HHS Public Access

Author manuscript

Mol Cell Endocrinol. Author manuscript; available in PMC 2019 March 05.

Published in final edited form as:

Mol Cell Endocrinol. 2018 March 05; 463: 131–141. doi:10.1016/j.mce.2017.10.015.

Gonadotropin regulation by pulsatile GnRH: signaling and gene expression

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Abstract

The precise orchestration of hormonal regulation at all levels of the hypothalamic-pituitary-gonadal axis is essential for normal reproductive function and fertility. The pulsatile secretion of hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by pituitary gonadotropes. GnRH acts by binding to its high affinity seven-transmembrane receptor (GnRHR) on the cell surface of anterior pituitary gonadotropes. Different signaling cascades and transcriptional mechanisms are activated, depending on the variation in GnRH pulse frequency, to stimulate the synthesis and release of FSH and LH. While changes in GnRH pulse frequency may explain some of the differential regulation of FSH and LH, other factors, such as activin, inhibin and sex steroids, also contribute to gonadotropin production. In this review, we focus on the transcriptional regulation of the gonadotropin subunit genes and the signaling pathways activated by pulsatile GnRH.

Keywords

Gonadotropin-releasing hormone; follicle-stimulating hormone; luteinizing hormone; G proteins; GnRH receptor; signal transduction

1. Introduction

Regulation of the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), is essential for normal mammalian sexual maturation and reproductive function (Marshall and Kelch, 1986). FSH and LH secretion from the gonadotrope is controlled primarily by the hypothalamic decapeptide, GnRH (Belchetz, Plant, Nakai et al., 1978). GnRH is synthesized in hypothalamic neurons and is secreted into the hypophyseal portal circulation to act primarily on the anterior pituitary. It binds to its G protein-coupled receptor, the gonadotropin-releasing hormone receptor (GnRHR), on the

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cell surface of a specific pituitary cell type, the gonadotrope cells, initiating downstream signaling that induces the production of these gonadotropins (Kaiser, Conn and Chin, 1997). LH and FSH, in turn, enter the peripheral circulation, acting at the ovaries and testes to regulate folliculogenesis, ovulation, spermatogenesis and steroidogenesis (Burger, Haisenleder, Dalkin et al., 2004). GnRH is released in a pulsatile manner and variations in GnRH pulse frequencies and amplitudes have differential effects on FSH and LH synthesis and release (Knobil, 1980, Savoy-Moore and Swartz, 1987, Wildt, Hausler, Marshall et al., 1981). FSH is preferentially stimulated at low GnRH pulse frequencies, whereas LH is preferentially stimulated at high GnRH pulse frequencies.

FSH and LH, are glycoprotein dimers that are comprised of two subunits, a common α-subunit (αGSU) and a distinct β-subunit (FSHβ or LHβ, respectively), which determines the biological specificity of the gonadotropins (Ciccone and Kaiser, 2009,Gharib, Wierman, Shupnik et al., 1990). The expression of the gonadotropin subunit genes is also dependent on GnRH pulse frequency. In rat models, *Fshb* gene expression is preferentially stimulated at low GnRH pulse frequencies (maximal at an interval of every 120 minutes) (Haisenleder, Dalkin, Ortolano et al., 1991, Kaiser, Jakubowiak, Steinberger et al., 1997, Dalkin, Haisenleder, Ortolano et al., 1989). Conversely, *Lhb* gene expression is preferentially stimulated at higher GnRH pulse frequencies (maximal at an interval of every 30 minutes) (Burger, Haisenleder, Aylor et al., 2008, Haisenleder, Burger, Walsh et al., 2008). Expression of *Cga*, the gene encoding αGSU, is stimulated by both pulsatile and continuous GnRH, with less frequency dependence (Weiss, Duca and Crowley, 1990, Ferris and Shupnik, 2006, Bedecarrats and Kaiser, 2003). The control of FSH and LH synthesis is closely correlated with the expression of the distinct β-subunits.

Many reproductive disorders are associated with disruption of GnRH, FSH, and/or LH signaling pathways (Seminara, Hayes and Crowley, 1998). For instance, persistently rapid GnRH pulses, which result in an increased LH:FSH ratio to contribute to excessive ovarian androgen production and ovulatory dysfunction, have been observed in polycystic ovarian syndrome (PCOS), a common disorder that affects 6%-10% of the female population of reproductive age (McCartney and Marshall, 2016, Dumesic, Oberfield, Stener-Victorin et al., 2015). This syndrome is also associated with cardiometabolic abnormalities (Baldani, Skrgatic and Ougouag, 2015), obesity, and impaired glucose tolerance (Wild, Carmina, Diamanti-Kandarakis et al., 2010, Blank, McCartney, Helm et al., 2007). Conversely, low GnRH pulse frequencies and abnormal serum gonadotropin levels are associated with hypothalamic amenorrhea (Reame, Sauder, Case et al., 1985, Marshall, Eagleson and McCartney, 2001). These examples highlight the importance of the proper functioning of HPG axis and of the differential control of FSH and LH secretion for normal reproductive function.

There are several excellent recent reviews of the signaling pathways activated by GnRH in the gonadotrope and of the regulation of gonadotropin subunit gene expression, but only a few focus on the differential regulation by GnRH pulse frequency (Thompson and Kaiser, 2014, Mugami, Kravchook, Rahamim-Ben Navi et al., 2017, Thackray, Mellon and Coss, 2010, Naor and Huhtaniemi, 2013, Coss, 2017, Stojilkovic, Bjelobaba and Zemkova, 2017). Several hypotheses regarding how gonadotropes decode patterns of pulsatile GnRH to

differentially regulate FSH and LH production have been proposed. However, the exact mechanisms remain to be fully elucidated. This review focuses on the signaling pathways activated by different GnRH pulse frequencies to result in differential regulation of gonadotropin subunit gene expression. Studies using pulsatile GnRH in an effort to more closely emulate physiological responses of the pituitary form the basis of this review.

2. Experimental Models for Studying Gonadotrope Function

Several experimental models have been used to study the hormonal regulation of the gonadotropes, including primary pituitary cell culture, *in vivo* animal models and immortalized cell culture.

Primary cultures of mixed pituitary cells generated by dispersion of fresh pituitary tissue are frequently employed. However, several limitations of these models need to be mentioned. First, the anterior pituitary gland is a heterogeneous population of secretory cells including gonadotropes, thyrotropes, somatotropes, lactotropes and corticotropes, as well as folliculostellate cells, which each secrete distinct hormones. Gonadotropes represent only 10-15% of the adenohypophyseal cell populations (Ooi, Tawadros and Escalona, 2004). Second, the gonadotropes and lactotropes are densely represented near the intermediate lobe in several species, suggesting a possible paracrine relationship (Bliss, Navratil, Xie et al., 2010, Denef, 2008). Moreover, folliculostellate cells can affect experimental outcomes as they produce paracrine factors, such as follistatin and PACAP (Thackray et al., 2010, Kawakami, Fujii, Okada et al., 2002, Winters and Moore, 2007), which can modulate gonadotrope responses. These paracrine relationships may be disrupted in dispersed pituitary cultures. Third, it is important to consider the endocrine environment at the time of pituitary harvest, such as the estrous stage of female mice, as these conditions may affect experimental results (Fallest and Schwartz, 1991).

To overcome these limitations, investigators have developed novel strategies for identifying and purifying gonadotropes from transgenic mouse models. For example, FSH-producing gonadotropes have been tagged *in vivo* with a transgenic cell surface antigen (H-2K^k) so that they can be purified *in vitro* by immunologically-based cell enrichment using H-2K^k-specific antibodies (Wu, Su, Safwat et al., 2004). As another strategy, researchers used a gene targeted approach to express yellow fluorescent protein (YFP) in gonadotropes and thereby help in their visualization and identification. Mice were generated which co-express Cre-recombinase with GnRHR (GnRHR-IRES-Cre, or GRIC, mice), thereby directing Cre-mediated expression of a YFP reporter allele specifically in the gonadotropes of these-in mice (Wen, Schwarz, Niculescu et al., 2008). Nonetheless, some limitations persist, such as difficulty in acquiring sufficient yields of purified gonadotropes to perform detailed characterization studies.

Many *in vivo* animal models have been used to study the synthesis, secretion and action of gonadotropins, including gonadectomized rats (Haisenleder et al., 1991, Dalkin et al., 1989) and gain- and loss-of-function mouse models (Kumar, 2016). Mice with gonadotrope-specific deletion of genes encoding transcription factors, such as cFos and steroidogenic factor 1 (SF-1) (Xie, Jonak, Kauffman et al., 2015, Zhao, Bakke, Krimkevich et al., 2001,

Zhao, Bakke and Parker, 2001, Tran, Zhou, Lafleur et al., 2013), or of other factors that are involved in the regulation of *Fshb* and *Lhb* transcription, such as ERK1/2 (Bliss, Miller, Navratil et al., 2009), have been developed. These animal models have provided the opportunity to study the effects of blocking specific signaling pathways *in vivo*.

The development of two murine gonadotrope-derived cell lines, αT3-1 and LβT2 cells, by the Mellon laboratory greatly benefited the studies of the hormonal signaling mechanisms that mediate expression of the gonadotropin genes. These immortalized cell lines were developed through targeted expression of SV40 T-antigen (Alarid, Windle, Whyte et al., 1996, Alarid, Holley, Hayakawa et al., 1998). αT3-1 cells represent immature gonadotropes and express only limited gonadotrope-associated proteins, including *Cga*, *Gnrhr*, and *Nr5a1* (encoding SF-1), but lack expression of *Fshb* and *Lhb* (Windle, Weiner and Mellon, 1990). In contrast, LβT2 cells are characterized by a more mature gonadotropic phenotype, as they express *Lhb* and *Fshb* and secrete LH and FSH in response to hormonal stimulation (Graham, Nusser and Low, 1999, Pernasetti, Vasilyev, Rosenberg et al., 2001, Turgeon, Kimura, Waring et al., 1996). These characteristics validate the LβT2 cell line as a representative model for studying gonadotrope physiology. LβT2 cells also express activin, follistatin, and inhibin as well as their receptors, and receptors for steroid hormones (Thackray, McGillivray and Mellon, 2006, Takeda, Otsuka, Otani et al., 2007, Lewis, Gray, Blount et al., 2000). Studies conducted with LβT2 cells and other gonadotrope-derived cell lines may lack the effects of paracrine factors produced by other pituitary cells. In addition, although LβT2 cells represent more mature gonadotropes than the αT3-1 cell line, they differ slightly from primary gonadotropes in their profile of expressed genes (Yuen, Choi, Pincas et al., 2012). However, the great majority of the regulated genes were the same. The observed differences in the expressed genes may be due to species differences, or may be the result of differences in experimental design, microarray used, sensitivity, or due to differences in paracrine factors.

Despite these limitations, LβT2 cells currently represent the most widely used *in vitro* cellular model for studying gonadotrope signaling. Since LβT2 cells have become a popular model for studying gonadotropin synthesis, the majority of data presented in this review are based on studies that used these immortalized gonadotropic cells.

3. The GnRH Receptor (structural and functional aspects)

The GnRHR is a member of the G protein-coupled receptor family (GPCRs), characterized by seven transmembrane domains linked by extracellular and intracellular loops (Stojilkovic, Reinhart and Catt, 1994, Re, Pampillo, Savard et al., 2010). GPCRs represent the largest and most complex group of integral membrane protein receptors in the human genome (Huang and Tesmer, 2011). They are classically divided into 3 main classes: class A Rhodopsin related receptors, class B Secretin and Adhesion related receptors, class C Glutamate related receptors and the Taste2, Frizzled and Vomeronasal related receptors (Naor and Huhtaniemi, 2013, Sharman, Mpamhanga, Spedding et al., 2011). The GnRHR belongs to the Rhodopsin family of GPCRs (Tsutsumi, Zhou, Millar et al., 1992).

In vertebrates, three cognate receptors or receptor-like sequences have been identified with distinct distributions and functions (Millar, 2005, Neill, Duck, Sellers et al., 2001). Only two types of GnRHR occur in mammals, though (Morgan and Millar, 2004). The mammalian type I GnRHR shares over 80% amino acid identity amongst rat, human, sheep and cow and pig (Millar, Lu, Pawson et al., 2004). The type II GnRHR is fully functional in monkeys, pigs and dogs, but absent in mice, sheep, and cows, as well as silenced in human and chimpanzee genomes (Millar, 2003, Hapgood, Sadie, van Biljon et al., 2005). The type I receptor is the type of receptor that is functional and predominant in the mammalian gonadotrope (in this review the term “GnRHR” refers to type I GnRHR). In some species, including humans, it is also expressed in reproductive tissues (e.g. breast, endometrium, ovary, prostate) and in tumors derived from these tissues (Cheung and Wong, 2008, Perrett and McArdle, 2013).

What is structurally unique in the mammalian GnRHR, compared to other GPCRs, is the lack of an intracellular cytoplasmic carboxyl-terminal tail (Finch, Green, Hislop et al., 2004, Davidson, Wakefield and Millar, 1994). The C-terminal tail normally plays a key role in rapid desensitization and receptor internalization (Ferguson, 2001). It is an important phosphorylation target of the GPCR kinases (GRKs). GRK-mediated phosphorylation generates a docking site for β-arrestin scaffolding proteins, which upon binding mediate rapid desensitization through uncoupling from G proteins and dynamin-dependent internalization of the receptor (Bliss et al., 2010, Perrett and McArdle, 2013, Bockaert, Marin, Dumuis et al., 2003). It has been demonstrated experimentally that the mammalian GnRHR is resistant to rapid desensitization upon GnRH stimulation and instead undergoes relatively slow internalization (Vrecl, Heding, Hanyaloglu et al., 2000, Finch, Caunt, Armstrong et al., 2009, Pawson, Faccenda, Maudsley et al., 2008). Furthermore, experiments fusing the C-terminal tail of various GPCRs to the mammalian GnRHR demonstrated rapid desensitization and internalization, like other members of the GPCR family (Heding, Vrecl, Bogerd et al., 1998, Hanyaloglu, Vrecl, Kroeger et al., 2001). Therefore, these results together establish that the absence of the C-terminal tail in the mammalian GnRHR accounts for its resistance to rapid desensitization and ligand-induced internalization, and the absence of recruitment of β-arrestin (McArdle, Davidson and Willars, 1999, Millar, Pawson, Morgan et al., 2008).

The atypical lack of GnRHR desensitization, compared to the other members of the GPCR family, suggests that different mechanisms occur that potentially modulate the cellular response to pulsatile GnRH, such as changes in receptor abundance or changes in the signaling pathways that are activated upon binding GnRH to its receptor. Several factors are believed to affect *Gnrhr* gene expression based on in vitro studies, such as gonadal steroids, activins and inhibins, but the most notable factor is GnRH itself (Nathwani, Kang, Cheng et al., 2000, Gregory and Kaiser, 2004). It has been demonstrated in rat pituitary cultures that *Gnrhr* gene expression is dependent on GnRH pulse frequency. *Gnrhr* mRNA levels are significantly increased at all pulse frequencies compared to untreated controls, with greater stimulation observed under conditions of high pulse frequency (e.g., every 30 minutes) (Kaiser et al., 1997). In addition, it has been shown that cell-surface GnRHR number in LβT2 cells follows similar pattern, with greater increases in number at higher frequencies of pulsatile GnRH (Bedecarrats and Kaiser, 2003). It has been shown that GnRH-regulated

Gnrhr expression is PKC-dependent and involves the MAPK signaling pathway (Bjelobaba, Janjic, Tavcar et al., 2016). Furthermore, at high cell surface densities of GnRHRs, *Lhb* expression is optimally stimulated, whereas *Fshb* expression is preferentially stimulated at lower cellular densities of the receptor (Kaiser, Sabbagh, Katzenellenbogen et al., 1995). These studies support a model in which the number of cell surface GnRHRs plays a critical role in the differential responses to various GnRH pulse frequencies.

4. Signaling Pathways Activated by Pulsatile GnRH

In the pituitary, GnRH acts by binding to the G protein-coupled GnRHR on the cell surface of the gonadotrope, inducing interaction of the receptor with heterotrimeric G proteins and catalyzing GTP-GDP exchange on the G protein α subunit (Lambert, 2008, Oldham and Hamm, 2008). Four Gα subfamilies have been identified in the mammalian genome: $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_{i/o}$ (Simon, Strathmann and Gautam, 1991). It is well established that the GnRHR interacts with Gα proteins to activate a variety of distinct signaling pathways. However, questions remain about precisely which G proteins are involved in GnRHR signaling in the gonadotrope, how each G protein contributes to signaling, and how the GnRH pulses are decoded to activate signaling cascades that preferentially induce FSH or LH production.

Initially, even before gonadotrope-derived cell lines became available, it was suggested that the GnRHR couples to a pertussis toxin-insensitive G protein (Gp, later renamed as Gq), based on studies performed in cultured rat pituitary cells (Naor, Azrad, Limor et al., 1986). Subsequently, studies in αT3-1, CHO-K1 and COS-7 cells enhanced the hypothesis that the GnRHR initiates signaling pathway(s) by coupling exclusively to $G\alpha_{q/11}$ (Hsieh and Martin, 1992, Grosse, Schmid, Schoneberg et al., 2000). On the other hand, studies in LβT2 cells indicated that both $G\alpha_{q/11}$ and $G\alpha_s$ were involved in GnRHR signaling (Liu, Usui, Evans et al., 2002). In addition, studies from the same laboratory noticed a differential desensitization of $G\alpha_s$ and $G\alpha_{q/11}$ in response to pulsatile GnRH stimulation (Tsutsumi, Mistry and Webster, 2010). In a more recent study, the expression and role of individual G proteins were investigated in LβT2 cells using siRNA and bacterial toxins. These studies indicated that GnRH signaling in gonadotrope cells involved primarily $G\alpha_{q/11}$ and $G\alpha_s$ (and to a lesser extent $G\alpha_{12/13}$ as well) and, more interestingly, that their depletion differentially affected GnRH-stimulated gonadotropin gene expression (Choi, Jia, Pfeffer et al., 2012). $G\alpha_{q/11}$ knockdown reduced GnRH-stimulated *Fshb* mRNA levels, while $G\alpha_s$ knockdown reduced GnRH-stimulated *Lhb* mRNA levels. However, these results were not entirely consistent with the effects on the promoter activity of *Fshb* and *Lhb*. Although the $G\alpha_{i/o}$ subfamily is expressed in LβT2 cells, it was not found to be involved in regulation of gonadotropin gene expression (Choi et al., 2012, Krsmanovic, Mores, Navarro et al., 2003).

Therefore, it appears that the GnRHR can activate multiple Gα subfamilies, a factor to be taken into consideration when studying the signaling pathways activated by GnRH. Several signaling pathways are activated and may be preferentially regulated by different GnRH pulse frequencies. In this review, we will focus primarily on protein kinase C/MAPK, calcium-calmodulin dependent kinases, calcineurin/NFAT, and cAMP/PKA pathways.

4.1 Protein kinase C/MAPK pathways

After the activation of $G\alpha_{q/11}$ proteins by the GnRHR, stimulation of phospholipase Cβ (PLCβ) occurs. PLCβ, in turn, cleaves phosphatidylinositol-4-5-bisphosphate (PIP_2) into inositol trisphosphate (IP3) and diacylglycerol (DAG) (Naor, 2009). IP3 stimulates Ca^{2+} release from endoplasmic reticulum stores into the cytosol, whereas DAG activates protein kinase C (PKC) (Tsutsumi and Webster, 2009). Some PKC isoforms (PKCα, βI, βII, γ) are activated by Ca^{2+} as well (Kishimoto, Mikawa, Hashimoto et al., 1989). Many investigators have shown that GnRH induces increased intracellular calcium levels and PKC activation, which can activate several mitogen-activated protein kinase (MAPK) cascades in gonadotropes (Bliss et al., 2010, Naor, 2009, Caunt, Finch, Sedgley et al., 2006). The MAPKs can translocate to the nucleus and activate several transcription factors (Murphy and Blenis, 2006).

Activation of the family members MAPK1/3 (extracellular signal-regulated kinase, ERK1/2), MAPK8/9 (c-Jun N-terminal kinase, JNK1/2) and MAPK14 (p38-α) has been reported to mediate GnRH-induced gonadotropin subunit transcription (Burger et al., 2004, Haisenleder et al., 2008, Naor and Huhtaniemi, 2013, Kanasaki, Purwana, Oride et al., 2012, Ando, Hew and Urano, 2001, Bernard, Fortin, Wang et al., 2010). For the purposes of this review, these family members will be referred to as ERK, JNK and p38, respectively. The ERK pathway is comprised of MAPK kinase kinase (Raf1), MAPK kinases (MEK1 and MEK2) and MAPKs (ERK1 and ERK2) (Pearson, Robinson, Beers Gibson et al., 2001). However, a study of mice with pituitary-targeted deletion of Raf1 demonstrated that Raf1 may not be mandatory for ERK signaling in gonadotropes (Bliss, Navratil, Xie et al., 2012). Moreover, it has also been shown that ERK1/2 activation can be promoted via a pathway that includes c-Src and Ras as well (Kanasaki et al., 2012). On the other hand, GnRH stimulates JNK and P38 pathways via activation of c-Src and Rac1/Cdc42 and subsequent activation of MAP4K (Harris, Bonfil, D et al., 2002). These molecules in turn activate MAPK kinases, MKK4/7 and MKK3/6, which subsequently activate JNK and p38, respectively (Naor, 2009, Pearson et al., 2001). These MAPK cascades, especially the ERK pathway, have been implicated in regulating *Fshb* and *Lhb* promoter activity in response to pulsatile GnRH (Kanasaki, Bedecarrats, Kam et al., 2005).

Studies in both perifused LβT2 cells and primary gonadotropes demonstrated more rapid onset and sustained patterns of ERK1/2 phosphorylation following stimulation by low rather than high GnRH pulse frequencies (Kanasaki et al., 2005, Haisenleder, Cox, Parsons et al., 1998). In addition, higher levels of nuclear phosphorylated ERK have been reported following low GnRH pulse frequency stimulation. The different patterns of ERK activation/inactivation in response to various GnRH pulse frequencies imply that ERK phosphorylation may be important for GnRH pulse frequency-dependent differential stimulation of *Fshb* and *Lhb* expression (Kanasaki et al., 2005). Many previous studies demonstrated the importance of the ERK-dependent transcription factor, early growth response-1 protein (Egr1), for *Lhb* expression, supporting this hypothesis (Dorn, Ou, Svaren et al., 1999, Fortin, Lamba, Wang et al., 2009, Lawson, Tsutsumi, Zhang et al., 2007, Lee, Sadovsky, Swirnoff et al., 1996, Wolfe and Call, 1999, Halvorson, Kaiser and Chin, 1999). In contrast, the role of ERK signaling in mediating the GnRH-induced *Fshb* expression is more controversial, although

ERK phosphorylation follows a pattern similar to that of *Fshb* expression. Studies using pharmacological inhibition of MEK1, the kinase that activates ERK1/2, demonstrated inhibition of GnRH-induced ovine, murine, rat and human *Fshb/FSHB* promoter activities in LβT2 cells (Burger et al., 2008, Bernard et al., 2010, Kanasaki et al., 2005, Bonfil, Chuderland, Kraus et al., 2004, Coss, Hand, Yaphockun et al., 2007, Coss, Jacobs, Bender et al., 2004). In addition, studies in perifused male rat primary pituitary cultures demonstrated that MEK1 inhibition attenuated GnRH-stimulated *Fshb* expression (Haisenleder et al., 1998). More recently, our group demonstrated that stimulation of both *Fshb* and *Lhb* gene expression by pulsatile GnRH was markedly attenuated by MEK1/2 inhibition at both high and low GnRH pulse frequencies (Kanasaki et al., 2005, Thompson, Ciccone, Zhou et al., 2016). Together, these data suggest a potential role of ERK1/2 in GnRH induction of both *Lhb* and *Fshb* gene expression. However, *in vivo* studies with gonadotrope-specific ERK1/2 double knock-out mice showed only partial impairment in *Fshb* expression and FSH secretion, with more marked effects on *Lhb* expression and secretion (Bliss et al., 2009). These data imply that ERK1/2 may be more important for LH than for FSH regulation *in vivo*, or that other GnRH-activated signaling pathways compensate for the loss of ERK1/2 function in these animals.

The distinct pattern of ERK phosphorylation in response to different GnRH pulse frequencies suggests a possible role of MAPK phosphatases (MKPs) as a mediator of this differential activity. MKPs are phosphatases that inactivate MAPKs through dephosphorylation of threonine and tyrosine residues (Kanasaki et al., 2012). Activation of ERK and JNK in pituitary gonadotropes promotes MKP1 and MKP2 (DUSP1 and DUSP4 respectively) activity, which then effectively inactivate ERK, JNK, and p38 (Zhang, Mulvaney and Roberson, 2001, Kondoh and Nishida, 2007, Franklin and Kraft, 1997). Studies in αT3-1 cells and *in vivo* in mice demonstrated an increase in MKP1 and MKP2 expression in response to GnRH (Zhang and Roberson, 2006). Furthermore, studies performed in perifused LβT2 cells showed increased MKP1 expression after high rather than low GnRH pulse frequency (Nguyen, Intriago, Upadhyay et al., 2010, Purwana, Kanasaki, Mijiddorj et al., 2011). This regulation pattern is distinct compared to ERK1/2 activation, which is more rapidly induced and more sustained at low frequency GnRH pulses (Kanasaki et al., 2005). Interestingly, knockdown of MKP1 in LβT2 cells promoted activation of ERK and *Lhb* expression by GnRH, while overexpression of MKP1 attenuated ERK and *Lhb* promoter activation (Nguyen et al., 2010).

Collectively these studies suggest a potential role of MKPs in a negative feedback loop to control MAPK activity in response to different GnRH pulse frequencies. Nevertheless, a study by Armstrong et al. using live cell imaging to track ERK2-GFP translocation in heterologous HeLa cells argues against this potential role. Using pharmacological and molecular genetic approaches to suppress MKP activity, they demonstrated little or no effect on the rapid and transient translocation of ERK2-GFP to the nucleus after pulsatile stimulation with GnRH (Armstrong, Caunt, Fowkes et al., 2010).

4.2 Calcium/Calmodulin-dependent kinase II (CaMK II) pathway

Binding of GnRH to the GnRHR initiates several intracellular signaling cascades, including calcium influx via L-type voltage-gated calcium channels and IP3-induced mobilization of calcium from intracellular stores (Duran-Pasten and Fiordelisio, 2013). The GnRH-induced rapid increase in intracellular calcium is essential for rapid gonadotropin secretion and for gonadotropin subunit gene expression (Naor, 2009). Previous studies have shown that blockade of Ca^{+2} channels prevents the increase in *Cga, Lhb* and *Fshb* expression in rat pituitary cells (Burger et al., 2004) and in *Lhb* promoter activity in LβT2 cells (Weck, Fallest, Pitt et al., 1998). Interestingly, perifusion studies in rat primary cells with a calcium channel agonist revealed a pulse frequency-dependent differential regulation of subunit gene expression similar to that induced by pulsatile GnRH. High frequency pulses preferentially stimulated *Lhb* while low frequency pulses favored *Fshb* (Haisenleder, Yasin and Marshall, 1997, Haisenleder, Workman, Burger et al., 2001).

The downstream mediators of calcium action within the gonadotrope have not been fully elucidated. Elevation of intracellular calcium levels is required for MAPK activation and for activation of certain PKC isoforms (Mugami et al., 2017, Roberson, Bliss, Xie et al., 2005). However, calcium also signals via the calcium/calmodulin-dependent kinase II (CaMK II) pathway (Roberson et al., 2005, Melamed, Savulescu, Lim et al., 2012). CaMK II, which is a common mediator of calcium signaling and pulse frequency decoder in many cell types, requires two molecules of calcium-bound calmodulin (Ca-CALM1) to bind to the enzyme in order to be activated (Ferris and Shupnik, 2006). The higher the frequency of Ca^{2+} influxes, the higher the percentage of CaMK II activated, likely because there is less time for Ca-CALM1 to dissociate from CaMK II while awaiting a second Ca-CALM1 molecule to bind (De Koninck and Schulman, 1998). In addition, it has been demonstrated that a single GnRH pulse can induce a rapid and transient increase in CaMK II activation in both primary pituitary and LβT2 cells. Another interesting finding is that inhibition of CaMK II inhibits *Cga, Lhb* and *Fshb* expression (Haisenleder, Burger, Aylor et al., 2003, Haisenleder, Ferris and Shupnik, 2003), suggesting a potential role of CaMK II in decoding GnRH pulse frequency within the gonadotrope.

On the other hand, it was reported that activation of CaMK II is upregulated by GnRH but not in a frequency-dependent manner (Burger et al., 2008). This observation does not exclude the potential role of CaMK II, but indicates that it is not the only mechanism involved. The rapid inactivation kinetics of CaMK II implies that extended CaMK II activity may occur preferentially at high GnRH pulse frequency and hence result in greater *Lhb* than *Fshb* expression (Thompson and Kaiser, 2014).

4.3 Calcineurin/NFAT pathway

In addition to CaMK II, another calcium/calmodulin target is the serine/threonine protein phosphatase, Calcineurin (Natarajan, Ness, Wooge et al., 1991, Lim, Luo, Koh et al., 2007). It has been demonstrated that Calcineurin is involved in the GnRH-induced derepression of FSHβ gene expression in αT3-1 gonadotrope cells, possibly by activation of Nur77 (Lim et al., 2007). More recently, the nuclear factor of activated T cells (NFAT), which is activated by Calcineurin, has been reported to be regulated by GnRH in αT3-1, LβT2 and HeLa cells

(Gardner, Maudsley, Millar et al., 2007, Armstrong, Caunt, Fowkes et al., 2009, Pnueli, Luo, Wang et al., 2011). This is of interest, as NFAT is a known transcriptional activator of Nur77 (Pnueli et al., 2011), indicating a possible role of Calcineurin/NFAT pathway in GnRH-induced *Fshb* expression. A study using a Calcineurin-specific inhibitor and siRNA for NFAT demonstrated that this signaling cascade is also necessary for the transcriptional regulation of the immediate early genes, *Jun*, *Fos* and *Atf3*, which are important for GnRH-regulated expression of *Cga* and *Fshb* (Binder, Grammer, Herndon et al., 2012). In contrast, induction of *Lhb* transcription by GnRH, which is dependent on Egr1 (Wolfe and Call, 1999), remained unaffected by changes in Calcineurin activation (Binder et al., 2012).

In addition, another study using the nuclear translocation of an emerald fluorescent protein-tagged NFAT (NFAT-EFP) as a downstream readout for the activation of the Calcineurin/NFAT pathway demonstrated translocation into the nucleus upon pulsatile GnRH stimulation in a dose- and frequency-dependent manner (Armstrong et al., 2009). The effect was reversible, but much slower in onset and in reversibility compared to ERK2-GFP (Armstrong et al., 2010, Armstrong et al., 2009). Pulsatile GnRH also caused a pulse frequency dependent activation of *Cga-, Lhb-, Fshb*-luciferase reporters, which was attenuated by the Calcineurin inhibitor, cyclosporine A. New mathematical models of GnRH signaling, which accurately reflect these experimental observations, demonstrate that the NFAT and ERK pathways can mediate genuine frequency decoding when they act in a cooperative manner at the transcriptome (Tsaneva-Atanasova, Mina, Caunt et al., 2012, Pratap, Garner, Voliotis et al., 2016).

4.4 cAMP/PKA/CREB pathway

The GnRHR can also activate the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/cAMP response element binding protein (CREB) pathway (Thompson and Kaiser, 2014). GnRH-stimulated cAMP production has been reported in rat pituitary cells, in LβT2 cells, and in many heterologous cell lines, including HeLa, GH3 and COS-7 cells (Perrett and McArdle, 2013, Cohen-Tannoudji, Avet, Garrel et al., 2012). The coupling mechanism between the GnRHR and the cAMP pathway has been studied extensively. It has been shown in LβT2 cells that GnRH stimulation can activate cAMP production via $G\alpha_s$ recruitment (Liu et al., 2002). In support of this mechanism, a recent study using cAMP and calcium biosensors in αT3-1 cells has demonstrated that GnRH increases cAMP production and that the GnRHR interacts with the proto-oncogene SET, which inhibits receptor coupling to the calcium signaling pathway and increases coupling to the cAMP pathway. Its action is probably mediated by preventing $G\alpha_{q/11}$ binding to the GnRHR (Avet, Garrel, Denoyelle et al., 2013). On the other hand, the same group showed that the PKC δ and ε isoforms can mediate cAMP production via activation of adenylate cyclase 5 and 7 in LβT2 cells, indicating that this pathway can also be activated via $G\alpha_{q/11}$ (Lariviere, Garrel, Simon et al., 2007). Several studies have demonstrated GnRH stimulation of PKA in rat pituitary cells and in LβT2 cells as well (Garrel, Simon, Thieulant et al., 2010, Grafer, Thomas, Lambrakos et al., 2009).

The majority of these studies, however, have used tonic GnRH treatment to activate signaling. Studies using pulsatile GnRH, which are closer to the physiological response of

the pituitary, are more limited. The Webster laboratory, using cAMP and PKA-dependent FRET reporters, monitored the dynamics of responses in LβT2 cells after pulsatile GnRH treatment. They demonstrated that pulses of GnRH, at both high and low frequencies, can cause pulses of cAMP elevation and PKA activation, which are rapid and transient with no obvious desensitization to subsequent pulses (Tsutsumi et al., 2010). Our group, using PKA inhibitors and measuring *Fshb* mRNA levels, *Fshb*-luciferase activity, and CREB phosphorylation, has shown that PKA activity and CREB phosphorylation are significantly increased to the greatest extent by low GnRH pulse frequencies. Moreover, after using a PKA inhibitor, both CREB phosphorylation and *Fshb* expression, but not *Lhb* expression, were attenuated at both high and low GnRH pulse frequencies, implicating PKA as an important mediator of GnRH stimulation of FSHβ production (Thompson, Ciccone, Xu et al., 2013). Despite minor differences, both of these studies support a role for the cAMP/PKA pathway in decoding pulsatile GnRH inputs.

5. Regulation of Gonadotropin Gene Expression by Pulsatile GnRH

The signaling pathways described previously affect *Cga*, *Fshb* and *Lhb* gene expression. These genes are differentially regulated by varying GnRH pulse frequencies, although the α-subunit is produced in excess, regardless of GnRH pulse frequency (Haisenleder et al., 1991, Dalkin et al., 1989). Therefore, the control of FSH and LH synthesis is closely correlated with the production of the distinct β-subunits. This section of this review will focus on transcription factors that are activated by pulsatile GnRH and mediate *Fshb* and *Lhb* expression.

5.1 *Lhb*

The structure of the *Lhb* gene promoter has been characterized to a great extent, providing much information about *Lhb* gene expression following GnRH stimulation. The proximal *Lhb* promoter region contains two binding sites for the immediate early gene protein Egr1, two binding sites for steroidogenic factor 1 (SF-1) and a homeodomain element, which is a binding site for Pitx1/Otx1 (Halvorson, Kaiser and Chin, 1996, Quirk, Lozada, Keri et al., 2001). The interactions among Egr1, SF-1 and Pitx1/Otx1 are required for GnRH induction of the promoter (Dorn et al., 1999, Tremblay and Drouin, 1999). The distal *Lhb* promoter region, which is necessary for full GnRH induction of the promoter, contains binding sites for Sp1 and NFY and an overlapping CArG box (Kaiser, Sabbagh, Chen et al., 1998, Weck, Anderson, Jenkins et al., 2000). The transcriptional co-activators SNURF and p300 connect the distal and proximal GnRH response regions of the *Lhb* promoter and may enhance Egr1 action (Mouillet, Sonnenberg-Hirche, Yan et al., 2004, Curtin, Ferris, Hakli et al., 2004).

Egr1 is a key factor regulating *Lhb* expression after pulsatile GnRH stimulation (Dorn et al., 1999, Fortin et al., 2009, Lawson et al., 2007). *In vivo* studies with Egr1 deficient mice demonstrated reduced expression of the *Lhb* gene and infertility (Lee et al., 1996, Topilko, Schneider-Maunoury, Levi et al., 1998). Pituitary-specific SF-1 knock-out mice are infertile as well and demonstrated markedly decreased LH levels, but treatment with high doses of GnRH induced *Lhb* expression (Zhao et al., 2001). Studies in perifused LβT2 cells demonstrated that *Egr1* expression is stimulated to a greater extent at high GnRH pulse

frequency, consistent with increased *Lhb* transcription at high GnRH pulse frequency (Kanasaki et al., 2005). In contrast, Nab1/2 (Ngfi-A binding proteins), corepressors of the Egr family, are preferentially stimulated at low GnRH pulse frequency (Lawson et al., 2007). The differential regulation of responses of Egr1 and Nab1/2 to varying frequencies of pulsatile GnRH suggest that regulation of Egr1 synthesis and bioavailability may play a role in the differential regulation of the *Lhb* gene following pulsatile GnRH stimulation (Tsutsumi and Webster, 2009) (Figure 1).

5.2 *Fshb*

In contrast to the *Lhb* gene promoter, the *Fshb* gene promoter has been less well characterized, due in part to the lack of ideal cellular models for study. The development of LβT2 cells, which produce both LHβ and FSHβ, provided a tool for the study of the *Fshb* gene promoter and hence a greater understanding of the gonadotropin regulation. Our group (Ciccone, Lacza, Hou et al., 2008) and others (Coss et al., 2004, Wang, Fortin, Lamba et al., 2008) have identified a GnRH-responsive element in the proximal *Fshb* promoter region, which is highly conserved in humans (Wang et al., 2008) and contains a partial cAMP response element (CRE)/AP1 site. We have demonstrated that the binding of USF1 and USF2 transcription factors mediate basal rat *Fshb* transcription, whereas GnRH stimulates CREB phosphorylation, which in turn binds to the homologous CRE/AP-1 half site and recruits the histone acetyltransferase CREB-binding protein (CBP) to the promoter (Ciccone et al., 2008). Several studies have demonstrated that GnRH-induced activation of MAPKs, including ERK1/2, JNK and p38, results in activation of members of the AP1 family, such as cFos, cJun and ATFs (Coss et al., 2004, Liu, Austin, Mellon et al., 2002, Xie, Bliss, Nett et al., 2005). The role of AP1 transcription factors in the stimulation of *Fshb* expression by GnRH is controversial. Miller's laboratory has extensively studied the role of AP1 sites in GnRH-induced *FSHB* transcription in vitro and in vivo. In vitro experiments demonstrated that GnRH signals through the AP1 sites to increase ovine *FSHB* transcription in gonadotropes (Strahl, Huang, Pedersen et al., 1997, Strahl, Huang, Sebastian et al., 1998). On the other hand, in vivo studies using mice called into question the importance of these AP1 sites in the GnRH regulation of the ovine *FSHB* promoter (Huang, Sebastian, Strahl et al., 2001).

Our group further explored the role of CREB in perifusion studies in vitro. It was demonstrated in LβT2 cells that the preferential *Fshb* expression at low GnRH pulse frequencies is dependent on CREB binding to the CRE/AP1 site (Ciccone, Xu, Lacza et al., 2010). In addition, we showed that CREB phosphorylation and PKA activity are increased to a greater extent at low GnRH pulse frequencies (Figure 2). Interestingly, PKA inhibition attenuated both CREB phosphorylation and *Fshb* expression, indicating that CREB phosphorylation is mediated by PKA (Thompson et al., 2013).

Mutation of this GnRH responsive element within the *Fshb* promoter resulted in loss of preferential *Fshb* expression at low GnRH pulse frequencies (Ciccone et al., 2010), leading to the hypothesis that activation of repressors of CREB and AP1 transcription factors at high GnRH pulse frequencies attenuates *Fshb* expression. The inducible cAMP early repressor (ICER) was identified as a potential repressor of *Fshb* expression. ICER expression and

synthesis are preferentially stimulated at high GnRH pulse frequencies, and ICER attenuates GnRH stimulation of *Fshb* expression by antagonizing the binding of CREB to the *Fshb* CRE site (Ciccone et al., 2010) (Figure 2). Our group demonstrated that induction of ICER by pulsatile GnRH is regulated by ERK1/2 (Thompson et al., 2016). Inhibition of *ICER* by inhibition of ERK1/2 activation did not augment the induction of *Fshb* expression at high GnRH pulse frequencies, however, likely because MEK1/2 inhibition attenuated *cFos* and *cJun* induction as well (Thompson et al., 2016). This observation suggests that stimulation of *Fshb* expression by pulsatile GnRH is in part driven by activation of AP1 transcription factors, which bind to the corresponding CRE/AP1 element (Coss et al., 2004). Interestingly, a study of *cFos* deficient mice showed markedly reduced *Fshb, Lhb, Cga and Gnrhr* mRNA levels, which did not recover after GnRH treatment (Xie et al., 2015). Thus, it is not clear if the reduction of *Fshb* expression is due to a direct effect of cFos on the *Fshb* promoter or due to a decrease in GnRHR levels. More recently, cJun dimerization protein 2 (Jdp2) has been identified as a novel repressor of GnRH-mediated *Fshb* induction. Studies in LβT2 cells and in Jdp2 null mice demonstrated that Jdp2 serves as negative regulator, directly by binding to the *Fshb* promoter to form a complex with cJun, thereby preventing cJun-cFos dimerization, and indirectly by reducing cJun expression (Jonak, Lainez, Roybal et al., 2017).

As mentioned, AP1 proteins are induced by GnRH. Mistry et al. demonstrated that cFos and cJun protein expression is increased at both high and low GnRH pulse frequencies, but to a greater extent at high GnRH pulse frequencies (Mistry, Tsutsumi, Fernandez et al., 2011). This is not consistent with the preferential *Fshb* expression at low GnRH pulse frequencies. However, other negative effectors of *Fshb* expression, SKIL and TGIF1, are also induced at high GnRH pulse frequencies. These repressors have been shown to bind to the *Fshb* gene promoter to inhibit any potential action of cFos and cJun (Mistry et al., 2011).

The signaling pathways activated to induce CREB, ICER, and AP1 proteins to decode GnRH pulse frequency and regulate FSH synthesis are not yet fully elucidated. In addition, it needs to be taken into consideration that many other factors contribute to the regulation of *Fshb* expression, such as activin, follistatin, steroids, VGF-derived peptide NERP1 and epigenetic modifications (Choi, Wang, Jia et al., 2016).

6. Conclusions

Normal reproductive function and fertility require the precise regulation of LH and FSH. Identifying the molecular mechanisms that regulate gonadotropin synthesis may help us to better understand the ovulatory and menstrual cycles, puberty, and even the pathophysiology of reproductive disorders such as polycystic ovarian syndrome. Several signaling pathways have been implicated in both LH and FSH synthesis. It appears that the GnRHR differentially activates multiple distinct signaling pathways in response to varying GnRH pulse frequencies. Considerable progress has been made during the past decade, but much more remains to be elucidated to fully understand the enigma of the GnRH pulse frequency decoder. More *in vivo* studies are necessary to confirm and refine results obtained in immortalized gonadotropic cell lines. *In vivo* studies using mice with gonadotrope-specific deletion of $G\alpha_s$ or $G\alpha_{q/11}$ will help to further elucidate the role of each G protein in

gonadotropin regulation. Furthermore, additional studies of the GnRHR are needed to understand the differential G protein coupling of the GnRHR upon pulsatile GnRH stimulation. In addition, other factors regulating gonadotropins, including inhibin, activin, follistatin, sex steroids, and other neuropeptides, as well as epigenetic contributions to regulation, need to be integrated into this model in order to fully understand the regulation of gonadotropins in health and disease.

Acknowledgments

We thank Dr. Rona Carroll for her contribution to the preparation of the manuscript and Dr. Iosif Pediaditakis for his contribution to the preparation of the figures. This work was supported by NIH R01 HD019938 to U.B.K.

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Highlights

1. Experimental Models for Studying Gonadotrope Function
2. The GnRH Receptor (Structural and Functional Aspects)
3. Signaling Pathways Activated by Pulsatile GnRH
4. Regulation of Gonadotropin Gene Expression by Pulsatile GnRH

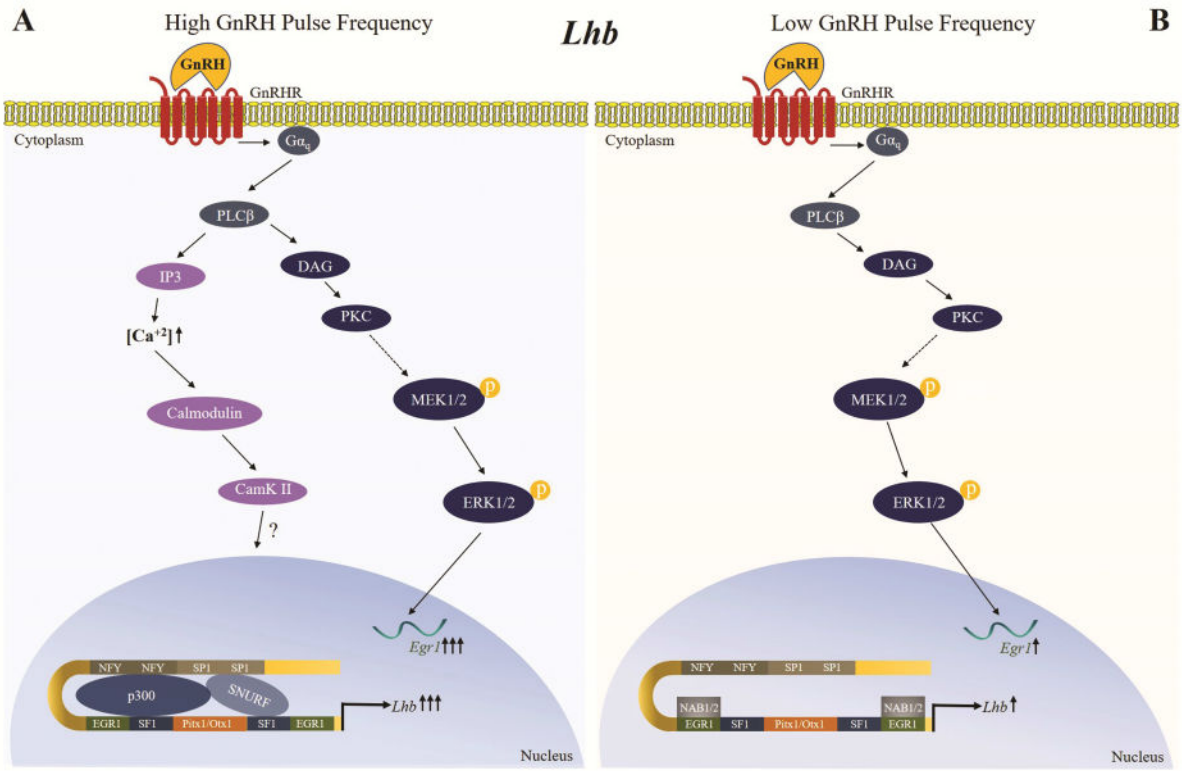

Figure 1. Proposed model of regulation of *Lhb* transcription by pulsatile GnRH

High and low GnRH pulse frequencies stimulate different signaling pathways to mediate the synthesis of transcription factors and repressors controlling *Lhb* transcription. **A)** At a high GnRH pulse frequency, the PKC/MAPK and Calcium/Calmodulin-dependent kinase II (CaMK II) pathways are activated, causing an increase in *Egr1* expression. Egr1 in turn increases activity of the *Lhb* promoter. **B)** At a low GnRH pulse frequency, the PKC/MAPK pathway is activated but the transient increase in *Egr1* expression is insufficient for maximal activation of the *Lhb* promoter, an effect further antagonized by increased expression of Nab1/2, corepressors of the Egr family, which are preferentially stimulated at low GnRH pulse frequency.

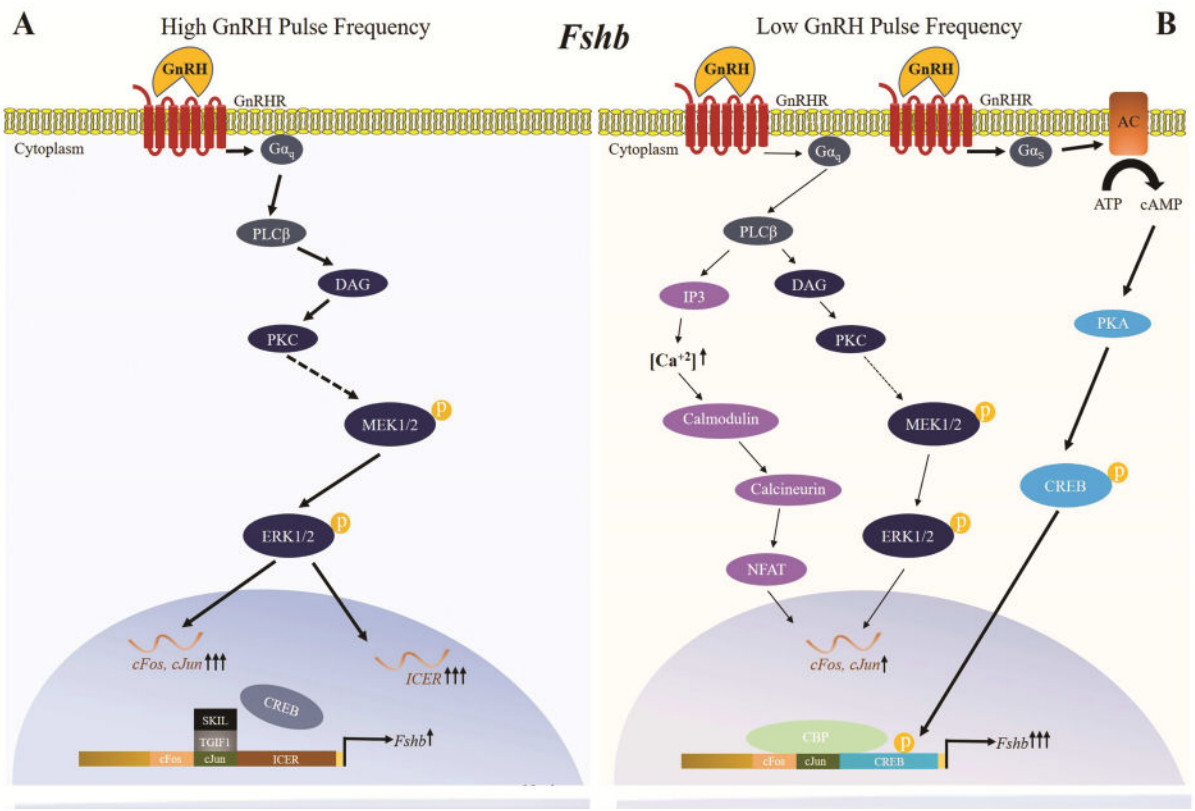

Figure 2. Proposed model of regulation of *Fshb* transcription by pulsatile GnRH

High and low GnRH pulse frequencies stimulate different signaling pathways that mediate the synthesis of transcription factors and repressors controlling *Fshb* transcription. **A)** At a high GnRH pulse frequency, the GnRHR preferentially couples to $G\alpha_{q/11}$ proteins, activating the PKC/MAPK pathway. This pathway stimulates ICER expression and synthesis. ICER attenuates GnRH stimulation of *Fshb* expression by antagonizing the binding of CREB to the *Fshb* CRE-AP1 site. SKIL and TGIF1 are also induced at high GnRH pulse frequencies and also bind to the *Fshb* gene promoter to prevent any potential action of cFos and cJun. **B)** At a low GnRH pulse frequency, the GnRHR preferentially couples to $G\alpha_s$ protein, activating the cAMP/PKA/CREB pathway (indicated by bold arrows). The phosphorylated CREB protein binds to the homologous CRE-AP1 site of the *Fshb* promoter and recruits the histone acetyltransferase CREB-binding protein (CBP), stimulating *Fshb* transcription. PKC/MAPK and Calcineurin/NFAT pathways are also activated, but to a lesser extent. They contribute to induction of *Fshb* transcription by stimulating cFos and cJun expression.