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Review

Gonadotropin-releasing hormone regulated transcription of gonadotropin subunit genes

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Summary. Two gonadotropins, luteinizing hormone and follicle-stimulating hormone, are synthetized and secreted by anterior pituitary gonadotropes and act on the gonads, controlling gametogenesis and sex hormone production. These hormones are glycoprotein polypeptides, composed of specific beta subunits and a common, alpha subunit. Both transcription and secretion of gonadotropins are regulated by gonadotropin-releasing hormone (GnRH), which is produced by a small number of hypothalamic neurons within the preoptic area and mediobasal hypothalamus. GnRH is released and reaches the pituitary in pulses, a pattern of secretion that is crucial for proper reproductive functions. This mini review covers mechanisms of transcriptional control of gonadotropin subunit genes by GnRH, predominantly focusing on *in vivo* experiments with mice and rats and *in vitro* experiments using primary pituitary cell cultures and immortalized pituitary cell lines derived from these species. We also provide an overview of the promoter regions of gonadotropin genes and major transcription factors involved in GnRH-driven expression of gonadotropin subunit genes.

Keywords: alpha polypeptide gene (*Cga*), Follicle-stimulating hormone beta subunit gene (*Fshb*), Gene transcription, Glycoprotein hormones, Gonadotrophs, Gonadotropin-releasing hormone (GnRH), Luteinizing hormone beta subunit gene (*Lhb*), Pituitary.

INTRODUCTION

The two principal pituitary gonadotropins in vertebrates are luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones are heterodimeric glycoproteins, consisting of an alpha and beta subunit. The alpha subunit (abbreviated as CGA and encoded by *Cga* gene) is common for both gonadotropins, as well as for thyrotropin-stimulating hormone, whereas the beta chains (abbreviated as LHB and FSHB and encoded by *Lhb* and *Fshb* genes, respectively) provide specificity for hormone actions (Pierce and Parsons 1981). Gonadotropins fundamentally control mammalian reproduction by acting on their receptors expressed in the ovaries and testicles, thus stimulating gametogenesis and steroidogenesis (McArdle and Roberson 2015).

LH and FSH synthesis and secretion occurs in gonadotropes, one of five secretory cell types located in the anterior pituitary gland. These processes are regulated differently in males and females during development and reproduction cycles (McArdle and Roberson 2015). The major regulator of gonadotropin gene expression, synthesis, and secretion is gonadotropin-releasing hormone (GnRH), a decapeptide produced in the hypothalamus and released in pulsatile manner from the median eminence (Herbison 2016). Gonadotropes are the only cell type in the anterior pituitary that expresses the GnRH receptor (GnRHR), which belongs to G-protein coupled receptor family of receptors. Gonadotropes also convey GnRH actions on gonadotropin subunit gene expression and hormone synthesis and release (Janjic et al. 2017).

Basal and GnRH-stimulated synthesis of gonadotropins relies on many processes, including transcription rate, RNA degradation and translation, posttranslational modifications,

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and storage of hormones in secretory vesicles. The stored hormones are released by constitutive exocytosis (predominantly FSH) and GnRH-regulated exocytosis (predominantly LH) (McArdle and Roberson 2015).

Here we focus on the mechanisms of gonadotropin subunit gene transcription. This review relies on a body of data collected using several experimental models, including *in vivo* experiments with mice and rats and *in vitro* experiments using primary pituitary cell cultures from these species, purified gonadotropes from transgenic mouse models, and murine gonadotrope-derived cell lines named αT3-1 and LβT2 cells.

GnRH-GnRHR SIGNALING NETWORK INVOLVED IN GONADOTROPIN SUBUNITS GENE TRANSCRIPTION

The multiple actions of GnRH in gonadotropes greatly depend on its pulsatile pattern of secretion from the hypothalamus. In primary rat pituitary cells, two GnRH pulses per hour alter the transcription of over 80 genes (Kucka et al. 2013). In a time-, dose-, and/or pulse frequency- specific manner, GnRH controls the expression of several genes: *i)* its own receptor gene *Gnrhr*; *ii)* the gonadotropin subunit genes *Lhb*, *Fshb* and *Cga*; *iii)* the expression of molecules that further influence gonadotropin subunit expression, like follistatin (Kakar et al. 2003; Kucka et al 2013).

More precisely, pulsatile secretion is required for effective GnRH-mediated gene expression, whereas continuous application of GnRH desensitizes transcription; pulse frequencies determine transcription in a gene-specific manner (Ferris & Shupnik 2006). Faster GnRH pulse frequencies (one per 0.5-1 h) were reported to favor *Lhb* transcription, whereas *Fshb* transcription is favored at the slowest pulse interval (one per 2-4 h). *Cga* is transcribed at a high GnRH frequency (one pulse every 8–30 min), but also at slower pulse frequencies (Haisenlender et al. 1991; Kaiser et al. 1997; Shupnik 1990). The mechanism for differential regulation of gonadotropins by varying GnRH pulse frequencies may also involve GnRHR density at the plasma membrane (Kaiser et al. 1995).

Depending on the pulse frequency, GnRH stimulates specific gonadotropin subunit gene transcription through immediate-early genes. These actions, mediated by GnRHR activation, involve a complicated intracellular signaling network (Fink et al. 2010). Activated GnRHR couples with $G\alpha_{q/11}$proteins, thus triggering phospholipase Cβ, which then cleaves phosphatidylinositol-4-5-bisphosphate into inositol trisphosphate (IP_3) and diacylglycerol (DAG). DAG further initiates mitogen activated protein kinases (MAPK) cascades through the activation of protein kinase C (PKC), ultimately leading to activation of several transcription factors, including Early Growth Response protein 1 (EGR1) and members of the Activator Protein 1 (AP-1) family, including Fos and Jun (Naor 2009). This signaling pathway is essential for regulation of the expression of gonadotropin subunits.

On the other hand, GnRH-induced Ca^{2+} released from intracellular stores by IP_3 is accompanied with Ca^{2+} influx through L-type calcium channels (Stojilkovic 2012), leading to the activation of a calcium/calmodulin dependent kinase II (CaMK II) pathway, as well as the activation of certain PKC isoforms (Stamatiades and Kaiser 2018). The CaMK II pathway is implicated in the control of gonadotropin subunit expression and pulse frequency decoding. In addition, calcineurin, another intracellular Ca^{2+} target, and its downstream partner nuclear factor of activated T cells (NFAT), have been implicated in controlling the expression of *Fshb* and *Cga*. This likely occurs through activation of Nerve growth factor IB (Nur77) and control of the transcription of immediate early genes like *Fos, Jun,* and cyclic AMP-dependent transcription factor (*Atf3*) (Lim et al. 2007; Binder et al. 2012; Stamatiades and Kaiser 2018).

It has been reported that by activation of other signaling pathways, e.g. through coupling with $G\alpha_s$, GnRHR could stimulate cAMP production, protein kinase A (PKA) activation, and the subsequent cAMP response element-binding protein (CREB) phosphorylation, which is allegedly associated with *Fshb* expression control (Duan et al. 2002; Liu et al. 2002; Thompson et al. 2013). It has yet to be clarified whether coupling of GnRHR and $G\alpha_s$ signaling pathway is a unique characteristic of immortalized pituitary gonadotropes or a common feature of GnRHR (Krsmanovic et al. 2003).

Other than activation of signaling pathways and recruitment of transcription factors, GnRHR activation is associated with dynamic changes in chromatin structure - histone modifications and nucleosome positioning (Melamed et al. 2018). The complexity of the GnRH-GnRHR signaling network is further potentiated by the actions of other paracrine, autocrine and endocrine factors that modulate gonadotropin subunits expression, synthesis and secretion (Vázquez-Borrego et al. 2018), but the mechanisms of their actions are not within the scope of this review. Rather, this review centers on GnRH actions on gonadotropin subunits gene transcription in rats and mice and major transcription factors (Table 1).

TRANSCRIPTIONAL REGULATION OF *Lhb*

Transcriptional factors EGR1, steroidogenic factor 1/ orphan nuclear receptor (SF-1 or Nr5a1), and a homeodomain protein Pitx1 have been suggested to be critical for controlling basal and/or GnRHR-regulated *Lhb* expression. The proximal *Lhb* promoter region contains two binding sites for EGR1 (GC- rich region) and two binding sites for SF-1

Table 1. Transcriptional factors, activators and repressors involved in basal and GnRH regulated gonadotropin subunits gene transcription.

Gene	Transcriptional factors	Activators	Repressors
Lhb	EGR1, SF-1, Pitx1 (Halvorson et al. 1996; Quirk et al. 2001) Sp1 (Kaiser et al. 1998)	p300and SNURF (Mouillet et al. 2004; Curtin et al. 2004)	Nab1, Nab2 (Lawson et al 2007)
Fshb	AP-1 (Ciccone et al. 2008); (Coss et al. 2004; Wang et al. 2008) NF-Y, SF-1 (Jacobs et al. 2003; Coss et al. 2004)	USF1, USF2 (Ciccone et al. 2008)	ICER (Ciccone et al. 2010) SKIL, TGIF1 (Mistry et al. 2011) JDP2 (Jonak et al. 2017)
Cga	SF-1 (Fowkes et al. 2002) Pitx1, Lhx3 (Xie et al. 2017)	Foxp3 (Jung et al. 2102)	DAX-1 (Fowkes et al. 2002)

on either side of the homeodomain element bound by Pitx1 (Halvorson et al. 1996; Quirk et al. 2001).

While GnRH induces *Egr1* expression, but not the expression of the other two (Kakar et al. 2003), the synergistic interaction of these three factors is necessary for maximal *Lhb* gene transcription (Tremblay and Drouin 1999). In *Egr1* deficient mice, the expression of the *Lhb* gene is reduced and resulting mice are infertile (Lee et al. 1996). Pituitary-specific *Nr5a1* knock-out mice are also infertile (Zhao et al. 2001).

Two putative binding sites have been identified within the distal region of the *Lhb* promoter for Specificity protein 1 (SP1), a three zinc-finger transcription factor (Kaiser et al. 1998). An upstream composite element (containing binding sites for SP1 and CArG proteins) acts to modulate and integrate the response of an additional downstream element (containing binding sites for SF-1 and EGR1) and both elements are required for maximum response to pulsatile GnRH (Weck et al. 2000). The transcriptional co-activators p300 (which coordinates the functional synergy between SF-1 and EGR1) and SNURF (which interacts with SP1 and SF-1) may mediate interactions between distal and proximal GnRH response regions of the *Lhb* promoter (Mouillet et al. 2004). Microarray analysis of gene expression in LβT2 cells treated with GnRH in a different pulsatile manner confirmed that *Egr1* is stably induced at high pulse frequency, while mRNAs for the *Egr1* corepressor genes, Ngfi-A binding proteins - *Nab1* and *Nab2*, are induced at a low pulse frequency. These authors demonstrated that Nab1/2 inhibits EGR1-mediated induction of the *Lhb* gene (Lawson et al. 2007).

Interestingly, several laboratories reported lack of pulsatile GnRH treatment on *Lhb* expression (Weiss et al. 1990; Ishizaka et al. 1992; Kucka et al. 2013).

TRANSCRIPTIONAL REGULATION OF *Fshb*

Since FSH is predominantly constitutively secreted, transcriptional regulation of *Fshb* should be a critical regulatory step for control of FSH levels in circulation. Other agonists, including activins and inhibins, also contribute to *Fshb* expression (McArdle and Roberson 2015).

Using LβT2 cells, Mellon's group identified specific regulatory elements within 398 bp of the proximal mouse *Fshb* promoter. These regulatory elements, involved in basal expression of the *Fshb* gene, bind SF-1 and nuclear factor Y (NF-Y), that interact both physically and functionally. Using the same cell model, the same group also localized GnRH-responsive region within the proximal part of promoter. In LβT2 cells, GnRH recruits cFos, FosB, cJun, JunB during 1 h and 3 h treatment and their different combinations bind to a novel AP-1 site, which consists of a half-site of the AP-1 consensus binding sequence and an adjacent CCAAT box. NF-Y and AP-1 interact and co-occupy this site *in vivo* following GnRH stimulation (Jacobs et al. 2003; Coss et al. 2004). In primary culture of rat pituitary cells and LβT2 cells, GnRH also induces transcription of AP-1 members *Fos* and *Jun* (Coss et al. 2004; Yuen et al. 2012).

However, Kaiser's group identified a GnRH-responsive element that contains a partial cAMP response element (CRE)/AP-1 site in the proximal region of rat *Fshb* promoter. They also showed that binding of upstream stimulating factors of USF families (USF 1 and USF2) increase basal transcription of rat *Fshb*. According to these authors, GnRH stimulates CREB phosphorylation, leading to occupation of the CRE/AP-1 half site and recruitment of histone acetyltransferase CREB-binding protein to the promoter, enhancing gene transcription. The authors also provided evidence of CREB binding to a CRE/AP-1 half site in the rat *Fshb* promoter after GnRH induction, with minimal AP-1 binding. Such discrepancies could originate from species-specific differences and need to be further investigated (Ciccone et al. 2008).

As we have previously mentioned, *Fshb* subunit gene transcription is preferentially stimulated at low GnRH pulse frequencies. In perifusion studies on LβT2 cells at low pulse frequencies, mutation of a CRE abolished induction of *Fshb* transcription (Ciccone et al. 2010), whereas PKA inhibition attenuated CREB phosphorylation and *Fshb* expression (Thompson et al. 2013). Activation of transcriptional repres-

sors, like inducible cAMP early repressor (ICER) may potentially explain the need for low GnRH pulse frequencies. ICER is undetectable under basal conditions, but its expression and synthesis are preferentially stimulated at high GnRH pulse frequencies. ICER production antagonizes CREB binding to CRE and attenuates *Fshb* transcription at high GnRH pulse frequencies (Ciccone et al. 2010). SKIL (ski-oncogene-like protein) and TGIF1 (TG-interacting factor 1) corepressors are also induced at high GnRH pulse frequencies, and bind to the *Fshb* promoter to inhibit cFos and cJun actions (Mistry et al. 2011). A novel repressor of GnRH mediated *Fshb* induction was recently discovered and named c-JUN-dimerization protein 2 (JDP2). Using LβT2 cells and JDP2 null mice, it was shown that JDP2 exhibits high basal expression and binds the *Fshb* promoter in complex with cJun, thus displacing cFos (Jonak et al. 2017).

TRANSCRIPTIONAL REGULATION OF *Cga*

CGA is one of the first proteins expressed in the pituitary during fetal development; this subunit is noticeable from E11.5 in the thyrotropes and gonadotropes of mice (Fowkes et al. 2002). In gonadotropes, the key regulator of *Cga* expression is GnRH. Unlike *Fshb* and *Lhb* expression, the expression of *Cga* seems to be triggered by both pulsatile and continuous GnRH input (Stamatiades & Kaiser 2018).

It has been suggested that three cell lines represent embryonic gonadotrope maturation stages: αT1-1 (gonadotrope progenitor cells), αT3-1 (immature gonadotropes), and LβT2 (mature gonadotropes). They express *Cga* and have an open chromatin status in the promoter region of this gene, meaning that at all stages of gonadotrope maturation the *Cga* promoter is accessible for transcription factors. One of the histone modifications related to active chromatin is H3 acetylation, found at the 5' end of the *Cga* gene in gonadotropes after GnRH input. GnRH also induces histone H3 phosphorylation, seen at promoter regions of gonadotropin genes, including *Cga* (Melamed et al. 2018).

Several studies have examined the *Cga* promoter region in mouse. An enhancer region (between -4.6 and -3.7 kb) upstream of the *Cga* transcription initiation site was identified as critical for high levels of expression in both gonadotrope and thyrotrope mouse cells (Kendall et al. 1994).

A number of *cis* elements regulating *Cga* expression have been found in the region 500 bp upstream of the transcription initiation site. Some of them have been identified, such as the LIM homeodomain protein (Lhx2) binding site (-342 to -329), Pitx1 binding site (-399 to -375), GnRH response element (GnRE) (-406 to -399), and binding sites for transcriptional activators SF-1 (-225 to -205) and GATA2/3 (-161 to -146). These regulatory elements are responsible for basal and/or GnRH-driven *Cga* transcription (Brinkmeier et al. 1998). Most experiments investigating transcriptional regulation of *Cga* expression were conducted either in αT3-1 or LβT2 cell lines. Fowkes et al. (2002) found that expression of *Cga* mRNA is reduced in LβT2 cell line compared to that previously reported in αT3-1 cells. This may be due to an increase in the DAX-1 (Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1): SF-1 ratio, suggesting that DAX-1 probably inhibits SF-1 mediated *Cga* transcription to some extent.

Recently, Xie et al. (2017) revealed that Pitx1 and Lhx3, the tissue-specific pituitary regulatory factors, are both able to bind to *Cga* promoter, but that Lhx3 is probably the one maintaining the expression of *Cga* from the early stages of development. The Forkhead box (Fox) family of transcriptional factors also plays a role in the regulation of *Cga* expression in LβT2 cell line, but it is not certain whether this factor is needed for regulation of *Cga* expression *in vivo* (Thackray 2014). Another Fox regulatory protein, Foxp3, may play a role in *Cga* mRNA expression. Namely, in male mice with a spontaneous mutation in the *Foxp3* gene, which results in a protein lacking a DNA binding domain, the expression of *Cga*, as well as other gonadotrope marker genes, was decreased (Jung et al. 2012).

CONCLUDING REMARKS

Despite the enormous body of work published on the transcriptional regulation of gonadotropin subunit genes, there are still quite a few questions that remain to be answered. While GnRH is the main controller in these processes, other endocrine, paracrine, and autocrine factors prevent unraveling the mystery of its pulse decoding. While extensive use of immortalized cell lines provides important information, relying only on such studies may lead to incomplete or even false conclusions about the processes that ultimately drive reproduction. Regardless of species-specific promoter regions, and thus differences in transcriptional control, deeper comprehension of gonadotropin subunits expression in rodents is necessary for a more profound understanding of reproduction processes in general.

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