

## Review

## GnRH signaling, the gonadotrope and endocrine control of fertility

Stuart P. Bliss, Amy M. Navratil, Jianjun Xie, Mark S. Roberson \*

Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, United States

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## ABSTRACT

Mammalian reproductive cycles are controlled by an intricate interplay between the hypothalamus, pituitary and gonads. Central to the function of this axis is the ability of the pituitary gonadotrope to appropriately respond to stimulation by gonadotropin-releasing hormone (GnRH). This review focuses on the role of cell signaling and in particular, mitogen-activated protein kinase (MAPK) activities regulated by GnRH that are necessary for normal fertility. Recently, new mouse models making use of conditional gene deletion have shed new light on the relationships between GnRH signaling and fertility in both male and female mice. Within the reproductive axis, GnRH signaling is initiated through discrete membrane compartments in which the receptor resides leading to the activation of the extracellular signal-regulated kinases (ERKs 1/2). As defined by gonadotrope-derived cellular models, the ERKs appear to play a central role in the regulation of a cohort of immediate early genes that regulate the expression of late genes that, in part, define the differentiated character of the gonadotrope. Recent data would suggest that in vivo, conditional, pituitary-specific disruption of ERK signaling by GnRH leads to a gender-specific perturbation of fertility. Double ERK knockout in the anterior pituitary leads to female infertility due to LH biosynthesis deficiency and a failure in ovulation. In contrast, male mice are modestly LH deficient; however, this does not have an appreciable impact on fertility.

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## 1. Introduction

The hypothalamic–pituitary–gonadal (HPG) axis consists of an intercommunicating set of neural and endocrine tissues that function as a highly integrated unit in the regulation of fertility. The basic organization of this physiological axis is highly conserved and underlies reproductive competence in a wide variety of diverse vertebrate species including birds, reptiles, fish, and mammals [2,76,140,168]. The gonadotrope cell of the anterior pituitary plays a particularly critical role within this system as the intermediary between the hypothalamic gonadotropin-releasing hormone (GnRH) signal and the germ cell reservoirs and steroid hormone productivity of the gonads. Expression of the GnRH receptor (GnRHR) is a defining characteristic of the gonadotrope. Binding of GnRH to its receptor triggers a complex array of intracellular signal transduction events within the gonadotrope; these signaling cascades orchestrate the overall physiological response of these cells to GnRH stimulation, culminating in the synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Thus, in both functional and anatomical senses, the GnRHR and the intracellular signal transduction sys-

tems to which it couples, form the fundamental interface between the “neuro-” and “endocrine” components of this neuroendocrine axis. The mechanisms underlying the response of the gonadotrope to hypothalamic GnRH are thus of great importance to our understanding of the molecular basis of reproductive function and fertility.

Signal transduction systems form the basic biochemical infrastructure by which cells mount specific and appropriate biological responses to a wide variety of extracellular or intracellular cues. Indeed, dysregulation of signaling processes contributes to many forms of disease including cancer, immunological disease and neuro-degenerative disorders, and targeted manipulation of signaling pathways is an area of great scientific as well as clinical therapeutic interest [20,182,298]. The signal transduction events activated by the GnRHR have themselves been the focus of extensive investigation for well over two decades (recently reviewed in detail by Naor in [199]). This work has generated a formidable body of knowledge regarding the nature and kinetics of GnRH-induced signaling networks and encompasses great advances in our understanding of GnRHR signaling in a variety of experimental systems. However, within the context of reproductive biology, significant challenges remain. The response of the differentiated gonadotrope to the GnRH signal in vivo occurs within the context of a complex and dynamic endocrine milieu. A meaningful understanding of the molecular basis of gonadotrope function therefore awaits systematic clarification of the functionality of GnRH-induced intracellular

\* Corresponding author. Address: Department of Biomedical Sciences, T4-018 Veterinary Research Tower, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, United States. Fax: +1 607 253 4495.

E-mail address: [msr14@cornell.edu](mailto:msr14@cornell.edu) (M.S. Roberson).

signal transduction pathways in vivo and their requirements for fertility. The development and refinement of sophisticated transgenic technologies offers a powerful tool by which to address these questions. Recent results utilizing this approach have begun to shed exciting new light on important facets of gonadotrope function in vivo. In this review, we will provide an overview of the mammalian HPG axis with a broad emphasis on the link between GnRH-induced intracellular signal transduction pathways and gonadotrope gene expression within the intact endocrine environment of the living animal.

## 2. The hypothalamic–pituitary–gonadal (HPG) axis

### 2.1. Overview of the functional organization of the mammalian HPG axis

In the classical view, the HPG axis is depicted as a tiered and linearly organized set of endocrine tissues that is dedicated to the regulation and support of reproductive activity. This axis consists of a small subset of hypothalamic neurons that express the decapeptide hormone GnRH, the gonadotrope cells of the anterior pituitary, and the gonads. Activation of this endocrine axis commences with the pulsatile secretion of GnRH from the hypothalamus. GnRH is delivered to the anterior pituitary via the hypophyseal portal circulation where it binds to the GnRHR on the surface of gonadotropes triggering the synthesis and secretion of the gonadotropins FSH and LH. The gonadotropins are dimeric glycoprotein hormones composed of distinct hormone-specific  $\beta$  subunits paired with a common  $\alpha$  subunit ( $\alpha$ GSU). The  $\beta$  subunits of FSH and LH, together with the  $\alpha$ GSU and the GnRHR comprise the essential genetic signature of the differentiated gonadotrope [241]. In the male, LH stimulates production of testosterone and androgen-binding protein by testicular Leydig cells and Sertoli cells, respectively. In the female, LH stimulates the production of androgens by the thecal cells that surround the growing ovarian follicle. During the terminal stages of follicular growth, LH also drives the production of progesterone from the granulosa cells of the preovulatory follicle. FSH binds to receptors on the surface of ovarian granulosa cells stimulating the expression of aromatase enzymes that convert thecal androgens to estradiol. In the male, FSH binds to receptors on the surface of Sertoli cells and functions in concert with testosterone to promote the proliferation of spermatogonia as well as the meiosis and postmeiotic development of germ cells.

Several animal models have provided insights into the functional organization of the HPG axis. The hypogonadal (HPG) mouse fails to produce GnRH due to a deletion mutation in the gene encoding the GnRH precursor peptide [174]. HPG mice never enter puberty and display a persistent hypogonadotropic–hypogonadal phenotype which is nevertheless able to be rescued by knockin of a functional GnRH gene [175]. Early studies in sheep demonstrated that surgical ablation of the pituitary stalk (hypothalamic–pituitary disconnection, HPD) led to dramatic decreases in pituitary secretion of the gonadotropins and secondary hypogonadism [38,39]. Pulsatile administration of GnRH to these animals reestablished corresponding and appropriate pulsatile gonadotropin secretion and fertility while continuous administration of GnRH led to virtual cessation of pituitary gonadotropin production, highlighting the fundamental importance of GnRH pulsatility for suitable gonadotrope cell function [37].

In mice, elimination of the gene for either the  $\beta$  subunit of LH, or the LH receptor, leads to infertility in both genders associated with marked decreases in gonadal steroid hormone production, along with defective spermatogenesis and late follicular developmental arrest [148,306]. FSH levels are normal in these mice and the

hypogonadal phenotype of the LH $\beta$  null mouse is able to be rescued by exogenous LH. In contrast, female mice lacking the gene for the  $\beta$  subunit of FSH are infertile due to a defect in ovarian follicular maturation; however, males remain fertile, despite decreased testicular size and sperm production [138]. Similarly, the phenotype of female mice lacking the FSH receptor (FSHR) is similar to that of the FSH $\beta$ -deficient animal (infertility due to follicular developmental arrest) [1,52]. However, in males, loss of the FSHR leads to a greater impairment of gonadal function than in FSH $\beta$  null mice, with significant decreases in Leydig cell numbers and levels of circulating testosterone [6]. It has been suggested that the more severe phenotype displayed by FSHR deficient animals as compared with those lacking FSH itself, is indicative of constitutive and physiologically important ligand-independent signaling activity of gonadal FSHR populations [6].

In addition to information gained from animal models, many naturally-occurring human diseases provide insight into the HPG axis function. Kallmann Syndrome (KS) is a form of hypogonadotropic–hypogonadism that results from a failure of migration of GnRH neurons from the olfactory epithelium to the hypothalamus during embryonic development and the consequent lack of hypothalamic GnRH production [77]. Individuals with KS typically are anosmic (deficient in the sense of smell) due to hypoplasia or aplasia of the olfactory tracts and in addition may display a variety of developmental abnormalities including dental or renal agenesis, palate defects, digital malformations and hearing loss [99]. Several specific mutations have been identified that collectively account for approximately 30% of reported cases of KS. The most severe form of the disease (KAL1) is an X chromosome-linked condition associated with a loss-of-function mutation in the *KAL1* gene that encodes the protein anosmin-1 [77,100]. Anosmin-1 is a secreted multidomain-containing glycoprotein that is thought to guide developing axonal projections during morphogenesis of the olfactory system in part through stabilization of growth factor–receptor complexes, especially complexes between fibroblast growth factors (FGFs) and their receptors (FGFRs) [55,260]. Since olfactory axonal tracts function as a pathway along which nascent GnRH neurons migrate from their origin in the olfactory epithelium to the hypothalamus, disturbances in the development of the olfactory system may interfere with the ability of GnRH neurons to populate the hypothalamus. This leads to GnRH deficiency and hypogonadotropic–hypogonadism. Loss-of-function mutations in *FGFR1* have also been associated with an autosomal dominant form of KS (KAL2); however this form of the disease is incompletely penetrant, indicating a more complex and probably oligogenic basis [56,240].

Mutations in the gene encoding the human GnRHR have been discovered that lead to hypogonadotropic–hypogonadism of variable severity [10,11,50,131,143] and reviewed in [9]. The impairment of intracellular inositol-phosphate accumulation associated with some GnRHR mutations is able to be rescued by molecular chaperones, indicating that misfolding and abnormal intracellular trafficking of newly synthesized receptor proteins and failure of receptor expression may underlie the effects of some mutations [145]. This is discussed in more detail below. Naturally occurring inactivating mutations in the genes for LH $\beta$  and FSH $\beta$  as well as their receptors have also been reported. These mutations are generally associated with hypogonadal phenotypes, and given their adverse effects on reproductive function, are understandably rare [4,14,144,269].

### 2.2. Hypothalamic secretion of GnRH

The release of GnRH from the hypothalamus is inherently pulsatile, and this pulsatility is required for appropriate gonadotropin production by the pituitary [12,172,172,278]. The importance of

GnRH pulsatility was first demonstrated in 1978 in experiments in Rhesus monkeys with surgically created lesions of the basomedial hypothalamus that eliminated the GnRH neurons [28]. Continuous administration of exogenous GnRH to these animals led to decreased production of gonadotropins and hypogonadism, while pulsatile administration of GnRH at hourly intervals restored gonadal function and reproductive cyclicity [12]. The suppression of pituitary responsiveness to GnRH following continuous exposure results at least in part from downregulation of GnRHR's at the level of the gonadotrope [160,176,204,209,283]. However, desensitization of signaling processes downstream of the GnRHR may also play a role [156,181,217,292]. Downregulation of gonadotrope responsiveness to GnRH through continuous hormone exposure effectively uncouples the gonads from pituitary regulation, allowing for precise clinical manipulation of germ cell maturation through the administration of exogenous gonadotropins. This general strategy forms the basis for the clinical use of GnRH analogs that are prescribed for the treatment of many forms of infertility, especially in females [169,236].

Both the amplitude and frequency of GnRH pulses are tightly regulated and, in the female, vary dramatically over the course of the reproductive cycle [172]. Rapid GnRH pulsatility has been shown to promote the synthesis of  $\alpha$ GUSU and LH $\beta$ , while slower GnRH pulsatility favors production of FSH $\beta$  [92]. Different pulse frequencies of GnRH stimulation also affect the kinetics of gonadotropin release from the pituitary, with higher pulse frequencies linked to selective secretion of LH against a background of more tonic and consistent secretion of FSH [92,116,220]. The resulting pulsatile discharge of gonadotropins, especially LH, from the gonadotrope leads to corresponding oscillations in plasma gonadotropin levels, the magnitude of which may further reflect the shorter circulatory half-life of LH as compared to FSH. The responsiveness of the gonadotrope to changes in hypothalamic GnRH pulsatility is critical for the generation of the preovulatory LH surge in the female, and the mechanisms underlying the ability of the gonadotrope to interpret GnRH pulsatility has received considerable attention in a number of basic scientific arenas [47,91,113,118,126,151,171,282]. In contrast, regulated fluctuations in GnRH pulsatility play a less significant role in male reproductive function. This sexual dimorphism with regards to cyclic variation in GnRH pulsatility is thought to be based in the neuro-anatomical circuitry involving the hypothalamic GnRH neurons and their various afferents. Significant gender dimorphism has been demonstrated with respect to the anatomical organization and functionality of the hypothalamic GnRH system; this dimorphism is established early in mammalian embryogenesis as a result of androgen exposure [41,43,97]. Whether any aspect(s) of gonadotrope responsiveness to differential GnRH pulse frequency reflects a similar intrinsic gender dimorphism remains an intriguing question. However, early studies in primates showed that castration can result in hypothalamic sensitivity to positive feedback effects of estrogen and female-like patterns of gonadotropin secretion in males. Thus, at least in primates, gender divergence in gonadotrope function appears to be a reversible effect of postnatal androgenic stimulation of the pituitary [120,205].

### 2.3. Feedback regulatory mechanisms in the HPG axis

The HPG axis is subject to both positive feed-forward and negative feedback regulation at several levels. At the level of the hypothalamus, early recognition of the pulsatile nature of GnRH secretion led to the notion of a central “pulse generator”, the inherent oscillatory activity of which controls the secretory rhythm of GnRH neurons [128]. Anatomically, this pulse generator is thought to be located predominantly within the suprachiasmatic nucleus of the hypothalamus. Interestingly, studies from dispersed hypothalamic neurons in primary culture suggest that pulsatile secretion

of GnRH may be an inherent property of the neurons themselves [187]. Moreover, populations of GnRH neurons may be capable of broad scale synchronization of GnRH pulsatility, and this synchronization does not appear to require direct intercellular contact [159]. GnRH neurons express the GnRHR, which couples to different intracellular heterotrimeric G-proteins in a manner apparently dependent on ligand dose [136]. These observations have led to a model in which low doses of GnRH may exert positive autocrine and paracrine feedback effects on neuronal GnRH secretion through GnRHR-G $\alpha$ s-induced increases in intracellular cyclic adenosine-monophosphate (cAMP) levels. The resulting increase in GnRH secretion subsequently propels dramatic further positive feedback effects by driving GnRHR-G $\alpha$ <sub>q/11</sub>-induced increases in intracellular calcium. However, once the concentration of extracellular GnRH reaches a sufficiently high (threshold) level, GnRHR-G $\alpha$ i-mediated signaling pathways are preferentially activated, leading to negative feedback suppression of secretory activity [123].

While the pulsatility of GnRH neurons is widely understood to represent an inherent cellular characteristic, the extent to which this property dictates the pattern of oscillatory release of GnRH in vivo is not clear. Furthermore, it is clear that the hypothalamic pulse generator is extensively modulated by a multitude of higher level inputs including photoperiod, environmental stress, metabolic state and nutritional status, as well as various endocrine mediators [8,24,83,132,153,185]. The central nervous system is thus constantly integrating and translating a vast diversity of environmental and physiological cues into a coordinated pattern of GnRH release from the hypothalamus. Gonadal steroid hormones play a particularly important role in feedback regulation of hypothalamic secretion of GnRH [60,84,154,216,218]. In the male, testosterone exerts negative feedback effects primarily on the release of GnRH from the hypothalamus [270]. Immunohistochemical analyses in rams suggest that, at least in this species, hypothalamic GnRH neurons do not express the androgen receptor [106]. Thus, the effects of testosterone and its metabolites are thought to be mediated by higher level afferent neuronal populations that likely include the kisspeptin neurons of the arcuate nucleus and the AVPV region [259]. In some species, a minor negative feedback effect may also be exerted at the level of the gonadotrope [270].

In the female, the feedback effects of gonadal steroids on HPG function are more complex, species-specific, and depend on the stage of the reproductive cycle. Studies in rats indicate that estrogen is capable of down-regulating the expression of GnRH within the hypothalamus, and that this effect is most pronounced during the follicular phase [213,214,312]. A direct inhibitory effect on the GnRH neuron mediated by the  $\beta$  isoform of estrogen receptor (ER $\beta$ ) has been reported [214]. However, the negative feedback effects of estrogen remain intact in ER $\beta$ -null mice, and more recent results indicate that negative feedback by estrogen during the early follicular phase is mediated through estrogen response element (ERE)-independent signaling of ER $\alpha$  [84]. In contrast, during the preovulatory period, the effects of estrogen on both the hypothalamus and the pituitary become positive [197]. Study of the positive effects of estrogen in sheep initially led to a model of periodic hypothalamic GnRH release that encompasses three distinct phases of activation, transmission, and surge [64]. The activation phase is now known to involve a positive indirect effect of estrogen on hypothalamic production of GnRH, mediated through ER $\alpha$  expressed by afferent kisspeptin-positive neurons of the rostral peri-ventricular region of the third ventricle that synapse on the GnRH neurons [63,105,214,254]. Increasing estrogen levels during the middle to late follicular phase leads to enhanced synthesis of kisspeptin within these afferent neurons; direct kisspeptin stimulation of hypothalamic GnRH neurons mediated through the kisspeptin receptor, KISS1R, (transmission phase) then leads in turn

to rapid and significant increases in hypothalamic GnRH secretion (surge phase) culminating in the massive preovulatory release of LH from the pituitary that is the definitive trigger of ovulation [42,64,105,115]. The rise in estrogen through the follicular phase has also been shown to exert positive feedback effects at the level of the pituitary where it primes the gonadotrope for the preovulatory LH surge [38,304].

In addition to the feedback effects mediated by estrogen, progesterone also exerts complex modulatory feedback effects at all levels of the HPG axis. The negative feedback effects of progesterone upon hypothalamic GnRH release during the luteal phase of the cycle are well established and play an important role in limiting the potential positive effects of luteal estrogen during the postovulatory phase. As with estrogen, the feedback effects of progestins are indirect, as GnRH neurons do not express the progesterone receptor (PR) [256]. Negative feedback effects of progesterone on GnRH release can occur during either the activation or transmission phases of the cycle of GnRH secretion, and kisspeptin-positive neurons of the suprachiasmatic nucleus appear to be a major target of progesterone action in this context [40,101,225,258]. Recent results suggest that progesterone may also exert direct negative feedback effects upon the release of GnRH from GnRH-producing cells through a rapid activation of membrane-associated PR, implicating signaling events at the plasma membrane of GnRH neurons as possible regulators of the surge phase [257]. However, the effects of progesterone on hypothalamic GnRH release may also be positive. Classic studies in rats have clearly established the importance of progesterone receptor activation for the preovulatory GnRH surge and in sheep, exogenous progesterone was shown to exert a priming effect on the hypothalamus, leading to higher GnRH levels during induced preovulatory GnRH surges, and delaying the onset of the GnRH surge relative to non-progesterone treated animals [27,65,135,188,219]. Recent work in rats indicates that astrocytes may function as a hypothalamic source of progesterone that facilitates the GnRH surge [188,189]. The synthesis of progesterone by hypothalamic astrocytes is itself positively regulated by estrogen [189]. This regulation involves rapid activation of plasma membrane-associated ER $\alpha$ , direct interaction of ER $\alpha$  with metabotropic glutamate receptors, and G $\alpha$ q-dependent increases in intracellular calcium. This provides an intriguing example of how estrogen may regulate biosynthetic processes through nonclassical plasma membrane-associated signaling and indirect modulation of plasma membrane ion channels [121]. As further illustration of the complex and integrated landscape of feedback regulation, expression of the PR has itself been shown to be induced by estrogen within peri-ventricular neurons of the hypothalamus, and exerts important positive regulatory effects on hypothalamic release of GnRH that may involve ligand-independent signaling. Indeed, elegant experiments involving systemic treatment with PR antagonists or intra-cerebroventricular administration of antisense oligonucleotides targeting the PR eliminated GnRH and LH surges in rats [32].

The mechanisms underlying the feedback effects of gonadal steroids on both the hypothalamic GnRH system as well as the gonadotrope have been explored in detail using a variety of transgenic models. Progesterone receptor null males are reproductively competent, while females are infertile and have complex reproductive phenotypes reflecting the diverse roles played by PR at all levels of the female HPG-genital tract axis [165]. Mice harboring a floxed PR allele allowing conditional deletion of both A and B isoforms of PR were recently described and promise to become a useful tool for conditional ablation of PR and analysis of both its transcriptional and signaling functions in either the pituitary or brain [102].

Mice lacking either kisspeptin or its receptor KISS1R display a hypogonadotropic-hypogonadal phenotype and are infertile.

Predictably, the phenotype of kisspeptin null mice is able to be at least partially rescued by administration of exogenous kisspeptin, while the KISS1R null animal is nonresponsive to this treatment [141]. The requirement for ER $\alpha$  in mediating the positive effects of estrogen are demonstrated in the divergent phenotypes of ER $\alpha$  and ER $\beta$ -null mice; ER $\alpha$  null mice are infertile and nonresponsive to estrogen feedback while ER $\beta$ -null mice are subfertile with impaired ovulatory capacity but remain sensitive to the positive feedback effects of estrogen [295]. The reproductive phenotype of the ER $\alpha$  null was further recapitulated in a neuronal-specific ER $\alpha$  knockout demonstrating that the positive feedback effects of estrogen upon GnRH release from the hypothalamus are exerted specifically upon neuronal afferents that regulate the activity of hypothalamic GnRH neurons [295]. Interestingly, in mice in which the wild-type ER $\alpha$  sequence was substituted with a mutant form of ER $\alpha$  possessing a defective DNA-binding domain, the negative feedback effects of estrogen were largely maintained while positive feedback effects were absent, indicating that positive feedback requires expression of ERE-regulated genes, while negative feedback is supported by non-canonical ERE-independent ER signaling [84]. At the level of the gonadotrope, the negative effects of ERE-independent estrogen signaling were subsequently shown to involve suppression of secretion, but not synthesis of LH, highlighting the importance of non-genomic ERE-independent ER signaling [85]. Recently, the role of ER $\alpha$  in the gonadotrope was further investigated using a pituitary-targeted conditional knockout of ER $\alpha$  [82]. Mice lacking ER $\alpha$  specifically in the pituitary showed a sexually dimorphic phenotype with males unaffected while females displayed irregular estrous cycles and were infertile. Interestingly, serum levels of the gonadotropins were not affected by the loss of ER $\alpha$  in the pituitary leaving the question of whether ER $\alpha$  functions primarily in a signaling or transcriptional capacity within the gonadotrope unanswered.

In addition to the gonadal steroids, the peptide hormones inhibin, activin, and follistatin play important roles in both gonadal and paracrine feedback regulation of gonadotrope function (reviewed in [87]). Inhibin is a dimeric glycoprotein consisting of a common  $\alpha$ -subunit paired with a distinct  $\beta$  subunit. Two  $\beta$  subunits have been identified within the reproductive axis (designated  $\beta_A$  and  $\beta_B$ ); two corresponding forms of inhibin exist, consisting of  $\alpha\beta_A$  and  $\alpha\beta_B$  dimers, respectively. Activins are related hormones that consist of dimers of inhibin  $\beta$  subunits. The monomeric  $\beta$  subunits of inhibin may pair in any combination, giving rise to three activin species,  $\beta_A\beta_A$ ,  $\beta_A\beta_B$ , or  $\beta_B\beta_B$ , referred to as activins A, B, or AB, respectively. Follistatin is an unrelated glycoprotein that modulates the bioactivity of activin dimers through direct interaction. The inhibins, activins, and follistatin were originally purified from gonadal tissue; however, they are now known to be variously expressed at all levels of the HPG axis as well as in several nonreproductive tissues including the liver and brain [129,232,239,246,280]. The  $\alpha$  and  $\beta_B$  subunits of inhibin/activin as well as follistatin are expressed in the pituitary, and while activin B and follistatin have been implicated in paracrine regulation of gonadotrope function, the gonads appear to be the predominant, if not the sole source of mature bioactive inhibin [68,231,232,293].

The inhibins and activins are members of the TGF $\beta$  family of mediators, and bind to the type II activin receptor, a transmembrane receptor with serine–threonine kinase activity. Binding of activin to the type II receptor leads to the recruitment of the type I receptor subunit and activation of the heterodimeric receptor–ligand complex. In contrast, binding of inhibin to the type II receptor occurs in conjunction with the inhibin coreceptor betaglycan, a cell surface proteoglycan, and prevents both activin interaction with the type II receptor and type I–II receptor dimerization [150]. Inhibin thus functions as a competitive inhibitor of activin. Follistatin binds activin within the extracellular compartment, including the

plasma, and neutralizes its activity by preventing receptor interaction [198].

The inhibin–activin–follistatin system functions primarily to modulate FSH production by the gonadotrope; however, this modulation may occur at several levels. Some evidence suggests that the expression of activin A and follistatin in the hypothalamus may constitute a local autocrine or paracrine feedback loop regulating secretion of GnRH [87,137,166]. Gonadotropes express the activin receptor and activin potently upregulates FSH expression in these cells in conjunction with GnRH. Activin may also regulate the expression of both FSH and LH indirectly by augmenting GnRH-induced homologous upregulation of the GnRHR [68,206].

Binding of activin to its cognate type II receptor leads to recruitment, dimerization, and trans-phosphorylation of the type I receptor. The receptor complex then leads to the activation of the receptor-associated SMADs 2 and 3, and their association with the common mediator SMAD 4. The SMAD complex undergoes nuclear translocation and functions as a transcriptional regulator through direct binding to DNA and recruitment of various coactivators [87]. In the gonadotrope, the most thoroughly characterized gene target of activin-induced SMAD signaling is FSH $\beta$ , and indeed, in some systems, activin has been shown to be a more potent inducer of FSH $\beta$  expression than GnRH itself [286]. In L $\beta$ T 2 cells, pretreatment with activin suppressed GnRH-induced activation of ERK, and enhanced activation of JNK and p38 activity indicating that upstream cross-regulation may take place between activin-induced SMAD signaling and other signaling pathways induced by GnRH [44,307]. However, functional interaction between these pathways appears to occur more prominently at the point of their convergence upon specific gene promoters (reviewed recently by [186,268]). As a notable example, activin-induced SMAD signaling and GnRH-induced upregulation of AP-1 have been shown to result in synergistic transcriptional activation of both the GnRHR and FSH $\beta$  through occupancy of SMAD and AP-1 response elements in the promoters of these respective genes [21,44,45,62,68,88,207,284].

### 3. Molecular basis of the response of the gonadotrope to GnRH

#### 3.1. Fundamental mechanisms underlying pituitary morphogenesis

The anterior lobe of the pituitary is composed of five discrete hormone-producing cell types; in addition to the gonadotrope, the corticotrope, thyrotrope, somatotrope, and lactotrope cells respond to distinct hypothalamic releasing factors and produce adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), growth hormone (GH), and prolactin (PRL), respectively. During embryogenesis, these hormone-producing cell lineages develop from a common primordium in a highly regulated spatial and temporal progression. The distinct phenotypes expressed by these cells vis-a-vis their hormone production have made the pituitary a useful model system in which to study basic mechanisms of cellular differentiation and organogenesis in vivo. A comprehensive review of pituitary development is beyond the scope of this review; however, several basic concepts related to the development of pituitary cell types are relevant to the experimental approaches and results described herein and are therefore outlined in brief. Pituitary development has been recently reviewed in detail in [311].

Proliferative expansion of the pituitary primordium and sequential appearance of lineage-committed cell types of the anterior portion of the gland occurs from e9.5 through approximately e13.5. These processes are dependent on tightly regulated spatiotemporal expression of numerous transcription factors [235,285]. The  $\alpha$ GSU is the first hormonal subunit gene to be expressed within the developing pituitary, appearing in the ventral region of

Rathke's pouch at e10.5 [311]. The distribution and timing of  $\alpha$ GSU promoter activation has been studied in transgenic mice using reporters linked to various portions of the  $\alpha$ GSU promoter. A 381 base pair fragment of the murine  $\alpha$ GSU promoter is sufficient to direct gonadotrope- and thyrotrope-specific reporter expression within the developing pituitary; however, higher levels of expression are obtained with the use of a 4.6 kb promoter region that incorporates an upstream enhancer element [122]. One study using this 4.6 kb  $\alpha$ GSU promoter fragment demonstrated reporter expression as early as e9.5 throughout the pituitary primordium [122]. The possibility of such early, promiscuous, or misregulated activation of this transgenic promoter fragment during development is important in relation to studies described below, in which in vivo pituitary-targeted genetic recombination was accomplished using this same 4.6 kb promoter to regulate expression of Cre recombinase.

#### 3.2. Developmental specification and differentiation of the gonadotrope lineage

The developmental events underlying fate specification of the gonadotrope lineage remain largely unclear. This is in contrast to other pituitary lineages such as the thyrotrope, somatotrope and lactotrope cells (the Pit-1 lineages) whose development and differentiation are completely dependent on expression of the POU domain-containing transcription factor Pit-1 [294]. The orphan nuclear receptor steroidogenic factor-1 (SF1) is first expressed at e13.5 and is the first gonadotrope-specific marker to appear within the developing gland [114]. Onset of expression of SF1 is occasionally cited as the defining event in gonadotrope specification. Indeed, pituitary-specific depletion of SF1 led to impaired expression of the gonadotropins as well as the GnRHR, and secondary hypogonadism [310]. However, this phenotype was able to be rescued by administration of exogenous GnRH suggesting that while SF1 is necessary for gonadotrope function, it is not required for developmental specification or differentiation of the gonadotrope lineage [310].

The gonadotrope is the last of the anterior pituitary cell types to undergo terminal differentiation as marked by the relatively late onset of expression of the genes encoding the  $\beta$  subunits of FSH and LH. The mechanisms underlying the timing of initial expression of these gonadotropin subunit genes are unknown. Activation of the murine FSH $\beta$  promoter first occurs at approximately e17.5 while LH $\beta$  biosynthesis commences approximately 24 h later. In conjunction with a cohort of relatively ubiquitous and cell type-*non*-specific transcription factors, SF1 plays a key role in activation of the promoters for all of the genes that comprise the genetic signature of the gonadotrope ( $\alpha$ GSU, FSH $\beta$ , LH $\beta$ , GnRHR), and is primarily responsible for restricting expression of FSH $\beta$ , LH $\beta$ , as well as GnRHR to the gonadotrope [7].

#### 3.3. Experimental models used in the study of gonadotrope cell function

More recent studies of GnRH-activated signal transduction pathways in the gonadotrope have relied upon and benefitted greatly from the  $\alpha$ T3-1 and L $\beta$ T 2 murine gonadotrope-derived cell lines derived by the Mellon laboratory. These cell lines were generated through targeted expression of SV40 large T antigen in developing pituitary cells [110,279,294]. Both lines express the GnRHR as well as the  $\alpha$ GSU. In addition, the L $\beta$ T 2 cell line expresses the  $\beta$  subunits of LH and FSH [86], and may thus represent a somewhat more differentiated gonadotrope phenotype.

Due to the restricted pattern of expression of the GnRHR as well as the specificity of GnRH action to gonadotropes, many aspects of gonadotrope function, particularly transcriptional responses to GnRH stimulation, can be readily examined in primary cultures

of mixed pituitary cells generated by enzymatic dispersal of fresh pituitary tissue. However, measurements of GnRH-induced changes in transcript levels in primary culture systems must be interpreted with caution, since expression of a gene of interest in cell types other than the gonadotrope will contribute to baseline transcript levels within an experimental unit, and can lead to underestimation of the effects of hormone stimulation on the sub-population of gonadotropes within that unit. Alternatively, protocols for purification of gonadotropes from dispersed pituitary tissue and their propagation in primary culture have been described; however, they are technically demanding [158]. Two intriguing new models for identification of gonadotrope cells *in vivo* make use of genetically modified mouse models. One approach has been to use the ovine FSH $\beta$  gene promoter regulating the expression of H-2K<sup>k</sup> gene, a major histocompatibility protein that can be expressed on the surface of FSH $\beta$  expressing cells such as gonadotropes [297]. Since the H-2K<sup>k</sup> protein is present on the cell surface, it can be effectively targeted using commercially available H-2K<sup>k</sup>-specific antibodies for immunologically-based cell enrichment. Alternatively, GRIC (GnRH receptor IRES Cre) mice have Cre recombinase knocked into the GnRHR gene locus along with an internal ribosome entry site (IRES) to facilitate Cre recombinase expression in cell types endogenously expressing the GnRHR [288]. When bred to reporter mice, the GRIC Cre expression effectively allows for fluorescent marker expression in gonadotropes. Using the GRIC model system along with fluorescent reporter mouse lines, future studies may focus on fluorescently-labeled gonadotropes which could be greatly enriched by methods such as flow cytometry.

Several genetically modified mouse models have been developed that shed light on the role of specific gene products within the pituitary. Constitutive gene ablation may result in a phenotype that reflects primary pituitary dysfunction. For example, despite its broad pattern of expression, mice lacking the immediate early gene *Egr1* display gender-specific infertility associated with a failure of LH production in the gonadotrope of female mice [146]. Alternatively, due to their more restricted pattern of expression, the promoters of genes encoding a number of the pituitary trophic hormones or cell type-specific receptors have been used to target transgene expression to specific pituitary cell lineages allowing conditional, Cre recombinase-mediated disruption of floxed alleles [16,33,34,82,201,288].

#### 3.4. Structural and functional aspects of the GnRH receptor

The GnRHR is a member of the large G-protein coupled receptor (GPCR) superfamily. Three forms of the GnRHR have been identified in vertebrates, designated types I, II, and III; three corresponding forms of GnRH (types I, II, and III) have also been identified [191]. The type I receptor is the predominant form expressed in the gonadotrope, and in some mammalian species including humans is also expressed in certain extra-pituitary tissues most notably the breast, gonads, prostate, and uterus [35]. The natural ligand for the type I receptor is type I GnRH; however, type II GnRH may also engage and activate the type I receptor [177]. Indeed, in several mammalian species, the type II GnRHR has been evolutionarily silenced at the genomic level. Thus, in humans, the biological effects of GnRH II or its analogs are thought to be mediated by the type I receptor [190].

The type I GnRHR was first cloned in 1992 from cDNA derived from the murine  $\alpha$ T3-1 gonadotrope-derived cell line [222,277]. Subsequent cloning of GnRHR's from human, rat, sheep, pig, and horse demonstrated that the receptor is highly conserved, with greater than 80% amino acid homology among these mammalian species [226]. The deduced sequence predicts a 327 (or, in some species, including humans, 328) amino acid (aa) receptor with seven

transmembrane domains, a 35 aa amino-terminal extracellular domain with 2 putative glycosylation sites, and a conspicuously short 1–2 aa carboxyl-terminal cytoplasmic domain. An additional glycosylation site is predicted within the first extracellular loop [226,277]. While the crystal structure of the GnRHR has not been determined, its homology with other members of the GPCR family, including rhodopsin, has allowed model-based predictions of the overall topology of the receptor, as well as the structural basis of its interaction with ligands [248]. Many of these predictions have been verified by site-directed mutagenesis studies in recombinant receptors expressed in cell culture systems. For example, alanine substitution of asparagine residues 18 or 102 (predicted glycosylation sites) led to decreased receptor glycosylation, and while this had no effect on ligand affinity, overall receptor expression was decreased, indicating a role for glycosylation of these residues in receptor stability or appropriate membrane targeting [48]. Similar methods have identified critical residues involved in ligand binding within the second and seventh transmembrane domains, as well as the third extracellular loop [72,73,127]. Insight into the structural basis of the interaction of the receptor with GnRH has facilitated the development of receptor antagonists, as well as high-affinity ligands that function as super-agonists [48]. Antide [N-Ac-D-Nal(2)<sup>1</sup>,d-pCl-Phe<sup>2</sup>,D-Pal(3)<sup>3</sup>,Lys(Nic)<sup>5</sup>,D-Lys(Nic)<sup>6</sup>,Lys(iPr)<sup>8</sup>, D-Ala<sup>10</sup>]GnRH and busarelin (des-GLY<sup>10</sup> [D-Ser(t-But)<sup>6</sup>] -LH-RH Ethylamide) are examples of GnRHR antagonist, and super-agonist, respectively, that are commonly used in molecular and cellular studies of gonadotrope function. Both compounds were used in the investigations described here.

The lack of a carboxyl-terminal intracellular domain renders the mammalian type I GnRHR a structurally and functionally unique member of the GPCR family [226]. The C-terminal tail domain of the prototypical GPCR is an important target for phosphorylation, typically mediated by a G-protein coupled receptor kinase (GRKs) [224]. C-terminal tail phosphorylation generates a docking site for members of the  $\beta$ -arrestin family of scaffolding proteins, which upon binding, mediate rapid desensitization and dynamin-dependent internalization of a receptor [163]. Lack of a C-terminal tail implies that the GnRHR may be resistant to rapid desensitization and internalization, and this has been confirmed experimentally for this receptor in a variety of settings [283,291,291,292]. In addition, numerous studies have demonstrated rapid desensitization and internalization of chimeric receptors in which the C-terminal tail of various GPCRs was fused to the C-terminus of the mammalian type I GnRHR [29,70,98,103,104,107,209,283]. Collectively, these results establish a causal link between the absence of the C-terminal tail and the delayed internalization kinetics of the type I GnRHR. Exposure of gonadotropes to GnRH does lead to receptor internalization; however, this process occurs more slowly than with other GPCR's, and appears to be independent of dynamin [108]. Indeed, the GnRHR has been described as a “naturally-occurring internalization deficient mutant” GPCR [180].

Appropriate plasma membrane expression of the GnRHR is of obvious importance for responsiveness of the gonadotrope to GnRH. In turn, surface expression of functional receptors requires proper intracellular folding, glycosylation, and trafficking of newly synthesized receptor proteins. Naturally-occurring mutations have been described in the human GnRHR gene that lead to hypogonadotropic-hypogonadism of variable clinical severity [269]. These mutations have been mapped to various locations throughout the length of the GnRHR coding sequence. Functional analysis of recombinant receptors harboring many of these mutations indicates that ligand binding and coupling to intracellular effectors are unimpaired, but that the mutant receptors undergo improper folding and either aberrant intracellular trafficking or premature degradation [145]. Loss of newly synthesized receptors due to stochastic processes of misfolding and premature degradation is

common for some membrane receptors, and underscores the dynamic nature of receptor biosynthesis and recycling [212,247]. In many instances, cell permeable ligand-mimetics may shift the equilibrium of this process in favor of effective receptor expression by improving the efficiency with which nascent receptors adopt a proper conformation during folding [211,287]. This has been shown for the GnRHR where treatment of cells with the peptidomimetic GnRH antagonist indole IN3 was able to establish GnRH responsiveness in cells expressing a variety of mutant receptors that otherwise were hypothesized to have undergone misrouting and premature degradation [145]. This has stimulated interest in the mechanisms underlying appropriate plasma membrane targeting of the human GnRHR as well as the development of pharmacological agents that would allow manipulation of cell surface receptor density by altering the efficiency of folding and trafficking.

### 3.5. GnRH-induced signal transduction in the gonadotrope

The signaling events initiated by engagement of the GnRHR have been the subject of considerable investigation and have recently been reviewed in detail [199]. As with other GPCR's, ligand binding induces a conformational change in the receptor that leads to dissociation and activation of a heterotrimeric G-protein which, in the pituitary, generally involves the  $G\alpha_{q/11}$  subunit [89]. While fluctuation of the receptor between active and inactive conformations is a fundamental process in GnRH signaling, recent work suggests that the receptor may be able to be stabilized in a number of different active conformations following the binding of different ligands [161,191,215]. The ability of these different conformations to lead to variable downstream signaling activities has led to the concept of ligand-induced selective signaling [191]. The molecular basis of selective signaling in response to specific ligands has been analyzed in the human GnRHR using point mutagenesis and domain swap techniques disclosing specific residues and extracellular domains that play key roles in determining ligand specificity [161,265]. Furthermore, elegant studies of the *Xenopus* GnRHR revealed a novel inside-out regulatory feedback mechanism in which agonist-induced activation of PKC led to an increase in the inherent affinity of the receptor for its ligand [31]. Interestingly, the human GnRHR was not subject to this feedback regulation; thus the significance of this inside-out signaling mechanism for gonadotrope responsiveness to hypothalamic GnRH remains unclear.

In the gonadotrope, GTP loading of  $G\alpha_{q/11}$  leads to activation of phospholipase (PLC) $\beta$ , and subsequent elaboration of the second messengers, phosphatidylinositol-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). Generation of IP<sub>3</sub> leads to an initial rapid rise in intracellular calcium concentration resulting from release of intracellular calcium stores. DAG leads to activation of PKC isozymes and contributes to a more sustained rise in intracellular calcium concentration, which derives from influx of extracellular calcium through L-type voltage gated channels [13]. These events are prerequisite for activation of mitogen-activated protein kinase (MAPK) activity, in particular the extracellular signal-regulated kinase (ERK) pathway [194] (see Fig. 1). The mitogen-activated protein kinase (MAPK) pathways are a highly conserved set of signal transduction cascades that mediate cellular responses to an enormous variety of environmental stimuli. In mammals, there are 4 predominant MAPK pathways: the ERK, jun-N-terminal kinase (JNK), p38, and ERK5/Big MAP kinase (ERK/BMK) pathways. The basic organization of these pathways is similar, consisting of a multi-level phosphotransfer system; activation of the pathway begins with phosphorylation of an upstream MAP kinase-kinase kinase (MAPKKK), which phosphorylates and activates an intermediate level MAP kinase kinase (MAPKK), which in turn activates the terminal MAP kinase (MAPK). In addition to these core kinases, numerous scaffolding and adaptor proteins have

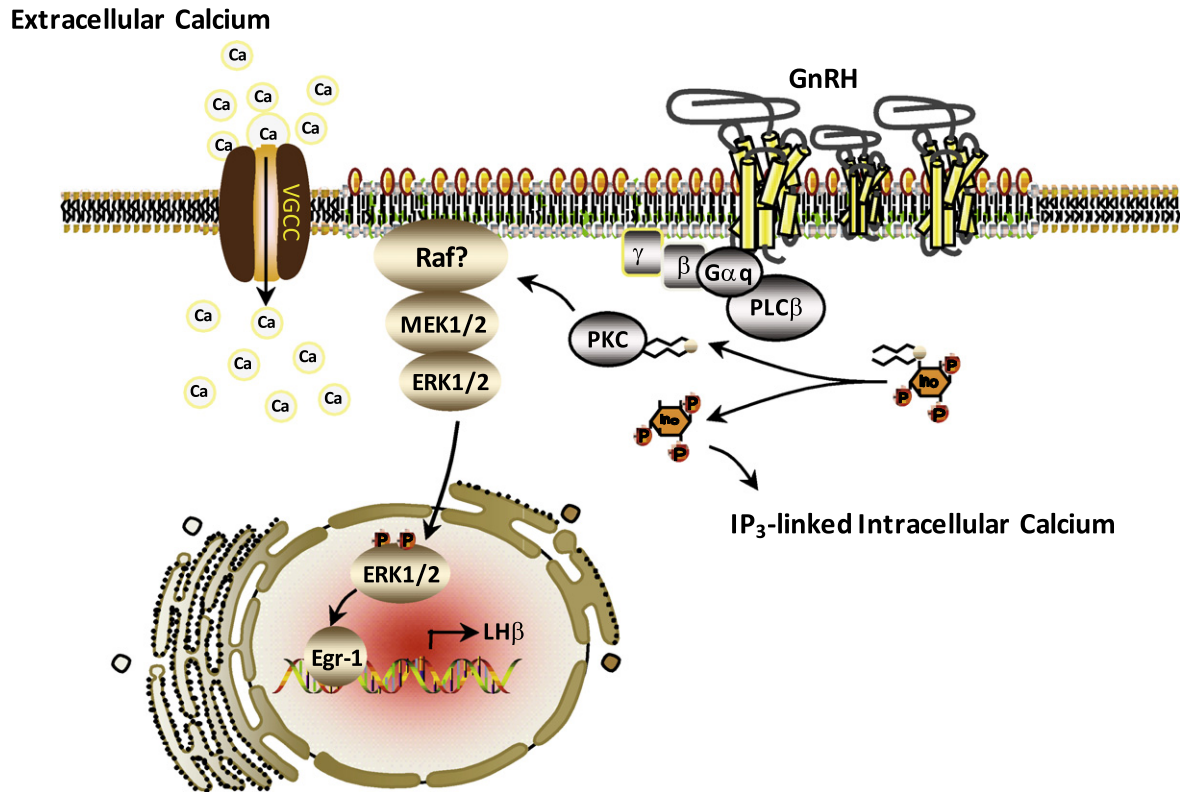
been shown to play important roles in the functional organization of this pathway [193]. Activated MAP kinases phosphorylate numerous substrates throughout the cell including elements of the transcriptional machinery, chromatin components, cytoskeletal structures, and other downstream enzymes [210,305].

#### 3.5.1. Membrane-associated signaling events

The delayed kinetics of GnRHR internalization underscores the importance of the plasma membrane as a platform for organization of the early signaling events that occur following ligand binding. This is in contrast to other GPCRs that undergo rapid internalization and that may nucleate active signaling complexes on the surface of endosomes or in association with cytoskeletal structures [80,183,184,252,274]. The GnRHR has been shown to couple to multiple G-proteins depending on cell type and experimental model, and controversy prevails regarding the relative importance of G-protein families for gonadotrope function. Studies based on over-expression of the GnRHR in heterologous cell systems indicate that the receptor is capable of interacting with both  $G\alpha_s$  and  $G\alpha_i$  subtypes; however, the *in vivo* relevance of these findings remains unclear [67,139,152]. Studies using the both  $\alpha$ T3-1 and L $\beta$ T 2 cell lines showed that GnRH stimulation leads to increases in intracellular cAMP; however, adenylate cyclase activation was apparently independent of  $G\alpha_s$  activation and was mediated by events downstream of  $G\alpha_{q/11}$  [89,157]. The ability of the GnRHR to couple to  $G\alpha_i$  in extra-pituitary tissues has been convincingly demonstrated, and signaling through  $G\alpha_i$  has been shown to play a role in regulating the pulse frequency of GnRH release from the hypothalamus [35,136,177]. However, the role of  $G\alpha_i$  signaling in the gonadotrope remains unclear. Overall, the signaling events initiated by GnRHR occupancy are thought predominantly to reflect the activation of  $G\alpha_{q/11}$  family members [89,112,250,251,261]. Notably,  $G\alpha_q$  and  $G\alpha_{11}$  appear to be redundant in their ability to mediate the effects of GnRHR activation since mice lacking either species of G-protein remain fertile [262].  $G\alpha_{q/11}$  comprises a group of pertussis toxin-insensitive palmitoylated GTP-binding proteins that lead to selective activation of PLC $\beta$  isoforms.

Detailed structural and functional analysis of protein domains within PLC $\beta$  indicate that these enzymes undergo constitutive and reversible membrane association mediated by the C-terminal region as well as the N-terminal plekstrin homology domain [58]. Activation of PLC $\beta$  does not involve membrane recruitment. Catalytic activity of PLC $\beta$  is stimulated by interaction with GTP-loaded  $G\alpha_{q/11}$  subunits, and leads to the elaboration of IP<sub>3</sub> and DAG within the plasma membrane. PLC $\beta$  also acts as a  $G\alpha_{q/11}$  GTPase activating protein (GAP) leading to negative feedback effects on the signaling input by stimulating hydrolysis of  $G\alpha_{q/11}$  bound GTP [36]. PLC $\beta$  isoforms are also present within the nucleus, where they catalyze a nuclear cycle of inositol-phosphate (IP) turnover that has been shown to be important in cellular responses to various mitogenic stimuli [173]. Interestingly, nuclear PLC $\beta$ 1 is a direct ERK substrate, and ERK-mediated phosphorylation of S982 within the C-terminal domain is required for PLC $\beta$ 1 activation by IGF-1 [302]. Of note, S982 resides within a regulatory region of the C-terminal domain that is also required for interaction with  $G\alpha_{q/11}$  [125]. Whether nuclear IP turnover plays any role in GnRH action, or whether GnRH-activated PLC isoforms are regulated by phosphorylation at the level of the plasma membrane is unknown.

In contrast to PLC $\beta$ , activation of PKC isoforms involves plasma membrane recruitment. Three major classes of PKC have been characterized, namely the conventional, novel, and atypical PKCs [273]. All are activated through the synergistic effects of DAG and phosphatidylserine. In addition, activation of the conventional PKCs requires calcium.  $\alpha$ T3-1 cells express a variety of PKCs representing all classes including the  $\alpha$ ,  $\beta$ ,  $\epsilon$ ,  $\theta$ , and  $\zeta$  isoforms [134,167,229]. Fractionation experiments indicated that the  $\alpha$ ,  $\epsilon$ ,



**Fig. 1.** Model for GnRH receptor (GnRHR) signaling within the pituitary gonadotrope that leads to LH biosynthesis. The GnRHR resides within discrete plasma membrane compartments (membrane rafts) characterized by increased presence of cholesterol (green within membrane) and sphingolipids (orange within membranes). Activation of the ERK signaling pathway occurs within the membrane rafts facilitated by local influx of extracellular calcium through voltage dependent calcium channels (VGCC).

and  $\zeta$  isoforms may be the major PKCs activated by GnRH in these cells [134].

In  $\alpha$ T3-1 cells, PKC activity is required for ERK pathway activation by GnRH; however the mechanism underlying this requirement is unknown [228,266]. Raf-1 kinase is widely believed to be the key upstream activator of the ERK signaling module in the gonadotrope, and direct activation of Raf-1 by PKC has been suggested as a mechanism by which GnRH-induced signaling events at the plasma membrane may link to an ERK cascade that ultimately converges on the nucleus [54,130]. Membrane recruitment is a critical step in the complex series of events that leads to Raf-1 activation; thus, recruitment and activation of Raf-1 by membrane-bound PKC is a conceptually attractive scenario. However, little evidence exists to support direct activation of Raf-1 by PKC. Furthermore, the importance of Raf-1 itself in GnRH-induced ERK activation has not been conclusively demonstrated, either in the  $\alpha$ T3-1 cells or L $\beta$ T 2 cell lines, or in vivo. The architecture of this portion of the GnRH signal transduction network, particularly the link between signaling activities at the plasma membrane and the ERK module, thus remain unclear.

### 3.5.2. Membrane compartments and the GnRHR

In contrast to the homogenous fluid mosaic model of cellular membrane organization originally proposed by S.J. Singer and G. Nicholson in 1972, contemporary views propose the plasma membrane to be a heterogeneous assembly of spatially and functionally distinct domains that may differ with respect to both lipid and protein composition [22,61,255]. This membrane raft hypothesis is predicated upon the notion that different lipid species within a membrane bilayer may differ in their affinities for each other and for membrane proteins, and that this may facilitate the formation of biochemically and biophysically distinct lipid domains for which

integral and peripheral membrane proteins may have different relative affinities. The GnRHR was shown to partition completely into low-bouyant density membrane fractions following sucrose density gradient centrifugation of both  $\alpha$ T3-1 cell homogenates and whole mouse pituitaries, suggesting that the receptor may have an inherent preference for a membrane raft environment [19,202] (see Fig. 1). Disruption of the biophysical properties of the plasma membrane through depletion of sterols uncoupled the GnRHR–G-protein complex from PLC $\beta$  and blocked the ability of GnRH to activate the ERK pathway. These observations led to the hypothesis that the ability of the GnRHR to signal appropriately is affected by the biophysical properties of the plasma membrane of the gonadotrope, and may require association with specialized plasma membrane microdomains such as cholesterol/sphingolipids enriched rafts.

Fluorescence recovery after photobleaching (FRAP) studies with fluorochrome labeled GnRHR showed that treatment with GnRH agonist, but not the GnRH antagonist, caused a dose-dependent decrease in the lateral mobility of receptors within the membrane [111]. Fluorescence resonance energy transfer (FRET) between receptors was not detected; however treatment with either GnRH agonist or antagonist caused a dose-dependent increase in FRET consistent with receptor dimerization [111]. This indicates that agonist-induced receptor clustering may facilitate coupling of the receptor with the downstream intracellular signaling apparatus. Consistent with this hypothesis, several key signaling intermediates known or presumed to play a role in the GnRHR–ERK pathway were also found to associate at least partially with low density membranes including G $\alpha$ q/11, calmodulin, isoforms of the 14–3–3 family of adaptor proteins, Raf-1, MEKs, and the ERKs themselves [19,202,227]. Thus, laterally segregated lipid microdomains within the plasma membrane of the gonadotrope may play an important



role in the regulation of signaling dynamics following ligand occupancy of the GnRH.

### 3.5.3. Calcium signaling in the gonadotrope

In the gonadotrope, GnRH induces a rapid biphasic elevation of intracellular calcium [263]. The initial sharp rise in cytosolic calcium reflects predominantly the release of calcium from intracellular storage depots, particularly the endoplasmic reticulum (ER). Following this initial spike, a more sustained phase of calcium elevation occurs, due primarily to influx of extracellular calcium via L-type voltage gated channels (VGCC; Fig. 1). Release of intracellular calcium from the ER is triggered by IP<sub>3</sub> through direct interaction with IP<sub>3</sub> receptors on the surface of the ER. Calcium influx through VGCC is dependent on the activity of PKC isozymes [195].

Calcium influx via VGCC is uniquely required for ERK activation in the gonadotrope, although the mechanisms underlying this requirement remain unclear [194,195]. In previous work from this laboratory, GnRH stimulation led to rapid calcium loading of the calcium sensing protein calmodulin, which was itself shown to be required for ERK activation independently of the calmodulin-dependent protein kinase CamKII [227]. In vitro reconstitution experiments indicated direct calcium-dependent interaction between calmodulin and Raf-1. In addition, calmodulin was also recovered from membrane rafts following sucrose density gradient centrifugation of  $\alpha$ T3-1 cell homogenates, suggesting that calmodulin may serve as a link between VGCC calcium and the ERK pathway, and that this linkage may be regulated through association with membrane rafts [227]. The cellular effects of calcium signals have long been known to be highly compartmentalized (calcium signaling via discrete microdomain) in a wide variety of excitable and nonexcitable cells [15]. Thus, our observations raise the intriguing possibility that the contribution of VGCC calcium to GnRH-induced ERK activation in the gonadotrope represents a similarly compartmentalized event, possibly organized through association with plasma membrane rafts.

Recent studies have also identified proline-rich tyrosine kinase 2 (Pyk2) as a calcium-dependent enzyme required for GnRH-induced ERK activation in both  $\alpha$ T3-1 and L $\beta$ T 2 cells. In L $\beta$ T 2 cells, Pyk2 was shown to act as a scaffold for the assembly of a signaling complex containing c-Src, Grb2, and Sos, and this complex was suggested to form a direct link between GnRH-induced calcium currents and canonical Ras-dependent activation of the ERK pathway [178]. Work from this laboratory also identified Pyk2 as a catalytic activity required for GnRH-induced ERK activation in  $\alpha$ T3-1 cells and showed that GnRH induces rapid phosphorylation of Pyk2 in mouse gonadotropes in vivo [300]. In agreement with previous results demonstrating a requirement for calmodulin for GnRH-induced ERK activation, this study documented a direct interaction between calcium-activated calmodulin and the catalytic domain of Pyk2 [227,300]. Pyk2 thus appears to play a key role in linking VGCC-mediated calcium signals with the ERK pathway in gonadotropes.

### 3.5.4. GnRH-induced ERK signaling

In the gonadotrope, GnRH stimulation leads to activation of the ERK, JNK, and p38 pathways [149,192,229,253]. Several studies link activation of these pathways to transcriptional regulation of the gonadotropin genes. A recent report indicates that ERK5/BMK is also activated by GnRH in the L $\beta$ T 2 cell line and may play a role in upregulation of FSH $\beta$  in the gonadotrope [151]. In the context of this review, we will focus on regulation of ERK activity by GnRH (Fig. 1).

The ERK pathway is involved in the transduction of signals emanating from a multitude of different cell surface receptors. The best characterized of these are the receptor tyrosine kinases (RTKs). Indeed, the ERK proteins were initially identified as kinase activities

induced in cells by RTK ligands including a variety of mitogenic peptides and insulin [17,264]. These ligands stimulate the ERK pathway through a classical mechanism involving activation of the low molecular weight GTPase Ras. The ability of ERKs to mediate the proliferative effects of RTK ligands has been demonstrated in a variety of settings, and has been the focus of intense investigation in the areas of basic cell biology as well as drug discovery and anti-cancer therapeutics [230,249]. The ERK pathway is also activated following engagement of many GPCR [289]. Agonists for these receptors include a wide variety of small molecules as well as numerous neurotransmitters, peptides hormones, cytokines, nucleotides and amino acids, ions, and lipids [238]. GPCRs are linked to a bewildering array of cellular functions. While the ERK pathway has been shown to be important in mediating the mitogenic effects of many GPCR ligands, ERKs also play a role in the specific responses of some highly differentiated cells to certain agonists [238,281].

The ability of the ERK pathway to elicit dramatically diverse biological responses in different contexts points to complex and varied mechanisms of signal interpretation that have evolved in cells. Early insights into ERK signal specificity came from studies of the PC12 rat pheochromocytoma cell line in which it was shown that sustained ERK activation induced by nerve growth factor (NGF) led to neuronal differentiation while transient ERK activation following EGF treatment elicited a non-differentiating proliferative response [170]. One established mechanism by which cells may interpret differences in ERK signal duration derives from study of the ERK-dependent immediate early response protein c-Fos. As a component of the dimeric transcription factor AP-1, c-Fos is capable of coupling ERK pathway activity with the expression of a wide variety of genes. Upregulation of c-Fos occurs rapidly following ERK activation; however, in the absence of post-translational modification, the newly synthesized protein undergoes rapid degradation. ERK-dependent phosphorylation of the C-terminus of c-Fos stabilizes the protein, prolonging its half-life and allowing its nuclear accumulation [196]. C-terminal phosphorylation also targets the protein for secondary ERK-dependent phosphorylation of internal residues 325 and 331, which enhances its transcriptional activation function [196]. However, this only occurs under conditions of relatively sustained ERK activation. These dual ERK-dependent phosphorylation events that sequentially stabilize and activate c-Fos provide an example of a simple but elegant mechanism by which cells may link ERK signals of variable duration with different transcriptional outputs.

Protein–protein interactions mediated through defined docking domains are also important in the establishment of ERK signal specificity. ERKs contain two key interaction domains: the common docking (CD) domain on the face of the protein opposite the catalytic site, and a separate interaction domain at the edge of the activation site [147,267]. The former binds to cognate ‘D’ domains of interactors such as ribosomal S6 kinases (RSK), MEK1/2, and certain MAP kinase phosphatases (MKPs)/dual-specificity phosphatases (DUSPs). The latter domain binds to the ‘docking for ERK [Phe-X-Phe]’ (DEF) domain of many ERK substrates including c-Fos and Egr1 [147,267]. Docking interactions are prerequisite for appropriate substrate recognition by ERKs and also guide the interaction of ERKs with various scaffolding and adaptor proteins. These interactions may contribute to signal specificity by confining ERK activity to particular subcellular compartments. For example, docking interactions underlie the nuclear translocation of activated ERKs that occurs in most cell types in response to various stimuli [23,124]. The MAPKs MEK 1 and 2 can play an important role in this process. The MEK proteins contain a nuclear export sequence that restricts them to the cytosol [78,79]. Under resting conditions, stable associations between MEKs and inactive ERKs mediated through D domain-CD domain interactions may tether ERKs within

the cytosolic compartment [78]. Upon pathway activation, however, the MEK–ERK association is destabilized allowing dissociation and nuclear translocation of activated ERKs.

In addition to their established tendency to undergo nuclear translocation, ERKs may also be recruited to extranuclear membranes, and may be activated within the context of membrane bound signaling complexes [271,275]. The DEF domain containing protein kinase suppressor of ras (KSR) is a scaffold protein that has been shown to facilitate Ras-dependent activation of membrane targeted ERKs through nucleation of signaling complexes containing all the core kinases of the ERK pathway [237]. KSR has also been shown to bind G $\beta\gamma$  dimers and to facilitate Ras-independent activation of membrane-associated ERKs following occupancy of certain GPCRs [46]. Docking interactions also underlie the organization of ERK signaling complexes by  $\beta$ -arrestins following desensitization and internalization of GPCRs [164]. Some data suggests that arrestin-associated complexes may serve to restrict activated ERKs to the cytosolic compartment thereby selectively targeting extranuclear substrates [164,272]. Similarly, formation of focal adhesions may involve the recruitment, activation, and retention of ERKs at sites of adhesion through interactions with p125-focal-adhesion kinase (FAK) or Pyk2 [71]. The activity of this pool of ERKs may be restricted to highly compartmentalized phosphorylation of cytoskeletal components of the adhesion complex.

### 3.5.5. Mechanisms of ERK activation by GPCR's

In the classical paradigm of GPCR signaling, ligand binding stabilizes the receptor in an active conformation leading to nucleotide exchange of its G-protein  $\alpha$  subunit and the dissociation of the  $\alpha$  subunit as well as the dimeric  $\beta\gamma$  subunit from the receptor. The free  $\alpha$  subunit then regulates the activity of various enzymatic effectors involved in the production of second messengers [81]. Historically, studies of GPCR signaling have emphasized the role of these receptors in highly differentiated cellular functions such as neurotransmission and excitation–contraction coupling. However, in recent years, appreciation of the role of GPCRs in cellular growth control has led to an interest in the mechanisms by which these receptors may activate established mitogenic pathways, in particular the ERK pathway [238].

The mechanisms of ERK activation by GPCRs vary considerably with both receptor and cell type. In some settings, GPCRs have been shown to cause phosphorylation and activation of RTKs such as the epidermal growth factor receptor (EGFR) or the platelet-derived growth factor receptor (PDGFR) in a process referred to as transactivation [243]. The most widely accepted view of RTK transactivation is based on the phenomenon of ectodomain shedding. According to this model, GPCR ligands lead to activation of ADAM-TS family transmembrane matrix metalloproteinases (MMPs) through the activity of various intracellular effectors such as calcium, reactive oxygen species, G $\beta\gamma$  dimers, PKC isoforms, and the non-receptor tyrosine kinases c-src and Pyk2. MMPs in turn catalyze the proteolytic release of membrane bound RTK ligands leading to conventional ligand-dependent “outside-in” activation of RTKs and canonical Ras-dependent activation of the Raf–MEK–ERK cascade [208,244]. One report demonstrated the importance of EGFR transactivation through ectodomain shedding for GnRH-induced ERK activation in  $\alpha$ T3-1 cells [233]. However, this may reflect a unique property of the specific cell clones used in those experiments as others, including ourselves, have been unable to demonstrate EGF responsiveness in  $\alpha$ T3-1 cells cultured in our laboratory. Similarly, c-Src has been implicated as a link between the GnRHR and the ERK pathway through the activation of Ras [13,178]. However, other reports indicate that GnRH-induced ERK activation is Ras-independent [223]. Thus, the mechanisms by which the unique GnRHR couples to the ERK module via ectodomain shedding and or activation of c-Src in vivo remain unclear.

The molecular architecture of this region of GnRH signaling cascade would therefore seem to be a promising area of investigation.

GPCRs may also regulate ERK activation through second messenger-dependent enzyme systems. For example, in the HEK293 cell model, activation of ERK through the endogenously expressed  $\beta(2)$ -adrenergic receptor was shown to involve cAMP-dependent activation of PKA, and subsequent activation of B-Raf via the small Ras-family GTPase Rap-1 [245].  $\beta$ -arrestins have been shown to be capable of linking numerous GPCRs to the ERK module through nucleation of signaling complexes containing internalized receptors along with all the core components of the ERK pathway [164]. Finally G $\beta\gamma$  dimers may couple to the ERK pathway independent of their cognate  $\alpha$ -subunit through the direct or indirect activation of the activity of phosphatidylinositol-3-kinase (PI3-kinase) or PLC $\beta$  [162].

In gonadotropes, GnRH stimulation leads to relatively transient activation of ERKs [155,200,228,253,266]. Studies on the mechanism of GnRH-induced ERK activation in gonadotropes have yielded conflicting results depending on the model systems employed. Nevertheless, some generalizations seem valid. Unlike other GPCRs, the GnRHR is not phosphorylated following ligand binding and the lack of a C-terminal tail precludes direct interaction with  $\beta$ -arrestins [109,291]. Therefore arrestin-mediated mechanisms of ERK activation are unlikely to apply [30]. In contrast, the requirement for extracellular calcium influx and activation of PKC isoforms for ERK activation has been widely demonstrated [195,200]. Previous work from this laboratory showed further that GnRH-induced ERK activation was blocked by inhibition of calmodulin [227]. Direct activation of Raf-1 by PKC is often cited as mechanism by GnRH may drive ERK pathway activation in a Ras-independent manner. This hypothesis derives largely from a study in which PKC was shown to phosphorylate Raf-1 in vitro. However, in light of the complex nature of Raf-1 activation, this hypothesis may be largely discounted [130]. Moreover, while Raf-1 is widely assumed to be the predominant upstream activator of the ERK pathway in gonadotrope cells, experimental evidence in support of this is lacking. Current views highlight the role of Raf-1 as an important regulator of apoptosis that is not required for ERK activation in all settings, and suggest that the alternative isoform B-Raf may be of greater overall importance as a MAPKKK than Raf-1 [5]. Indeed, within the pituitary, activation of the ERK pathway in both corticotrope and somatotrope cells following stimulation with the GPCR ligands corticotropin-releasing hormone (CRH) or growth hormone-releasing hormone (GHRH) does not involve Raf-1, but proceeds through a pathway involving G $\alpha$ s, PKA, Rap-1 and B-raf [133,234]. Similar studies of the role of Raf kinase isoforms regulating ERK activation in gonadotropes following GnRH stimulation have not been reported.

## 4. Signal transduction and the response of the gonadotrope to variation in GnRH pulse frequency

### 4.1. Cell signaling and integration of GnRH pulse frequency

Over two decades, clinical observation and experimental study have confirmed the requirement for pulsatile GnRH stimulation for appropriate gonadotrope function and fertility [12,172,172,278]. Excessively low GnRH pulsatility leads to hypothalamic amenorrhea and infertility in women, while chronically high GnRH pulsatility contributes to infertility associated with the polycystic ovarian syndrome. Further, specific variations in GnRH pulse frequency are essential to the normal estrous cycle in females [96,221]. Lower frequency GnRH stimulation predominates over the follicular phase of the cycle favoring production and secretion

of FSH and follicular recruitment and growth, while a precisely coordinated transition to higher frequency stimulation is required to drive the preovulatory LH surge [92]. While pulsatile hormone release is a common feature of many endocrine tissues, the striking responsiveness of the gonadotrope to GnRH pulse frequency has made this cell an attractive model for investigation of the mechanisms underlying stimulus-frequency interpretation [282].

While the specific biochemical processes by which the gonadotrope mounts specific responses to changing GnRH pulse frequencies have not been clarified, selective activation and termination of signaling activity is thought to play a major role in translating a given GnRH pulse frequency into selective expression of a given gene [69]. Calcium signaling has been implicated in pulse frequency interpretation in the gonadotrope [25,94]. Exposure of perfused rat pituitary cells to pulses of the calcium channel agonist BayK8644 revealed highly specific transcriptional response patterns of the gonadotropin subunits, with more rapid calcium pulse frequencies leading to preferential expression of  $\alpha$ GSU and LH $\beta$ , while slower pulse frequencies caused preferential expression of FSH $\beta$  [95]. Indeed the patterns of gonadotropin transcriptional response in this system were very similar to the patterns of response seen with GnRH stimulation. Activation of calmodulin by calcium loading constitutes an important signaling hub in the gonadotrope, and results from our laboratory show that calmodulin loading is required for ERK activation by GnRH [227]. Calmodulin loading also leads to rapid activation of calcium/calmodulin-dependent kinase II (CamK II) in the gonadotrope [90,93]. GnRH has been shown to induce oscillatory calcium signals in the gonadotrope, and the activation of CamK II is sensitive to both the frequency and amplitude of intracellular calcium oscillations [49,276]. The activation of CamK II is not itself dependent on GnRH pulse frequency; however, the rapid deactivation kinetics of CamK II suggest that sustained CamK II activity may occur preferentially under conditions of rapid GnRH pulsatility, which is consistent with a role for this enzyme in mediating differential responses to variable and physiologically relevant GnRH pulse frequencies [25,90,93]. Therefore, CamK II is an attractive candidate molecule with respect to the ability of the gonadotrope to decode GnRH pulse frequency.

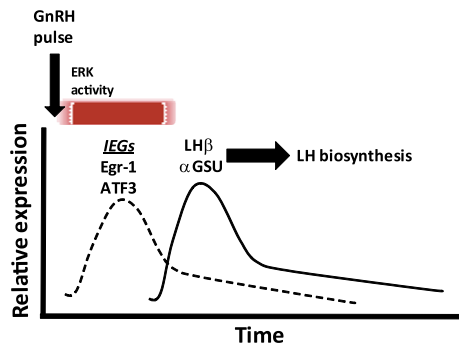
The MAP kinase pathways, most notably the ERK signaling system, have also been implicated in GnRH pulse frequency interpretation in the gonadotrope [25,26,117]. In perfused L $\beta$ T 2 cells as well as primary gonadotropes, slower pulse frequency of GnRH stimulation led to a more rapid onset and sustained pattern of ERK activation as compared with higher frequency stimulation. In addition, slower pulse frequencies resulted in higher levels of nuclear phospho-ERK, suggesting that ERK-dependent transcriptional processes may be enhanced under conditions of lower pulse frequency [117]. Interestingly, pharmacological inhibition of ERK signaling in perfused L $\beta$ T 2 cells as well as in primary pituitary cell cultures blocked the stimulatory effect of GnRH on both FSH $\beta$  and LH $\beta$  transcription [117]. The ERK-dependence of LH $\beta$  transcription is consistent with many previous studies showing the importance of the ERK-dependent transcription factor Egr1 for LH $\beta$  expression [57,74,142,146,296]. In contrast, the role of ERK signaling in mediating upregulation of FSH $\beta$  by GnRH is less established and the observed requirement for ERK activity for FSH $\beta$  expression may appear difficult to reconcile with our data showing that FSH $\beta$  expression remains largely intact in mice with ERK-deficient gonadotropes [18]. However, the perfusion system in which ERK-dependence of FSH $\beta$  expression was observed was designed to test the effects of GnRH in isolation from other endocrine signals, particularly activin. Thus, whether our results represent an inherent ERK-independence of FSH $\beta$  expression in differentiated gonadotropes in vivo, or whether a collateral activin-mediated mechanism supporting FSH $\beta$  expression is at play in this setting is unclear.

#### 4.2. Intracellular negative feedback and phosphatase activity

The mechanism by which slower GnRH pulse frequency leads to more sustained ERK activation may ultimately be linked not only to feed-forward activation kinetics, but also to intracellular feedback processes of ERK signal termination. The dual-specificity phosphatases (DUSPs) are a heterogeneous family of regulatory phosphatases that bind and dephosphorylate MAP kinases through D-domain-dependent interactions. As such, the DUSPs have received much attention as negative regulators of MAP kinase signaling [3,51,51,53,119,308]. Microarray analysis showed that DUSPs 1 and 4 (MKP1 and 2) are upregulated following GnRH stimulation in L $\beta$ T 2 cells [299]. Results from this laboratory have shown further that DUSPs 1 and 4 are upregulated by GnRH both in  $\alpha$ T3-1 cells and in vivo and that this upregulation is correlated with decreases in both ERK and JNK activity [308]. DUSP 4 upregulation in  $\alpha$ T3-1 cells was itself dependent upon ERK activation and Egr1 expression, pointing to a potential feedback loop underlying termination of the ERK signal [309]. We have observed that DUSP4 transcript levels are not increased in response to GnRH stimulation in primary pituitary cell cultures from mice with pituitary-targeted deletion of ERK 1 and 2, as they are in cells from control animals (S Bliss & M Roberson, unpublished results). However, more recent results using heterologous expression of the GnRHR in HeLa cells have challenged this notion, showing that dephosphorylation of GnRH-activated ERK, while dependent on de novo protein synthesis, is not affected by combined suppression of DUSPs 1, 2, and 4 accomplished through the use of siRNA [3]. Interestingly, in this same system, the more sustained ERK activation that occurred following specific activation of PKC was sensitive to the effects of DUSP knockdown. Interpretation of our own studies in  $\alpha$ T3-1 cells as well as results in HeLa cells is complicated by potential cell-type specificity and the in vitro nature of the studies. Indeed it is notable that the murine DUSP knockout models that have been reported to date (involving DUSPs 1, 2, 10, and 14) are all fertile [242]. Thus, the potential for functional redundancy between DUSP family members is likely a complicating issue in the determination of the role of phosphatase shaping of the magnitude and duration of the ERK response to GnRH stimulation.

#### 5. Importance of ERK signaling for gonadotrope function and fertility

In gonadotropes, a substantial fraction of active ERK undergoes rapid nuclear translocation, and numerous studies using a variety of cellular models point to the importance of ERK signaling in GnRH-induced transcriptional responses [59,75,155,178,194,300,301]. The immediate early gene response to GnRH stimulation was examined by microarray analysis in the L $\beta$ T 2 cell line where 28 immediate early genes were shown to be significantly upregulated within 60 min of GnRH exposure [299]. The role of ERK signaling in the induction of several of these genes, including c-Fos, Egr1, and LRG21/ATF3, is established [59,155,227] (Fig. 2). As a component of the dimeric transcription factor AP-1, c-Fos has been implicated in GnRH-induced expression of FSH $\beta$ , as well as in homologous upregulation of the GnRHR [44,45,284,290]. Egr1 plays an established role in the transcriptional upregulation of LH $\beta$ , and also contributes to the expression of the dual specificity phosphatase MKP2/DUSP4 [296,309]. ERKs have also been shown to contribute to GnRH-induced transcriptional upregulation of the  $\alpha$ GSU through putative phosphorylation of Ets family transcription factors associated with the  $\alpha$ GSU promoter [228]. ATF3 may also enhance expression of at least the human  $\alpha$ GSU through dimerization with c-Jun [301]. ERK signaling is thus linked to the expression of the full complement of genes (GnRHR, FSH $\beta$ , LH $\beta$ ,  $\alpha$ GSU) that comprise the



**Fig. 2.** Temporal relationship between transient ERK activation, immediate early gene (IEGs) upregulation and activation of late genes that lead to LH biosynthesis following a pulse of GnRH.

genetic signature of the gonadotrope. Whether ERK signaling contributes to the expression of other members of this immediate early gene repertoire in the gonadotrope remains to be determined.

Despite the vast amount of data that has been published regarding the organization and function of the ERK signaling pathway in a variety of cellular models, little is known about the importance of ERK signaling for the function of differentiated cells in vivo. This is particularly true with respect to GnRH-induced ERK activation in the gonadotrope. Much of the data on ERK signaling in the gonadotrope is based on the assumption that ERK1 and ERK2 are functionally redundant; thus the question of whether ERK1 and ERK2 serve differential roles in these cells remains largely unexplored. However, it has been shown that ERK1 and 2 can serve quite distinct roles in some differentiated cell types [179]. In addition, while the ERK1 null mouse is viable and fertile and displays only subtle phenotypic defects, attempts to address the in vivo requirements for ERK signaling generally have been complicated by the embryonic lethality of the ERK2 null genotype [203,303].

To address the requirement of ERK signaling for the function of differentiated gonadotropes in vivo, and to evaluate the functional redundancy between ERK1 and ERK2 in these cells, our laboratory recently developed a murine model of gonadotrope-targeted ERK pathway ablation [18]. This model was generated by crossing  $\alpha$ GSU-Cre expressing animals with mice harboring a floxed allele at the ERK2 locus. In a background of normal ERK1 alleles, both male and female mice with ERK2-deficient gonadotropes (conditional ablation) were viable and fertile. In light of the lack of reproductive phenotype in the ERK1 null mouse, these results indicated that either ERK1 or ERK2 are capable of supporting gonadotrope function in a redundant manner in vivo. Crossing the gonadotrope-targeted ERK2 conditional knockout onto the ERK1 null background generated ERK1/2 compound knockouts at significantly less than the expected Mendelian frequency, suggesting that early and promiscuous expression of the Cre recombinase (as outlined earlier) may have impacted viability in a subset of these animals. However, in compound knockouts that survived to adulthood, males were reproductively normal, while females demonstrated anovulatory infertility. Exogenous replacement of gonadotropins effectively rescued the anovulatory phenotype. These studies also made use of the increased frequency of GnRH pulsatility following gonadectomy (classical ablation studies effectively removing gonadal steroid negative feedback) to examine if GnRH hyperstimulation of the gonadotrope resulted in normal upregulation of gonadotropin subunit genes and gonadotropin secretion in the absence of ERK signaling. The female infertility reflected primarily a lack of both basal and inducible synthesis of LH $\beta$  and LH secretion, and this defect was in turn linked to a failure in the ability of gonadotropes to upregulate Egr1 in response to GnRH. ERK signaling was also shown to be required for normal

expression of the common  $\alpha$ GSU in this paradigm. In contrast, expression of FSH $\beta$  and the GnRHR transcripts were relatively maintained in ERK-deficient animals of both genders following castration. Interestingly, despite interruption of the ERK pathway in thyrotropes, ERK1/2 compound null animals of both genders remained euthyroid, indicating that ERK signaling is not required for thyrotrope function. These results indicated that GnRH-induced ERK signaling is essential for fertility in the female mouse, and the sexually dimorphic requirement for this signaling pathway appeared to reflect the importance of ERK activity and Egr1 inducibility in mediating the transcriptional response of the gonadotrope to high frequency GnRH stimulation, the subsequent accumulation of LH within the gonadotrope necessary for the generation of the LH surge at the time of ovulation. Accumulation of high levels of LH within the male gonadotrope is simply not necessary to maintain normal male reproductive function.

## 6. Conclusions and future directions

Over the past two decades, a great deal of mechanistic detail has emerged from research examining GnRHR behavior and GnRH actions in pituitary gonadotropes leading to the induction of a complex signaling network largely attributable to the creation and utility of gonadotrope cell lines such as  $\alpha$ T3-1 and L $\beta$ T 2 cells. The GnRHR is a truly unique member of the GPCR superfamily, playing a central role in the regulation of gonadotrope cell function. The GnRHR is an exclusive and constitutive occupant in discrete membrane rafts and productive signaling at least to the ERK pathway requires this compartmentalized membrane association and organization. This need is clearly compatible with the need for putatively localized calcium signals supporting ERK activation. However, integration of ERK signaling in gonadotropes in response to GnRH at the level of PKC and specific MAPKKK involved is not currently clear and remains the focus of our continued efforts. Future studies will be greatly augmented with our ability to isolate responses to GnRH and ERK signaling using the GRIC-Cre mouse line both to facilitate gene excision exclusively in gonadotropes as well as visualize gonadotrope populations with the use of fluorescent reporter mouse lines. Collectively these studies are leading toward elucidation of a more complete understanding of the immediate early gene repertoire contributing to late expression of gene products that define the behavior and identity of the gonadotrope.

One of the goals of this review was to outline the importance of GnRH-induced ERK signaling within the gonadotrope of the anterior pituitary. However, the role and requirement of ERK signaling within each tiered level of the HPG axis is of considerable importance. This is exemplified by the recent elegant studies of a double ERK conditional knockout within ovarian granulosa cells recently reported by the Richards group [66]. These studies provide intriguing evidence that gonadotropin-induced ERK signaling within ovarian granulosa cells is required for resumption of meiosis by the oocyte, ovulation and subsequent luteinization of follicle cells following ovulation. Like our studies of GnRH-induced ERK activation and upregulation of Egr1 activity regulating LH $\beta$  subunit expression in the anterior pituitary, LH-induced ERK activity and subsequent induction of C/EBP $\beta$  activity underlies a critical pathway directing ovulation and luteinization within the ovary. Thus, ERK activity is required for normal fertility at both the pituitary and ovarian levels within the HPG axis. Future studies will focus on a possible role of ERKs within hypothalamic neurons and germ cells themselves. Clearly the enticing prediction is that ERK signaling integrates control of fertility at several levels with the HPG axis, at least in female mice.

While GnRH signaling through the ERK pathway is clearly central to endocrine regulation of fertility, this signaling cascade

reflects only a small portion of the global signaling network induced by a pulse of GnRH. Studies focusing on the role and requirement of JNK and p38 MAPK signaling may well be highly informative and set the framework for pharmacological and therapeutic manipulations to both inhibit and enhance fertility.

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