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Regulation of Reproduction via Tight Control of Gonadotropin Hormone Levels

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Abstract

Mammalian reproduction is controlled by the hypothalamic-pituitary-gonadal axis. GnRH from the hypothalamus regulates synthesis and secretion of gonadotropins, LH and FSH, which then control steroidogenesis and gametogenesis. In females, serum LH and FSH levels exhibit rhythmic changes throughout the menstrual or estrous cycle that are correlated with pulse frequency of GnRH. Lack of gonadotropins leads to infertility or amenorrhea. Dysfunctions in the tightly controlled ratio due to levels slightly outside the normal range occur in a larger number of women and are correlated with polycystic ovaries and premature ovarian failure. Since the etiology of these disorders is largely unknown, studies in cell and mouse models may provide novel candidates for investigations in human population. Hence, understanding the mechanisms whereby GnRH regulates gonadotropin hormone levels will provide insight into the physiology and pathophysiology of the reproductive system. This review discusses recent advances in our understanding of GnRH regulation of gonadotropin synthesis.

Keywords

Luteinizing hormone; Follicle-stimulating hormone; Differential regulation; GnRH pulse frequency; Reproduction

1. Physiology and pathophysiology of LH and FSH

Gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are synthesized by the anterior pituitary gonadotropes and secreted into the circulation to regulate gonadal function. Every secretory pulse of LH from the pituitary corresponds to a pulse of GnRH from the hypothalamus (Levine et al., 1982). On the other hand, FSH secretion is not entirely regulated by GnRH and most of FSH is constitutively released (Culler and Negro-Vilar, 1987; Levine and Duffy, 1988). In females, LH and FSH

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concentration fluctuates throughout the menstrual or estrous cycle and the levels are firmly controlled to assure reproductive fitness and new waves of steroidogenesis and gametogenesis (Pierce, 1988). Pulsatile LH secretion maintains basal level of this hormone in the circulation and steroidogenesis throughout the cycle. LH increases steroid hormone production via induction of steroidogenic enzymes in interstitial Leydig cells and theca cells in males and females, respectively. In females, LH exhibits a 5–10 fold mid-cycle increase that causes ovulation. FSH in males induces androgen binding protein and contributes to spermatogenesis through interaction with testosterone. In females, FSH stimulates aromatase expression in follicle granulosa cells, which increases estrogen production and follicular growth. FSH increases together with LH during the preovulatory surge and exhibits a separate, second increase that is necessary for folliculogenesis.

LH and FSH are critical for reproductive fitness and thus, survival of the species. LH is required for pubertal development and gonadal function, and a lack results in hypogonadism and infertility in both sexes in humans and mouse models (Huhtaniemi, 2006; Huhtaniemi et al., 2006; Ma et al., 2004). FSH in humans is also critical for reproductive function in both genders, since its deficiency results in absent or incomplete pubertal development in women, relatively normal pubertal development but azoospermia in men, and infertility in both women and men (Lamminen et al., 2005). Female mice deficient in FSH have a block in folliculogenesis prior to the antral stage resulting in infertility, while males have impaired reproductive function due to lower sperm count, but are not infertile (Kumar et al., 1997).

LH and FSH are glycoprotein heterodimers, each comprised of a common α subunit (*Cga*, or αGSU) and a unique β subunit (Pierce and Parsons, 1981). αGSU is minimally regulated by hormones, since it is expressed at the sufficiently high basal level (Chin and Boime, 1990). Unique β-subunits provide biological specificity and are limiting components in the mature hormone synthesis, whose amounts are regulated at the transcriptional level of the β-subunits (Kaiser et al., 1997b; Papavasiliou et al., 1986). Pulsatile GnRH secretion increases transcription of LHβ subunit (Burger et al., 2009; Haisenleder et al., 2008; Haisenleder et al., 1988). Although FSH can be secreted without a corresponding GnRH pulse, FSHβ transcription is dependent on GnRH. In mice lacking GnRH (Mason et al., 1986) or GnRH receptor (Wu et al., 2010), FSH levels, as well as LH levels, are 60%–90% lower in males and females. GnRH injection in rats with low endogenous GnRH increased FSHβ transcription 4 fold (Dalkin et al., 2001). This level of increase is comparable to the changes in FSH concentration in the circulation throughout the cycle. Therefore, while concentration of LH in the circulation is regulated at both the level of β subunit transcription and GnRH-stimulated secretion, FSH concentration is regulated primarily at the level of β subunit transcription. Regulation of gonadotropin synthesis and differential expression is not elucidated at the mechanistic level. Herein, we discuss recent findings since our previous review (Thackray et al., 2010), concentrating on the regulation of β-subunit transcription by GnRH. Our review may shed light on differential regulation and point to remaining gaps in our knowledge, providing basis for future investigations that may lead to novel therapeutic treatments of reproductive disorders discussed below.

1.1. Differential regulation of LH and FSH synthesis

Both gonadotropins are synthesized in the same gonadotrope cells, but differences in the relative amounts occur at specific stages of the menstrual or estrous cycle. As illustrated above, LH exhibits a single increase during the preovulatory surge, while FSH increases two times during the menstrual or estrous cycle. The question of how are these two hormones differentially regulated in the same gonadotrope population, by the same hormonal milieu, has been an area of intense investigation. Differential levels of LH and FSH may stem from differences in secretion, since LH is secreted strictly through the GnRH-regulated secretory pathway while FSH is constitutively secreted (Levine and Duffy, 1988).

Alternatively, post-translational modifications of these hormones contribute to differences in the half-life that would ultimately affect concentration in the circulation. Namely, LH and FSH are N-linked glycosylated proteins, containing N-acetylglucosamine residue linked to an amide group of an asparagine (Asn) amino acid in the peptide chain (Green et al., 1986). N-linked glycosylation of LH and FSH is critical for their bioactivity and non-glycosylated forms serve as antagonists (Fares, 2006). This is likely due to necessity of glycosylation for dimerization of α and β subunits and subsequent glycohormone secretion (Wang et al., 2016). LH and FSH have many molecular variants depending on the differences in their oligosaccharide chains. Alkalinity/acidity of variants, and their half-life in the circulation, depends in part on sialylation and sulfation of terminal galactose. There is a shift to less basic variants of LH at the evening of proestrus and to more basic variants of LH after ovariectomy (De Biasi et al., 2002), while FSH is shifted to less acidic variant at midcycle (Wide and Bakos, 1993), implying endocrine regulation of glycosylation enzymes' activity (Damian-Matsumura et al., 1999; Ulloa-Aguirre et al., 2001). Furthermore, human FSH is primarily siaylated while LH is sulfated, a difference that contributes to their variable clearance rates (Green et al., 1986). Since the enzyme that adds the sulfate, sulfotransferase, does not recognize specific peptide sequence but requires only tri-saccharides (Ulloa-Aguirre et al., 1999), enzyme synthesis or activity represents another layer of potential differential regulation of gonadotropin hormones synthesized in the same cell.

Lastly, β-subunit transcription precedes changes in hormone concentration in the circulation, and fluctuations in mRNA levels in the gonadotrope correlate with concentration of the hormones. Therefore, transcriptional regulation of β-subunits may be a critical point that determines levels of gonadotropin hormones. A midcycle surge of GnRH elicits a surge in both LH and FSH, resulting in ovulation of the mature follicle in response to LH (Jones, 1997). LHβ gene is induced five fold and FSHβ transcription increases four fold in the afternoon of proestrus prior to the onset of the surge (Butcher et al., 1974; Ortolano et al., 1988; Zmeili et al., 1986). In rodents, during the morning of estrus, a secondary FSH raise occurs without a concomitant increase in LH (Besecke et al., 1997; Woodruff et al., 1996). FSHβ mRNA is induced three fold during this time (Halvorson et al., 1994; Ortolano et al., 1988). This secondary increase in FSH is essential for follicular development for the subsequent estrous cycle (DePaolo et al., 1979; Hoak and Schwartz, 1980; Papavasiliou et al., 1986). In humans, the FSH levels in the serum increase in the late luteal phase through the mid follicular phase of the menstrual cycle. This FSH increase corresponds to a time when the next cohort of follicles is being recruited during folliculogenesis. Thus, although

responding to the same hormonal milieu, synthesis of LH and FSH is differentially regulated during the estrous or menstrual cycle and this regulation appears to occur primarily at the level of the β subunits transcription. This differential expression of two gonadotropin hormones from the same cell in response to the same endocrine stimuli is critical for reproductive fitness.

1.2. Polycystic ovary syndrome (PCOS)

Higher than normal levels of gonadotropins in females also lead to reproductive pathologies, albeit less severe than a lack of hormone. Most notably, polycystic ovary syndrome (PCOS) is characterized by an increase specifically in LH (Chang, 2007; Hall et al., 1998; Morales et al., 1996), and premature ovarian failure (POF) or primary ovarian insufficiency is associated with an increase in FSH (Chand et al., 2010; Goswami and Conway, 2007). The etiology of PCOS and POF is not clear; it may stem from dysregulation of GnRH pulsatility, pituitary responsiveness, or ovarian hormone production. Endocrine feedback loops cause difficulty in pin-pointing the primary site of dysregulation.

PCOS affects 10% of reproductive age women, yet much is unknown regarding the cause and pathophysiology (De Leo et al., 2016). This is complicated by the fact that the syndrome is categorized with heterogeneous characteristics and patients present with a combination of several symptoms such as chronic or frequent anovulation, polycystic ovaries, hyperandrogenism, hirsutism, acne, obesity, and insulin resistance (Goodarzi et al., 2011). Elevated LH levels are observed in 55%–75% of the women with PCOS, leading to elevated LH/FSH ratio (De Leo et al., 2016). Since obesity is often comorbid with PCOS, and obesity causes reduced LH levels (Mihalca and Fica, 2014; Pasquali et al., 2002; Rosenfield and Bordini, 2010), PCOS women with normal LH levels may have competing influences of obesity and PCOS per se, that keep the LH within the normal range. In a support of this supposition, lean PCOS patients have elevated LH levels (Pangaribuan et al., 2011; Sahin et al., 2014). Recent genomic studies correlated polymorphism in the LHβ gene to the occurrence of PCOS (El-Shal et al., 2016), implicating dysregulation in LHβ transcription, and possibly higher LH levels, among the causes of PCOS. Surprisingly, another genome wide association study correlated polymorphism in FSHβ promoter with PCOS, implicating changes in FSH levels with this disorder, maybe through altered estrogen synthesis (Hayes et al., 2015).

Preponderance of animal studies point to prenatal or early hyperandrogenemia as a contributing cause of PCOS (Abbott et al., 2008; Abbott et al., 2013; Kauffman et al., 2015; Manikkam et al., 2008; Roland et al., 2010; Veiga-Lopez et al., 2008). High insulin levels, with or without obesity, in PCOS patients may be a cause of hyperandrogenimia since insulin in synergy with LH elicits ovarian androgen production (recently reviewed in (Rosenfield, 2013)). Subsequently, hyperandrogenemia may diminish progesterone inhibition of GnRH pulse frequency, causing rapid pulsatile secretion of LH and further increase in ovarian androgen production, correlating regulation of LH frequency and amplitude to PCOS (Moore et al., 2015; Pielecka et al., 2006).

1.3. Premature ovarian failure (POF)

Premature ovarian failure (POF) or primary ovarian insufficiency is a disorder affecting 1% of reproductive age women that lose ovarian function before the age of 40. Majority of POF cases are idiopathic, while 10% are acquired due to chemotherapy, autoimmunity, or toxicity, and 20% have known genetic causes (Rossetti et al., 2016). Fragile X mutation or pre-mutation carriers comprise a bulk of individuals with known genetic causes of POF (Macpherson and Murray, 2016). The rest have mutations in autosomal genes, such as FOXL2, FSH receptor, aromatase and others, which were associated with POF (Laven, 2016).

However, as mentioned, in 70% of patients POF is idiopathic. They are diagnosed usually after ovarian function has ceased, since they seek care due to infertility or amenorrhea. At that time it is not clear if increased FSH stems from a deficiency in ovarian inhibin and is the result of a lack of negative feedback, or if increased FSH was a contributing factor (Laven, 2016). Thus, there is disagreement whether the high FSH level in POF is preceded by diminished ovarian inhibin level; or alternatively, high FSH leads to recruitment of larger number of follicles into a growing pool in every cycle in younger women resulting in early depletion. In the Fragile X syndrome mouse model, FSH is elevated prior to the loss of ovarian function, strengthening the possibility that high FSH may be a cause and not a consequence (Lu et al., 2012). Recently, it was determined that increased levels of FSH in the circulation in other mouse models, due to overexpression or re-trafficking of FSH to the GnRH-regulated secretory pathway, resulted in hemorrhagic ovaries and 5-fold more corpora lutea, implicating higher FSH levels in greater follicle recruitment (Kumar et al., 1999; Wang et al., 2014). Mouse model of constitutively active FSH receptor also experiences accelerated loss of follicular reserves (Peltoketo et al., 2010). Furthermore, polymorphism in the human FSHβ promoter, which regulates β subunit expression, increases FSH levels and has been associated with early menarche and premature menopause in women (He et al., 2010). Another genome-wide association study reported that polymorphism in the 5′ region of the FSHβ gene is also associated with higher FSH levels in the circulation, premature puberty, early natural menopause, and dizygotic twinning (Mbarek et al., 2016).

Therefore, although feedbacks complicate pin-pointing the primary site of dysregulation, genomic evidence ties polymorphism in the FSHβ gene promoter to POF, indicating that regulation of FSHβ subunit expression may contribute significantly to the etiology, at least in some affected individuals. Alternatively, mutations in transcription factors that influence FSHβ transcription, such as FOXL2, or signaling pathways that lead to induction of FSHβ mRNA may contribute to POF. Given that 70% of POF patients develop this disorder from unknown causes, studies using mouse models or model cell lines, may provide potential candidates to investigate etiology in human population.

2. GnRH receptor signaling pathways that regulate gonadotropin gene expression

The complexity of GnRH signaling provides a variety of regulatory avenues for gonadotropin gene regulation (Fig. 1). Molecular studies that identified signal transduction pathways activated by GnRH receptor (GnRHR) binding have been facilitated with the development of model cells lines. Whole animal studies, although critical for understanding of the HPG axis within the endogenous hormonal setting, can be inconclusive as illustrated above, due to hormonal feedback and the possible effects on different levels of the axis. Primary pituitary cells are frequently employed to study hormonal effects on gonadotropin synthesis. However, they are not conducive to analyses at the molecular levels due to scarcity of gonadotropes that comprise around 10% of the mixed cells population in the anterior pituitary. Two immortalized clonal cell lines were developed in the Mellon laboratory using targeted tumorigenesis with SV40 T-antigen, which contributed greatly to delineating GnRH signaling pathways and transcriptional regulation of β-subunits (Alarid et al., 1998; Alarid et al., 1996). The αT3-1 cell line created using the 5′ regulatory regions of α-GSU approximates an immature gonadotrope cell. It expresses α-GSU and GnRH receptor, but not β-subunits. On the other hand, the LβT2 cell line created using LHβ promoter, displays a more mature phenotype since it expresses LHβ and FSHβ and secretes LH and FSH in response to hormonal cues (Graham et al., 1999; Pernasetti et al., 2001).

Because it may underlie molecular causes of subfertility, pituitary responsiveness to GnRH leading to induction of gonadotropin expression is an important area of investigation. GnRH binding to its G protein-coupled receptor (GPCR), expressed on the gonadotropes of the anterior pituitary, activates the synthesis of the gonadotropin hormones, LH and FSH, and stimulates their secretion (Seeburg et al., 1987; Vale et al., 1977). GnRH is secreted in a pulsatile fashion into the hypophysial-portal system, while long-term tonic exposure to GnRH adversely affects gonadotrope function (Belchetz et al., 1978; Burrin and Jameson, 1989; Shupnik, 1990). Frequency of GnRH pulses changes throughout the menstrual and estrous cycle, modulated by the upstream regulatory neuronal network. Kisspeptin, secreted by several neuronal populations, is a crucial neuropeptide that synchronizes GnRH neuron secretion and stimulates GnRH release (Semaan and Kauffman, 2010). GnRH exhibits high pulse frequency in proestrus during the preovulatory surge (Harris and Levine, 2003; Levine et al., 1985; Levine and Ramirez, 1982). Several seminal studies analyzing β subunits transcription in the rat in vivo determined that fast pulse frequency of GnRH favors LHβ synthesis, while FSHβ expression is more highly induced by low pulse frequency (Burger et al., 2002; Dalkin et al., 1989; Haisenleder et al., 1991; Kaiser et al., 1997b; and recently reviewed in Thompson and Kaiser, 2014). However, the mechanisms of gonadotrope responsiveness to varied pulse frequencies are only beginning to emerge.

Although a member of the G protein-coupled seven transmembrane domain receptors, GnRHR has a unique structure (Neill, 2002; Tsutsumi et al., 1992). Unlike the other family members, it lacks the intracellular carboxyl-terminal tail and contains several sequence differences in regions otherwise highly conserved (Kaiser et al., 1997a; Sealfon et al., 1997; Stojilkovic et al., 1994; Tsutsumi et al., 1992). The absence of the C-terminal tail that

normally mediates desensitization through β-arrestin association and encapsulation into clathrin-coated vesicles renders the receptor resistant to this regulatory mechanism. Thus, the receptor does not internalize as rapidly as other GPCRs (Blomenrohr et al., 1999; Hislop et al., 2001; McArdle et al., 2002; Pawson et al., 2008; Willars et al., 1999), leading to longer signaling, which raises a question of intracellular negative feedback that stops the signal. Since gonadotropin gene expression is dependent upon GnRH pulses, the gonadotropes must maintain sensitivity to subsequent GnRH pulses by resolving the effect of a prior pulse. Several steps of the GnRH signaling pathways therefore contain negative regulators, in addition to molecules that propagate the signal, which will be discussed below. This rapid adaptation and maintenance of responsiveness underlies the ability of gonadotropes to properly express LH and FSH and regulate reproductive function.

2.1. G-proteins

Ligand binding to the GnRHR causes activation of trimeric G proteins. Gαq/11 isoform is activated in a model of immature gonadotrope, αT3 cells, while both Gαq/11 and Gαs are involved in GnRHR signaling in a model of mature gonadotrope LβT2 cells and in primary rat cultures. Some involvement of Gαi and Gα12/13 was shown as well, but without a clear downstream effect (Liu et al., 2002; Liu et al., 2003; Naor et al., 2000; Sim et al., 1995; Stanislaus et al., 1998). GnRHR coupling to various G proteins has been reviewed of late (Naor and Huhtaniemi, 2013) and it won't be discussed in depth here. Although GnRH signaling has been reviewed recently as well (Perrett and McArdle, 2013), in this review we will focus on how these various signaling pathways and downstream effectors contribute to differential expression of gonadotropin β subunits.

Recently the functional significance of activation of Gαq/11 and Gαs trimeric G proteins by GnRH was assessed in LβT2 using siRNA. The study demonstrated that endogenous FSHβ mRNA induction requires Gαq/11, while maximal LHβ involves Gαs (Choi et al., 2012). Surprisingly, Gαs activity may diminish FSHβ expression, via induction of inhibin α (Choi et al., 2012). Thus, engagement of either Gαq/11 or Gαs may lead to differential expression of gonadotropin β subunits.

Activation of Gαs normally leads to cAMP-PKA signaling, but downstream targets of this pathway at the level of the LHβ promoter are unknown. Elegant study by Webster lab demonstrated that GnRH signal has a unique kinetics of cAMP generation and PKA activation compared to other stimuli that activate the same pathway (i.e. Pituitary adenylate cyclase-activating polypeptide (PACAP, (Tsutsumi et al., 2010)). More importantly, the study demonstrated that Gαs-cAMP-PKA module is not susceptible to desensitization (Tsutsumi et al., 2010).

More commonly, GnRHR couples to Gαq/11 and activates phospholipase Cβ (PLC), causing a creation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG; Stojilkovic et al., 1994). IP3 formation and IP3 receptor activation leads to an increase in intracellular calcium concentration via calcium mobilization from intracellular stores. Increase in DAG and intracellular calcium concentration leads to activation of PKC and calcium calmodulin kinase II (CamKII), respectively. Gαq/11-PLC-DAG/Calcium signaling module desensitizes at several steps after GnRH stimulation: at DAG generation and at calcium increase

(Tsutsumi et al., 2010). The latter may be due to the loss of IP3 receptors via downregulation that are necessary for calcium mobilization (McArdle et al., 1999; McArdle et al., 2002; McArdle et al., 1996). Thus, fast pulse frequency of GnRH from the hypothalamus may desensitize Gαq/11-DAG and lead to lower FSHβ expression, while Gαs does not desensitize and leads to increased LHβ. The question whether Gαs signal is less sensitive or not sustained during low pulse frequency, which results in lower LHβ than FSHβ needs to be elucidated. It has been postulated that GnRH may switch to Gαs-cAMP signal only during preovulatory surge to engage new set of signaling targets (Lariviere et al., 2007). Indeed, fast pulse frequency increases receptor concentration on the cell surface (Bedecarrats and Kaiser, 2003). This higher receptor number may allow coupling to and engaging Gαs in addition to Gαq/11, which induces higher levels of LHβ necessary for a rapid increase of LH in the circulation and ovulation, while low pulse frequency and Gαq/11 activation may be sufficient for lower level of LHβ induction, as well as FSHβ, throughout the rest of the cycle to stimulate steroidogenesis and folliculogenesis (Fig. 2).

2.2. Calcium

GnRH receptor activation causes an increase in intracellular calcium concentration. The calcium increase stems either from mobilization from intracellular calcium stores or from influx through the calcium channels (Mulvaney et al., 1999; Van Goor et al., 1999); recently reviewed in (Stojilkovic, 2012; Stojilkovic et al., 2010). As discussed above, increase in calcium from intracellular stores occurs through IP3 formation, and is sensitive to desensitization via downregulation of IP3 receptors (McArdle et al., 1999; McArdle et al., 2002; McArdle et al., 1996). Consistent with this, using αT3 cells, it was demonstrated that extracellular calcium influx leads to more sustained increase in calcium than mobilization from intracellular stores (Mulvaney and Roberson, 2000). Calcium channels may be activated by GnRH through PKC (Stojilkovic et al., 1991). Pulsatile stimulation of the calcium channel by a pharmacological activator in the rat pituitary cells exhibits the same effect on the gonadotropin gene expression as GnRH pulses, with higher pulse frequencies favoring LHβ transcription, and lower pulse frequencies preferring FSHβ transcription (Haisenleder et al., 2001).

Increase in calcium concentration activates CamKII, and CamKII is activated by GnRH irrespective of pulse frequency (Burger et al., 2008) and plays a role in induction of both β-subunits (Ely et al., 2011; Haisenleder et al., 2003b). Consistent with a lack of frequency dependence, CamKII activation can be achieved with either source of calcium, extracellular or from internal stores in LβT2 cells (Haisenleder et al., 2003b). Nuclear factor of activated T-cells (NFAT) is another putative target of calcium increase via calciuneurin that may be activated by GnRH to induce immediate early gene promoters (Binder et al., 2012). Calcium causes calmodulin/calcineurin-dependent nuclear NFAT translocation, but a lack of desensitization of this pathway implies that while NFAT may mediate GnRH action, it is not a decoder of GnRH pulse frequency (Armstrong et al., 2009). Additionally, the effect of NFAT on gonadotropin β-subunit expression has not been demonstrated. Thus, CamKII maybe a critical signaling molecule activated by calcium.

2.3. MAPK

GnRHR ligand binding also leads to activation of three branches of the mitogen-activated protein kinase (MAPK) pathway: ERK1/2, JNK, and p38 (Liu et al., 2002; Roberson et al., 1995; Sundaresan et al., 1996). Mechanisms of MAPK activation are not completely clear. GnRHRs are found in low-density membrane microdomains called lipid rafts, which are enriched in cholesterol and sphingolipids and may allow assembly of multiprotein signaling complexes (Navratil et al., 2003). In αT3 cells and mouse pituitary cells, GnRHRs co-localize with a proportion of total cellular ERK2 in lipid rafts (Bliss et al., 2007), which may provide a partial explanation of ERK2 activation by GnRH. Activation of ERK1/2 by GnRH also involves calcium. ERK1/2 responds to pulsatile GnRH, and lower pulse frequency leads to longer, sustained activation in LβT2 mature gonadotrope cell line, which may correlate ERK1/2 activation to FSHβ expression (Kanasaki et al., 2005). Additionally, low pulse frequency of GnRH causes high amplitude of ERK1/2 activation that is necessary for FSHβ transcription in vivo in rats (Burger et al., 2008; Haisenleder et al., 1998). Extracellular calcium influx through voltage gated calcium channel may be necessary for ERK1/2 activation in αT3 cells (Mulvaney et al., 1999). The study demonstrating that PKC stimulation leads to voltage gated calcium channel activation (Stojilkovic et al., 1991), may explain why ERK1/2 phosphorylation is PKC dependent (Ely et al., 2011). However, ERK1/2 solely may not be a true frequency decoder due to different kinetics of activation and deactivation (Armstrong et al., 2010). The mathematical model postulated by this group (Armstrong et al., 2011) implies cooperative action of two or more pathways leading to two or more transcription factors that coordinately induce the expression of their target gene.

The p38 branch of the MAPK pathway is also activated by GnRH via PKC and calcium (Mugami et al., 2017; Roberson et al., 1999). p38 can be activated by both extracellular calcium influx and from intracellular stores in αT3 cells, while in LβT2 p38 activation requires calcium from intracellular stores. Pulse dependency of p38 activation has not been analyzed. p38 activation is involved in FSHβ induction by GnRH in LβT2 cells (Bonfil et al., 2004; Coss et al., 2007; Ely et al., 2011; Wang et al., 2008), but it is not critical in vivo in rats (Haisenleder et al., 2008). Calcium from intracellular stores leads to JNK activation in αT3 cells (Kraus et al., 2001; Mulvaney and Roberson, 2000). Whether these distinct calcium sources: extracellular, leading to ERK1/2 activation and intracellular, leading to JNK activation play a role in more mature LβT2 cells or primary gonadotrope as well is yet to be determined. GnRH activation of ERK1/2, p38 and JNK involves several PKC isoforms in both αT3 and LβT2 cells, primarily PKCα, PKCε and PKCδ (Dobkin-Bekman et al., 2010; Kratzmeier et al., 1996; Liu et al., 2003; Mugami et al., 2017). However, a specific isoform which selectively activates ERK1/2 but not JNK has not been identified, to indicate the isoform necessary for voltage gated calcium channel activation and extracellular calcium influx. Furthermore, understanding of the significance of the studies delineating Gαs versus Gαq/11 signaling modules is complicated by findings that these same PKCε and PKCδ that are normally activated by Gαq/11 maybe be involved in cAMP accumulation, in addition to cAMP accumulation by Gαs activation (Lariviere et al., 2007). More importantly, PKC isoform-specific roles in gonadotropin gene expression were not examined.

While ERK1/2 may be activated longer or more highly with low pulse frequency of GnRH, regulation of MAPK activity occurs at the level of dephosphorylation as well. MAPKs are deactivated by a family of MAPK phosphatases (MKPs), which is a subfamily of a large dual-specificity phosphatase (DUSP) family. Studies analyzing MPKs/DUSPs are complicated due to multiple members that are ubiquitously expressed and lack substrate specificity. MKP1/DUSP1 and MKP2/DUSP4 are induced by GnRH in αT3 cells (Roberson et al., 1995; Zhang and Roberson, 2006). MKP1/DUSP1 is also phosphorylated by GnRH and, since localized specifically in the nucleus, it may provide rapid cessation of nuclear ERK1/2 activity specifically and maintain sensitivity to GnRH stimulation. (Nguyen et al., 2010). MKP1/DUSP1 is induced with high pulse frequency of GnRH (Purwana et al., 2011), which may explain sustained ERK1/2 activation with low pulse frequency.

In summary, GnRH-stimulated calcium increase from two different sources, intracellular stores or extracellular influx, activates different downstream signaling pathways. Since one of them, intracellular via IP3, can be desensitized, calcium sources may lead to differential regulation of downstream gene targets; however this remains to be examined. Receptor concentration may also play a role. Fast pulse frequency leads to higher receptor concentration, which is correlated to higher LHβ transcription. Therefore, at low pulse frequency, GnRHR couples to Gαq/11 and activates CamKII that induces both LHβ and FSHβ transcription. Activation of PKC additionally causes influx of extracellular calcium, which leads to sustained activation of ERK1/2 and preferential induction of FSHβ to a higher level than LHβ. At high pulse frequency, GnRHR number is augmented, and this high receptor number allows for coupling to both Gαs and Gαq/11, and while Gαq/11 desensitizes, sustained signaling via Gαs module induces higher expression of LHβ via unknown mechanism (Fig. 2).

3. GnRH regulation of LHβ and FSHβ Transcription

The specific gonadotropin β subunits are expressed at low basal levels and are differentially induced through pulses of GnRH (Bedecarrats and Kaiser, 2003; Haisenleder et al., 1991; Shupnik, 1996; Weiss et al., 1990). GnRH regulates expression of the specific β subunits through immediate-early genes. Early growth response 1 (EGR1) is an intermediary gene that regulates GnRH induction of LHβ, while activating protein 1 (AP1) transcription factor, comprised of a heterodimer of FOS and JUN isoforms, mediates induction of FSHβ by GnRH (Fig. 3).

3.1. GnRH regulation of LHβ transcription

Immediate-early gene that regulates LHβ induction by GnRH is EGR1 and is the only transcription factor binding the LHβ promoter that is dramatically induced by GnRH (Tremblay and Drouin, 1999). EGR1 is the most highly induced gene in the gonadotrope by GnRH in LβT2 model of mature gonadotrope when analyzed by microarrays (Kakar et al., 2003; Lawson et al., 2007; Wurmbach et al., 2001). EGR1 is also induced in rat primary pituitary cells following GnRH stimulation (Kucka et al., 2013). Confirming a role of EGR1 and lack of compensation by other family members (EGR2, EGR3, and EGR4), the EGR1 null mice are infertile and lack expression of the LHβ gene (Lee et al., 1996). EGR1 also

organizes a complex on the LHβ promoter to bring forth coordinated expression of this gene. In the proximal LHβ promoter region, two EGR1 sites are located adjacent to sites for steroidogenic factor 1 (SF1; or NR5A1) on either side of a homeodomain element (HD) bound by Ptx1 (Halvorson et al., 1996; Quirk et al., 2001). This location allows for a complex formation that integrates pituitary specific (SF1), basal (Ptx1) and GnRH-induced (EGR1) expression. Synergistic interaction between SF1, EGR1 and Ptx1 is essential for maximal LHβ gene expression (Dorn et al., 1999; Tremblay and Drouin, 1999). EGR1-organized complex interacts with the Sp1 sites in the distal region (Kaiser et al., 1998), in a spatially-dependent manner (Kaiser et al., 2000; Weck et al., 2000). Interaction is likely facilitated by the co-activators, SNURF (Curtin et al., 2004) and p300 (Mouillet et al., 2004). β-catenin may play a role in GnRH induction of LHβ via interaction with this complex through SF-1 (Gardner et al., 2007; Salisbury et al., 2007). However, recent studies addressing a role of β-catenin in the gonadotrope *in vivo* demonstrated a lack of any role for β-catenin in LHβ expression and LH levels in the circulation, in either gonadotrope specific deletion of β-catenin, or gonadotrope specific stabilization of β-catenin, which increases its levels in the cell (Boerboom et al., 2015).

Mechanisms involved in the responsiveness to pulsatile GnRH treatment and frequency-dependent expression of the LHβ gene have also been analyzed. Namely, maximal LHβ expression is achieved with high pulse frequency characteristic of pre-ovulatory period. This allows for a high level of LHβ gene expression, which then dimerizes with non-limiting α-subunit and during preovulatory surge of GnRH increases rapidly LH levels in the circulation that are necessary for ovulation. EGR1 intermediate gene which induces LHβ in response to GnRH, similarly to other immediate early genes, is not only rapidly activated, but is rapidly degraded as well. The short half-life allows for strong temporal regulation of gene targets. To address the importance of pulsatile GnRH treatment, it was postulated that proteasome function is critical for LHβ responsiveness to GnRH treatment, since GnRH leads to ubiquitination of EGR1 and cycling of this factor on and off of the LHβ promoter (Walsh and Shupnik, 2009). Cycling of EGR1 may explain the importance of pulsatile GnRH compared to tonic treatment that inhibits gonadotropin expression.

Explanation of higher induction with high pulse frequency of GnRH characteristic of the preovulatory proestrus period, may lie in the differential expression of intermediates. High pulse frequency upregulates SF1 mRNA in rat pituitary cells (Burger et al., 2011), which, although SF1 induction is much lower than of EGR1, combined with already high basal level, is sufficient to form a complex with highly induced EGR1 with any frequency. Another mechanisms is differential regulation of positive and negative regulators of transcription. Sustained induction of EGR1 specific repressor proteins from the Nab family at low pulse frequency may explain the necessity for high pulse frequency of GnRH for LHβ responsiveness (Lawson et al., 2007). High pulse frequency of GnRH may be necessary to allow for accumulation of EGR1 protein in order to overcome repression by Nabs.

As mentioned previously, β-subunits are expressed at a very low basal level which may indicate active repression until a signal is applied that would de-repress the promoter at the appropriate time. Repressors that bind inactive promoters normally function via recruitment of histone deacetylase (HDAC) enzymes that remove active histone acetylation marks and

prevent transcription. GnRH stimulus leads to changes in histone acetylation in the αT3 model of immature gonadotrope that normally do not express β-subunits (Lim et al., 2007). This may designate developmental activation of β-subunit transcription via chromatin remodeling, which occurs after gonadotropes acquire responsiveness to GnRH. Developmental and prepubertal regulation of gonadotropin subunits by GnRH is supported by a recent thorough study that demonstrated strong correlation and synchronization of GnRHR expression with gonadotropin β-subunit expression in female and in male rats (Bjelobaba et al., 2015). We recently demonstrated that GnRH stimulus elicits an increase in histone acetylation in mature LβT2 cells (Jonak et al., 2017), which may suggest that chromatin remodeling occurs with each pulse. Remodeling of chromatin that modulates gene expression occurs upon recruitment of the coactivator proteins to the DNA. EGR1 interacts with SF1 and Ptx1 and forms the active transcriptional complex that recruits coactivator CBP. Coactivator CBP has histone acetyltransferase activity (HAT), which acetylates histones and leads to activation of transcription (Korzus et al., 1998). CBP is indeed necessary for LHβ expression and normal fertility in vivo (Miller et al., 2012). This scenario points to rapid changes in repression and activation of the promoter via transcriptional repressors, chromatin remodeling and changes in histone acetylation (Fig. 3).

Signaling pathways triggered by GnRH receptor binding, discussed above, that activate gonadotropin genes transcription also induce transcription of immediate early genes. As demonstrated previously, GnRH induction of both gonadotropin β subunits is dependent on CamKII activation (Burger et al., 2008; Haisenleder et al., 2003a). We determined that GnRH induction of both EGR1 and cFOS immediate early genes occurs through an increase in intracellular calcium concentration and activation of CamKII. CamKII activity leads to phosphorylation of the SRF transcription factor (Ely et al., 2011). The EGR1 promoter contains four SRF binding sites (Witham et al., 2013), compared to the single site in the cFOS promoter that is responsive to GnRH (Ely et al., 2011). Higher induction of EGR1 than cFOS in response to GnRH may stem from four response elements in the promoter. Thus, this mechanism may be responsible for higher responsiveness of LHβ gene, a target of EGR1, to GnRH during the high pulse frequency preovulatory period, since at that time interpulse interval is not lengthy enough to allow immediate early gene turnover and protein degradation (Fig. 4).

3.2. GnRH regulation of FSHβ transcription

GnRH induces FSHβ by upregulating immediate early genes that belong to AP1 family of transcription factors, which subsequently bind the only site in the FSHβ promoter that conveys GnRH responsiveness (Coss et al., 2004). AP1 is a heterodimer of FOS and JUN transcription factors, which are activated rapidly and transiently by various stimuli and growth factors in numerous cell types (Karin et al., 1997). GnRH induces cFOS, FosB, cJUN and JunB but not JunD, and the combination of these factors bind the AP1 site in FSHβ promoter (Coss et al., 2004). Transcriptome analysis revealed that AP1 members are highly induced by GnRH in LβT2 cells (Wurmbach et al., 2001) and primary rat gonadotrope cells (Yuen et al., 2012). Similarly to EGR1, AP1 organizes a complex to integrate basal expression through interaction with NFY, and activin induction through interaction with activin-regulated SMAD3 and FOXL2 (Coss et al., 2007; Roybal et al.,

2014; Wang et al., 2008). cFOS deficient mice survive until early adulthood indicating possible compensation by FosB for survival, but are infertile, hypogonadotropic, and have similar ovarian phenotype as FSHβ knockouts, indicating necessity of cFOS for reproductive function (Xie et al., 2015). β-catenin may play a role in GnRH-induction of FSHβ expression, via its cofactor, breast cancer metastasis-suppressor 1-like (Brms1L, (Wang et al., 2013). In vivo studies using mice with gonadotrope specific knockdown of β-catenin, mice failed to show the role of β-catenin in FSHβ induction. On the other hand, male mice with β-catenin mutant that prevents its degradation and allows for accumulation in the gonadotrope, exhibit reduced FSHβ mRNA expression and diminished FSH levels due to an increase in pituitary follistatin expression (Boerboom et al., 2015).

Since AP1 family members are induced by a variety of stimuli in other cell types, but are sufficient for activation of FSHβ transcription, it is critical to understand specificity of GnRH induction of immediate early genes. Analysis of pathways and transcription factors activated solely by GnRH to induce cJUN in gonadotrope cells, provide critical insight into GnRH regulation of its target gene, the gonadotropin subunit FSHβ. We determined that GnRH is the sole regulator of cJUN in the mature LβT2 cells, which occurs through GnRH phosphorylation of ATF2 transcription factor by p38, the same pathway responsible for GnRH induction of cJUN and FSHβ in LβT2 cells (Bonfil et al., 2004; Lindaman et al., 2013). Of four p38 isoforms, p38β and p38δ are sufficient for cJUN induction, demonstrating additional specificity of signaling via this branch (Lindaman et al., 2013). On the contrary, FSHβ induction by GnRH in primary rat pituitary cells does not involve p38 (Haisenleder et al., 2008), likely because cJUN component of the AP1 complex is present at some basal level in the gonadotrope cells, which is sufficient for heterodimerization with tightly regulated and highly induced cFOS. To induce cFOS, GnRH uses neurotransmitter-like signaling and the CamKII pathway, rather than a growth factor signaling via MAPK. This provides the specificity of GnRH signaling to regulate FSHβ gene expression in the gonadotrope cells (Ely et al., 2011; Stojilkovic et al., 2010). The same signaling pathways are necessary for induction of both cFOS and FSHβ, and cFOS induction precedes that of FSHβ. As with EGR1 described above, GnRH induces cFOS primarily through an increase in intracellular calcium concentration and activation of CamKII, which then leads to phosphorylation of the SRF transcription factor. The necessity for CamKII in rat pituitary cultures for both LHβ and FSHβ (Haisenleder et al., 2003a), may stem from CamKII involvement in both EGR1 and cFOS induction (Ely et al., 2011; Witham et al., 2013). Although receptors for several growth factors that induce cFOS in other cell lines are expressed in the gonadotrope, these factors are not sufficient to induce cFOS, and therefore are not involved in the regulation of FSHβ, likely because they do not cause an increase in intracellular calcium concentration that is necessary to activate CamKII and though it SRF. Presence of the single SRF site in the cFOS promoter, compared to four in the EGR1 promoter, may explain lower responsiveness of the cFOS gene than the EGR1 gene to GnRH, either when induction was analyzed using a reporter (Ely et al., 2011) or endogenous mRNA level (Wurmbach et al., 2001). Downstream, since both EGR1 and cFOS are induced rapidly and transiently, it may lead to lower FSHβ, a target of cFOS, than LHβ, a target of EGR1, at high pulse frequency. However, induction of immediate early genes does not explain higher induction of FSHβ at low pulse frequency.

To answer dependence of FSHβ expression on pulsatile GnRH, several studies analyzed repressors that may explain frequency responsiveness of the FSHβ promoter. Skil and Tgif1 repressors that downregulate FSHβ induction are induced at the higher level with high pulse frequency, thus antagonizing cFOS (Mistry et al., 2011). Another repressor, ICER is also induced by GnRH at the high pulse frequency and binds to the AP1 site in the FSHβ promoter, thus repressing FSHβ expression at high pulse frequency (Ciccone et al., 2010). Importance of either Skil, Tgif1 or ICER has not been examined in vivo. We recently identified Jun-dimerizing protein 2 (JDP2) as another repressor that binds the same AP1 site and interacts with cJUN thereby displacing cFOS (Jonak et al., 2017). JDP2 belongs to the AP1 superfamily since it contains homologous DNA binding domain and the leucine zipper through which it heterodimerizes with cJUN, but lacks a transactivation domain to form a transcriptionally active complex (Aronheim et al., 1997). JDP2 is expressed at the high basal level and is not highly induced transcriptionally, but its repressive function may be regulated by phosphorylation (Katz et al., 2001). However, frequency dependence of JPD2 phosphorylation status remains to be explored. Consistent with a JDP2 role in FSHβ repression in the cells, female mice deficient in JDP2 exhibit higher expression of FSHβ, augmented FSH concentration and consequently higher estrogen in the circulation, and increased recruitment of ovarian follicles to the growing pool. JDP2 nulls also exhibit early puberty, increased litter size and cessation of reproductive function at younger age, implicating higher FSHβ expression to premature reproductive senescence (Jonak et al., 2017). Cessations of reproduction 70 days earlier in JDP2 null mouse is proportional to 8-year earlier menopause in women over respective reproductive lifespans. A complete lack of gonadotropins results in infertility in affected individuals. However, larger number of individuals have pathologies such as POF, with hormone levels slightly outside the physiological range. Since 70% of POF causes are idiopathic, repressors may provide candidates for investigation in affected individuals.

Repressors may underlie the lower levels of FSHβ at high pulse frequency, but these findings still do not explain higher levels of FSHβ than LHβ at low pulse frequency. We uncovered a mechanism that may function during a period of low GnRH pulse frequency to extend the half-life of the cFOS protein specifically, allowing sufficient accumulation of this factor to increase transcription of its target gene FSHβ (Reddy et al., 2013). This leads to selective induction of FSH and its secondary rise, which is necessary for follicular growth. GnRH induces rapidly both EGR1 and cFOS, but specifically decreases the rate of cFOS degradation, by triggering cFOS protein phosphorylation through the ERK1/2 pathway (Fig. 4). Role of ERK1/2 in GnRH induction of FSHβ that has been shown in rat primary pituitary cells (Haisenleder et al., 1998), may stem not from direct role of ERK1/2 on FSHβ transcription, but from increased half-life of the cFOS protein than induces FSHβ (Reddy et al., 2013). Preferential activation of ERK1/2 in vivo in GnRH treated rats with low pulse frequency, also confirms its role in FSHβ specifically (Burger et al., 2008). Phosphorylation extends the half-life of cFOS protein, which is normally rapidly degraded. Confirming the role of phosphorylation in promoting increased protein activity, cFOS mutant which cannot be phosphorylated by GnRH has a shorter half-life and induces lower expression of the FHSβ promoter than wild-type, phosphorylatable cFOS (Reddy et al., 2013). Our study stressed the role of post-translational modification in the regulation of gonadotropin gene

expression by GnRH. This modification may cause higher levels of FSH during the time of low GnRH pulse frequency to stimulate folliculogenesis.

4. Crosstalk of GnRH with other hormones that regulate gonadotropins

Another possible mechanism of differential regulation is interaction of GnRH signaling pathways and other hormones that regulate gonadotropin levels. Gonadal steroid hormones regulate expression of gonadotropin β subunits and pituitary responsiveness to GnRH, by modulating the levels of induction by GnRH-induced signaling. Pituitary responsiveness to GnRH is regulated by paracrine factors expressed in the pituitary itself.

4.1. Gonadal hormones

Inhibin and activin are members of TGFβ superfamily. Regulation of FSH secretion is their initially identified and thus most studied role (Makanji et al., 2014). Inhibin decreases FSH secretion, while activin stimulates it. Gonads are the primary source of circulating inhibin, since after gonadectomy inhibin levels are undetectable. Activin, although originally purified from follicular fluid, has low circulating level and primarily functions in the paracrine or autocrine manner. Thus, activin role in gonadotropin synthesis will be discussed below. Inhibin is a heterodimer of α and β subunits, whereas activin is ββ homodimers. Thus, they share β subunits and availability of α subunit and the preferential assembly of αβ heterodimers over ββ dimers may be one mechanism of inhibin to antagonize activin. More likely, functional antagonism of activin signaling by inhibin is achieved through the competitive binding to the common receptor on the cell surface via the shared β subunit. Subsequently, inhibin binding prevents recruitment of activin signaling receptor, but instead recruits betaglycan, which prevents signaling (Chapman and Woodruff, 2003). Therefore, primary role of inhibin in FSH synthesis is to antagonize autocrine activin function.

Although gonadal steroid hormone feedback primarily targets the hypothalamus, modulation of pituitary responsiveness by steroid hormones has been reported. Estrogen likely does not directly regulate FSHβ gene expression, but induction of LHβ gene expression by estrogen in gonadotropes has been reported (Shupnik et al., 1989a; Shupnik et al., 1989b). The mechanism may involve either ER binding to an imperfect estrogen response element in the distal rat LHβ promoter (Shupnik and Rosenzweig, 1991; Shupnik et al., 1989b); or ER recruitment to the LHβ promoter through interactions with SF1 and Ptx1 (Luo et al., 2005). Estrogen has been shown to potentiate GnRH induction of LHβ indirectly by enhancing the expression of EGR1 and suppressing the expression of ZEB, a transcriptional repressor that binds the rat LHβ promoter (Kowase et al., 2007).

Progesterone and testosterone decrease LHβ expression and increase FSHβ induction (Thackray et al., 2009; Thackray et al., 2006; Thackray and Mellon, 2008). Testosterone and other androgens have been reported to repress LHβ gene expression, in both castrated, GnRH antagonist-treated rats (Burger et al., 2004; Wierman and Wang, 1990) and primary pituitary cell culture (Winters et al., 1992). Androgens also reduced GnRH-stimulated LH section via alterations of calcium signaling (Ortmann et al., 1998). Androgen treatment suppressed GnRH-induced transcription of the rat and ovine LHβ promoters, through a

direct protein-protein interaction between the androgen receptor (AR) and Sp1 (Curtin et al., 2001), or between AR and SF1 (Jorgensen and Nilson, 2001), respectively.

Both basal and GnRH-induced FSHβ mRNA levels increased in rats in vivo and in primary cells treated with progesterone (Attardi and Fitzgerald, 1990; Kerrigan et al., 1993). Progesterone antagonists blocked FSH secretion and FSHβ expression during the preovulatory FSH surge (Ringstrom et al., 1997) and the secondary FSH rise (Knox and Schwartz, 1992; Szabo et al., 1998). Like progesterone, androgens upregulate FSHβ mRNA levels. Castrated GnRH antagonist-treated rats exhibited a selective increase in FSHβ mRNA upon treatment with testosterone (Burger et al., 2004; Dalkin et al., 1992; Paul et al., 1990; Wierman and Wang, 1990). Suppression of LHβ and induction of FSHβ by progesterone and androgens may contribute to differential regulation of β-subunits.

4.2. Autocrine and paracrine regulation by pituitary hormones

Gonadotropes comprise 5–15% of the cells in the anterior pituitary (Ooi et al., 2004). Anterior pituitary contains four other types of secretory cells including thyrotropes, somatotropes, lactotropes and corticotropes, as well as folliculostellate cells. Many hormones secreted from these cells have paracrine effects on gonadotrope cells (reviewed in (Denef, 2008). Folliculostellate cells have been reported to rapidly overgrow other pituitary cells in culture (Kawakami et al., 2002) and can impact the experimental outcome since they produce paracrine factors such as follistatin and PACAP (reviewed in (Winters and Moore, 2007, 2011).

PACAP was initially identified as hypothalamic factor that increases pituitary adenylate cyclase signaling as its name suggests. PACAP is also secreted in the pituitary by gonadotropes and folliculostelate cells and works in the autocrine or paracrine manner to modulate response to GnRH (Counis et al., 2007). GnRH increases the expression of PACAP receptor in the gonadotrope and in turn, PACAP potentiates the effect of GnRH on β-subunit expression likely via cAMP pathway crosstalk (Purwana et al., 2010). Some inconsistencies that exist in the literature regarding a role of PACAP in the gonadotrope, maybe due to the level or PACAP receptor expression in different models (Fowkes et al., 2003b). Alternatively, they can possibly be explained by crosstalk of pathways, since with cotreatment the pathway may be augmented, while pretreatment may lead to downregulation and inability to respond to a subsequent signal (Fowkes et al., 2003a; Larivière et al., 2008). The fact that pretreatment with either PACAP of GnRH diminishes the effect of the other, can likely be explained by downregulation. This hypothesis is strengthened by a study in which transgenic male mice with permanently elevated PACAP levels have delayed puberty and decreased gonadotropin levels (Moore et al., 2012).

Prolactin from the lactotropes diminishes pituitary responsiveness to GnRH by decreasing LH secretion (De Paul et al., 2000; Tortonese et al., 1998). Lactotropes, which comprise 20% of pituitary cells, form a honey-comb pattern in the anterior pituitary surrounding the gonadotropes, allowing for a close contact and paracrine function by prolactin (Noda et al., 2001). Gonadotropes express prolactin receptor (Tortonese et al., 1998) and prolactin has been shown to diminish the LH secretion and expression in response to GnRH, with no effect on basal levels (Garcia et al., 1985; Hodson et al., 2012; Smith, 1982).

Follistatin is a functional antagonist of TGFβ superfamily that binds activin member of the family with the highest activity (Makanji et al., 2014). It binds activin at the several residues that are necessary for activin binding to its receptor, thus inhibiting its function. Follistatin, synthesized by folliculostellate cells and gonadotropes themselves, represses FSH synthesis, likely by antagonizing endogenous, autocrine activin (Bilezikjian et al., 2004). Follistatin also diminishes GnRH induction of the FSHβ gene (Coss et al., 2007), again likely due to synergistic effect of autocrine activin with GnRH as discussed below.

Activin stimulates secretion of FSH and activates FSHβ transcription. Activin is synthesized by gonadotropes themselves and functions in the autocrine manner, while inhibin and follistatin, as discussed above, regulate FSH primarily by antagonizing activin. The acute GnRH-independent increase in FSHβ transcription after ovariectomy is due to a lack of inhibin to antagonize autocrine activin (Burger et al., 2001). This is true as well in younger women with decreased ovarian function or menopausal women that experience an increase primarily in FSH, as discussed as the beginning of this manuscript. Since FSH is mostly constitutively secreted, transcriptional regulation may be a critical regulatory step of FSH levels by activin. Activin functions via SMAD transcription factors and induces FSHβ transcription via SMAD3 phosphorylation. Activin-regulated SMAD3 and 4 are necessary for FSHβ expression in vivo (Fortin et al., 2014a; Fortin et al., 2014b). Bernard lab (Lamba et al., 2009) and our lab (Corpuz et al., 2010) identified FOXL2 as a critical component in activin induction of the FSHβ gene. FOXL2 overexpression in gonadotropes can potentiate activin induction and its expression can confer activin responsiveness to FSHβ in heterologous cells where this promoter is normally refractory to activin induction. Mice deficient in FOXL2, both whole body knockout or gonadotrope-specific knockdown, have lower FSHβ level (Justice et al., 2011; Roybal et al., 2014; Tran et al., 2013)). Various FOXL2 mutations have been identified in patients with an autosomal dominant disorder, BPES, which is characterized by distinctive eyelid abnormalities and POF (Crisponi et al., 2001; De Baere et al., 2001; De Baere et al., 2002). Considering that POF is caused by mutations of the FOXL2 gene, which induces FSHβ, and that this reproductive defect is associated with higher FSH levels in the circulation, it is intriguing to postulate again a role of FSH in the development of POF.

Activin also increases pituitary responsiveness to GnRH (Coss et al., 2007; Coss et al., 2010; Fortin et al., 2015; Gregory and Kaiser, 2004). Cotreatment of activin and GnRH results in the synergistic induction of FSHβ. p38 appears to be a point of crosstalk between different signaling pathways since it is more highly activated after GnRH and activin co-treatment of LβT2 cells than GnRH treatment alone (Coss et al., 2007; Zhang et al., 2006). Synergy is accomplished through a complex formation of GnRH-induced AP1 and activin-regulated SMAD3. We showed that FOXL2, with activin-phosphorylated SMAD3, also plays a role in synergistic induction of FSHβ by GnRH and activin through interactions with the cJUN component of the AP1 complex that is necessary for GnRH responsiveness (Roybal et al., 2014). Another member of TGFβ family, GDF9, previously considered specifically expressed in oocytes is expressed in primary pituitary cultures and LβT2 cells. GDF9 is implicated in induction of FSHβ and synergism with GnRH, which may also contribute to differential expression (Choi et al., 2014). GDF9 also activates SMAD transcription factors, but whether the same complex plays a role in synergism with GnRH remains to be

determined. The synergistic induction is specific for FSHβ gene and may contribute to the differential expression of the gonadotropin subunits. It may occur when ovarian inhibin levels decrease, allowing activin, or GDF9, to synergize with GnRH, at a time coinciding with the secondary peak of FSH, which is necessary for follicular development (Woodruff et al., 1996).

5. Conclusion

GnRH differentially regulates gonadotropin β-subunits expression, the limiting components of the mature hormone. Each pulse of GnRH induces a new wave of transcription of immediate early genes, EGR1 that leads to induction of LHβ, and cFOS (and more stable, but less regulated, cJUN) that leads to induction of FSHβ (Fig. 3 and Fig. 4). Both EGR1 and cFOS have labile mRNAs and equally unstable proteins, exhibiting rapid turnover and degradation to allow for a tight temporal regulation of gene targets. With increasing pulse frequencies of GnRH in the preovulatory period, EGR1 and cFOS accumulate to overcome repressors (Nabs and JDP2 for example, respectively) and allow expression of their respective targets. Enabled by EGR1 and cFOS complex organizing facilities, expression of β-subunits is augmented. Due to four response elements in its promoter, EGR1 is induced at the higher level than cFOS leading to higher induction of LHβ than FSHβ with fast pulse frequency. Responsiveness to GnRH fast pulse frequency is likely maintained by increased receptors concentration that allows for an engagement of Gαs that is less susceptible to desensitization, in addition to Gαq/11. Both LHβ and FSHβ heterodimerize with non-limiting αGSU and are secreted under pulsatile GnRH, increasing their concentration in the circulation. During the low pulse frequency, specific phosphorylation of the cFOS protein via ERK1/2 extends its half-life, while EGR1 is degraded during each interpulse interval preventing accumulation. Under the endogenous activin tone, activin-regulated factors, such as SMAD3 and FOXL2, interact with cFOS/AP1 complex to synergistically induce FSHβ. FSHβ dimerizes with abundant αGSU to form a mature hormone that is subsequently constitutively secreted to stimulate folliculogenesis. This mechanism may explain preferential induction of FSHβ during the low pulse frequency of GnRH.

The conundrum of how a single hormone, GnRH, interacting with a single receptor can differentially activate gonadotropin β-subunit genes in the same cell has not been answered completely, but significant progress has been made in our understanding of molecular mechanisms that regulate differential expression. Given that pathologies such as PCOS or POF may arise due to aberrations in the narrowly controlled levels, further insight is crucial to our understanding of regulation of reproduction at the level of the gonadotrope. Future studies may lead to the development of potential therapeutics for pathologies caused by dysregulation of the tight differential regulation.

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Abbreviations

AP1	activator protein 1
AR	androgen receptor
CamKII	calcium calmodulin kinase II
ChIP	chromatin immunoprecipitation
DAG	diacyl glycerol
EGR1	early growth response 1
ER	estrogen receptor
ERK	extracellular signal regulated kinase
FSH	follicle-stimulating hormone
GWAS	genome-wide association study
GSU	glycoprotein subunit
GnRH	gonadotropin-releasing hormone
HD	homeodomain element
IP3	inositol triphosphate
JNK	Jun N-terminal kinase
LH	luteinizing hormone
HPG	hypothalamic-pituitary-gonadal
MAPK	mitogen-activated protein kinase
MKP	MAP kinase phosphatase
PACAP	pituitary adenylate cyclase-activating polypeptide
PCOS	polycystic ovary syndrome
POF	premature ovarian failure
POI	primary ovarian insufficiency
PKC	protein kinase C
SBE	smad-binding element
SRF	serum response factor
SF1	steroidogenic factor 1

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Highlights

1. Gonadotropin hormone levels are regulated by variations in GnRH pulse frequency
2. GnRH-induced transcription of their unique β-subunits controls gonadotropin levels
3. GnRH signaling cross-talks with gonadal and pituitary factors to regulate β-subunits
4. GnRH induces LHβ via induction of EGR1 factor and FSHβ via induction of cFOS and cJUN
5. Signaling pathways and factors respond differently to varied GnRH pulse frequencies

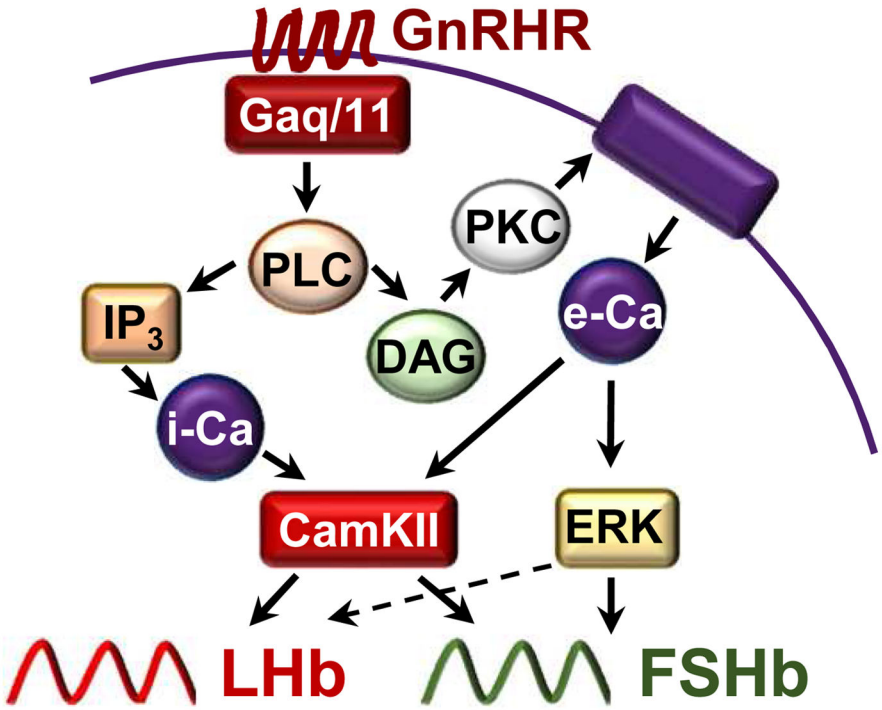

Figure 1. GnRH signaling pathways

Following binding of GnRH, GnRH receptor (GnRHR) primarily couples to Gαq/11 and activates PLC signaling that leads to formation of inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 causes an increase in the intracellular calcium (i-Ca) by mobilization from intracellular stores, while DAG activates PKC, which in turn leads to ERK1/2 MAPK activation. Calcium influx (e-Ca) occurs through voltage-gated calcium channels (VGCC), whose activation may involve PKC. Increase in intracellular calcium from either source activates CamKII, which is necessary for induction of both LHβ and FSHβ. Sustained increase of extracellular calcium causes higher activation of ERK1/2 and preferentially increase of FSHβ.

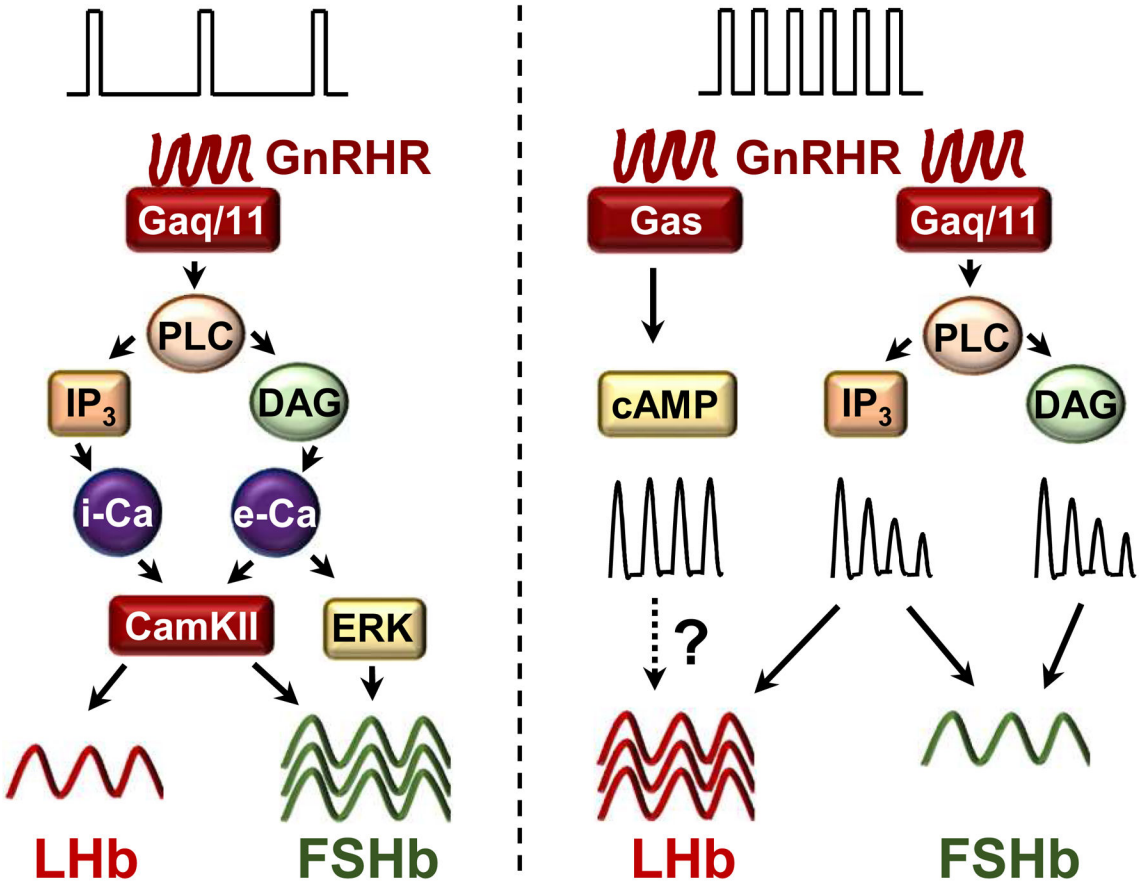

Figure 2. GnRH signaling pathways that differentially regulate LHβ and FSHβ

A - Left, During low pulse frequency of GnRH, classical GnRH signaling, as described in Figure 1, activates both LHβ and FSHβ transcription. Higher activity of ERK1/2, likely caused by sustained influx of extracellular calcium, activates specifically FSHβ and contributes to higher levels of FSHβ during low pulse frequency. **B - Right,** During high pulse frequency of GnRH in proestrus, GnRHR engages Gαs in addition to Gαq/11. This may occur due to increased receptor concentration that is correlated with higher LHβ induction. Gαs is necessary for maximal induction of LHβ via unknown mechanisms, indicated with a question mark. Gαs signaling module is resistant to desensitization, while both IP3 receptors and DAG formation in the Gαq/11 signaling module desensitize with high pulse frequency. This leads to higher LHβ levels than FSHβ with high pulse frequency of GnRH.

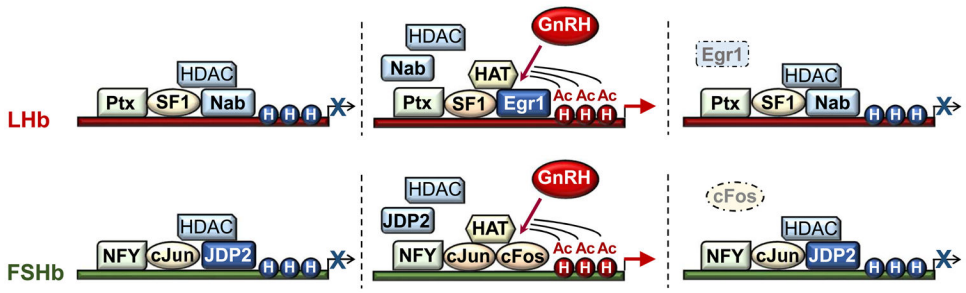

Figure 3. Transcriptional regulation of β-subunits by GnRH

GnRH activates transcription of gonadotropin β-subunits via intermediates, immediate early genes; EGR1 that induces LHβ (top) and cFOS that induces FSHβ following heterodimerization with cJUN that forms the AP1 transcription factor (bottom). These GnRH-induced genes organize complexes on their respective target gene promoters to integrate basal (Ptx1 for LHβ and NFY for FSHβ) and gonadotrope-specific factors (SF1). Under basal conditions (left panel), β subunit expression remains low due to repressors, Nab for LHβ, and JDP2 or ICER for FSHβ that recruit histone deacetylases (HDAC). Following EGR1 and cFOS induction by GnRH (middle panel), they replace Nab and JDP2 respectively, to induce β-subunit expression. Formation of the active transcriptional complex recruits histone acetyltransferase (HAT) to remodel histones (H). EGR1 and cFOS have very short half-lives and are rapidly degraded. This allows repressor recruitment and removal of active histone marks in the promoter until the following pulse of GnRH (right panel).

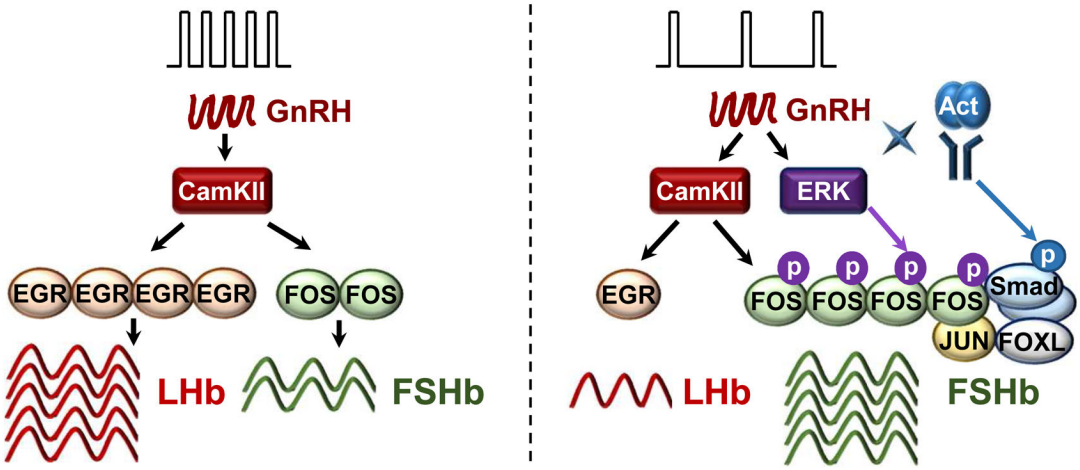

Figure 4. Differential regulation of β-subunits expression by GnRH

A – Left, During fast pulse frequency of GnRH in proestrus, EGR1 and cFOS accumulate to overcome repressors. Both EGR1 and cFOS induction by GnRH is dependent on CamKII activation of SRF transcription factor. Since the EGR1 promoter has four SRF response elements, compared to one in the cFOS promoter, EGR1 is more highly induced than cFOS, which leads to higher induction of LHβ than FSHβ during high pulse frequency of GnRH. **B - Right,** During low pulse frequency of GnRH, EGR1 is degraded throughout the interpulse interval due to its short half-life. GnRH signaling via ERK1/2 specifically phosphorylates cFOS protein (indicated with “p”) and increases its half-life, which allows accumulation of cFOS. GnRH and activin (Act) synergistically induce FSHβ, indicated with “x”. GnRH-induced FOS/AP1 complex interacts with activin-regulated SMAD3/SMAD4/FOXL2 complex, which leads to synergistic induction of FSHβ and increase of FSH in the circulation during low pulse frequency of GnRH.