suppresses activation of inflammatory kinases and gonadotropin expression in pituitary gonadotropes. Biol Repr. 84: 466-75.

Abstract of the Dissertation

The Molecular Mechanism Underlying the Gondaotropin Releasing Hormone
Pulse Sensitivity of Follicle Stimulating Hormone Beta Subunit Gene

by

Devendra S. Mistry

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2011

Professor Nicholas Webster, Chair

Gonadotropin synthesis and release is dependent upon pulsatile stimulation by the hypothalamic neuropeptide GnRH. Generally, slow GnRH pulses promote FSH production while rapid pulses favor LH, but the molecular mechanism underlying this pulse sensitivity is poorly understood. In this study, we develop and test a model for FSHβ regulation in mouse LβT2 gonadotropes. We observed that positive regulators of FSHβ expression, such as c-fos and c-jun, were upregulated at slower pulse frequencies than a number of potential negative

regulators, such as the co-repressors SnoN, CREM and TGIF1. These latter co-repressors reduced FSHβ promoter activity whether driven by transfection of individual transcription factors or by treatment with GnRH and activin. Overexpression of Smad binding or phosphorylation defective SnoN and TGIF mutants, however, failed to repress FSHβ promoter activity. Knock down of the endogenous repressors SnoN and TGIF, but not CREM, increased FSHβ promoter activity driven by continuous GnRH or activin. ChIP analysis showed that c-fos, SnoN and TGIF1, and to a lesser degree c-jun, occupy the FSHβ promoter in a cyclical manner following GnRH stimulation. Overexpression of corepressors SnoN or TGIF repressed induction of the FSHβ promoter at the slow GnRH pulse frequency, but had little effect at the fast pulse frequency. In contrast, knock down of endogenous SnoN or TGIF selectively increased FSHβ mRNA at the fast GnRH pulse frequency. Finally, relative to corepressors, stimulators have greater FSHβ promoter occupancy at slow GnRH pulse frequency which favors greater FSHβ mRNA production. Therefore, we propose a potential mechanism by which production of gonadotropin FSHβ is modulated by positive transcription factors and negative corepressors with different pulse sensitivities.

Chapter 1. The Significance of the Gonadotropin Releasing Hormone in Maintaining Normal Reproductive Function

1.1 GnRH and GnRH Neurons

The Gonadotropin Releasing Hormone (GnRH) is a decapeptide (Figure 1). It is synthesized from a 92 amino acid preprohormone coded by the *GNRH1* gene located on chromosome 8 (1). GnRH is an important reproductive hormone and is crucial for the normal function of the hypothalamic pituitary gonadal (HPG) axis.

GnRH producing neurons originate in olfactory placode during the development (2, 3). From there, they migrate to the olfactory bulb and finally into the fetal hypothalamus (4-9). Due to such migration during development, cell bodies of GnRH neurons can be found throughout the medial septum and hypothalamus (10-12). Even though GnRH neurons are scattered through such vast region of brain, they are interconnected *via* their long dendritic connections, which allow them to secrete GnRH in a synchronized manner (12).

While the precise mechanism by which GnRH neurons are regulated by the other neuronal population is still under investigation, recent studies suggest that a subpopulation of neurons, abbreviated as KNDy neurons, located in the

Arcuate nucleus (AC) of the brain are involved in the regulation of the GnRH neurons (13, 14). KNDy subpopulation is conserved amongst a range of species ranging from rodents to humans (13-17). KNDy neurons are so named due to their production of kisspeptin, neurokinin B and dynorphin peptides, all of which can regulate GnRH secretion (13, 18-25). KNDy neurons are interconnected with one another and also have projections on the GnRH neuron cell bodies in the mediobasal hypothalamus and the neurosecretory terminals in median eminance (14, 16, 26-31). KNDy neurons regulate GnRH synthesis by their projections on the GnRH neuron cell bodies and GnRH secretion through their interaction with GnRH neuron terminals in the median eminence (14, 32-34). In addition to KNDy neurons, Kiss-1 neurons in Anteroventral Periventricular nucleus (AVPV) can also regulate GnRH neurons (35-37).
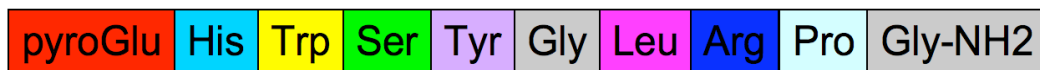
Figure 1: The Decapeptide GnRH.

The amino acid sequence of the decapeptide GnRH after it is cleaved from its precursor.

1.2 The Hypothalamic Pituitary Gonadal Axis

GnRH is secreted in a pulsatile manner from the hypothalamic GnRH neurons directly into the hypophyseal portal system at the median eminence (37-39). The portal blood carries GnRH to the pituitary where it stimulates gonadotropes. Gonadotrope cells are one of the five major cell types in the anterior pituitary. As their name implicates, they specialize in production of the gonadal regulatory hormones, the gonadotropins. Upon stimulation with GnRH, the gonadotropes produce and secrete the gonadotropins, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) in an episodic manner (40) (Figure 2). LH and FSH are crucial for both the male and female reproduction as they play an important role in gonadal development, spermatogenesis and the female reproductive cylce (37-45).

Once secreted from the pituitary into the circulation, these gonadotropins function primarily by acting on gonads to stimulate the production of steroidal hormones, such as estrogen and progesterone, and peptide hormones, such as activin, inhibin and follistatin. These gonadal hormones provide negative and/or positive feedback to both the hypothalamus and the pituitary (24, 46-48). Although the precise hypothalamic and pituitary feedback mechanism of these gonadal hormones is still under investigation, the studies so far suggest that steroidal hormones can regulate the gonadotropin levels by adjusting the GnRH

pulse frequency by their direct or indirect impact on Kiss-1 and KNDy neurons in hypothalamus and by directly acting on the pituitary gonadotropes; the peptide hormones, activin, inhibin and follistatin, on the other hand mainly act at the level of the pituitary (17, 22, 24, 25, 47, 48).
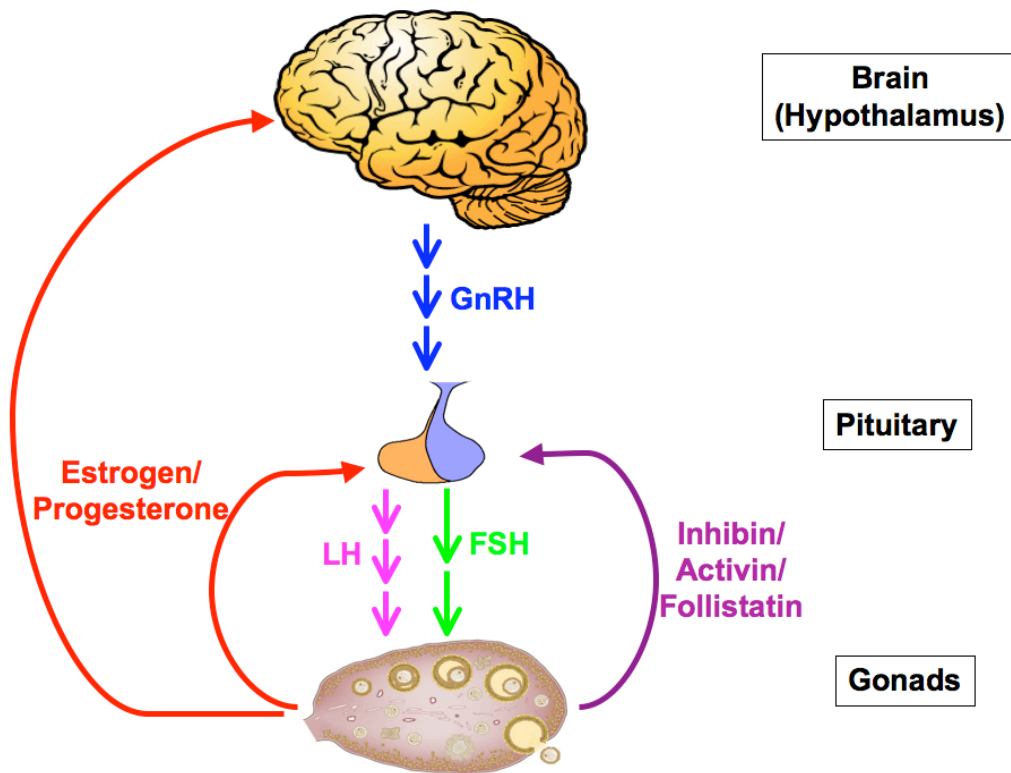
Figure 2: The Hypothalamic-Pituitary-Gonadal (HPG) Axis.

GnRH, secreted from hypothalamic GnRH neurons, stimulates the pituitary gonadotropes leading to the secretion of the LH and FSH gonadotropins. LH and FSH acts on the gonads, which in turn, produce steroidal hormones, such as estrogen and progesterone, and peptide hormones, such as activin, follistatin and inhibin.

1.3 GnRH Pulse Regulation of Gonadotropins

As discussed earlier, GnRH is secreted in a pulsatile manner from hypothalamic GnRH neurons (38, 39). GnRH pulse frequency varies throughout the different stages of the female reproductive cycle and changes in pulse frequency correlate with changes in LH and FSH gonadotropins levels (49-51). LH and FSH are heterodimeric glycoproteins (52). They both share a common alpha glycoprotein subunit (α-GSU), but have different beta subunits, Luteinizing Hormone β (LHβ) and Follicle Stimulating Hormone β (FSHβ) (Figure 3a) (52).

Secretion of GnRH in a pulsatile manner is important for gonadotropin production as chronic exposure to GnRH or its analogs leads to a reversible suppression of gonadotropin release in arcuate nucleus lesioned monkeys (39, 53). Physiologically, the rate of GnRH pulse frequency varies from one GnRH pulse every 30 min to one pulse every ≥ 120 min, according to the studies performed in humans, monkeys, ewes and rodents (36, 51, 54-61).

Interestingly, a great number of *in vitro* studies, utilizing gonadotropins subunit promoter based assays and the measurements of subunit mRNA or primary transcripts induction in gonadotropes or gonadotrope based cell lines, as well as many *in vivo* studies, measuring actual LH and FSH hormone secretions, indicates that different GnRH pulse frequencies regulate the synthesis of these

subunits differentially. While expression of GSU-α subunit is observed at all of the pulse frequencies, it is synthesized more at the faster pulse frequencies (62-67). The production of beta subunits is more frequency dependent. LHβ subunit is mainly synthesized at the fast pulse frequency of 30 min (Figure 3b). FSHβ subunit, on the other hand, is preferentially synthesized at the slow pulse frequency of $\geq$ 120 min (Figure 3b) (62-70).

As α subunit shows little preference in terms of its synthesis by different GnRH pulse frequencies and also as beta subunit grants the hormone biological specificity, the synthesis of the specific beta subunit is the rate-limiting step for production of LH and FSH gonadotropins (62, 71). Therefore, to understand GnRH pulse regulation of LH and FSH, it is crucial to investigate the mechanism by which GnRH regulates the transcription of LHβ and FSHβ subunit genes.
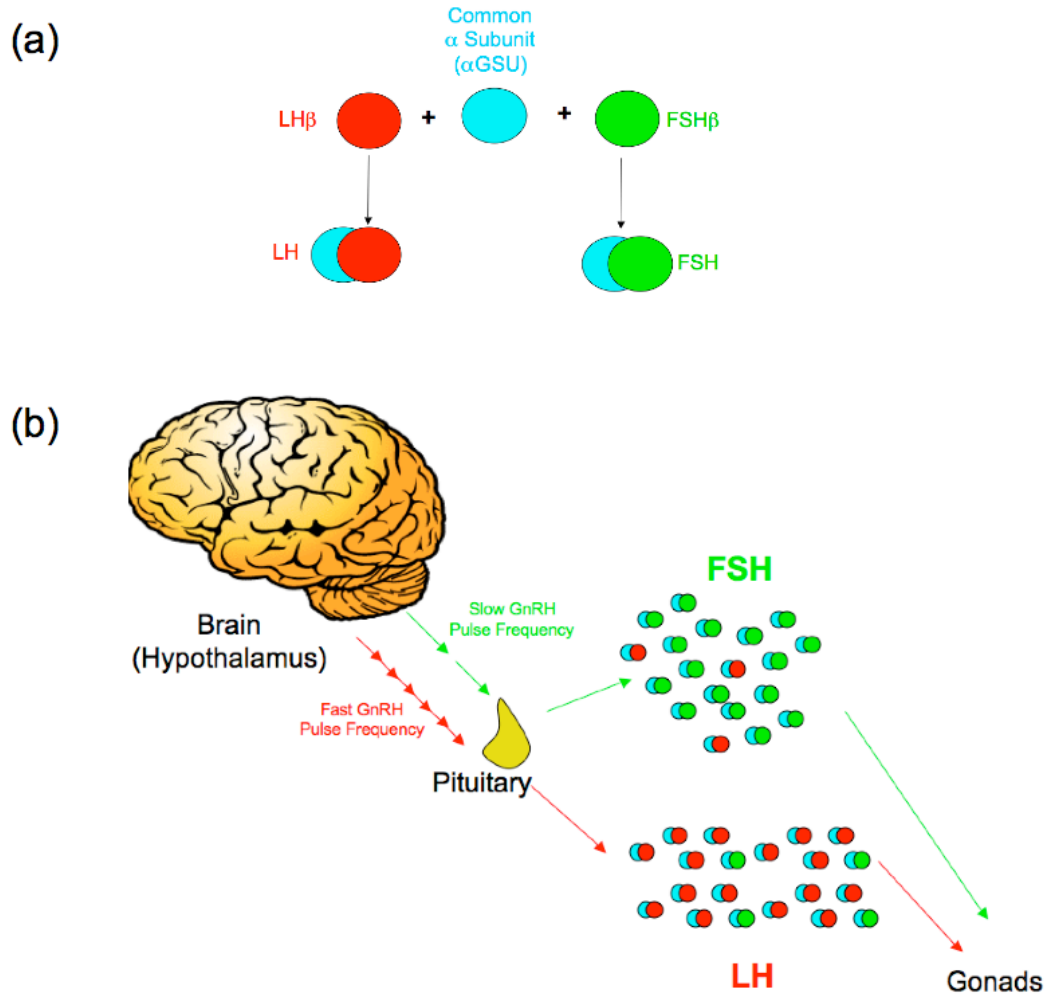
Figure 3: The Gonadotropin Subunits.

(a) LH and FSH are heterodimeric glycoproteins. They have a common α subunit, α GSU and unique β subunits, LHβ and FSHβ. Synthesis of the β subunit is the rate-limiting step for the production of the LH and FSH hormones. (b) Fast GnRH pulse frequency preferentially promotes LH over FSH production while the slow GnRH pulse frequency promotes FSH over LH production.

1.4 Significance of the HPG Axis

As the HPG axis regulates the production of the reproductive hormones, its function is critical for fetal development, puberty, the menstrual cycle, pregnancy, post-partum, and menopause (48). Disruption of hormonal function at hypothalamic, pituitary or gonadal level in the HPG axis can have adverse physiological and reproductive effects.

At the hypothalamic level, GnRH neuronal migration during the development, their networking with other regulatory neurons and proper GnRH secretion is important for the maintanance of the HPG function. For instance, abnormal GnRH neuronal migration causes Kallmann Syndrome (72, 73). Patients with this syndrome, along with congenital anosmia or hyposmia, suffer from hypogonadotropic hypogonadism (73, 74). The hypogonadotropic hypogonadism condition is characterized by low circulating gonadotropins levels, reduced gonadal size and subfertility or infertility in both sexes. A point mutation (L148S) in the kisspeptin receptor GPR54 reduces its functional responses and is also associated with the development of hypogonadotropic hypogonadism in humans and mice (75, 76). In addition, such mutation also leads to pubertal delay in both species (76). Production of GnRH is crucial for the proliferation and development of pituitary gonadotropes. *Hpg* mice, harboring a deletion in the GnRH gene, have fewer gonadotropes, reduced gonadotropin levels and are

infertile (43, 77).

At the level of pituitary, the HPG hormal feedback to gonadotropes and physiologically relevant secretion of gonadotropins by gonadotropes is crucial for the normal reproductive function. Mutations in the human GnRH receptor gene that affect GnRH receptor's ligand binding ability and function also cause hypogonadotropic hypogonadism underscoring the significance of gonadotropes regulation by GnRH (77-79). Furthermore, females suffering from Polycystic Ovarian Syndrome (PCOS) have abnormally high LH and low FSH levels (80). Such irregularities in gonadotropins secretion by pituitary gonadotropes may be due to improper feedback from gonads and hypothalamus (81). This condition is associated with infertility or subfertility along with other symptoms such as menstrual irregularity, hirsutism, acne and more (82-89).

At the gonadal level, gonadal stimulation by gonadotropins and proper secretion of gonadal hormones by gonads is vital for the gonadal feedback to the hypothalamus and thus fuctioning of the HPG axis. Mutations, abnormalities and polymorphism in FSH receptor have been linked to conditions such as ovarian hyperstimulation syndrome, 46XX gonadal dysgenesis and infertility (90-93). Mutations and abnormalities in LH receptors in contrast have been linked to pseudohermaphroditism, hypospdias, micropenis and infertility (94-96). Finally, excessive production of steroidal hormones by gonads is also associated with PCOS (97-99).

Other physiological abnormalities can also have impact upon HPG axis and can lead to aberrant reproductive syndromes. For example, high androgen and estrogen levels observed in obese females are linked to the PCOS (99-101). In conditions such as hyperinsulinemia, an increase in GnRH pulse frequency results in increased LH and ovarian androgen levels which are also linked with PCOS (99, 102, 103). Chronic malnutrition and excessive caloric expenditure can also have adverse effects on reproductive axis. Excessive fasting and exercise are associated with functional hypothalamic amenorrhea (FMA). Reduction in metabolic fuel can be detected by hindbrain and hypothalamic neurons (37, 104, 105). These neurons can either directly or indirectly communicate with GnRH neurons and inhibit GnRH secretion (14, 37, 104, 105). Aging can also lead to detrimental effects on the HPG axis. In women, levels of gonadotropins and GnRH pulse frequency progressively decrease after menopause and pituitary becomes less responsive to GnRH (37, 106).

Chapter 2. Female Reproductive Cycle: Humans and Rodents

2.1 The Menstrual Cycle

The menstrual cycle is an important reproductive event in the women and female apes as it maintains their fertility by the periodical release of eggs from the ovaries. While length of a cycle varies from cycle to cycle and from female to female, the mean length of a cycle is calculated to be 29.1 days in women (107). During the menstrual cycle, the females undergo significant hormonal changes causing noticeable physiological changes (49, 51, 57) (Figure 4). Physiologically, shedding of the uretarine wall causes menstrual bleeding once every cycle. This event is referred to as menstruation and marks the beginning of a cycle. Midway through the cycle a small rise in body temprature also occurs.

At the cellular levels, one of the organs where morphological and physiological changes are most apparent is in the ovaries. More specifically, under the influence of different HPG hormonal changes, ovarian structures, such as follicles, go through unique alterations (108). Based on such observed changes, the menstrual cycle can be divided into two different phases: the follicular phase and the luteal phase.
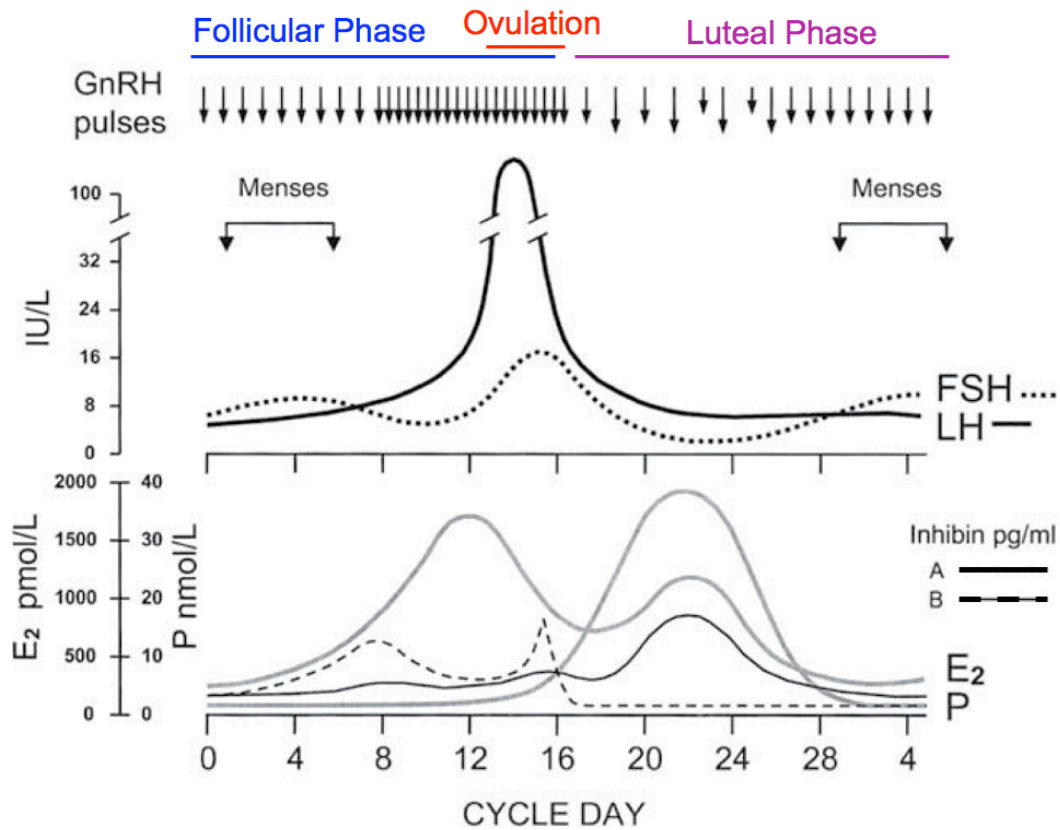
Figure 4: The Hormonal Changes During the Menstrual Cycle.

During the early follicular phase of the menstrual cycle, the high FSH levels correlate with the slow GnRH pulse frequency and low Inhibin and steroidal hormone levels. Moving towards mid to late follicular phase a rise in Inhibin A levels correlates with a drop in FSH levels. During this time a rise in Estradiol (E_2) and increase in GnRH pulse frequency correlates with a surge in LH levels, which triggers the process of the ovulation. During the early luteal phase, increase in Estradiol, Progesterone (P) and Inhibin B as well as slowing of GnRH pulse frequency correlates with drop in LH and FSH gonadotropin levels. Towards the late luteal phase, drop in gonadal hormones E_2, P and Inhibin B as well as slow GnRH pulse frequency correlates with increase in FSH levels.

2.1.1 The Follicular Phase

The follicular phase is the period when the folliculogenesis occurs. During the early follicular phase, estrogen, progesterone and inhibin levels are low, GnRH pulse frequency is slow and FSH levels are high (Figure 4). The FSH promotes the growth of the follicles (49). As the follicular phase progresses, there is a rise in Inhibin B levels and a drop in FSH levels (49, 109, 110). During this time, the follicles grow under the influence of FSH and compete with one another for dominance. Only the dominant follicle continues to grow as the phase progresses and upon reaching its maturity is referred to as an antral or Graafian follicle (111).

During the late follicular phase, the estrogen levels increase which correlates with an increase in the GnRH pulse frequency (36, 37, 51). Increase in GnRH pulse frequency, in turn, correlates with a surge in LH levels (Figure 4). The LH surge triggers the ovulation process and thus, the ovum is released from the Graafian follicle (49, 111, 112).

2.1.2 The Luteal Phase

Post ovulation, the remaining follicular granulosa cells luteinize and form the corpus lutea. Thus, this post-ovulatory phase is termed the luteal phase. During early to mid luteal phase, a rise in the estrogen and progesterone levels is observed (50, 113-115). Along with this rise in steroidal hormones, a rise in Inhibin A is also observed during this time (110, 116). This rise in gonadal hormones correlates with reduction in the GnRH pulse frequency as well as a drop in the LH and FSH gonadotropins levels (49-51).

As the menstrual cycle progresses towards the late luteal phase, a decline in the gonadal hormones is observed which corresponds with a rise in the FSH levels (49, 50). Interestingly, no major changes are observed in the levels of activin hormone throughout the menstrual cycle.

2.1.3 Hormonal Action During the Menstrual Cycle

While role of various HPG hormonal changes in regulating the progression of menstrual cycle is still a major area of study, great developments have been made by almost a century worth of research.

During the early follicular phase, high FSH levels stimulate the development of the follicles (111). A follicle consists of an oocyte, surrounded by granulosa cells, which in turn are bordered by the theca cells. During the early phases of follicle development, the granulosa cells mainly express the FSH receptors (FSHR) while the theca cells express the LH receptor (LHR) (111). High FSH during the early follicular phase stimulates the FSHR on the granulosa cells, leading to granulosa cell proliferation and differentiation. It also leads to formation of antrum fluid in developing follicles (110, 111, 117). Antrum fluid contains Inhibin, which is produced by granulosa cells under the influence of androgens (110). Inhibin can antagonize the activin function by reducing activins bioavailability (118-120). As activin stimulates FSH production and to a much lesser degree LH production from the pituitary gonadotropes, such increase in inhibin during the mid to late follicular phase reduces FSH levels (46, 48, 67, 121-125).

Theca cells upon LH stimulation can produce androgens. Granulosa cells

contain aromatase enzyme, which converts the androgen testosterone, from theca cells, into estradiol (126). FSH stimulates aromatase activity and steroid secretion (126). Activin enhances FSH stimulated aromatase activity and steroid secretion and during the early follicular phase and also upregulates FSHR on the granulosa cells (124, 127-129). Estrogen, produced through increased aromatase activity, can act on both pituitary and hypothalamus (14, 37, 48, 50).

Increase in estrogen production activates Kiss-1 neurons in AVPV (36). Kiss neurons, in turn, stimulates the GnRH neurons and increases GnRH pulse frequency (36, 37). Increased stimulation of gonadotropes by fast GnRH pulse frequency increases LH production and secretion by gonadotropes (40, 54, 56, 69, 130). This surge in LH can trigger the ovulation process as LH increases synthesis of mediators that degrade extracellular matrix on apex of follicles and vascular changes favorable for the ovulation (131). LH also acts on granulosa cells LHR, which were induced by FSH during follicular phase (132, 133). Activation of LHR halts the proliferation of the granulosa cells (134).

After ovulation, the remaining granulosa and theca follicle cells undergo a transformation and produce corpus lutea (135). The corpora lutea secrete progesterone, estradiol and inhibin A (50). As before, progesterone and estradiol can act at both, the level of hypothalamus and pituitary to slow GnRH pulse frequency and to reduce the gonadotropins levels, while inhibin reduces FSH levels by antagonizing the function of activin at the pituitary level (14, 50, 110). If

the oocyte is fertilized, trophoblast cells from the blastocyst secrete human chorionic gonadotropins (hCG) upon implantation (136). hCG stimulates the corpus luteum to secrete progesterone, which maintains thick lining of the uterine endometrium and provides area rich in blood vessels for zygote development (50, 135, 136). However, if the oocyte released by ovulation is not fertilized, the corpus luteum decays and stops secreting progesterone. Without progesterone, the outer lining of the uterus degenerates and falls off (50, 135, 136). This event is referred to as menses.

2.2 The Estrous Cycle

The estrous cycle in the rodents is equivalent to the menstrual cycle in humans. One major difference between estrus and menstrual cycle is that in estrus cycle, the endometrium is reabsorbed, compared to menstrual cycle where the endometrium is shed (137). Also, the length of an estrous cycle in rodents is shorter (about a week) compared to the length of a menstrual cycle in humans (about a month) (137). Furthermore, estrous cycle in rodents also slightly varies from species to species. For example, an estrous cycle in rats lasts 4 days, but in mice it lasts 4-7 days. Also, compared to rats, mice cycles are more irregular (138). Based on hormonal and physiological changes observed, the estrous cycle is divided into 4 phases: proestrus, estrus, metestrus and diestrus (137).

Proestrus stage is equivalent to mid to late follicular phase of the menstrual cycle. During morning of proestrus, inhibin and follistatin levels are high while LH and FSH gonadotropins levels are low (48, 139). As the proestrus day progresses, a rise in estradiol level along with an increase in GnRH pulse frequency and amplitude is detected (48, 137, 140). At the night of proestrus, a surge in LH and FSH is observed (48, 137, 139, 141). And similar to menstrual cycle, the ovulation accompanies the LH surge (48, 137).

Afterwards during the estrus stage, estradiol levels remain elevated, but

inhibin and follistatin levels drop (48, 137, 140). Also, GnRH pulse frequency becomes slower and a secondary surge in FSH is detected (142, 143). Like in menstrual cycle, this secondary surge in FSH is essential for the follicle development in rodents (48, 71, 144, 145). Thus, the estrus stage in rodents is similar to the late luteal phase in humans.

During the metestrus stage, estradiol levels fall and remain low and finally during diestrus stage, they begin to rise (137, 140). LH and FSH levels remain low during both of these stages (137, 141). These two stages are equivalent to early to mid follicular phase of the menstrual cycle.

Chapter 3. The Molecular Mechanism Underlying the GnRH Pulse Sensitivity of a Gonadotrope

3.1 GnRH Receptor Mediated Signaling

GnRH receptor (GnRHR) is a G protein coupled receptor (GPCR). It belongs to the rhodopsin family of seven-transmembrane GPCR family and is mainly expressed on the pituitary gonadotropes (38, 146-148). Interestingly, unlike other GPCRs, GnRHR lacks an intracellular cytoplasmic, tail so it is internalized relatively slowly and is not subject to desensitization (149). Upon stimulation with GnRH, GnRHR can couple with Gs and Gq/11 in the gonadotrope cell lines while in other cell lines it has also been shown to interact with Gi and G12/13 (150-153).

GnRHR coupling with Gq/11 activates phospholipase Cβ (PLCβ) (154) (Figure 4). Activated PLC hydrolyzes intracellular phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). DAG, on the other hand, activates protein kinase C (PKC) mediated downstream signaling (154, 155). IP3 binds to the IP3 receptor on the endoplasmic reticulum and releases intracellular calcium (Ca^{2+}), which activates calcium calmodulin kinase II (CaMKII) mediated downstream signaling (156) (Figure 5). GnRHR

also induces CaMKII downstream signaling by activation of the L-type Calcium channels and allowing influx of the extracellular Ca^{2+} (37, 157-161). Such GnRH induced Ca^{2+} mobilization can also trigger the secretion of the gonadotropins (162). GnRHR coupling with Gs activates adenylate cyclase and increases intracellular cyclic adenosine monophosphate (cAMP) levels (Figure 5). cAMP, in turn, activates Protein Kinase A (PKA) and the downstream signaling (153, 155, 163) (Figure 5).

Our recent *in vivo* live cell imaging study demonstrates that Gs and Gq/11 pathways are differentially stimulated by pulsatile and continuous GnRH in the mouse pituitary gonadotrope cell line, the LβT2 cells (164). Using flurescence resonance energy transfer (FRET) reporters, we showed that continuous GnRH treatment leads to transient increase in cAMP levels and PKA activation while prolonged increase in DAG and Ca^{2+} levels (164). We also showed when cells are treated with pulsatile GnRH, each pulse activates cAMP and PKA reporters; however, the DAG and Ca^{2+} reporters are only activated with the initial few pulses and then their signal is quickly desensitized indicating a possible negative feedback loop through possible downstream signaling by PKC or CaMKII (164). Therefore, it is possible that each pathway may have a unique role in the GnRH pulse frequency and amplitude sensing mechanism of a gonadotrope.

Activation of the GNRHR also leads to downstream activation of three major mitogen activated protein kinase (MAPK) pathways: p38, extracellular

signal regulated kinase 1/2 (ERK1/2) and c-jun N-terminal kinase (JNK) (Figure 5) (155, 165-168). While the precise mechanism of the specific pathways that induce LH and FSH gonadotropins production by GnRH is still under investigation, studies so far reveal that GnRH can activate ERK and JNK, leading to induction of a major stimulator of the LHβ promoter Egr-1 (155, 169-171). Inhibition of p38 MAPK pathway blocks GnRH stimulation of the FSHβ promoter (48, 172). More specifically, inhibition of the p38 MAPK, either through inhibitors or through dominant mutant, reduces GnRH induction of c-fos and Activin stimulated c-terminal phosphorylation of the Smad 3, both of which stimulates FSHβ promoter (173). In addition to p38, inhibition of Erk also leads to reduced c-fos levels (155). The specific mechanism of pulsatile GnRH mediated gonadotropins induction is still not understood.
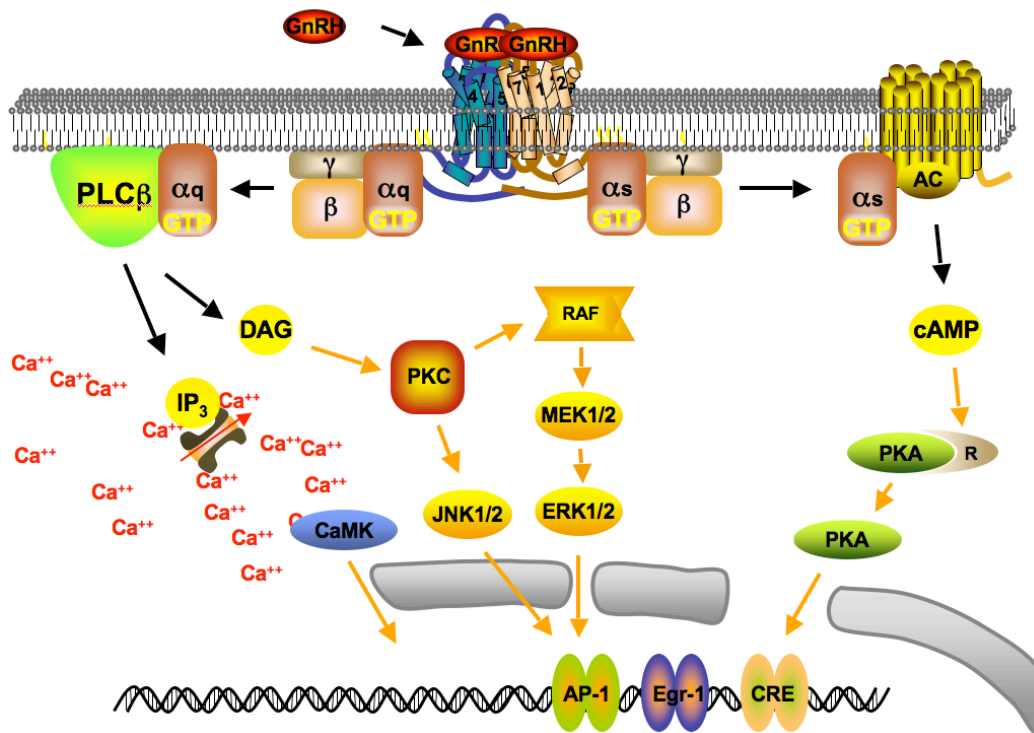
Figure 5: The GnRH Receptor Mediated Signaling.

GnRH can stimulate GnRH receptor and can lead to activation of both the Gs and Gq pathways. Activation of Gs pathway results in the downstream induction of cAMP and activation of PKA enzyme. Activation of Gq pathway, on the other hand, results in activation of PLC and increase in DAG and IP_3 levels. DAG activates PKC and downstream PKC mediated cascade while IP_3 raises intracellular Ca^{2+} levels and thus activates CaMK mediated downstream cascade.

3.2 Regulation of LHβ Subunit Gene

GnRH mediated LHβ induction is dependent on the two distinct region of the LHβ promoter. In the proximal region of the LHβ promoter, two early growth response 1 (Egr1) binding sites in tandem with two steroidogenic factor 1 (SF-1) binding sites surrounding the homeodomain (HD) element are located (48, 174, 175). In the heterologus cells, pituitary specific HD protein, Ptx1, occupies the HD site while in the LβT2 gonadotrope cells the same site is occupied by the Otx1 related HD protein (48, 176, 177). GnRH induction of LHβ gene is dependent on Egr-1, SF-1 and Ptx-1 regulation of the LHβ proximal promoter region through their synergetic interaction (176, 178). In addition, GnRH treatment of the gonadotropes accumulated β-catenin in the gonadotrope nucleus where it interacts with SF-1 and increases SF-1/Egr-1 stimulation of the LHβ promoter (48, 179, 180). The binding sites for Sp1 and NFY as well as an overlapping CArG elements are located on the distal region of the LHβ promoter (181, 182). Based on some recent studies, it is proposed that co-activator SNURF and the scaffolding protein p300 bridge the proximal and distal LHβ promoter and increases Sp-1 interaction with Egr-1/SF-1/HD leading to LHβ promoter stimulation (48, 182-185). The binding sites for majority of these transcription factors are conserved across mammalian species (186).

Unfortunately, most of the studies investigating LHβ promoter are done

using continuous GnRH treatment. More recently though, using a pulse perifusion system to administer GnRH in pulsatile manner, we demonstrated that the rat LHβ promoter is positively regulated by transcription factors Egr1 and Egr2 and these stimulators are expressed at high pulse frequencies (69). In addition, the transcription factor SF1 is expressed under basal conditions and low pulse frequencies and also stimulates LHβ promoter (69). Furthermore, the corepressors Dax1, Nab1 and Nab2 are induced at slow GnRH pulse frequencies and are able to repress LHβ promoter induction mediated by SF1, Egr1 and Egr2 (69). Finally, Nab2 and Dax1 selectively repress LHβ promoter stimulation by a fast GnRH pulse frequency of 30 min compared to a slow GnRH pulse frequency of 120 min (69). These observations allow us to propose a model where LHβ mRNA production is repressed at slow GnRH pulse frequencies due to expression of Nab and Dax corepressors. However, increased expression of positive transcription factors Egr1/2 at high GnRH pulse frequencies can overcome Nab repression leading to increased production of LHβ mRNA (Figure 6).

The interaction of Egr1 and SF1 transcription factors with LHβ promoter is quite dynamic. A recent study demonstrated that in the presence of GnRH, the transcription factors SF-1 and Egr1 get ubiquitinated and undergo proteasomal degradation (187). Such turn over causes Egr-1 and SF1 to cycle on and off on LHβ promoter in response to GnRH treatment (187). The periodicity of SF-1 and Egr1 for LHβ promoter occupancy is 30 minutes, the same time interval as that of a fast GnRH pulse frequency (187). Therefore, GnRH pulse frequency specific

production and cycling of LHβ promoter regulators provides an explanation for the LHβ promoter's responsiveness to GnRH pulses and GnRH frequency dependent induction of LH.

In addition to GnRH, LHβ promoter is also regulated by activin. Activin stimulates activin receptor mediated activation of Smad 2/3 heterodimers by phosphorylating them. The Smad 2/3 heterodimer forms complex with Smad 4 and is localized to the nucleus (Figure 7) (188, 189). The nuclear localized Smad complex can stimulate the activin-responsive element (ARE) located on the proximal LHβ promoter region (125). As co-treatment of GnRH and Activin to LβT2 cells leads to additive increase, it is likely that GnRH and activin stimulate the LHβ promoter by separate and independent molecular mechanism (48, 170, 173).

Other than GnRH and Activin, steroidal hormones estrogen and progesterone also regulate LHβ promoter, but their mechanism of action for most part is still under the investigation. Estrogen receptor (ER) can bind to an imperfect estrogen response element (ERE) at -1189 in the distal LHβ promoter region or can be recruited through SF-1 and Ptx1 (190, 191). Estrogen treatment of LβT2 gonadotropes induces Egr1 and represses LHβ promoter repressors ZEB transcription, so estrogen may also indirectly stimulate LHβ promoter (192). Progesterone binds to mouse proximal LHβ promoter region -180 to +40 and

represses the rat LHβ promoter utilizing its proximal -300 to -150 region (193). Progesterone also represses basal as well as GnRH and activin stimulated LHβ gene expression; however, like estrogen, its precise mechanism of action is still not well understood.
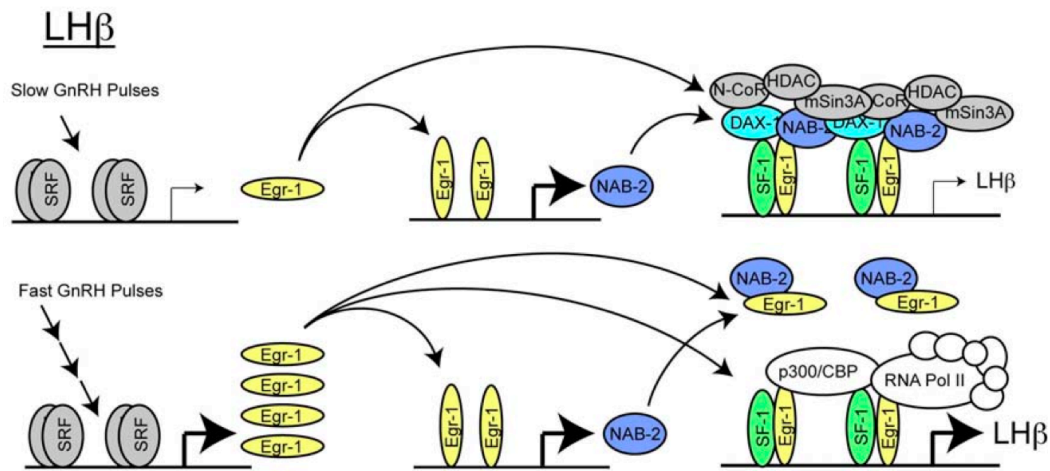
Figure 6: The Model for the GnRH Pulse Regulation of the LHβ Subunit Gene.

At the slow GnRH pulse frequency, there is mainly induction of the corepressor Nab-2, which represses LHβ promoter. On the other hand, by the fast GnRH pulse frequency there is preferential induction of the LHβ promoter stimulatory transcription factor Egr-1. Increased levels of Egr-1 titrate out Nab-2 corepressors leading to greater stimulation of LHβ promoter at the fast GnRH pulse frequency.

3.3 Regulation of FSHβ Subunit Gene – The Stimulators

Like LHβ promoter, all GnRH, activin and steroidal hormones can regulate the FSHβ promoter; however, unlike LHβ promoter, greater degree of synergy is observed in FSHβ promoter regulation by these different pathways, making it a bit more complicated to study. Deletion of the *GnRH* gene in the hypogonadal (*hpg*) mice abrogates the LH and reduces the FSH levels by 60-90% indicating, unlike LH the production of FSH is not completely dependent on the GnRH (194).

It has been reported that activation of the GnRH receptor mediated signaling induces Activator Protein 1 (AP-1) complexes in the gonadotropes. The AP-1 complexes consist of dimers of various Fos isoforms, such as c-fos, FosB, Fra-1 and Fra-2, and Jun isoforms, such as c-jun, JunB and JunD. A Fos family member can only make an AP-1 complex by heterodimerizing with a Jun family member. Jun family members, on the other hand, can also homodimerize and make an AP-1 complex (195, 196). These AP-1 complexes have been shown to bind to a specific region on various promoters called AP-1 consensus site, composed of bases TGA(G/C)TCA. Upon binding to this region, the AP-1 complex can activate transcription of the targeted promoters (172, 197-203). Two consensus AP-1 sites located at -120 and -83 in the ovine FSHβ (oFSHβ) promoter has been proposed to be important for FSHβ expressions due to their

interaction with c-jun and GnRH's inability induce oFSHβ promoter upon their mutation in the heterologus Hela cells; however, one of those two sites is not conserved in mouse, rat or human FSHβ promoters (197). Furthermore, mice carrying transgene for the mutation in both of these sites showed similar response to GnRH as the wildtype mice (202).

A novel AP-1 half-site, composed of bases GTCA, is also located between positions -72 and -69 in the proximal FSHβ promoter region and is conserved in mouse, rat, ovine, bovine and human (172). This half AP-1 site is crucial for GnRH-mediated FSHβ promoter induction as the FSHβ stimulators c-fos and c-jun can directly bind to this site and mutation in this site reduces GnRH as well as fos-jun mediated stimulation of FSHβ promoter (172). The AP-1 half-site is juxtaposed to a CCAAT box. Since the nuclear factor Y (NF-Y) is binds both with the AP-1 factors and the CCAAT box, it is proposed to stabilize AP-1 complex on the AP-1 half-site.

E-box, composed of bases CANNTG, and partial cAMP responsive element (CRE) site, composed of bases GGTCA, located on the proximal rat FSHβ promoter are equivalent to the mouse promoter CCAAT box and AP-1 halfsite respectively (204). The binding of upstream stimulating factors (USF) proteins to the E box is important for basal rat FSHβ promoter stimulation while GnRH stimulated phosphorylated cAMP response element binding (CREB)

protein interaction with the partial CRE site is proposed to be crucial for GnRH mediated FSHβ promoter stimulation (204).

FSHβ subunit gene, like LHβ, is also stimulated by activin in addition to GnRH. As a matter of fact, activin is a potent stimulator of FSHβ gene in the rodents. The FSHβ promoter contains three activin responsive elements (205-210). The ARE located at -267 in the mouse FSHβ promoter is a consensus smad binding element (SBE), consisting bases GTCTAGAC, but it is not preserved in humans and its mutation only reduces the activin responsiveness of the FSHβ pomoter (173, 211). The other two AREs, including a half SBE site composed of bases AGAC at -153 on murine promoter, are conserved in ovine, bovine, porcine, humans, mouse and rat. These sites are stimulated through their interaction with Smad using tethering protein Pbx and Prep and mutation in either of these sites completely abolishes activin response of the mouse FSHβ promoter (209, 210). Alternatively, recent studies also suggest that in addition to Smads, a member of forkhead family of transcription factor FoxL2 can regulate FSHβ promoter utilizing the SBE halfsite as well as other regions on the proximal FSHβ promoter in response to the activin (47, 211, 212). Unlike LHβ, GnRH and activin can lead to synergistic expression of FSHβ subunit gene. Based on a recent study, such such synergy is due to crosstalk of both pathways at p38 MAPK level and utilized AP-1 and Smad sites located in the proximal FSHβ promoter region (173).

The steroidal hormones, estrogen and progesterone, are also involved in FSHβ regulation, but the precise mechanism underlying is still under the investigation. Estrogen has little effet on FSHβ gene since estrogen treatment of estrogen receptor transfected LβT2 cells does not alter FSHβ gene expression (213). Estrogen effect on FSHβ may be indirect as estrogen receptor α (ERα) knockout mice have increased activin B in the pituitary (214). Progesterone has stimulatory effect on the FSHβ gene since progestin, a synthetic progestogin with similar effects as progesterone, induces rat and ovine FSHβ promoter stimulation in respective primary cultures and also induces FSHβ gene expression in the anterior pituitary (215-217). Furthermore, progesterone receptor (PR) can bind to six putative hormone responsive element (HRE) sites containing -500 and -95 region on murine FSHβ promoter (213). Activin seems to enhance steroid response on FSHβ promoter as activin and progestin co treatment of LβT2 cells leads to a synergistic increase in murine FSHβ promoter (218).
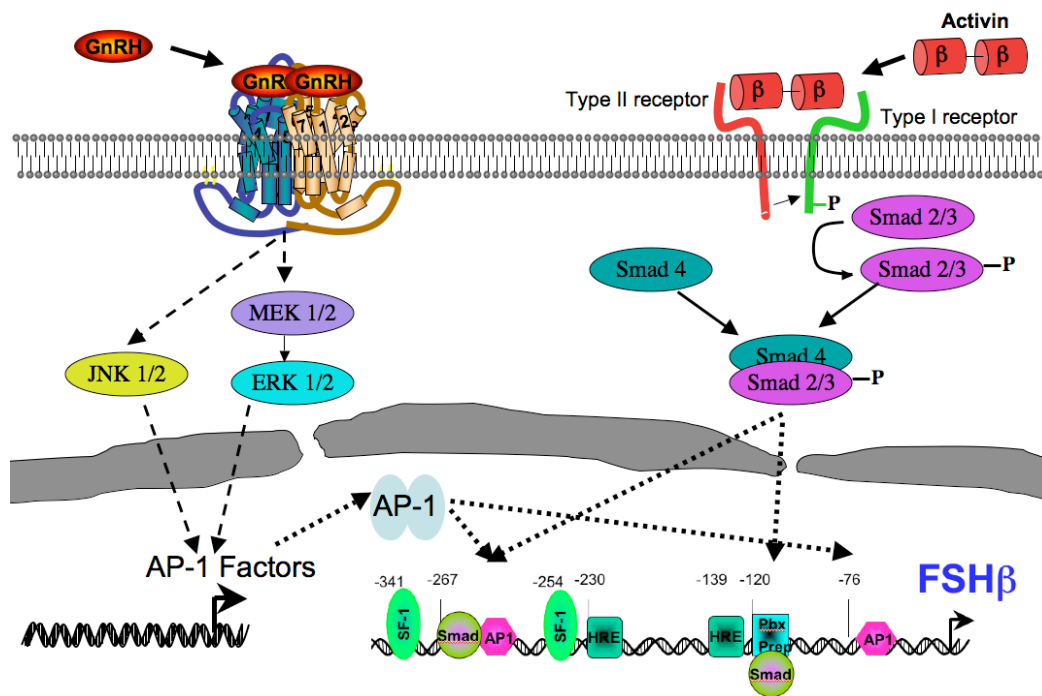
Figure 7: GnRH and Activin Stimulation of the FSHβ Promoter.

GnRH induces Fos and Jun AP-1 factors. AP-1 factors form homodimeric (Jun/Jun) or heterodimeric (Fos/Jun) AP-1 complex, which can bind to FSHβ promoter and stimulate it. Activin activates Smad signaling pathway, which leads to formation of Smad heterotrimeric (Smad2/3/4) complex and its nuclear localization. Upon its nuclear localization Smads can also bind to and stimulate FSHβ promoter.

3.4 Regulation of FSHβ Subunit Gene – The Corepressors

While many studies have focused on how GnRH and the other hormones stimulate FSHβ promoter using continuous hormonal treatment, the mechanisms underlying GnRH pulse sensitivity of the FSHβ promoter is not understood. One of the ways pulsatile GnRH may regulate FSHβ gene expression is by differentially regulating expression of FSHβ regulatory stimulators and corepressors by different GnRH pulse frequencies as it does LHβ promoter. While FSHβ stimulators have been targeted by many studies, the role of different corepressors in FSHβ promoter regulation remains unknown.

A recent study reported that the corepressor Inducible cAMP Early Repressor (ICER) is selectively induced at the fast GnRH pulse frequency of 30 min when FSHβ expression is low (219). ICER is an isoform of CREM and is induced by alternative intronic promoter P2 of the *CREM* gene (220, 221). While different isoforms of CREM act as stimulators or corepressors, ICER essentially contains DNA binding domain of the CREM and is a potent repressor of CRE (220). ICER is involved in GnRH pulse regulation of FSHβ promoter *via* competing with CREB for the partial CRE site on the rat proximal FSHβ promoter (222). However, CREB deficient mice don't have detectable changes in FSH raising the possibility that it may be due to functional redundancy by other family members such as cAMP response element modulator (CREM) or

activating transcription factor (ATF) or due to CREB regulation of rat FSHβ promoter in a species specific manner (223)

Smad-mediated transcription is also subject to negative regulation. Ski and SnoN (also known as SkiL) are members of the ski family of nuclear proto-oncogenes, which interfere with TGF-β mediated Smad signaling in various cell lines, but their role in pituitary gonadotropes has not been investigated (224-227). Both SnoN and Ski bind to Smad 2, 3 and 4 (Figure 8) (225). Binding to both receptor regulated Smads and the common partner Smad 4 is essential for Ski and SnoN mediated repression, as well as transformation of embryonic fibroblasts (225). Both SnoN and Ski have C-terminal coiled coil domains through which they can homodimerize with themselves or heterodimerize with each other (Figure 8) (228). Heterodimerization of Ski and SnoN is preferred over homodimeriztion and deletion of the domain critical for dimer formation reduces transcriptional repression by SnoN (229). Binding of SnoN and Ski to Smad proteins results in disruption of the active heterotrimeric Smad complex and removal of the coactivators p300/CBP from the Smad proteins (230, 231). Additionally, SnoN and Ski can associate with Histone Deacetylase Complex 1 (HDAC1) through binding to NcoR-1 and mSin3A leading to repression of their target genes (232, 233).

Corepressors TG-interacting factor 1 (TGIF1) and 2 (TGIF2) can also associate with Smads and again repression of Smad-dependent transcription by

TGIFs involves the recruitment of HDACs instead of the coactivator p300 into the Smad complex (Figure 8) (234-236). Association of c-jun is also important for TGIF1 mediated repression of Smad signaling (237). Repression of target genes by corepressors SnoN, Ski and TGIFs through their association with Smads and c-jun makes the differential pulse sensitivity of SnoN, Ski and TGIF of particular relevance to GnRH and activin regulation of FSHβ.
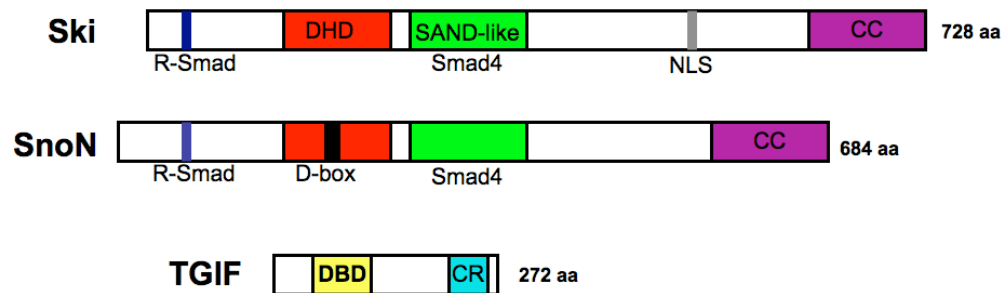
DHD: Dachshund homology domain
SAND: Sp100, AIRE1, NucP41/75 and DEAF1
R-Smad: Smad 2/3 binding region
Smad4: Smad 4 binding region
NLS: Nuclear localization sequence
D box: Degradation box
CC: Coiled Coil Region
DBD: DNA binding domain
CR: Conserved Repression domain

Figure 8: Potential Corepressors of the FSHβ Promoter.

Ski and SnoN are members of the Ski family of oncoproteins. They both can bind with Smad 2 and Smad 3 (R-Smad) as well as Smad 4 (Co-Smad). In addition, they can also form homodimers and heterodimers by the coiled coil domain located at their C-terminus. TGIF can also bind with R-Smads and Co-Smad. In addition, TGIF can bind to c-jun. TGIF has a DNA binding domain located near its N-terminus and a conserved repression domain located near its C-terminus.

Chapter 4. Material And Methods

4.1 Materials

GnRH was purchased from Sigma Chemical Co. (St. Louis, MO) or the National Hormone and Peptide Program (Los Angeles, CA). Activin A was purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal horseradish peroxidase-linked anti-rabbit antibodies, anti-SnoN (sc-9141), anti-Ski (sc-9140), anti-CREM (sc-440), anti-TGIF (sc-9084), anti-c-fos (sc-52), anti-c-jun (sc-1694), anti-fra-1 (sc-183), anti-FosB (sc—48) and anti-β-tubulin (sc-9104) as well as ChIP grade versions of c-fos, c-jun, TGIF and SnoN antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SnoN (sc-36519), TGIF (sc-36660), CREM (sc-37701) and control (sc-37007) siRNAs were also purchased from Santa Cruz. Protein G-Dynabeads Immunoprecipitation Kit (Invitrogen 100.07D), DMEM and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA) and Cellgro (Mediatech, Inc., Manassas, VA). The human wildtype HA-tagged TGIF expression vector was obtained from addgene (addgene.org). The human, TGIF1 mutant (Threonine 235/239 to Valine) and control vector were obtained from Dr. Joan Massague (Sloan Kettering Cancer Institute, New York, NY). Human TGIF2 was obtained from Dr. David Wotton (University of Virginia, Charlottesville, VA). Human

wild-type and smad binding deficient mutant SnoN and control vector were obtained from Dr. Kunxin Luo (University of California, Berkeley, CA). CREB, DCREB, KCREB and MCREB expression vectors were obtained from Dr. Jane Reusch (University of Colorado Health Sciences Center, Aurora, CA). The -1.5Kb mouse FSHβ promoter-luciferase construct (-1.5Kb mFSHβ-luc) was obtained form Dr. Pamela Mellon (UCSD). AP1 and SBE multimer-luciferase constructs were obtained from Dr. Djurdjica Coss (UCSD). Expression vectors for mouse c-fos, c-jun, Fra-1, FosB, JunB, JunD, SnoN, TGIF1, Ski, CREM and pCMVSPORT6 were purchased from ATCC (ATCC.org). All other reagents were purchased from either Sigma or Fisher Scientific (Pittsburgh, PA).

4.2 LβT2 Cell Culture

Mouse pituitary gonadotrope derived cell line, LβT2 cells were maintained in a monolayer culture in DMEM supplemented with 10% fetal bovine serum (FBS), Glutamax (Gmx) and penicillin-streptomyocine (PS) antibiotics in 175 cm^3 flasks, which in turn were kept in a humidified 10% CO_2 atmosphere 37°C incubator. Once confluent, cells were washed once with phosphate buffered saline (PBS) buffer. They were removed from the flask wall using trypsin and then suspended in 37°C prewarmed DMEM/FBS/Gmx/PS. Cells were spun down at 3000rpm using a centrifuge. The soup media was discarded to remove trypsin and then resuspended in fresh 37°C prewarmed DMEM/FBS/Gmx/PS. Cells were counted using hemocytometer and then seeded into new flasks to maintain the culture. Alternatively, they were seeded into 100mm, 150mm, 6 well or 12 well plates for the experiments.

4.3 Primary Pituitary Cultures

Primary pituitary cells were obtained as described before with minor modifications (238, 239). Briefly, anterior pituitaries were rapidly removed from 18-day old male rats, placed in freshly prepared DMEM/Gmx, cut into small pieces and incubated in 0.25% trypsin for 30 min. After addition of DNAse and fetal bovine serum, the fragments were dispersed into individual cells in freshly prepared Krebs-Ringer bicarbonate buffer without Ca^{2+} or Mg^{2+} and filtered through a 70 μm cell strainer (BD Biosciences, Bedford, MA). Pituitary cells (400,000 per well) were plated in 12-well plates coated with poly-L-lysine (PLL) in DMEM (low glucose) supplemented with 10% horse serum, 2.5% fetal calf serum, 1% minimum essential medium Eagle nonessential amino acids, fungizone, and gentamicin (GIBCO). The following day, cells were washed with serum-free medium DMEM with high glucose-F12 supplemented with 0.1% BSA) and stimulated with 100 nM GnRH for the indicated times. RNA was extracted from cells and Q-PCR performed as described above using appropriate primers for rat SnoN and TGIF.

4.4 Microarray Data Analysis

Microarray data from a previous study was analyzed to determine how known FSHβ transcription factors were induced in perifused LβT2 cells treated with 10 and 100 nM, continuous and pulsatile GnRH (69). Briefly, cells, starved for 16h in serum free media, were treated with 10 or 100nM, 5 min GnRH pulse for 0, 1, 2, 4 or 8 times over the period of 4 h in a perifusion system. In addition, one group of cells was treated continuously with 10 or 100nM GnRH for 4 h. The experiment was repeated twice.

RNA was extracted using Trizol and purified using RNeasy. 10ug RNA from each condition was analyzed on Affymetrix mouse MU74Av2 genechips in duplicates. The raw value was derived from Affymetrix Microarray Suite software (MAS 5) and was imported into VAMPIRE for analysis. A Bonferroni multiple testing correction was used to identify genes with most robust changes and for the selected genes data were normalized and plotted as fold basal change.

4.5 Single Pulse, Multiple Pulses and Continuous GnRH Treatment in Static Culture

LβT2 cells were maintained as described before. For single pulse experiments, LβT2 cells were grown to confluence in 6-well or 12-well plates (BD Biosciences, San Jose, CA), washed once with serum free medium, and incubated in fresh serum-free medium for 24 h. Cells were stimulated with either 10 nM or 100 nM GnRH for 5 min then GnRH-containing medium was removed and fresh 37°C serum-free medium was added. At the indicated times, the medium was removed and cells were washed once with PBS. Cells were harvested for RNA or protein extraction at various times thereafter. For continuous GnRH treatment, the same protocol was followed except that the culture medium was not changed after GnRH addition.

For multiple pulses in static culture, LβT2 cells were grown to confluence in 6-well plates, washed once with serum-free medium, and incubated in fresh serum-free medium for 24 h. Cells were stimulated with either 1 nM or 10 nM GnRH, or with vehicle, for 5 min at 30 min or 120 min intervals for 6 h. Every 30 min, serum-free medium was removed and fresh warmed serum-free medium was added to all three groups irrespective of GnRH stimulation to ensure all groups were treated equally and to correct for autocrine stimulation by factors secreted by the LβT2 cells. After 6 h, medium was removed and cells were washed once with

PBS before harvesting for RNA or protein extraction. For pulse kinetic experiments, cells were treated with pulses as above, given one last 5 min pulse at 6 h then harvested at the indicated times after the last synchronized pulse.

4.6 Quantitative RT-PCR

RNA was extracted from LβT2 cells using RNA-Bee (Tel-Test, Friendswood, TX). First-strand cDNA was synthesized using a high capacity cDNA synthesis kit (Roche Applied Science, Indianapolis, IN). Samples were run in 20 μL reactions on a MJ Research Chromo4 instrument using iSYBR Green (BioRad, Hercules, Ca) and sequence-specific primers for c-fos, c-jun, FosB, Fra1, TGIF, SnoN, CREM, Ski and FSHβ (Table 1). Ct values were extracted by manually setting the threshold midway between basal and maximum fluorescence on a log10 scale. Gene expression levels were calculated after normalization to the housekeeping gene, GAPDH or M36B using the ΔΔCt method and expressed as relative RNA levels compared with the control.

Table 1: Primers.

The table contains a list of primers used for PCR and qPCR.

Ski-fwd	GAGAAAGAGACGTCCCCACA
Ski-rev	AAAGCTCTTGTAGGAGTAGAAGCTG
snoN-fwd	GACAGGGAGCCCGAGTATG
snoN-rev	CCGCTCCTGTCTGAGTTCAT
Tgif1-fwd	GAAACCCCAGCTTCACCTCT
Tgif1-rev	GCCAGATGCTGCAACAAG
Tgif2-FWD	GGCTCTATCTGCACCGCTAC
Tgif2-REV	CGGGAGATGGTGAACTGATT
Crem-fwd	GCTGAGGCTGATGAAAAACA
Crem-rev	GCCACACGATTTTCAAGACA
ICER-Fwd	CTGCCTCACCAGGAAG
ICER-Rev	CAGCTCCAGCTTGAGAGTTG
mFSHb-Fwd	CCACTTGGTGTGCGGGCTACTG
mFSHb-rev	GTGTAGAGGGAGTCTGAGTGGCGGGC
c-fos-Left	AGGGAGCTGACAGATACACTCC
c-fos-Right	TGCAACGCAGACTTCTCATC
c-jun-Left	TGCCACCGAGACTGTAAAGA
c-jun-Right	CCTGTGCGAACTGGTATGAG
JunB-Left	TACCACGGAGGGAGAGAAAA
JunB-Right	TTGGCAGCTGTGCGTAAA
JunD-left	TCTTGGGCTGCTCAAACTC
JunD-Right	CCCGTTGGACTGGATGAT
Fra1-Left	TGCAGAAACCGAAGAAAGGA
Fra1-Right	TTCTCATCCTCCAATTTGTCG
m36B4-FWD	ACCCTGAAGTGCTCGACATCACAG
m36B4-REV	GCAGGGGCAGCAGCCGCAAATGC
mouseF-GAPDH	CATGGCCTTCCGTGTTCCTA
mouseR-GAPDH	CCTGCTTCACCACCTTCTTGAT
Rat TGIF-fwd	GCTCCGGCTTCCCAACTGCC
Rat TGIF-rev	GCCAGAGGCCGCTGACGATG
Rat SnoN-fwd	TGACCACGGTCCTCAGACCCA
Rat SnoN-rev	GACTGGGACGCAGCAGAGGC
Rat Cyclo-fwd	GTCTCTTTTCGCCGCTTGCT
Rat Cyclo-rev	TCTGCTGTCTTTGGAACTTTGTCTG
FSHb-prom-1-FWD	TGATACTGATGGGTTCCTCCA
FSHb-prom-1-REV	GAATCCTGATAGAAATGGGTATATGAA
FSHb-prom-2-FWD	CACAGCCCATAGGAACAAGA
FSHb-prom-2-REV	CCAAAGCAGTCTAAATGCCA
FSHb-prom-3-FWD	GGTGTGCTGCCATATCAGATTCGG
FSHb-prom-3-REV	GCATCAAGTGCTGCTACTCACCTGTG

4.7 Western Blotting

LβT2 cells were lysed on ice in SDS sample buffer (50 mM Tris; 150mM NaCl; 0.25% Deoxycholic acid; 1% Nonidet P-40; 1nM EDTA; 0.05% protease inhibitor; 0.04% β-mercaptoethanol; 1x NuPAGE LDS sample buffer), boiled for 5 min to denature proteins, and sonicated for 5 min to shear the chromosomal DNA. Equal volumes (10-20 μl) of these lysate were separated by SDS-PAGE on 10 or 12 % gels, electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA). The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline-Tween (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween 20). Blots were incubated with primary antibodies in blocking buffer for 60 min at room temperature and then incubated with horseradish peroxidase-linked secondary antibodies followed by chemiluminescent detection using Pico Luminescent Substrate (Thermo Scientific, Rockford, IL). Films were scanned and data were quantified by densitometry (Carestream Molecular Imaging Software). Intesity of each sample band was normalized to the respective β-tubulin band. This ratio was plotted as basal or time-zero fold change.

4.8 Chromatin Immunoprecipitation Assay (ChIP)

ChIP was performed as previously described (172). Briefly, LβT2 cells were grown on 150 mm tissue culture plates to confluency. They were treated with a single pulse, multiple pulses or continuous GnRH as indicated. Cells were crosslinked using 1% formaldehyde, lysed and sonicated to produce the chromatin lysate. Protein G coated dynabeads were prepared and conjugated with indicated antibodies according to manufacturer's protocol. The chromatin lysate was incubated with conjugated dynabeads overnight. The beads containing the antigen-antibody complex were washed successively with low-salt buffer, high salt buffer, LiCl wash buffer, then finally 3 times with dynabead buffer. Chromatin was eluted using the elution buffer provided with the dynabeads and then TE buffer at 65 ˚C and constant agitation. The cross-linking was reversed by heating the samples at 65 ˚C overnight. The samples were then treated with Proteinase K and DNA was purified using a PCR purification kit (Qiagen). PCR was performed on immunoprecipitated samples as well as input samples using three different sets of primers targeting the proximal FSHβ promoter region (Table 1). Data were quantified by densitometry (Carestream Molecular Imaging Software) and intensities of bands produced by three different sets of primers were averaged. Sample band intensities were normalized to respective input bands and to the mean input intensity. As a negative control in the initial experiments, unconjugated beads were used to immunoprecipitate the FSHβ

promoter region. No detectable bands were observed upon PCR of these samples

(data not shown).

4.9 Perifusion, Transfections and Promoter Activity Assays

LβT2 cells were routinely maintained in 175 cm^2 flasks in DMEM-supplemented with 10% FBS at 37°C with 5% CO_2. In the perifusion experiments, 12-15 x 10^7 cells were plated on a 1 ml bed volume of cytodex 3 microcarrier beads in a 10 cm Petri dish with DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin and grown for 5 days in an atmosphere of 5% CO_2. Subsequently, cells were washed once in fresh serum-free DMEM and transfected with 4 μg of a 2:1:1 ratio of mouse -1.5Kb FSHβ-luc, co-repressor expression vector or pCMVSPORT6 control, and CMV-βgal. Transfections were performed using Fugene 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations. After 16 h, cells were loaded into the perifusion chambers as described in previous publication (69) and pulsed with 10 nM GnRH at given pulse frequencies. Two ng/ml activin was supplemented every 120 min to maintain basal FSHβ promoter activity. After the perifusion, cells were lysed in 100 mM PBS containing 0.1% Triton X-100, vortexed for 30 sec, and clarified by centrifugation at 14,000g. Cell lysates were assayed for luciferase (Luciferase Assay System, Promega) and β-galactosidase (Galacto-Light Tropix, Bedford, MA) activity according to manufacturer's instructions in a 96-well plate using a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA)

For monolayer transfections, LβT2 cells were plated in 6 or 12-well plates. Transfection was performed using Fugene 6 reagent or Transfast (Promega Corp., Madison, WI) following manufacturer's protocol. For most experiments, each well was transfected with 500 ng of -1.5Kb mFSHβ-luc, 50 ng of tk-lacZ, and 50 ng of each expression vector for 12-well plates or 1.5 μg of -1.5Kb mFSHβ-luc, 150 ng of tk-lacZ, and 150 ng of each expression vector for 6-well plates. For human wildtype and mutant SnoN and TGIF, 215 ng of expression vectors were used per well in 6-well plates. Empty vectors were used to balance DNA mass as necessary. Control experiments contained the empty pGL3 reporter plasmid. The following day, the cells were switched to serum-free DMEM supplemented with 0.1% BSA. Twenty-five ng/ml activin was added to specified wells in various experiments. After overnight incubation, the cells were treated with vehicle or single pulse of 100 nM GnRH for 6 h. For experiments where no GnRH or activin is used, cells were harvested after specified time with vehicle treatment. Cell lysates were assayed for luciferase and β-galactosidase activity as above.

4.10 siRNA Knockdowns

LβT2 cells were seeded into 12-well culture plates 24 h before transfection. The cells (60–70% confluence) were transfected with 100 nM siRNA as well as -1.5Kb mFSHβ-luc, tk-lacz, smad3, c-fos and/or c-jun expression vectors using 2 μl Fugene HD (Roche) using similar ratios of plasmids as above. The cells were cultured for the given time and harvested. For GnRH-Activin experiments, the medium was replaced with fresh serum-free medium after the given siRNA treatment. Twenty-five ng/ml activin was added when applicable. The next day 100 nM GnRH was added to the indicated groups. Six hours after GnRH treatment, cells were washed once with PBS and assayed for Luciferase-β-galactosidase activity or immunoblotted for protein expression as described above. For multiple pulse experiments, siRNA was electroporated into cells using a Microporator at 1300 V pulse amplitude, 20 ms pulse width and 2 pulses (BTX/Harvard Apparatus, Holliston, MA).

4.11 Statistical Analysis

Data were analyzed using Prism software using Annova for multiple group comparisons with the significance threshold set at p<0.05. The significance of differences between individual conditions was determined using the Tukey post hoc tests again with p<0.05. Student's t test was used for specific pair-wise comparisons with significance being p<0.05. Linear regression analysis was performed using Prism. The r^2 value for the fit was determined and the line was tested for significant non-zero slope at a significance value of p<0.05. All the experiments were repeated at least three times unless otherwise indicated.

Chapter 4, in part, is a reprint of the material as it appears in Gonadotropin-Releasing Hormone Pulse Sensitivity of Follicle Stimulating Hormone-Beta Gene Is Mediated by Differential Expression of Positive Regulatory Activator Protein 1 Factors and Corepressors SKIL and TGIF1 2011. Mistry, Devendra et al. Molecular Endocrinology, 2011. The dissertation author was the primary investigator and author of this paper.

Chapter 5. The Differential Expression of FSHβ Promoter Regulatory Transcription Factors and Corepressors by the Different GnRH Pulse Frequencies

5.1 GnRH Pulse Frequency Mediated Induction of AP-1 Factors and Corepressors mRNA

We examined our pulse perifusion microarray data from a previously published study to determine how known FSHβ transcription factors were induced in perifused LβT2 cells treated with continuous or pulsatile GnRH (69). While reanalyzing the microarray data, we paid particular attention to pulsatile and continuous GnRH stimulated changes in the AP-1 subunits, Smads and CRE regulatory factors as they are involved in FSHβ promoter regulation. We also focused on *Ski*, *Sno* and *Tgif* family members as we speculated that they might also be involved in FSHβ promoter regulation based on their ability to interact with FSHβ promoter stimulatory transcription factors.

With 10 nM GnRH pulses, only JunD was significantly increased by a single pulse as well as multiple pulses and continuous treatment (Figure 9a). The other AP-1 subunit genes, c-fos, c-jun, JunB, FosB and Fra-1, were only significantly induced at the highest GnRH pulse frequency of 30 min or by the continuous GnRH treatment. Similarly, corepressors TGIF1, TGIF2, CREM and

SnoN were significantly induced only by the highest GnRH pulse frequency or by continuous treatment (Figure 9a).

At the higher 100 nM GnRH pulse amplitude, all of the AP-1 subunit genes (c-fos, c-jun, FosB, JunB, Fra-1, JunD) as well as the corepressor SnoN and CREM were significantly increased by both slow and fast GnRH pulse frequencies in addition to by continuous GnRH treatment (Figure 9b). However, a significant increase in the corepressor TGIF1 was only seen at faster GnRH pulse frequencies or continuous GnRH treatment (Figure 9b). Similar pulse sensitivity was observed for the related family member TGIF2. The Smad2, Smad3, Smad4, CREB, ATF2 and Ski genes were constitutively expressed regardless of the pulse frequency or amplitude (Figure 9 and data not shown).
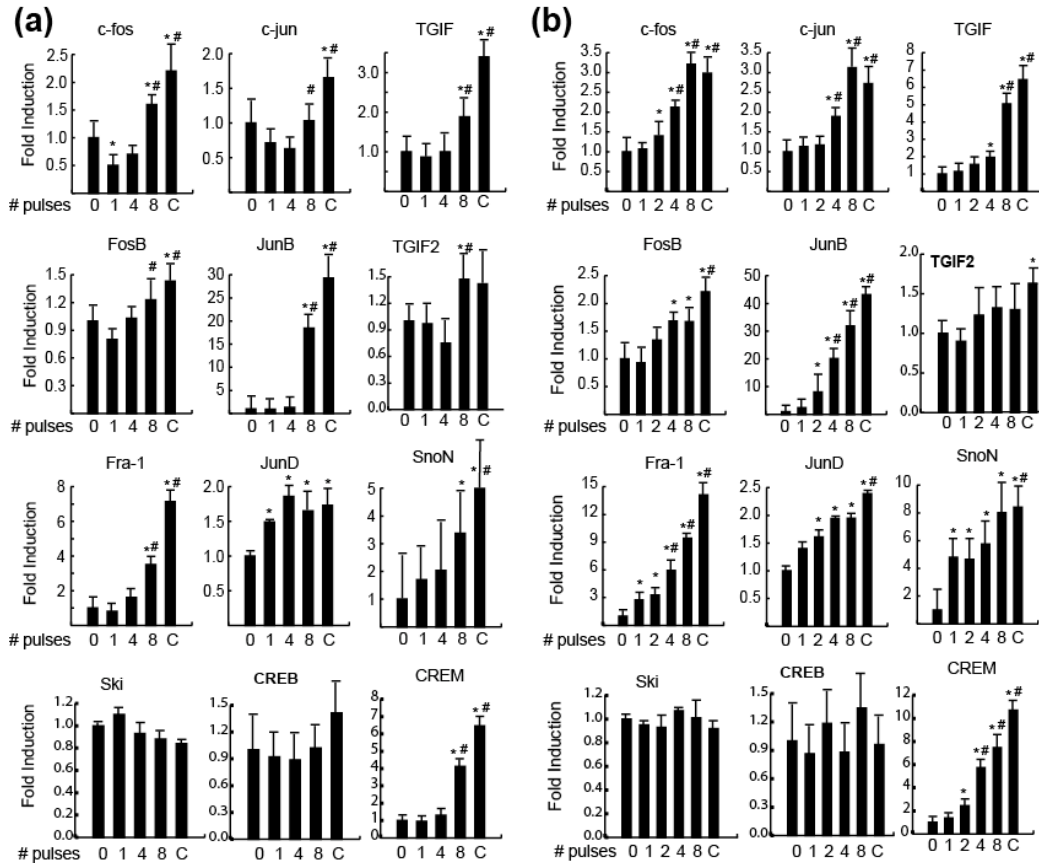
Figure 9: Induction of AP-1 factors and corepressors mRNA by GnRH.

Duplicate sets of RNA isolated from perifused LβT2 cells, pulsed with one, two, four, or eight pulses of 10 nM or 100 nM GnRH or treated with vehicle or 10 nM or 100 nM continuous GnRH over 4 h, were assayed on Affymetrix MU72Av2 microarrays and compared using VAMPIRE (69). Panel (a) shows normalized expression of selected transcription factors and co-repressors by pulses of 10 nM GnRH. Panel (b) shows normalized expression of selected transcription factors and co-repressors by pulses of 100 nM GnRH. The number of GnRH pulses given over 4 h is shown bellow each graph: 0 = vehicle treatment; 1= GnRH single pulse treatment; 2 = 120 min GnRH pulse frequency; 4 = 60 min GnRH pulse frequency; 8 = 30 min GnRH pulse frequency; c = continuous GnRH treatment. * indicates significant difference (p < 0.05) compared to respective vehicle treated control. # indicates significant difference (p < 0.05) compared to a single GnRH pulse for (a) or to the 120 min GnRH pulse frequency for (b).

5.2 The Kinetics of AP-1 Factors and Corepressors mRNA Induction by a GnRH
Single Pulse

We had previously shown that the difference in the pulse sensitivity of
Egr1 stimulator and Nab corepressor at the LHβ promoter correlated with their
respective mRNA stabilities (69). To address the possibility that corepressor
genes may be rapidly degraded making their induction undetectable at slower
GnRH pulse frequencies, LβT2 cells were stimulated with a single 5 min pulse of
10 nM or 100 nM GnRH and relative mRNA expression was checked by QPCR at
different times up to 4 h post stimulation. Pulses of both 10 and 100 nM GnRH
led to the induction of the AP-1 factors c-fos, c-jun, FosB and Fra1 (Figure 10),
and also the corepressors SnoN, CREM and TGIF1 (Figure 11). There was no
detectable change in Ski levels as expected from our microarray data (Figure 11).

A single GnRH pulse led to the rapid induction of c-fos, which peaked at
30 min of GnRH treatment. The highest induction of c-jun and Fos B mRNA
occurred at 60 min of GnRH treatment. The induction of Fra-1 was noticeably
slower with peak expression at 90 min and expression was maintained up to 4 h
(Figure 10). Pulses of 10 nM and 100 nM GnRH led to different amplitudes of
AP-1 subunit induction. The lower amplitude pulse of 10 nM GnRH led to a 4-
fold induction of c-jun, a 6-fold induction of Fra-1, a 40-fold induction of c-fos
and a 100-fold induction of FosB. The higher amplitude pulse of 100nM GnRH

led to a 7-fold induction of c-jun, a 33-fold induction of Fra-1, a 100-fold induction of c-fos and a 500-fold induction of FosB (Figure 10).

A single GnRH pulse led to maximum induction of SnoN, CREM and TGIF at 60 min, 120 min and 90 min of stimulation, respectively. The 10 nM pulse led to a 6-fold induction of SnoN and a 2.5-fold induction for TGIF and CREM, whereas the 100 nM GnRH pulse led to a 12-fold induction of SnoN, a 5-fold induction of CREM and a 6-fold induction of TGIF (Figure 11).

To verify that SnoN and TGIF are induced in a more physiologically relevant model, we measured SnoN and TGIF in primary pituitary cultures from 18 days old male rats after stimulation with 100 nM GnRH for 1 and 2 h. TGIF1 was significantly induced at 2 h while SnoN was significantly induced at both 1 and 2 h demonstrating that these genes are GnRH-sensitive in mixed primary cultures (Figure 12). While their mRNA induction was relatively lower than that observed in LβT2 gonadotropes, it was to be expected, as the gonadotropes is only 5-10% of the total population of the anterior pituitary cells. The other cell types in the pituitary may also express these genes, which may lower the magnitude of increase detected.

Since ICER is derived from an alternatively spliced form of CREM gene and was also shown to be involved in the GnRH pulse regulation of FSHβ promoter, we also checked ICER induction by a single pulse of 100 nM GnRH to

compare with CREM (219). Both CREM and ICER were maximally induced at 90 min by single pulse of GnRH and did not have any difference in terms of the pattern of their induction suggesting that our observed increase in ICER is due to increase in CREM (Figure 13).
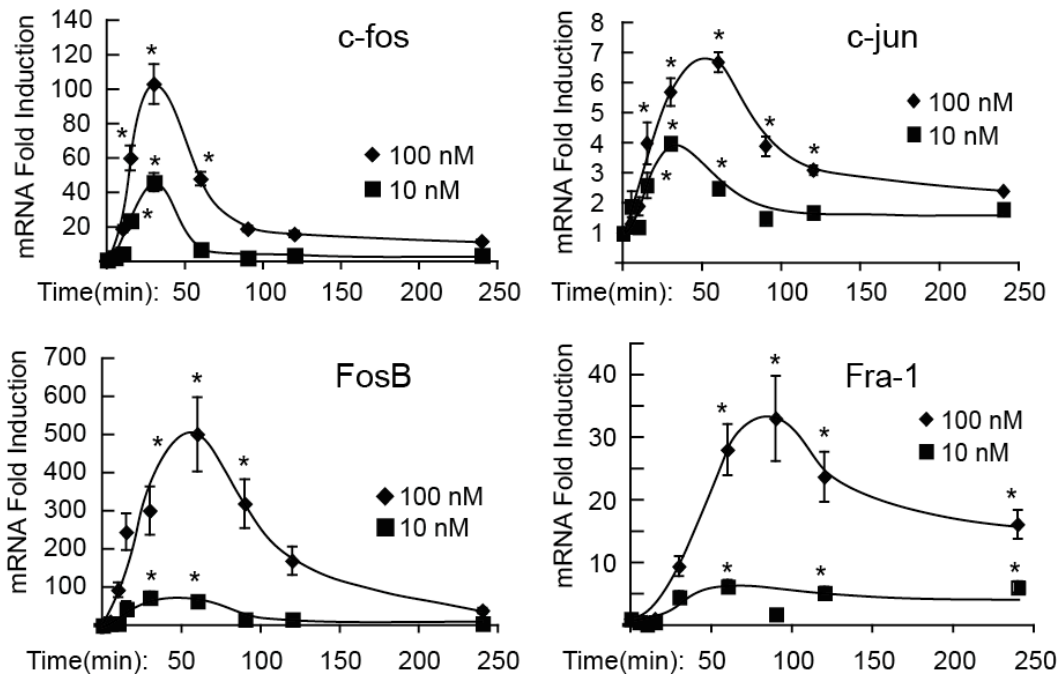
Figure 10: Induction of AP-1 Factors mRNA by a Single Pulse of GnRH.

LβT2 cells were serum starved for 24 h and then were stimulated with a single 5 min pulse of 10 or 100nM GnRH for the indicated time. mRNA was extracted and QPCR was performed using specific primers. The data represent the means and SE of at least two independent experiments. * indicates significant difference ($p < 0.05$) compared to respective zero time point.
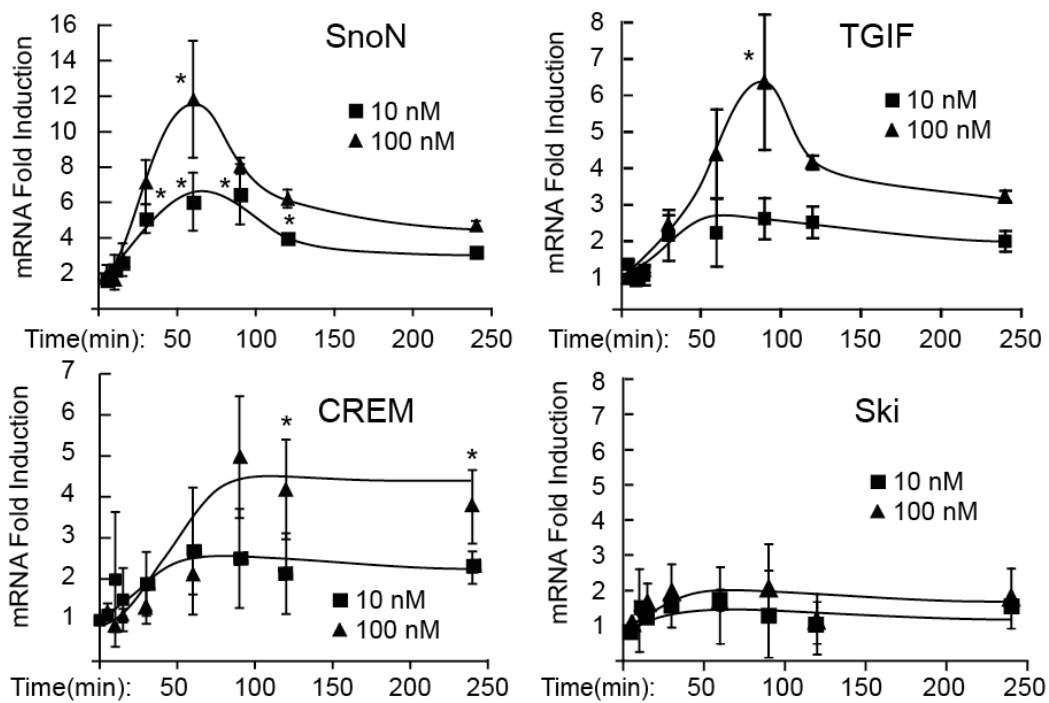
Figure 11: Induction of Corepressors mRNA by a Single Pulse of GnRH.

LβT2 cells were serum starved for 24 h and then were stimulated with a single 5 min pulse of 10 or 100nM GnRH for the indicated time. mRNA was extracted and QPCR was performed using specific primers. The data represent the means and SE of at least two independent experiments. * indicates significant difference (p < 0.05) compared to respective zero time point.
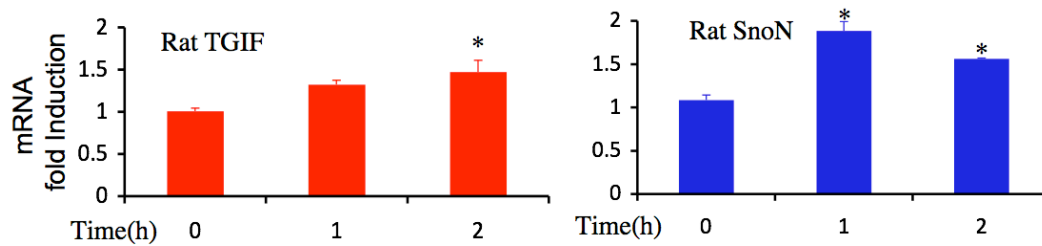
Figure 12: Induction of Corepressors mRNA in the Primary Pituitary Culture by a Continuous GnRH.

18 day old male rat derived primary pituitary cells were treated with 100nM GnRH for given time, mRNA was extracted and qPCR was performed to check rat SnoN and rat TGIF gene expression. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to 0 time point.
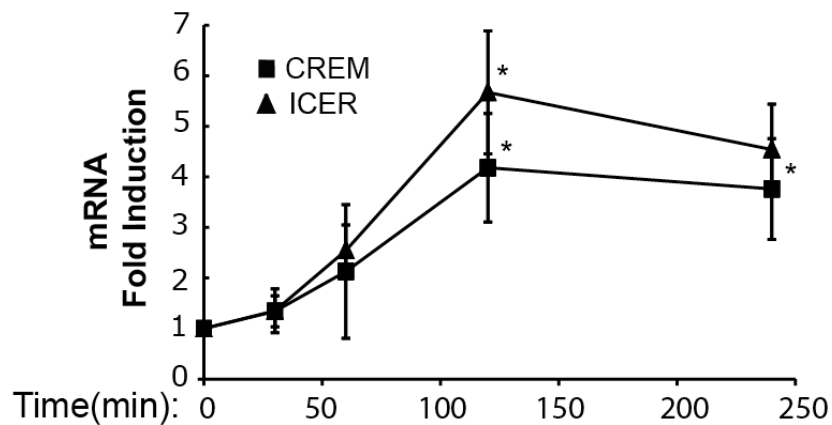
Figure 13: Comparison of CREM and ICER mRNA Induction by a GnRH Single Pulse.

LβT2 cells were serum starved for 24 h and then were stimulated with a single 5 min pulse of 100nM GnRH for given time. mRNA was extracted and qPCR was performed using sequence specific primers for CREM and ICER. The data represents means and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to basal.

5.3 The Kinetics of AP-1 factors and Corepressors Protein Induction by the Continuous GnRH Treatment

Having observed induction of AP-1 factors and corepressors at the mRNA level upon GnRH treatment by both microarray and QPCR, we then investigated induction at the protein level. Cells were stimulated with continuous 100 nM GnRH and protein expression assessed over 6 h. C-fos and c-jun were induced maximally at 2 h of GnRH treatment (Figure 14a), which is consistent with the maximal mRNA induction at 30-60 min via QPCR (Figure 10), and protein expression was still detectable at 6 h. Multiple bands were detected for both c-fos and c-jun. We believe the upper bands represent the phosphorylated versions of c-fos and c-jun, phosphorylated at the carboxyl and amino termini, respectively, as has been shown previously (155, 240-242). We confirmed this for c-jun protein by blotting with a phospho-c-jun (Ser73) antibody, which only detected the upper band (Figure 15a). The lower unphosphorylated form of c-fos is induced initially but then the upper phosphorylated form predominates at later times. The converse is true for c-jun, the early-induced form corresponds to the phosphorylated form, but at later times the unphosphorylated form predominates (Figure 14a). GnRH stimulated maximal induction of Fra-1 and Fos B proteins at 4-6 h similar to the mRNA results (figure 14a).

The corepressors SnoN and TGIF1 were induced maximally at 4 h and expression was maintained for 6 h (Figure 14b), which was consistent with the maximal mRNA induction observed at 90 min (Figure 11). The TGIF1 antibody detected two bands that are likely to be unphosphorylated (lower band) and phosphorylated (upper band) forms of TGIF1 based on prior published data and also based on the expression of HA tagged human TGIF1 in the LβT2 gonadotropes (Figure 15b) (243). GnRH increased both forms of TGIF1. In contrast to the other proteins, only a slight induction of CREM was detected at 6 h (Figure 14b). Finally, as expected from our mRNA data, no changes were detected in the Ski protein levels by the continuous GnRH treatment (Figure 14b). A representative β-tubulin blot is shown to demonstrate equal loading.

(a) Continuous 100nM GnRH

c-fos
c-jun
Fra-1
FosB

β-tubulin

Time: 0   0.5   1   2   4   6 h

(b) Continuous 100nM GnRH

SnoN
TGIF
CREM
Ski

β-tubulin

Time: 0   0.5   1   2   4   6 h
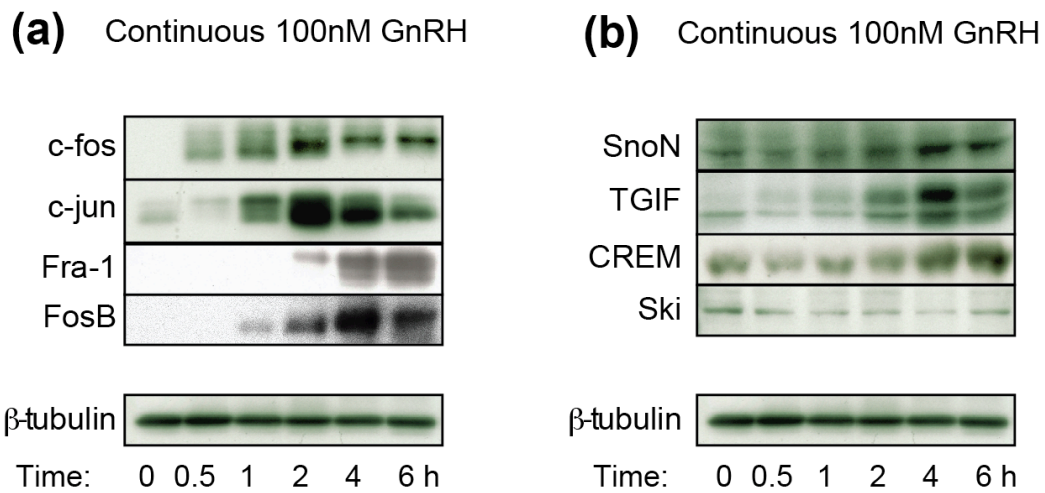
Figure 14: Induction of AP-1 Factors and Corepressors Proteins by Continuous GnRH.

LβT2 cells were serum starved for 24h. Afterwards, they were treated with 100nM GnRH continuously for the given time. Cells were harvested and westerns were performed using the indicated AP-1 factors (a) and corepressors (b) antibodies. Blots were stripped and reblotted for β-tubulin to ensure equal loading.

(a)

(b)
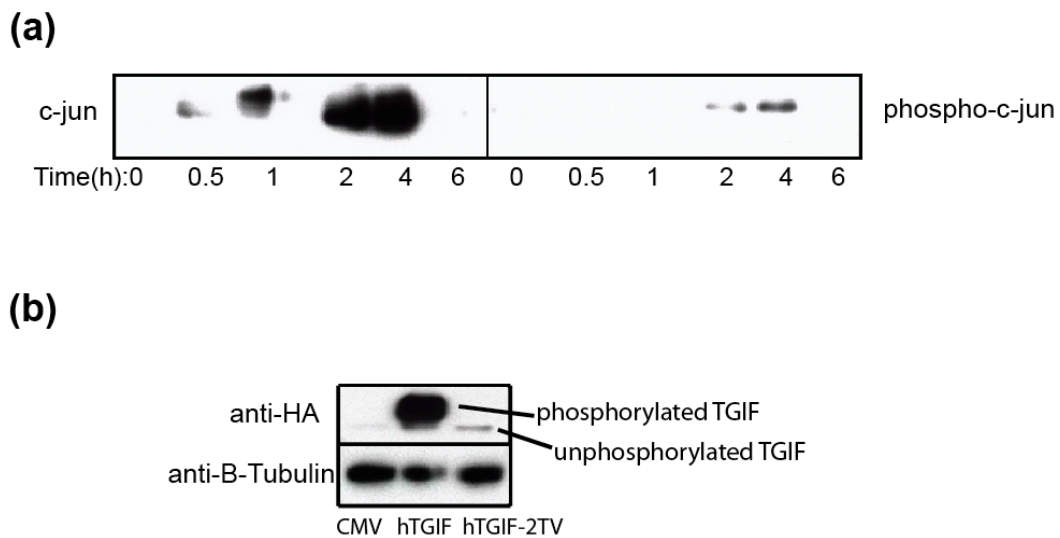
Figure 15: Phosphorylation of c-jun and TGIF.

(a) LβT2 cells were serum starved for 24h and then treated with 100nM continuous GnRH for given time. Westerns were performed on duplicates of these samples. One group was blotted by c-jun antibody (left) and other was blotted by phospho-Ser73-c-jun antibody (right). (b) LβT2 cells were transfected with either HA-tagged wildtype or 2TV mutant human TGIF1 or respective control vector. 2 days past transfection cells were lysed and westerns were performed with anti-HA or anti-β-tubulin antibody.

5.4 The Kinetics of AP-1 factors and Corepressors Protein Induction by a GnRH Single Pulse

We then checked whether a single 100 nM GnRH pulse would induce these transcription factors. Both c-fos and c-jun were induced maximally at 2 h similar to the continual treatment, but dropped rapidly thereafter (Figure 16a). Fra-1 and FosB were induced at later times, 4-6 h, and expression was maintained similar to the continual treatment (Figure 16a).

Maximal induction of the corepressors TGIF and SnoN occurred at 2-4 h following a single pulse of GnRH, but CREM did not respond to a single GnRH pulse (Figure 16b). The level of the Ski protein remained unchanged upon both single pulse and continuous GnRH treatment in agreement with the mRNA data (Figure 16b). A representative β-tubulin blot is shown to demonstrate equal loading.

(a) 5 min 100nM GnRH pulse

c-fos
c-jun
Fra-1
FosB

β-tubulin

Time: 0 0.5 1 2 4 6 h

(b) 5 min 100nM GnRH pulse

SnoN
TGIF
CREM
Ski

β-tubulin

Time: 0 0.5 1 2 4 6 h
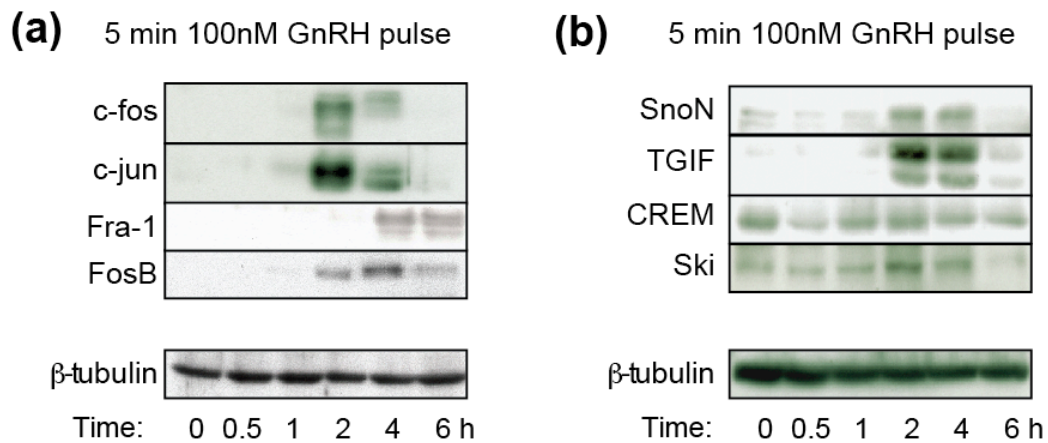
Figure 16: Induction of AP-1 Factors and Corepressors Proteins by a Single Pulse GnRH.

LβT2 cells were serum starved for 24h. Afterwards, they were treated with a 5min 100nM GnRH single pulse and then harvested at the given time. The westerns were performed using the indicated AP-1 factors (a) and corepressors (b) antibodies. Blots were stripped and reblotted for β-tubulin to ensure equal loading.

5.5 Differential Protein Induction of AP-1 Factors and Corepressors with Pulsatile

GnRH Treatment

After confirming that both AP-1 factor and corepressor proteins were

induced by GnRH, we tested the response of these proteins to multiple pulses of

GnRH. LβT2 cells were treated with 5 min pulses of 1 nM and 10 nM GnRH

every 30 or 120 min for 6 h (Figure 17). The lower doses of GnRH were used for

the multiple pulse experiments to prevent desensitization. C-fos and c-jun were

induced at both the pulse frequencies, but showed greater induction at the faster

frequency (Figure 18a). FosB was induced almost equally by both the fast and

slow GnRH pulse frequencies (Figure 18a).

In contrast, Fra-1, SnoN and TGIF1 were only induced at the faster GnRH

pulse frequency and were not induced at the slower pulse frequency (Figure

18a,b). CREM protein was only weakly induced by the faster pulse frequency at a

concentration of 1 nM GnRH (Figure 18b). As expected, Ski protein levels did

not change with GnRH treatment in agreement with our mRNA data (Figure 18b).

In most cases, treatment with 10 nM GnRH led to a higher induction c-fos, c-jun,

TGIF1 and SnoN protein expression than 1 nM GnRH treatment (Figure 18).

Chapter 5, in part, is a reprint of the material as it appears in

Gonadotropin-Releasing Hormone Pulse Sensitivity of Follicle Stimulating

Hormone-Beta Gene Is Mediated by Differential Expression of Positive Regulatory Activator Protein 1 Factors and Corepressors SKIL and TGIF1 2011. Mistry, Devendra et al. Molecular Endocrinology, 2011. The dissertation author was the primary investigator and author of this paper.
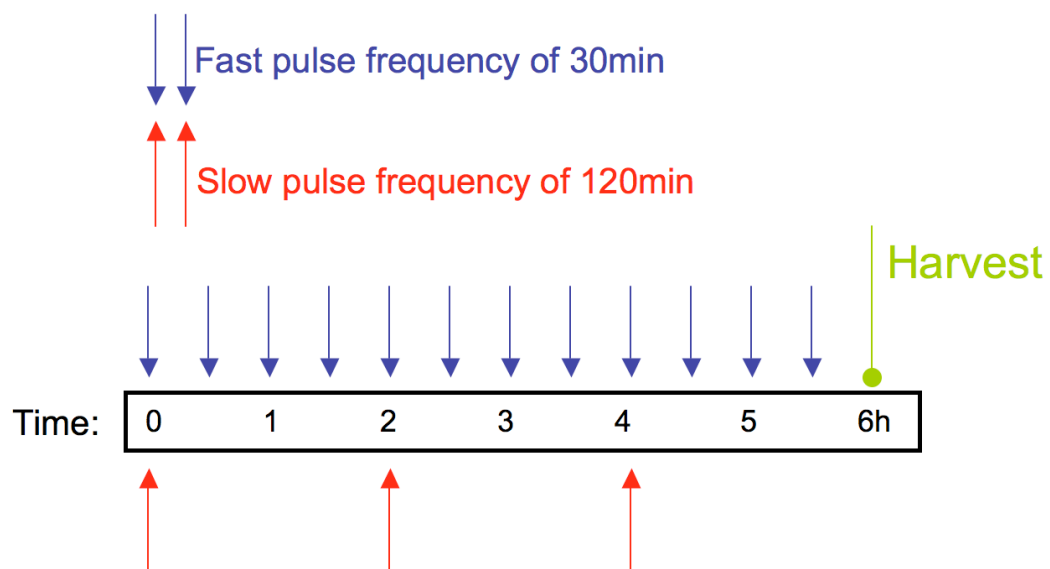
Figure 17: GnRH Pulse Treatment Scheme#1.

LβT2 cells were serum starved for 24h. Afterwards, they were treated with 1 or 10nM GnRH pulses at the interval of 30 min (fast pulse frequency) or 120 min (slow pulse frequency) for total of 6h. Cells were harvested at 6h and used for westerns or RT-qPCR.
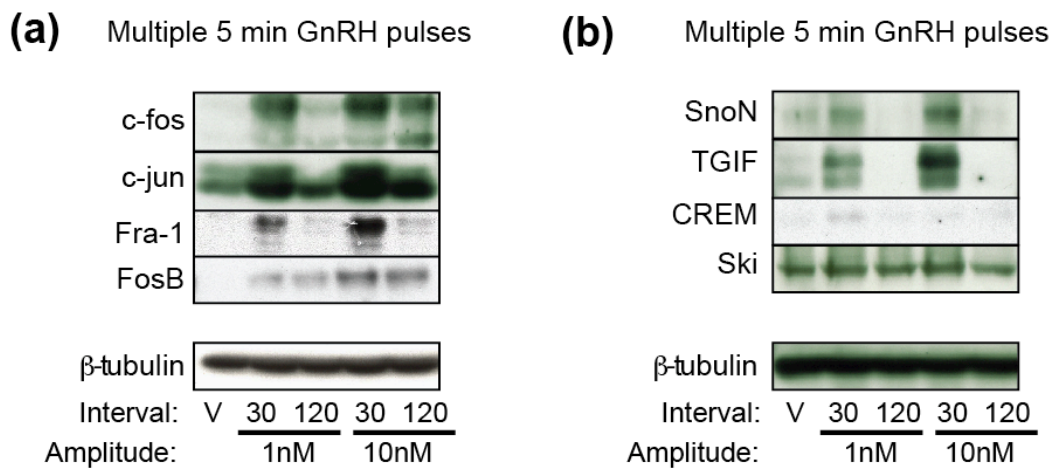
Figure 18: Induction of AP-1 Factors and Corepressors Proteins by Multiple Pulses of GnRH.

LβT2 cells were serum starved for 24h. Afterwards, they were treated with 1 or 10nM pulsatile GnRH for 6h at the indicated frequency (as shown in Figure 17). Cells were harvested and westerns were performed using the indicated AP-1 factors (a) and corepressors (b) antibodies. Blots were stripped and reblotted for β-tubulin to ensure equal loading.

Chapter 6. Repression of the FSHβ Promoter by TGIF, SnoN, Ski and CREM

6.1 Repression of the the FSHβ Promoter by the Overexpression of Corepressors

6.1.1 Repression of AP-1 Factor - Stimulated FSHβ Promoter Activity by SnoN, Ski, TGIF and CREM Overexpression

We have shown that GnRH pulses lead to differential induction of transcriptional activators and corepressors. To test whether these corepressors could repress the FSHβ gene promoter, a mouse -1.5 Kb FSHβ promoter-luciferase reporter (mFSHβ-luc) was co-transfected with TGIF, SnoN or Ski expression vectors into LβT2 cells. None of the co-repressors reduced basal FSHβ promoter activity (Figure 19). As GnRH induces the FSHβ promoter via the AP-1 site, we stimulated promoter activity by co-transfecting expression vectors for c-fos, Fra-1, c-jun, JunB, or JunD individually, or in combination. Transfection of vectors for c-fos, c-jun or Fra-1 led to a 1.8-fold increase in FSHβ promoter activity, but JunB had no effect. Co-transfection of mouse TGIF1, SnoN, or Ski reduced FSHβ promoter activity to basal levels (Figure 19).

When transfected in combination, c-fos and c-jun led to a 3.8-fold increase in FSHβ promoter activity, while c-jun and Fra-1, or c-fos and JunB

cotransfection led to a 2.5-fold increase in the FSHβ promoter activity (Figure 19). Cotransfection of c-fos and Fra-1 with JunB did not lead to a greater induction than seen with Fra-1 alone suggesting that JunB is not a partner for Fra-1. JunD transfection did not increase promoter activity alone and had no additive effect in combination with c-fos or Fra-1 (Figure 20). Cotransfection of SnoN or Ski reduced FSHβ promoter activity stimulated by c-fos and c-jun or JunB, but did not reduce promoter activity significantly when stimulated by Fra-1 and c-jun or JunB (Figure 19). TGIF1 was only able to repress when promoter activity was stimulated by Fra-1 or c-fos in combination with JunB, but not with c-jun (Fig. 4b). Interestingly, TGIF1 is known to physically interact with c-jun (237). It is possible that TGIF1 can repress AP-1 driven FSHβ promoter activity with endogenous levels of c-jun, but overexpression of c-jun may titrate TGIF1 from the AP-1 complex on the promoter preventing its repressive effect.
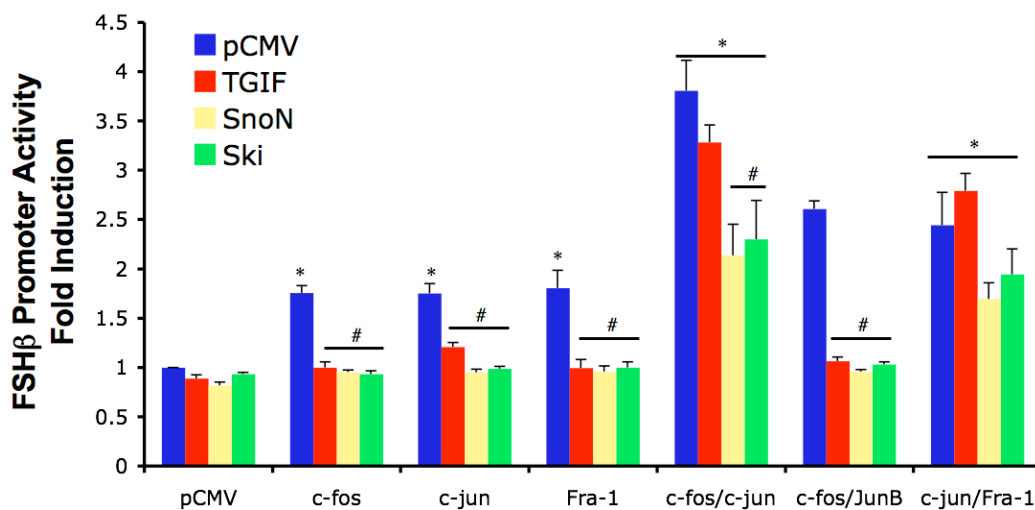
Figure 19: Repression of Ap-1 Factors Stimulated FSHβ Promoter Activity by TGIF, SnoN and Ski.

LβT2 cells were co-transfected with -1.5Kb mFSHβ-luc, tklacZ, expression vectors for c-fos, c-jun, Fra-1 and JunB, and with expression vectors for corepressors SnoN, Ski and TGIF or pCMVSPORT6. Cells were harvested 2 days post transfection and the luciferase assay was performed. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) from respective AP-1 stimulated group.
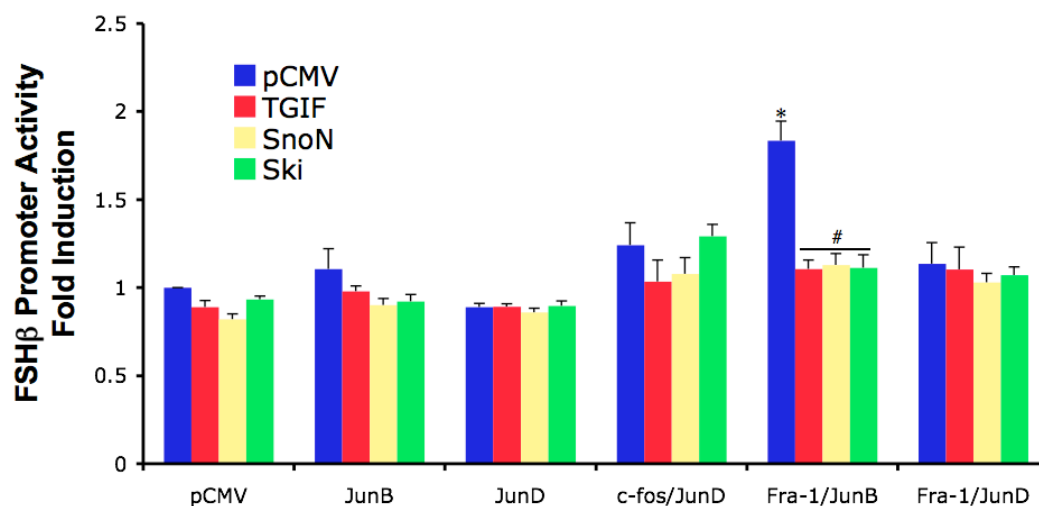
Figure 20: JunB and JunD Fails to Stimulate the FSHβ Promoter.

LβT2 cells were co-transfected with -1.5Kb mFSHβ-luc, tklacZ, expression vectors for JunB, JunD, c-fos, and Fra-1, and with expression vectors for corepressors SnoN, Ski and TGIF or pCMVSPORT6. Cells were harvested 2 days post transfection and the luciferase assay was performed. Data represent the mean and SE of at least three independent experiments. * indicates significant difference (p < 0.05) compared to vehicle treated control group. # indicates significant difference (p < 0.05) from respective AP-1 stimulated group.

6.1.2 Repression of Smad - Stimulated FSHβ Promoter Activity by TGIF, SnoN
and Ski Overexpression

As SnoN, TGIF1 and Ski are known to form complexes with Smads, we
then tested whether these corepressors can repress FSHβ promoter activity
stimulated by Smad proteins. LβT2 cells were transfected with expression
vectors for Smad2, Smad3 or Smad4 alone or in combination, along with the
mFSHβ-luc reporter gene and tklacZ. Smad3 transfection increased FSHβ
promoter activity 3-fold, but Smad2 had no effect (Figure 21). Smad4
transfection alone did not increase promoter activity, but Smad3 and Smad4 co-
transfection increased promoter activity 5-fold. Cotransfection of SnoN or Ski
reduced Smad3 and Smad3/4 stimulated promoter activity to basal levels (Figure
21). TGIF1 also repressed FSHβ promoter activity, but not as strongly as Ski or
SnoN. Thus, the corepressors SnoN, Ski and TGIF1 repress both AP-1 and
Smad-stimulated FSHβ promoter activity.
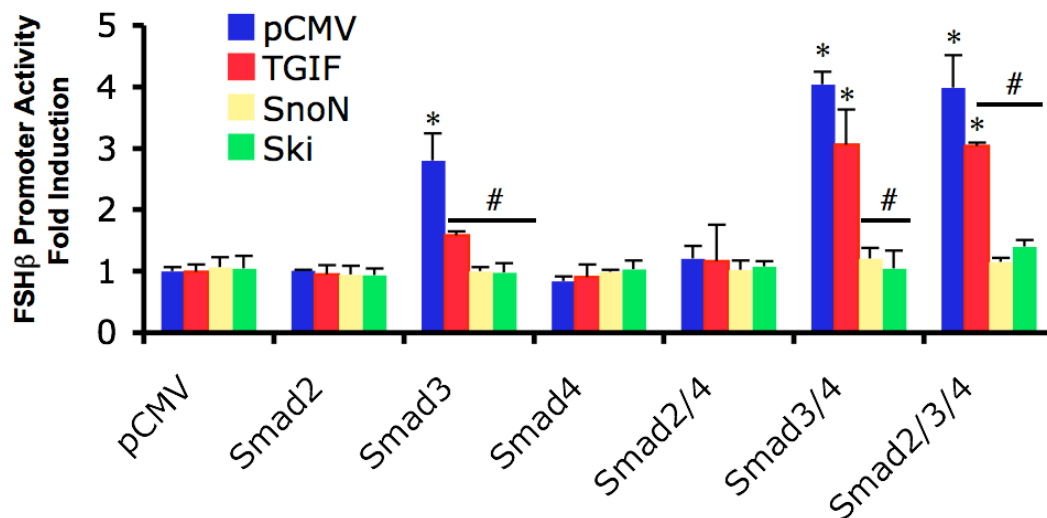
Figure 21: Repression of Smad Stimulated FSHβ Promoter Activity by TGIF, SnoN and Ski.

LβT2 cells were co-transfected with -1.5Kb mFSHβ-luc, tklacZ, expression vectors for Smad2, Smad3 and/or Smad4 and with expression vectors for corepressors SnoN, Ski and TGIF or pCMV as indicated. Cells were harvested 2 days post transfection and the luciferase assay was performed. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) from respective Smad stimulated group.

6.1.3 Repression CREB Stimulated FSHβ Promoter Activity by CREM Overexpression

Mutation of the conserved CRE on the FSHβ promoter reduces GnRH-stimulated activation of the rat FSHβ gene promoter, therefore we wanted to investigate whether CREB and its corepressor CREM were important for GnRH regulation of the mouse FSHβ promoter (204). LβT2 gonadotropes were transfected with the -1.5Kb mFSHβ-luc reporter construct along with expression vectors for CREB or a constitutively active mutant DIEDML-CREB (DCREB), with or without CREM. DCREB has substitution of 6 non conserved amino acids from sterol responsive element binding protein (SREBP) into the activation domain of the CREBD, making it high affinity phosphorylation independent CREB binding protein (CBP) binder (244). Increased interaction with CBP allows DCREB to stimulate CRE more strongly than CREB (244). Transfection of CREB and DCREB seem to induce FSHβ promoter activity 2-3 fold, but the induction was not significant (Figure 22). Nevertheless, when CREM was cotransfected with either CREB or DCREB, FSHβ promoter activity was reduced down to basal levels (Figure 22).
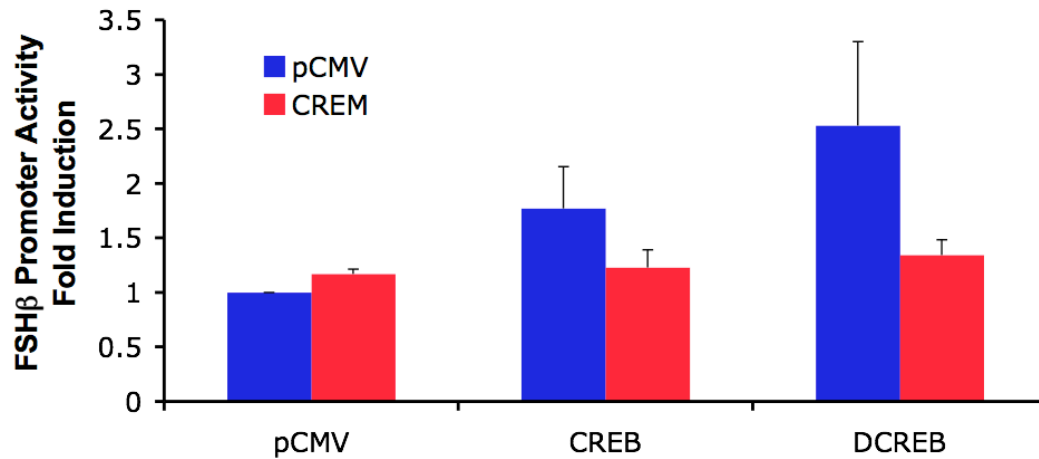
Figure 22: Repression of CREB Stimulated FSHβ Promoter Activity by CREM.

LβT2 cells were transfected with -1.5Kb mFSHβ-luc, tklacZ, expression vectors for CREB or DCREB, and expression vectors for CREM or pCMV control vector. Cells were harvested 2 days post transfection and the luciferase assay was performed. Data represent the mean and SE of at least three independent experiments.

6.1.4 Repression of GnRH and Activin - Stimulated FSHβ Promoter Activity by

TGIFs, SnoN and Ski Overexpression

Next, we tested the effect of these corepressors on GnRH and/or Activin-

stimulated FSHβ promoter activity. LβT2 cells were transfected with or without

expression vectors for the corepressors along with the -1.5Kb mFSHβ-luc reporter

construct (Figure 23). In the absence of the corepressors, both activin treatment

(25 ng/ml, 16 h) and GnRH (100 nM, 6 h) treatment increased in FSHβ promoter

activity 3 to 4 fold (Figure 23). Activin and GnRH co-treatment induced FSHβ

promoter activity 7-fold. Co-transfection of either SnoN or Ski reduced FSHβ

promoter activity induced by GnRH, activin or both (Figure 23). In contrast,

TGIF1 only reduced promoter activity following activin and GnRH cotreatment,

but did not have a significant repressive effect on cells stimulated with GnRH or

activin individually (Figure 23). Human TGIF2 also repressed GnRH and

Activin-stimulated FSHβ promoter activity in similar manner to mouse TGIF1,

but it also repressed the basal FSHβ promoter activity (Figure 24).
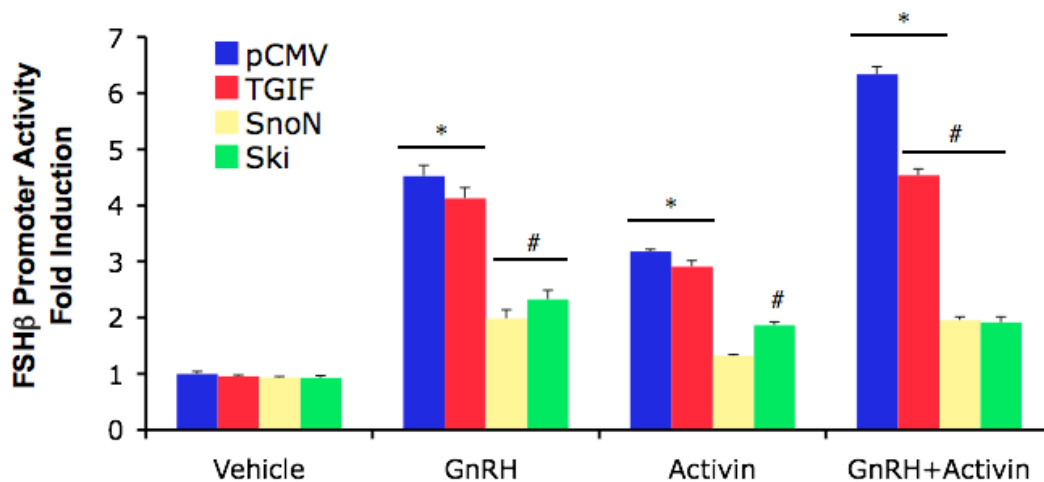
Figure 23: Repression of GnRH and Activin Stimulated FSHβ Promoter Activity by TGIF, SnoN and Ski.

LβT2 cells were transfected with -1.5Kb mFSHβ-luc, tklacZ, and expression vectors for corepressors SnoN, Ski and TGIF or pCMVSPORT6. One day post transfection, cells were starved 16 h with serum free medium in the presence or absence of 25 ng/ml activin A. Afterwards, 100nM GnRH was added for 6 h and FSHβ promoter activity was measured. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) from respective GnRH and Activin stimulated group.
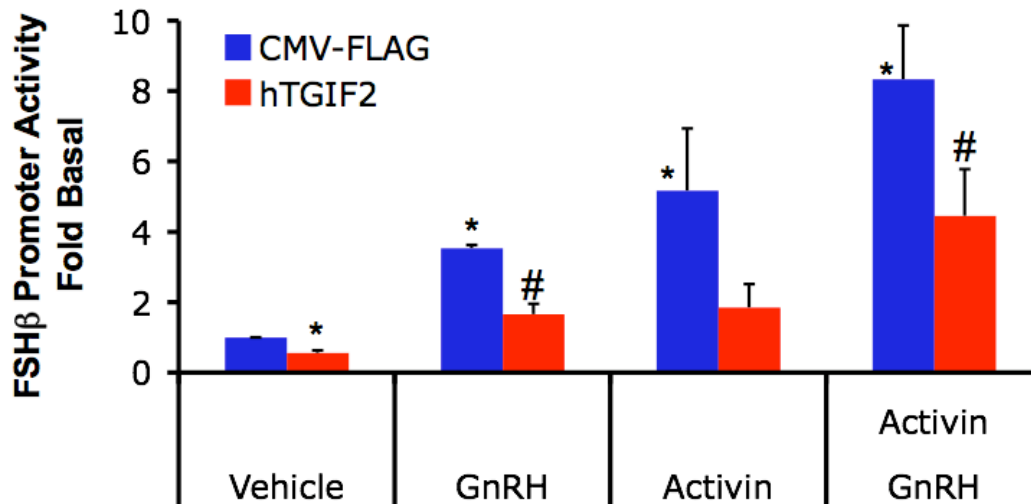
Figure 24: Repression of GnRH and Activin Stimulated FSHβ Promoter Activity by human TGIF2.

LβT2 cells were co-transfected with pCMV control or hTGIF2 along with -1500 mFSHβ promoter-luc and tklacZ as indicated. Transfected cells were starved for 24h with serum free media and then treated with 100nM GnRH for 6h, 25ng/ml activin for overnight or both as before. After the treatment cells were harvested and the promoter activity was measured. Data represents means and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) from respective GnRH and/or Activin treated pCMV transfected control groups.

6.1.5 Repression of GnRH and Activin Stimulated FSHβ Promoter Activity by CREM and CREB Mutants Overexpression

Finally, we performed similar experiments to test whether CREM or dominant negative forms of CREB could repress FSHβ promoter activity mediated by GnRH, activin or both. Expression vectors for CREM, KCREB, or MCREB were transfected with the -1.5Kb mFSHβ-luc reporter construct. KCREB and MCREB are dominant negative mutants of CREB, KCREB having a point mutation in its DNA binding motif such that it heterodimerizes with endogenous CREB and reduces its binding to CRE, and MCREB having a point mutation in its phosphorylation site (S133A) such that it can bind to CRE site, but can not bind to coactivator CBP (245). Treatment with GnRH (100 nM, 6 h) and/or activin (25 ng/ml, 16 h) stimulated the FSHβ promoter as before (Figure 25). Transfection of CREM or the dominant negative mutants KCREB and MCREB reduced promoter activity stimulated by GnRH alone and both GnRH and activin, but not by activin alone consistent with the known requirement of the CRE solely for GnRH induction of FSHβ (Figure 25).
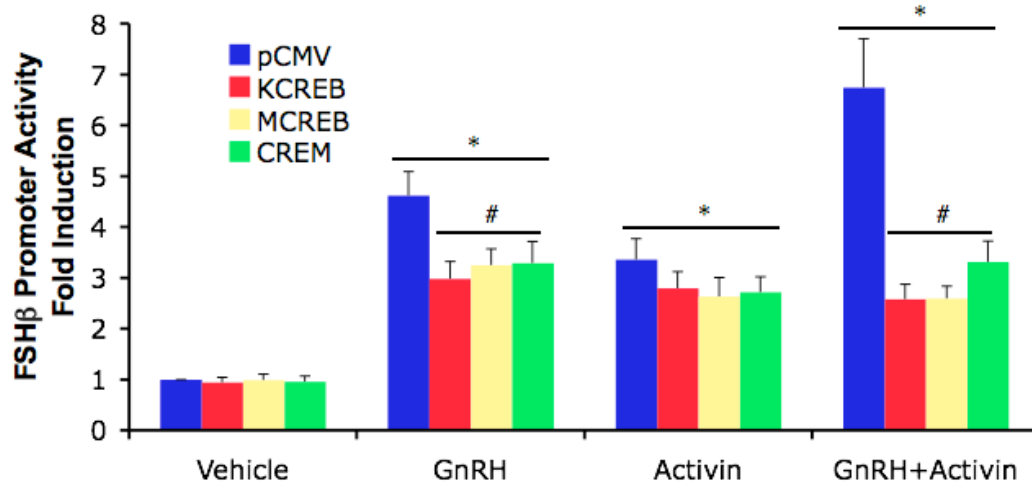
Figure 25: Repression of GnRH and Activin Stimulated FSHβ Promoter Activity by CREM and CREB mutants.

LβT2 cells were transfected with -1.5Kb mFSHβ-luc, tklacZ, and expression vectors for CREM, KCREB and MCREB or pCMVSPORT6. One day post transfection, cells were starved 16 h with serum free medium in the presence or absence of 25 ng/ml activin A. Afterwards, 100nM GnRH was added for 6 h and FSHβ promoter activity was measured. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) from respective GnRH and Activin treated group.

6.2 Increase in the FSHβ promoter Activity by the Knockdown of the Corepressors

6.2.1 Knockdown of SnoN, TGIF or CREM Increases FSHβ Promoter Activity

The experiments above demonstrated that overexpression of the corepressors SnoN, Ski, CREM or TGIF1 represses FSHβ promoter activity. To test whether the endogenous corepressors regulate the FSHβ promoter, SnoN, TGIF and CREM were knocked down using siRNA.

We first tested the functional effect of SnoN knockdown on Smad3-stimulated FSHβ promoter activity as SnoN binds to Smad3 (225). There was no increase in basal FSHβ promoter activity at 24 or 48 h, but we observed a significant increase in basal activity at 72 h of SnoN knockdown. Similarly, SnoN knockdown increased Smad3-stimulated FSHβ promoter activity at both 48 and 72 h, resulting in a 6-fold stimulation of FSHβ promoter activity at 72 h (Figure 26).

We then tested the functional effect of the TGIF knockdown on c-fos/c-jun-stimulated FSHβ promoter activity as TGIF binds to c-jun (237). TGIF knockdown increased basal FSHβ promoter activity at 48 and 72 h and also increased c-fos/c-jun-stimulated promoter activity at 48 and 72 h (Figure 27).

Finally, we tested the functional effect of the CREM knockdown on DCREB-stimulated FSHβ promoter activity. While the CREM knockdown did not increase basal promoter activity, it significantly increased DCREB-stimulated promoter activity at both 42 and 72 h (Figure 28).
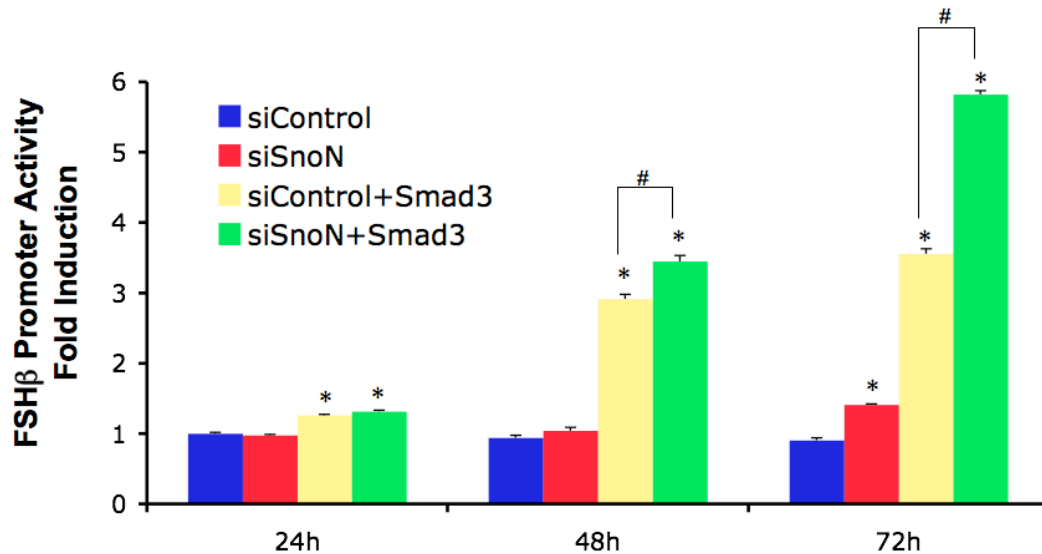
Figure 26: Knockdown of SnoN Increases Smad3 Stimulated FSHβ Promoter Activity.

LβT2 cells were cotransfected with -1.5Kb mFSHβ-luc, tklacZ, expression vectors for Smad3, and siRNAs against SnoN or control siRNAs. Afterwards, cells were harvested at the indicated times and FSHβ promoter activity was measured as before. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) between designated groups.
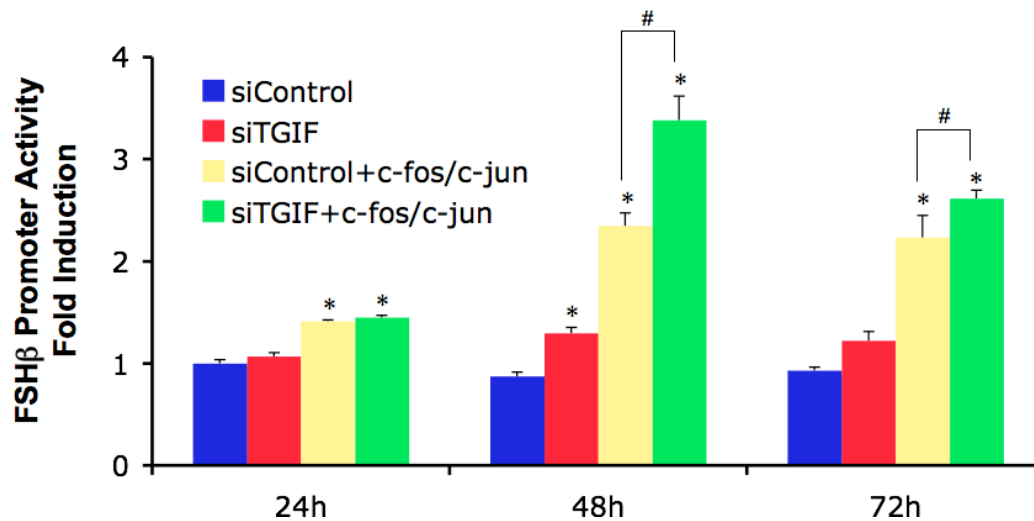
Figure 27: Knockdown of TGIF Increases AP-1 Stimulated FSHβ Promoter Activity.

LβT2 cells were cotransfected with -1.5Kb mFSHβ-luc, tklacZ, expression vectors for c-fos/c-jun, and siRNAs against TGIF or control siRNAs. Afterwards, cells were harvested at the indicated times and FSHβ promoter activity was measured as before. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) between designated groups.
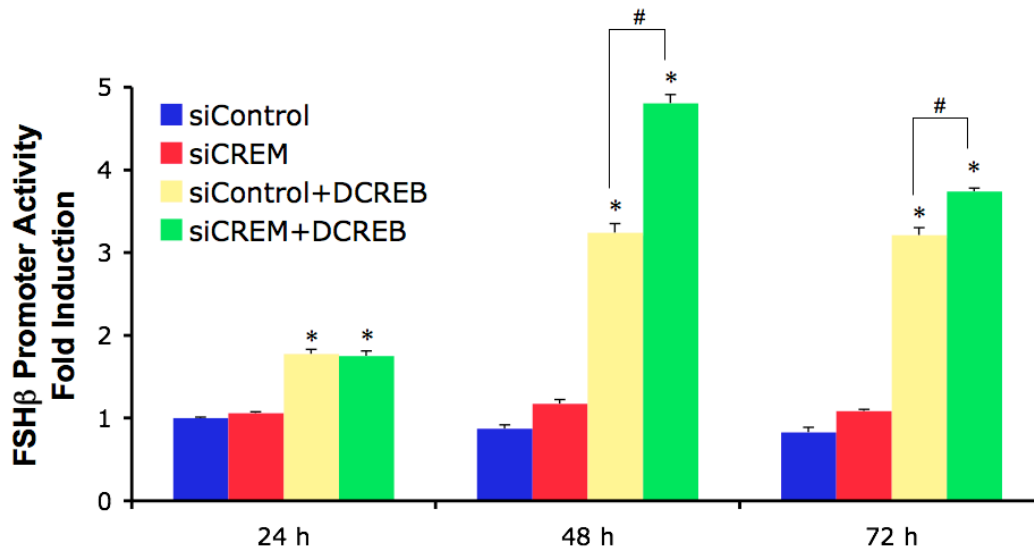
Figure 28: Knockdown of CREM Increases DCREB Stimulated FSHβ Promoter Activity.

LβT2 cells were cotransfected with -1.5Kb mFSHβ-luc, tklacZ, expression vectors for DCREB, and siRNAs against CREM or control siRNAs. Afterwards, cells were harvested at the indicated times and FSHβ promoter activity was measured as before. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) between designated groups.

6.2.2 The Effect of TGIF1, SnoN and CREM Knockdown on GnRH and Activin
Stimulation of FSHβ promoter

Having established the functional effect of TGIF1, SnoN and CREM
knockdowns on AP-1, Smad and DCREB-stimulated promoter activity, we tested
the effect of their knockdown on GnRH and activin-stimulation. Treatment with
GnRH (100 nM, 6 h), activin (25 ng/ml, 16 h) or both led to increase in FSHβ
promoter activity as before (Figure 29a, b). Knockdown of SnoN resulted in
further stimulation of FSHβ promoter activity by GnRH, Activin or both (Figure
29a, b). Interestingly, TGIF1 knockdown only increased promoter activity to a
noticeable degree when cells were co-treated with both GnRH and activin (Figure
29a, b). This last observation is consistent with the earlier finding that TGIF1
overexpression only reduced promoter activity when stimulated by both GnRH
and activin. Unexpectedly, CREM knockdown did not increase promoter activity
stimulated by GnRH or Activin (Figure 29a, b) indicating that endogenous CREM
does not regulate induction of FSHβ by GnRH or activin.
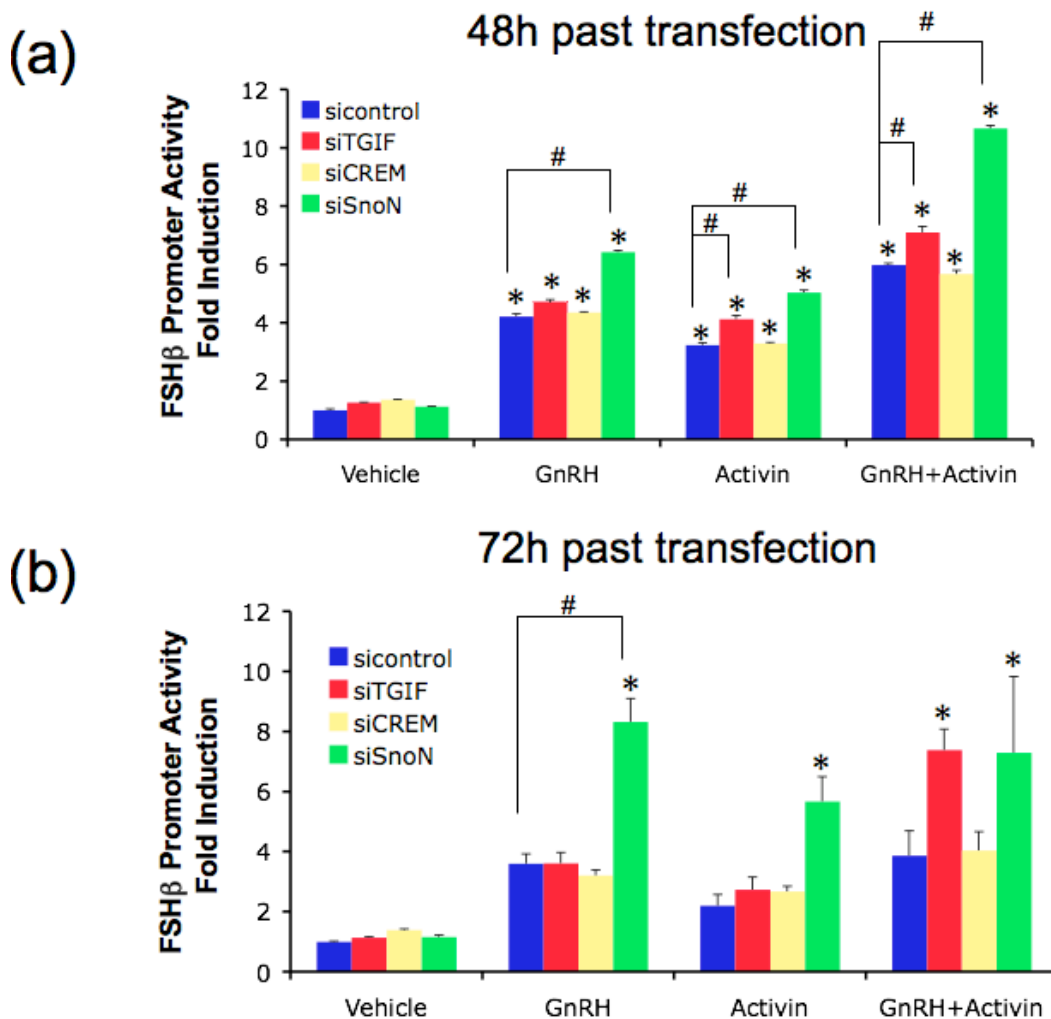
Figure 29: Knockdown of SnoN and TGIF Increases GnRH and Activin Stimulated FSHβ Promoter Activity.

In panel (a), LβT2 cells were cotransfected with the -1.5Kb mFSHβ-luc and tklacZ plasmids and siRNAs against corepressors TGIF, SnoN, CREM or control. The day after transfection, cells were starved with serum-free medium in the absence or presence of 25 ng/ml activin A, then treated with 100 nM GnRH for 6 h. Afterwards, cells were harvested 48 h post transfection and FSHβ promoter activity was measured as before. In panel (b), similar experiments were performed as in panel (a) except that treatments were started one day later and cells harvested 72 h post transfection. Data represent the means and SE of at least three independent experiments. * indicates significant difference (p < 0.05) compared to vehicle treated control group. # indicates significant difference (p < 0.05) between designated groups.

6.3 Loss of function TGIF and SnoN Mutants Do Not Repress the FSHβ Promoter

As an alternative strategy to confirm that TGIF1 and SnoN are involved in repression of FSHβ promoter, LβT2 cells were transfected with the -1.5Kb mFSHβ-luc reporter and expression vectors for mutant corepressors. For TGIF, the mutant (hTGIF-2TV) contains threonine to valine substitutions at positions 235 and 239 in human TGIF, residues that are sites for ERK-dependent phosphorylation (235, 243). The SnoN mutant (hSnoN-S3,4W) contains alanine substitutions at amino acids 85-88 and 318 (Figure 30). This mutant is deficient in Smad2 and Smad3 binding due to mutation of residues 85-88 and deficient in Smad4 binding due to mutation of tryptophan 318 (225).

Overexpression of human TGIF1 reduced FSHβ promoter activity in response to GnRH, activin or both similar to that seen by mouse TGIF1, reaching significance for both GnRH and activin (Figure 31). Unexpectedly, transfection of cells with the hTGIF-2TV mutant increased FSHβ promoter activity in GnRH, activin, or co-treated cells compared to respective control groups suggesting that the hTGIF-2TV mutant acts as a dominant negative to inhibit endogenous mouse TGIF1.

Wild-type human SnoN reduced FSHβ promoter activity significantly when cells were stimulated with both GnRH and activin similar to mouse SnoN.

As expected, the mutant hSnoN-S3,4W did not repress promoter activity compared to respective treated control, suggesting that binding to the Smads is required for repression (Figure 32).

Chapter 6, in part, is a reprint of the material as it appears in Gonadotropin-Releasing Hormone Pulse Sensitivity of Follicle Stimulating Hormone-Beta Gene Is Mediated by Differential Expression of Positive Regulatory Activator Protein 1 Factors and Corepressors SKIL and TGIF1 2011. Mistry, Devendra et al. Molecular Endocrinology, 2011. The dissertation author was the primary investigator and author of this paper.
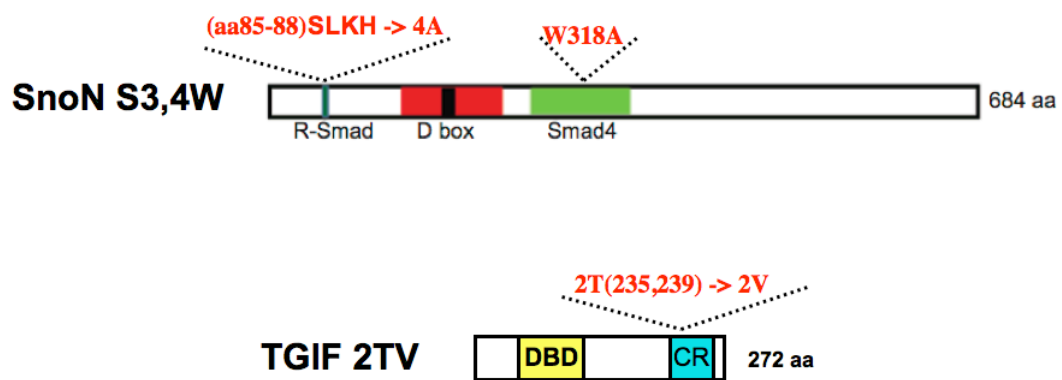
Figure 30: SnoN and TGIF Mutants.

Pictorial diagrams describing human SnoN and TGIF mutants. SnoN mutant has residues in its Smad 2/3 binding site (amino acids 81-84) and Smad 4 binding site (Tryptophan 318) mutated to the Alanine. This mutant SnoN S3,4W is unable to bind to Smad 2, Smad 3 or Smad 4. Human TGIF1 mutant on the other hand has mutation in conserved repression domain. It has its Threonine 235 and 239 mutated to Alanine. Such Mutant is unable to be phosphorylated on those residues and less active and less stable than wild type TGIF.
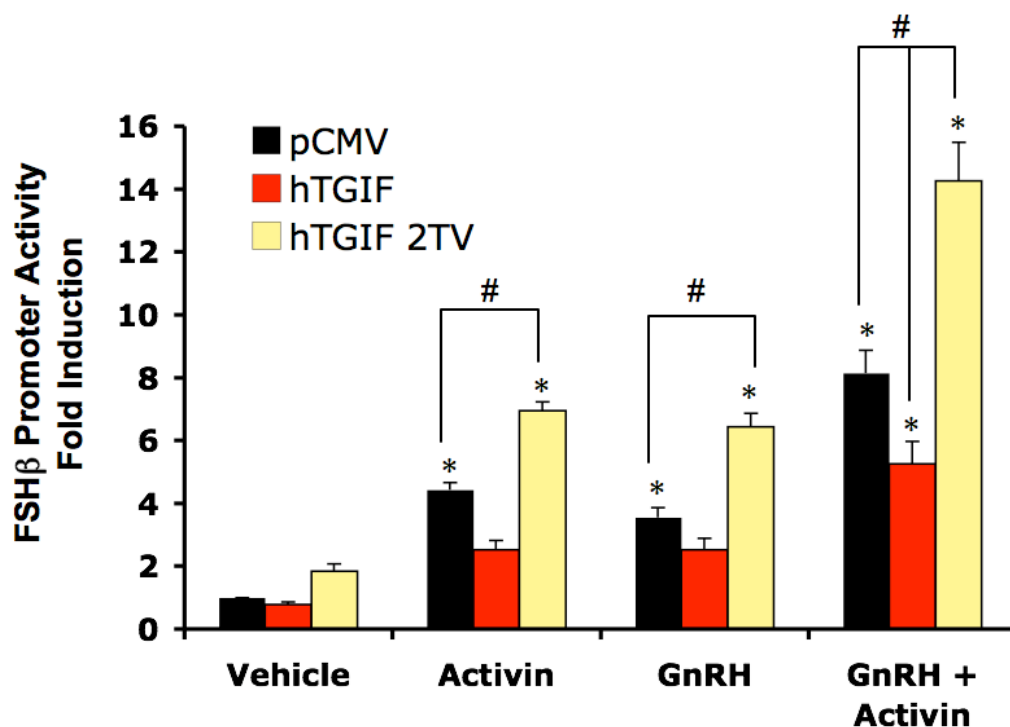
Figure 31: TGIF Mutant Does Not Repress FSHβ Promoter.

LβT2 cells were cotransfected with -1.5Kb mFSHβ-luc, tklacZ, and expression vectors for human wild-type TGIF, mutant TGIF-2TV or pCMV vector. The day after transfection, cells were starved 16 h with serum free medium in the absence or presence of 25 ng/ml activin A then treated with 100nM GnRH for 6 h. FSHβ promoter luciferase activity was measured and corrected for beta-galactosidase activity as before. Data represent the mean and SE of at least three independent experiments. * indicates significant difference ($p < 0.05$) compared to vehicle treated control group. # indicates significant difference ($p < 0.05$) between designated groups.
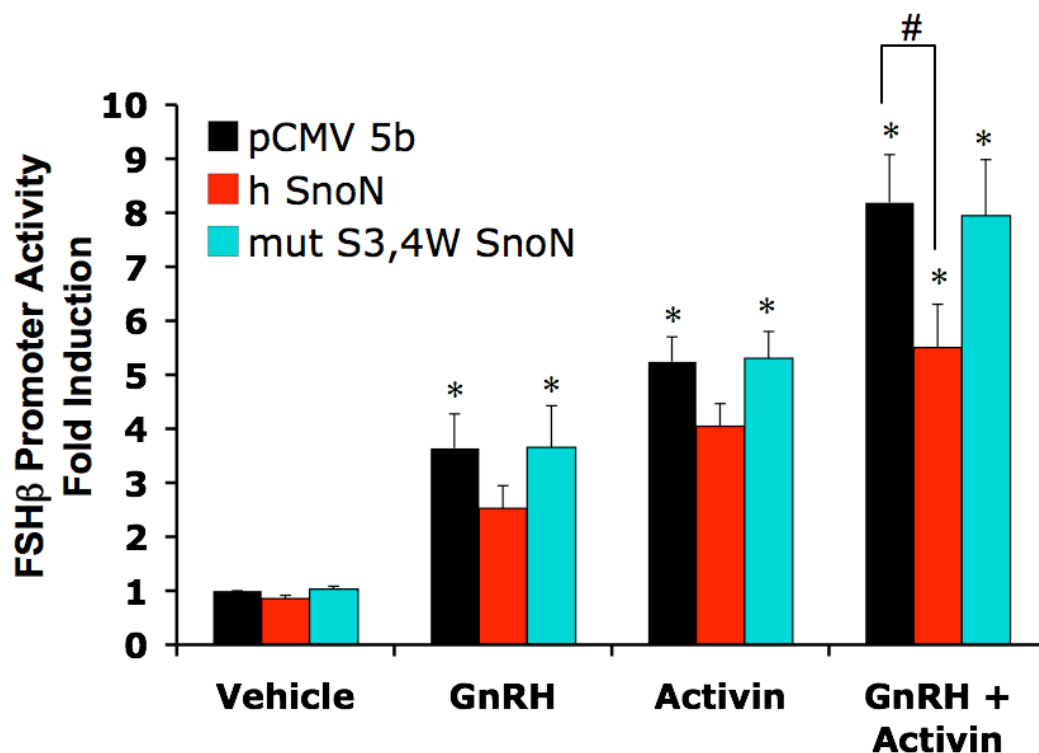
Figure 32: SnoN Mutant Does Not Repress FSHβ Promoter.

LβT2 cells were cotransfected with -1.5Kb mFSHβ-luc, tklacZ, and expression vectors for human wild-type SnoN, mutant SnoN-S3,4W or pCMV vector. The day after transfection, cells were starved 16 h with serum free medium in the absence or presence of 25 ng/ml activin A then treated with 100nM GnRH for 6 h. FSHβ promoter luciferase activity was measured and corrected for beta-galactosidase activity as before. Data represent the mean and SE of at least three independent experiments. * indicates significant difference (p < 0.05) compared to vehicle treated control group. # indicates significant difference (p < 0.05) between designated groups.

Chapter 7. Modulation of SnoN and TGIF Expression Alters the Sensitivity of the FSHβ Subunit Gene to GnRH Pulses

7.1 FSHβ mRNA Induction by Pulsatile GnRH in LβT2 Gonadotropes

To verify that the LβT2 cells respond appropriately to GnRH pulse frequency, we treated cells with 5 min pulses of 1 nM and 10 nM GnRH at 30 and 120 min intervals for 6 h (Figure 33). FSHβ mRNA expression was measured on total RNA by QPCR. At the lower pulse amplitude (1 nM GnRH), the slower GnRH pulse frequency led to a higher induction of FSHβ mRNA in agreement with the differential expression observed in other cells *in vitro* and in intact animals *in vivo* (62, 246). Interestingly, a higher amplitude GnRH pulse (10 nM GnRH) led to similar differential FSHβ mRNA expression, but the overall induction of FSHβ mRNA was less at both fast and slow pulse frequencies (Figure 33).
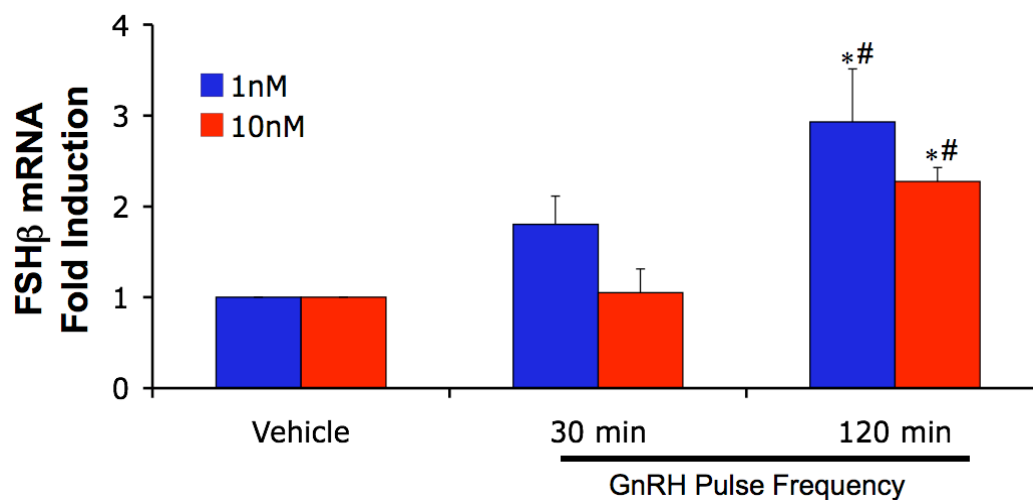
Figure 33: FSHβ mRNA Induction by the Pulsatile GnRH.

LβT2 cells were treated with 5 min pulses of 1 and 10 nM GnRH at intervals of 30 and 120 min over 6 h (As described in the Figure 17). Total RNA was extracted and QPCR performed using FSHβ subunit specific primers. Data represent the means and SE of at least 3 independent experiments. * indicates significant difference ($p<0.05$) compared to vehicle-treated group. # indicates significant difference ($p<0.05$) compared to respective 30 min GnRH pulse frequency treated group.

7.2 Overexpression of SnoN or TGIF Selectively Represses Slow GnRH Pulse Frequency Stimulated FSHβ Promoter Activity

Having verified that the LβT2 cells respond appropriately to GnRH pulse frequencies, we tested whether SnoN and TGIF are involved in GnRH pulse regulation of FSHβ. We focused on SnoN and TGIF1 for this study, rather than CREM, as these are the repressors that appear to mediate GnRH and activin effects in our earlier studies. The mFSHβ-luc reporter was transfected into LβT2 cells on cytodex beads along with expression vectors for SnoN or TGIF1 or pCMV-SPORT6 control. Cells were treated with 5 min pulses of 10 nM GnRH in perifusion culture at 30 or 120 min pulse intervals for 6 h. A perifusion cultures was used for the promoter transfections as we have previously shown that the response of the promoter to GnRH pulses is better in perifusion than static culture. Since endogenous activin is important for basal FSHβ promoter expression and is removed by the perifusion system, 2 ng/ml activin was added to pulse-treated and control groups every 120 min to support basal promoter activity.

Both 30 and 120 min GnRH pulse frequencies increased FSHβ promoter activity in control cells transfected with pCMVSPORT6 (Figure 34). Like the endogenous FSHβ gene, the 120 min pulse frequency tended to give higher induction than the 30 min frequency but the difference was not significant (Figure 34). No repression of FSHβ promoter activity by TGIF1 and SnoN was observed

at the faster pulse frequency (Figure 34), but both TGIF and SnoN significantly reduced FSHβ promoter activity at the slower frequency, which is consistent with our earlier observation that endogenous SnoN and TGIF proteins are induced at the high GnRH pulse frequency so overexpression would have less effect (Figure 34).
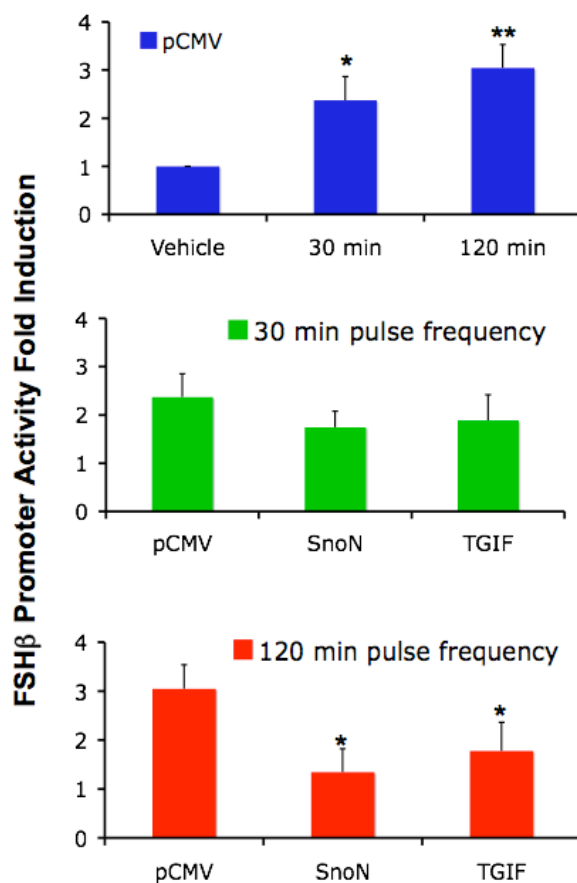
Figure 34: Repression of Pulsatile GnRH Stimulated FSHβ Promoter Activity by Overexpression of SnoN and TGIF.

LβT2 cells were cotransfected with -1.5Kb mFSHβ-luc, tklacZ, and expression vectors for mTGIF, mSnoN or control pCMVSPORT6 vector. Cells were grown on cytodex beads, then one-day post transfection, the beads were loaded into perfusion columns and starved for 16 h with serum free medium. Afterwards, cells were treated with 5 min pulses of 10nM GnRH in a pulse perifusion system at intervals of either 30 min or 120 min for 6 h. Two ng/ml activin A was added to all of the groups at an interval of 120 min to maintain basal promoter activity. At the end of 6 h, FSHβ promoter activity was measured as before. Upper panel shows the results of different pulse frequencies in the control transfected cells. * indicates significant (p<0.05) increase relative to vehicle control. ** indicates significant (p<0.01) increase relative to vehicle control. Middle panel shows the results of TGIF or SnoN overexpression on FSHβ induction by GnRH at 30 min pulse intervals. Lower panel shows the results of overexpression of TGIF and SnoN on FSHβ induction by GnRH at 120 pulse intervals. * indicates significant decrease (p<0.05) relative to pCMV transfected cells.

7.3 Knockdown of TGIF or SnoN Selectively Increases Fast GnRH Pulse Frequency Stimulated FSHβ mRNA Induction

To confirm the role of the endogenous corepressors in GnRH pulse regulation of FSHβ, SnoN or TGIF expression were knocked down in LβT2 cells. We verified initially that the siRNAs decreased protein expression then assessed FSHβ gene induction (Figure 35).

Cells were transfected with scrambled control, TGIF or SnoN siRNA and were treated with fast and slow GnRH pulse frequencies in static culture for total of 6 h. Pulses in static culture were used, rather than perifusion culture, as we have found that the response of the endogenous gene is more robust. As before, 2 ng/ml activin was added to media to maintain basal FSHβ gene expression. For cells transfected with control siRNA, slow GnRH pulses caused a significantly higher induction of FSHβ mRNA compared to cells exposed to fast pulses, as expected from previous experiments (Figure 36). When cells transfected with SnoN or TGIF siRNA were treated with fast GnRH pulses, the SnoN and TGIF knockdown groups significantly increased FSHβ mRNA induction (Figure 36). In contrast, when these cells were treated with slow GnRH pulses, no significant difference was observed compared to control siRNA transfected cells. The knockdown would only be expected to be functional at the high GnRH pulse frequency when TGIF1 and SnoN are induced.

To ensure that addition of activin did not alter the differential induction of c-fos, c-jun, SnoN and TGIF, we assessed transcription factor induction in these cells (Figure 37a). While activin slightly increases the basal levels of these transcription factors, it did not alter the differential induction of SnoN and TGIF by fast and slow GnRH pulse frequencies (Figure 37b).

Chapter 7, in part, is a reprint of the material as it appears in Gonadotropin-Releasing Hormone Pulse Sensitivity of Follicle Stimulating Hormone-Beta Gene Is Mediated by Differential Expression of Positive Regulatory Activator Protein 1 Factors and Corepressors SKIL and TGIF1 2011. Mistry, Devendra et al. Molecular Endocrinology, 2011. The dissertation author was the primary investigator and author of this paper.
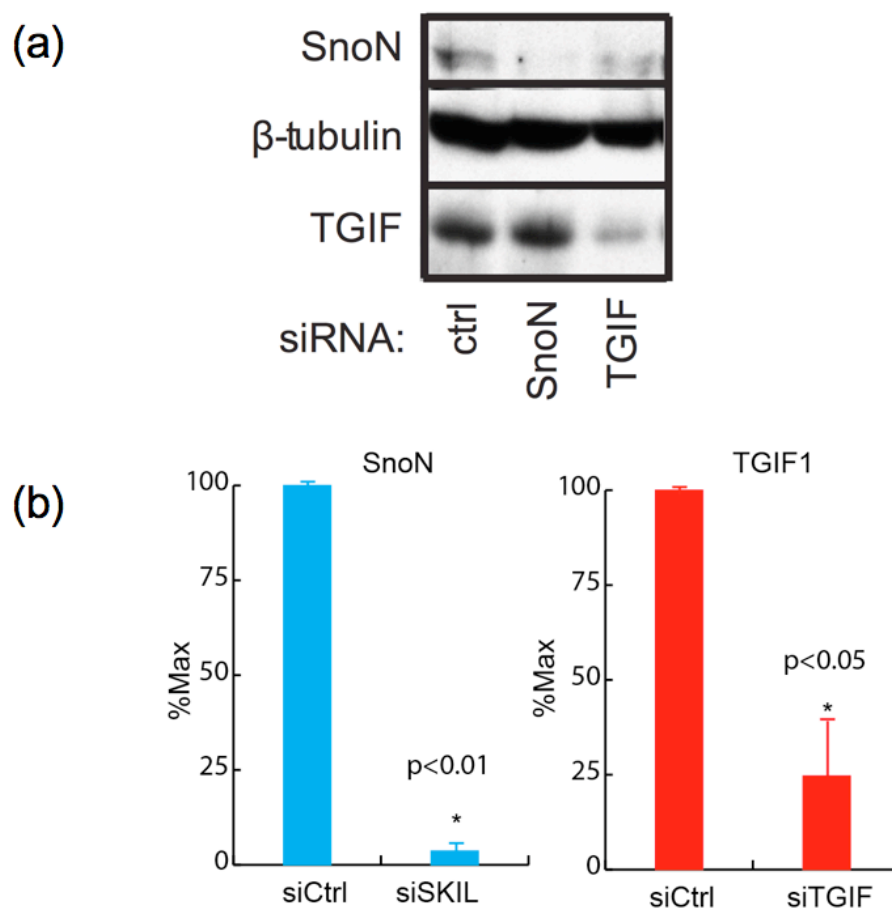
Figure 35: Knockdown of the SnoN and TGIF.

In panel (a), LβT2 cells were electroporated with the indicated siRNAs using a microporator. Forty-eight hours later, cells were treated with 100 nM GnRH for 4 h to induce TGIF1 and SnoN. Protein expression was measured by immunoblotting using the indicated antibodies. (b) Quantification for panel (a) TGIF1 and SnoN knockdown blots. SnoN and TGIF1 band intensities were normalized with respective β-Tubulin band intensities. Data are plotted as %max. * indicates significant difference (p < 0.05) compared to control siRNA transfected group.
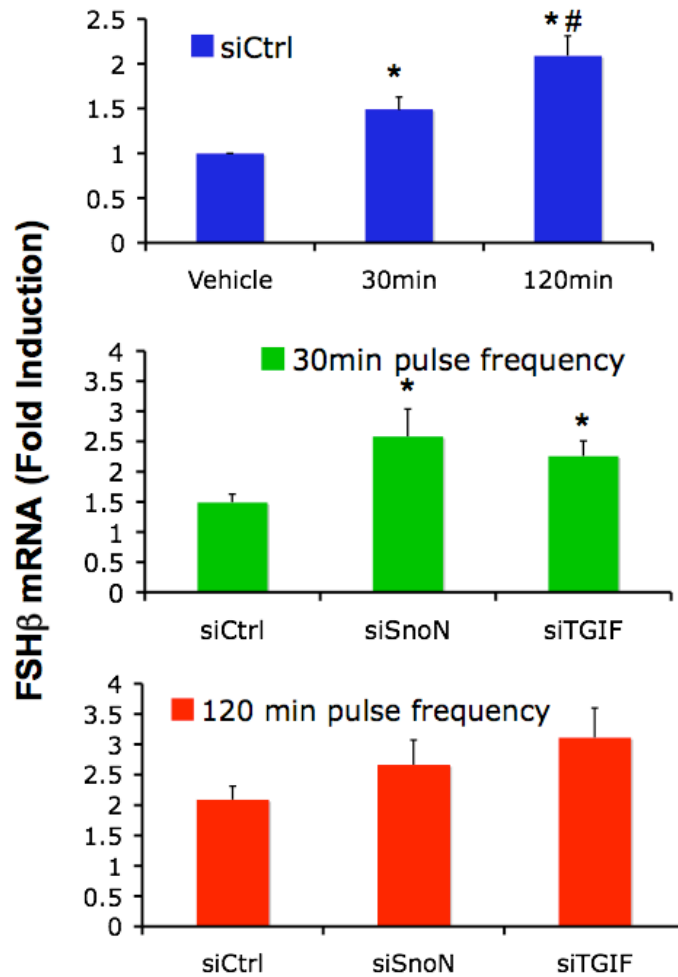
Figure 36: The Effect of SnoN and TGIF Knockdown on the FSHβ mRNA Induction by the Pulsatile GnRH.

In the upper panel, serum-starved LβT2 cells that had been electroporated with the control siRNA were treated with fast or slow 10 nM GnRH pulses for 6 h in static culture. RNA was extracted and RT-QPCR was performed for FSHβ mRNA expression. * indicates significant difference from vehicle treated control ($p < 0.05$). # indicates significant difference from 30 min pulse frequency treatment ($p < 0.05$). In the middle panel, serum- starved LβT2 cells electroporated with the indicated siRNAs were treated with 10 nM GnRH pulses every 30 min for 6 h. FSHβ mRNA expression was measured as before. * indicates significant difference from control siRNA transfected group ($p < 0.05$). In the lower panel, serum- starved LβT2 cells electroporated with the indicated siRNAs were treated with 10 nM GnRH pulses every 120 min for 6 h. The FSHβ mRNA expression was measured as before. Data represent the mean and SE of at least three independent experiments.
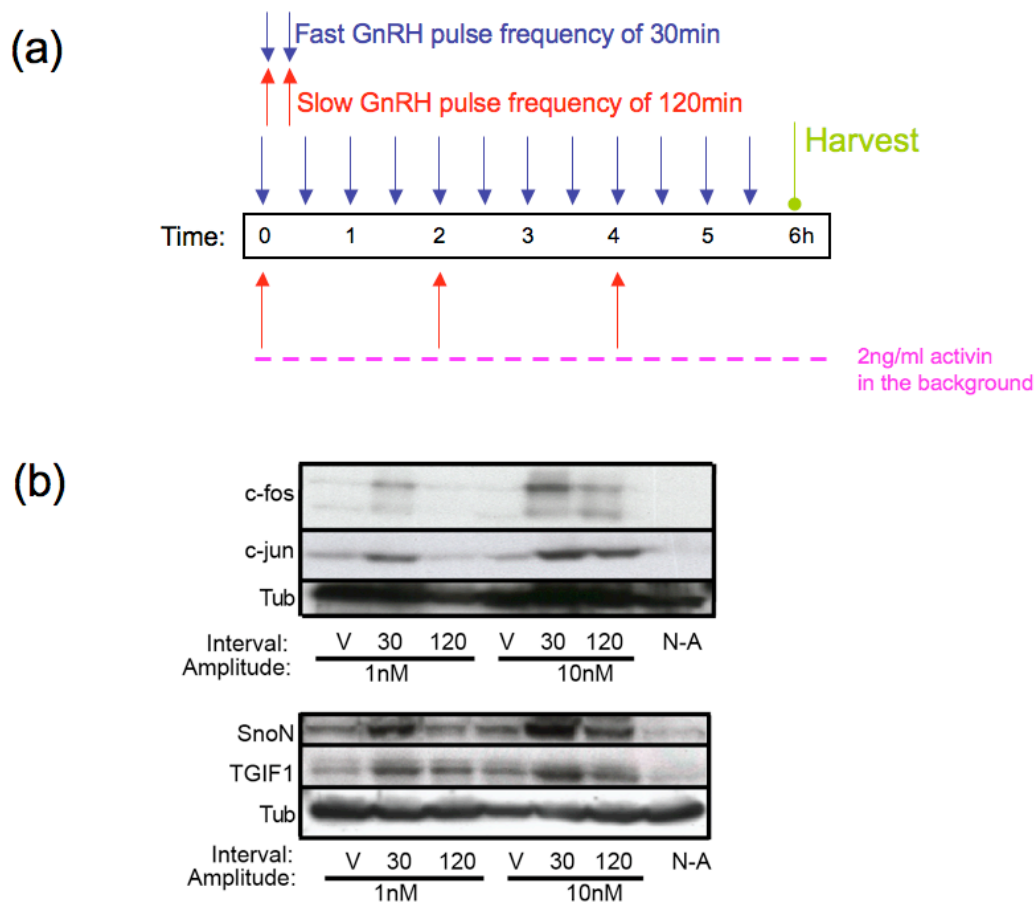
Figure 37: Protein Induction by Pulsatile GnRH in Presence of Activin.

(a) A scheme describing the new GnRH and Activin cotreatment regime. LβT2 cells were treated with 5 min pulses of 1 and 10 nM GnRH at intervals of 30 and 120 min over 6 h (As described in the Figure 17), but this time in presence of 2ng/ml of activin. (b) LβT2 cells were treated with fast and slow GnRH pulse frequency as described in panel (a) with 2ng/ml activin in the background. "N-A" represents negative control without the GnRH or activin treatment. As before, westerns were performed using the indicated antibodies. Blots were reblotted with β-tubulin for loading control. The experiment was repeated at least two times.

Chapter 8. FSHβ Promoter Occupancy of the Stimulators and Corepressors In Response to GnRH

8.1 Occupancy of the c-fos, c-jun, SnoN and TGIF on the FSHβ promoter in Response to Continuous GnRH Treatment

The overexpression and knockdown experiments implicated c-fos, c-jun, SnoN and TGIF in the regulation of the FSHβ promoter. To investigate whether these are direct effects on the FSHβ promoter we measured promoter occupancy by chromatin immunoprecipitation (ChIP). Initially, LβT2 cells were stimulated with 100 nM GnRH over 6 h then chromatin was precipitated using antibodies to c-fos, c-jun TGIF and SnoN.

All four factors bound to the FSHβ promoter but their occupancy changed over the 6 h of GnRH stimulation (Figure 38a). Binding of c-fos and TGIF appears to increase after GnRH treatment but SnoN binding appears high initially and then decreases. The ChIP data for c-fos, c-jun, SnoN and TGIF1 using three independent PCR products from multiple experiments were quantified to determine the dynamic changes over the time course (Figure 38b). Inspection of the quantified data indicated that the stimulatory transcription factor c-fos, the two co-repressors SnoN and TGIF1, and to a lesser degree c-jun bound to the

111

promoter in a cyclical manner. While c-jun didn't seem to oscillate on FSHβ

promoter as much as other transcription factors, over the time it did have a

significant increase in FSHβ promoter occupancy (Figure 38b).
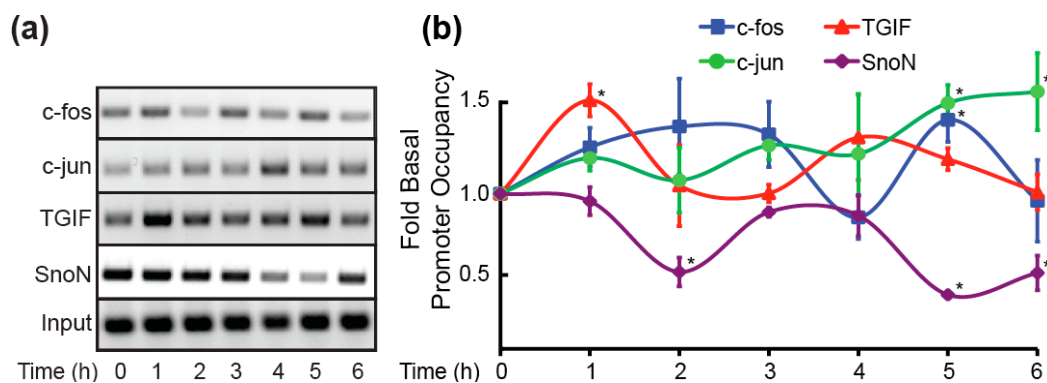
Figure 38: Cyclical Promoter Occupancy by c-fos, c-jun, SnoN and TGIF in Response to the Continuous GnRH.

In panel (a), LβT2 cells were serum-starved for 24 h then treated with continuous 100 nM GnRH for the indicated times. Cells were cross-linked, lysed, and the chromatin sonicated. A ChIP assay was performed using antibodies to c-fos, c-jun, TGIF1 and SnoN. Precipitated chromatin was amplified by PCR using three independent sets of primers to the proximal FSHβ promoter. A representative PCR reaction is shown. Amplification of input DNA is shown as a control for chromatin recovery. Panel (b) shows the quantified results for the ChIP assay. Results are the mean of at least three experiments. Each experiment was performed with three independent pairs of PCR primers. Data were normalized to respective inputs and then to their respective 0 time points. * indicates significant difference from respective 0h ($p < 0.05$).

8.2 Occupancy of the c-fos, c-jun, SnoN and TGIF on the FSHβ promoter in Response to a Single Pulse of GnRH

We then studied occupancy of the FSHβ promoter in response to a single 100 nM GnRH pulse (Figure 39). The ChIP data were quantified, normalized and averaged as before. A single GnRH pulse caused a peak of c-fos occupancy on the FSHβ promoter at 2 h and, in contrast to the results with constant GnRH, also caused a peak of c-jun occupancy at 4 h (Figure 39). TGIF1 occupancy of the FSHβ promoter oscillated following a single pulse, being high initially, low at 2 h, high at 4 h then low again at 6 h similar to the periodicity seen with constant GnRH. SnoN occupancy did not oscillate following a single pulse but rather declined gradually over 6 h (Figure 39).
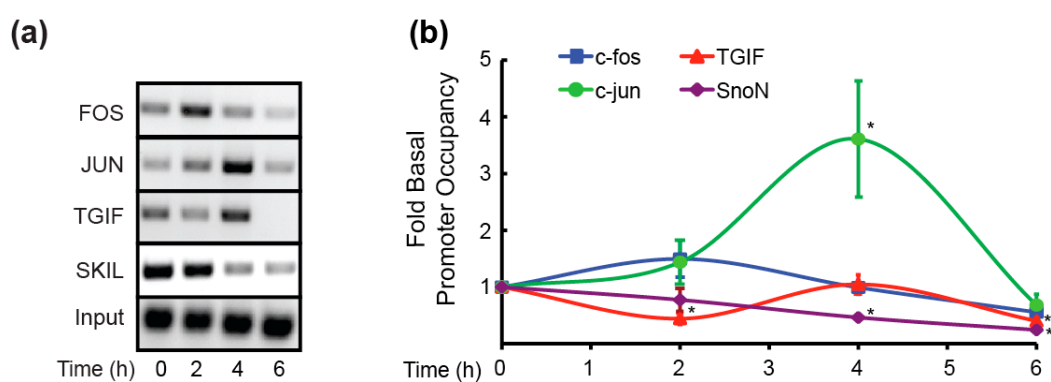
Figure 39: Promoter Occupancy by c-fos, c-jun, SnoN and TGIF in Response to the Single Pulse GnRH.

In panel (a), serum-starved LβT2 cells were treated with a single 100 nM GnRH pulse for the indicated times. The ChIP assay was performed as before. The panels show representative PCR reactions. Panel (b) shows the quantification of ChIP following a single pulse of GnRH. Data were normalized and analyzed as before. * indicates significant difference from respective 0 h (p <0.05). Data represent the mean and SE of at least two independent experiments.

8.3 The Kinetics of c-fos, c-jun, SnoN and TGIF1 Protein Induction, Their Occupancy on the FSHβ Promoter, and Induction of the FSHβ mRNA in Response to Multiple Pulses of GnRH

8.3.1 The Kinetics of c-fos, c-jun, SnoN and TGIF1 Protein Induction in Response to Multiple Pulses of GnRH

Our previous GnRH pulse treatment regime would not allow us to correctly determine the kinetics of protein expression and FSHβ promoter occupancy of the FSHβ regulatory transcription factors since in that scheme the cells were harvested at different interval from the last fast and slow pulse of GnRH (Figure 17). Therefore, to perform such pulse kinetics experiments, we developed a new scheme where LβT2 cells were treated with 5 min GnRH pulses (1 or 10 nM) every 30 or 120 min for 6 h. Medium was changed on all cells every 30 min to control for effects due to autocrine stimulation by secreted factors. After 6 h, the cells received a final 5 min pulse of GnRH then were harvested immediately or at 30, 60 or 120 min after that last pulse (Figure 40).

We first checked the GnRH pulse kinetics of the FSHβ regulatory protein induction. For both 1 nM and 10 nM GnRH pulses, c-fos and c-jun proteins were induced at 30-60 min in agreement with data from static cultures. Immediately after the last pulse, cells that had been pre-conditioned at the GnRH high pulse

frequency showed higher basal levels of c-fos and c-jun consistent with the earlier data, but also showed prolonged expression of c-fos and c-jun at 120 min post final pulse compared to the cells pre-conditioned with slow GnRH pulses (Figure 41, 42).

The co-repressor TGIF1 was induced only in cells pre-conditioned by fast 1 nM GnRH pulses, but at the higher 10 nM pulse amplitude some induction was seen in cells pre-conditioned with slow GnRH pulses (Figure 41, 43). With 1 nM GnRH pulses, SnoN was barely induced and there was no difference between fast and slow pulse frequencies, but at the higher 10 nM pulse amplitude, SnoN was strongly induced in cells pre-conditioned with fast GnRH pulses with negligible induction at slow pulse frequency (Figure 41, 43). A representative β-tubulin blot is shown to demonstrate equal loading.
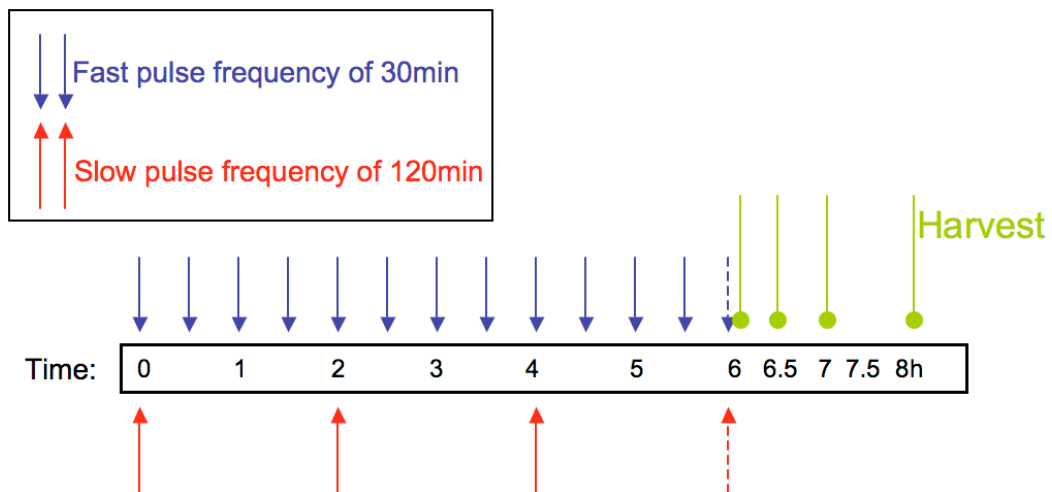
Figure 40: GnRH Pulse Kinetics Experiment Scheme.

As before (Figure 17), LβT2 cells were treated with the fast GnRH pulse frequency of 30 min or the slow GnRH pulse frequency of 120 min with 1 or 10nM GnRH pulse amplitude for 6h. Unlike previous experiments (Figure 17), cells are treated with one more synchronizing pulse of GnRH at 6h time point and are harvested immediately, 30 min, 60 min or 120 min after the last pulse of GnRH.
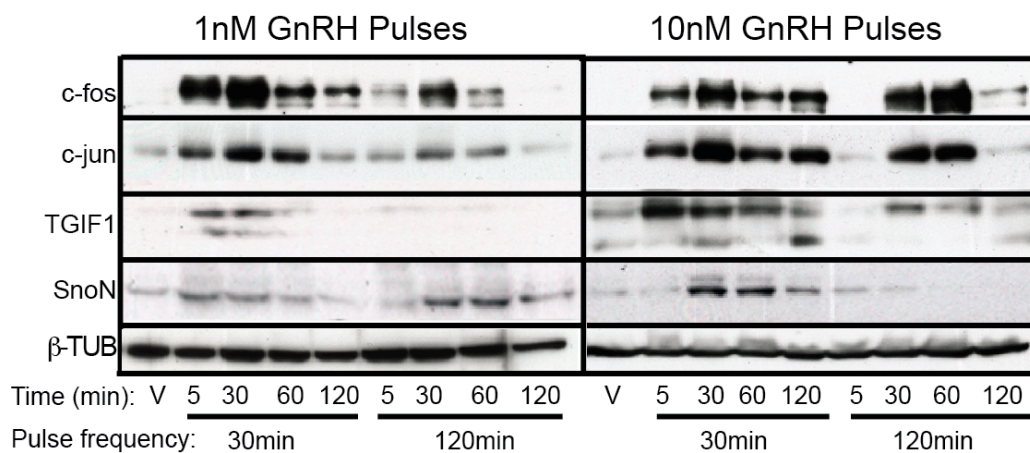
Figure 41: Kinetics of the c-fos, c-jun, SnoN and TGIF Protein Induction in Response to the Pulsatile GnRH.

LβT2 cells were serum-starved for 24 h then treated with 5 min pulses of 1 or 10 nM GnRH every 30 or 120 min for 6 h. After 6 h, cells were treated with one final synchronizing pulse of GnRH then harvested immediately or 30, 60 or 120 min after the last pulse as shown in Figure 40. Westerns were performed using the indicated antibodies. The experiment was repeated at least 3 times. Representative blots are shown. Blots were reblotted for β-tubulin for loading control and were quantified by densitometry.
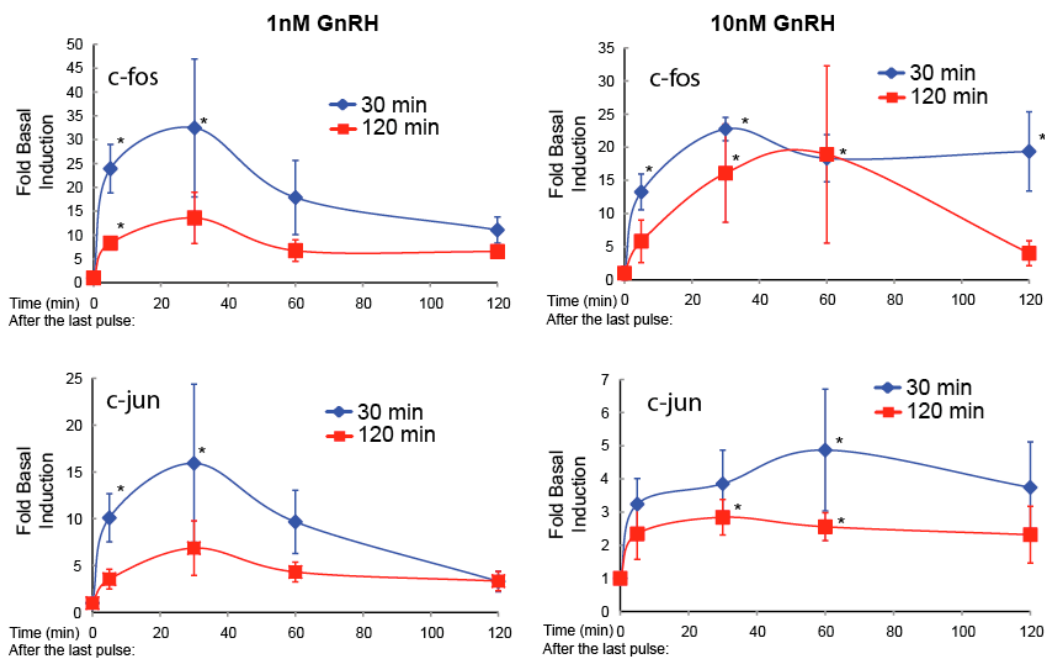
Figure 42: Quantification of the Stimulators Protein Induction from Figure 41.

Sample band intensities were normalized to respective β-tubulin band intensities and data were plotted as fold basal. * indicates significant difference ($p < 0.05$) compared to basal.
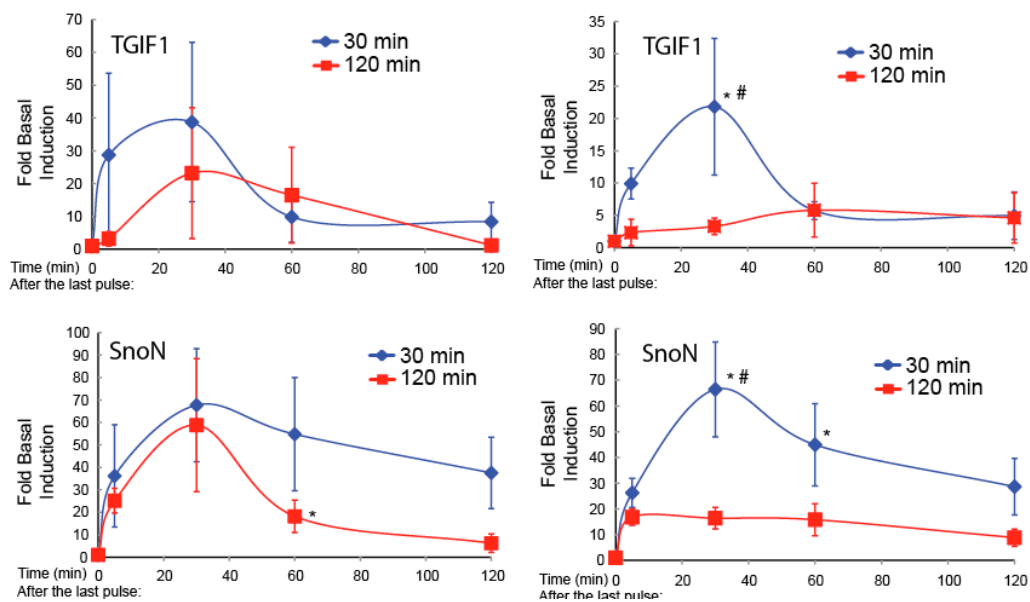
Figure 43: Quantification of the Corepressors Protein Induction from Figure 41.

Sample band intensities were normalized to respective β-tubulin band intensities and data were plotted as fold basal. * indicates significant difference (p < 0.05) compared to basal and # indicates significant difference (p < 0.05) compared to respective slow pulse treated time point.

8.3.2 Occupancy of the c-fos, c-jun, SnoN and TGIF on the FSHβ promoter in Response to GnRH Multiple Pulses Treatment

The promoter occupancy by these transcription factors was then studied in cells exposed to multiple 10 nM GnRH pulses using the same experimental design (Figure 40). We only used 10 nM GnRH as it gave more robust changes in protein expression. ChIP data were quantified and averaged from multiple experiments as before and were normalized to cells treated with pulses of vehicle.

All four proteins showed changes in promoter occupancy over the 120 min time course following the final pulse. For cells pre-conditioned with fast GnRH pulses, promoter occupancy by TGIF1 and c-fos were closely correlated but in cells pre-conditioned with slow GnRH pulses this correlation was less apparent (Figure 44). Occupancy by c-jun and SnoN was also correlated, but did not change significantly (Figure 44). In cells pre-conditioned with fast GnRH pulses, promoter occupancy by both c-fos and TGIF1 is elevated compared to vehicle-pulsed cells and then decreases following the pulse before increasing again at 120 min (Figure 44). In cells pre-conditioned with slow GnRH pulses, basal occupancy by c-fos and TGIF1 is again elevated relative to vehicle-pulsed cells, but occupancy by c-fos transiently decreased at 30 min and rebounded at 60 and 120 min, whereas occupancy by TGIF1 decreased gradually over time.

As our model is that FSHβ promoter activity is determined by the balance of the positive and negative factors, we normalized the promoter occupancy of c-fos and c-jun to either TGIF1 or SnoN. Relative promoter binding of c-fos to either TGIF1 or SnoN is higher in cells that have been pre-conditioned with slow GnRH pulses (Figure 45, Table 2). The relative binding of c-jun to TGIF1 or SnoN is also increased (Figure 45, Table 2).
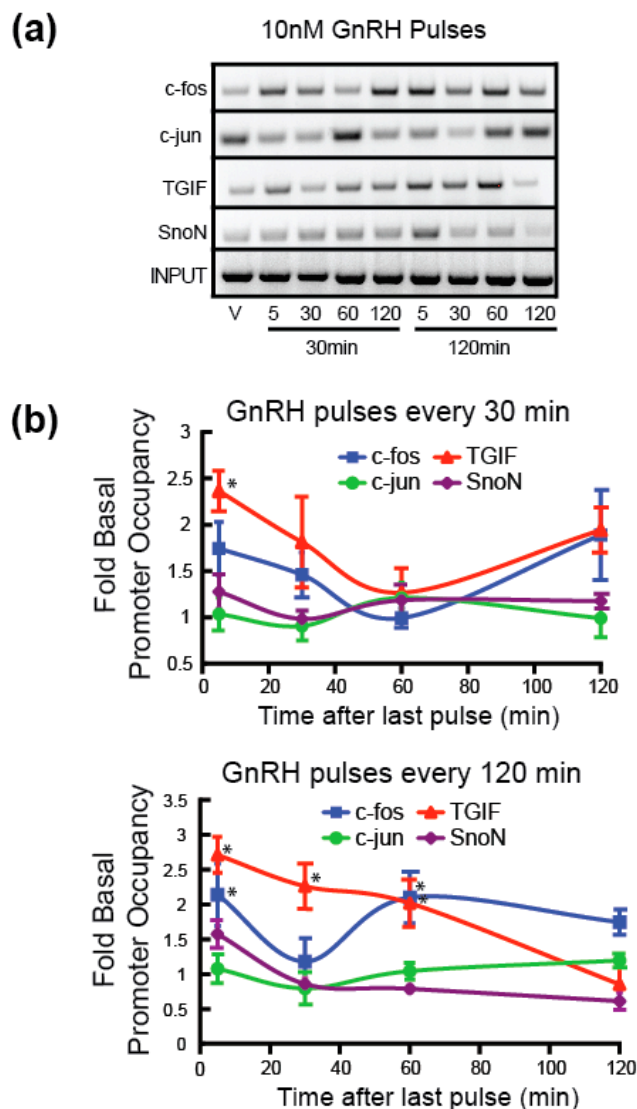
Figure 44: Kinetics of Stimulators and Corepressors Promoter Occupancy in Response to the Multiple GnRH Pulses.

In panel (a), serum-starved LβT2 were treated with 5 min pulses of 10 nM GnRH every 30 or 120 min for 6 h as in panel and were harvested immediately or 30, 60 or 120 min after the last pulse as described in Figure 40. The ChIP assay was performed in triplicate as before using the indicated antibodies. The panels show representative PCR reactions. Panel (b) shows the quantification of the ChIP data following a multiple pulses of GnRH. Data were normalized to respective inputs, then to vehicle-treated cells. * indicates significant difference from respective 0 time point (p <0.05). Data represent the mean and SE of at least two independent experiments.
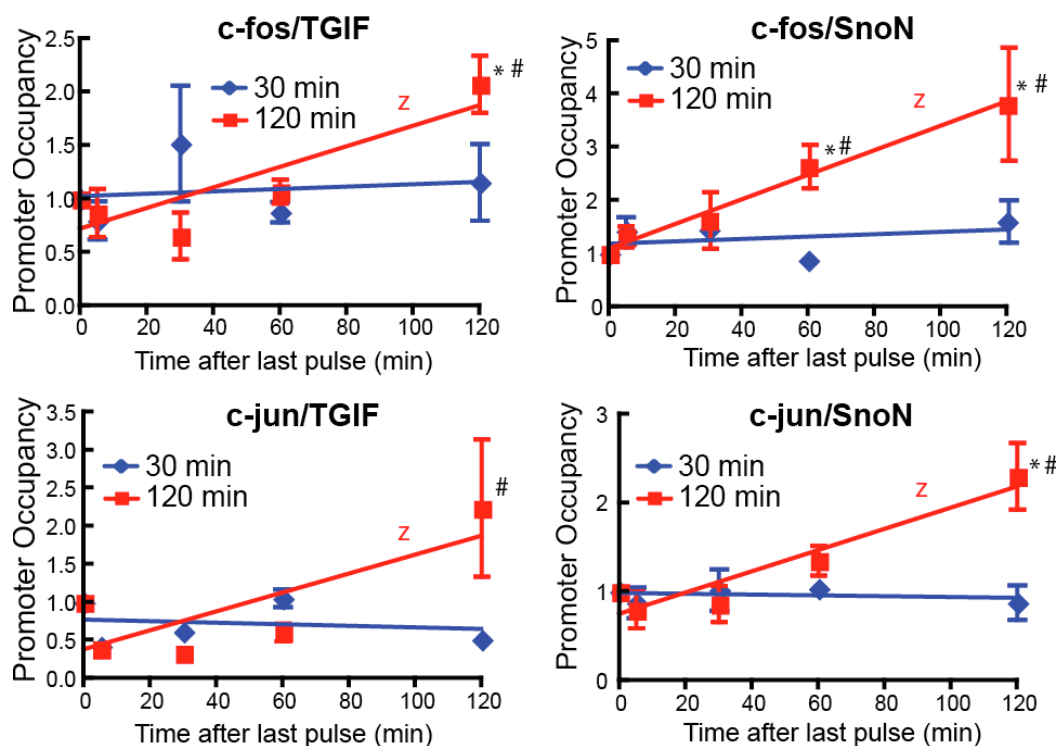
Figure 45: Relative FSHβ Promoter Occupancy of the Stimulators Compared to the Corepressors in Response to the Multiple GnRH Pulses.

The ChIP data from Figure 44 are plotted as c-fos/TGIF, c-fos/SnoN, c-jun/TGIF or c-jun/SnoN promoter occupancy ratios. # indicates significant difference compared to the respective 30 min time point ($p < 0.05$). * indicates significant difference compared to the 0 time point ($p < 0.05$). Z indicates significant line fit through regressional analysis ($p < 0.01$).

8.3.3 The Kinetics of the FSHβ Induction mRNA in Response to Multiple GnRH Pulses

Finally, the induction of FSHβ mRNA by the final GnRH pulse in cells preconditioned with multiple pulses was also measured to test if there is a correlation between transcription factor induction, promoter binding and mRNA synthesis. LβT2 cells were preconditioned with GnRH pulses at fast or slow frequencies, given the final synchronizing pulse as before, then total RNA was extracted immediately or after 30, 60 or 120 min. FSHβ mRNA was elevated immediately after the pulse in cells pre-treated with slow GnRH pulses in agreement with our earlier results (Figure 46, Table 2). Additionally, FSHβ mRNA was further elevated in the period after the pulse in cells previously exposed to slow 1 or 10 nM GnRH pulses, but did not increase further in cells pre-conditioned with fast GnRH pulses (Figure 46, Table 2).

Chapter 8, in part, is a reprint of the material as it appears in Gonadotropin-Releasing Hormone Pulse Sensitivity of Follicle Stimulating Hormone-Beta Gene Is Mediated by Differential Expression of Positive Regulatory Activator Protein 1 Factors and Corepressors SKIL and TGIF1 2011. Mistry, Devendra et al. Molecular Endocrinology, 2011. The dissertation author was the primary investigator and author of this paper.
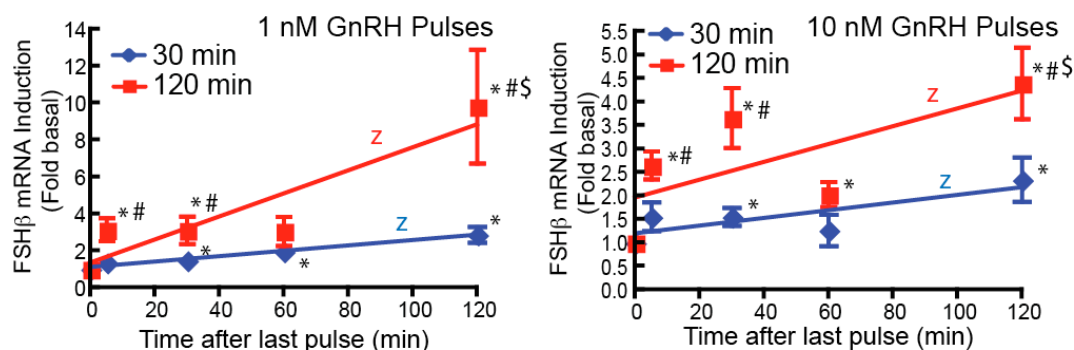
Figure 46: Relative FSHβ mRNA Induction by the Fast and Slow GnRH Pulse Frequencies.

Starved LβT2 cells were treated with multiple pulses of 1 or 10 nM GnRH as above. Total RNA was harvested at the indicated times and QPCR was performed for FSHβ. * indicates significant difference from respective vehicle treatment ($p < 0.05$). # indicates significant difference from respective fast pulse treated time point ($p < 0.05$). $ indicates significant difference between indicated points ($p < 0.05$). Z indicates significant line fit through regressional analysis ($p < 0.01$). Data represent the mean and SE of at least three independent experiments.

Table 2: Supplementary Information for the Figures 45 and 46.

R square and P values for lines in Figure 45 and 46.

Figure 45	R square value		P value	
Pulse Frequency	Fast (30 min)	Slow (120 min)	Fast (30 min)	Slow (120 min)
c-fos/SnoN	0.03	0.39	0.36	0.0002
c-fos TGIF1	0.004	0.42	0.73	<0.0001
c-jun/SnoN	0.003	0.49	0.77	<0.0001
c-jun/TGIF1	0.02	0.23	0.45	0.008
Figure 46	R square value		P value	
Pulse Frequency	Fast (30 min)	Slow (120 min)	Fast (30 min)	Slow (120 min)
1nM Amplitude	0.36	0.28	0.0001	0.0002
10nM Amplitude	0.13	0.2	0.01	0.001

Chapter 9. Discussion

9.1 Model for GnRH Pulse Regulation of the FSHβ Subunit Gene

We have shown in this study that differential GnRH pulse frequencies and amplitudes regulate known FSHβ transcriptional activators and also found evidence for differential corepressor expression that can modulate FSHβ promoter activity. GnRH stimulates the FSHβ promoter via AP-1 complexes composed of dimers of bZIP transcription factors such as c-fos and c-jun. We observed a gradual increase in the mRNA for and jun family members with increasing GnRH pulse frequency, but a sharp increase in corepressors TGIF1, SnoN and CREM at the highest pulse frequency. This was corroborated at the protein level, where c-fos and c-jun proteins were expressed at both fast and slow GnRH pulse frequencies, but SnoN, TGIF1 and CREM were only detected at the faster GnRH pulse frequency. In addition, we showed through a combination of overexpression and knockdown that SnoN and TGIF1 could repress FSHβ promoter activity in response to constant GnRH and activin, and could also eliminate the ability of the FSHβ promoter and gene to distinguish GnRH pulse frequency in a pulsed culture system. On the basis of these findings, we propose a model for GnRH pulse regulation of FSHβ in which induction of stimulatory AP-1 factors at a slow GnRH pulse frequency leads to increased transcription of FSHβ but as the GnRH

pulse frequency increases, the corepressors SnoN and TGIF1 are induced to dampen further FSHβ induction (Figure 47). Our experimental observations are consistent with this model and explain why increased expression of AP-1 family members at a higher GnRH pulse frequency does not result in greater induction of, or even reduces, FSHβ mRNA. Forced overexpression of SnoN or TGIF1 selectively represses FSHβ promoter activity in response to slow GnRH pulses because the endogenous genes are not expressed under these conditions, but overexpression has little effect at the higher GnRH pulse frequency as endogenous SnoN and TGIF1 are expressed. Conversely, knockdown of SnoN or TGIF only increases FSHβ mRNA in response to a fast GnRH pulse frequency where the endogenous SnoN and TGIF1 genes are expressed.
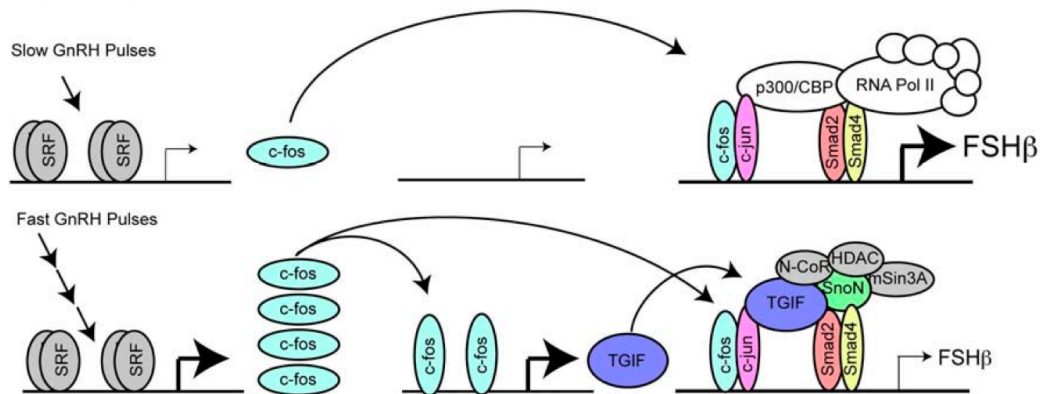
Figure 47: Model for the GnRH Pulse Regulation of the FSHβ Gene.

The transcription factors c-fos and c-jun are induced at slower GnRH pulse frequencies but the co-repressors SnoN and TGIF1 do not change. This leads to an increased ratio of promoter occupancy by stimulatory transcription factors to inhibitory co-repressors causing increased FSHβ mRNA induction. As the GnRH pulse frequency increases there is additional induction of the co-repressors SnoN and TGIF1, so the ratio of promoter occupancy by stimulatory transcription factors to inhibitory co-repressors does not change. The presence of the co-repressors on the FSHβ promoter dampens induction of the FSHβ mRNA at the faster GnRH pulse frequency.

9.2 Cyclic FSHβ Promoter Occupancy of FSHβ Promoter Regulators

We observed furthermore that c-fos, TGIF1, SnoN, and to a lesser degree c-jun, bind to the proximal FSHβ promoter region in cyclical manner with a periodicity of approximately 2 h similar to the optimal pulse frequency for FSHβ gene induction. Cyclical promoter occupancy has been demonstrated for EGR1 and SF1 on the LHβ gene with a periodicity of 30 min, which again correlates with the optimal pulse frequency for LHβ gene induction (187). It is possible that these periodicities of binding underlie the optimum pulse sensitivities of the two genes as the cycles of binding due to each pulse create positive reinforcement of the signal.

Our data also point to a disconnection between protein expression and promoter binding. For example, in cells exposed to fast GnRH pulses, c-fos, c-jun, TGIF1 and SnoN are all induced following the final pulse, but promoter binding of both c-fos and TGIF1 decreases over the same period before rebounding, and occupancy by c-jun and SnoN do not change. It is possible that the elevated basal protein levels due to the prior pulses are sufficient to saturate binding to the FSHβ promoter so the further increase in protein has no effect. The observed decrease in promoter occupancy by c-fos and TGIF1 following the last pulse may reflect a signaling event rather than a change in expression. Indeed, phosphorylation of c-jun on Ser63/73 or Thr91/93 by the Jun-N-terminal kinases

(JNKs) triggers degradation of c-jun and c-fos (247, 248). JNKs are robustly activated by GnRH which may serve to trigger promoter clearance followed by renewed binding as the JNK signal wanes.

The situation in cells exposed to slow GnRH pulses is quite different. Basal expression of the four factors due to the prior pulses is low and only c-fos and c-jun are strongly induced following the last pulse. Promoter binding of c-fos shows a transient decrease and then recovers quickly, again likely reflecting a JNK mediated promoter clearance, but TGIF1 binding decreases continually over time, perhaps reflecting that TGIF1 protein is not induced under these conditions so there is less protein to allow repopulation of the promoter.

Comparing promoter binding of the positive transcription factors to the co-repressors provides perhaps the best representation of the data. The relative binding of c-fos/c-jun to TGIF1/SnoN reveals that the ratio of positive to negative factors increases over time in cells exposed to slow GnRH pulses but does not change in cells exposed to fast pulses. This change in relative occupancy correlates closely with the increase in FSHβ mRNA following the final pulse as would be predicted by our model.

How these transcription factors cycle on the FSHβ promoter is an important question. Ubiquitin-mediated proteasomal degradation of EGR1 and SF1 was shown to be important for cycling on the LHβ promoter. The

degradation of c-fos is more complicated as it is subject to both ubiquitin-dependent and ubiquitin-independent degradation by the proteasome (249). A major C-terminal destabilization element in monomeric c-fos is an unstructured domain that directs ubiquitin-independent degradation by the 20S proteasome core. Phosphorylation of this domain on Ser362/Ser374 by ERK/Rsk1/2, or formation of a c-jun/c-fos heterodimer prevents degradation by the ubiquitin-independent pathway (249). When the C-terminal destabilization element is removed, ubiquitin-dependent degradation via the 26S proteosome can be observed due to a N-terminal destabilization element. As mentioned earlier, JUN and FOS degradation is triggered by phosphorylation of JUN but the ubiquitinated residues have not been identified (247, 248).

9.3 The Role of Different AP-1 Subunits in the Regulation of FSHβ Promoter

While we have focused our ChIP analysis on c-fos and c-jun, the dynamics of binding to the AP-1 site are likely very complex. Multiple AP-1 isoforms are induced by GnRH and can bind to this site by EMSA, including c-fos, c-jun, junB and FosB. We found dramatic differences in the kinetics of mRNA and protein induction for AP-1 family members that may alter the composition of the AP-1 complex on FSHβ promoter. Following a GnRH pulse, the initial complex may comprise c-fos and c-jun, but these subunits may be replaced by other AP-1 subunits including JunB, JunD, or FosB at intermediate times, and Fra-1 at later time point.

Not all AP-1 subunits, for example JunB and JunD, support FSHβ induction so the changing composition of the AP-1 complex may modify the kinetics of FSHβ induction. Such dynamics may also be important for TGIF1 and SnoN-mediated repression of FSHβ promoter. For example, TGIF1 shows differential effects when c-jun or JunB are present. TGIF1 can repress c-fos mediated FSHβ promoter activity in the absence or presence of JunB, but not in the presence of overexpressed c-jun. Further ChIP studies with the other subunits will be necessary to unravel the dynamics of the composition of the AP-1 complex on the FSHβ promoter.

9.4 Mechanism Underlying Corepressors Regulation of FSHβ Promoter

9.4.1 Regulation by SnoN and Ski

Although we have shown that the corepressors SnoN, Ski, CREM and TGIF1 repress FSHβ expression, the mechanisms underlying their action are not fully understood. Both SnoN and Ski interact with Smad proteins (232, 233) and both repress target genes by binding to NCoR-1 and mSin3A recruiting Histone Deacetylase Complex 1 (HDAC1) and displacing the transcriptional co-activator p300/CBP (230-233). Interestingly, SnoN and Ski can also repress AP-1 or GnRH-stimulated FSHβ promoter activity although neither of these proteins has been shown to bind AP-1 directly (250, 251).

Repression by SnoN and Ski may further depend on post-transcriptional modification of these proteins. For example, SnoN can be modified by SUMOylation on residues 50 and 383 via a specific SUMO E3 ligase, which leads to repression of TGF-β signaling in a promoter specific manner (252, 253). Ski and SnoN are also subject to ubiquitination and proteasomal degradation that limits their repressive effect. Whether such mechanisms are involved in repression of GnRH and activin-mediated induction of the FSHβ promoter remains unknown. While Ski was not induced by GnRH, it may still be important for GnRH pulse frequency mediated repression of FSHβ as Ski preferentially

forms a heterodimer with SnoN and such heterodimers are more active than individual Ski or SnoN homodimers (228, 229).

9.4.2 Regulation by TGIF

Unlike SnoN and Ski, TGIF1 was only able to modulate FSHβ promoter activity when both GnRH and activin were added. TGIF1 can interact and form complexes with both c-jun and the Smads (237); therefore, it is possible that TGIF1 requires activation of both GnRH and activin signaling pathways to trigger occupancy of both the AP-1 site and the SBEs to have its repressive effects. Like SnoN and Ski, TGIF1 is known to associate with HDACs through their interaction with NCoR1 and mSin3a and to prevent recruitment of p300/CBP (236, 254).

Repression by TGIF is also under post-translational control. TGF-β activation of the Ras-Mek-Erk pathway in HaCaT and Cos cells leads to phosphorylation of TGIF1 on multiple sites (235, 243). Two C-terminal threonine residues are phosphorylated by ERK and increase protein stability, possibly by interfering with ubiquitination and proteasomal degradation (46). GnRH stimulation of LβT2 gonadotropes induces two bands of different molecular weights recognized by the anti-TGIF1 antibody. Based on previous publications, we believe that the upper band is the phosphorylated and the lower band the unphosphorylated form of TGIF1. Phosphorylation of TGIF1 does appear to be important for its repression of the FSHβ promoter because the TGIF-2TV mutant, which lacks the two C-terminal phosphorylation sites, was unable to

repress FSHβ promoter activity like wild-type. On the contrary, this mutant led to increased promoter activity possibly indicating a dominant negative effect on the endogenous TGIFs. The protein stabilizing effect of TGIF1 phosphorylation was also seen in our overexpression studies. Transfection of wild-type TGIF1 led to a large increase in the slower migrating, phosphorylated form of TGIF1. In contrast, transfection of the 2TV mutant led to expression of low levels of a faster migrating band. The importance of ERK phosphorylation of TGIF and the known activation of the Ras-Mek-Erk pathway by GnRH, raises the interesting question of whether GnRH alters the phosphorylation state of TGIF and its ability to repress FSHβ transcription.

9.4.3 Regulation by CREM

Contrary to our expectations, while overexpression of CREM reduced FSHβ promoter activity, knockdown of CREM had no effect. A recent study has demonstrated that ICER, an alternatively spliced product of the CREM gene, regulates FSHβ promoter pulse sensitivity through binding to the CRE site in the proximal FSHβ promoter (222). The ICER splice variant is transcribed from the internal P2 promoter of the CREM gene. The isoform contains a short unique 5' sequence and a larger 3' sequence that is common to all CREM isoforms and contains the C-terminal DNA binding domain (255). The probes and primers used to detect CREM expression by microarray and QPCR target the conserved 3' region so measure all CREM isoforms including ICER. We have also measured ICER isoform expression using specific primers and find that both CREM and ICER mRNA are induced similarly.

The induction of CREM and ICER proteins is less clear. We see robust induction of CREM at 4-6 h of continual GnRH treatment that is similar to the published time course for ICER. The induction with GnRH pulses is much weaker in our study and we only see increases in CREM protein following 6 h of 1 nM GnRH pulses at a 30 min pulse interval. This is in contrast to the strong induction of ICER following pulsing with 10 nM GnRH for 20 h (219). It is possible that the longer period of perfusion with GnRH pulses allows for a more

robust increase in protein. Both studies demonstrated that CREM or ICER overexpression represses FSHβ promoter activity in transfected LβT2 cells. Ciccone et al. demonstrated that transfected ICER binds to the FSHβ promoter by ChIP although they did not demonstrate binding of endogenous ICER. We show that CREM, and dominant negative CREB mutants, specifically block GnRH stimulation of FSHβ, whereas Ciccone et al. use over-expression of CREB coupled with GnRH stimulation to show the repression by ICER in static culture.

Where the two studies differ is that we are not able to see an effect of CREM/ICER knockdown on GnRH-stimulated FSHβ promoter activity, whereas the other study sees an increase in promoter activity with fast GnRH pulses. Our knockdown approach uses a mixture of three synthetic siRNAs and we verified a functional knockdown by measuring DCREB stimulation of the FSHβ promoter whereas Ciccone et al. use a short hairpin RNA targeted against a conserved region of ICER (219). The two studies also use different FSHβ promoters, we use 1.5 Kb of the mouse promoter, whereas the earlier study used only the proximal -140 bp rat promoter which might account for some of the differences in the results.

9.5 Animal Models

The support for the importance of these transcription factors and co-repressors in reproduction comes from studies in knockout mice. Knocking out c-fos causes female infertility with increased numbers of atretic follicles, but it is unclear whether the defect is central or in the ovary (256). In contrast, knockout of JunD caused male infertility and a decrease in FSH but females were not affected (257). Knockout of other AP-1 subunits, such as c-jun, JunB, Fra-1 and Fra-2, causes embryonic or perinatal lethality, which precludes a reproductive assessment (258). The FosB knockout survives, but females show a severe nurturing defect and reduced litter sizes (259). Mouse knockouts of any the Smad proteins are embryonic lethal with multiple developmental defects, but deficiency of the activin typeIIA receptor (*Acvr2*) results in female infertility and delayed fertility in males with decreased FSH, but normal LH (260, 261). The phenotype is very similar to the FSHβ knockout with a block in folliculogenesis at the pre-antral stage (261).

The analysis of the roles of SnoN, Ski, CREM and TGIF in reproduction *in vivo* has been complicated by lethality and redundancy. Three lines of SnoN knock out mice have been generated. One line was generated by removal of amino acids 1-362 and this knockout is embryonic lethal (262). The other two lines were produced by 5' deletion or by removal of first exon (263). The 5'

deletion produced a hypomorph allele reducing, but not eliminating SnoN production while removal of the first exon yielded homozygous null mice. These mice were viable and fertile, but had T-cell development defect due to increased TGFβ signaling and reduced fertility on a 129SvJ background (263). It is not clear why such a discrepancy exists in these three SnoN knockout lines. Ski knockout mice suffer from exencephaly, craniofacial and skeletal abnormalities and die perinatally, which precludes an assessment of fertility (264). Male CREM knockout mice are infertile due to round spermatid maturation arrest, but no reproductive phenotype has been reported for the females (265). The TGIF1 knockout female mice do have a reproductive phenotype with reduced litter size and litter number that is due to problems in vascularization in the placenta, which is similar to but less severe than the Fra-1 and JunB knockouts (266). It is unclear, however, whether the TGIF1 knockout mice have a central reproductive phenotype too. A more robust phenotype might have been expected based on our findings, however it is possible that TGIF2 may share functional redundancy with TGIF1 and may still repress FSHβ promoter activity in the absence of TGIF1.

9.6 Conclusion

In this study, we demonstrate that not only AP-1 transcription factors, such as c-fos, c-jun, FosB and Fra-1, but also the corepressors, SnoN and TGIF1, can be induced at both mRNA and protein levels upon the GnRH treatment of LβT2 gonadotropes. Furthermore, we show that the corepressors SnoN and TGIF are only induced by the fast GnRH pulse frequency when there is relatively lower FSHβ mRNA production. The stimulators c-fos and c-jun, on the other hand are induced not only by fast but also slow GnRH pulse frequency when there is relatively high FSHβ mRNA induction. We also ensure SnoN and TGIF1's physiological relevance by demonstrating their GnRH stimulated mRNA induction in the rat primary pituitary cultures.

We not only show that GnRH differentially induces these AP-1 factors and co repressors, but we also demonstrate their functional role in the regulation of the FSHβ promoter. More specifically, we show that the overexpression of the corepressors, SnoN and TGIF reduces c-fos and c-jun AP-1 factor – stimulated, Smad – stimulated as well as GnRH and activin – stimulated FSHβ promoter acitivity. The opposite is also true as knockdown of SnoN and TGIF increases basal as well as c-fos and c-jun, Smads and GnRH and activin stimulated FSHβ promoter activity. Furthermore, by use of TGIF and SnoN mutants we also show

that GnRH stimulated phoshorylation of the TGIF as well as the binding of Smads to SnoN are cruicial for their repression of the FSHβ promoter.

We also show that overexpression of SnoN or TGIF selectively repress FSHβ promoter stimulation by the slow GnRH pulse frequency while the knockdown of SnoN or TGIF selectively increase GnRH stimulated FSHβ mRNA induction by the fast GnRH pulse frequency. Together these two pieces of data support our hypothesis that both SnoN and TGIF are involved in the GnRH pulse regulation of the FSHβ subunit gene.

Finally, we demonstrate that like LHβ regulators SF-1 and Egr-1, FSHβ regulators c-fos, SnoN, TGIF1 and to a lesser degree c-jun, have cyclical promoter occupancy in response to GnRH. Furthermore, FSHβ promoter stimulators c-fos and c-jun have greater FSHβ promoter occupancy relative to the corepressors SnoN and TGIF by the slow GnRH pulse frequency when the FSHβ mRNA levels are relatively high. Therefore, we show that c-fos, c-jun, SnoN and TGIF1 regulate the FSHβ promoter by their direct impact on in response to pulsatile GnRH.

In brief, we, for the very first time, illustrate how fast and slow GnRH pulse frequencies differntially regulate the FSHβ subunit gene by differentially inducing FSHβ stimulatory AP-1 factors and corepressors and by having them

occupy FSHβ promoter in GnRH pulse frequency specific manner. Our discovery provides an explanation for the observation that high FSH levels correlate with the slow GnRH pulse frequecncy during menstrual cycle.

9.7 Future Directions

In future, we would like to further study the molecular mechanism underlying the SnoN and TGIF mediated repression of FSHβ gene (Figure 48). More specifically, we would like to investigate whether SnoN and TGIF can interact with AP-1 subunits, Smads, HDACs, other repressors or some new partners in the gonadotrope cells. We are also interested in investigating through which specific sites on FSHβ promoter these corepressors are utilizing their repressive function.

We would also like to study the role of SnoN and TGIF *in vivo*. We plan to create transgenic mice either overexpressing wild type or mutant form of corepressors, specifically, in the pituitary gonadotropes using the Cre-lox strategy (Figure 49). Using such mice, it would be interesting study the overexpression of TGIF 2TV mutant in the pituitary gonadotropes as it seemed to act as dominant negative mutant and led to further increase in FSHβ promoter activity in LβT2 cells stimulated by GnRH, activin or both.

We and others have demonstrated that the LβT2 gonadtropes form neurite like formations upon GnRH treatment (267, 268). We hypothesize that such formations are important for the gonadotrope-gonadotrope communication as well as secretion of LH and FSH gonadotropins. We would like to study such

formations by generating mice that overexpress yellow fluroscent protein (YFP) specifically in the gonadtropes using the Cre-lox system and performing *in vivo* imaging of the pituitary of such mice. We also plan to study different micro-stuctures in such neurite like formations through high-resolution transmission electron microscopy (TEM) (Figure 50). A recent study indicates that SnoN and its isoform SnoN2 are involved in regulation of the rat granule neuronal branching and positioning (269). Therefore, it would also be interesting to investigate the role of SnoN in such neurite like formation in the gonadotrope cells.

Chapter 9, in part, is a reprint of the material as it appears in Gonadotropin-Releasing Hormone Pulse Sensitivity of Follicle Stimulating Hormone-Beta Gene Is Mediated by Differential Expression of Positive Regulatory Activator Protein 1 Factors and Corepressors SKIL and TGIF1 2011. Mistry, Devendra et al. Molecular Endocrinology, 2011. The dissertation author was the primary investigator and author of this paper.
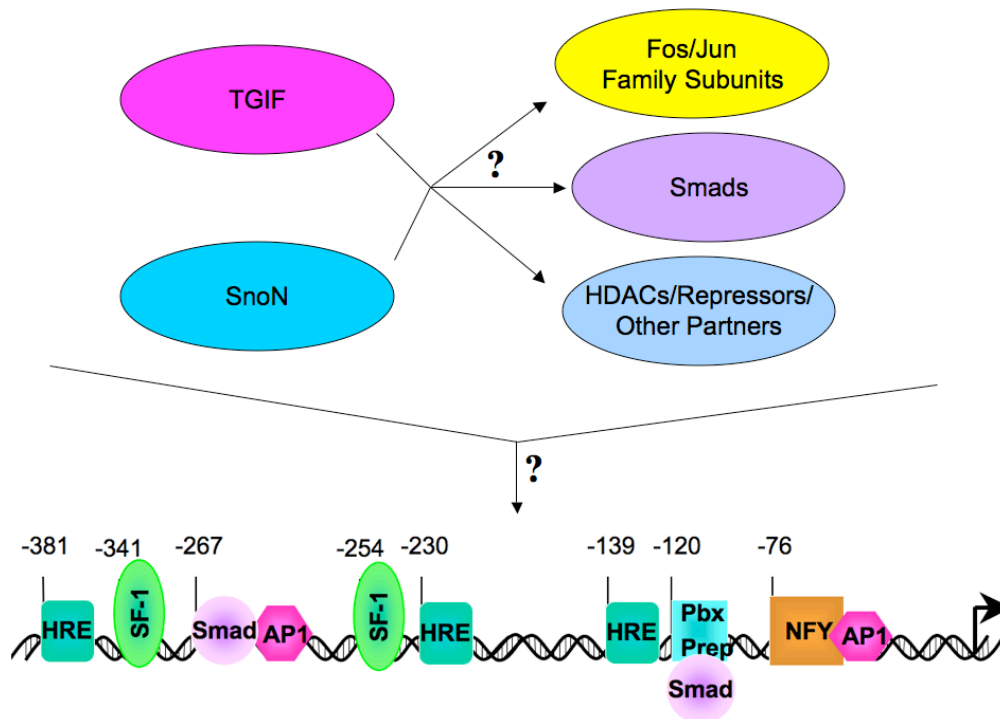
Figure 48: Future Goals – Investigating the Potential Binding Partners and FSHβ Promoter Binding Sites for SnoN and TGIF.

Based on our results as well as previous studies, SnoN and TGIF interaction with AP-1 subunits, Smads, HDACs and other corepressors is speculated. It is also possible that they may interact with previously not know factors. Also, TGIF and SnoN may interact with AP-1 binding sites, SBEs or other sites on FSHβ promoter.

CMV	Lox-stop-Lox	WT/Mutant Snon/TGIF	SV40-PolyA

X

LHβ-promoter	CRE
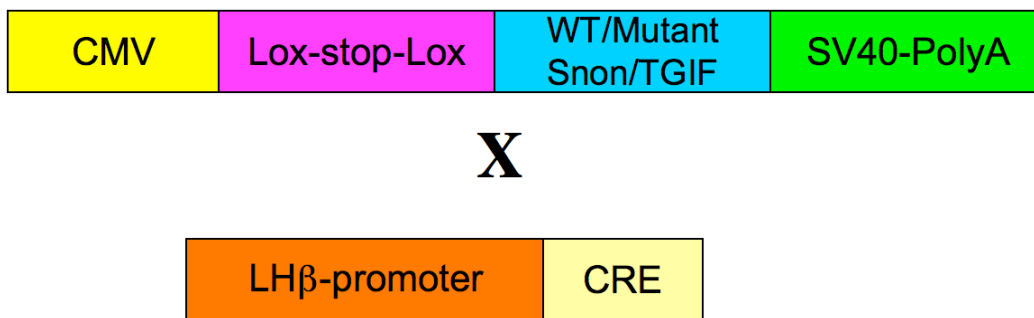
Figure 49: Future Goals – Investigating the Role of TGIF and SnoN *in vivo*

A strategy depicting generation of the proposed transgenic mice. Flox controlled wild type or mutant corepressor gene expressing mice will be crossed with LHβ promoter regulated Cre gene expressing mice to produce the mice overexpressing the wildtype or mutant corepressors specifically in the gonadotropes of the pituitary.
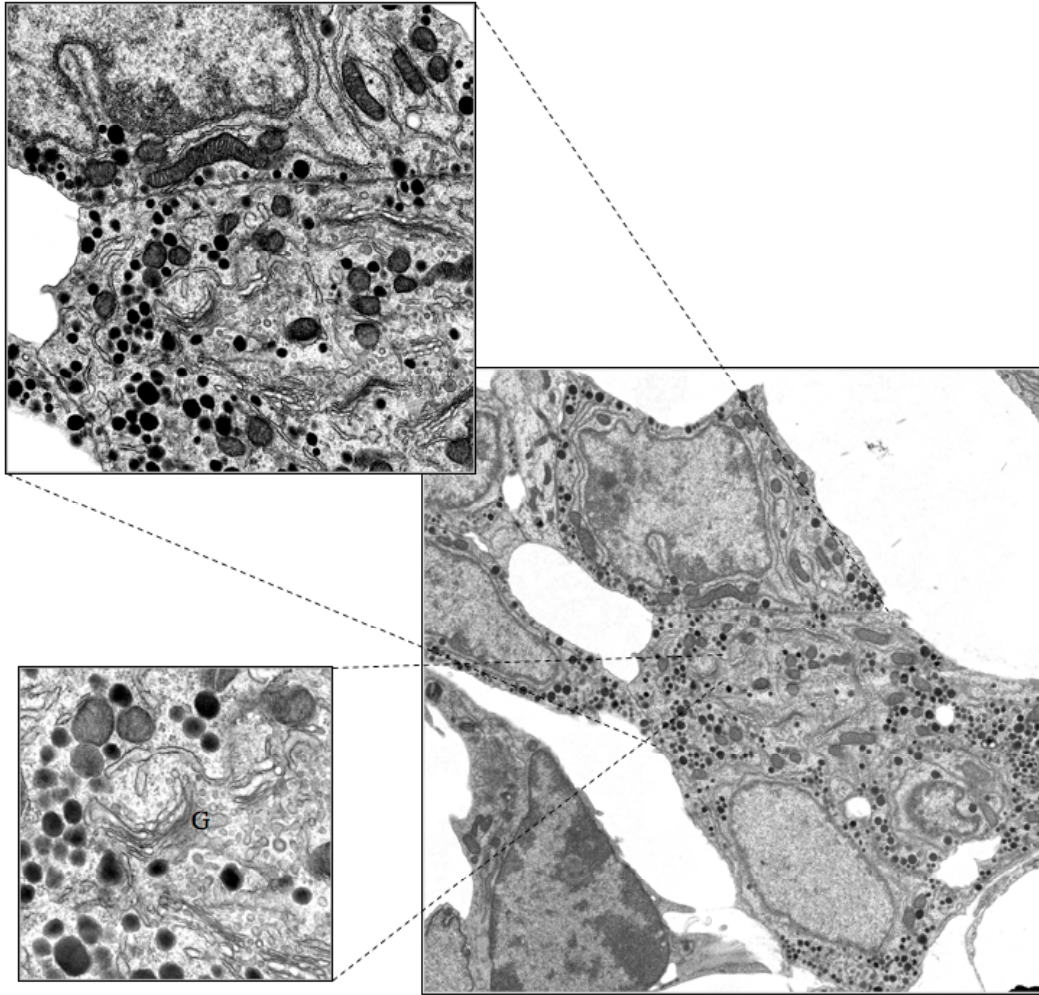
Figure 50: Future Goals – A Picture of Potential Mouse Gonadotrope-Gonadotrope Junction.

A mouse pituitary was fixed with gluteraldehyde and post-fixed with osmium tetroxide. It was stained with urenyl acetate and embedded in durcupan. Approximately 50-80nM thin slices were obtained, were post-stained with urenyl acetate and lead and finally observed under a transmission electron microscope. The gonadotropes were distinguished from other cell types by their large size, shor profiles of rough endoplasmic reticulum, c-shaped golgi and irregular distribution of the electron dense vesicles. The magnified pictures show potential gonadotrope-gonadotrope junctions and c-shaped golgi structure (G).

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