GONADOTROPIN-RELEASING HORMONE RECEPTOR SIGNALING AND FUNCTIONS

EDITED BY: Ivana Bjelobaba, Stanko S. Stojilkovic and Zvi Naor
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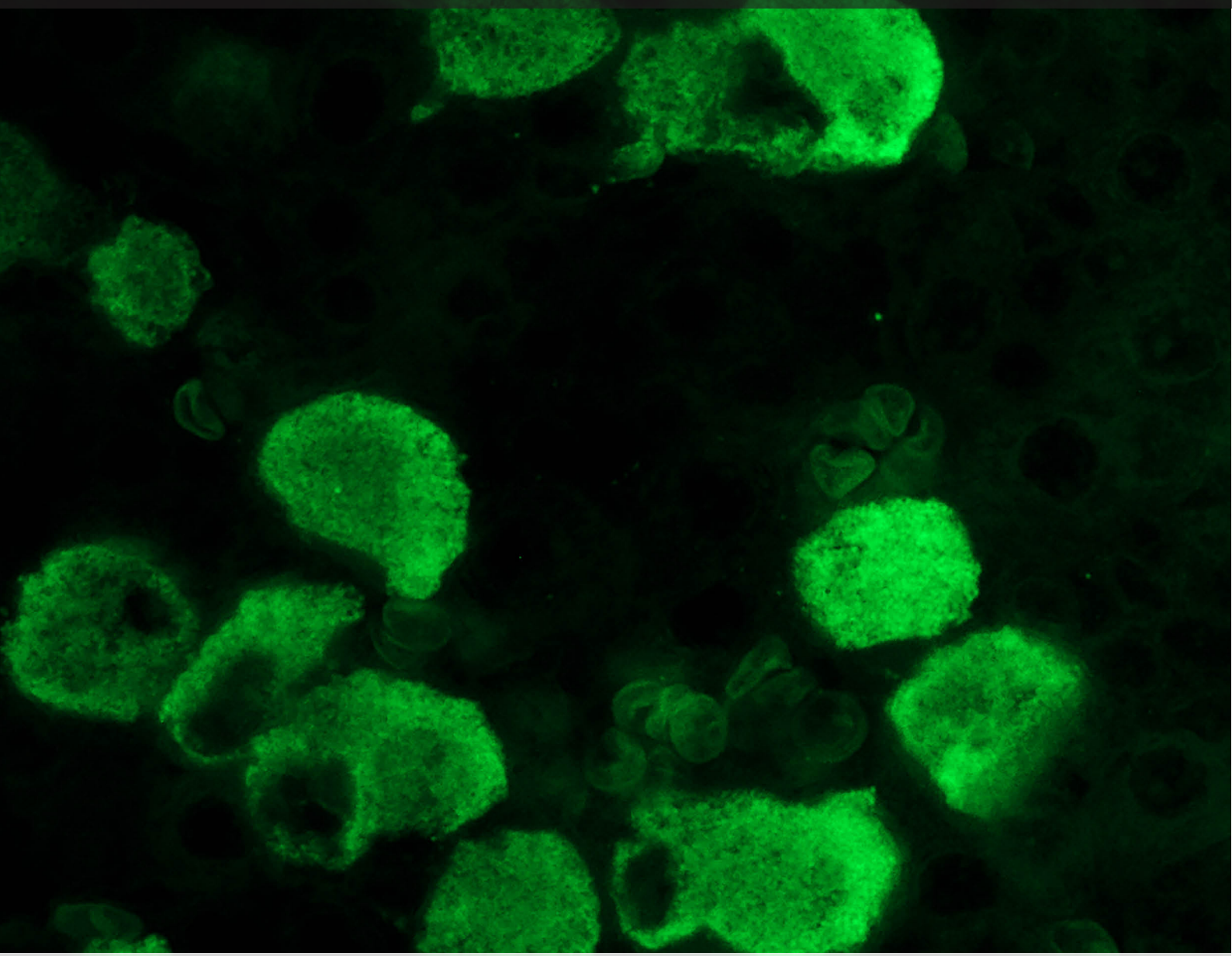

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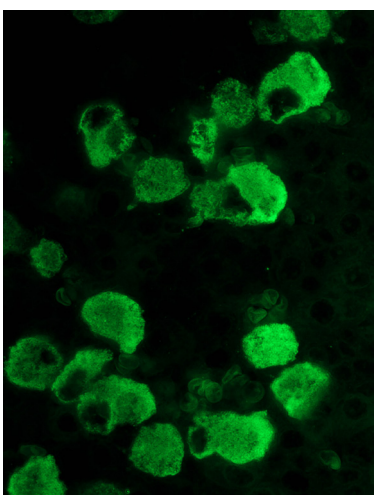
GONADOTROPIN-RELEASING HORMONE RECEPTOR SIGNALING AND FUNCTIONS

Topic Editors:
Ivana Bjelobaba, University of Belgrade, Serbia
Stanko S. Stojilkovic, Eunice Kennedy Shriver National Institute of Child Health and Human Development, United States
Zvi Naor, Tel Aviv University, Israel



Adult male rat gonadotrophs in the anterior pituitary gland, immunostained for LHβ gonadotropin subunit. Gonadotrophs are the only cells in the pituitary gland that express Gonadotropin-releasing hormone receptor.

Image: Ivana Bjelobaba.

This eBook provides a comprehensive overview of our current knowledge on Gonadotropin-releasing hormone receptor evolution, structure, signaling and functions. Apart from review articles, it comprises exciting new research, as well as hypotheses and perspectives, all of which are valuable in guiding our further research in this field.

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Table of Contents

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EDITORIAL
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Editorial: Gonadotropin-Releasing Hormone Receptor Signaling and Functions

Ivana Bjelobaba[1], Stanko S. Stojilkovic[2] and Zvi Naor[3]*

[1] *Department of Neurobiology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Belgrade, Serbia,*
[2] *National Institute of Child Health and Human Development, National Institute of Health, Bethesda, MD, United States,*
[3] *Department of Biochemistry and Molecular Biology, Tel Aviv University, Tel Aviv, Israel*

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Editorial on the Research Topic

Gonadotropin-Releasing Hormone Receptor Signaling and Functions

The hypothalamic decapeptide gonadotropin-releasing hormone, termed GnRH or GnRH1, and its receptor expressed in pituitary gonadotrophs, termed GnRHR or GnRHR1, play a central role in vertebrate reproduction. In gonadotrophs, GnRHR activation leads to $InsP_3$-dependent oscillatory calcium signaling and protein kinase C activation, accompanied with periodic changes in electrical activity and voltage-gated calcium influx. These receptors also trigger multiple lipid-derived messengers and mitogen-activated protein kinases activation, ultimately controlling transcription of numerous genes and gonadotropin secretion. Unlike other vertebrate GnRHRs and all other G-protein coupled receptors, mammalian GnRHRs lack a C-terminal tail, which makes them more resistant to desensitization and internalization. This peculiarity led to research of GnRHR trafficking and subsequent identification of mutations that are affecting human fertility. Also, the GnRH ligand–receptor system became an important target in assisted reproductive technologies in humans and domestic animals and in some cancers, prostate cancer in particular.

GnRHRs appear early in the evolution of invertebrates; their natural ligands are beginning to emerge, and their functions are not necessarily related to reproduction. Diverse forms of GnRH and GnRHR have also been identified in vertebrates, including GnRH2 and its receptor GnRHR2. The vertebrate GnRHR is also found in extrapituitary sites, including central nervous system, reproductive tissues, and cancer cells derived from such tissues. The enhanced interest for the extrapituitary GnRH ligand–receptors systems also comes from the findings that they mediate antiproliferative and/or proapoptotic effects and may, therefore, be directly targeted in cancer therapy. This collection of original research articles, reviews, perspectives, and hypotheses and theories summarizes well our current knowledge of GnRHR evolution, structure and regulation, mechanisms of pulsatile GnRH release, the roles of GnRH ligands and receptors in cellular functions, and practical application of this knowledge.

In vertebrates, activation of GnRHR in gonadotrophs and gonadotropin release relies on pulsatile GnRH secretion from the hypothalamus, but the mechanism underlying these pulses is still not well characterized. In her review, Constantin summarizes the current knowledge of the physiology of hypothalamic GnRH neurons in terms of excitability and secretion and the proposed mechanisms for synchronization of electrical activity to generate pulsatile GnRH release.

The crystal structure of GnRHR is not yet resolved, which limits our understanding of GnRH binding to its receptors in gonadotrophs and their subsequent activation. In their review article, Flanagan and Manilall discuss how the crystal structures of related GPCRs could help in clarification

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Hubert Vaudry,
Université de Rouen, France

****Correspondence:***
Ivana Bjelobaba
ivana.bjelobaba@ibiss.bg.ac.rs

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of GnRHR structure, ligand binding to the receptor ectodomain and transduction of signals to cytoplasmic domain and heterotrimeric $G_{q/11}$ proteins.

Pituitary gonadotrophs are excitable cells and various voltage- and ligand-gated and related channels provide a background pathway for spontaneous firing of action potentials and calcium signaling. Activation of GnRHR and other calcium-mobilizing receptors in these cells leads to calcium release from endoplasmic reticulum through IP_3R channels coupled with a rapid gonadotropin secretion. This is followed by switch in the pattern of firing of action potentials from tonic single spiking to periodic plateau bursting, the latter being essential for sustained calcium signaling and gonadotropin release. Overview of these mechanisms and ion channels in gonadotrophs is given by Stojilkovic et al.

Two articles in this issue deal with GnRHR-mediated cell shape remodeling. Edwards et al. nicely summarize the gonadotroph cell network and plasticity *in vivo*. GnRH engages the actin cytoskeleton to not only increase cell movement but also causes membrane remodeling events in the form of membrane ruffles, filopodia, and lamellipodia, in order for the cell to gain access to the vasculature. The authors stress that the mechanisms of actin polymerization and activation of mitogen-activated protein kinases are still elusive.

On the other hand, Rahamim-Ben Navi et al. show that GnRH-induced membrane bleb formation in immortalized LβT2 gonadotrophs and mouse primary pituitary cells depends on ERK1/2 signaling. GnRHR, c-Src, ERK1/2, FAK, paxillin, and tubulin appear to be accumulated in the blebs, which the authors consider as yet another form of gonadotroph cell plasticity and as a normal response of cells to GnRH. Furthermore, members of the signalosome, which was previously described by the authors, migrate to the blebs, which are apparently involved in cell migration.

Three articles provide details about GnRH-induced gene expression in gonadotrophs. The article by Janjic et al. describes basal and regulated GnRHR gene transcription in mammalian gonadotrophs, and the role of GnRH in regulated transcription. In rat and mouse, GnRH-induced transcription of genes relies primarily on the protein kinase C signaling pathway, with subsequent activation of mitogen-activated protein kinases. In contrast to pulsatile, continuous GnRH application shuts off regulated but not basal transcription, suggesting that different branches of this signaling pathway control transcription.

GnRH–GnRHR signaling pathways regulate the expression of various other genes and evidence implies that this signaling also influences the chromatin organization of target genes. Melamed et al. integrate the latest findings on GnRH-induced alterations in the chromatin of gonadotroph signature genes. The authors stress that further characterization of epigenome of gonadotropin genes may have implications in fertility drug development as well as in cancer biology.

Tackling the exact same question and using combination of a novel gel bead-in-emulsion drop-seq method and genome-wide chromatin accessibility state, Ruf-Zamojski et al. bring us a sneak peek into epigenetic and single-cell transcriptional landscapes of LβT2 gonadotrophs during GnRH stimulation. The authors provide useful data sets, and report a putative FSH beta gene enhancer identified as highly open chromatin. Interestingly, while great variability in basal and GnRH-induced gene expression of individual cells was observed, the authors report no influence of cell cycle stage on the gene response to GnRH.

Two articles give further guidance on GnRH/GnRHR signaling in pituitary gonadotrophs from the physiological point of view. Odle et al. discuss the role of leptin in reproduction and bring us two hypotheses derived from the experiments in which leptin receptor gene expression in gonadotrophs was manipulated. The authors propose that the cyclic changes in pituitary GnRHR expression create a mechanism by which these cells are activated only when environmental conditions are optimal. They further suggested that leptin's role in the permissive regulation of the reproductive cycle depends on timed events that involve multiple interactive target cells in the hypothalamo–pituitary–gonadal axis.

Terasaka et al. argue that reactive oxygen species (ROS) were often overlooked when it comes to our understanding of GnRHR signaling in gonadotrophs. Indeed, ROS can interfere with different GnRHR-induced signaling events, including MAPK activation. Moreover, the authors show that the monounsaturated fatty acid oleate can induce mitochondrial ROS in LβT2 cells. The authors conclude that appreciating ROS signaling in gonadotrophs may give us insights into integration of stress signaling and the reproductive axis.

In contrast to GnRH/GnRHR, the roles of GnRH2/GnRHR2 remain largely unexplored; reflecting the absence of expression or function of this receptor in many mammals, including the most used experimental animals, rat and mouse. In their review article, Desaulniers et al. give an overview of GnRHR2 expression across the mammalian species, its structure and up-to-now revealed biological functions, which are diverse and not related to pituitary gonadotroph functions. The brain receptors may have a role in coordination of interactions between nutritional status and sexual behavior, whereas the gonadal receptors may contribute to the control of reproduction by stimulating steroidogenesis.

GnRH–GnRHR signaling pathway is also operative in other peripheral tissues. For example, in women GnRH influences gastrointestinal motility, while in rat prolonged GnRH treatment induces enteric neuron cell death. The review article by Ohlsson discusses the roles and potential mechanisms of GnRH action on the level of gastrointestinal tract. The author proposes further investigation for definitive confirmation of the presence of GnRHR(s) in the gut.

We now know more on GnRHR signaling in cancer cells and it is clear that signal transduction in different cancer cell lines does not include classical players of the GnRHR signaling cascade in gonadotrophs, as described in a review article by Gründker and Emons. Antiproliferative effects of GnRH and its analogs in cancer cells seem to rely on coupling of the receptor to $G_{i/o}$ signaling pathway and activation of a phosphotyrosine phosphatase.

Finally, Sakai et al. review is focused on structure and biological functions of GnRH, adipokinetic hormone, corazonin, and their related peptides in invertebrates. While GnRH regulates some aspects of reproduction in mollusks, it is clear that all of these interrelated peptides have other biological roles in invertebrates.

The authors advocate that further research in invertebrates, including sequence analyses in different species, will give us more information on evolutionary processes and biological significance.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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Progress and Challenges in the Search for the Mechanisms of Pulsatile Gonadotropin-Releasing Hormone Secretion

*Stephanie Constantin**

Cellular and Developmental Neurobiology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, United States

Fertility relies on the proper functioning of the hypothalamic–pituitary–gonadal axis. The hormonal cascade begins with hypothalamic neurons secreting gonadotropin-releasing hormone (GnRH) into the hypophyseal portal system. In turn, the GnRH-activated gonadotrophs in the anterior pituitary release gonadotropins, which then act on the gonads to regulate gametogenesis and sex steroidogenesis. Finally, sex steroids close this axis by feeding back to the hypothalamus. Despite this seeming straightforwardness, the axis is orchestrated by a complex neuronal network in the central nervous system. For reproductive success, GnRH neurons, the final output of this network, must integrate and translate a wide range of cues, both environmental and physiological, to the gonadotrophs *via* pulsatile GnRH secretion. This secretory profile is critical for gonadotropic function, yet the mechanisms underlying these pulses remain unknown. Literature supports both intrinsically and extrinsically driven GnRH neuronal activity. However, the caveat of the techniques supporting either one of the two hypotheses is the gap between events recorded at a single-cell level and GnRH secretion measured at the population level. This review aims to compile data about GnRH neuronal activity focusing on the physiological output, GnRH secretion.

Keywords: gonadotropin-releasing hormone release, preovulatory surge, gonadotropin-releasing hormone pulsatility, kisspeptin, electrophysiology, calcium imaging

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Alfonso Abizaid, Carleton University, Canada

Reviewed by:
Daniel J. Spergel, Yale University, United States
Zvi Naor, Tel Aviv University, Israel

***Correspondence:**
Stephanie Constantin
constantinss@mail.nih.gov

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INTRODUCTION

Fertility and its onset, puberty, are integrated phenomena. A complex network in the central nervous system (CNS), conveying physiological and environmental signals, converges onto neurons secreting gonadotropin-releasing hormone (GnRH). GnRH leads the hormonal cascade, driving gonadotrophs to secrete gonadotropins, which in turn control the gonads, i.e., steroidogenesis and gametogenesis in both sexes and ovulation in females. Thus, GnRH neurons are the output of the CNS for fertility, integrating and encoding cues into a signal readable by gonadotrophs, GnRH. However, GnRH neurons are not an on/off switch but a precise rheostat. GnRH secretion is pulsatile, with changes in amplitude and frequency over time. Yet, the mechanisms by which GnRH neurons generate pulses are unknown. This review summarizes recent data about GnRH neurons with a focus on secretion and the difficulty of answering this fundamental question.

Undoubtedly, cells upstream of GnRH neurons contribute to fertility by helping to provide the homeostatic conditions necessary for survival. However, in this review, the word *fertility* simply refers to the ability to generate gametes and offspring in optimal breeding conditions.

NEUROANATOMICAL DISTRIBUTION OF GnRH CELL BODIES

Gonadotropin-releasing hormone neurons derive from the olfactory placodes (1, 2) and migrate into the hypothalamus during prenatal development [reviewed in Ref. (3, 4)]. GnRH processes then extend toward the median eminence (ME) (5). This embryonic feature shapes the GnRH neuronal distribution (6). In mouse and rat, the distribution, centered around the preoptic area (POA) and the *organum vasculosum laminae terminalis* (OVLT), respectively (7, 8), is largely confined to the rostral forebrain. In monkey, it expands caudally to the mediobasal hypothalamus (MBH) (9, 10). However, data suggest that the location of the cell bodies is not important to trigger luteinizing hormone (LH) and promote fertility, as long as GnRH nerve terminals reach the hypophyseal portal system. In hypogonadal mice bearing a deletion in the *GnRH* gene (11), transplantation of fetal POA in the rostral third ventricle restores spermatogenesis (12) and pregnancies (13). Similarly, in female monkeys with lesioned MBH, menstrual cycles are restored with transplantation of olfactory placodes in the third ventricle (14). Notably, the pregnancies in mice receiving transplants are initiated by reflex, not spontaneous, ovulation (15), but still indicate gametogenesis and an ovulatory surge occur (16). Two possibilities, extrinsic to GnRH neurons, might explain the absence of spontaneous ovulation in transplanted mice: the required inputs (1) cannot reach transplanted GnRH neurons in their abnormal location and/or (2) are reduced/absent in hypogonadal mice (17). In contrast, in female monkeys, cyclicity was recovered since the inputs were present, i.e., the hypothalamic–pituitary–gonadal (HPG) axis was functional before its disruption. The next section addresses the distinct mechanisms for GnRH secretion leading to ovulation and gametogenesis.

GnRH SECRETION AND FERTILITY

Gonadotropin-releasing hormone neurons have two modes of secretion: surge triggering ovulation, restricted to females, and pulses regulating gametogenesis and sex steroidogenesis, in both sexes. In rat, 90% of GnRH neurons project outside the blood–brain barrier as indicated by Fluorogold retrograde labeling (18). In mouse, only 64% of GnRH neurons are labeled in intact animals but hormonal manipulation labels 88% (19). Unfortunately, peripheral injection of Fluorogold does not discriminate the uptake site. In addition to the ME (20), GnRH neurons exhibit branched processes beyond the blood–brain barrier into the OVLT (21). Thus, the hypophysiotropic proportion of the GnRH population is unknown. Lectin wheat germ agglutinin applied onto the ME reveals an uptake in up to 59% of GnRH neurons (22). While the majority of GnRH neurons probably connect to the ME, a specific number might be irrelevant since few GnRH neurons are needed to acquire and maintain fertility (12, 13, 23). Some GnRH neurons may project to other brain areas, in addition to or instead of the ME and OVLT, and may control additional functions (24, 25).

Puberty

Puberty is the developmental time an organism acquires its reproductive capacity. Physiologically, puberty coincides with activation of the HPG axis [reviewed in Ref. (26, 27)]. Although this review is not about puberty, I introduce kisspeptin-expressing neurons here (28–30), since puberty onset requires direct contacts onto GnRH neurons, *via* kisspeptin receptor (GPR54) (31).

Kisspeptin neurons are localized in two hypothalamic areas: rostral periventricular area of the third ventricle (RP3V) and the arcuate nucleus (ARC). Both subpopulations express the estrogen receptor alpha and the expression of *Kiss1* gene is sensitive to circulating sex steroids (32, 33). GnRH neurons do not express estrogen receptor alpha (34, 35) and cannot directly integrate gonadal steroid feedback (36). Hence, the role of kisspeptin neurons goes beyond puberty, contributing to fertility throughout life (37). Estradiol has opposite effects on *kiss1* gene expression in the RP3V and ARC in rodents (32, 33). This divergence serves the two GnRH secretory modes. Although the anatomical and functional segregation of the two kisspeptin subpopulations is not obvious in other species (38), rodents help decipher the mechanisms for surge and pulses.

Preovulatory GnRH Surge

The neurobiology of the preovulatory GnRH surge is reviewed in detail (39, 40). Only a subset of GnRH neurons generates the abrupt release of GnRH into the hypophyseal portal system. In rodents, activated GnRH neurons are immunocytochemically identified by immediate early genes (41, 42). In rat and mouse, ~40% of GnRH neurons, express cFos at the time of the surge (41, 43). Although the OVLT area contains most of the cFos-expressing GnRH neurons, they are found anywhere on the continuum caudal to the OVLT (41, 43). cFos-labeled GnRH neurons exhibit higher spine density (44), indicating increased inputs at the time of the surge. Furthermore, GnRH neurons display entwined dendrites with shared synapses (45), revealing common inputs, despite scattered cell bodies.

Although the surge is not regulated by a single neuronal population (40) and involves cells at the ME (46), kisspeptin is a powerful stimulator of GnRH neurons (47, 48) and direct inputs to GnRH neurons, *via* GPR54, is necessary (31). The RP3V neuronal subpopulation, larger in females and upregulated by estradiol (32), plays a critical role in the activation of GnRH neurons involved in the surge (49). Notably, although physiologically the preovulatory surge is only observed in females, it is not an intrinsic ability of female GnRH neurons but the consequence of female-specific inputs to GnRH neurons. RP3V kisspeptin neurons undergo sex-specific neonatal (50) and prepubertal (51) development, orchestrated by testosterone and estradiol, respectively. Hormonal perturbations altering the sexual dimorphism of RP3V result in LH surges in males and loss of LH surge in females (52).

GnRH Pulsatility

Gonadectomy releases the HPG axis from sex steroid negative feedback and reveals regular GnRH pulses (53–56). Pulsatility is a critical feature of GnRH secretion and is required for LH secretion (57, 58), underlying LH pulses (59). To date, GnRH pulsatility is still a confounding fact: how do scattered GnRH neurons synchronize to generate pulses? Two possibilities for a pulse generator exist: intrinsic, i.e., GnRH neurons generate pulses on their own or extrinsic, i.e., GnRH neurons are driven by other cell type(s). In the first scenario, synchronization requires connectivity between GnRH neurons, direct or indirect. In the second scenario, synchronization requires connections from a pulse generator to GnRH neurons.

Intrinsic Pulse Generator

The hypothesis of an intrinsic pulse generator comes from *in vitro* models for GnRH neurons: (1) mouse cell lines obtained by immortalization, GT1 (60), and (2) primary GnRH cells maintained in organotypic cultures of olfactory placodes, i.e., nasal explants (61–64). Without CNS inputs, these models exhibit pulsatile release of GnRH [GT1 (65–67); nasal explants (64, 68–70)]. One caveat is that nasal explants contain GABAergic and glutamatergic neurons that influence GnRH neuronal activity (71, 72). In both models, GnRH neurons exhibit action potentials (APs) (71, 73, 74) and fluctuations of intracellular calcium concentration ($[Ca^{2+}]_i$) (75–77), concomitant with bursts of APs (78). GnRH neurons in nasal explants also exhibit synchronized $[Ca^{2+}]_i$ oscillations (76, 77), supporting connectivity between GnRH neurons.

In immortalized cell lines, gap junctions mediate electrical coupling between GnRH neurons (79–81). Both $[Ca^{2+}]_i$ waves across GT1 cells (82) and pulsatile GnRH release (80) are gap junction dependent. However, this mechanism might be an adaptation of GT1 cells (83) since *in vivo* data reject coupling between GnRH neurons (84, 85). However, gap junctions between GnRH neurons (84) and surrounding cells (86) could allow signal propagation from one GnRH neuron to another and contribute to the synchronicity. In nasal explants, non-neuronal cells exhibit $[Ca^{2+}]_i$ oscillations (87) and blocking gap junctions impairs GnRH secretion (86). Hypothetically, if GnRH neurons were electrically connected *in vivo*, electrical activation of a subpopulation of GnRH neurons should propagate through the entire population and evoke an all-or-none GnRH/LH secretory response. However, a linear relationship exists between the number of optogenetically activated GnRH neurons and amplitude of LH pulse, refuting the hypothesis (88).

The alternative to electrical coupling is chemical coupling. GT1 cells on two coverslips within the same chamber exhibit a GnRH secretion profile identical to that of single coverslips, suggesting synchronization through diffusible molecules (66), such as adenosine triphosphate (ATP) or nitric oxide (NO). *In vivo* GnRH neurons express P2X purinoreceptors (89, 90). In nasal explants, ATP contributes to synchronization of GnRH neurons *via* P2X receptors (91), but not basal GnRH neuronal activity (72). In agreement, ATP facilitates, but does not evoke, GnRH release from isolated MBH (92). No physiological data support or refute the role of ATP. While NO contributes to pulsatile secretion at the ME *ex vivo* (93, 94), NO is released at the time of the surge *in vivo* (95, 96). Notably, GnRH neurons do not express NO synthase (NOS) (97), but NO might contribute to the synchronicity of GnRH neurons by modulating their firing in the POA (98). Both NO actions in the ME, *via* endothelial NOS (99, 100), and in the POA, *via* neuronal NOS (98, 101, 102), provide examples of cooperative microenvironments. However, GnRH neurons do not initiate the signal and the second possibility of other cell type(s) driving GnRH neurons dominates.

Extrinsic Pulse Generator

In vitro ARC–ME fragments exhibit pulsatile release of GnRH (103) and *in vivo* data support the role of the ARC in GnRH pulsatility (104). Increases in the frequency of multiunit activity (MUA) in the ARC are concomitant with LH pulses (105–107). The nature of the cells generating MUA volleys is unknown, but GnRH neurons or GnRH *en passant* fibers are not responsible for them. Estradiol-triggered GnRH surges (107) or kisspeptin-evoked GnRH secretion (108) do not trigger MUA volleys.

Mentioned earlier, the ARC kisspeptin subpopulation is proposed as a pulse generator [reviewed in Ref. (109)]. This subpopulation is not sexually dimorphic and is downregulated by sex steroids (32, 33). These kisspeptin neurons are the central players of an autoregenerative pulsing model. The two peptides they co-express, neurokinin B and dynorphin, are autocrine modulators providing on-/off-switches (110–112). Unfortunately, the model seems incomplete: (1) ARC kisspeptin neurons unequivocally provide an on-switch for GnRH neurons (113), but not an off-switch; kisspeptin evokes long-lasting electrical and calcium responses in GnRH neurons (47, 48, 114, 115), yet only a short activation produces a LH surge (88), (2) neurokinin B evokes GnRH secretion in kisspeptin knockout mice (116), and (3) neurokinin B is present in kisspeptin neurons in humans but dynorphin is not, thus the pulse generator might be species dependent (117). The model might be more convoluted since ARC and RP3V kisspeptin neurons are interconnected (118), co-express glutamate or GABA, respectively (119) and ARC kisspeptin neurons activate GnRH neurons by stimulating RP3V kisspeptin neurons *via* glutamatergic release (112). Thus, the mystery of GnRH pulses remains.

FROM GnRH NEURONAL ACTIVITY TO GnRH SECRETION

Elucidating how pulsatile GnRH secretion occurs is the key to understanding reproductive neuroendocrinology. However, measuring GnRH secretion is difficult. The GnRH neuronal population is small and a subset generates a pulse, therefore the amount of released GnRH is near threshold detection, even with sensitive radioimmunoassay (70). In addition, access to the hypophyseal portal system requires complex surgery and apparatus (53, 120–122), incompatible with the mouse. Finally, a half-life of GnRH is only 2–4 min. Thus, LH secretion, amplifying and diffusing the GnRH signal to the systemic circulation, is commonly used as a mirror of GnRH secretion (121, 123). However, pulsatile GnRH/LH release requires serial sampling and even LH measurements are hardly achievable with mouse

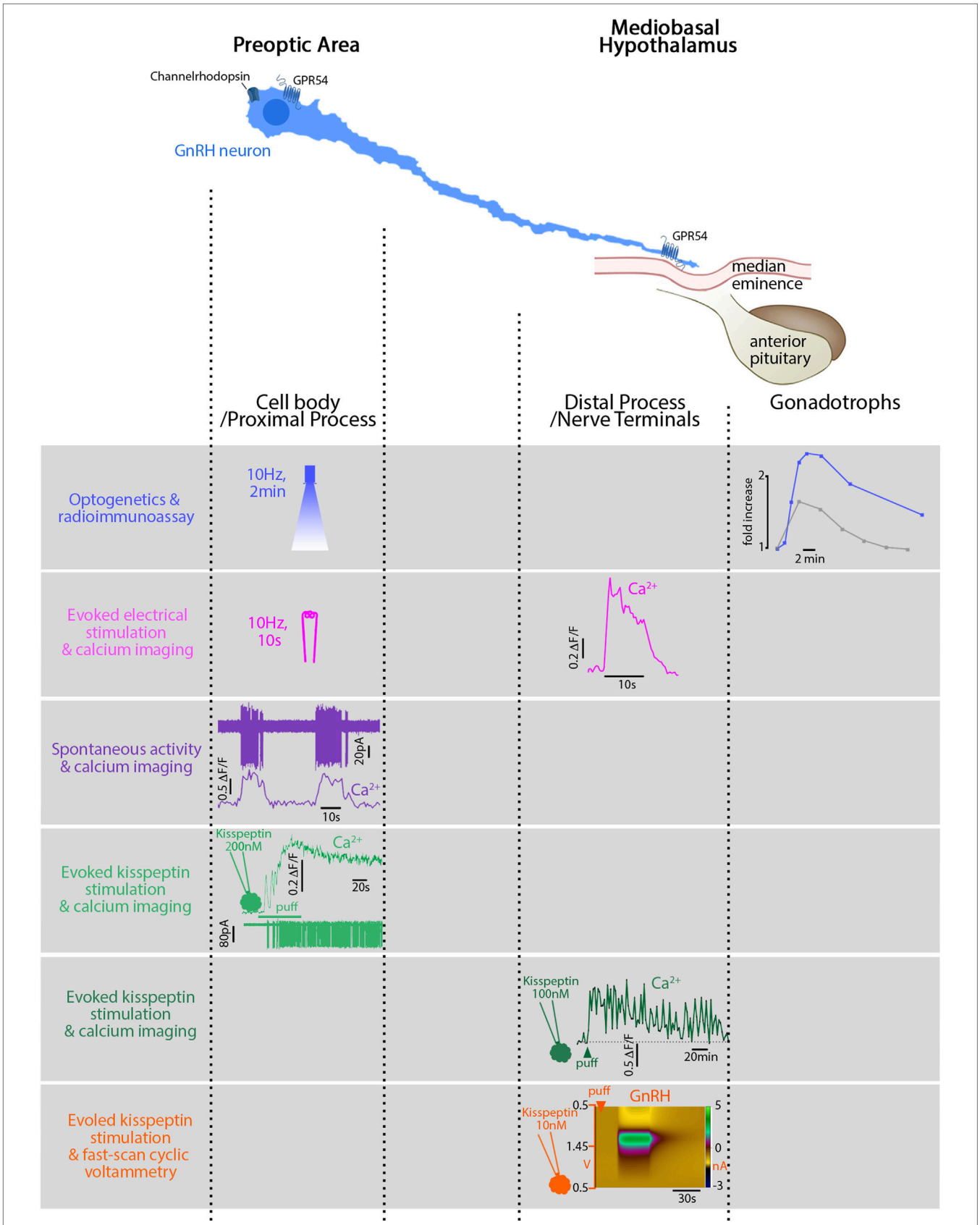

FIGURE 1 | Continued

FIGURE 1 | Continued
Relationship between electrical activity, intracellular calcium, and secretion in gonadotropin-releasing hormone (GnRH) neurons in mice. GnRH neurons can be divided into two main functional segments: cell body/proximal process, mainly in the preoptic area (POA), and distal process/nerve terminals, in the mediobasal hypothalamus. First row: blue light, flashed in the POA, electrically activates GnRH neurons expressing channelrhodopsin *in vivo*. The functional read-out reflecting GnRH secretion is the luteinizing hormone (LH) increase (blue trace) in the systemic circulation evoked by the gonadotrophs. The gray trace shows a spontaneously occurring LH pulse, much smaller. Second row: electrical stimulation of GnRH fibers activates GnRH neurons expressing genetically encoded calcium sensor GCaMP3 *in vitro* and evokes a calcium rise in the nerve terminals. Note the stimulus-restricted calcium increase. Third row: spontaneous action currents at the cell body evoke simultaneous rises in $[Ca^{2+}]_i$. Note: the difference between the frequency of spontaneous events (10 s, every 30 s) and the frequency of spontaneously occurring LH pulses [every 21 min in ovariectomized mice (124)]. Fourth row: Kisspeptin, locally applied at the cell body, binds to its cognate receptor, GPR54, and evokes a long-lasting calcium rise and train of action potentials (APs). Fifth row: Kisspeptin, locally applied at the nerve terminals, evokes a long-lasting calcium rise (>60 min) in GnRH neurons expressing genetically encoded calcium sensor GCaMP6s but no APs are required. Sixth row: Kisspeptin, locally applied at the nerve terminals, evokes secretion (~1 min). Figures adapted with permission of the authors [row 1 (88); row 2–5 (115); row 6 (141)].

blood volume (124). At a cellular level, the first challenge is anatomical: preserving the connectivity with relevant inputs (125), GnRH cell morphology (126), and tracking a neuron within the complexity of the ME (20). The second challenge is technical as methods for detection of quantal secretion are not applicable to GnRH neurons: (1) synaptically coupled neurons are recorded simultaneously in brain slices [reviewed in Ref. (127)], but GnRH neurons lack downstream partners, (2) patch clamp measurement of capacitive current is limited to soma and isolated nerve terminals (128, 129), therefore does not reflect hypophysiotropic GnRH secretion, and (3) fast-scan cyclic voltammetry (FSCV) is restricted to electrochemically active small neurotransmitters [reviewed in Ref. (130)].

Since techniques directly monitoring secretion cannot be applied to GnRH neurons, the alternative is to rely on the relationship between electrical activity, voltage-gated calcium channels, calcium, and secretion [reviewed in Ref. (131)] and use electrophysiology and calcium imaging of the GnRH cell bodies to assess GnRH secretion indirectly. The hypothesis of pulsatile secretion being intrinsic to GnRH neurons led to studies of electrical properties in GnRH neurons {GT1 cells (73); nasal explants (71); *ex vivo* GnRH neurons [reviewed in Ref. (40, 132, 133)]}. Although most GnRH neurons display autonomous firing of APs, firing is heterogeneous among GnRH neurons (126), far from an oscillatory activity that could trigger pulses every ~20 min (124). Even *in vivo* GnRH neurons exhibit heterogeneous behavior (114). The search for changes in the firing pattern *ex vivo*, i.e., increases in firing rate occurring at the same frequency as GnRH pulses, is rather inconclusive (134–136). Notably, in addition to intrinsic properties, it is assumed that each GnRH neuron contributes to consecutive GnRH pulses. Although experimentally activated GnRH neurons can trigger multiple LH pulses (88), this assumption has yet to be proven.

Simultaneous recording from multiple GnRH neurons bypasses this assumption and shows *in vitro* relationships between synchronized $[Ca^{2+}]_i$ oscillations and GnRH pulses (70) or frequency of $[Ca^{2+}]_i$ oscillations and GnRH secretion (137). Recently, optogenetic activation of GnRH neurons defined the firing of GnRH neurons triggering LH secretion *in vivo* (88) (**Figure 1**). However, the predicament to linking an electrical event to a secretion, at a single-cell level, is the resolution for the detection of GnRH release. Calcium dynamics in GT1 cells correlate with FM1-43 uptake, i.e., secretion (138), but this observation cannot be extrapolated to native GnRH neurons with complex morphology and where GnRH release occurs from cell bodies and fibers (139, 140).

Modified FSCV, applicable to GnRH, is a step forward, providing secretion data from one to few GnRH neurons (140). It supports, at a smaller scale, the relationship between APs and secretion: increased firing rate evoked by hormonal status, recorded at the cell body (142), correlates with increased secretion, at the ME (140), highlighting the regulation of firing activity. Most importantly, it allows subcellular measurements and shows a site-specific regulation of GnRH release (141). Different regulation of somatodendritic and nerve terminal release is known in magnocellular neurons (143), but a new insight in GnRH neurons. In the POA (at bundles of proximal processes), increases in $[Ca^{2+}]_i$ evoked by sarco/endoplasmic reticulum calcium-ATPase blocker evoke GnRH release. While in the ME (at nerve terminals), APs must accompany such increases to evoke GnRH release (141). In addition, locally applied inositol triphosphate receptor blocker prevents kisspeptin-evoked GnRH release in the ME but not in the POA. In contrast, locally applied calcium channel blocker prevents kisspeptin-evoked GnRH release in the POA but not in the ME, where calcium and sodium channel blockers are necessary (141).

Subcellular electrophysiology and calcium imaging identify different functions at different locations in GnRH neurons (20, 115, 144) (**Figure 1**). APs initiate in the proximal process (144) and patterning occurs at the cell soma (145). APs propagate along the process (144) and elicit temporally restricted calcium rises at the nerve terminals (115). The activation of GnRH neurons in the POA triggering a GnRH/LH pulse *in vivo* illustrates this phenomenon (88). However, the straightforwardness stops with electrical stimuli. GnRH neurons become versatile when exposed to ligands. GnRH projections exhibit unique properties allowing local depolarizations to reshape APs along the way to the ME (20). Applied at the cell body, kisspeptin evokes a calcium rise, accompanied by APs (115). Although the calcium rise at the cell body is independent of firing (48, 115, 141), APs will travel and evoke a spike-dependent calcium rise at the nerve terminals (115). Applied at the nerve terminals, kisspeptin evokes a local calcium rise, independent of APs (115), and triggers GnRH secretion, even when sodium channel blockers are present (141).

Until today, the conundrum was with kisspeptin producing both a massive surge and timely restricted pulses. However, subcellular regulation in GnRH neurons provides new hypotheses for GnRH secretion (**Figure 2**). RP3V kisspeptin neurons

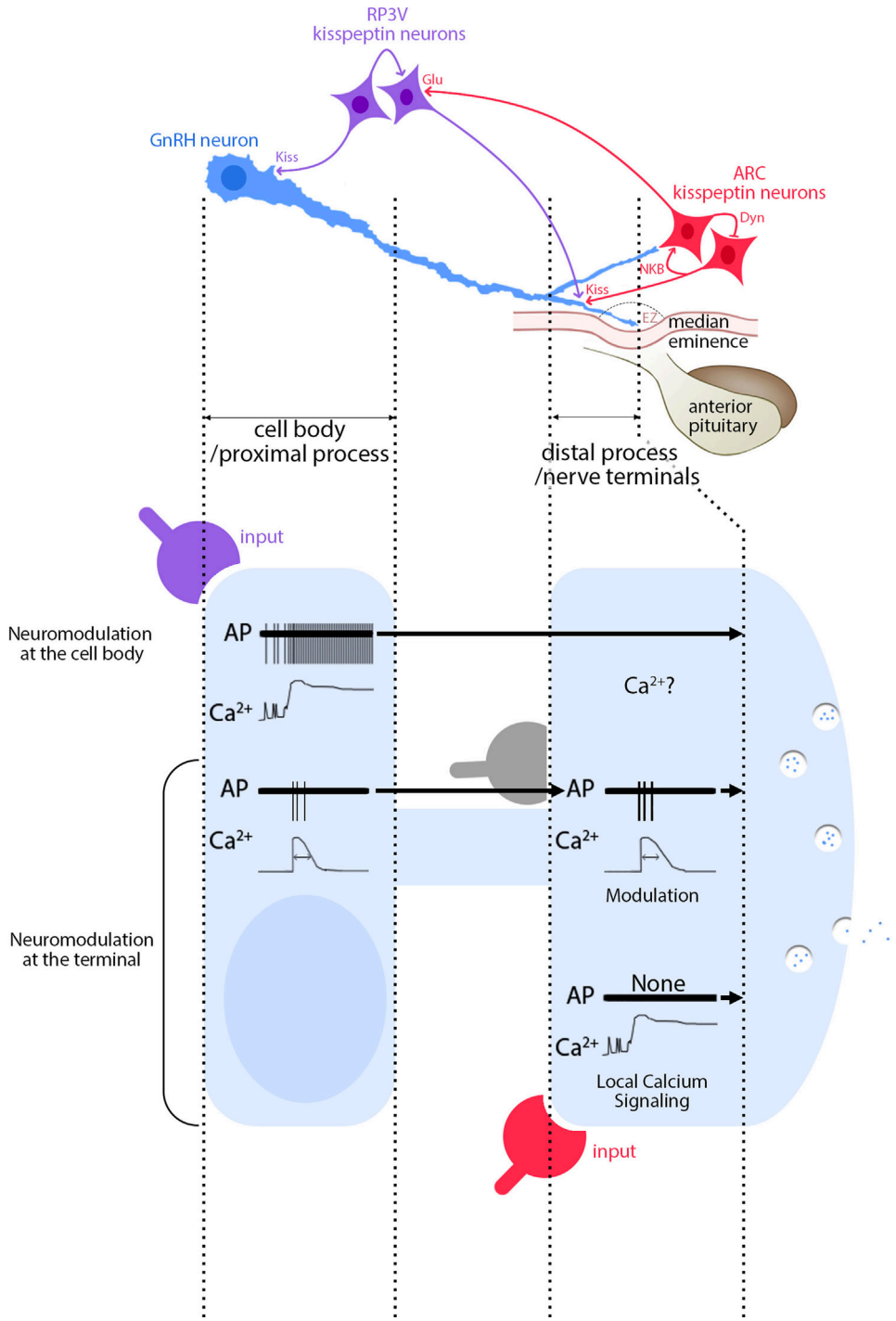

FIGURE 2 | Functional consequences of segment-specific signaling in gonadotropin-releasing hormone (GnRH) neurons. Kisspeptin/GnRH signaling: GnRH neurons (blue) receive inputs from RP3V kisspeptin neurons (purple) at the cell body and at the distal process, outside the external zone (EZ) of the median eminence. In contrast, inputs from ARC kisspeptin neurons (red) are seen only at the distal process. Inputs from ARC to RP3V kisspeptin neurons are glutamatergic. Activity of ARC kisspeptin neurons relies upon an autoregulatory loop involving neurokinin B and dynorphin A. High magnification view of events: signals evoked at GnRH soma from RP3V kisspeptin neurons (purple) produce a long-lasting calcium rise and a train of action potentials (APs) that travel toward the nerve terminals (top traces). Based on **Figure 1** (second row), an AP-dependent calcium rise would be expected, unless the calcium rise *via* GPR54 is autoregenerative, travels along the process and therefore would be AP independent. In contrast, signaling evoked at GnRH nerve terminals from ARC kisspeptin neurons (red) produces a long-lasting calcium rise, without APs (bottom traces). In addition, APs, accompanied by AP-dependent calcium rises, travel toward the nerve terminals and modulatory inputs such as glutamate (gray) can reshape the APs and possibly the concomitant calcium rises (middle traces). The traces are schematic and do not have scale bars.

innervating the GnRH cell body (118) probably initiate different response than ARC kisspeptin neurons innervating the nerve terminals (118), thus regulating GnRH secretion differently. For example, kisspeptin evokes a long-lasting calcium rise in nerve terminals (>60 min) (115) but FSCV detects GnRH release for ~1 min (141). Possibly, non-secreting calcium-dependent vesicle dynamics might follow calcium-evoked GnRH secretion at the nerve terminals (115, 129, 146). FSCV indicates kisspeptin-evoked secretion at the ME is specifically regulated and an increase in $[Ca^{2+}]_i$ is not the only requirement (141). Exocytosis involves many protein–protein interactions regulated by second messengers and phosphorylation (147). Kisspeptin triggers a complex signaling pathway (48, 148) that might allow it to define the relationship between calcium and secretion at GnRH nerve terminals.

CONCLUSION

Our knowledge of the physiology of GnRH neurons is ever evolving and we should remain as naïve as possible when studying them. As in many other fields, the knowledge is limited by techniques and none of the "classical" tools available in neuroscience are readily usable for GnRH neurons. Even nowadays, the knowledge of GnRH neurons still suffers from the anatomical intricacy of the system. However, with creativity and tenacity, knowledge about GnRH neurons builds up and common assumptions fall: the simple bipolar GnRH neuron displays arborized distal processes, the scattered cell bodies are reunited with entwined dendrites, GnRH is released at the cell body, the processes become dendrons with merged features of axon and dendrite, and neuronal inputs relocate into differentially regulated GnRH neuron segments. I am positive the list will continue to grow as we try and understand the mechanism(s) underlying pulsatile GnRH secretion.

What do we need to unravel the mystery behind GnRH secretion? I believe the next step is to tailor genetic tools to target genetically encoded sensors such as GCaMP6s and pHuji, to GnRH neurons for simultaneous imaging of calcium dynamics and secretory vesicle fusion. This should allow for the deciphering of their precise relationship and the investigation of how intracellular signaling pathways downstream of GPCRs and other receptors can modulate this relationship.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work approved it for publication.

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REVIEW
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Gonadotropin-Releasing Hormone (GnRH) Receptor Structure and GnRH Binding

Colleen A. Flanagan and Ashmeetha Manilall*

Faculty of Health Sciences, School of Physiology, University of the Witwatersrand, Johannesburg, South Africa

Gonadotropin-releasing hormone (GnRH) regulates reproduction. The human GnRH receptor lacks a cytoplasmic carboxy-terminal tail but has amino acid sequence motifs characteristic of rhodopsin-like, class A, G protein-coupled receptors (GPCRs). This review will consider how recent descriptions of X-ray crystallographic structures of GPCRs in inactive and active conformations may contribute to understanding GnRH receptor structure, mechanism of activation and ligand binding. The structures confirmed that ligands bind to variable extracellular surfaces, whereas the seven membrane-spanning α-helices convey the activation signal to the cytoplasmic receptor surface, which binds and activates heterotrimeric G proteins. Forty non-covalent interactions that bridge topologically equivalent residues in different transmembrane (TM) helices are conserved in class A GPCR structures, regardless of activation state. Conformation-independent interhelical contacts account for a conserved receptor protein structure and their importance in the GnRH receptor structure is supported by decreased expression of receptors with mutations of residues in the network. Many of the GnRH receptor mutations associated with congenital hypogonadotropic hypogonadism, including the $Glu^{2.53(90)}$ Lys mutation, involve amino acids that constitute the conserved network. Half of the ~250 intramolecular interactions in GPCRs differ between inactive and active structures. Conformation-specific interhelical contacts depend on amino acids changing partners during activation. Conserved inactive conformation-specific contacts prevent receptor activation by stabilizing proximity of TM helices 3 and 6 and a closed G protein-binding site. Mutations of GnRH receptor residues involved in these interactions, such as $Arg^{3.50(139)}$ of the DRY/S motif or $Tyr^{7.53(323)}$ of the N/DPxxY motif, increase or decrease receptor expression and efficiency of receptor coupling to G protein signaling, consistent with the native residues stabilizing the inactive GnRH receptor structure. Active conformation-specific interhelical contacts stabilize an open G protein-binding site. Progress in defining the GnRH-binding site has recently slowed, with evidence that $Tyr^{6.58(290)}$ contacts Tyr^{5} of GnRH, whereas other residues affect recognition of Trp^{3} and $Gly^{10}NH_2$. The surprisingly consistent observations that GnRH receptor mutations that disrupt GnRH binding have less effect on "conformationally constrained" GnRH peptides may now be explained by crystal structures of agonist-bound peptide receptors. Analysis of GPCR structures provides insight into GnRH receptor function.

Keywords: gonadotropin-releasing hormone receptor, G protein-coupled receptor, receptor structure, receptor activation, ligand binding

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Ivana Bjelobaba, University of Belgrade, Serbia

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Honoo Satake, Suntory Foundation for Life Sciences, Japan

****Correspondence:***
Colleen A. Flanagan
colleen.flanagan@wits.ac.za

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH) regulates reproduction by binding and activating GnRH receptors on pituitary gonadotrope cells, which synthesize and secrete the gonadotropins, LH, and FSH. The gonadotropins act on the gonads to stimulate gametogenesis, gonadal cell proliferation, and production of the gonadal steroids. GnRH secretion is suppressed during childhood and increases at puberty, when increased production of gonadotropins and gonadal steroids initiate sexual development. Disruption of GnRH receptor function disrupts reproduction and mutations of the GnRH receptor gene disrupt or delay pubertal development, resulting in congenital hypogonadotropic hypogonadism (cHH) (1, 2). This central role in regulation of reproduction has made the GnRH receptor a target for treatment of infertility and of sex steroid-dependent hyperplasias, including uterine fibroids, endometriosis and prostatic cancer, where gonadal steroid production is decreased by administration of GnRH antagonists or high doses of GnRH agonists, which downregulate receptor expression (3–5). Agonist binding to the GnRH receptor activates the $G_{q/11}$ family of heterotrimeric G proteins. Activated GTP-bound $G\alpha_{q/11}$ subunits activate phospholipase Cβ, which catalyzes production of the second messengers diacylglycerol and inositol trisphosphate, which initiate the cellular signaling pathways that culminate in gonadotropin synthesis and secretion (3, 6, 7). Although the GnRH receptor is also reported to transiently activate G_s proteins in the LβT2 gonadotrope cell line (3, 8, 9) and inhibit cell growth *via* the inhibitory G_i proteins, no direct GnRH receptor activation of $G\alpha_i$ or $G\alpha_s$ could be shown in a range of cell lines (10–12) and it has been proposed that GnRH-stimulated activation of G_i or G_s proteins may be downstream of activation of the $G_{q/11}$ proteins (6, 12). The mammalian (type 1) GnRH receptor does not activate β-arrestin-dependent signaling (3, 6, 7, 13), suggesting that all effects of the GnRH receptor may be mediated by activation of $G_{q/11}$ proteins.

The GnRH receptor belongs to the G protein-coupled receptor (GPCR) family, which constitutes the largest family of membrane proteins in the human genome (14, 15). The GPCRs regulate physiological systems ranging from vision and olfaction through neurotransmission and immunology in addition to endocrine systems. Physiological ligands that activate GPCRs range from cations (Ca^{2+}), small molecule neurotransmitters and immune modulators to peptide and protein hormones, cytokines and even light, which changes the 11-*cis*-retinal prosthetic group of rhodopsin from a covalently bound inverse agonist (an antagonist that actively stabilizes inactive receptor conformations) to an agonist. In spite of their diverse physiological functions and ligands, all GPCRs share a common molecular function, which consists of transducing an extracellular signal across a biological membrane *via* a change in receptor protein conformation (16–18). This conserved function is supported by a conserved protein structure that consists of an extracellular amino-terminus, a bundle of seven membrane-spanning α-helical segments connected by three intracellular and three extracellular loops and a cytoplasmic carboxy-terminus (16, 19, 20). No crystal structure of the GnRH receptor has yet been reported, but much can be learned about its structure and how it conveys the extracellular GnRH-binding signal to intracellular signaling pathways by studying the structures of related GPCRs that have been crystallized and combining this with biochemical studies. This review will focus on understanding of the structure of the GnRH receptor and ligand binding that has arisen since the last major review (13) with emphasis on the application of recently described GPCR structures and how these may inform mechanisms of GnRH receptor structure, activation and ligand binding.

PRIMARY STRUCTURES OF GnRH RECEPTORS

Based on conserved amino acid sequence features (**Table 1**), the GnRH receptor is a class A GPCR. Class A is the largest and best-studied class of GPCR proteins and includes rhodopsin, adrenergic and other monoamine neurotransmitter receptors and many peptide and protein-binding receptors. The membrane-spanning segments of GPCRs are most conserved, whereas the loops and termini are more variable (19). To facilitate comparison of amino acid residues of the GnRH receptor with equivalent residues of other class A GPCRs, the Ballesteros and Weinstein numbering system (21) will be used. Residues are numbered relative to the most conserved residue in each transmembrane (TM) segment, which is designated .50, preceded by the TM segment number and followed, where relevant, by the amino acid sequence number in the receptor in parenthesis. For example Asp^{319} of the human GnRH receptor is designated $Asp^{7.49(319)}$, because it immediately precedes the most conserved residue in TM7, $Pro^{7.50(320)}$. The equivalent residue of the mouse receptor is $Asp^{7.49(318)}$.

This review will focus on the mammalian type 1 GnRH receptor, which is characterized by absence of a cytoplasmic carboxy-terminal tail (13, 40) that accounts for the lack of arrestin-dependent desensitization, internalization, and signaling. Many systems of nomenclature have been used for GnRH receptor subtypes, largely because of the unclear relationship between the tailless mammalian receptors and the other GnRH receptors, all of which have carboxy-terminal tails (13, 41, 42). The discovery that some lower vertebrates have tailless GnRH receptors that are structurally and functionally similar to mammalian receptors (43) has now provided some consensus (40, 44–46). All of the tailless GnRH receptors are designated type 1 and all of the tailed GnRH receptors, type 2.

Human GnRH receptors have all of the highly conserved Ballesteros and Weinstein reference residues, except for the acidic $Asp^{2.50}$ in TM2, which is substituted with uncharged Asn (**Table 1**; Figure S1 in Supplementary Material). Mutation of $Asn^{2.50(87)}$ to the normal Asp disrupted GnRH receptor expression (23, 25), confirming the functional importance of the substitution. The type 1 GnRH receptors also have variations of the highly conserved amino acid sequence motifs. In TM7 the NPxxY motif ($Asn^{7.49}$-$Pro^{7.50}$-x-x-$Tyr^{7.53}$ where x represents any amino acid) is changed to **D**PxxY (**Asp**$^{7.49}$-$Pro^{7.50}$-$Leu^{7.51}$-$Ile^{7.52}$-$Tyr^{7.53}$). Mutation of $Asp^{7.49}$ to Asn reversed the disruption of GnRH receptor expression caused by mutation of $Asn^{2.50}$ to Asp, suggesting these resides might be close to each other in the three-dimensional structures of class A GPCRs (25). The CWxPY motif in TM6 is preserved

TABLE 1 | Highly conserved amino acid residues and motifs in class A GPCRs and equivalent residues in type 1 and type 2 GnRH receptors.

Conserved GPCR residue or motif	Function in GPCRs	Reference	Residue in human type 1 GnRH receptor	Residue in type 2 GnRH receptors	Function in GnRH receptors	Reference
$Asn^{1.50}$	Part of the conformation-independent conserved interhelical network	(19, 20)	$Asn^{1.50(53)}$	$Asn^{1.50}$	Structural[a]	(23)
	Part of the water-mediated polar networks	(22)				
$Asp^{2.50}$	Part of the conformation-independent conserved interhelical network	(19, 20)	$Asn^{2.50(87)}$	$Asp^{2.50}$	Structural	(23, 25)
	Part of the water-mediated polar networks	(22)				
	Binding of Na^+	(24)				
$Asp^{3.49}$–$Arg^{3.50}$–$Tyr^{3.51}$ (DRY)	Part of the ionic lock Interacts with G proteins	(26–28)	$Asp^{3.49(138)}$–$Arg^{3.50(139)}$–$Ser^{3.51(140)}$ (DRS)	$Asp^{3.49}$–$Arg^{3.50}$–$Xaa^{3.51}$ (DRx)	Structural and activation of cellular signaling	(29, 30)
$Trp^{4.50}$	Part of the conserved conformation-independent interhelical network	(19, 20)	$Trp^{4.50(164)}$	$Trp^{4.50}$		
$Pro^{5.50}$	Part of the transmission switch	(22, 26, 31, 32)	$Pro^{5.50(223)}$	$Pro^{5.50}$		
$Cys^{6.47}$–$Trp^{6.48}$–x–$Pro^{6.50}$–$Tyr^{6.51}$ (CWxPY)	Part of the conformation-independent conserved interhelical network	(19, 20)	$Cys^{6.47(279)}$–$Trp^{6.48(280)}$–$Thr^{6.49(281)}$–$Pro^{6.50(282)}$–$Tyr^{6.51(283)}$	$Cys^{6.47}$–$Trp^{6.48}$–$Thr^{6.49}$–$Pro^{6.50}$–$Tyr^{6.51}$	Structural and ligand-binding affinity.	(34–38)
	Part of the conserved intramolecular water-mediated polar networks	(22)				
	Forms an exaggerated kink that opens the G protein-binding pocket when TM6 rotates	(33)				
$Asn^{7.49}$–$Pro^{7.50}$–x–x–$Tyr^{7.53}$ (NPxxY)	Part of the conformation-independent conserved interhelical network	(19, 20)	$Asp^{7.49(319)}$–$Pro^{7.50(320)}$–$Leu^{7.51(321)}$–$Ile^{7.52(322)}$–$Tyr^{7.53(323)}$ (DPxxY)	$Asp^{7.49}$–$Pro^{7.50}$–x–x–$Tyr^{7.53}$ (DPxxY)	Structural, possible Na^+ counter-ion, activation of cellular signaling	(23, 25, 36, 39)
	Part of the conserved intramolecular water-mediated polar networks	(22)				
	Forms conformation-specific interhelical interactions	(17)				

A brief summary of the key functions of highly conserved residues revealed by structures of class A GPCRs is provided with a listing of the functions of equivalent GnRH receptor residues based on functional (site-directed mutagenesis) studies.

[a]*Structural effects relate to effects on cell surface expression of mutant receptors. Prior to development of technology to "rescue" expression, mutants that were not expressed could not be studied further.*

as $Cys^{6.47}$-$Trp^{6.48}$-$Thr^{6.49}$-$Pro^{6.50}$-$Tyr^{6.51}$, whereas the DRY motif at the cytosolic end of TM3 is DRS ($Asp^{3.49}$-$Arg^{3.50}$-$Ser^{3.51}$) (**Table 1**; Figure S1 in Supplementary Material).

Type 1 GnRH receptors have a $Glu^{2.53(90)}$ residue in TM2, which has risen to prominence because a cHH-associated $Glu^{2.53(90)}Lys$ mutation disrupts membrane expression of the receptor, but treatment with a pharmacoperone [small-molecule membrane-permeable GnRH receptor antagonists that act as templates for folding of nascent receptor proteins (34, 35, 47)] rescues expression of the mutant receptor, both *in vitro* and in knock-in transgenic mice (34, 47, 48). In other class A GPCRs the equivalent residue is mostly large and hydrophobic (Leu, Val, or Phe) (49) and is $Ile^{2.53}$, $Val^{2.53}$, or $Met^{2.53}$ in type 2 GnRH receptors (13, 43), suggesting that the carboxyl side chain of $Glu^{2.53(90)}$ may not be required.

The functional importance of the highly conserved $Tyr^{5.58}$ residue was revealed by crystal structures of active rhodopsin (33, 50). Type 1 GnRH receptors have $Asn^{5.58}$, but all tailed GnRH receptors have the conserved $Tyr^{5.58}$ (13, 40, 43). In most class A GPCRs a conserved large aliphatic amino acid, $Ile^{3.40}$, forms part of a group of conserved residues referred to as the "core triad" (22) or "transmission switch" (26, 31, 32), which changes configuration during receptor activation. GnRH receptors have a small $Ala^{3.40(129)}$ residue, which is also present in type 2 GnRH receptors.

THE THREE-DIMENSIONAL STRUCTURE OF THE GnRH RECEPTOR

Ligands interact with the variable extracellular half of GPCR molecules. The membrane-spanning domain conveys the ligand-binding signal to the cytosolic surface of the receptor, which interacts with the G protein (20). In order to specifically transduce an agonist signal across a cellular membrane, a GPCR must exist in a silent state that does not activate G proteins. Once agonist binds, the receptor must undergo transition to a state that binds and activates G proteins located on the opposite side of the membrane. Thus, agonist ligands, such as GnRH, can be thought of as allosteric activators of GPCRs, enabling them to catalyze G protein activation (16, 18, 51).

Current theories of receptor activation posit that GPCRs exist in an equilibrium of inactive "R" and activated "R*" conformations, with the equilibrium balanced toward the R conformations in the absence of ligand. The R conformations cannot activate G proteins, are stabilized by binding of inverse agonist (antagonist)

ligands and have low affinity for agonist ligands. In contrast, R* conformations bind and activate G proteins, have high affinity for agonist ligands and are stabilized by binding of agonists and/or G proteins (16, 52–54). Thus, agonist binding induces or stabilizes one or more active GPCR conformation(s), which activate G protein signaling. Similarly, G protein binding increases the binding affinity of the receptor for agonist (16, 54, 55). Until recently, the structural correlates of the R and R* conformations were unknown, but a flurry of technical innovations has recently allowed X-ray crystallographic determination of the structures of rhodopsin and then other GPCRs. Initial structures were bound to inverse agonists and thus represent inactive R conformations. These were followed by crystal structures of agonist-bound GPCRs that were partially active, whereas, in most cases, cocrystallization of agonist-bound receptors with a G protein or a G protein mimetic (antibody or truncated G protein) was required to achieve fully active GPCR structures (16, 27, 31, 32, 56). The GPCR crystal structures reveal the differences between the R and R* conformations, although they do not provide dynamic information about the activation process. We will attempt to use information from the structures of class A GPCRs that have been crystallized to understand GnRH receptor structure.

Conformation-Independent Intramolecular Interactions

G protein-coupled receptor crystal structures show that the three-dimensional structures of GPCRs are more conserved than the amino acid sequences (15, 19, 20, 26, 57). The convergence of diverse amino acid sequences to a common structure allowed considerable plasticity in the evolutionary development of the diverse GPCR family (17, 19, 57). The crystal structures have defined the relative positions of the known highly conserved amino acids and of the conserved amino acid sequence motifs and shown that the highly conserved $Pro^{5.50}$, $Pro^{6.50}$, and $Pro^{7.50}$ residues in TM5, TM6, and TM7 cause bends in the α-helices that are not classical proline kinks (32, 58, 59).

The TM6 Proline Kink

The exaggerated bend angle around $Pro^{6.50}$, in the CWxPY motif in TM6, is stabilized by a water molecule that makes hydrogen bonds to the $Cys^{6.47}$ and $Tyr^{6.51}$ residues of the CWxPY motif and to a residue in TM7 in GPCR structures (33, 58, 60–62). The importance of this structure in the GnRH receptor is supported by no less than three cHH-associated mutations of the CWxPY motif. The $Pro^{6.50(282)}$Arg mutation of the GnRH receptor completely disrupts receptor function, which cannot be recovered by pharmacoperone treatment (36), showing that the proline kink is essential for GnRH receptor expression. The $Cys^{6.47(279)}$Tyr mutant GnRH receptor and a laboratory-produced $Cys^{6.47(279)}$Ala mutant showed no measurable GnRH binding and severely decreased cellular responses to GnRH stimulation that were recovered after pharmacoperone treatment of cells transfected with the mutant receptor (1, 35, 63, 64). This suggests that the $Cys^{6.47(279)}$ mutations disrupt receptor folding during biosynthesis, but the rescue shows that its effect is less destructive than mutating the $Pro^{6.50(282)}$. A $Tyr^{6.51(283)}$His GnRH receptor mutation causes cHH and results in no measurable function *in vitro* (65).

Conformation-Independent Interhelical Contacts Form a Conserved Scaffold

The overall GPCR fold (the relative positions of the seven TM segments) is stabilized by ~200–260 non-covalent intramolecular contacts (hydrogen bonds, van der Waals interactions, etc.) and by a network of hydrogen-bonded water molecules in the interior of the TM domain. Depending on methodology, 24–40 interhelical contacts between topologically equivalent loci (positions) are present in all active and inactive class A GPCR structures (17, 19, 20). These conserved conformation-independent interhelical contacts determine the overall GPCR structure, forming a conserved "scaffold," on which conformational changes can occur. Some of the conserved interhelical contacts are required for protein folding and insertion into the membrane during biosynthesis. The conserved interhelical contacts involve many of the residues that are highly conserved in class A GPCRs (Table S1 in Supplementary Material) but also involve residues in topologically equivalent loci, where the amino acids are not conserved (19, 20, 57). Most of the conserved conformation-independent interhelical interactions are located toward the central and cytoplasmic side of the TM domain (20) (Figure S2 in Supplementary Material), consistent with the emerging recognition that these are the areas in which GPCR structure is most conserved, whereas the extracellular side of the TM barrel is less conserved, because of the need to accommodate diverse ligands (18).

Gonadotropin-releasing hormone receptor residues topologically equivalent to the residues in conserved conformation-independent interhelical contacts are listed in Table S1 in Supplementary Material, with the predicted interhelical contacts and effects of previously reported mutations of the residues on GnRH receptor expression and function. Many mutations result in undetectable receptor function, consistent with disruption of cell surface GnRH receptor expression or severe misfolding of the receptor protein. The disruption of functional receptor expression confirms the importance of the residues for GnRH receptor structure and indicates that the conserved interhelical contacts constitute part of the GnRH receptor structure, similar to their roles in other GPCRs.

The complete disruption of GnRH receptor expression when $Asn^{2.50(87)}$ in TM2 was substituted with Ala or Asp (Table S1 in Supplementary Material) supports a role for the $Asn^{2.50(87)}$ residue in stabilizing GnRH receptor structure. However, GPCR crystal structures show that $Asp^{2.50}$ makes conserved contacts with residues in TM1 and TM7, but it is only connected to $Asn^{7.49}$ *via* the water-mediated hydrogen bond network (19, 58). Based on the conserved structural scaffold, $Asn^{2.50(87)}$ of the GnRH receptor contacts $Asn^{1.50(53)}$ and $Pro^{7.46(316)}$, whereas $Asp^{7.49(319)}$ does not form any conserved conformation-independent contacts (Table S1 in Supplementary Material).

cHH-Associated GnRH Receptor Mutations Affect Conserved Conformation-Independent Interhelical Contacts

Many cHH-associated GnRH receptor mutations involve residues that constitute conserved interhelical contacts in the crystallized GPCR structures. These include the $Glu^{2.53(90)}$Lys,

Glu$^{2.53(90)}$Asp, Ala$^{4.57(171)}$Thr, Cys$^{6.47(279)}$Tyr, Tyr$^{6.51(283)}$His, Tyr$^{6.52(284)}$Cys, Pro$^{7.50(320)}$Arg, and Tyr$^{7.53(323)}$Cys mutations. Most cHH-associated mutant receptors are poorly expressed *in vitro* (Table S1 in Supplementary Material), consistent with the mutations disrupting the structural scaffold of the receptor. Disruption of interhelical contacts would destabilize receptor protein folding, resulting in fewer correctly folded receptor molecules being transported to the cell membrane or decreased residence time of less stable receptor proteins once they get to the cell membrane. Pharmacoperones act as templates for folding of nascent receptor proteins, stabilizing biosynthesis, and increasing protein expression (34, 35, 47). In most cases, pharmacoperone treatment of cells transfected with cHH-associated mutant GnRH receptors, enhanced mutant receptor expression (Table S1 in Supplementary Material) and the "rescued" receptors showed wild type-like function. This supports a role for the mutated residues in stable folding and cell surface expression of the GnRH receptor (34, 35, 47).

The late Michael Conn's laboratory and others proposed that the Glu$^{2.53(90)}$ side chain of the GnRH receptor forms a interhelical salt-bridge with Lys$^{3.32(121)}$ in TM3 and that the cHH-associated Glu$^{2.53(90)}$Lys mutation disrupted receptor biogenesis and folding by breaking this salt bridge (66–69). Although Glu$^{2.53(90)}$ is conserved in type 1 GnRH receptors, type 2 GnRH receptors and other class A GPCRs have large hydrophobic residues at position 2.53 (13, 43, 49, 57). The lack of conservation suggests that the acidic side chain of Glu$^{2.53(90)}$ may not be necessary for type 1 GnRH receptor structure and that the effect of the Glu$^{2.53(90)}$Lys mutation may result from disruptive effects of introducing Lys, rather than lack of Glu. This is supported by a mutation of Glu$^{2.53(90)}$ to uncharged Gln, which had no effect on receptor function (70) and a conservative Glu$^{2.53(90)}$Asp, which is associated with cHH (Table S1 in Supplementary Material). A Glu$^{2.53(90)}$Ala mutation resulted in undetectable GnRH receptor function (68), but we have not found any report of the effect of the Glu$^{2.53(90)}$Asp mutation, which would formally test the salt bridge hypothesis.

The conserved interhelical contacts in GPCR structures predict that Glu$^{2.53(90)}$ interacts with Ser$^{3.35(124)}$ (Table S1 in Supplementary Material). Interaction of Glu$^{2.53(90)}$ with Ser$^{3.35(124)}$ is supported by an automated (unbiased) structural homology model of the GnRH receptor (71), which shows Glu$^{2.53(90)}$ close to Ser$^{3.35(124)}$, whereas Lys$^{3.32(121)}$ points away, toward Asp$^{2.61(98)}$ (**Figure 1**). A Ser$^{3.35(124)}$Asp mutation resulted in undetectable binding, suggesting that the mutation caused receptor instability by introducing close apposition of two carboxyl side chains (37). Thus, it is likely that the cHH-associated Glu$^{2.53(90)}$Lys mutation disrupts GnRH receptor expression by disrupting the conserved interhelical contact with the 3.35 locus, rather than disrupting a salt-bridge with Lys$^{3.32(121)}$.

Another cHH-associated GnRH receptor mutant, Tyr$^{6.51(283)}$His, also showed undetectable function *in vitro* (65). Since Tyr$^{6.51}$ forms a conserved interhelical contact with the residue in the 7.39 locus, the Tyr$^{6.51(283)}$His mutation may disrupt receptor structure by disrupting an interhelical contact of Tyr$^{6.51(283)}$ with Phe$^{7.39(309)}$ in TM7 of the GnRH receptor (Table S1 in Supplementary Material).

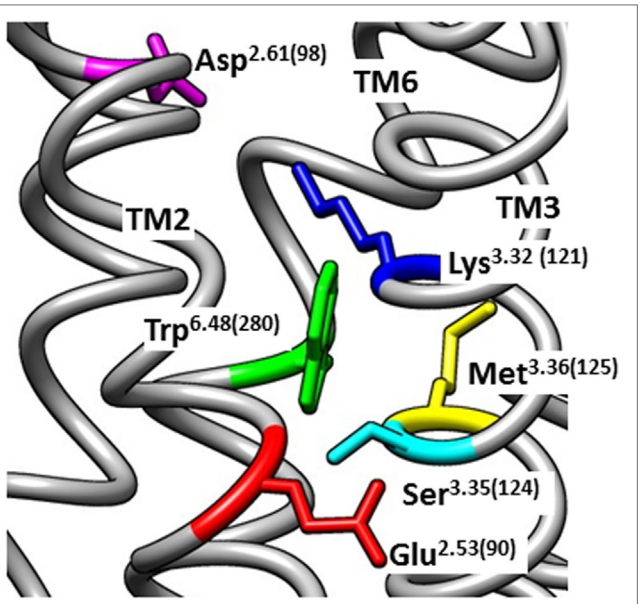

FIGURE 1 | Homology model of the inactive human gonadotropin-releasing hormone (GnRH) receptor. The model was downloaded from the GPCRdb website (www.gpcrdb.org/structure/homology_models) (71) and viewed using the UCSF Chimera software package (72) to show the spatial positioning of Glu$^{2.53(90)}$ (red) relative to the neighboring residues Ser$^{3.35(124)}$ (light blue), Lys$^{3.32(121)}$ (dark blue), Asp$^{2.61(98)}$ (magenta), Met$^{3.36(125)}$ (yellow), and Trp$^{6.48(280)}$ (green). Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

Role of Trp$^{6.48(280)}$ of the CWxPY Motif in the Conserved Structural Scaffold of the GnRH Receptor

The Trp$^{6.48(280)}$ residue in the CWxPY motif was proposed to directly contact the Trp^{3} residue of the GnRH peptide (69, 73). Systematic mutagenesis of Trp$^{6.48(280)}$ to Ala, His, Ser, Gln, and Met disrupted GnRH receptor expression, as did mutation of Trp$^{6.48(279)}$ of the rat GnRH receptor to Arg or Ser (74). However, once expression of the mutant receptors was recovered, using a pharmacoperone, the mutant receptors displayed unchanged ligand-binding affinity and signaling (37, 38, 73). This shows that Trp$^{6.48(280)}$ does not directly contact GnRH, but is important for GnRH receptor structure. Trp$^{6.48(280)}$ likely forms conserved interhelical contacts with Met$^{3.36(125)}$ and Ala$^{7.42(312)}$ of the GnRH receptor (Table S1 in Supplementary Material), which would be disrupted by the mutations.

The Inactive Receptor Structure

About half of the intramolecular interactions differ between the inactive and active GPCR structures. The GnRH receptor residues equivalent to the residues that form conserved conformation-specific interhelical contacts are listed in **Table 2**. These conformation-specific interhelical contacts depend on reassignment of the interacting amino acid pairs during the activation process (17, 19) (**Figure 2**). This section will describe the key features of the inactive GPCR structures and discuss the evidence for similar structural features in the GnRH receptor.

TABLE 2 | GnRH receptor residues potentially involved in conserved conformation-specific interhelical contacts.

GnRH receptor residue	Inactive conformation-specific interhelical contacts	Active conformation-specific interhelical contacts	GnRH receptor mutations	Effects of mutations	Reference
Phe$^{1.53(56)}$	Phe$^{1.53(56)}$–Tyr$^{7.53(323)}$				
Leu$^{2.43(80)}$		Leu$^{2.43(80)}$–Gly$^{7.54(324)}$	Leu$^{2.43(80)}$Ala	Decreased expression and decreased agonist potency	(76)
Met$^{3.43(132)}$	Met$^{3.43(132)}$–Phe$^{6.40(272)}$ Met$^{3.43(132)}$–Ala$^{6.41(273)}$	Met$^{3.43(132)}$–Asp$^{7.49(319)}$ Met$^{3.43(132)}$–Tyr$^{7.53(323)}$	Met$^{3.43(132)}$Ala	Undetectable,[a] expression, and signaling rescued by pharmacoperone[b]	(77)
Ile$^{3.46(135)}$	Ile$^{3.46(135)}$–Thr$^{6.37(269)}$	Ile$^{3.46(135)}$–Tyr$^{7.53(323)}$	Ile$^{3.46(135)}$Ala Ile$^{3.46(135)}$Leu Ile$^{3.46(135)}$Val	Undetectable Increased coupling efficiency Undetectable	(29)
Arg$^{3.50(139)}$	Arg$^{3.50(139)}$–Thr$^{6.37(269)}$	Arg$^{3.50(139)}$–Phe$^{6.40(272)}$	Arg$^{3.50(139)}$His Arg$^{3.50(139)}$Lys Arg$^{3.50(139)}$Gln Arg$^{3.50(139)}$Ala Arg$^{3.50(139)}$Cys	Undetectable, cHH, rescued by pharmacoperone Undetectable Increased expression uncoupled Decreased expression and coupling cHH, decreased expression, rescued by pharmacoperone, decreased coupling	(47, 78, 79) (29) (30)
Leu$^{5.55(228)}$		Leu$^{5.55(228)}$–Ala$^{6.41(273)}$			
Asn$^{5.58(231)}$		Asn$^{5.58(231)}$–Phe$^{6.40(272)}$			
Ile$^{5.62(235)}$		Ile$^{5.62(235)}$–Thr$^{6.37(269)}$			
Met$^{6.36(268)}$	Met$^{6.36(268)}$–Tyr$^{7.53(323)}$				
Thr$^{6.37(269)}$	Ile$^{3.46(135)}$–Thr$^{6.37(269)}$ Arg$^{3.50(139)}$–Thr$^{6.37(269)}$	Ile$^{5.62(235)}$–Thr$^{6.37(269)}$	Thr$^{6.37(269)}$Met	cHH, undetectable	(80)
Phe$^{6.40(272)}$	Met$^{3.43(132)}$–Phe$^{6.40(272)}$ Phe$^{6.40(272)}$–Asp$^{7.49(319)}$	Arg$^{3.50(139)}$–Phe$^{6.40(272)}$ Asn$^{5.58(231)}$–Phe$^{6.40(272)}$	Phe$^{6.40(272)}$Ala Phe$^{6.40(272)}$Leu Phe$^{6.40(272)}$Glu Phe$^{6.40(272)}$Lys Phe$^{6.40(272)}$Tyr	Decreased expression Increased expression Undetectable Undetectable Decreased expression	(77) (81)
Ala$^{6.41(273)}$	Met$^{3.43(132)}$–Ala$^{6.41(273)}$	Leu$^{5.55(228)}$–Ala$^{6.41(273)}$			
Asp$^{7.49(319)}$	Phe$^{6.40(272)}$–Asp$^{7.49(319)}$	Met$^{3.43(132)}$–Asp$^{7.49(319)}$	Asp$^{7.49(318)}$Asn (M)[c] Asp$^{7.49(318)}$Ala (M) Asp$^{7.49(318)}$Glu (M) Asp$^{7.49(318)}$Leu (M)	Decreased coupling efficiency Decreased coupling efficiency Decreased expression Undetectable	(23, 25, 39)
Tyr$^{7.53(323)}$	Phe$^{1.53(56)}$–Tyr$^{7.53(323)}$	Met$^{3.43(132)}$–Tyr$^{7.53(323)}$ Ile$^{3.46(135)}$–Tyr$^{7.53(323)}$	Tyr$^{7.53(323)}$Ala Tyr$^{7.53(322)}$Phe (M) Tyr$^{7.53(323)}$Cys	Uncoupled Increased coupling efficiency Decreased coupling efficiency	(36, 39, 77)
Gly$^{7.54(324)}$		Leu$^{2.43(80)}$–Gly$^{7.54(324)}$			

GnRH receptor residues equivalent to those that form inactive or active conformation-specific interhelical contacts in class A GPCR structures (17, 19) are listed with summaries of the effects of mutagenesis.

[a]*Undetectable indicates no measurable function mostly due to lack of expression.*

[b]*Rescued by pharmacoperone indicates that pharmacoperone pretreatment of cells increased ligand binding or cellular signaling.*

[c]*Mutations in mouse GnRH receptors are indicated by (M).*

The main features of inactive GPCR structures include a "closed" G protein-binding pocket, a water-mediated hydrogen bond network and a hydrophobic barrier separating the water network in the ligand-binding pocket from the G protein-binding pocket (17, 18, 22, 32, 33, 56, 75).

Interactions That Stabilize the Closed G Protein-Binding Pocket

The inactive rhodopsin structure showed a salt bridge between Arg$^{3.50}$ of the D/ERY motif at the cytoplasmic end of TM3, and Glu$^{6.30}$ at the cytosolic end of TM6 (28, 82). This "ionic-lock" interaction stabilizes the inactive GPCR conformation by drawing the cytoplasmic ends of TM3 and TM6 together (26, 83, 84). However, the salt bridge between Arg$^{3.50}$ and Glu$^{6.30}$ cannot provide a universal mechanism for stabilizing inactive GPCR conformations, because Glu$^{6.30}$ is not conserved (83). The GnRH receptor has Arg$^{6.30(262)}$, which clearly cannot form a salt bridge with Arg$^{3.50(139)}$. Nevertheless, mutations show that both Arg residues are important for GnRH receptor structure and function. The cHH-associated Arg$^{6.30(262)}$Gln mutation decreased ligand binding and cellular signaling (85), which was recovered when cells were treated with pharmacoperone (1, 47), suggesting that the Arg$^{6.30(262)}$ side chain forms an intramolecular interaction that stabilizes folding of the unoccupied GnRH

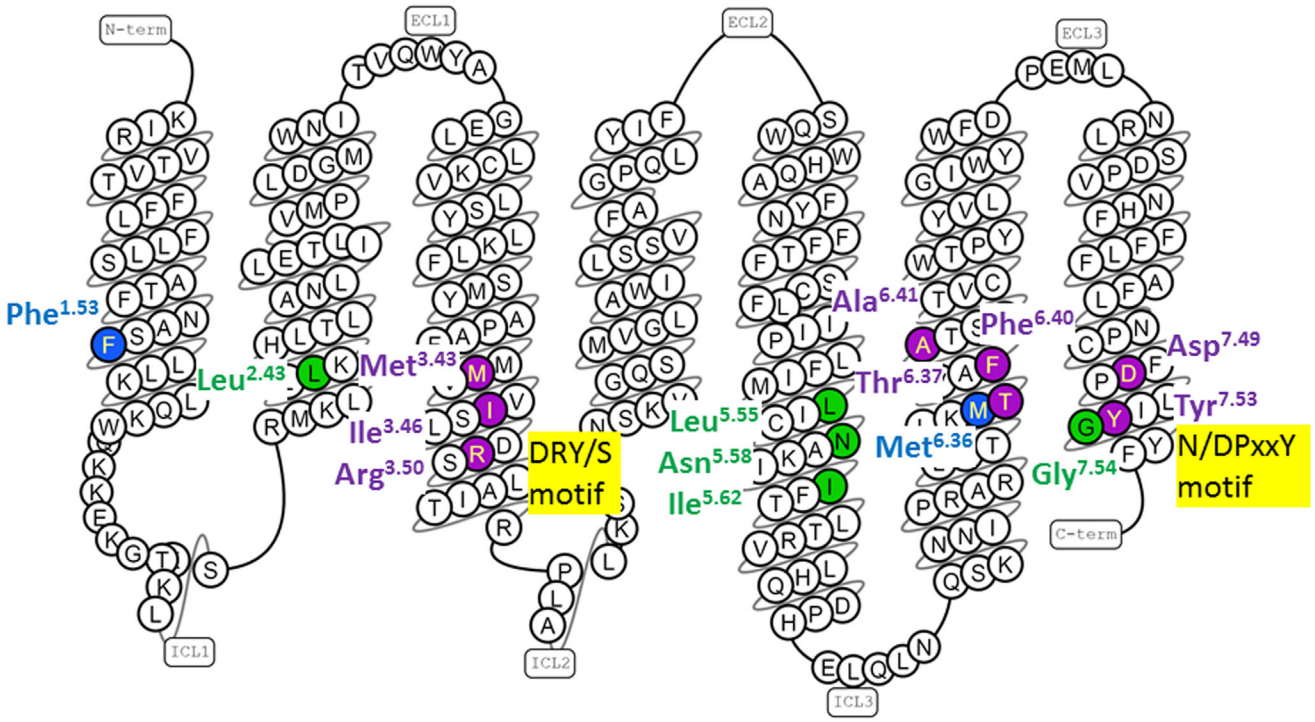

FIGURE 2 | Human gonadotropin-releasing hormone (GnRH) receptor residues topologically equivalent to G protein-coupled receptor (GPCR) residues that form conserved conformation-specific interhelical contacts. The snake diagram of the GnRH receptor amino acid sequence was downloaded from GPCRdb (www.gpcrdb.org/structure/homology_models) (71). GnRH receptor residues topologically equivalent to residues that form conserved conformation-specific contacts (17, 19) only in inactive GPCR structures (blue); only in active GPCR structures (green) and with different partners in inactive and active GPCR structures (purple) are shown.

receptor. Since GPCRs are biosynthesized in cellular compartments that are inaccessible to endogenous ligands, receptors are likely synthesized in inactive conformations. Inactive conformation-specific interhelical contacts may thus stabilize receptor biosynthesis, so mutations that disrupt these contacts may also disrupt expression.

Mutation of $Arg^{3.50(139)}$ in the DRY/S motif to other basic amino acids, His or Lys, resulted in no measurable receptor function, consistent with disruption of a structurally important interaction. An $Arg^{3.50(139)}$Gln mutation increased GnRH receptor expression and decreased activation of cellular signaling (29). This suggests that the Gln side chain mimics an interhelical interaction of $Arg^{3.50(139)}$ that stabilizes receptor folding and stabilizes the inactive receptor conformation. The importance of the $Arg^{3.50(139)}$ side chain for GnRH receptor structure is also supported by cHH-associated $Arg^{3.50(139)}$His and $Arg^{3.50(139)}$Cys mutations, which are poorly expressed (30, 78), but rescued by pharmacoperone treatment (**Table 2**) suggesting that the mutations disrupt biosynthesis of the unoccupied (inactive) GnRH receptor. Since $Arg^{3.50(139)}$ cannot form an ionic lock in the GnRH receptor, it must form a different contact. GPCR structures show a conserved inactive conformation-specific interhelical contact of $Arg^{3.50}$ with the 6.37 locus (19). Thus, the $Arg^{3.50(139)}$ side chain may stabilize the inactive GnRH receptor conformation by forming an interhelical hydrogen bond with $Thr^{6.37(269)}$ (**Table 2**), which may be enhanced in the $Arg^{3.50(139)}$Gln mutant receptor.

Many conserved inactive conformation-specific interhelical contacts involve residues in TM3 and TM6 (19), suggesting that, like the ionic lock, they maintain close proximity of the cytoplasmic ends of TM3 and TM6. $Met^{3.43(132)}$, conserved as a large hydrophobic residue in most GPCRs, is likely to stabilize the inactive GnRH receptor conformation by interacting with $Phe^{6.40(272)}$ and $Ala^{6.41(273)}$ in TM6 (**Table 2**). Mutation of $Met^{3.43(132)}$ and four mutations of $Phe^{6.40(272)}$ decreased GnRH receptor expression, suggesting that they disrupt receptor biogenesis. In contrast, the $Phe^{6.40(272)}$Leu mutation increased receptor expression (**Table 2**; Figure S3 in Supplementary Material) (77, 81), suggesting that the $Phe^{6.40(272)}$Leu mutation enhances the TM3-TM6 interhelical contact with $Met^{3.43(132)}$ and so enhances expression of the inactive GnRH receptor.

A more recent study, using five pairs of GPCR structures found that only one TM3–TM6 inactive conformation-specific contact is conserved (17). This interaction between the 3.46 and 6.37 loci is the key contact that defines the "closed" conformation of the G protein-binding site and prevents G protein binding (17). In the GnRH receptor, these residues are $Ile^{3.46(135)}$ and $Thr^{6.37(269)}$ (**Table 2**). Mutation of $Ile^{3.46(135)}$ to Ala and Val both completely ablated GnRH receptor function (29). In the context of the inactive GPCR crystal structures, the Ala and Val side chains are likely too short to "fulfill the distance criteria for contact formation" (17). Substitution of $Ile^{3.46(135)}$ with Leu, which has a large branched aliphatic side chain, partially preserved GnRH

receptor expression and increased coupling efficiency (29). Thus, the Ile$^{3.46(135)}$Leu mutation may destabilize the inactive conformation of the GnRH receptor by weakening the Ile$^{3.46(135)}$–Thr$^{6.37(269)}$ interhelical contact and favoring formation of the R* active conformation. A cHH-associated Thr$^{6.37(269)}$Met mutation resulted in no measurable function in several assay systems (80). This result confirms the importance of Thr$^{6.37(269)}$ in GnRH receptor structure, but provides no information about its function in receptor conformation.

The second key inactive conformation-specific interhelical contact orients Tyr$^{7.53}$ of the NPxxY motif in TM7 toward TM1, where it contacts the residue in the 1.53 locus, keeping the Tyr$^{7.53}$ side chain away from the interior of the TM domain (17, 19). The corresponding residues of the GnRH receptor are Phe$^{1.53(56)}$ in TM1 and Tyr$^{7.53(323)}$ in the N/DPxxY motif in TM7 (**Table 2**).

Conserved Water-Mediated Hydrogen Bond Networks and the Sodium Ion-Binding Pocket

Higher resolution GPCR structures revealed internal water molecules that form a conserved network, with hydrogen bonds connecting the conserved amino acids in different helices, including Asn$^{1.50}$, Asp$^{2.50}$, Trp$^{6.48}$ of the CWxPY motif and residues of the NPxxY motif. The water molecules both stabilize the GPCR structural fold and mediate transition between conformational states, by forming many "low energy switches," which can be broken and reconfigured during GPCR activation (22, 26, 33, 50, 86). The GnRH receptor likely has a network of intramolecular water molecules between its highly conserved amino acids and mutations that disrupt GnRH receptor expression or function, may do so by disrupting the water-mediated intramolecular network.

The hydrophilic network in inactive GPCRs also includes a sodium ion, which makes a conserved contact with Asp$^{2.50}$ and stabilizes the inactive receptor conformation. Cations decrease GnRH receptor agonist binding (87). Since type 1 GnRH receptors do not have Asp$^{2.50}$, the nearby Glu$^{2.53(90)}$ and Asp$^{7.49(319)}$ (of the N/DPxxY motif) residues may provide negative charges that enhance cation binding, as suggested for the PAR1 receptor (22, 24, 88, 89).

The Hydrophobic Barrier and Transmission Switch

All inactive class A GPCR structures have a layer of hydrophobic amino acids on the cytosolic side of Asp$^{2.50}$ that separates the water molecules in the ligand- and G protein-binding pockets. The barrier stabilizes the inactive GPCR conformation and consists of conserved class A GPCR residues, including some that also form conserved inactive conformation-specific interhelical contacts (26, 33, 75). The partially overlapping "central hydrophobic core," consisting of the conserved Phe$^{6.44}$ and conserved hydrophobic residues in the 3.43 and 6.40 loci (90), the "core triad" consisting of Phe$^{6.44}$, Pro$^{5.50}$ and the hydrophobic residue at position 3.40 (22) and the "transmission switch," consisting of the hydrophobic residue at position 3.40, Pro$^{5.50}$, Leu$^{5.51}$, and Phe $^{6.44}$, which is one helical turn away from Trp$^{6.48}$ of CWxPY motif, all couple the ligand-binding pocket to the hydrophobic barrier (26, 31, 32). The GnRH receptor has hydrophobic residues in the loci associated with the hydrophobic barrier and the transmission switch, so it is likely that the GnRH receptor has a hydrophobic barrier that stabilizes its inactive conformation.

The Active Receptor Structure

The diverse GPCR ligands trigger a variety of receptor-specific molecular changes to initiate receptor activation (31, 91). The changes must converge to generate a structurally conserved G protein-binding pocket that can be recognized by- and activate a G protein that interacts with many GPCRs (17, 18). Structural features that define the activated GPCR conformation include rotation of TM6, changed interfaces of TM3, TM5, and TM6, opening of the hydrophobic barrier, movement of the Tyr$^{5.58}$ (Asn$^{5.58(231)}$ in type 1 GnRH receptors) and Tyr$^{7.53}$ side chains toward the interior of the TM bundle and opening of a cytoplasmic surface cleft that allows G protein access and binding (20, 31, 32, 56, 90, 91).

Rotation of TM6 and Activation of the Transmission Switch

In spite of the diversity of ligand-binding surfaces many ligands contact highly conserved residues, particularly Trp$^{6.48}$ and Tyr$^{6.51}$ of the CWxPY motif. In the active structure of rhodopsin retinal isomerization induces movement of Trp$^{6.48}$, which causes rotation of TM6, without changing either the rotamer angle of the Trp$^{6.48}$ side chain or the hinge angle of the proline kink. The exaggerated bend angle of Pro$^{6.50}$ amplifies movement of the cytoplasmic end of TM6, which moves outward, away from TM3 (33). Similar rotation of the cytoplasmic end of TM6 was seen in all fully active GPCR structures (22, 27, 56, 92, 93).

Rotation of TM6 also changes its interhelical contacts with TM3 and TM5. The Phe$^{6.44}$ side chain moves toward TM5, where it rearranges the Leu$^{5.51}$ and Pro$^{5.50}$ residues and moves the conserved Leu$^{3.40}$ of TM3 away from TM5, thus triggering the "transmission switch" (26, 31). Similar activation of the transmission switch *via* agonist-induced movement of Trp$^{6.48}$ is seen in the A_{2A}-adenosine and μ-opioid receptors (22, 31). The transmission switch changes the conformation of the proline kink of TM5 and rotates the conserved Tyr$^{5.58}$ side chain (near the cytoplasmic end) inwards. It also changes the position of TM3, rotating it and moving it slightly toward the extracellular of side the TM domain (90).

Active structures of other GPCRs showed similar outward movement of the cytosolic end of TM6 but showed no agonist contact with Trp$^{6.48}$ (31, 93–98), so TM6 movement must be achieved *via* different mechanisms. In the β_2-adrenergic receptor, agonist binding at the extracellular end of TM5 causes movement of Pro$^{5.50}$, which moves Ile$^{3.40}$ in TM3 and Phe$^{6.44}$ in TM6, thus opening the core triad, triggering the transmission switch and rotating the cytoplasmic end of TM6 away from the helix bundle (26, 31). A similar opening of the core triad residues occurs in the μ-opioid receptor, except that the agonist binds to two residues (the 3.32 and 3.36 loci) in TM3, resulting in movement of Ile$^{3.40}$, which activates the core triad (22). Mutation of the Lys$^{3.32(121)}$ residue in TM3 of the GnRH receptor decreased binding of GnRH agonists but not antagonists (99), suggesting that GnRH interaction with Lys$^{3.32(121)}$ initiates activation. Although mutagenesis experiments

do not support a role for Trp$^{6.48(280)}$ in ligand binding or activation of the GnRH receptor (38), there is evidence that GnRH contacts Tyr$^{6.58(290)}$ at the extracellular end of TM6 (100) and that Tyr$^{6.51(283)}$ of the CWxPY affects ligand-binding affinity (37), suggesting that these residues may initiate rotation of TM6.

Reconfiguration of the Water-Mediated Polar Network and Opening of the Hydrophobic Barrier and G Protein-Binding Pocket

The water-mediated polar network in active GPCR structures differs from that of inactive structures. Agonist-induced movement of the extracellular ends of the TM helices rearranges water molecules at the extracellular side of the TM domain and opens the hydrophobic barrier, which allows formation of a continuous water channel between the ligand-binding pocket and the cytoplasmic surface of the receptor. This changes the conformation of TM7 and causes rotation of Tyr$^{7.53}$ away from its interaction with TM1 toward the center of the TM bundle (22, 32, 33, 50, 75, 90, 96, 101, 102). The rearranged water molecules link Arg$^{3.50}$ with Tyr$^{5.58}$ and Tyr$^{7.53}$ in a water-mediated interhelical network that can be considered the "open" conformation of the ionic lock that stabilizes the active receptor conformation (22, 28, 96). Movements of TM6 and TM7 collapse the sodium ion-binding pocket, making it too small to accommodate the ion (24, 88, 97) and the cation moves toward the cytoplasm through the open hydrophobic barrier (24). Mutation of the water-associated Arg$^{3.50(139)}$, Asp$^{7.49(318)}$ and Tyr$^{7.53(322)}$ residues decreases GnRH receptor coupling efficiency (23, 29, 30, 39, 77, 103) indicating that they have roles in the active receptor conformation, which may be mediated by the water network.

Rotation of TM6 and opening of the hydrophobic barrier break the inactive conformation-specific interhelical contacts and form new active conformation-specific interhelical contacts. The ionic lock opens and the Arg$^{3.50}$ side chain moves into the space vacated by TM6 where it orients toward Tyr$^{5.58}$ of TM5. Arg$^{3.50}$ and Tyr$^{5.58}$ form new interhelical contacts with the hydrophobic residue in position 6.40 (19, 22, 28). The GnRH receptor has Asn$^{5.58(231)}$, which is smaller than Tyr. The NTSR1 neurotensin receptor also has Asn$^{5.58}$ and an "active-like" NTSR1 structure shows a hydrogen bond between Asn$^{5.58(257)}$ and Arg$^{3.50(167)}$ (104), which suggests that Asn$^{5.58(231)}$ stabilizes the open ionic lock in the GnRH receptor.

On activation Met$^{3.43}$ breaks its inactive conformation-specific interhelical contact with the residues in positions 6.40 and 6.41. The release of the hydrophobic side chain in position 6.41 allows it to contact the hydrophobic residue in position 5.55, forming one of two key active conformation-specific interhelical contacts (17, 19). In the GnRH receptor these residues are Leu$^{5.55(228)}$ and Ala$^{6.41(273)}$ (**Table 2**). One helical turn closer to the cytoplasmic side of the receptor, the Ile$^{3.46}$ side chain breaks its inactive conformation-specific contact with the position 6.37 side chain and forms a new interhelical contact with Tyr$^{7.53}$, forming the second key active conformation-specific interaction (17, 19). The breaking of the TM3-TM6 contacts opens a cleft that allows G protein access and releases the position 6.37 residue to make a conserved interaction with the G protein (17, 27) (Figure S3 in Supplementary Material). In the GnRH receptor these residues are Ile$^{3.46(135)}$, Thr$^{6.37(269)}$ and Tyr$^{7.53(323)}$ (**Table 2**). Mutation of Ile$^{3.46(135)}$ to Leu increased GnRH receptor coupling efficiency (29), suggesting that the Leu side chain may favor interaction with Tyr$^{7.53(323)}$ over interaction with Thr$^{6.37(269)}$, thus favoring the active conformation. A role for Tyr$^{7.53(323)}$ in stabilizing the active GnRH receptor conformation is supported by the Tyr$^{7.53(323)}$Ala mutant, which did not activate cellular signaling (39, 77).

LIGAND-BINDING INTERACTIONS

Gonadotropin-releasing hormone is a decapeptide with the sequence pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}NH$_2$. The amino-terminal residues, pGlu^1, His^2, and Trp^3, determine agonist activity, but the carboxy-terminal residues, particularly Arg^8, are necessary for high affinity binding to the GnRH receptor (4, 13, 105). Although the GnRH peptide is conformationally flexible, the predominant conformer consists of a β-turn that brings the amino- and carboxy-termini close together. This conformation is stabilized by substituting the achiral Gly^6 residue of the GnRH peptide with D-amino acids, which increase receptor-binding affinity, whereas L-amino acids decrease affinity (4, 13, 105). In the absence of a GnRH-receptor crystal structure, alanine-scanning mutagenesis and molecular models have identified potential intermolecular contacts. However, most have not been validated by biochemical studies to distinguish indirect disruption of the GnRH-binding surface (Figure S4 in Supplementary Material). We will discuss potential GnRH-receptor contacts in the context of peptide-bound GPCR structures and recent GnRH receptor mutagenesis studies.

The Consensus Ligand-Binding Pocket in the GnRH Receptor

In spite of the diversity of GPCR ligand-binding pockets, Venkatakrishnan et al. identified a consensus ligand-binding pocket consisting of topologically equivalent residues at positions 3.32, 3.33, and 3.36 in TM3, 6.48 and 6.51 in the CWxPY motif, and 7.39 in TM7. These residues include two conserved interhelical contacts, 3.36–6.48 and 6.51–7.39, which couple the ligand-binding pocket to the conserved GPCR structure (20). Structures of peptide-bound GPCRs show that the sections of the peptides that are required for agonist activity, the carboxy-termini of endothelin-1 and apelin and the amino-termini of chemokines, penetrate the TM cores of their receptors and interact with subsets of the consensus ligand-binding residues, whereas other parts of the peptides bind outside of the core (93, 106–109). This suggests that the amino-terminal residues of GnRH may interact with the consensus-binding pocket.

Lys$^{3.32(121)}$

The endothelin-1 peptide penetrates the TM core of the ET$_B$-endothelin receptor and contacts the consensus-binding residues, Trp$^{6.48}$ and Leu$^{6.51}$ and Gln$^{3.32}$ (107). The smaller peptide agonist NTS$_{8–13}$ binds closer to the extracellular surface of the NTSR1 neurotensin receptor, but may contact the consensus Arg$^{3.32(149)}$ residue (104). Mutation of the equivalent GnRH receptor residue, Lys$^{3.32(121)}$, to Gln or Ala decreased GnRH affinity and signaling, but had minimal effect on binding of a peptide antagonist, which

had modified amino-terminal residues. This led to a conclusion that $Lys^{3.32(121)}$ may form a hydrogen bond with the aromatic rings of His^2 or Trp^3 of GnRH (37, 99). Subsequent models proposed that $Lys^{3.32(121)}$ contacts $pGlu^1$ or His^2 (37, 68, 110–113) but, in the absence of further experiments, it remains uncertain whether $Lys^{3.32(121)}$ directly contacts GnRH or initiates receptor activation.

$Trp^{6.48(280)}$

Agonist peptide ligands bound to the NTSR1 neurotensin, US28 viral chemokine and apelin receptors do not penetrate deeply enough to contact $Trp^{6.48}$ of the CWxPY motif (93, 104, 106) and mutagenesis of $Trp^{6.48}$ had minimal effects on NTSR1 receptor function (114). Molecular models suggested that $Trp^{6.48(280)}$ of the GnRH receptor interacts with Trp^3 of the GnRH peptide (13, 69, 74, 111). However, mutations of $Trp^{6.48(280)}$ had minimal effects on GnRH affinity or cellular signaling (37, 38), indicating that it does not directly contact GnRH. Since TM3 has central roles in ligand binding, the conserved interhelical network and the hydrophobic core, movement of TM3 may provide an alternative molecular pathway to $Trp^{6.48}$-mediated activation of the transmission switch in the GnRH and NTSR1 receptors (20, 57).

$Tyr^{6.51(283)}$ and $Phe^{7.39(309)}$

The hydrophobic residue in position 6.51 contacts the ligand in the ET_B-endothelin and apelin receptors (106, 107), whereas the conserved $Glu^{7.39}$ of chemokine receptors is a key determinant of chemokine binding and receptor activation (109). A $Tyr^{6.51(283)}$Phe mutation in the GnRH receptor and mutations of $Phe^{7.39(309)}$ to Leu or Gln decreased GnRH-binding affinity (37). A recent computational model suggests that $Phe^{7.39(309)}$ may contact Trp^3 of GnRH (110). Together, these data are consistent with the mutations disrupting ligand binding by breaking an interhelical contact between residues that may also contact the ligand, but more experiments are needed.

In summary, it is possible that amino-terminal residues of the GnRH peptide contact some of the consensus ligand-binding residues, $Lys^{3.32(121)}$, $Tyr^{6.51(283)}$, and $Phe^{7.39(309)}$, but not $Trp^{6.48(280)}$. It remains uncertain whether GnRH binds to a largely extracellular surface of the receptor like $NTS_{8–13}$ (104) or penetrates the TM core like the peptide ligands of the apelin and chemokine receptors (106, 109).

GnRH Interactions Outside of the Consensus Ligand Pocket

The Amino Terminus, TM2, and Extracellular Loop 1

A molecular model predicted that $Arg^{1.35(38)}$ in the amino terminus of the GnRH receptor is close to the carboxy-terminal Pro^9-$Gly^{10}NH_2$ of GnRH. Mutations of $Arg^{1.35(38)}$ decreased GnRH-binding affinity, but had lesser effects on binding of [Pro^9-NHEt]-GnRH, which lacks $Gly^{10}NH_2$ (112). The results support a hydrogen bond contact between $Arg^{1.35(38)}$ and $Gly^{10}NH_2$, but show that both the geometry and charge of the $Arg^{1.35(38)}$ side chain are important for additional inter- or intramolecular interactions. $Asn^{2.65(102)}$ in TM2 has similar functions in distinguishing $Gly^{10}NH_2$ of GnRH (13, 115). These studies suggest that the carboxy-terminus of GnRH may locate close to both $Arg^{1.35(38)}$ and $Asn^{2.65(102)}$ (Figure S4 in Supplementary Material). Systematic mutagenesis of $Asp^{2.61(98)}$ of the GnRH receptor, combined with ligand modification, showed that the $Asp^{2.61(98)}$ side chain determines receptor recognition of His^2 of the GnRH peptide, *via* a hydrogen bond, whereas the charge of the $Asp^{2.61(98)}$ side chain may configure the surface of the ligand-binding pocket by forming an interhelical salt bridge with $Lys^{3.32(121)}$ (13, 116). Thus, residues in the amino-terminus and extracellular ends of TM1 and TM2 of the GnRH receptor appear to affect GnRH binding *via* direct contacts with amino- and carboxy-terminal residues of the peptide and *via* intramolecular interactions that affect the shape of the ligand-binding surface.

TM6, Extracellular Loop 3, and TM7

Molecular models of the GnRH receptor showed contact of $Tyr^{6.58(290)}$, two helical turns toward the extracellular end of TM6 from the CWxPY motif, with the Tyr^5 side chain of the GnRH peptide. Systematic mutagenesis showed that both the hydroxyl group and the aromatic ring of the $Tyr^{6.58(290)}$ side chain contribute to high affinity binding of GnRH, but had less effect on binding of [Ala^5]-GnRH, consistent with the hydroxyl group of $Tyr^{6.58(290)}$ interacting with the aromatic ring of Tyr^5 of the peptide. The receptor mutations also decreased GnRH potency in signaling assays more than they decreased binding affinity, showing that the $Tyr^{6.58(290)}$ side chain has an additional role in coupling agonist binding to receptor activation (100). The $Tyr^{6.58(290)}$–Tyr^5 interaction may initiate movement and rotation of TM6 in the GnRH receptor.

Mutation of $His^{7.36(305)}$ at the extracellular end of TM7 in the mouse GnRH receptor to non-polar amino acids decreased GnRH-binding affinity, suggesting loss of a hydrogen bond interaction. Ligand modification suggested a $His^{7.36(305)}$-Trp^3 hydrogen bond contact. However, mutation of $His^{7.36(305)}$ to polar amino acids had no effect on ligand-binding affinity, making a direct interaction with the ligand unlikely. Molecular modeling showed that $His^{7.36(305)}$ made only intramolecular contacts with the amino terminus of the receptor, whereas Trp^3 of GnRH was oriented near the consensus ligand-binding residue, $Phe^{7.39(308)}$. This suggests that $His^{7.36(305)}$ forms an interhelical contact that positions $Phe^{7.39(308)}$ to form π–π contact with Trp^3 of the peptide (110). The cHH-associated GnRH receptor mutation, $Thr^{32}Ile$, which decreases ligand-binding affinity (63), is immediately adjacent to the $His^{7.36(305)}$ interhelical contacts and may disrupt the interhelical contact.

Mammalian GnRH has a basic Arg^8 residue, which is important for binding to type 1 GnRH receptors. Mutation of the acidic $Asp^{7.32(302)}$ residue to uncharged Asn decreased binding affinity of GnRH, but had no effect on binding of peptides with uncharged Gln^8. This suggests that $Asp^{7.32(302)}$ forms a salt bridge contact with Arg^8 of the peptide. However, "conformationally constrained" GnRH peptides, in which the high affinity β-turn was stabilized by a D-amino acid in position 6, retained high affinity binding in the absence of $Asp^{7.32(302)}$ or Arg^8 or both. Since the Arg^8 side chain also contributes to stabilizing the β-turn in the native GnRH peptide, it was concluded that the interaction of $Asp^{7.32(302)}$ with Arg^8 induces the high-affinity peptide conformation (70, 117).

Conformationally Constrained GnRH Peptides

Although it was hypothesized that the $Asp^{7.32(302)}$-Arg^{8} interaction induced the high affinity conformation of GnRH on binding to the receptor, mutation of many different GnRH receptor residues causes a similar large decrease in binding affinity of native GnRH, but much smaller decreases in affinity for conformationally constrained GnRH peptides (37, 68, 100, 115). So the ability of constrained peptides to overcome the ligand-binding affinity effects of receptor mutations is not specific to the $Asp^{7.32(302)}$-Arg^{8} interaction. In the active GPCR conformations that have increased agonist-binding affinity, a "cap" forms over the extracellular surface of the ligand-binding pocket and increases agonist affinity by hindering dissociation of the ligand and trapping it in the binding pocket (51). In peptide receptors the larger ligand extends beyond the TM-binding pocket, so it cannot be capped. Nevertheless, the extracellular sides of agonist-bound peptide receptors, such as the NTSR1 neurotensin and μ-opioid receptors, move inwards and it has been suggested that this movement "pinches" the peptide ligand, increasing its affinity by hindering its dissociation (51). Comparison of the ET_B-endothelin receptor structures with and without endothelin-1 showed that the peptide induces inward movement of the extracellular ends of the TM helices "tightening" the ligand pocket (107). Extrapolating to the GnRH receptor, conformationally constrained peptides may be more compact than GnRH before contacting the receptor and hence enhance narrowing of the ligand-binding pocket *via* multiple contacts with the ligand-binding pocket. The tightening would overcome mutation-induced loss of individual contacts.

CONCLUDING REMARKS

Although only direct determination will confirm the GnRH receptor structure, growing numbers of other GPCR structures provide insight into common features likely to be shared by the GnRH receptor. GPCR structures can be used to hypothesize mechanisms by which agonist binding is coupled to G protein activation, which must be tested by dynamic methods, such as site-directed mutagenesis and functional analysis, regardless of availability of directly determined structures. Identification of the conformation-independent interhelical contact network has provided explanations for decreased expression of many cHH-associated mutant GnRH receptors, whereas the conserved conformation-specific interhelical contacts begin to explain how conserved residues mediate receptor activation. In spite of the diversity of ligand-binding surfaces, recent agonist-bound peptide-binding GPCRs suggest that ligand contacts in TM3 may trigger receptor activation and they may explain the high affinity of conformationally constrained GnRH peptides.

AUTHOR CONTRIBUTIONS

CF conceived the project and wrote the manuscript. AM wrote a preliminary review as part of her MSc dissertation, which was partly used in the current project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fendo.2017.00274/full#supplementary-material.

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Ion Channels of Pituitary Gonadotrophs and Their Roles in Signaling and Secretion

Stanko S. Stojilkovic[1], Ivana Bjelobaba[2] and Hana Zemkova[3]*

[1] Section on Cellular Signaling, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, United States, [2] Institute for Biological Research "Siniša Stanković", University of Belgrade, Belgrade, Serbia, [3] Department of Cellular and Molecular Neuroendocrinology, Institute of Physiology Academy of Sciences of the Czech Republic, Prague, Czechia

Gonadotrophs are basophilic cells of the anterior pituitary gland specialized to secrete gonadotropins in response to elevation in intracellular calcium concentration. These cells fire action potentials (APs) spontaneously, coupled with voltage-gated calcium influx of insufficient amplitude to trigger gonadotropin release. The spontaneous excitability of gonadotrophs reflects the expression of voltage-gated sodium, calcium, potassium, non-selective cation-conducting, and chloride channels at their plasma membrane (PM). These cells also express the hyperpolarization-activated and cyclic nucleotide-gated cation channels at the PM, as well as $GABA_A$, nicotinic, and purinergic P2X channels gated by γ-aminobutyric acid (GABA), acetylcholine (ACh), and ATP, respectively. Activation of these channels leads to initiation or amplification of the pacemaking activity, facilitation of calcium influx, and activation of the exocytic pathway. Gonadotrophs also express calcium-conducting channels at the endoplasmic reticulum membranes gated by inositol trisphosphate and intracellular calcium. These channels are activated potently by hypothalamic gonadotropin-releasing hormone (GnRH) and less potently by several paracrine calcium-mobilizing agonists, including pituitary adenylate cyclase-activating peptides, endothelins, ACh, vasopressin, and oxytocin. Activation of these channels causes oscillatory calcium release and a rapid gonadotropin release, accompanied with a shift from tonic firing of single APs to periodic bursting type of electrical activity, which accounts for a sustained calcium signaling and gonadotropin secretion. This review summarizes our current understanding of ion channels as signaling molecules in gonadotrophs, the role of GnRH and paracrine agonists in their gating, and the cross talk among channels.

Keywords: gonadotrophs, gonadotropin-releasing hormone, voltage-gated channels, ligand-gated channels, electrical activity, calcium signaling, luteinizing hormone secretion

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****Correspondence:***
Stanko S. Stojilkovic
stojilks@mail.nih.gov

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INTRODUCTION

Gonadotrophs are the anterior pituitary cell lineage specialized for synthesis and release of two gonadotropins, such as follicle-stimulating hormone and luteinizing hormone (LH) (1). In addition to genes encoding beta subunits of gonadotropins, *Fshb* and *Lhb* (2, 3), gonadotrophs are defined by at least two other genes not expressed in other secretory pituitary cell types, such as gonadotropin-releasing hormone (GnRH) receptor (GnRHR) gene (*Gnrhr*) (4) and dentin matrix protein 1 gene

(5). Together with thyrotrophs, gonadotrophs express the *Cga* gene encoding the α glycoprotein subunit (6). Ontogenetically, the lineage commitment is associated with the expression of the orphan nuclear receptor NR5A1, a transcriptional factor that also plays a role in the expression of gonadotroph-specific genes in the postnatal animals (7).

Gonadotrophs are neuron-like; they express numerous voltage-gated sodium (Na_v), calcium (Ca_v), potassium (K_v), and chloride channels at the plasma membrane (PM), and fire action potentials (APs) spontaneously (8). These cells also express ligand-gated ion channels at PM, which activation by hypothalamic and intrapituitary ligands leads to increase in firing frequency and facilitation of Ca^{2+} influx and hormone release (9). The function of gonadotrophs is regulated by several Ca^{2+}-mobilizing receptors capable of modulating electrical activity and AP-dependent Ca^{2+} influx and hormone release (10). The main Ca^{2+}-mobilizing receptor for these cells is GnRHR, signaling through heterotrimeric $G_{q/11}$ proteins (11), which α subunit activates phospholipase C-β1, leading to generation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (12) and release of Ca^{2+} from endoplasmic reticulum (ER) through IP_3 receptor (IP_3R) channels (9).

Here, we focus on the role of ion channels in electrical/Ca^{2+} signaling and Ca^{2+}-controlled cellular functions in gonadotrophs. We will first review the expression and roles of voltage-gated channels in spontaneous excitability and accompanied Ca^{2+} influx in these cells, followed by description of additional channels contributing to facilitation or modulation of excitability of these cells. These include the hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels, acetylcholine (ACh)-gated receptor (AChR) channels, γ-aminobutyric acid (GABA)-gated A-type receptor ($GABA_AR$) channels, and ATP-gated receptor (P2XR) channels, all expressed at PM, and IP_3R channels expressed at ER membranes.

SIGNALING BY VOLTAGE-GATED CHANNELS

The superfamily of voltage-gated ion channels of more than 140 members, including Na_v, Ca_v, K_v, and numerous less selective channels, is one of the largest groups of signal transduction proteins (13). These channels are also expresses in gonadotrophs and account for spontaneous and receptor-controlled electrical and Ca^{2+} signaling (9).

Nine members of Na_v channels are expressed in mammals, which contribute to the initiation and propagation of APs (14). The inward Na_v current has been identified in rat (15, 16), ovine (17), fish (18, 19) and mouse native (20, 21), and immortalized gonadotrophs (22, 23). **Figure 1A** shows traces of Na_v currents in cultured rat gonadotrophs. It appears that the level of Na_v channel expression is greater in these cells than in other secretory anterior pituitary types (16). Voltage-insensitive Na^+ conductance is also present in all endocrine pituitary cells, including gonadotrophs (24, 25).

The Ca_v channels have a dual role in excitable cells: they generate inward currents that can initiate APs and are also critical for coupling of electrical signals on PM with physiological intracellular events by generating intracellular Ca^{2+} signals. There are 10 members of these channels that exhibit different electrophysiological and pharmacological properties (26). Pituitary gonadotrophs express at least inactivating T-type and non-inactivating L-type Ca_v currents, as documented in cultured cells from rat (16), mouse (21), ovine (27), fish (19), as well as in αT3-1 immortalized gonadotrophs (22). **Figure 1B** shows a representative trace of Ca_v current in rat gonadotrophs.

The K_v channels are composed of at least four functional classes: fast activating delayed rectifier, slow activating delayed rectifier (including M channels), A-type K_v channels, and ether-a-go-go-gene channels (28). **Figure 1C** illustrates total K_v currents in rat pituitary gonadotrophs, which are driven by several K_v channels. αT3-1 gonadotrophs (22) and native goldfish (19), rat (16) and ovine (29) gonadotrophs express delayed rectifiers, which expression is controlled by estrogens (29). The A-type K_v channels are also expressed in αT3-1 cells (22) as well as in native fish (18, 19, 30), frog (31), and rat (16, 32, 33) gonadotrophs. In rats, the expression level of these channels is much higher in gonadotrophs than somatotrophs (16). Functional M-type channels are expressed in mouse gonadotrophs and GnRH through a still uncharacterized signal cascade inhibits these channels (34). Moreover, our transcriptome study implies that a pulsatile GnRH application downregulates the expression of *Kcna2* (K_v 1.2) and *Kcnh6* (ether-a-go-go), while it upregulates *Kcnk10* (outward rectifier) and Na^+/Ca^{2+} exchanger *Slc24a3*, indicating that GnRHR may indirectly be involved in regulation of cell excitability (5).

Calcium-activated K^+ channels (K_{Ca}) are composed of two families: three small-conductance K^+ (SK) channels and one intermediate-conductance channel are members of the first family and the high-conductance K^+ (BK) channels belong to the second family. These channels are activated by elevation in cytosolic Ca^{2+} and play a critical role in control of firing properties of excitable cells (35), including pituitary cells (36). The expression of SK channels is well documented in fish (37), rat (38, 39), mouse (40), and ovine gonadotrophs (17), and the level of their expression is dependent on estradiol (20). Whole-cell current recordings confirmed the presence of BK current in several pituitary cell types but not in gonadotrophs (16).

Gonadotrophs also express the hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels (41), which are permeable to both K^+ and Na^+ and play a critical role in cardiac rhythmicity (42). As their name indicates, HCN channels are activated by voltage (**Figure 1D**) and cyclic nucleotides. Rat gonadotrophs and other pituitary cell types also express the cation-conducting transient receptor potential (TRP) cation-like channels (43), initially characterized by their role in *Drosophila* phototransduction (44). Mouse gonadotrophs express TRPC5 subtype of these channels, which are activated by GnRH and promote Ca^{2+} influx (45). Finally, Ca^{2+}-activated non-selective cationic currents are present in rat gonadotrophs, but the nature of these channels has not been identified (46).

The expression of voltage-gated channels in gonadotrophs makes them electrically excitable cells, i.e., capable of exhibiting regenerative and propagated APs spontaneously or in response to stimulation. In general, the membrane potential (V_m) of

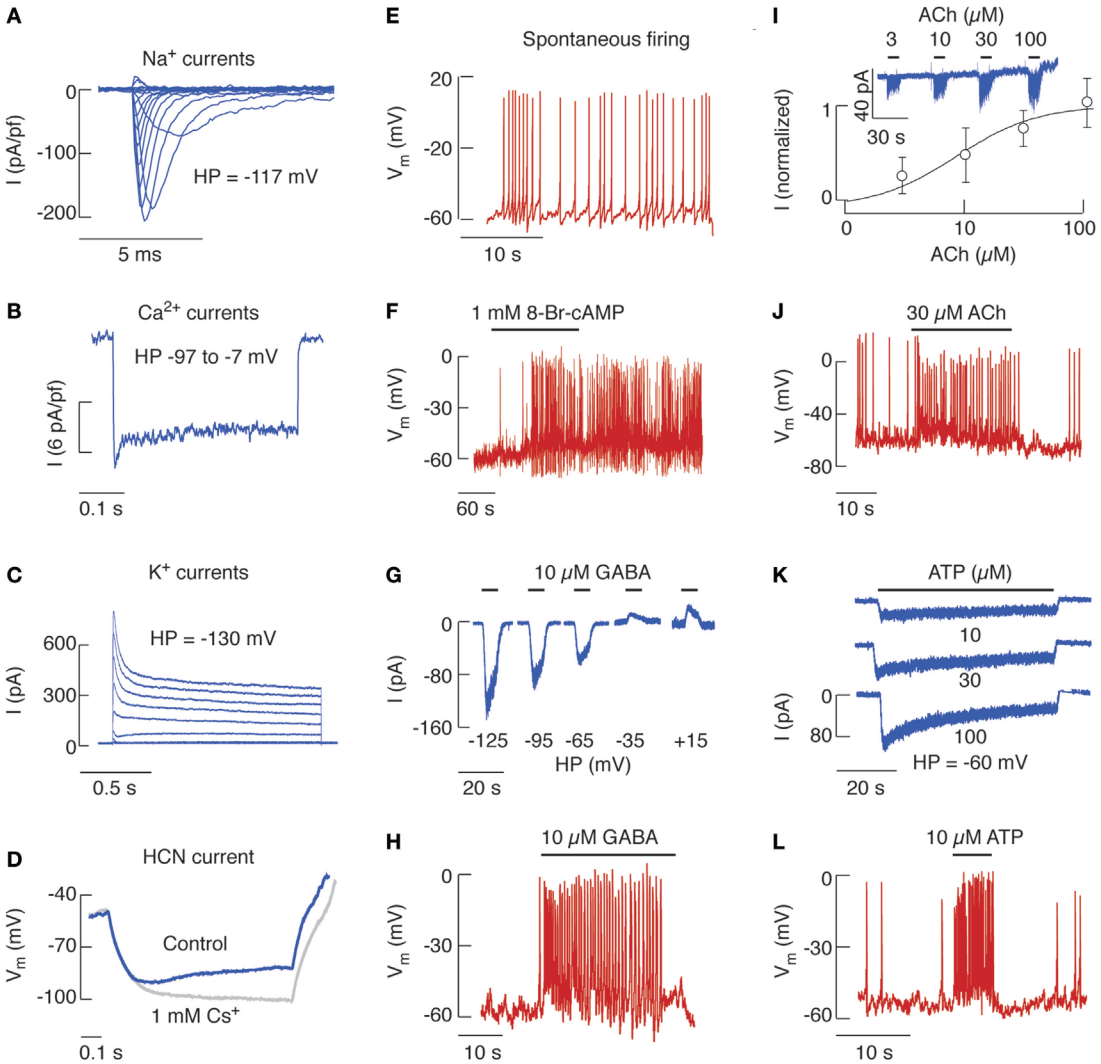

FIGURE 1 | The expression of voltage-gated and ligand-gated ion channels in identified rat pituitary gonadotrophs. **(A)** Voltage-gated sodium current traces elicited by 100 ms voltage steps from holding potentials (HPs) of −117 mV. **(B)** Voltage-gated calcium current traces elicited by 400 ms voltage-steps. **(C)** Voltage-gated potassium current traces elicited by 1.5 s voltage steps. **(D)** Cells expressing HCN current display inward rectification in response to hyperpolarizing current pulses of −5 pA that are suppressed by 1 mM Cs^+, a blocker of HCN channels. **(E)** Spontaneous electrical activity can be observed in about 50% of cultured gonadotrophs. **(F)** 8-Br-cAMP stimulates electrical activity. **(G)** γ-aminobutyric acid (GABA)-induced current recorded using gramicidin-perforated patch from cells held at different membrane potentials. **(H)** GABA-stimulated electrical activity. **(I)** Concentration-dependent effects of acetylcholine (ACh) on the amplitude of nicotinic current. (Top) Representative traces. (Bottom) Mean ± SEM values. **(J)** ACh-induced electrical activity. **(K)** Concentration-dependent effects of ATP on peak P2XR current response. **(L)** ATP-induced firing of action potentials. Derived from data shown in Ref. (16, 41, 50, 52, 63, 67); no permission is required from the copyright holder.

single gonadotrophs in culture is not stable but fluctuates from resting potentials of −60 to −50 mV due to spontaneous activity of hyperpolarizing and depolarizing channels. When the depolarization waves reach the threshold level, gonadotrophs fire tall and narrow APs (**Figure 1E**), with spiking frequency of ~0.7 Hz, amplitude of more than 60 mV, and half-width of about 50 ms (47). Ovine gonadotrophs also fire single APs spontaneously (17). In contrast to gonadotrophs, other pituitary cell types predominantly exhibit bursting pattern of spontaneous electrical activity, i.e., periodic depolarized potentials with superimposed small-amplitude spikes (47–50).

Depolarizing currents are pacemaking currents, accounting for a gradual reduction of PM resting potential toward the threshold for AP firing, and spike depolarization currents, accounting for the upstroke of an AP. The nature of channels contributing to pacemaking depolarization in gonadotrophs is not well characterized. The ongoing work is focused on the potential role of background Na^+ (24) and TRP channels (43) in this process. The cell permeable cAMP analog 8-Br-cAMP initiates AP firing in quiescent gonadotrophs (**Figure 1F**) and increases the frequency of spikes in spontaneously firing cells (41), an action consistent with the expression of HCN channels (**Figure 1D**) and/or protein

kinase A-mediated phosphorylation of some other channels in gonadotrophs (51).

The main function of Na_v channels is to depolarize cells and generate the upstroke of the AP, controlling the firing amplitude in excitable cell. In gonadotrophs, they act in conjunction with Ca_v channels to generate APs (17) or Ca_v channels are exclusively responsible for the spike depolarization (52). Simultaneous measurements of V_m and $[Ca^{2+}]_i$ showed that the bulk Ca^{2+} levels are low (50–100 nM) in spontaneously spiking gonadotrophs, in contrast to spontaneously bursting lactotrophs, somatotrophs and GH_3B_6 cells, which generate much higher (300–1,200 nM) and clearly oscillatory Ca^{2+} transients (48, 52, 53). In gonadotrophs, AP-driven Ca^{2+} influx is below the threshold needed to trigger exocytosis (52), whereas the bursting type of electrical activity in lactotrophs and somatotrophs accounts for high basal hormone secretion (48, 52). Because in intact tissue pituitary cell lineages are organized as complex networks (54–56), further studies are needed to characterize the excitatory and secretory patterns in pituitary cells with preserved tridimensional structure.

SIGNALING BY LIGAND-GATED RECEPTOR CHANNELS

Ligand-gated receptors channels are activated by chemical signals (ligands) rather than to changes in the V_m. These proteins are typically composed two different domains: a pore forming transmembrane domain and an extracellular domain containing the ligand binding site. There are three families of these channels: the Cys-loop family of channels activated by ACh, 5-HT, GABA, and glycine (57), glutamate-gated receptor-channels (58), and ATP-gated purinergic P2XR channels (59). Pituitary gonadotrophs express $GABA_AR$, nicotinic AChR, and P2X2R channels (9).

γ-Aminobutyric acid is acting through $GABA_AR$ and $GABA_CR$ channels permeable to Cl^-; in the central nervous system, GABA usually silences electrical activity and Ca^{2+} signaling (60). However, in gonadotrophs GABA and muscimol, a $GABA_AR$ agonist, increase intracellular Ca^{2+}, suggesting that chloride-mediated depolarization activates Ca_v channels. Furthermore, the $GABA_AR$ channel reversal potential for chloride ions is positive to the baseline V_m (**Figure 1G**), and the activation of these channels results in depolarization of cells and initiation of AP firing (**Figure 1H**) and stimulation of *Fshb* and *Lhb* expression (61) and LH release (62). The lower expression of cation/chloride transporter KCC2 in rat pituitary cells probably accounts for the depolarizing nature of $GABA_AR$ channels in cultured gonadotrophs (63).

The binding of nicotine, ACh, or other ligands to AChR channels stimulates cation (Na^+ and K^+ and for some neuronal subtypes Ca^{2+} as well) influx through a channel and generally results in membrane depolarization. Seventeen subunits of nicotinic AChR have been identified and were shown to assemble into a variety of receptor subtypes (64, 65). We have shown recently the expression of β2, β1, α9, and α4 mRNAs in cultured rat pituitary cells and β2, α4, and α1 in immortalized LβT2 mouse gonadotrophs. We also showed the expression of β2 subunit protein in gonadotrophs (50). These cells express nicotinic AChR channels capable of generating an inward current (**Figure 1I**) and facilitating electrical activity (**Figure 1J**) and Ca^{2+} influx (not shown). We also found that GnRHR stimulation downregulates gene expression of both α4 and α9 subunits (5, 50), suggesting that the expression of nicotinic AChR in gonadotrophs *in vitro* compensates for the loss of GnRH stimulation.

ATP is not only an intracellular molecule but is also released by cells and acts as an extracellular ligand for P2XR family of channels, composed of three subunits, each composed of a large ectodomain, two transmembrane domains and the N- and C-terminus facing the cytoplasm (59). In intact gonadotrophs, ATP-induced extracellular Ca^{2+}-dependent rise in cytosolic Ca^{2+} (66). In voltage-clamped cells, extracellular ATP-induced non-oscillatory current composed of rapidly depolarizing, slowly desensitizing, and rapidly deactivating phases, with the peak amplitudes and the rates of current desensitization determined by ATP concentration (**Figure 1K**). In current-clamped gonadotrophs, ATP induces a rapid depolarization that initiated firing of APs in quiescent cells, an increase in the frequency of firing in spontaneously active cells (**Figure 1L**), and a transient stimulation of LH release (67). The biophysical and pharmacological investigations suggested that gonadotrophs express the P2X2R subtype of these channels (67). Consistent with this conclusion, the full size and several splice forms of P2X2 subunit were identified in pituitary gland (68).

ATP is released by GnRH-secreting GT1 cells and cultured pituitary cells and metabolized by ectonucleotidase (69). Furthermore, GnRH increases ATP release in cultured pituitary cells (66). In accordance with these observations, it has been shown that ATP is co-secreted with GnRH from the median eminence into the hypophyseal-portal vasculature in ovariectomized sheep and that gonadotrophs have intrinsic ability to metabolize ATP in the extracellular space (70). This is consistent with the autocrine actions of extracellular ATP, where this molecule amplifies GnRH-induced Ca^{2+} signaling and LH secretion by activating P2X2Rs (67, 70). Pituitary cells other than gonadotrophs also express pannexin-1 and -2 channels (71), which contribute to ATP release in the extracellular medium in cultured pituitary cells (72). Thus, ATP and its degradation products ADP and adenosine may serve as paracrine factors to provide a cross talk between cell lineages within the pituitary gland *via* P2X2R (67), P2X4R (73), G-protein-coupled P2YRs (74), and adenosine receptors (75). By physical association with P2XRs, pannexin-1 may also provide a mechanism for autocrine control of functions of pituitary cell types expressing both proteins (76).

SIGNALING BY CHANNELS EXPRESSED IN ER MEMBRANES

Two families of structurally and functionally similar Ca^{2+} release channels, ryanodine receptors and IP_3Rs, are expressed in the ER membrane. Ryanodine receptors account for intracellular transduction and translation of PM electrical signals by Ca^{2+}-induced Ca^{2+} release from ER, whereas IP_3Rs are activated by Ca^{2+}-mobilizing receptors. In non-excitable cells, the IP_3R-induced depletion of ER-Ca^{2+} stores facilitates Ca^{2+} influx

through store-operated Ca^{2+}-conducting PM channels. Two proteins, named stromal-interacting molecule and Orai, are critical for this Ca^{2+} entry pathway (77). IP_3Rs are expressed in all secretory pituitary cells as indicated by ability of numerous Ca^{2+}-mobilizing agents to trigger Ca^{2+} release from ER (9). In contrast, no conclusive evidence was presented about the expression and role of ryanodine receptors and Orai channels in gonadotrophs and other secretory pituitary cell types (36).

The Ca^{2+}-mobilizing pathway is operative in gonadotrophs and is activated by GnRH as well as by pituitary adenylate cyclase-activating peptides, endothelins, ACh, vasopressin and oxytocin (50, 78–80). Among pituitary cells, a unique characteristic of mammalian gonadotrophs is the oscillatory pattern of Ca^{2+} release through IP_3Rs. **Figure 2A** illustrated GnRH-induced Ca^{2+} oscillations. In contrast, αT3-1 (**Figure 2B**) and LβT2 gonadotrophs (not shown) release Ca^{2+} in a non-oscillatory manner when stimulated with GnRH (81, 82). GnRH-induced calcium signaling is also non-oscillatory in fish pituitary cells (83) as well as in rat Leydig cells (**Figure 2B**) (84). In rat gonadotrophs, the frequency of Ca^{2+} oscillations is determined by GnRH concentration and varies between 3 and 20 pulses per minute (85, 86). In neonatal rat gonadotrophs, GnRH-induced, but not IP3-stimulated, Ca^{2+} oscillations are inhibited by melatonin (87–90).

Gonadotropin-releasing hormone-induced Ca^{2+} oscillations have profound effects on electrical activity of these cells. In current-clamped gonadotrophs, GnRH-induced a transient hyperpolarization, followed by a bursting pattern episode of tall electrical spikes (**Figure 2C**). When the membrane was voltage-clamped, GnRH-induced current oscillations were observed (**Figure 2D**) (91, 92). Patterns of Ca^{2+} and current oscillations are highly comparable in the same cell and current oscillations coincide with transient hyperpolazation of PM. It is well established that Ca^{2+}-activated SK channels account for coupling from the ER to PM in rat gonadotrophs (16, 38, 46, 93), whereas BK channels may also contribute to such coupling in mice gonadotrophs (40). In non-oscillatory αT3-1 gonadotrophs, GnRH stimulates L-type Ca^{2+} channels, leading to protein kinase C-dependent ERK activation (94), a process that requires dynamin GTP-ase activity (95).

The physiological relevance of bursting electrical activity in GnRH-stimulated gonadotrophs has been shown in voltage-clamped cells. By controlling the holding potential (HP) of the cell, this procedure provides a way to control the Ca^{2+} influx rate. In hyperpolarized cells with silent Ca_v channels, GnRH-induced current oscillations persist for about 5 min, reflecting a gradual depletion of the ER Ca^{2+} content. However, when the HP was more depolarized, many Ca_v channels are open and GnRH-induced current oscillations last much longer (**Figure 2E**), indicating that voltage-gated Ca^{2+} influx sustains signaling (91). Facilitation of Ca^{2+} influx through P2X2R channels also increases amplitudes of sustained GnRH-stimulated current oscillations (**Figure 2F**), a finding consistent with effect of ATP on GnRH-induced V_m oscillations and LH release (67).

The gating properties of IP_3R channels in gonadotrophs were not studied directly, and our understanding of kinetics of opening and closing is based on analysis of GnRH/IP_3-induced Ca^{2+}/current oscillations. IP_3 is needed to initiate the ER-dependent Ca^{2+} signaling, oscillations in intracellular IP_3 are not required to generate oscillatory Ca^{2+} release as documented by injection of non-metabolizable IP_3 analogs, and the concentration of IP_3

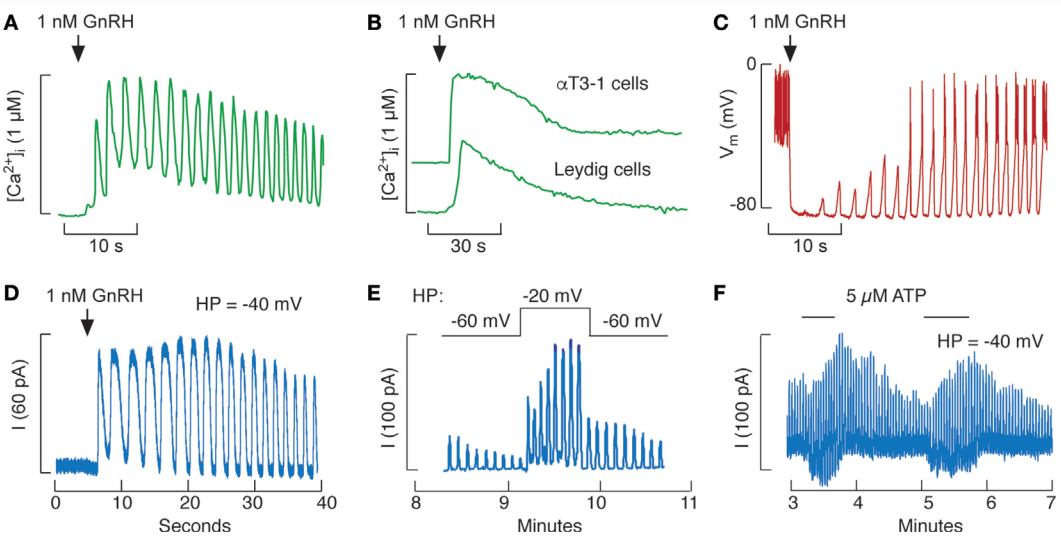

FIGURE 2 | Influence of Ca^{2+} mobilization on excitability of pituitary gonadotrophs. **(A,B)** Gonadotropin-releasing hormone (GnRH)-induced calcium oscillations in rat pituitary gonadotrophs **(A)** and non-oscillatory calcium signals in immortalized αT3-1 pituitary gonadotrophs and testicular Leydig cells **(B)**. **(C,D)** GnRH-induced membrane potential **(C)** and small-conductance K^+ (SK) current **(D)** oscillations in rat gonadotrophs. **(E,F)** Increase in the amplitude of GnRH-induced SK current oscillations by depolarization **(E)** and activation of P2X2R channels by extracellular ATP **(F)** in rat gonadotrophs. Current oscillations were initiated by 0.1 nM GnRH **(E)** and 1 nM GnRH **(F)**. Calcium recordings were done in intact Indo-1-loaded cells **(A,B)**, whereas electrophysiological recordings were done in nystatin-perforated cells voltage-clamped **(D–F)** or in current-clamped cells **(C)**. V_m, membrane potential; I, SK current; HP, holding potential. Derived from data shown in Ref. (36, 50, 63, 91); no permission is required from the copyright holder.

underlines the frequency of spiking (96). Furthermore, cytosolic Ca^{2+} influences IP_3-dependent Ca^{2+} release in these cells bidirectionally, stimulatory at lower concentrations and inhibitory at higher concentrations. The rapid stimulatory effect of Ca^{2+} on IP_3-depenent Ca^{2+} release is shown by phase resetting of GnRH-induced oscillations by a brief pulse of voltage-gated Ca^{2+} entry (97). The inhibitory effect of high Ca^{2+} concentrations on GnRH-induced Ca^{2+} oscillations was also shown (98).

INTERCELLULAR SIGNALING BY GAP JUNCTION CHANNELS

Secretory cells are not randomly spread throughout the pituitary gland but represent very organized three-dimensional network structures critical for the proper cell-type function (54, 99). Tridimensional imaging also suggested that pituitary gonadotrophs form a homotypic network (55). These and other pituitary cells express connexin-43 (100). In general, coupling of cells through connexin gap junctions provides a pathway for the passage of ions, metabolites, small molecules, and second messengers from cell to cell, without exposure to the extracellular environment (101, 102). However, the roles of connexins in synchronization of gonadotroph activity in intact tissue have not been systematically investigated.

CONCLUSION

This short review clearly indicates the complexity in expression and role of PM and ER channels in gonadotrophs. Various voltage-gated and related channels provide a background pathway for spontaneous firing of APs and Ca^{2+} signaling. In contrast to other secretory pituitary cells, spontaneous electrical activity is not coupled to exocytosis, i.e., Ca^{2+} signals generated by APs are subthreshold. However, the excitability of gonadotrophs is facilitated by activation of nicotinic AChRs, $GABA_AR$, and P2X2Rs, and the accompanied Ca^{2+} signals can trigger gonadotropin secretion. Activation of GnRHR and other Ca^{2+}-mobilizing receptors in gonadotrophs leads to Ca^{2+} release from ER through IP_3R channels coupled with a rapid LH secretion, and switch in the pattern of firing of APs from tonic single spiking to periodic plateau bursting, the latter being essential for sustained Ca^{2+} signaling and LH secretion. Further studies are needed to detail the role of ion channels in intracellular signaling cascade, gene expression, Ca^{2+} secretion coupling, and mechanism of synchronous activation of gonadotrophs in intact tissue.

AUTHOR CONTRIBUTIONS

All the authors participated in writing; SS prepared figures.

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Functional Role of Gonadotrope Plasticity and Network Organization

*Brian S. Edwards[1], Colin M. Clay[2], Buffy S. Ellsworth[3] and Amy M. Navratil[1]**

[1] Department of Zoology and Physiology, University of Wyoming, Laramie, WY, United States, [2] Department of Biomedical Science, Colorado State University, Fort Collins, CO, United States, [3] Department of Physiology, Southern Illinois University Carbondale, Carbondale, IL, United States

Gonadotrope cells of the anterior pituitary are characterized by their ability to mount a cyclical pattern of gonadotropin secretion to regulate gonadal function and fertility. Recent *in vitro* and *in vivo* evidence suggests that gonadotropes exhibit dramatic remodeling of the actin cytoskeleton following gonadotropin-releasing hormone (GnRH) exposure. GnRH engagement of actin is critical for gonadotrope function on multiple levels. First, GnRH-induced cell movements lead to spatial repositioning of the *in vivo* gonadotrope network toward vascular endothelium, presumably to access the bloodstream for effective hormone release. Interestingly, these plasticity changes can be modified depending on the physiological status of the organism. Additionally, GnRH-induced actin assembly appears to be fundamental to gonadotrope signaling at the level of extracellular signal-regulated kinase (ERK) activation, which is a well-known regulator of luteinizing hormone (LH) β-subunit synthesis. Last, GnRH-induced cell membrane projections are capable of concentrating LHβ-containing vesicles and disruption of the actin cytoskeleton reduces LH secretion. Taken together, gonadotrope network positioning and LH synthesis and secretion are linked to GnRH engagement of the actin cytoskeleton. In this review, we will cover the dynamics and organization of the *in vivo* gonadotrope cell network and the mechanisms of GnRH-induced actin-remodeling events important in ERK activation and subsequently hormone secretion.

Keywords: gonadotropin-releasing hormone receptor, actin cytoskeleton, extracellular signal regulated kinase signaling, gonadotrope cell signaling, luteinzing hormone, network dynamics

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****Correspondence:***
Amy M. Navratil
anavrati@uwyo.edu

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INTRODUCTION

Gonadotrope cells are a population of endocrine cells located in the anterior pituitary that are responsible for regulating the reproductive axis (1, 2). Gonadotropin-releasing hormone (GnRH) is synthesized in hypothalamic neurons and secreted in a pulsatile manner toward the fenestrated capillaries in the median eminence. Following release, GnRH is transported *via* the hypophysial portal vessels to the anterior pituitary where it binds to the GnRH receptor (GnRHR) located on gonadotrope cells. Stimulation of the GnRHR culminates in the synthesis and secretion of four main gene products: the common glycoprotein α-subunit, the hormone-specific luteinizing hormone (LH) β subunit, follicle-stimulating hormone (FSH) β-subunit, and the GnRHR (1–3). The

Abbreviations: GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; LH, luteinizing hormone; FSH, follicle-stimulating hormone; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; Arp 2/3, actin-related protein 2 and 3; mTORC2, mammalian target of rapamycin complex 2.

heterodimeric glycoproteins, LH and FSH, are then released into systemic circulation where they regulate gonadal development and function by stimulating steroidogenesis, gametogenesis, folliculogenesis, and ovulation (4, 5).

Depending on the phenotypic markers used to identify gonadotropes, the population undergoes dynamic changes in both size and numbers depending on the stage of the estrous cycle (6–8). For example, gonadotropes are thought to represent approximately 5–7% of total anterior pituitary cells during diestrus but can increase upwards to 15% in proestrus (8, 9). Additionally, evidence suggests that gonadotropes are a heterogeneous population of cells that can be classified as small, medium, and large (10–12). Gonadotropes that are large are bihormonal and enriched during estrus (13). Gonadotrope cells are also organized in homotypic and heterotypic cellular networks that can adapt to changing physiological conditions to generate coordinated hormone pulsatility (14–16). Examples of adaptable mechanisms in gonadotropes include cell morphology, migration, and positioning to vasculature; all of which requires a dynamic actin cytoskeleton.

The actin cytoskeleton plays an important role in cell division, motility, and intracellular trafficking of vesicles. The actin cytoskeleton has been extensively studied in the nervous system where it is important in synaptic morphology, function, vesicle mobilization, and recycling (17–20). Similarly, in secretory cells such as gonadotropes, an intact actin cytoskeleton is important in the regulated release of vesicular hormones and the replenishment of these vesicles with reserve vesicles (21–23). Thus, gonadotrope network organization and plasticity is essential to the optimization of proper reproductive function. In this review, we will highlight gonadotrope population networks and organization, GnRH-mediated actin reorganization events, and functionally linking these events with mitogen-activated protein kinase (MAPK) activation and subsequent gonadotropin secretion.

GONADOTROPE DEVELOPMENT AND ORGANIZATION

The anterior pituitary is a complex endocrine gland that secretes multiple hormones to control homeostasis, growth, lactation, and reproduction. It is composed of five distinct endocrine cell types: gonadotropes, thyrotropes, corticotropes, somatotropes, and lactotropes (24). During murine development, organogenesis of the pituitary commences at embryonic day (e) 9.0 with a focal dorsal invagination of somatic oral ectoderm (Rathke's pouch) to form the anterior and intermediate lobes (25). Lineage commitment and differentiation of pituitary cells initiates at e12.5 in a sequential manner and are orchestrated by combinatorial expression of cell type-specific transcription factors, epigenetic modifications, and cell–cell interactions (24, 26, 27). Gonadotrope cells are the last of the anterior pituitary cell lineages to undergo terminal differentiation with expression of the *Lhb* transcript occurring on e16.5, then *Fshb* on e17.5. Gonadotropes begin to become clustered and are localized to the central mediolateral region by e18.5 (14, 24, 27).

During development, it has been suggested that organization of latter differentiating anterior pituitary endocrine cell types (i.e., gonadotropes) are directed by earlier developing endocrine cell types (14). Indeed, corticotropes, which have been detected in mice at e13.5, are thought to direct the differentiation and clustering of gonadotropes (14, 15). The organization of the heterotypic network between gonadotropes and corticotropes occurs along the ventral surface of the anterior pituitary and is thought that these cells maintain direct contact throughout adulthood. In contrast, the homotypic network of gonadotropes develops along the dorsal surface of the anterior pituitary with little contact with other endocrine cell types (14). Interestingly, pituitaries that are deficient in corticotropes, *Tpit*$^{-/-}$ pituitaries display a decrease in gonadotrope cell volume and an increase in gonadotrope number due to an alternate cell fate adopted by their common precursor (28). A role for inter-connected networks was also highlighted between lactotropes and gonadotropes where ablation of gonadotropes resulted in modifications of lactotrope development and organization (29). Thus, network inter-connectivity between endocrine cell types may act as a scaffold that serves to organize and establish gonadotrope networks.

Postnatally, gonadotrope populations have been shown to be homogenously distributed throughout (lateral, caudal, rostral) the anterior pituitary when imaging whole-mount preparations of entire pituitary glands from prepubertal mice (30). However, following reproductive maturation, there is an increased density of gonadotropes in the rostral region relative to the lateral and ventral regions of the anterior pituitary. Furthermore, postpubertal gonadotrope populations have been characterized as being organized in string like clusters on both the ventral and dorsal surfaces of the anterior pituitary (14). Thus, plasticity within the gonadotrope population may be key for mounting appropriate responses to fluctuating hormone levels that occur as mice transition from pre- to postpuberty. Toward this end, priming gonadotropes with long-term estradiol treatment increased cellular plasticity and responsiveness to GnRH (30). Interestingly, the population of gonadotropes as a whole in sexually mature mice also display a high degree of plasticity depending on the physiological demands. This is demonstrated in lactating mice where gonadotropes reside in clusters in the lateral and ventral areas and not in the rostral region (30). Taken together, it is clear that gonadotrope networks exhibit a continuous plasticity that is pertinent to producing a proper response to changing physiological conditions.

GONADOTROPE PLASTICITY *IN VIVO*

A primary goal of cellular secretory elements of endocrine glands is directed secretion of hormone into the blood stream. As such, endocrine cells are often embedded in connective tissue surrounded by rich vascular networks. In particular, it has long been evident that gonadotrope cells display considerable surface area in close apposition to capillary endothelium (14, 30). Such an arrangement presumably allows for efficient and robust delivery of gonadotropin into the circulation. It is reasonable to predict that gonadotrope "priming" reflects multiple events that include enhanced GnRH responsiveness, mobilization of secretory granules and, perhaps, increased apposition of the basolateral

secretory surface area of gonadotropes. Collectively then, each of these would contribute to placing both gonadotropes and secretory granules in the most effective position for maximal release of hormone in response to GnRH stimulation. According to this paradigm, GnRH not only elicits exocytosis of secretory granules from gonadotropes but also contributes to organizing these cells in the most favorable spatial orientation to achieve the rapid and pronounced increase in circulating gonadotropin concentrations.

Gonadotropes are characterized by their ability to mount a cyclical pattern of hormone secretion, an event critical in the production of the preovulatory LH surge in females (2, 3, 31, 32). Previous evidence suggests that these cells display both structural and functional plasticity throughout the female reproductive cycle (6, 9, 30). Under conditions of GnRH and, perhaps, steroid stimulation, morphological rearrangements of gonadotropes are elicited leading to the development of cellular processes or projections that extend toward capillary sinusoids. As early as 1985, Dr. Gwen Childs noted that GnRH stimulated gonadotropes developed processes during peak LH secretory episodes (7). Osamura and colleagues in Japan also demonstrated a similar phenomenon based on three dimensional reconstructions of pituitary vasculature and endocrine cells (33, 34). Previous live cell studies of *ex vivo* pituitary slices have shown that gonadotropes display a high degree of plasticity in the face of neuroendocrine stimulation (30, 35). GnRH exposure to murine pituitary slices leads cell processes and spatial repositioning of GFP-labeled gonadotropes using the *ex vivo* paradigm (35). The stimulation-dependent plasticity displayed by gonadotropes is thought to lead to increased association between gonadotropes and the microvasculature of the pituitary (30). Spatial positioning of gonadotropes reveals a much closer proximity to vasculature when compared to corticotropes (14), and there is evidence that gonadotropes can have a close spatial association with more than one blood vessel through multiple cellular projections. It should be noted that the GnRH-induced cellular projections extending toward blood vessels contain LH secretory granules, which may increase the secretory impact of gonadotropes (36).

Gonadotropin-releasing hormone-induced plasticity in gonadotropes creates transient cellular structures in the form of lamellipodia, membrane ruffles, and filopodia. The actin cytoskeleton supports these membrane remodeling events by assembling actin monomers to form filamentous actin (17, 37, 38). We have previously found that GnRH induces rapid dynamic engagement of the actin cytoskeleton within 1 min of treatment (35). However, pretreatment with a pharmacological disruptor of the actin cytoskeleton, jasplakinolide (Jas), blunts GnRH-induced membrane remodeling events (35). Not only does the actin cytoskeleton play an important role in structural support and cell migration but it is also important for coordinating the trafficking and release of secretory vesicles in endocrine cells. We have previously shown that upon GnRH stimulation of primary murine gonadotrope cells, there is an approximate 3.5-fold increase in LH secretion (36). In contrast, GnRH stimulation to primary murine pituitary cells that are pretreated with Jas results in a significant reduction in LH secretion with no difference compared to vehicle (36). Thus, the GnRH-mediated plasticity is critical in maintaining physiological levels of LH and to spatially align responsive gonadotropes in close proximity to the pituitary vasculature for secretory events.

Gonadotrope plasticity is also pertinent in establishing an organized network throughout the anterior pituitary. Network organization is a critical aspect in the maintenance of reproduction as gonadotropes must orchestrate hormone secretory events in the face of changing physiological demands. In order for proper gonadotrope organization, it is thought that communication and interaction between endocrine and non-endocrine networks is an underlying mechanism. Specifically, folliculostellate cells predominantly communicate through gap junctions and paracrine and autocrine signaling with endocrine cells in the anterior pituitary (15, 39). Additionally, the number of gap junctions between folliculostellate cells and the altered morphological relationship with hormonal cells in the anterior pituitary also provides additional evidence of functional plasticity in this non-hormonal cell type (40–42). Overall, the large-scale gonadotrope reorganization and interaction with non-hormonal cells may be the key in mounting a proper response to changing physiological conditions through connections with one or more blood vessels *via* their protrusions.

GONADOTROPE SIGNALING TO ACTIN

Gonadotropin-releasing hormone actions are modulated through the GnRH receptor (GnRHR), a G-protein-coupled receptor found on the plasma membrane of gonadotropes. Upon activation, the GnRHR undergoes a conformational change that promotes the activation of the heterotrimeric G-proteins, specifically, $G\alpha_{q/11}$. Activation of $G\alpha_{q/11}$ activates phospholipase Cβ1, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 interaction with the IP_3 receptor induces an elevation of intracellular Ca^{2+} from the endoplasmic reticulum, while DAG activates one or more isoforms of PKC (43, 44) that initiate Ca^{2+} influx through activation of voltage-gated L-type Ca^{2+} channels (VGCCs) (45–47). These upstream events underlie GnRH activation of extracellular signal-regulated kinase (ERK), the MAPK predominantly involved in regulating LHβ synthesis (48–50).

The actin cytoskeleton is a dense meshwork of protein polymers that undergoes cycles of assembly and disassembly and is regulated by a number of actin-associated proteins (37). Cortactin is a filamentous actin-binding protein that acts as an actin-scaffolding protein to mediate actin polymerization (51, 52). Cortactin mediates actin polymerization by binding actin-related protein (Arp) 2/3 complex, a nucleating factor that serves to facilitate actin filament branching, through a three amino acid motif in its amino terminus (52). Furthermore, cortactin is a target of multiple tyrosine and serine/threonine kinases (53, 54). Our laboratory has previously shown that cortactin activation is required for GnRH-induced plasticity in αT3-1 gonadotropes, and that src-induced tyrosine phosphorylation of cortactin is key in facilitating association of Arp3 to effectively engage the actin cytoskeleton (36).

In addition to regulating actin polymerization, cortactin may also serve as a functional link between intracellular signaling cascades and actin assembly events (53–55). Interestingly, disrupting

the actin cytoskeleton with Jas resulted in a loss of GnRH-induced ERK phosphorylation. However, GnRH-induced cell movement and projections is not inhibited by the MAPK kinase 1 inhibitor PD98059. Collectively suggesting that in αT3-1 cells, ERK activation is not a prerequisite for actin reorganization, but an intact actin cytoskeleton is required in the activation of ERK (35, 36). Consistent with this work, HEK293 cells expressing the GnRHR showed altered cellular morphology and cytoskeletal reorganization following treatment with GnRH. In addition, activation of ERK was significantly reduced following cytoskeletal disruption (56). The precise mechanism of how actin engagement impacts ERK activation remains unclear although data suggests that PKC is working downstream of the actin cytoskeleton. In support of this, direct activation of PKC with phorbol 12-myristate 13-acetate was not sufficient to induce cytoskeletal remodeling suggesting that PKC is working downstream of the actin cytoskeleton to facilitate activation of ERK in gonadotropes. Recent work also supports the notion that actin reorganization may be important for GnRH-mediated opening of L-type calcium channels (47)—the key calcium signal leading to ERK activation (46, 47).

Dynamin, a large GTPase and proline-rich domain-containing protein, possesses mechanochemical properties important in membrane remodeling events and fission (57). Many of these functions of dynamin appears to be associated with remodeling of the actin cytoskeleton (58); however, the mechanism by which it does so remains unclear. Overexpression of dominant-negative dynamin mutant proteins impaired in hydrolyzing GTP (K44A) perturbs many F-actin-rich cellular structures (59–61). Consistent with this data, αT3-1 cells transfected with K44A resulted in a loss of GnRH-induced actin remodeling events (62). Our group also demonstrated that pharmacological inhibition of dynamin GTPase activity, using both dynasore and dyngo, not only perturbed GnRH-induced actin reorganization but also significantly suppressed ERK activation (63). Thus, highlighting the importance of dynamin GTPase activity in actin reorganization and subsequent MAPK activation. In addition, the actin-binding protein, cortactin, not only enhances dynamin GTPase activity but also binds dynamin through its C-terminal SH3-domain (64). It is also well known that dynamin and cortactin colocalize in podosomes (65), membrane ruffles (66), and actin comets (67). Similarly, our group highlighted that upon GnRH stimulation, cortactin and dynamin are redistributed and become colocalized in areas indicative of high actin reorganization in αT3-1 cells (63). In addition to regulating Tyr phosphorylation of cortactin, there is also evidence that src induces Tyr phosphorylation of dynamin (62, 68). Thus, GnRH-induced gonadotrope plasticity may be modulated through the interaction of dynamin and cortactin to effectively engage the actin cytoskeleton to subsequently regulate PKC activation, VGCC opening, and ERK phosphorylation (47, 63). Clearly, the functionality and mechanism by which dynamin regulates gonadotrope plasticity warrants further investigation.

Although our group and others have started to unravel the signaling intermediates, GnRH utilizes to engage the actin cytoskeleton, identification of the full cohort of intermediates remains unclear. Recent work suggests that mammalian target of rapamycin (mTOR) also signals to the actin cytoskeleton to regulate cellular morphology both *in vitro* and *in vivo* (69, 70). mTOR is a serine/threonine protein kinase that forms two distinct complexes, mTORC1 and mTORC2. Our recent work using the LβT2 gonadotrope cell line establishes a specific role for mTORC2 in regulating membrane remodeling events (71). Pharmacological inhibition of mTORC2-blunted GnRH-mediated actin reorganization and similarly attenuated activation of ERK and LHβ gene expression (71). Although we have established an additional key intermediate linking GnRHR signaling to actin remodeling and ERK activation, the upstream signaling molecules regulating activation of mTORC2 in LβT2 cells remains unknown. It has been previously demonstrated that the Rho GTPase, Rac1, binds to and activates mTORC2 and also facilitates localization to the plasma membrane (72). GnRH also modulates LβT2 cell morphology and migration through Rho family members (73). Thus, Rac1 is likely a strong candidate involved in mediating mTORC2 activation and subsequent engagement of the actin cytoskeleton in gonadotrope cells. Taken together, GnRH-mediated actin cytoskeletal reorganization is controlled by multiple signaling networks to insure proper reproductive functioning.

CONCLUSION

The gonadotrope population displays profound plasticity that is present during late stages of embryological development and continues into adulthood (14, 15). The plasticity in the population is not only dependent on fluctuating hormone levels and reproductive status but also other endocrine cellular networks acting as a guidance scaffold. However, the gonadotrope plasticity in an individual cell is dependent on an intact dynamic actin cytoskeleton that is directed by multiple signaling intermediates. The actin cytoskeleton in gonadotropes serves a critical function in maintaining competence of the hypothalamic–pituitary–gonadal axis and mammalian fertility. We highlighted that GnRH engages the actin cytoskeleton to not only increase cell movement but also causes membrane remodeling events in the form of membrane ruffles, filopodia, and lamellipodia to potentially gain increased access to the pituitary vasculature (30). We suggest that cortactin and dynamin form an actin remodeling protein complex that functionally links neuroendocrine stimulation and actin polymerization (35, 36, 63). We also underscore mTORC2 as an additional signaling intermediate important in regulating membrane remodeling events and subsequent MAPK activation in gonadotropes (71). However, despite our data emphasizing gonadotrope plasticity and associated proteins; the mechanisms by which actin polymerization results in activation of ERK upstream of PKC remains largely undefined.

AUTHOR CONTRIBUTIONS

All authors have contributed to the writing of this review, and all have read and approved the final manuscript.

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GnRH Induces ERK-Dependent Bleb Formation in Gonadotrope Cells, Involving Recruitment of Members of a GnRH Receptor-Associated Signalosome to the Blebs

Liat Rahamim-Ben Navi[1], Anna Tsukerman[2], Alona Feldman[2], Philippa Melamed[2], Melanija Tomić[3†], Stanko S. Stojilkovic[3], Ulrich Boehm[4], Rony Seger[5‡] and Zvi Naor[1§]*

[1] Department of Biochemistry and Molecular Biology, Tel Aviv University, Tel Aviv, Israel, [2] Faculty of Biology, Technion-Israel Institute of Technology, Haifa, Israel, [3] National Institute of Child Health and Human Development, National Institute of Health, Bethesda, MD, United States, [4] Department of Pharmacology and Toxicology, University of Saarland School of Medicine, Homburg, Germany, [5] Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel

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***Correspondence:**
Zvi Naor
zvin@tauex.tau.ac.il

†Melanija Tomić, Deceased (May She Rest in Peace). This manuscript is dedicated to Melanija Tomić.

‡Incumbent of the of the Yale S. Lewine and Ella Miller Lewine Professorial chair for cancer research.

§Incumbent of the Abraham E. Kazan Chair in Structural Biology.

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We have previously described a signaling complex (signalosome) associated with the GnRH receptor (GnRHR). We now report that GnRH induces bleb formation in the gonadotrope-derived LβT2 cells. The blebs appear within ~2 min at a turnover rate of ~ 2–3 blebs/min and last for at least 90 min. Formation of the blebs requires active ERK1/2 and RhoA–ROCK but not active c-Src. Although the following ligands stimulate ERK1/2 in LβT2 cells: EGF > GnRH > PMA > cyclic adenosine monophosphate (cAMP), they produced little or no effect on bleb formation as compared to the robust effect of GnRH (GnRH > PMA > cAMP > EGF), indicating that ERK1/2 is required but not sufficient for bleb formation possibly due to compartmentalization. Members of the above mentioned signalosome are recruited to the blebs, some during bleb formation (GnRHR, c-Src, ERK1/2, focal adhesion kinase, paxillin, and tubulin), and some during bleb retraction (vinculin), while F-actin decorates the blebs during retraction. Fluorescence intensity measurements for the above proteins across the cells showed higher intensity in the blebs vs. intracellular area. Moreover, GnRH induces blebs in primary cultures of rat pituitary cells and isolated mouse gonadotropes in an ERK1/2-dependent manner. The novel signalosome–bleb pathway suggests that as with the signalosome, the blebs are apparently involved in cell migration. Hence, we have extended the potential candidates which are involved in the blebs life cycle in general and for the GnRHR in particular.

Keywords: GnRH, GnRH receptor, ERK, gonadotropes, blebs, signalosome

INTRODUCTION

GnRH interaction with the GnRH receptor (GnRHR) in pituitary gonadotropes is a key step in reproduction (1, 2) (for reviews). The GnRHR is a unique member of the GPCR family, lacking a c-terminal tail (3, 4). The signaling of the GnRHR is complex and includes interaction with heterotrimeric G proteins (G-proteins) primarily *via* the Gq and/or G_{11} (5), stimulation of cyclic adenosine monophosphate (cAMP), protein kinase A, prostaglandins (PGs) (2), Ca^{2+}-calmodulin (6–8), protein

kinase C isoforms (PKCs), and mitogen-activated protein kinases (MAPKs) (2, 9). The signaling pathways culminate in luteinizing hormone (LH) and follicle-stimulating hormone synthesis and release (1–9).

Mitogen-activated protein kinase cascades in mammals include ERK1/2 (p42 and p44), JNK1/3, p38 (α, β, γ, δ), and ERK5 (10, 11). MAPKs act by sequential phosphorylation and activation of their kinase components (10, 11). MAPKs translocate to the nucleus and activate transcription factors; however, they can also reside and act in the cytosol (10, 11). MAPKs participate in GnRH-induced transcriptional control of the gonadotropin subunits and the GnRHR genes (2, 12–28).

GnRH receptor-associated protein–protein complexes and actin cytoskeletal remodeling events have been described (29–32). We have previously demonstrated the presence of such a complex (signalosome) that seems to reside in microtubules and focal adhesions (FAs) (33). Members of the signalosome included the GnRHR, Ras–MEK–ERK, PKCs, focal adhesion kinase (FAK), paxillin, vinculin, and tubulin (Figure S1 in Supplementary Material). We have proposed that the role of the signalosome is to sequester a pool of GnRH-activated ERK1/2 in the cytosol for the phosphorylation of FAK and paxillin at FAs, to mediate cell migration, as recently proposed for GnRH-stimulated gonadotropes (34, 35).

Cell membrane blebs are dynamic protrusions that are implicated in apoptosis, cytokinesis, and cell movement (36). The blebs are formed by depolymerization of the actin cortex, which leads to rapid bleb formation as a result of the cell internal hydrostatic pressure (36). Blebs expand up to 2 μm from the cell membrane and are defined by a spherical morphology (36). Blebs have highly dynamic life cycle that roughly lasts 1–2 min; rapid bleb expansion, a short static phase; and retraction of the blebs (36–39). Initial expansion of the blebs does not involve actin polymerization, which distinguishes plasma membrane bleb from all other known cell protrusions such as lamellipodia and filopodia (36–39). Actin is subsequently polymerized at the bleb cortex to halt bleb expansion and actomyosin contractility is generated to retract the blebs (40). The contractility for bleb retraction is provided by signaling through Rho-ROCK-myosin. In this cascade, Rho-GTP activates its effector kinase Rho-associated kinase (ROCK) that directly phosphorylates myosin light chain, which then induces actomyosin contraction (36, 41).

Here, we show that GnRH induces bleb formation in the immortalized LβT2 pituitary gonadotrope cells, a process requiring active ERK1/2 and Rho-ROCK but not active c-Src. Members of the above described signalosome are also present in the blebs during bleb formation, stabilization, or retraction, suggesting that they were recruited separately to the blebs. We also confirmed the findings in rat- and mouse-isolated gonadotropes. Hence, we have extended the potential candidates which are involved in the blebs life cycle in general and the GnRHR in particular.

MATERIALS AND METHODS

Materials

Medium, serum, and antibiotics for cell cultures are from Biological Industries (Kibbutz Beit Ha'Emek, Israel). GnRH and PMA were obtained from Sigma (St. Louis, MO, USA). EGF was purchased from Prospec (East Brunswick, NJ, USA). U0126, SB203580, 8-Br-cAMP, mouse monoclonal anti-doubly phosphorylated-ERK1/2 antibodies, and rabbit polyclonal antibodies to general ERK were obtained from Sigma-Aldrich (Rehovot, Israel). jetPRIME Transfection reagent was obtained from polyplus transfection (Illkirch, France). GnRH antagonist (cetrorelix acetate) was from Merck (NJ, USA). The ROCK inhibitor Y-27632 was from Cayman Chemical Company (Ann Arbor, MI, USA). Secondary horseradish peroxidase-conjugated goat anti mouse antibodies or goats anti rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). GFP-ERK2 and c-Src-GFP were kindly provided by Dr. Rony Seger, and Vinculin-GFP was kindly provided by Dr. Benny Geiger (Weizmann Institute of Science, Rehovot, Israel). GnRHR-mCherry construct was kindly provided by Dr. Colin Clay (Colorado State University, USA). Paxillin-GFP and FAK-GFP were kindly provided by Dr. Kenneth Yamada (NIH, USA). Actin-YFP was kindly provided by Dr. Ilan Tsarfaty, and EMTB-3XGFP was kindly provided by Dr. David Sprinzak (Tel-Aviv University, Israel).

Cell Culture

LβT2 cells (kindly provided by Prof. P. Mellon UCSD, USA) were grown in DMEM supplemented with 10% FCS, streptomycin (100 μg/ml), penicillin (100 U/ml), and 5% glutamine. Cells were maintained in humidified atmosphere of 5% CO_2 and at 37°C. At 70–80% confluence, the cells were serum starved overnight in DMEM with 0.1% FCS, and stimulants were added in DMEM. Cells were washed twice with ice-cold PBS and overlaid with lysis buffer (20 mM Tris–HCl pH 7.5, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5% Tryton x-100, 50 mM β-glycerophosphate, 30% glycerol, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF), followed by centrifugation (15,000 × *g*, 15 min, 4°C). The supernatants were collected, and aliquots were separated on 10% SDS-PAGE, followed by Western blotting.

Live Cell Imaging

LβT2 cells were plated on 35 mm glass-bottom plates and were transfected with 1 μg of GnRHR-mCherry along with 1 μg of one of the complex proteins constructs (ERK2–GFP, FAK–GFP, etc.) by using the jet PRIME™ transfection reagent. Approximately 30 h after transfection, the cells were serum starved (0.1% FCS) for 16 h and later stimulated with various ligands and inhibitors as indicated. Images were acquired at 10-s intervals using Leica TCS STED microscope (Leica, Wetzlar, Germany) using the 63 objective. Cells were kept in a microscope stage incubator at 37°C in a humidified atmosphere of 5% CO_2 throughout the experiment. Data and image analysis was performed using ImageJ (NIH, Bethesda, MD, USA).

Cell Migration Assay

LβT2 cells (1×10^5) were trypsinized and resuspended in starvation medium (0.1% FCS) and plated in Matrigel (1:150 dilution)-coated transwell inserts with or without the MEK inhibitor, U0126 (25 μM). Lower chambers contained starvation medium with 10 nm GnRH. After 24 h, cells were fixed in 2.5%

glutaraldehyde for 15 min and washed with DDW. Cells were stained with 0.1% methylene blue for 60 min. Cells that did not migrate to the underside of the membrane were scraped off using a cotton swab. Migrated cells were observed under a microscope and counted from ten random fields.

Primary Culture of Anterior Pituitary Cells

Post-pubertal female Sprague-Dawley rats obtained from Taconic Farms (Germantown, NY, USA) were euthanized by asphyxiation with CO_2, and the anterior pituitary glands were removed after decapitation. The procedure was approved by the NICHD Animal Care and Use Committee (#14-041). The methods were carried out in accordance with the approved guidelines. Pituitary tissue was cut into 1 mm^3 pieces, treated with trypsin (20 μg/ml diluted in PBS + 0.3% BSA medium) for 15 min at 37°C, and followed by addition of a pinch of DNAase and 2.6 mg/ml trypsin inhibitor. Mechanical dispersion of cells was done in calcium-deficient PBS medium. Dispersed anterior pituitary cells were plated on poly-L-lysine coated 25 mm circular coverslips (Thomas Scientific, Swedesboro, NJ, USA) at 7×10^5 cells/coverslip density and cultured overnight in medium-199 containing Earle's salts and supplemented with 10% horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Life Technologies). At least an hour prior to experiments, the medium was changed to Krebs-Ringer containing 2.5 μM Fura-2 AM (Life Technologies). The coverslips were then washed in Krebs-Ringer medium and mounted on the stage of an inverted Observer-D1 microscope (Carl Zeiss, Oberkochen, Germany) with an attached ORCA-ER camera (Hamamatsu Photonics, Hamamatsu City, Japan) and a Lambda DG-4 wavelength switcher (Sutter, Novato, CA, USA). Hardware control and image analysis was performed using Metafluor software (Molecular Devices, Downingtown, PA, USA). Experiments were performed with a 63× oil-immersion objective while alternatively recording transmitted light image and the image at 380 nm excitation beam. There were approximately 20 mixed pituitary cells in the field, and the gonadotropes were identified by their intracellular Ca^{2+} response to GnRH, i.e., rapid decrease of 380 nm-induced fluorescence intensity, followed by a slower increase. After that, blebbing was analyzed. The images were further analyzed using ImageJ (NIH, Bethesda, MD, USA).

Preparation of Primary Gonadotrope Cultures from GRIC-Ai9 Mice

In order to confirm our findings from the gonadotrope-derived cell line, we have prepared primary gonadotropes from transgenic mice that carry a fluorescent signal in their gonadotropes, the GRIC/Ai9 mice. GRIC mice express Cre recombinase driven by the promoter of the GnRHR gene. Therefore, when Ai9 mice are crossed with GRIC mice the stop cassette is excised, which activates constitutive expression of dTomato in the gonadotropes. These mice thus allow identification and sorting of the gonadotrope cells. Animals were held and handled after protocol approval by the Technion IACUC and in accordance with their guidelines and regulations. We prepared primary gonadotrope culture from heterozygous mice created by breeding GRIC females with Ai9 males. Sexually mature female heterozygous mice were sacrificed, their pituitaries removed, and pituitary cells were prepared as previously described (42, 43). The gonadotropes were collected from the total pituitary population based on their fluorescence, using a FACS Aria 2 sorter. Following sorting, the cells were plated on glass-bottom plates for 12 h in fresh medium (DMEM 10% FCS). At least an hour prior to experiments, cells were serum starved (0.1% FCS), later stimulated with various ligands and inhibitors. Images were acquired using Leica TCS STED microscope (Leica, Wetzlar, Germany) using the 63 objective. Cells were kept in a microscope stage incubator at 37°C in a humidified atmosphere of 5% CO_2 throughout the experiment. Data and image analysis was performed using ImageJ (NIH, Bethesda, MD, USA).

Statistical Analysis

Results from three or more experiments were expressed as mean ± SEM. Where appropriate, data were subjected to statistical analysis by Student's *t*-test, or by one- or two-way ANOVA, depending on the experimental design. Values of $p < 0.05$ were considered statistically significant.

RESULTS

GnRH Induces Bleb Formation and GnRHR Is Present in the Blebs

Time-lapse confocal microscopy of GnRH-treated LβT2 cells showed that GnRH induces bleb formation (**Figure 1A**) (see also Video S1 in Supplementary Material). The blebs appear within ~2 min and last for at least 90 min at the apparent turnover rate of ~2–3 blebs/min. In order to further investigate the involvement of the GnRHR in bleb formation, LβT2 cells were transfected with GnRHR-mCherry and then treated with GnRH for 30 min. Under basal conditions, GnRHR was observed in the membrane (**Figure 1B**), while after GnRH treatment, GnRHR decorated the blebs membrane, with no difference between blebs expansion and retraction. Preincubation with the GnRH antagonist (cetrorelix acetate), abolished bleb formation by GnRH (**Figures 1C,D**), confirming that bleb formation is mediated by the GnRHR. In addition, the cells returned to pretreatment morphology after removal of GnRH indicating that the process is reversible. Retreatment with GnRH (30 min) 6 h later resulted in a "priming effect," which is defined as an increase in cells response to the second exposure to GnRH compared with the first. Indeed, the second exposure to GnRH elevated the percentage of blebbing cells (**Figure 2**).

ERK1/2 Accumulates in the Blebs and Is Involved in Bleb Formation

ERK1/2 activation by GnRH in LβT2 cells was reported to involve PKC, Ca^{2+} influx, dynamin, and c-Src (20, 44, 45). Previous studies in our laboratory have examined the kinetics of ERK1/2 activation in response to GnRH treatment in LβT2 cells. GnRH treatment resulted in a rapid and robust activation of ERK1/2 with a peak 5 min after stimulation and decline but still detectable after 90 min (44). It is thought that RTK and GPCR ligands induce a rapid translocation of ERK1/2 to the nucleus to

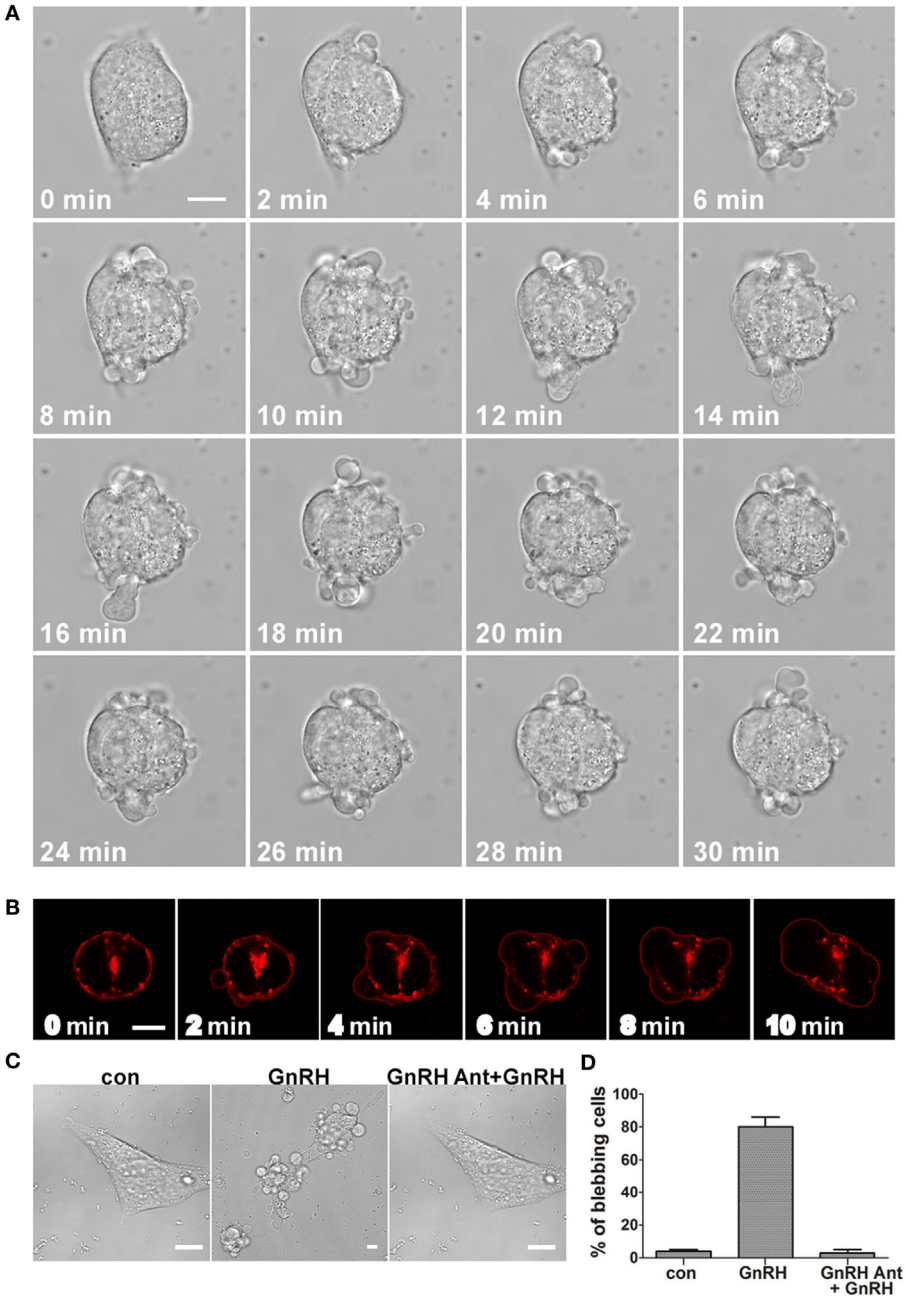

FIGURE 1 | **GnRH induces bleb formation in LβT2 cells and GnRH receptor (GnRHR) is present in the blebs**. **(A)** Images from a confocal microscopy time-lapse movie of serum-starved LβT2 gonadotrope cells treated with GnRH (10 nM) (0–30 min). The treatment resulted in bleb formation. The *scale bar* is 10 μm. **(B)** GnRHR is present in the blebs. Serum-starved LβT2 cells were subjected to time laps confocal microscopy. Addition of GnRH (10 nM) (0–10 min) to LβT2 cells transfected with GnRHR-mCherry resulted in bleb formation, while GnRHR is present in the blebs. The *scale bar* is 5 μm. **(C)** GnRHR mediates the formation of the blebs by GnRH. Serum-starved LβT2 cells were incubated with GnRH (10 nM) (30 min), or preincubated first with 100 nM GnRH antagonist (cetrorelix acetate) for 30 min followed by GnRH (10 nM) (GnRH antagonist + GnRH) for additional 30 min. Cells were imaged using confocal microscope and *the scale bar* is 10 μm. **(D)** Quantitation of blebbing cells in control, GnRH and GnRH antagonist + GnRH. Images of at least 10 fields were taken for each treatment, and the *bars* are mean ± SEM from 3 experiments.

phosphorylate and activate transcription factors (10, 11). In order to understand the involvement of ERK1/2 in bleb formation, we followed the cellular localization of ERK1/2 in response to GnRH treatment. LβT2 cells were transfected with GnRHR-mCherry and ERK2-GFP and then treated with GnRH for 15 min. Time-lapse confocal microscopy showed that GnRH-induced ERK1/2 accumulation in the blebs within 1 min (**Figure 3A**) (see also Video S2 in Supplementary Material). A line intensity profile

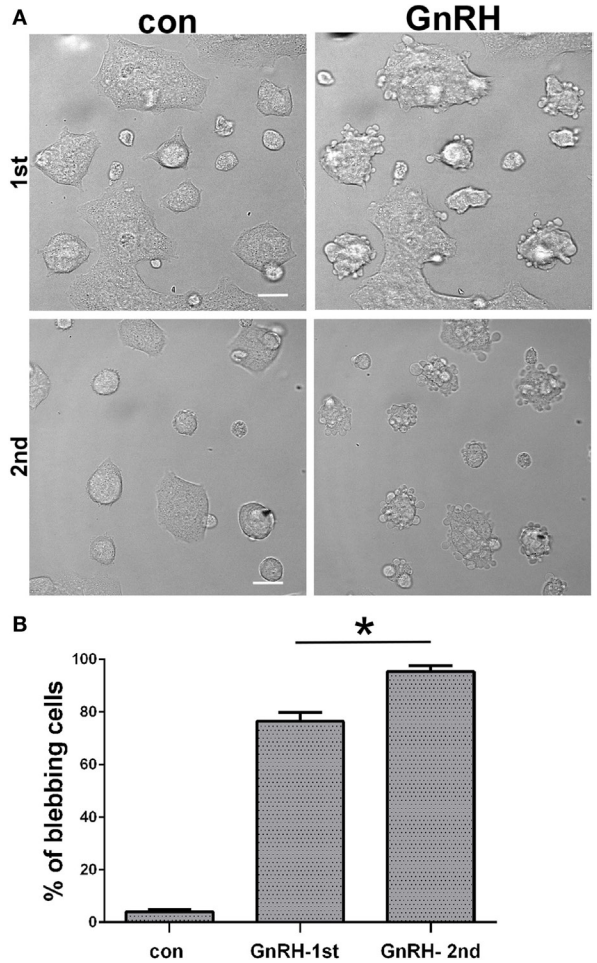

FIGURE 2 | **Priming effect of GnRH upon blebs formation**. **(A)** Serum-starved LβT2 cells were incubated for 30 min with 10 nM GnRH and analyzed for blebs formation as above. Another group of cells were first incubated for 30 min with 10 nM GnRH, at the end of which GnRH was removed by a wash. Six hours later, a second dose of 10 nM of GnRH was added to the cells. Both groups of cells were imaged using confocal microscopy. The *scale bar* is 20 μm. **(B)** Quantitation of blebbing cells in control, GnRH (first treatment) and GnRH (second treatment). Images of at least 10 fields were taken for each treatment, and the *bars* are mean ± SEM. *p-Value ≤0.05 vs. GnRH first exposure.

across the cell was obtained (**Figure 3B**) and intensity profiles shown on the right demonstrate accumulation of ERK1/2 in the blebs. Quantitation of mean fluorescence intensity showed higher values of ERK1/2 in the blebs vs. intracellular area (without the blebs area) (**Figure 3C**). Moreover, preincubation with the MEK inhibitor U0126 strongly inhibits GnRH-induced bleb formation (**Figure 3D**). Also, preincubation with the MEK inhibitor U0126 strongly reduced GnRH-induced cell migration (data not shown) suggesting that the bleb formation may be involved in cell migration. Pretreatment with SB203580, a p38 inhibitor did not attenuate the bleb formation (**Figure 3E**). Quantitation confirmed that compared to GnRH treated cells, U0126 reduced cell blebbing and SB203580 had no significant effect (**Figure 3E**). In addition, fluorescence intensity was measured across the cells and the profiles shown in line graphs on the right indicates that ERK2 accumulates in the blebs even in the presence of the p38 inhibitor (**Figure 3F**).

ERK1/2 Activation Is Required, but Not Sufficient for Bleb Formation

Epidermal growth factor (EGF) plays important roles in proliferation, differentiation, and migration *via* stimulation of the ERK1/2 signaling pathway (46). Moreover, Bonfil et al. (20) reported that ERK1/2 activation by GnRH in LβT2 cells is mediated by PKC, Ca^{2+} influx, dynamin, and c-Src, and not *via* transactivation of the EGFR. PMA is a PKC activator, which mimics the action of the naturally occurring DAG by binding to the C1 region of PKC, thus activating the enzyme (47–49). PMA mimicked the activation of ERK1/2 by GnRH (44). In addition, GnRH stimulates cAMP production in LβT2 gonadotrope cells *via* PKCδ (50). We therefore examined the effect of the various ligands on ERK1/2 activation and bleb formation since we have shown above that active ERK1/2 is required for bleb formation (**Figure 3**). Addition of EGF resulted in rapid activation of ERK1/2 with a peak after 5 min, similar to the effect of GnRH (33) (**Figure 4A**). LβT2 cells were treated with EGF for 30 min, and time-lapse confocal microscopy revealed minimal bleb formation (**Figure 4B**). GnRH was then added to the EGF-pretreated cells and significant elevation of bleb formation was observed (**Figures 4B,C**). The ligands induced ERK1/2 activation in the rank order of: EGF > GnRH > PMA > cAMP (**Figure 4D**). Later, we examined the effect of the ligands on bleb formation. cAMP and PMA induced relatively small amount of blebs (**Figure 4E**). The rank order for bleb formation differs from that obtained for ERK1/2 activation and is: GnRH > PMA > cAMP > EGF (**Figures 4C,E**). Therefore, we propose that ERK1/2 activation is required, but not sufficient for bleb formation. Furthermore, the data suggest compartmentalization of the ERK1/2 signal to the blebs in a ligand-dependent manner, since GnRH-activated ERK1/2, was preferentially sorted also to the blebs.

RhoA–ROCK Is Involved in GnRH-Induced Bleb Formation

The Rho family members RhoA, Rac1, and Cdc42 are implicated in actin cytoskeleton rearrangements. Godoy at el. (35) showed that GnRH activates Rho family members and increases cell motility. We therefore examined whether RhoA/ROCK is involved in GnRH-induced bleb formation. Preincubation with the ROCK inhibitor Y27632 abolished GnRH-induced bleb formation (**Figures 4F–H**). The findings indicate that RhoA–ROCK is involved in GnRH-induced bleb formation.

c-Src Is Present in the Blebs

Our previous studies showed that GnRH activates ERK1/2 in LβT2 gonadotrope cells in a c-Src-dependent manner (20). The modular SH1, SH2, and SH3 and kinase domains of the Src family tyrosine kinases allow these domains to act as scaffolds for diverse signaling proteins (51). Since c-Src is a member of the signalosome (33), we followed its cellular localization in

response to GnRH treatment. LβT2 cells were transfected with c-Src-GFP and GnRHR-mCherry and later treated with GnRH for 30 min (**Figure 5A**). Time-lapse confocal microscopy showed that c-Src is present in the blebs, with no difference between expansion and retraction of the blebs (**Figure 5A**). Furthermore, the data show the colocalization of c-Src and the GnRHR in the blebs (**Figure 5B**). Inhibition of c-Src activity by PP2, which we have shown previously (20), had no effect on bleb formation (**Figure 5C**), suggesting that active c-Src is not required for bleb formation by GnRH. Thereafter, we have chosen to follow other members of the signalosome known to interact with c-Src in relation to their presence in the blebs.

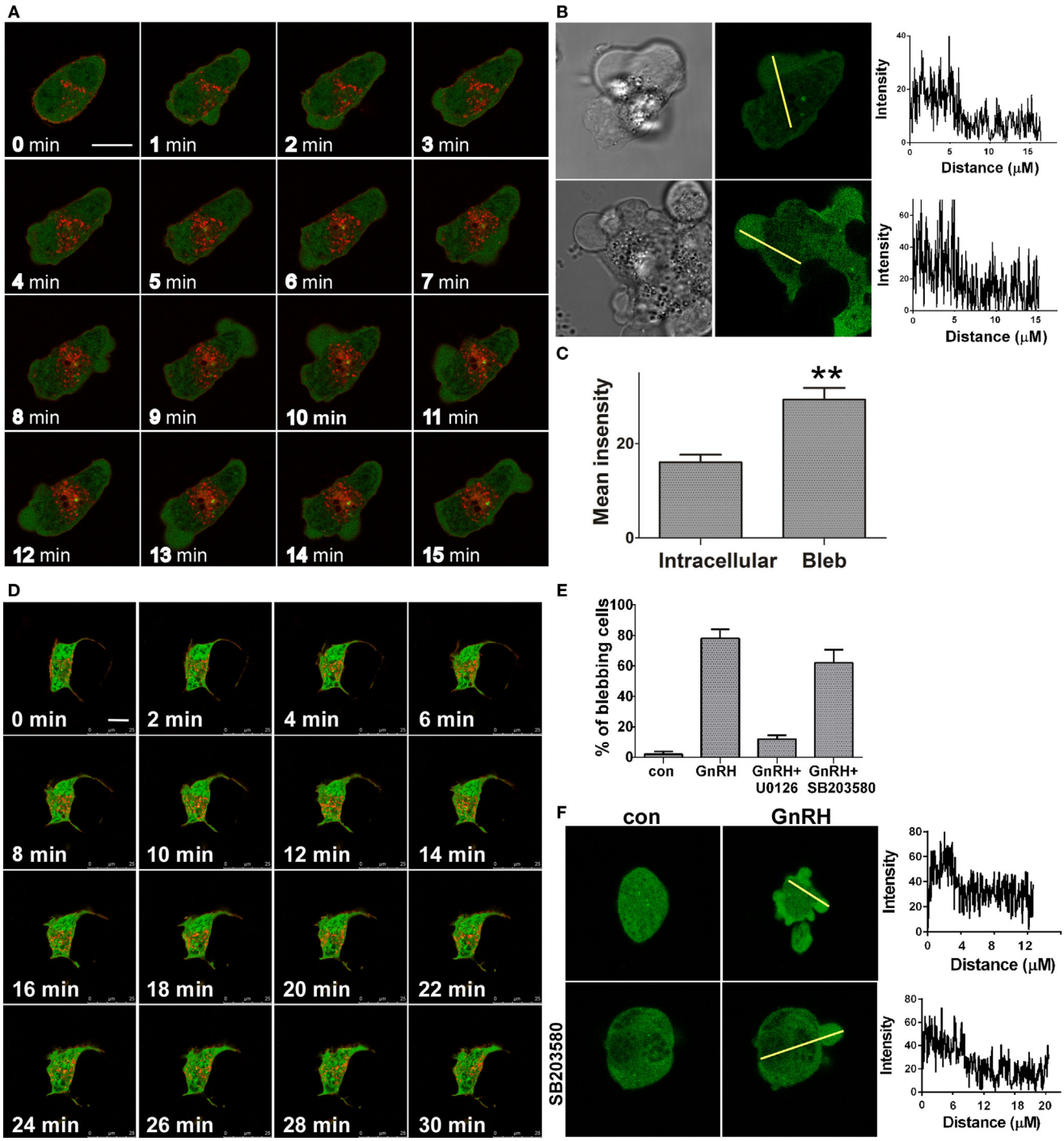

FIGURE 3| Continued

FIGURE 3 | Continued

ERK1/2 is present and involved in bleb formation. **(A)** Addition of GnRH (0–15 min, 10 nM) to serum-starved LβT2 cells transfected with GnRH receptor (GnRHR)-mCherry and ERK-GFP resulted in bleb formation, while ERK1/2 accumulates in the blebs. The *scale bar* is 10 μm. **(B)** Blebs images after GnRH treatment including differential interference contrast (DIC) and fluorescent images of ERK2-GFP. A line intensity profile across the cells was obtained and intensity profiles are shown on the right. **(C)** Bars show mean ± SEM of fluorescence intensity of the blebs vs. intracellular area from multiple scanning of each cell from at least five experiments. ***p*-Value ≤0.01. **(D)** Addition of the MEK selective inhibitor U0126 (25 μM) 20 min prior to GnRH (0–30 min, 10 nM) to serum-starved LβT2 gonadotrope cells transfected with GnRHR-mCherry and ERK2-GFP abolished bleb formation. Similar results were observed in two other experiments. The *scale bar* is 10 μm. **(E)** ERK1/2, but not p38MAPK, is involved in bleb formation. Serum-starved LβT2 gonadotrope cells were pretreated with U0126 or SB203580 (MEK and p38MAPK selective inhibitors, respectively) at 25 μM for 20 min prior to GnRH (10 nM, 30 min). Quantitation of the percentage of blebbing cells is shown. Images of at least 10 fields were taken for each treatment, and the *bars* are mean ± SEM from 3 experiments. **(F)** Pretreatment with SB203580 did not attenuate GnRH-induced ERK1/2 accumulation in the blebs as indicated by fluorescence intensity measurements. Serum-starved LβT2 cells transfected with ERK-GFP and were pretreated with or without SB203580 (25 μM) for 20 min prior to GnRH (10 nM, 30 min). A line intensity profile across the cells was obtained, and intensity profiles are shown on the right.

Vinculin Is Present in the Blebs

Vinculin is a scaffold protein, which binds to actin filament and is localized to FAs (52). Vinculin controls and regulates FA formation and cell migration. It is known that vinculin regulates survival and motility *via* ERK1/2 by controlling the accessibility of paxillin for FAK interaction (53). Since vinculin is a member of the signalosome and binds paxillin, we examined its involvement in bleb formation in response to GnRH treatment by live imaging microscopy. LβT2 cells were transfected with GnRHR-mCherry and vinculin-GFP and then treated with GnRH for 30 min. Time-lapse confocal microscopy showed that vinculin accumulates in the blebs (**Figure 5D**). However, unlike ERK1/2, c-Src, FAK, and paxillin (see below), vinculin was not present in the initial bleb expansion and was detected after the blebs were stabilized (**Figure 5E**). Therefore, we assume that vinculin is involved in bleb retraction.

FAK Is Present in the Blebs

Focal adhesion kinase is a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of proliferation and migration of normal and tumor cells. FAK associates with integrin receptors and recruits a number of SH2- and SH3-domain-containing proteins to the site of this interaction, thus forming a signaling complex that transmits signals from the extracellular matrix to the cell cytoskeleton (54). Since FAK binds c-Src and paxillin and is a member of the signalosome (33), we followed its presence in bleb formation. LβT2 cells were transfected with GnRHR-mCherry and FAK-GFP and then treated with GnRH for 30 min (**Figure 6A**). Time-lapse confocal microscopy showed that FAK is present in the blebs, while the GnRHR decorates the membrane but most of the receptors are retained in the cells as observed by others (55). Fluorescence intensity was measured across the cells and the profiles shown in line graphs on the right indicate that FAK accumulates in the blebs (**Figure 6B**). In addition, histograms show higher mean fluorescence intensity in the blebs vs. intracellular area (without the blebs area) (**Figure 6C**).

Paxillin Is Present in the Blebs

Paxillin, a multi-domain adapter protein, belongs to the FAs protein family and is known to interact with Ras, c-Src, tubulin, vinculin, and FAK (members of the signalosome) and is targeted to FAs *via* its LIM 1–4 domains (56, 57). It is thought that c-Src phosphorylation of Tyr118 of paxillin creates an ERK1/2-binding site. The activated paxillin then binds to Raf and MEK to activate ERK1/2. Moreover, the interaction with paxillin partially prevents ERK1/2 nuclear translocation, indicating that the task of restricting ERK1/2 in the cytosol is apparently carried out at least in part by paxillin. ERK1/2 phosphorylation of paxillin on Ser/Thr residues facilitates paxillin association with FAK (58, 59). Paxillin, together with FAK, is essential for cell spreading and migration (60, 61) as we have suggested for the signalosome (33). As indicated paxillin binds c-Src, FAK, and ERK1/2 and is a member of the signalosome, and therefore, we followed the cellular localization of paxillin in response to GnRH treatment. LβT2 cells were transfected with GnRHR-mCherry and paxillin-GFP and then treated with GnRH for 30 min (**Figure 6D**). Time-lapse confocal microscopy showed that paxillin accumulates in the blebs. Intensity profiles across the cells were obtained (**Figure 6E**), and graphs shown on the right demonstrate accumulation of paxillin in the blebs. Quantitation of mean fluorescence intensity (**Figures 6F**) shows higher values in the blebs vs. intracellular area (without the blebs area) as described above for FAK.

α-Tubulin, but Not Microtubules, Is Present in the Blebs

Microtubules are an important part of the cytoskeleton and play a vital role in many cellular processes, such as intracellular transport, mitosis, meiosis, and motility. Microtubules are composed of α–β-tubulin heterodimers (62). Since α-tubulin binds paxillin (57) and ERK1/2 (63) and is a member of the signalosome, we followed its presence in the process of bleb formation. LβT2 cells were transfected with GnRHR-mCherry and EMTB-3XGFP, which is the microtubule binding domain of ensconsin (EMTB) fused to 3GFP molecules, allowing microtubules visualization. Then, cells were treated with GnRH for 30 min. Time-lapse confocal microscopy showed the presence of microtubules fibers in the cells but not in the blebs, while α-tubulin was present in the blebs (**Figures 7A,B**).

Actin Is Present in GnRH-Induced Blebs Retraction

The actin cytoskeleton is a central structure for various intracellular processes, such as vesicle transport, cell shape, cell division,

motility, cell signaling, and morphogenesis (64). Furthermore, actin depolymerization and polymerization are involved in blebs life cycle (39, 40). Moreover, actin is involved in GnRH to ERK1/2 signaling (30, 34, 65). To examine the present of actin in GnRH-induced bleb formation, LβT2 cells were transfected with actin-YFP and then treated with GnRH for 30 min. Actin is not present in the blebs during blebs expansion. However, we could detect actin at the steady phase of the blebs and during

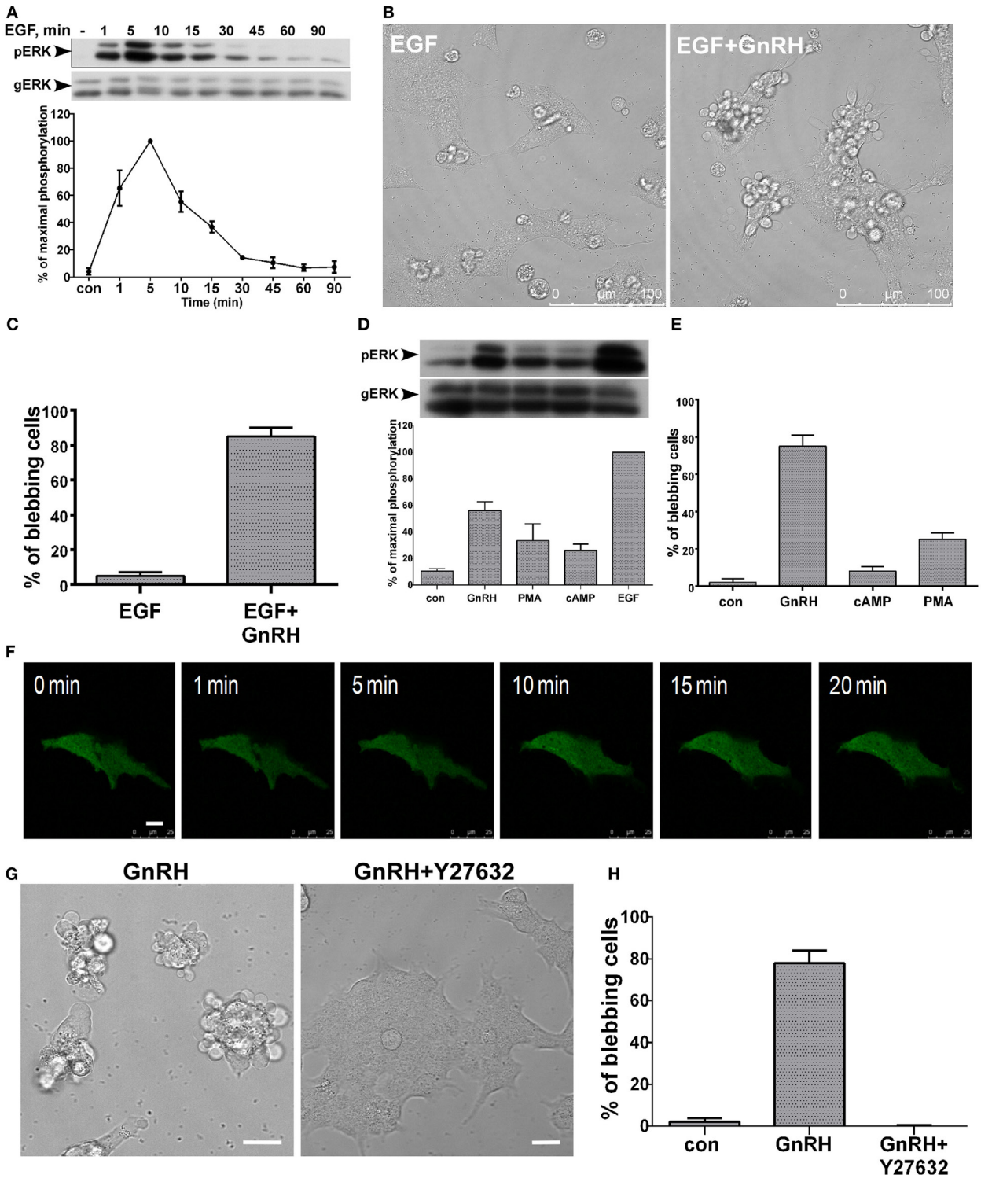

FIGURE 4 | **Continued**

FIGURE 4 | **Continued**
(A–E) ERK1/2 activation is required, but not sufficient for bleb formation. **(A)** Serum-starved LβT2 were treated with epidermal growth factor (EGF) (10 ng/ml, 0–90 min). Thereafter, cell lysates were analyzed for ERK1/2 activity by Western blotting using an antibody for phospho-ERK1/2. Total ERK (gERK) was detected with polyclonal antibody as a control for sample loading. Results are shown as mean ± SEM of maximal phosphorylation. A representative blot is shown and similar results were observed in two other experiments. **(B)** Addition of EGF (10 ng/ml, 30 min) to LβT2 cell resulted in minimal bleb formation. Then, addition of GnRH (30 min, 10 nM) resulted in marked elevation of bleb formation. **(C)** Quantitation of blebbing cells in EGF and EGF + GnRH treatment are shown. Images of at least 10 fields were taken for each treatment, and the *bars* are mean ± SEM from 3 experiments. **(D)** Serum-starved LβT2 gonadotrope cells were treated with GnRH (10 nM), PMA (50 nM), 8-Br-cAMP (1 mM), or EGF (10 ng/ml) for 5 min. Cell lysates were analyzed for ERK2 activity by Western blotting using an antibody for phospho-ERK1/2. Total ERK (gERK) was detected with a polyclonal antibody as a control for sample loading. Results are shown as mean ± SEM of maximal phosphorylation from three experiments. **(E)** Quantitation of the percentage of blebbing cells after GnRH (10 nM), PMA (50 nM), or 8-Br-cAMP (1 mM) treatment (30 min). Images of at least 10 fields were taken for each treatment, and the *bars* are mean ± SEM from 3 experiments. **(F–H)** RhoA–ROCK are involved in GnRH-induced bleb formation. **(F)** LβT2 gonadotrope cells were transfected with ERK2-GFP, while 30 h after transfection cells were serum-starved, and preincubated with Y-27632 (a ROCK selective inhibitor, 10 μM) for 20 min prior to GnRH (20 min, 10 nM). Y-27632 abolished bleb formation. The *scale bar* is 10 μm. **(G)** Images of DIC from a confocal microscope of LβT2 gonadotrope cells. Serum-starved LβT2 cells were pretreated with or without Y-27632 (10 μM) for 20 min prior to GnRH (30 min, 10 nM). The *scale bar* is 20 μm. **(H)** Quantitation of blebbing cells in control, GnRH, and GnRH + Y-27632 (added 20 min before GnRH) treatment as in panel **(G)**. Images of at least 10 fields were taken for each treatment, and the *bars* are mean ± SEM from 3 experiments.

the retraction (**Figure 7C**). The results are interesting since actin is not present in the signalosome (33), suggesting that blebs member's proteins are not restricted to those present in the signalosome.

GnRH Induces ERK1/2-Dependent Bleb Formation in Primary Cultures of Rat Pituitary Cells and Isolated Mouse Gonadotropes

The data shown above confirmed that GnRH induces ERK1/2-dependent bleb formation in LβT2 cells. To determine whether this effect is also evident in primary rat pituitary cells in culture, dissociated rat pituitary cells were prepared and the gonadotropes were identified by their intracellular Ca^{2+} response to GnRH. Addition of GnRH resulted in bleb formation (**Figure 8A**). Quantitation of the percentage of blebbing cells showed that GnRH treatment resulted in bleb formation and ERK1/2 inhibition by U0126 significantly reduced bleb formation by GnRH (**Figure 8B**). We then isolated mouse pituitary gonadotropes by the use of FACS-sorted cells from adult GRIC/Ai9 mice and kept them in culture (42, 43, 66, 67). Time-lapse confocal microscopy revealed that GnRH-induced bleb formation (**Figures 8C–E**). In addition, preincubation of the cells with the MEK inhibitor, U0126, abolished bleb formation induced by GnRH (**Figures 8D,E**). Quantitation of the percentage of blebbing cells confirmed that GnRH induced ERK1/2-dependent bleb formation in primary gonadotropes in culture (**Figure 8E**).

DISCUSSION

Following the fate of ERK1/2 in LβT2 cells transfected with GnRHR-mCherry and ERK2-GFP and treated with GnRH, we noticed bleb formation in the cells. The blebs appear within ~2 min at a turnover rate of ~2–3 blebs/min and last for at least 90 min. The formation of the blebs is GnRHR-dependent since the GnRH antagonist (cetrorelix acetate) abolished bleb formation. Interestingly, retreatment with GnRH (30 min) 6 h later resulted in a priming effect, which is defined as an increase in cells response to the second exposure to GnRH compared with the first (**Figure 2**). A priming effect of the LH response to GnRH has been observed (68). The mechanism of the priming effect is under investigation.

A "Funnel Paradox" exists, namely, how is signal specificity maintained, while most of the receptor tyrosine kinases (RTKs) (of the 90 tyrosine kinases, 58 are receptor type) and GPCRs (>800) act *via* MAPKs with different biological responses. The most likely explanation is the presence of scaffold proteins and signaling complexes (signalosomes) (69, 70) that bring together different MAPK cascade members and their substrates and target them to specific sites in a spatio/temporal fashion (71, 72). Indeed, a signaling platform for ERK1/2 activation by GnRH including the GnRHR, c-Raf kinase, Ca^{2+}-calmodulin, and ERK1/2 that was localized to low-density membrane microdomains (lipid rafts) has been proposed (29). Another complex including FAK and c-Src at FAs has been reported to be involved in ERK1/2 activation by GnRH in HEK 293 cells stably expressing the GnRHR (30).

In search of c-Src-interacting proteins, we came upon a large protein–protein complex associated with the GnRHR, a signalosome (33). The presence of FAK, paxillin, vinculin (residence of FAs), and tubulin led us to suggest that the signalosome resides in microtubules at the boundaries of FAs (33). We have shown that the role of the signalosome is to sequester a pool of GnRH-activated ERK1/2 in the cytosol for the phosphorylation of FAK and paxillin at FAs to mediate cell migration as recently proposed for GnRH-stimulated gonadotropes (34, 35). It is thought that RTK and GPCR ligands induce translocation of ERK1/2 to the nucleus to phosphorylate and activate transcription factors (10, 11). In the present work, we found a link between the signalosome and the blebs, suggesting that as with the signalosome, the blebs may be involved in cell migration.

ERK1/2 accumulated in the blebs, and we assume that ERK1/2 migrated from the signalosome to the blebs, since both are associated with an active membrane pool of ERK1/2. In support of this notion is the observation that various members of the signalosome were also found in the blebs. Since the signalosome is preformed and unlike the blebs is not dependent on GnRH (33), members of the signalosome were most likely

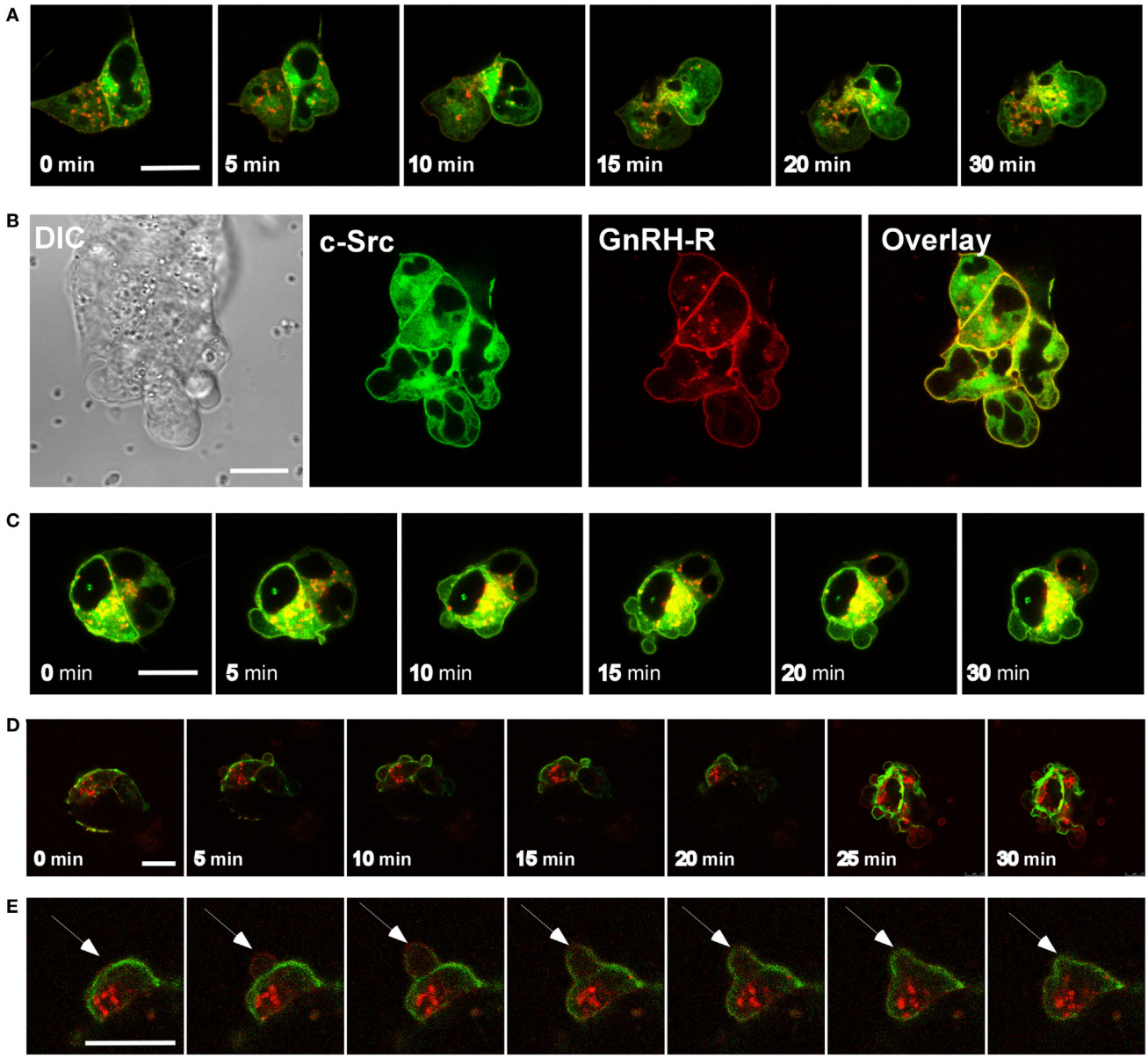

FIGURE 5 | **c-Src and vinculin are present in the blebs**. **(A)** Addition of GnRH (0–30 min, 10 nM) to serum-starved LβT2 cells transfected with c-Src-GFP and GnRH receptor (GnRHR)-mCherry resulted in bleb formation, while c-Src is present in the blebs. The *scale bar* is 10 μm. **(B)** Blebs time laps of DIC and fluorescent images of Src-GFP, GnRHR-mCherry, and overlay showing colocalization of c-Src and the GnRHR in the blebs. The *scale bar* is 10 μm. **(C)** Serum-starved LβT2 cells transfected with c-Src-GFP and GnRHR-mCherry were pretreated with the c-Src inhibitor PP2 (10 μM) for 30 min. Thereafter, GnRH (10 nM) was added for 30 min. Similar results were observed in two other experiments. The *scale bar* is 10 μm. **(D)** Addition of GnRH (0–30 min, 10 nM) to serum-starved LβT2 cells transfected with GnRHR-mCherry and vinculin-GFP resulted in bleb formation, while vinculin is present in the blebs. The *scale bar* is 10 μm. **(E)** Unlike c-Src, ERK1/2, focal adhesion kinase, and paxillin (see below), vinculin was recruited to the blebs during stabilization and retraction. Addition of GnRH (10 nM) to serum-starved LβT2 cells transfected with GnRHR-mCherry and vinculin-GFP resulted in bleb formation, single blebs were monitored every 10 seconds. The *scale bar* is 10 μm.

recruited to the blebs. Also, formation of the blebs requires active ERK1/2 as evident by the use of the MEK1/2 inhibitor, U0126, which abolished bleb formation (**Figures 3D–E**). Interestingly, the use of the MEK1/2 inhibitor, U0126, which abolished bleb formation abolished also cell migration (data not shown). However, since the MEK inhibitor is not a specific bleb inhibitor, further studies are required to link bleb formation to gonadotrope migration.

Epidermal growth factor is a member of a family of peptide growth factors that activates the EGF receptors (EGFR). EGFR signaling pathway plays important roles in proliferation, differentiation, and migration of a variety of cell types, especially in

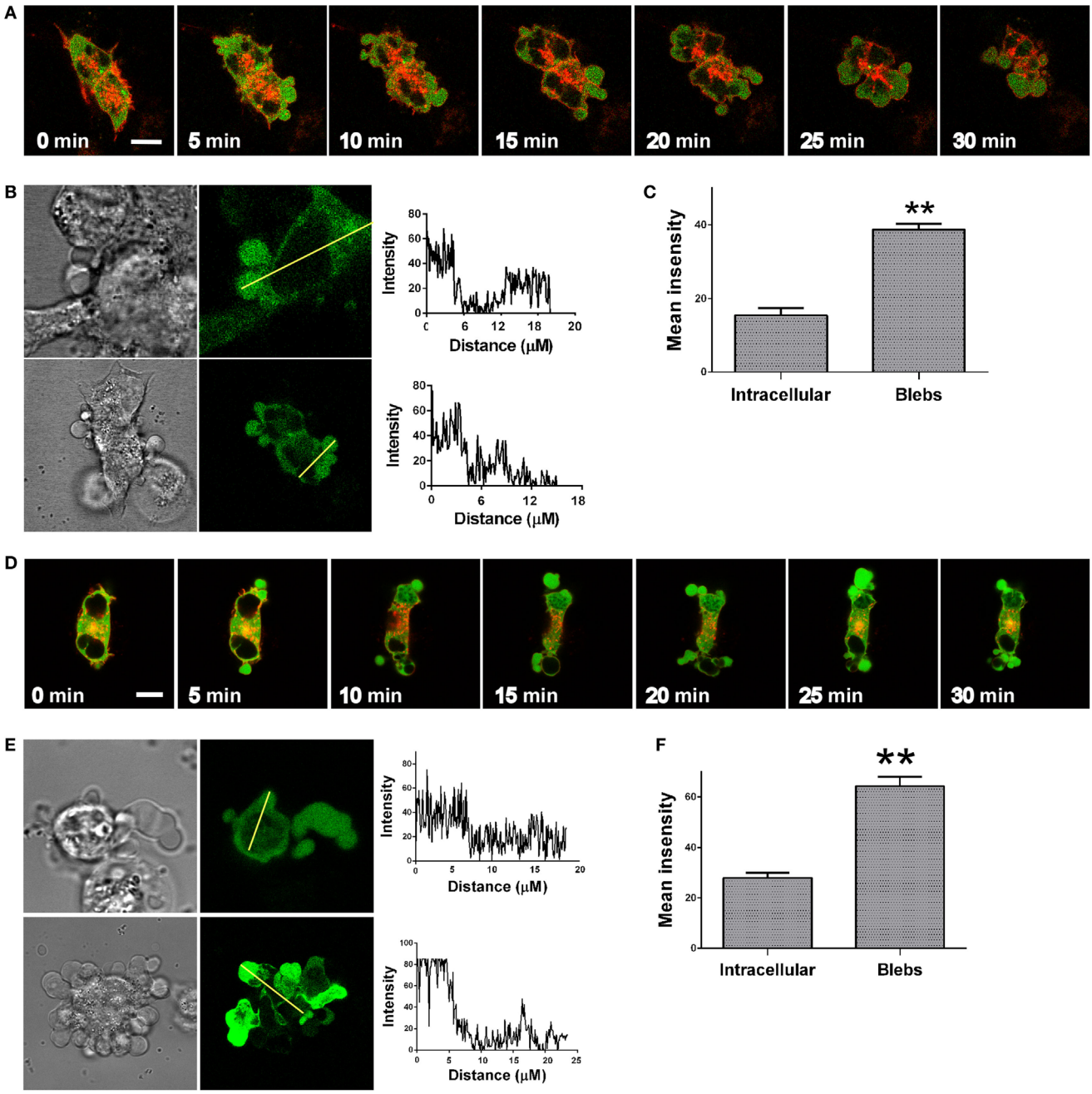

FIGURE 6 | **Focal adhesion kinase (FAK) and paxillin are present in the blebs**. **(A)** Addition of GnRH (0–30 min, 10 nM) to serum-starved LβT2 cells transfected with GnRH receptor (GnRHR)-mCherry and FAK-GFP resulted in bleb formation, while FAK is present in the blebs. The *scale bar* is 10 μm. **(B)** Blebs images after GnRH treatment, including DIC and fluorescent images of FAK-GFP. Fluorescence intensity was measured across the cells and intensity profiles are shown on the right. **(C)** Quantitation of FAK-GFP mean fluorescence intensity in the blebs vs. intracellular area from multiple scanning of each cell from at least five experiments. The *bars* are mean ± SEM. ***p*-Value ≤0.01. **(D)** Addition of GnRH (30 min, 10 nM) to serum-starved LβT2 cells transfected with GnRHR-mCherry and paxillin-GFP resulted in bleb formation, while paxillin accumulates in the blebs. The *scale bar* is 10 μm. **(E)** Blebs images after GnRH treatment, including DIC and fluorescent images of paxillin-GFP. A line intensity profile across the cells was obtained and intensity profiles are shown on the right. **(F)** Histograms show mean ± SEM of fluorescence intensity of the blebs vs. intracellular area from multiple scanning of each cell from at least five experiments. ***p*-Value ≤0.01.

epithelial cells (46). In addition, EGF is known to stimulate the ERK1/2 signaling pathway (46). Although the following ligands: EGF > GnRH > PMA > cAMP stimulate ERK1/2 in LβT2 cells, they produced little or no effect on bleb formation as compared to the robust effect of GnRH (GnRH > PMA > cAMP > EGF). The results indicate that ERK1/2 is required but not sufficient for bleb formation possibly due to compartmentalization of ERK1/2 in a ligand-dependent manner (**Figures 4A–E**).

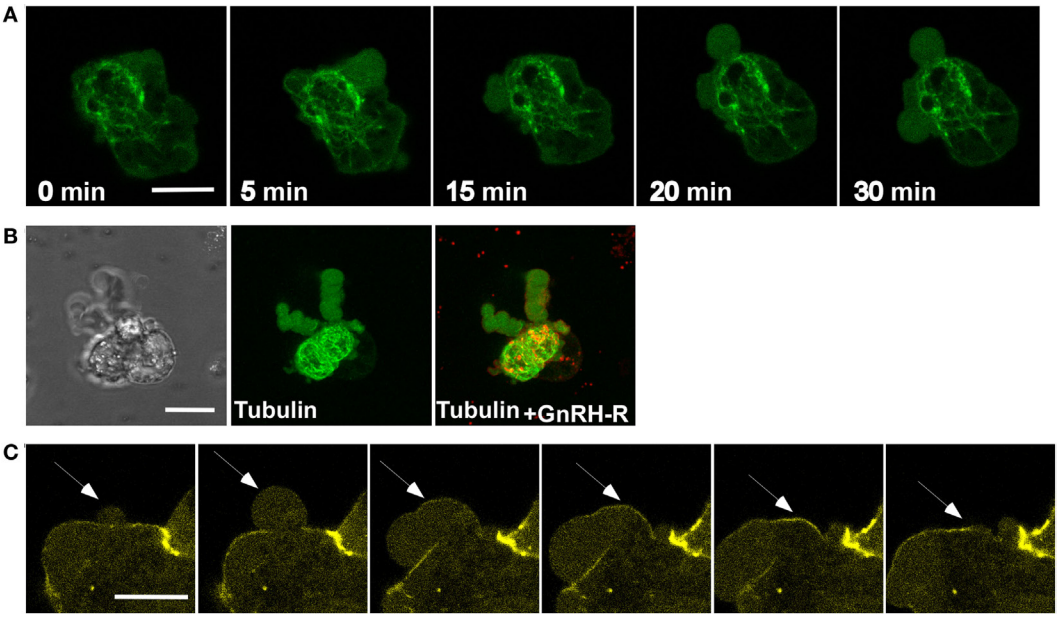

FIGURE 7 | **Tubulin and actin, but not microtubules, are present in the blebs**. **(A)** Images from a confocal microscopy time-lapse movie of serum-starved LβT2 cells transfected with GnRH receptor (GnRHR)-mCherry and EMTB-3XGFP (the microtubule-binding domain of ensconsin (EMTB) fused to three GFP molecules, allowing microtubules visualization) and treated with GnRH (30 min, 10 nM). Bleb formation was noticed, while microtubules are not present in the blebs. The *scale bar* is 10 μm. **(B)** Blebs images after GnRH treatment, including DIC and fluorescent images of GnRHR-mCherry and EMTB-3XGFP, supporting the data observed in panel **(A)**. The *scale bar* is 10 μm. **(C)** Actin is involved in bleb retraction. Addition of GnRH (30 min, 10 nM) to serum-starved LβT2 cells transfected with actin-YFP resulted in bleb formation. Actin is recruited to the blebs after they are stabilized and is best observed during blebs retraction (see arrows). The *scale bar* is 10 μm.

The Rho family members RhoA, Rac1, and Cdc42 are small GTPases known to regulate actin cytoskeleton rearrangements. Godoy at el. (35) showed that GnRH inhibits p250RhoGAP expression in LβT2 cells. Hence, GnRH activates Rho family members, induces cytoskeletal rearrangements, and increases cell motility (35). The contractility for bleb retraction is provided by signaling through RhoA–ROCK–myosin. In this cascade, RhoA-GTP activates its effector kinase ROCK that directly phosphorylates myosin light chain, which then induces actomyosin contraction (38, 41). We therefore used the ROCK inhibitor Y27632 to examine its involvement in bleb formation. Indeed, Y27632 abolished bleb formation implicating the RhoA/ROCK signal in the process (**Figures 4F–H**).

GnRH receptor, c-Src, ERK1/2, FAK, paxillin, and tubulin, members of the above mentioned signalosome (33), accumulated in the blebs. On the other hand, vinculin was not present in the initial bleb expansion and was detected in the static phase. In addition, we could detect actin only at the steady phase of the blebs and during the retraction. Since vinculin is a known actin-binding protein, we assumed that vinculin and actin are involved in the static phase and in GnRH-induced bleb retraction, as indeed was the case. In addition, the activated ERK1/2 can phosphorylate myosin light chain kinase in a c-Src–FAK-dependent manner to further increase actomyosin contractility, which regulates adhesion disassembly and promote cell migration (73). Also, c-Src to FAK signaling and phosphorylation of FAK and paxillin *via* the activated ERK1/2, as observed in the signalosome (33), lead to FAs turnover at the cell front and cell migration (73–75), hence more blebs, supporting the signalosome–bleb pathway.

A major member of the signalosome is c-Src (33), and we have shown previously that GnRH activates c-Src and ERK1/2 activation is c-Src dependent (20). By virtue of its modular SH1, SH2, and SH3 domains, the soluble tyrosine kinase can act as scaffold for diverse signaling proteins (51). Time-lapse confocal microscopy identified c-Src in the blebs during expansion and retraction of the blebs (**Figures 5A,B**). Surprisingly, inhibition of c-Src activity by PP2 (**Figure 5C**) had no effect on bleb formation, suggesting that unlike ERK1/2, active c-Src is not required for bleb formation and that unlike the signalosome inactive c-Src is present in the blebs. Furthermore, since GnRH activates ERK1/2 *via* active c-Src (20), it is possible that active ERK1/2 migrated to the blebs from the signalosome or other cellular compartment and there is no further activation of ERK1/2 by GnRH *via* c-Src in the blebs. Alternatively, as we have previously shown, GnRH can also activate ERK1/2 in a c-Src-independent fashion (20) and this pool of ERK1/2 may reside in the blebs.

Previous studies have shown that GnRH stimulates remodeling of the cytoskeleton in gonadotrope-derived cell lines, primary cultures of dissociated pituitary cells (ovine and murine) and intact living pituitary that leads to the formation of lamellipodia and filopodia and increased cell migration (30, 34, 65). In addition, GnRH signaling to ERK requires actin polymerization (30, 34) and ERK inhibition did not inhibit the formation of lamellipodia and filopodia by GnRH (65).

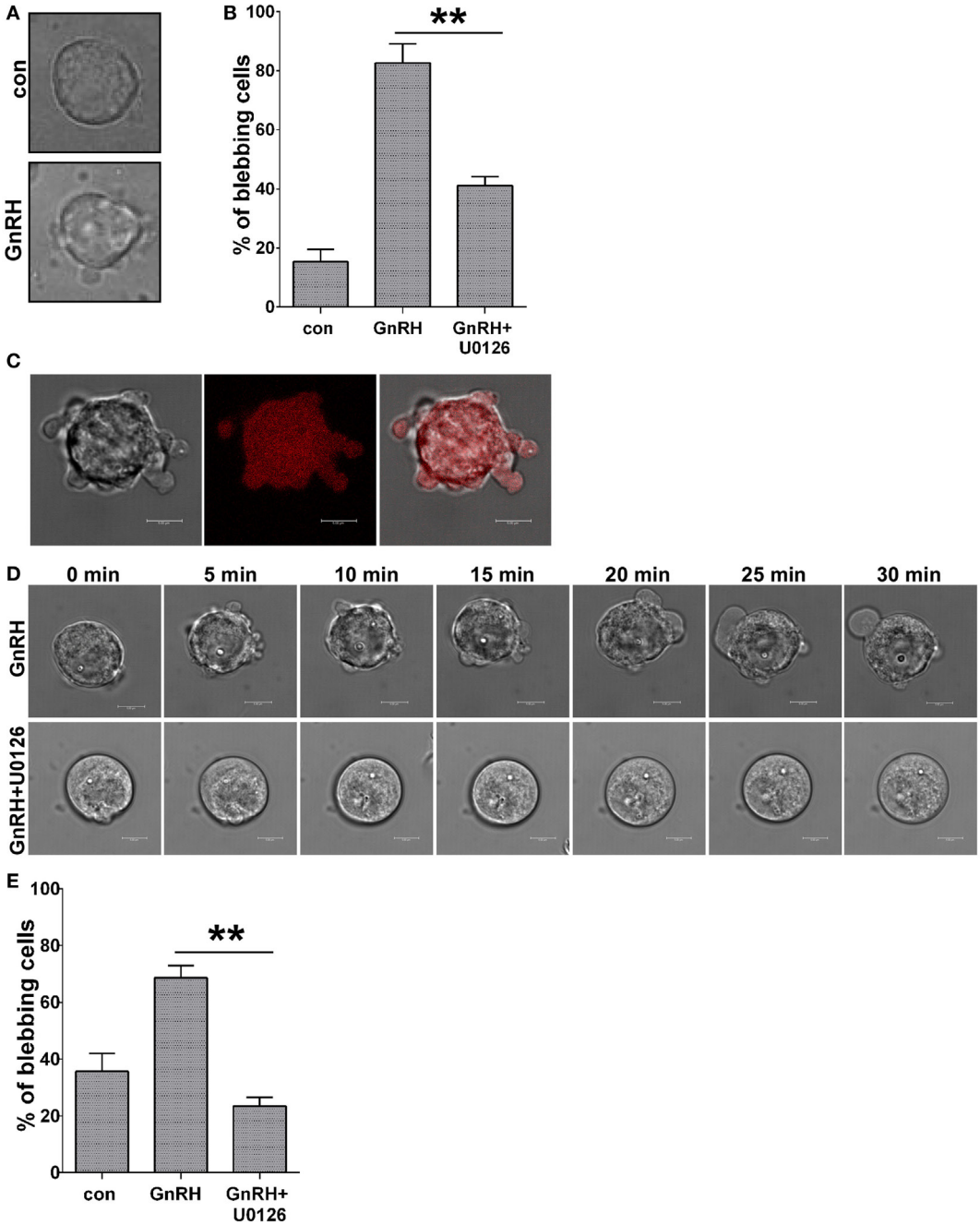

FIGURE 8 | GnRH induces ERK1/2-dependent bleb formation in primary cultures of rat pituitary cells and isolated mouse gonadotropes. **(A)** DIC images from a confocal microscope of dissociated rat pituitary cells. Culture rat pituitary cells were prepared, and gonadotropes were identified by their Ca^{2+} response as described in Section "Materials and Methods." The cells were treated with GnRH (10 nM) for 30 min. **(B)** Quantitation of the percentage of blebbing cells from dissociated rat pituitary cells. The cells were pretreated with U0126 (10 μM) for 30 min, followed by GnRH (10 nM) for additional 30 min. Data are mean ± SEM from three experiments. **(C)** DIC and fluorescent images from a confocal microscope of FACS-purified primary gonadotrope cells from adult GRIC/Ai9 mice treated with GnRH (30 min, 10 nM). The *scale bar* is 5 μm. **(D)** Images from a confocal microscopy time-lapse movie of FACS-purified primary gonadotrope cells. The cells were pretreated with or without U0126 (25 μM) for 30 min, followed by GnRH (10 nM) for additional 30 min. The *scale bar* is 5 μm. **(E)** Quantitation of the percentage of blebbing cells from panel **(D)** is presented (at least 30 cells for each experiment) and data are mean ± SEM. ***p*-value ≤0.01.

Common to the above studies is that the dynamic remodeling of the actin cytoskeleton was upstream to ERK1/2 activation in the GnRHR signaling network. On the other hand, in another system, activation of the ERK signaling pathway was required for the induction of actin polymerization and subsequent lamellipodium formation (76).

Our observations reported here differ from the above in particular in terms of signaling from the GnRHR to ERK1/2 and the blebs. Here, we show that GnRH induces blebs formation, which differ from lamellipodia or filopodia which are dependent on polymerizing actin filaments (36), while blebs growth is pressure driven, and not due to actin polymerization. In addition, we have shown that ERK1/2 inhibition strongly inhibits GnRH-induced bleb formation and cell migration. Hence, we propose that blebs formation is downstream to ERK1/2 activation in GnRHR signaling.

We emphasize here the signalosome–blebs pathway, suggesting that both are involved in cell migration. This is based on several lines of evidence; members of the signalosome are also found in the blebs; we have proposed that the role of the signalosome is to sequester a pool of active ERK1/2 to phosphorylate and activate FAK and paxillin at FAs to mediate cell migration (33) as shown for GnRH-stimulated gonadotropes (34, 35). Here, we show that bleb formation is dependent on active ERK1/2; hence, the potential link to the signalosome as a provider of active ERK1/2 in the vicinity of the membrane. Assuming that members of the signalosome migrated to the blebs, it is not clear if they migrated as a multi-protein complex, or separately. In support of the second assumption is the observation that some proteins migrated during bleb formation (GnRHR, c-Src, ERK1/2, FAK, and paxillin) and some during bleb stabilization and retraction (vinculin). The results lend support to the notion that the signalosome members were recruited separately to the blebs. In addition, although actin was present in the blebs, we could not detect actin in the signalosome (33), suggesting that blebs member's proteins are not restricted to those found in the signalosome.

Importantly, we have confirmed that the blebs are formed in a more accurate physiological setting, as they were seen in cultured primary rat pituitary cells, in which the gonadotropes (5–10% of pituitary cells) (**Figures 8A,B**) were identified by their intracellular Ca^{2+} response to GnRH. They were also apparent in cultured FACS-sorted mouse primary gonadotropes from adult GRIC/Ai9 mice (**Figures 8C–E**). (42, 43, 66, 67) and in both cell models; we could demonstrate that the GnRH-induced bleb formation was dependent on active ERK1/2. We have thus established that this is a normal response of the gonadotropes to GnRH.

ETHICS STATEMENT

Ethical consideration were approved for the use of animals in this study as stated in the text.

AUTHOR CONTRIBUTIONS

LN carried most of the experiments, and AT, AF, and MT carried some of the experiments. PM, SS, UB, and RS participated in the design of the experiments. ZN participated in the design of the experiments and in the preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2017.00113/full#supplementary-material.

VIDEO S1 | **GnRH induces bleb formation in LβT2 cells, and GnRH receptor is present in the blebs**. Time-lapse movie of serum-starved LβT2 gonadotrope cells treated with GnRH (10 nM) (0–30 min). The treatment resulted in bleb formation.

VIDEO S2 | **ERK1/2 is present and involved in bleb formation**. Time-lapse movie of serum-starved LβT2 cells transfected with GnRH receptor-mCherry and ERK-GFP and treated with GnRH (0–15 min, 10 nM). GnRH stimulated bleb formation, while ERK1/2 accumulated in the blebs.

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Intrinsic and Regulated Gonadotropin-Releasing Hormone Receptor Gene Transcription in Mammalian Pituitary Gonadotrophs

*Marija M. Janjic[1], Stanko S. Stojilkovic[2] and Ivana Bjelobaba[1]**

[1] Department of Neurobiology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Belgrade, Serbia, [2] Section on Cellular Signaling, Eunice Kennedy Shiver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, United States

The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH), acting *via* its receptors (GnRHRs) expressed in pituitary gonadotrophs, represents a critical molecule in control of reproductive functions in all vertebrate species. GnRH-activated receptors regulate synthesis of gonadotropins in a frequency-dependent manner. The number of GnRHRs on the plasma membrane determines the responsiveness of gonadotrophs to GnRH and varies in relation to age, sex, and physiological status. This is achieved by a complex control that operates at transcriptional, translational, and posttranslational levels. This review aims to overview the mechanisms of GnRHR gene (*Gnrhr*) transcription in mammalian gonadotrophs. In general, *Gnrhr* exhibits basal and regulated transcription activities. Basal *Gnrhr* transcription appears to be an intrinsic property of native and immortalized gonadotrophs that secures the presence of a sufficient number GnRHRs to preserve their functionality independently of the status of regulated transcription. On the other hand, regulated transcription modulates GnRHR expression during development, reproductive cycle, and aging. GnRH is crucial for regulated *Gnrhr* transcription in native gonadotrophs but is ineffective in immortalized gonadotrophs. In rat and mouse, both basal and GnRH-induced *Gnrhr* transcription rely primarily on the protein kinase C signaling pathway, with subsequent activation of mitogen-activated protein kinases. Continuous GnRH application, after a transient stimulation, shuts off regulated but not basal transcription, suggesting that different branches of this signaling pathway control transcription. Pituitary adenylate cyclase-activating polypeptide, but not activins, contributes to the regulated transcription utilizing the protein kinase A signaling pathway, whereas a mechanisms by which steroid hormones modulate *Gnrhr* transcription has not been well characterized.

Keywords: basal transcription, regulated transcription, gonadotrophs, gonadotropin-releasing hormone, gonadotropin-releasing hormone receptor

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****Correspondence:***
Ivana Bjelobaba
ivana.bjelobaba@ibiss.bg.ac.rs

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INTRODUCTION

The gonadotropin-releasing hormone (GnRH) receptor (GnRHR) is a member of a G protein-coupled receptor family (1). The receptor is expressed in pituitary gonadotrophs of all vertebrates, as well as in other tissues (2). The main signal transduction pathways of activated GnRHR in gonadotrophs is phospholipase C-β1-mediated phosphatidylinositol hydrolysis, thereby

generating inositol-1,4,5-trisphosphate and diacylglycerol (3). Inositol-1,4,5-trisphosphate binds to its receptor at the endoplasmic reticulum membrane, leading to oscillatory Ca^{2+} release and Ca^{2+}-dependent modulation of electrical activity (4). Diacylglycerol alone or together with Ca^{2+} activates protein kinase C (PKC) family of enzymes (5), whereas mitogen-activated protein kinases (MAPK) (6), phospholipase D (7), and phospholipase A2 (8) are PKC downstream signaling proteins. The coupling of GnRHRs to the synthesis of follicle-stimulating hormone (FSH) and synthesis and release of luteinizing hormone (LH) is critical for the establishment of hypothalamic–pituitary–gonadal axis, as these hormones regulate steroidogenesis and gametogenesis. In turn, gonadal hormones exhibit feedback effects at hypothalamic GnRH neurons and pituitary gonadotrophs (6).

The pituitary GnRHR number depends on developmental and reproductive stage and determines their responsiveness to GnRH. The receptor number is regulated, at least in part, at the transcriptional level (9). Cloning of GnRHR cDNA from numerous species facilitated investigations of GnRHR gene (*Gnrhr*) transcription. In general, transcription of the *Gnrhr* in gonadotrophs *in vitro* occurs in the absence (basal) and presence (regulated) of GnRH stimulation (2). The differences in the regulation of *Gnrhr* expression in mammalian species reflect differences in the promoter region of the gene (9, 10). The common aspect of regulated transcription of this gene is up- and downregulation by GnRH, depending on the pattern of application (11–13). Other hormones also contribute to regulation of *Gnrhr* transcription.

Here, we will mainly discuss *Gnrhr* transcription in the most frequently used mammalian models: rats, mice, sheep, and immortalized αT3-1 and LβT-2 gonadotrophs. We will first review the literature about *in vivo* GnRHR mRNA levels during development, aging and reproductive stage, followed by a brief description of rat and mouse *Gnrhr* structure and promoter region, basal vs. regulated activities, homologous upregulation of gene expression, and effects of gonadal and adrenal steroid hormones and other ligands on transcriptional activity of this gene.

IN VIVO VARIATIONS IN *Gnrhr* EXPRESSION

Developmental profile of *Gnrhr* expression in rats is depicted in **Figure 1A**. In females, *Gnrhr* expression increases rapidly over the first 2 weeks of development, followed by a transient decline and secondary rise in 7–8 weeks of age. In males, it increases gradually until 5 weeks of age (14–16), followed by a decline toward a steady expression at the adult age (11). The peak of *Gnrhr* expression during development correlates well with expression of gonadotropin subunit genes *Lhb*, *Fshb*, and *Cga* in both sexes (16) as well as with greater LH and FSH secretion in females, but not in males

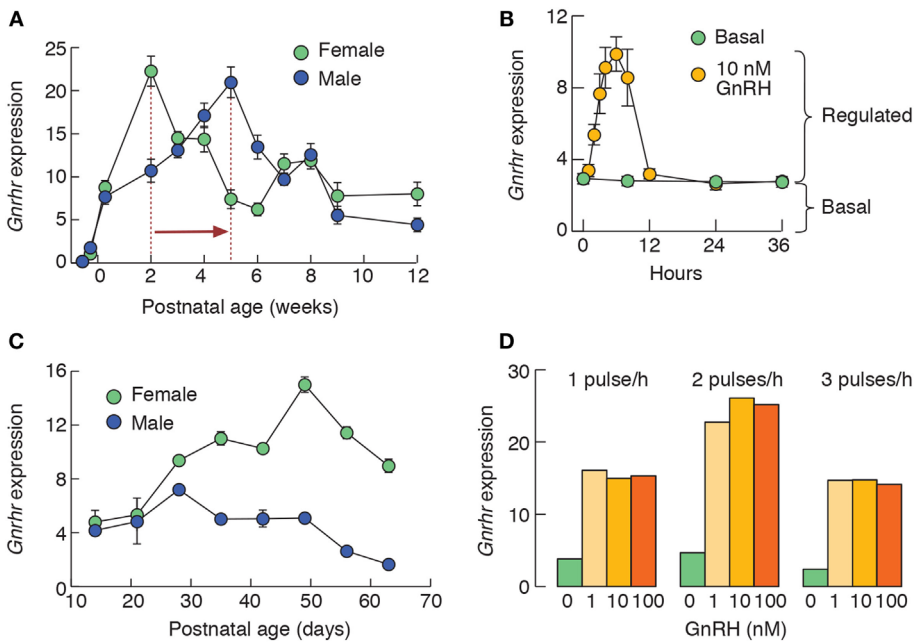

FIGURE 1 | *In vivo* and *in vitro* expression patterns of rat pituitary *Gnrhr*. **(A)** Female and male developmental profiles of *Gnrhr* expression *in vivo*. Notice the differences in the peak of *Gnrhr* expression in females and males, as indicated by vertical dotted lines and a horizontal arrow. **(B)** Gonadotropin-releasing hormone (GnRH)-induced *Gnrhr* expression in 2-day-old static cultures of anterior pituitary cells from 7-week-old females. Cells were cultured in the absence or in continuous presence of 10 nM GnRH. Notice that desensitization of GnRH-induced *Gnrhr* expression does not affect basal expression. **(C)** The amplitude of GnRH-induced (10 nM continuously for 6 h) *Gnrhr* expression in female and male pituitary static cultures obtained from animals of different age is sex specific, in contrast to comparable levels of expression of this gene in both sexes *in vivo* **(A)**. **(D)** GnRH-induced *Gnrhr* expression in perifused pituitary cells from rat females. Cells were stimulated with 1, 10, or 100 nM GnRH for 1 × 5 min/hour, 2 × 5 min/hour, and 3 × 5 min/hour during 6 h. Notice that 1 nM GnRH was sufficient to induce maximum in response. This figure is derived from data published in Ref. (11, 16, 20); no permission is required from the copyright holder.

(17). These data are in accordance with the reports on GnRHR concentration and binding capacity during rat ontogeny (18, 19).

Gnrhr expression is downregulated in aged male rats (21), probably reflecting impaired GnRH secretion from the hypothalamus, because pituitary response to GnRH remains operative (22). However, in middle aged ovariectomized female rats, *Gnrhr* expression levels were lower than in young ovariectomized animals and the pituitary response to a steroid-induced gonadotropin surge was also impaired (23).

Gnrhr expression in the rat pituitary changes significantly during estrous cycle (24–26). Pituitary GnRHR mRNA content is relatively high on the mornings of diestrus I and diestrus II and declines sharply in the afternoons of diestrus days. However, higher *Gnrhr* expression can again be observed in the late evening of diestrus II (26). During proestrus, a sharp rise in *Gnrhr* expression occurs between morning and noon, followed by oscillation in expression until 17:00 h, when a second peak can be observed (25). It should also be noted that maximal binding of D-Ala^6-GnRH, a synthetic GnRH analog, occurs at diestrus II as well, indicating that the maximal number of GnRHRs during the cycle is reached before proestrus (27). Estrous is characterized by low *Gnrhr* expression (24, 25). In general, the changes in pituitary *Gnrhr* levels correlate well with GnRH content and *Gnrh* expression in the hypothalamus (25, 26). In sheep, GnRHR mRNA expression and GnRH binding increase over the luteal phase and decline after the preovulatory LH surge, reaching the lowest levels 24 h after estrous (28–30).

Ovariectomy in rats and mice reduces the pituitary GnRHR numbers (31, 32), combined with marked upregulation of GnRHR mRNA (33). Interestingly, in ovariectomized rats, hypothalamic *Gnrh* and pituitary *Gnrhr* expression levels fluctuate during the day (26). In castrated male rats, there was a rise in mRNA, and receptor number, GnRHR affinity for GnRH, and gonadotropin secretion, which was, at least partly, prevented by a testosterone replacement therapy (33–38). Similarly, castration induces upregulation of *Gnrhr* expression in sheep (39). By contrast, castration was shown to induce a fall in mouse pituitary GnRHR numbers (40).

Rat pituitary responsiveness to GnRH remains low until 12th day after conception and then rises to reach maximum on the first day postpartum (41). We also noticed lower aplitude of GnRH-induced expression of dentin matrix protein 1 in gonadotrophs from pregnant female rats (20). These data imply that GnRHR mRNA content changes during pregnancy in rat, although this was not investigated. By contrast, pregnancy does not induce changes in GnRHR numbers or mRNA levels in sheep, suggesting that other mechanisms account for a fall in maternal pituitary responsiveness (42). Number of GnRHRs (43–45) as well as *Gnrhr* expression levels (46) are low during lactation in rat (probably reflecting diminished GnRH secretion from the hypothalamus), but rise rapidly after pup removal (45, 46).

THE STRUCTURE OF *Gnrhr* PROMOTER REGION

The 5′-flanking sequences of rat and mouse *Gnrhr* promoter have been isolated and characterized (47–50). In these species, *Gnrhr* gene is present as a single copy, positioned on chromosome 14 and 5, respectively, and contains three coding exons and two introns (10). Both promoters share strong homology over the region 1.2 kb upstream of the ATG codon (50). In this region, two identical response *cis*-elements of the mouse promoter are present in the rat *Gnrhr* promoter, a canonical activating protein 1 and steroidogenic factor 1 (also present in the ovine promoter; SF1 or NR5A1) (9). The rat promoter contains two additional response elements that are held responsible for functional differences between rat and mouse promoter: an imperfect cAMP response element, suspected to convey pituitary adenylate cyclase-activating peptide (PACAP) actions, and an element confined to −252/−245, that binds a protein yet unidentified, termed SF1 adjacent protein or SAP. All of these elements are required to mediate the gonadotroph-specific activity (51–53). An element termed the *Gnrhr* activating sequence, which could confer activin actions in mice, is also present in the rat promoter, but it is inactive (54). Comparing to the mouse promoter, where all known response elements fall in the proximal region, an additional regulatory region containing *Gnrhr*-specific bipartite enhancer (GnSE) is situated on a more distal part of the rat promoter. Thus, for the full gonadotroph-specific activity of the rat promoter, additional distal elements within the −1,150/−750 bp region are required. Two major response elements located at positions −994/−960 and −871/−862 are responsible for GnSE action (51, 52). Maximal GnSE activity requires the presence of SF1 response element located in the proximal domain. Both GnSE elements bind LIM-homeodomain proteins LHX3 and ISL1 and this seems to be crucial for gonadotroph-specific expression of the gene (9, 52, 55). For the detailed structure of rat and mouse promoters, see Ref. (9). The functional properties of the ovine *Gnrhr* promoter region were not investigated in details; however, the analysis of the 5′-UTR indicates that different mechanisms evolved for pituitary specific expression of *Gnrhr* in sheep and rodents (56).

BASAL AND GnRH-REGULATED *Gnrhr* EXPRESSION

Several lines of evidence indicate that *Gnrhr* expression is inherent to gonadotrophs. Some functional receptors must be present in gonadotrophs in Kallmann syndrome patients to explain how GnRH administration restores pituitary and gonadal functions (57). In agreement with this, *Gnrhr* expression is detectable and functional GnRHRs are present in the rat gonadotrophs *in vitro* even after prolonged period of GnRH absence (58). Furthermore, prolonged continuous GnRH application does not completely stop *Gnrhr* transcription (**Figure 1B**) (11). Finally, naïve (never stimulated) αT3-1 and LβT-2 cells express functional Ca^{2+}-mobilizing GnRHRs (59, 60).

In rat, mouse, and sheep, the main positive regulator of *Gnrhr* transcription is GnRH itself (11–13, 61), depending on the pattern of GnRH application. **Figure 1B** illustrates that continuous stimulation of rat pituitary cells induces a transient induction of *Gnrhr* transcription, with maximal response at 6 h (11, 13). Longer GnRH stimulation leads to downregulation in *Gnrhr* transcription (11, 62). Therefore, it is reasonable to postulate that

pulsatile GnRH stimulation is required not only for gonadotropin subunit expression, but also for the proper regulation of *Gnrhr* expression (63, 64). In the rat pituitary cells, 6 h application of GnRH in two pulses per hour, each lasting 5 min, provides the highest amplitude of response (**Figure 1D**). By contrast, immortalized gonadotrophs do not respond to GnRH application with upregulation in *Gnrhr* transcription (11, 65). This could reflect their embryonic origin or the side-effects of immortalization procedure. However, short GnRH stimulation increases GnRHR binding in αT3-1 membranes, without apparent effect on *Gnrhr* expression (66). Continuous GnRH application in αT3-1 also does not affect GnRHR mRNA levels, but downregulates GnRHR numbers (65). Thus, GnRHR signaling also engages translational regulation. Interestingly, GnRHR signaling induces remodeling of ribosome content in LβT-2 cells (67).

We also noticed that basal and GnRH-induced *Gnrhr* expression depends on the age and sex of rats used for pituitary cell preparation when cells are cultivated in the absence of steroid hormones. Although the relationship between basal and GnRH-stimulated transcriptional activity is comparable in both sexes, the amplitude of response to GnRH increases in female from juvenile to adult stage, but this is not the case with male rat cells (**Figure 1C**) (11). It is interesting to speculate that epigenetic modifications may have a role in the observed differences, although *Gnrhr* promoter regions in mouse and rat are not rich in cytosine–phosphate–guanine islands (68).

Gonadotropin-releasing hormone-induced *Gnrhr* expression relies, at least partially, on PKC activation and subsequent MAPK phosphorylation. The localization of the GnRHR in the lipid rafts (69) is important for activation of these signaling pathways (70, 71). The roles of different PKC isoforms in activation of the "classical" MAPK signaling pathways, composed of extracellular signal-regulated kinase (ERK1/2 and ERK5), c-Jun N-terminal kinases (JNK1/2) and p38, were characterized in immortalized gonadotrophs (72–75), but not in native gonadotrophs. MAPKs activate Fos and Jun proteins, which form a complex that binds to the AP1 site. GnRH itself also induces *Fos*, *Jun*, and *Junb* transcription in the rat gonadotrophs (20, 76). GnRH-induced *Gnrhr* expression in dispersed rat pituitary cells seems to depend mostly on ERK1/2 pathway, with a small but significant involvement of p38 and ERK5 (11). Intriguingly, although JNK1/2 was shown to play a critical role in GnRH induction of the *Gnrhr* expression in αT3-1 cells (77), inhibition of JNK1/2 had no effect on basal or GnRH-stimulated *Gnrhr* expression in the primary cultures (11). Whether this means that, in the rat gonadotrophs, Jun proteins are activated trough alternative pathways or that they are already active in a manner sufficient to induce transcription, remains to be elucidated.

Basal *Gnrhr* transcription also depends on PKC–MAPK signaling pathway (11). However, the existence of basal *Gnrhr* expression during continuous GnRH application could be explained by the fact that the signaling pathways downstream of PKC may also be activated by other factors, whose signaling converges to MAPKs. Indeed, increased Ca^{2+} influx, which in gonadotrophs is also stimulated by PKC (78), is sufficient to induce *Gnrhr* transcription (11), which may imply the possible role of calmodulin in activation of MAPKs (79). Also, portions of ERK1/2 and p38 are phosphorylated and therefore active under basal conditions in immortalized gonadotrophs (74, 80). Although infertile, female ERK1/2 knockout mice also retain *Gnrhr* expression in the pituitary (81), indicating that basal *Gnrhr* expression only partially relies on this pathway, at least in the mouse. Accordingly, cFos-deficient mice show an aberrant, but not completely abolished *Gnrhr* expression (82). In addition, in the rat pituitary cells, ERK inhibition cannot eliminate GnRH-induced *Gnrhr* transcription completely (11).

DEPENDENCE OF *Gnrhr* EXPRESSION ON PACAP AND ACTIVINS

Pituitary adenylate cyclase-activating peptide from hypothalamus may reach the pituitary, but could also be synthetized in the pituitary by gonadotrophs and folliculostellate cells (83), i.e., it could act as an autocrine/paracrine regulator of gonadotrophs by activating its PAC1 receptor expressed in these cells (84). Like GnRH, PACAP activates Ca^{2+} release in inositol-1,4,5-trisphosphate-depedent manner (85), but also increases cAMP production, leading to an activation of protein kinase A (86). A high pulse frequency PACAP administration to LβT-2 cells induced *Gnrhr* transcription (87) and in αT3-1 cells with a rat *Gnrhr* construct, dibutyryl-cAMP increased promoter activity (49). On the other hand, PKA stimulation by forskolin failed to induce *Gnrhr* transcription in LβT-2 cells (11). Although a bipartite element in the rat *Gnrhr* promoter was identified and termed as PACAP response elements I and II (53), the role of PACAP in regulation of *Gnrhr* expression in rat, mouse, and sheep gonadotrophs should be further investigated.

Activin-A stimulates GnRHR synthesis in pituitary cells from juvenile female rats. This effect could not be abolished by inhibin (88) and probably is posttranscriptional; unlike mouse, rat promoter region does not contain a functional activin response element (10). Although activin A, alone or in synergy with GnRH was shown to influence *Gnrhr* transcription upregulation in αT3-1 cells (89, 90), activin receptor II is not required for *Gnrhr* expression in mice (91). For more details on *in vitro* and *in vivo* actions of activins, see Ref. (92).

Prolonged inhibin treatment of the rat pituitary cells cuts the number of GnRHR in half (93), while in ovine pituitary cell culture, 48 h inhibin treatment increases GnRHR binding (94). Continuous microinfusion of inhibin downregulates GnRHR mRNA levels in immature male rats, but this effect could not be observed in adult animals (15).

DEPENDENCE OF *Gnrhr* TRANSCRIPTION ON STEROID HORMONES

In intact rats and sheep, serum estradiol correlates well with increased GnRHR numbers (27, 95), suggesting stimulatory effect of this steroid on transcriptional and/or posttranscriptional events. In contrast to estradiol, progesterone suppresses *Gnrhr* transcription and downregulates pituitary responsiveness to GnRH in mammals (94, 96–98). Progesterone treatment also reduces GnRHR mRNA levels after LH surge in estradiol primed

TABLE 1 | Up- and downregulation of *Gnrhr* expression by hypothalamic, intrapituitary, gonadal, adrenal hormones, and factors.

	Upregulation	Downregulation	No effect
Rats *in vivo*	GnRH, E2 (122)	P (96), Cetrorelix (123), Inhibin (15)	
Rat pituitary cells	GnRH (11, 20)		E2, P (108)
Mouse pituitary cells	GnRH (11)		
Mouse LβT-2 cells	Dexamethasone (111, 119), PACAP (87), Activin-A (119)		GnRH (11), E2 (111)
Mouse αT3-1 cells	GnRH (77), Triptorelin (110), Dibutyryl-cAMP (49), Activin-A (89)	E2, P (110)	GnRH (65)
Sheep *in vivo*	GnRH (61), E2 (62, 116, 124)	P (98)	Cortisol (115, 116)
Ovine pituitary cells	E2 (106)		

Triptorelin, GnRHR agonist; Cetrorelix, GnRHR antagonist; E2, estradiol; P, progesterone; T, testosterone; PACAP, pituitary adenylate cyclase-activating polypeptide; GnRH, gonadotropin-releasing hormone.
Numbers in parentheses indicate the corresponding references.

ovariectomized female rats (96). Furthermore, it was suggested that a decrease in progesterone, rather than an increase in estradiol, during luteolysis is responsible for the increase in GnRHR mRNA and GnRHR number in the ovine pituitary (99–101). In male rats, there was a negative correlation between GnRHR-binding capacity and testosterone levels in serum (18, 19), further suggesting that androgen treatment also inhibits *Gnrhr* transcription/posttranscriptional events. However, these *in vivo* experiments could not dissociate between the direct effects of gonadal steroid hormones on *Gnrhr* transcription from the indirect effects mediated by modulation of GnRH secretory pattern. Gonadotrophs from castrated rats showed fewer GnRH-induced spike–plateau Ca^{2+} responses than cells obtained from intact rats (102), which could be reversed by treatment with a testosterone analog, thus implying its direct effect (102, 103).

The estradiol regulation of the GnRHR numbers in sheep was extensively studied [for review, see Ref. (104)]. *In vivo* administration of estradiol in orchidectomized sheep increased GnRHR mRNA content (105). Similarly, in ovine pituitary cultures, prolonged estradiol treatment increased the number of GnRHRs (106) and *Gnrhr* expression, which was greatly attenuated by progesterone (94, 107). Addition of progesterone alone also reduced GnRHR binding (94). By contrast, neither estradiol nor progesterone affect basal *Gnrhr* expression in the female rat pituitary cells, while progesterone inhibits GnRH-induced *Gnrhr* expression (108). In αT3-1 cells, estradiol reduced GnRHR numbers and mRNA (109, 110), but did not affect *Gnrhr* expression in LβT-2 cells (111).

However, an estradiol responsive element is not present within rat or mouse and ovine *Gnrhr* promoter (9, 10, 56) and rat *Gnrhr* promoter region does not contain the progesterone binding element (9, 10). It was suggested that estradiol effect on *Gnrhr* transcription occurs through membrane associated estrogen receptor-α (112), while mechanism(s) of progesterone action remain unclear.

It is well established that adrenal glucocorticoids affect reproduction (113), but the role of endogenous glucocorticoids in the regulation of *Gnrhr* expression in rats and mice has not been systematically investigated (114). However, continuous infusion of cortisol did not change *Gnrhr* expression in orchidectomized sheep, although it reduced the amplitude of estradiol-induced *Gnrhr* expression upregulation (115, 116). Studies in rats showed that corticosterone and cortisol do not have an effect on GnRHR numbers (117, 118). Dexametasone stimulated *Gnrhr* expression in LβT-2 cells (111, 119). In mouse *Gnrhr* promoter, an activating protein 1 containing site was identified as a mediator of dexamethasone induced transcription (120, 121).

CONCLUSION

Gnrhr transcription is a functional marker of differentiated gonadotrophs. It occurs in the absence of any stimuli and is regulated by several hormones (**Table 1**). The main regulator of transcription of this gene is hypothalamic GnRH and pulsatile GnRH exposure is needed to sustain this process. Transcription is also facilitated by PACAP in an autocrine/paracrine manner, while activins are unlikely to play a physiological role in *Gnrhr* transcription. Steroid hormones influence *Gnrhr* transcription through regulation of GnRH secretion and directly, through a largely uncharacterized mechanisms. The mouse immortalized αT3-1 and LβT-2 cells remain, to this day, the best characterized gonadotroph cell model, although data obtained using these cells do not always correlate with findings in primary mouse and rat pituitary cells. Further studies are needed to elucidate signaling pathways accounting for control of *Gnrhr* transcription, especially in sheep. This includes the possible effects of gonadectomy or steroid hormone application on MAPK signaling.

AUTHOR CONTRIBUTIONS

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Multifaceted Targeting of the Chromatin Mediates Gonadotropin-Releasing Hormone Effects on Gene Expression in the Gonadotrope

Philippa Melamed, Majd Haj, Yahav Yosefzon, Sergei Rudnizky, Andrea Wijeweera, Lilach Pnueli and Ariel Kaplan*

Faculty of Biology, Technion—Israel Institute of Technology, Haifa, Israel

Gonadotropin-releasing hormone (GnRH) stimulates the expression of multiple genes in the pituitary gonadotropes, most notably to induce synthesis of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), but also to ensure the appropriate functioning of these cells at the center of the mammalian reproductive endocrine axis. Aside from the activation of gene-specific transcription factors, GnRH stimulates through its membrane-bound receptor, alterations in the chromatin that facilitate transcription of its target genes. These include changes in the histone and DNA modifications, nucleosome positioning, and chromatin packaging at the regulatory regions of each gene. The requirements for each of these events vary according to the DNA sequence which determines the basal chromatin packaging at the regulatory regions. Despite considerable progress in this field in recent years, we are only beginning to understand some of the complexities involved in the role and regulation of this chromatin structure, including new modifications, extensive cross talk, histone variants, and the actions of distal enhancers and non-coding RNAs. This short review aims to integrate the latest findings on GnRH-induced alterations in the chromatin of its target genes, which indicate multiple and diverse actions. Understanding these processes is illuminating not only in the context of the activation of these hormones during the reproductive life span but may also reveal how aberrant epigenetic regulation of these genes leads to sub-fertility.

Keywords: gonadotropin-releasing hormone, gonadotrope, luteinizing hormone, follicle-stimulating hormone, chromatin, histone, transcription, gene

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****Correspondence:***
Philippa Melamed
philippa@tx.technion.ac.il

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH) regulates the expression of multiple gonadotropic genes [e.g., Ref. (1–4)], to control population size (5–7), differentiation (8), morphology, and migration (9–11) as well as response to other regulatory hormones [e.g., Ref. (12–15)]. The GnRH receptor (GnRHR)-induced activation of MAP- and other kinase pathways (16–18), culminates in expression and/or activation of gene-specific transcription factors [e.g., Ref. (19–25)], allowing them to bind the DNA and stimulate transcription, often *via* recruitment of coactivators which catalyze chromatin modifications [e.g., (19, 26, 27)]. However, some MAPKs are associated with the chromatin, where they phosphorylate histones (28, 29), and GnRH also targets several chromatin and DNA-modifying genes directly [Ref. (8, 19, 30, 31); **Figure 1**], indicating much broader mechanisms for moderating chromatin organization.

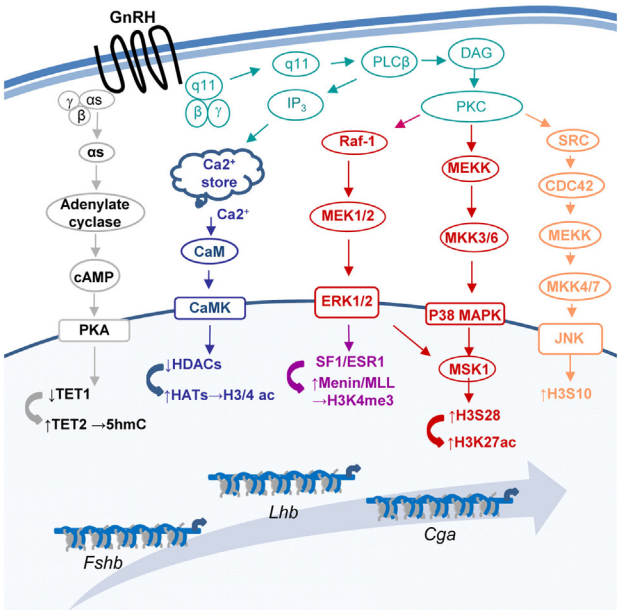

FIGURE 1 | Some of the pathways through which gonadotropin-releasing hormone (GnRH) modifies the chromatin at the three gonadotropin subunit genes. GnRH binds its receptor (GnRHR) to activate a number of pathways that modify the chromatin and lead to changes in expression of the genes encoding the common gonadotropin α-subunit (*Cga*) and the hormone specific β-subunits of luteinizing hormone (*Lhb*) and follicle-stimulating hormone (*Fshb*).

Although the nucleosome is usually highly stable, chromatin structure is dynamic, and this plays a role in determining the accessibility of regulatory DNA, *via* chromatin modifications which alter nucleosome behavior (32). Many histone modifications occur on the N-terminal tails; some affect contact with DNA through altering histone charge, while others "write" a signal which is recognized by protein effectors ["readers" (33, 34)]. However, chromatin-modifying complexes often comprise multiple components with activities to both "read" and "write" various modifications including those on DNA, as well as ATP-dependent remodeling enzymes that reposition or reorganize the nucleosomes to facilitate transcription initiation and also transition of RNAPII through nucleosomes. Such a diversity of distinct enzymes in a single complex allows for sophisticated dialog and cross talk (33, 35, 36).

This short review will highlight the multiple ways through which GnRH targets the chromatin at the gonadotropin genes which, in addition to clarifying the regulation of these genes during development, should lead to greater understanding of how aberrant epigenetic regulation of these genes might underlie fertility problems.

HISTONE ACETYLATION AND DEACETYLATION

By neutralizing the positive charge of lysines on histone N-terminal tails, acetylation at this residue disrupts histone–DNA interactions to make chromatin more accessible, and is thus commonly found at active regions of the genome. Accordingly, basal expression levels of the three gonadotropin genes in partially differentiated gonadotrope-precursor αT3-1 cells closely correlate with levels of H3 acetylation and inversely with H3 occupancy (31). Although H3 and H4 undergo acetylation at various lysines, their differential acetylation at the N-terminus may have a redundant role in transcription such that, in certain contexts, the cumulative charge neutralization influences the transcriptional outcome of a gene more than the acetylation of any specific lysine (37, 38). However, acetylated lysines can be recognized by bromodomain proteins, including multiple chromatin-modifying and remodeling enzymes, such that this modification may well function as a specific recognition site for additional transcriptional activators (39). GnRH increases gonadotrope H3 acetylation, seen both globally and at the 5′ end of the *Cga* gene which encodes the gonadotropin common α subunit, indicating that this comprises part of the regulatory mechanism of GnRH-induced upregulation of gene expression (31). Histone acetylation is catalyzed by the histone acetyl transferase (HAT) activity of several common transcriptional coactivators, some of which have been shown to mediate hormonally-induced expression of the gonadotropin genes (19, 26, 40).

The opposing activity is executed by histone deacetylases (HDACs) which repress expression of the gonadotropin β-subunit genes in gonadotrope precursor cells (22, 41). Exposure of these cells to GnRH allows de-repression of the *Lhb* and *Fshb* genes as a result of activation of calmodulin-dependent kinases, which phosphorylate class II HDACs associated with the gene promoters, leading to their nuclear export (17, 22, 41). A similar mechanism may be responsible for the repression of *Fshb* in the more fully differentiated LβT2 cell line, as GnRH or an HDAC inhibitor facilitated its expression quite specifically, indicating repression by HDACs, which is overcome by GnRH (42, 43).

Both HAT and HDAC enzymes are characteristically found in large multiprotein complexes whose recruitment may follow other chromatin modifications, while they often also recruit additional modifying enzymes to these *loci* to provide elaborate cross talk (44). In partially differentiated gonadotropes, SIN3A and SMRT corepressors were found at the *Fshb* gene promoter, together with class I and class II HDACs. GnRH treatment caused loss of SIN3A and HDAC association, and various components were displaced following knockdown of HDAC4 or either of the co-repressors, suggesting their central roles in this complex (22). The nature of the repressive HDAC complex at the *Lhb* gene is less clear, but as this gene is regulated by DNA methylation and by TET1 (8), additional modifying enzymes are clearly involved: HDAC-containing complexes are often recruited by methylated DNA-binding proteins (MBPs) or DNA methyl transferases (DNMTs), and the HDAC-containing PRC2 complex contains also EZH2 which represses transcription by catalyzing H3K27me3 (35, 45).

On the other hand, HATs are often associated with other chromatin-activating enzymes, including additional HATs and chromatin-remodeling enzymes, as well as chromodomain proteins that bind H3K4me3. The chromodomain-helicase–DNA-binding domain protein 1 (CHD1) in yeast SAGA/SLIK HAT

complexes is recruited to gene promoters in this way, thereby facilitating HAT activity (46) while also altering nucleosomal stability or turnover. Notably, CHD1 is found at the active *Cga* promoter, but its levels were significantly reduced after disruption of enhancer function, and it was not found at the *Lhb* gene promoter (47, 48). This drop in CHD1 association with the *Cga* gene was accompanied by a drop in histone acetylation and H3K4me3 levels which suggests a common pathway/complex of recruiting these enzymes, although the exact mechanism has still to be elucidated. We did not measure histone phosphorylation in this context, but histone acetylation has also been linked to phosphorylation, since discovery that the HAT GCN5 binds preferentially phosphorylated H3S10 which couples these modifications in EGF-induced transcription (49–51).

HISTONE PHOSPHORYLATION

Given that GnRHR signaling involves activation of several MAPKs, it is not surprising that GnRH induces histone phosphorylation, seen globally and at the gonadotropin promoters (31). Some of the MAPKs activated by GnRH, including JNK, can target histone phosphorylation directly (28, 29) and this kinase is responsible for H3S10p at the *Cga* promoter. The phosphorylation of H3S28 at the *Cga* 5′end is *via* GnRH-activation of MSK1, which is a downstream target of ERK and p38 MAPK (31).

By introducing a positive charge, this phosphorylation would be expected to destabilize the DNA–histone interactions and thus aide in passage through the nucleosome (32). However, the role of H3 phosphorylation in transcriptional regulation is still not fully understood, as it is seen at both repressed and active genes (52, 53). Notably, phosphorylation of H3S10 on the *Cga* gene 5′ end/promoter, even though it is increased in response to GnRH, appears to have little function on on-going and/or hormonally upregulated *Cga* transcription, as no effect was noted when its basal levels were reduced by over 90%. However, H3S28p at the first nucleosome in the transcribed region appears to play a role in elongation, presumably facilitating RNAPII transition through this nucleosome, which is likely aided by GnRH-induced H3K27ac (31).

H3S10 or S28 phosphorylation may also alter the affinity of additional chromatin binding proteins to their targets, as reported above for GCN5 (49, 54). At the *Cga* gene, H3K9ac appeared independent of H3S10p, while inhibition of H3S28p was accompanied by a drop in global levels of H3K27ac, although H3S18ac was unaffected (31). In other contexts, H3S10p plays a more crucial role in the activation of repressed genes, as it can trigger the displacement of the HP1γ repressor, and facilitate recruitment of the SWI/SNF chromatin-remodeling enzyme Brg1 and RNAPII (55, 56). The interpretation of histone phosphorylation thus appears to be highly context specific, involving cross talk with neighboring histone residues to determine the precise outcome.

HISTONE METHYLATION

The mono-, di- or trimethylation of specific lysines by histone methyltransferases and demethylases distinguishes transcriptionally active from inactive chromatin domains [reviewed by Ref. (57)]. At the gonadotropin genes, levels of H3K4 trimethylation (H3K4me3) which marks the 5′ ends and/or promoters of all actively transcribed genes and is essential for transcription initiation (58), correlate well with basal expression levels and increase following GnRH exposure (27). Mammals have six distinct complexes capable of catalyzing this modification, with multiple subunits affording different mechanisms of recruitment and regulation (59, 60). The mixed-lineage leukemia (MLL)-COMPASS-like complex 1/2 is recruited to the gonadotropin genes during their upregulation by GnRH and is responsible for the GnRH-induced increase in H3K4me3 at all three gonadotropin promoters (27). Unique among the Set1/COMPASS-like complexes, the MLL1/2 complex contains menin (60), which interacts with various gene-specific transcription factors including ERα (61, 62) and Sf-1 (27). The GnRH-induced association of menin with the β-subunit genes, is dependent on Sf-1. Sf-1 recruits ERα to the *Lhb* promoter, after GnRH-induced modification of both factors. Both of these factors appear to play roles in the recruitment of the MLL1/2 complex and thus also H3K4me3 at these genes (12, 21, 27).

The GnRH-induction of H3K4me3 at the gonadotropin genes alters nucleosomal occupancy, and there was increased association of H3 at the promoters following inhibition of menin which was not overcome by exposure to GnRH (27). H3K4me3 was previously reported to play a role in maintaining low nucleosomal occupancy, likely due to its ability to bind chromatin-remodeling enzymes such as CHD1 and ISWI (46, 63, 64). As described above, it also helps recruit HAT complexes (65, 66) and TFIID (67). However, whether the menin-dependent loss of H3 at the gonadotropin gene promoters following GnRH exposure involves such a mechanism is not yet clear.

Trimethylation of H3K36 (H3K36me3) in the coding regions of all three gonadotropin genes also increases following exposure to GnRH (27). This is likely a direct consequence of increased transcription rates as it is catalyzed by Set2 which is recruited by the elongating S2p form of RNAPII [reviewed by Ref. (68)], and the increased level of this modification at the gonadotropin genes correlates with elevated association of RNAPII (27). H3K36me3 is reported to have a number of roles including regulating histone exchange (69), suppression of initiation through recruitment of DNMT3b and intragenic DNA methylation (70), RNA processing (71), chromatin organization (72), and others, which have yet to be explored in this context.

H2B UBIQUITINATION

Monoubiquitinated H2B at lysine 120 (H2BK120ub) is generally associated with actively transcribed genes, as first reported in yeast, although its precise function in mammals has been controversial. It is reportedly required for recruitment of the Set1/COMPASS complex to gene promoters due to its recognition by one of the complex subunits. This ubiquitin is later removed by a component of the SAGA HAT complex to allow recruitment of the kinase that phosphorylates RNAPII at S2, thus signaling promoter escape and elongation (68, 73). However, it appears that for many mammalian genes H2BK120ub is found primarily in the transcribed region where it plays a role in the reassembly

of nucleosomes in the wake of elongating RNAPII (74–76). The presence and requirement of H2BK120ub at mammalian gene promoters has been disputed, perhaps in part due to the different chromatin organization in the various genes studied and the associated diverse transcription dynamics (see below), and possibly also to earlier experimental protocols which mapped its exact genomic location with poorer resolution.

The apparent lack of requirement for H2BK120ub at some mammalian promoters is likely due to the more numerous complexes that can catalyze H3K4me3 than found in yeast. As described above, these complexes contain distinct subunits which allow recruitment of the lysine methyl transferase (KMT) complex through diverse proteins including transcription factors. Accordingly, there is no apparent correlation between levels of promoter H2BK120ub and expression levels of the three gonadotropin genes, nor with levels of H3K4me3 at their promoters. However, GnRH induces a major increase in H2BK120ub levels globally, and specifically in the coding regions of the gonadotropin genes, in keeping with the changes in coding region H3K36me3 described above (27). The fact that this elevation in H2BK120ub was also noted globally suggests that it is a common event at the multiple genes upregulated by GnRH.

HISTONE CITRULLINATION

Histone citrullination, in which histone tail arginine residues are converted by peptidylarginine deiminase (PAD) enzymes to citrulline has been observed but is still poorly understood. The citrullination is thought to induce chromatin decondensation, and was recently shown to be particularly crucial for transcriptional activation during early embryonic development (77, 78). Notably, PAD family members are highly expressed in female reproductive tissues, have been indicated to play a critical role in female reproduction, and a correlation was seen particularly between PAD2 expression levels, and stages of the estrous cycle (79). Moreover, GnRH was reported to induce PAD2 nuclear localization in gonadotropes, where it stimulates citrullination of H3 at R2, R8, and R17. The inhibition of this activity was seen to blunt the GnRH stimulatory effect on the gonadotropin β-subunit genes, suggesting that histone citrullination mediates part of the GnRH effect, although it has yet to be shown whether it targets the gonadotropin genes directly and the exact mechanism involved (30).

THE DNA-MODIFYING TET ENZYMES

DNA methylation is generally considered a stable mechanism to repress gene expression, often in concert with repressive histone modifications. However, since the discovery of the TET family of enzymes (80, 81), it has become clear that methylated cytosines (5mC) can be hydroxylated to 5hmC, often imparting a very different function as the 5hmC modified-DNA is not recognized similarly by some of the 5mC-binding proteins (82) which can thus lead to de-repression through "functional demethylation." The maintenance DNMT, DNMT1 also binds 5hmC DNA with much lower affinity than to 5mC DNA, leading to a passive demethylation in replicating cells (83, 84). More recently, the TET enzymes were noted to catalyze additional modification of 5hmC, to bases that are quickly removed by the base-excision repair mechanism, in a form of active demethylation (85, 86). Despite this potential of the TET enzymes to relieve inhibition of gene expression, TET1 is also sometimes found in repressor complexes in association with other inhibitory chromatin-modifying enzymes (87–89).

In this context, TET1, which is highly expressed in gonadotrope-precursor cells, was found to repress the *Lhb* gene in association with promoter H3K27 methylation, possibly playing a role in recruitment of the KMT enzyme to this locus. *Tet1* expression is downregulated to allow the precursor gonadotropes to complete their differentiation, following exposure to GnRH and also in response to estrogens or androgens *via* steroid receptors that bind the *Tet1* promoter (8). With developmental or experimentally-induced downregulation of TET1, it is replaced at the *Lhb* gene promoter by TET2 which hydroxymethylates the methylated CpGs on this gene promoter so, in concert with the GnRH-activated transcription factors, facilitating *Lhb* expression (8). In this way, the exposure of the partially differentiated gonadotrope precursors to GnRH promotes their final differentiation via downregulation of *Tet1* and the ensuing elevation in gonadal steroids provides the feedback to keep *Tet1* repressed.

NUCLEOSOMAL ORGANIZATION AND REMODELING

For all three gonadotropin genes, nucleosome levels drop following GnRH treatment, reflecting histone displacement (27, 31). However, the organization of the chromatin at the *Cga* and *Lhb* gene promoters in functional gonadotropes differs markedly, in accordance with their distinct expression levels and means of regulation. In the gonadotropes, the *Cga* proximal promoter, similar to that of many other highly expressed genes but unlike its state in non-gonadotropes, is exposed and accessible to transcription factors, such that transcription may be initiated quite easily (48). Upregulation of *Cga* expression by GnRH is, therefore, likely directed primarily at the level of RNAPII promoter escape and elongation such that after exposure to GnRH it is quickly released from the promoter (27, 48). This is reflected in much higher levels of RNAPII at the *Cga* than *Lhb* promoter in unstimulated mature gonadotropes, and the fact that the first nucleosome in the coding region, characteristic of genes with high RNAPII occupancy (90), is positioned 10 bp further downstream than at the *Lhb* gene. RNAPII transition through this nucleosome is clearly facilitated by the incorporation of a histone H2A variant which allows greater nucleosomal mobility (48), and likely further enhanced by GnRH-induced modifications targeting the histones in this nucleosome (**Figure 2**).

This organization of the *Cga* gene differs fundamentally from that of *Lhb* whose proximal promoter is packaged into a nucleosome (**Figure 2**) that encompasses binding sites of Sf-1, Pitx-1, and Egr-1, which activate this gene (91, 92). The initiation of transcription must thus require reorganization to allow access of these factors to the DNA, likely facilitated by the incorporation of

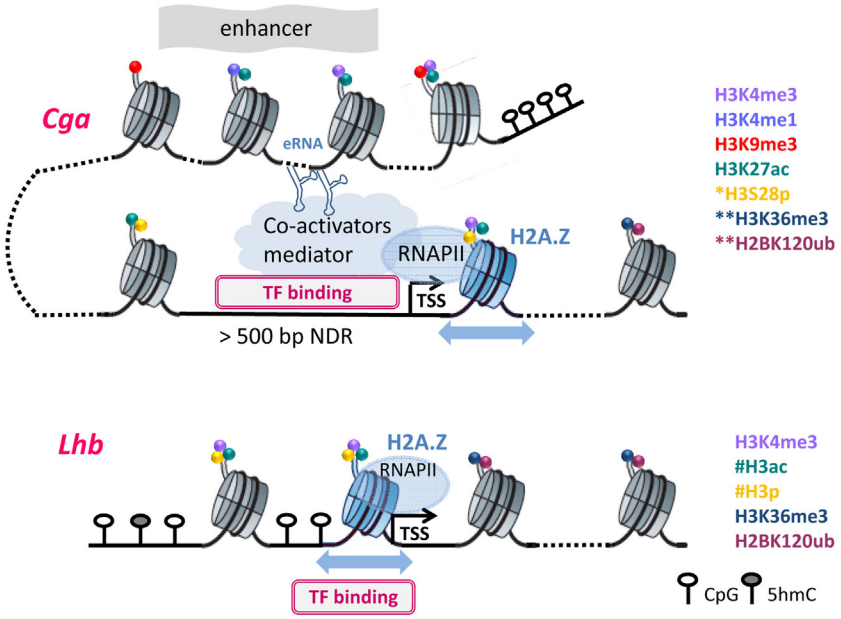

FIGURE 2 | The different chromatin organization necessitates distinct modifications to facilitate transcription: Chromatin organization at *Cga* and *Lhb* regulatory regions are shown in their active states, with DNA and histone modifications as noted. *S28p was examined only at the proximal region of *Cga*; **at the downstream nucleosomes, only H3K36me3 and H2K120Bub were assessed; #histone acetylation and phosphorylation are inferred but were not measured directly on the *Lhb* promoter. Furthermore, the nucleosomal density on the *Lhb* proximal promoter precluded precise mapping of H3K4me3. Dotted lines represent DNA packaged into additional nucleosomes that are not shown, and the blue arrows represent nucleosome mobility due to H2A.Z incorporation. Further details can be found in the text.

H2A.Z at the *Lhb* promoter (48, 93). The binding of TFs to sites that are buried inside the nucleosome is thought to be modulated by the thermally driven spontaneous "breathing" (94). This involves partial wrapping and unwrapping of the DNA at the entry and exit to nucleosome which can expose the binding site, such that its ability to bind is also a function of the distance of the binding site from the nucleosome dyad (95, 96). These fluctuations, which are typically much faster than rates of nucleosome repositioning, likely work together with increased nucleosome mobility to facilitate the initial access of Sf-1 and/or Pitx-1 to their binding sites (93). Binding of either of these "pioneer" factors would destabilize the nucleosome both through the binding itself and the recruitment of the histone-modifying enzymes described above, as well as quite possibly ATP-dependent chromatin-remodeling enzymes. Clearly there is much more work to be done in order to understand the various components and their intricate roles in the activation of this gene, and also whether the nucleosomes at the *Fshb* gene promoter are similarly organized and modified following GnRH exposure.

CONCLUDING COMMENTS

The organization of the chromatin at the gonadotropin genes and its GnRH-induced modifications that facilitate transcription is crucial in understanding how these genes are activated during the reproductive lifespan, but may also have implications in non-pituitary GnRHR-expressing cancer cells. There is increasing indication, however, that much larger distal genomic regions function to determine expression of specific genes, as shown for the eRNA that regulates *Cga* chromatin (47, 97). The likelihood that genes are regulated by a variety of distal enhancers in various scenarios (97), points to highly complex gene-regulation in distinct developmental, hormonally activated and pathological contexts, while emphasizing the importance of the chromatin architecture in extensive genomic regions. As the epigenome is susceptible to external perturbations, elucidation of the full complement of elements that regulate gonadotropin gene expression and their chromatin organization will further our understanding of abnormal gonadotropin levels and the ensuing pituitary-origin reproductive disorders, while opening the way for epigenetic targeting as a basis for fertility drug development and treatment.

AUTHOR CONTRIBUTIONS

All authors contributed to this review, and all have read and approved the final manuscript.

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Regulatory Architecture of the LβT2 Gonadotrope Cell Underlying the Response to Gonadotropin-Releasing Hormone

*Frederique Ruf-Zamojski[1], Miguel Fribourg[1], Yongchao Ge[1], Venugopalan Nair[1], Hanna Pincas[1], Elena Zaslavsky[1], German Nudelman[1], Stephanie J. Tuminello[2], Hideo Watanabe[2], Judith L. Turgeon[3] and Stuart C. Sealfon[1,4]**

[1] Department of Neurology, Center for Advanced Research on Diagnostic Assays, Icahn School of Medicine at Mount Sinai, New York, United States, [2] Department of Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, Icahn School of Medicine at Mount Sinai, New York, United States, [3] University of California, Davis, Davis, CA, United States, [4] Departments of Neuroscience and Pharmacological Sciences, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, United States

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Zvi Naor,
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***Correspondence:**
Stuart C. Sealfon
stuart.sealfon@mssm.edu

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The LβT2 mouse pituitary cell line has many characteristics of a mature gonadotrope and is a widely used model system for studying the developmental processes and the response to gonadotropin-releasing hormone (GnRH). The global epigenetic landscape, which contributes to cell-specific gene regulatory mechanisms, and the single-cell transcriptome response variation of LβT2 cells have not been previously investigated. Here, we integrate the transcriptome and genome-wide chromatin accessibility state of LβT2 cells during GnRH stimulation. In addition, we examine cell-to-cell variability in the transcriptional response to GnRH using Gel bead-in-Emulsion Drop-seq technology. Analysis of a bulk RNA-seq data set obtained 45 min after exposure to either GnRH or vehicle identified 112 transcripts that were regulated >4-fold by GnRH (FDR < 0.05). The top regulated transcripts constitute, as determined by Bayesian massive public data integration analysis, a human pituitary-relevant coordinated gene program. Chromatin accessibility [assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq)] data sets generated from GnRH-treated LβT2 cells identified more than 58,000 open chromatin regions, some containing notches consistent with bound transcription factor footprints. The study of the most prominent open regions showed that 75% were in transcriptionally active promoters or introns, supporting their involvement in active transcription. *Lhb*, *Cga,* and *Egr1* showed significantly open chromatin over their promoters. While *Fshb* was closed over its promoter, several discrete significantly open regions were found at −40 to −90 kb, which may represent novel upstream enhancers. Chromatin accessibility determined by ATAC-seq was associated with high levels of gene expression determined by RNA-seq. We obtained high-quality single-cell Gel bead-in-Emulsion Drop-seq transcriptome data, with an average of >4,000 expressed genes/cell, from 1,992 vehicle- and 1,889 GnRH-treated cells. While the individual cell expression patterns showed high cell-to-cell variation, representing both biological and measurement variation, the average expression patterns correlated well with bulk RNA-seq data. Computational assignment of each cell to its precise

cell cycle phase showed that the response to GnRH was unaffected by cell cycle. To our knowledge, this study represents the first genome-wide epigenetic and single-cell transcriptomic characterization of this important gonadotrope model. The data have been deposited publicly and should provide a resource for hypothesis generation and further study.

Keywords: LβT2, gonadotrope, gonadotropin-releasing hormone, chromatin accessibility mapping, transcription profiling, single-cell transcriptomics

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) plays a key role in the control of reproduction in mammals. Secreted by the hypothalamus in a pulsatile fashion, GnRH acts *via* its receptor (GnRHR) to trigger the synthesis and release of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gonadotropes. In turn, the gonadotropins regulate gametogenesis and steroidogenesis in the gonads. The gonadotropins are composed of a common glycoprotein hormone α subunit (CGA) and a specific β subunit (LHβ or FSHβ). The frequency of GnRH pulse release varies at different stages of reproductive life, e.g., during puberty and the female menstrual cycle. GnRH pulse frequency differentially regulates gonadotropin subunit gene expression and gonadotropin secretion (1). While *Lhb* gene expression is preferentially induced by high-frequency GnRH pulses, low-frequency pulses favor *Fshb* expression (2, 3).

The immortalized LβT2 gonadotrope cells have been used extensively as an *in vitro* model for the study of gonadotropin gene regulation and GnRH signaling. The cell line was developed through targeted tumorigenesis in mice carrying the rat LHβ regulatory region linked to the SV40 T-antigen oncogene (4–6). LβT2 cells have some functional characteristics of mature gonadotropes, as they express *Cga*, *Gnrhr*, and *Lhb*. The cell line responds to pulsatile GnRH stimulation by upregulating *Lhb* and *Gnrhr* and secreting LH. In the presence of steroid hormones, LβT2 cells further increase the LH secretory response to GnRH pulses as well as the levels of *Lhb* and *Gnrhr* mRNAs (6). In addition, LβT2 cells induce *Fshb* under either activin A (7, 8) or GnRH pulse stimulation (3), with the level of *Fshb* being influenced by both pulse frequency and average concentration of GnRH (9). While LβT2 cells exhibit an increase in intracellular calcium and exocytosis in response to GnRH stimulation (5, 6), they differ from mature anterior pituitary cells in that they lack a characteristic large-amplitude calcium oscillatory response to GnRH (10). In addition, continuous GnRH stimulation does not induce *Gnrhr* gene expression, which is in contrast with rat pituitary cells (11).

Previous studies in LβT2 cells showed that GnRH activates a complex cell signaling network that rapidly induces the expression of early genes such as *Egr1*, *c-Fos*, and *c-Jun* (12–14), whose products consecutively activate the transcription of gonadotropin subunit genes. Over the past two decades, a number of studies in the LβT2 cell line have implicated various pituitary factors in gonadotropin subunit gene regulation. These factors include secreted peptides such as bone morphogenetic proteins, pituitary adenylate cyclase-activating polypeptide, growth differentiation factor 9, VGF nerve growth factor inducible (15–19) [for review, see Ref. (20)], as well as transcription factors (TFs) such as AP1 (Fos/Jun heterodimer), SF1, and Egr1 (14, 21–23). Nevertheless, the molecular mechanisms underlying the gonadotrope response to GnRH and the decoding of the GnRH pulse signal are not fully understood.

Recent advances in high-throughput sequencing technologies have enabled researchers to solve key questions about gene regulation both at the chromatin and at the transcriptome levels. Hence, mapping of "open" chromatin regions using the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) allows the detection of putative DNA regulatory regions that are likely bound by TFs (24, 25). Correlating the transcriptome (measured by RNA-seq) with a map of open chromatin may identify transcriptional regulatory elements that are involved in the GnRH response. Furthermore, as distinct cells within a cell population display significant variations in RNA expression, analysis of cell-to-cell variability of gene expression can deepen our understanding of cell population complexity and transcriptome dynamics by isolating transcriptomic heterogeneity (e.g., cell cycle status) that is concealed in cell population studies [for reference, see Ref. (26)] and providing insight into the cellular variation in gene expression levels and induction. Gel bead-in-emulsion (GEM) Drop-seq is a droplet-based single-cell (SC) RNA-seq method that can profile thousands of individual cells per sample with high sensitivity (27–29).

An unbiased epigenomic and SC transcriptomic study of LβT2 gonadotrope cells has not been reported. Here, we generated a global mRNA expression profile and a genome-wide atlas of accessible chromatin in GnRH-stimulated LβT2 cells by analyzing RNA-seq data (30) and ATAC-seq data (**Figure 1A**), respectively. An integrative analysis of the transcriptome and open chromatin data identified key GnRH-regulated genes along with putative *cis*-regulatory elements. We also analyzed cell-to-cell variability of the transcriptome of GnRH-stimulated LβT2 cells and the effects of cell cycle state on this response using GEM Drop-seq (**Figure 1B**).

Abbreviations: ATAC-seq, assay for transposase-accessible chromatin with high-throughput sequencing; FSH, follicle-stimulating hormone; GIANT, genome-scale integrated analysis of gene networks in tissues; GEM, gel bead-in-emulsion; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; SC, single cell; TF, transcription factor; TSS, transcription start site.

MATERIALS AND METHODS

Cell Culture and Treatment

Gonadotropin-releasing hormone was purchased from Bachem (Torrance, CA, USA). LβT2 cells were obtained from Dr. Pamela

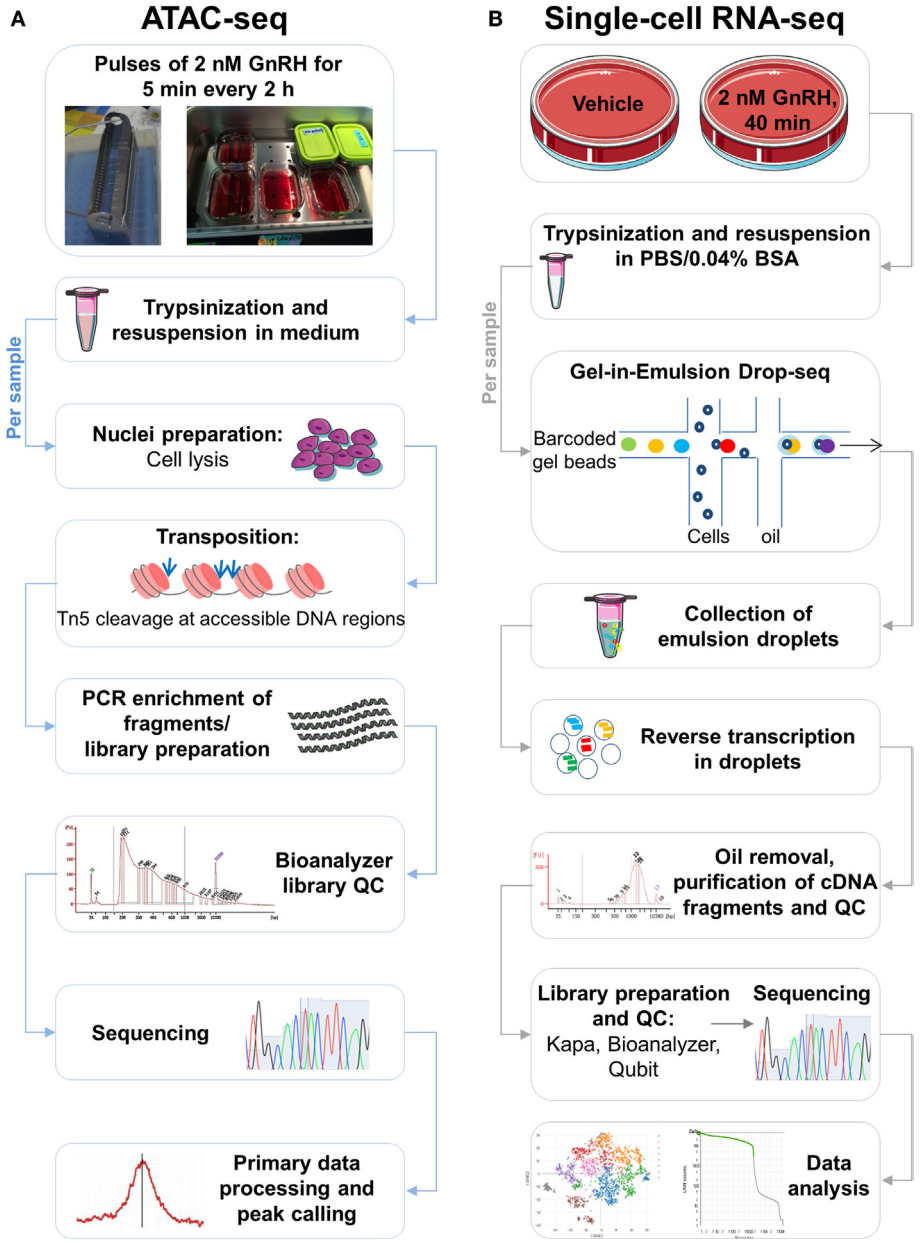

FIGURE 1 | Schematic of the study. **(A)** Assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) workflow. Following treatment with pulses of gonadotropin-releasing hormone (GnRH), LβT2 cells cultured in coverslips placed in racks [see in Ref. (9)] were harvested by trypsinization and resuspended in medium, as indicated. Cell samples were lysed, generating crude nuclei preparations. Next, the transposition reaction was performed in the presence of Tn5 transposase, which cuts and ligates adapters at DNA regions of increased accessibility. The quality of the transposed DNA fragment libraries was assessed using a Bioanalyzer. Libraries were sequenced, mapped to the genome, and processed bioinformatically, and accessible genomic regions were detected (peak calling). **(B)** Single-cell (SC) RNA-seq [gel bead-in-emulsion (GEM) Drop-seq] workflow. Following treatment with GnRH or vehicle, cultured LβT2 cells were harvested by trypsinization and resuspended in a PBS BSA-containing buffer. Cell samples were loaded on a microfluidic chip, combined with reagents and barcoded gel beads to form Gel beads in Emulsion (GEMs), and then mixed with oil. The resulting emulsions were collected and reverse transcribed in SC droplets. Oil was removed, and barcoded cDNA was amplified, purified, and subject to quality control (QC) assessment. Amplified cDNA was incorporated into libraries, which were subsequently subject to QC evaluation using Kapa, Bioanalyzer, and Qubit. Libraries were pooled for sequencing and demultiplexed for subsequent analyses.

Mellon (University of California, San Diego, CA, USA). Cells were cultured at 37°C in DMEM (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS; Gemini, Calabasas, CA, USA) in a humidified air atmosphere of 5% CO_2. Cells were frozen in freezing medium containing 70% DMEM, 20% FBS, and 10% DMSO (Sigma) and maintained in liquid nitrogen. Cell line authentication was achieved by comparing our cells with an early passage aliquot of LβT2 cells provided by

Dr. Mellon and used as a standard reference (Idexx Bioresearch, Columbia, MO, USA). Our results confirmed that our LβT2 cells were *Mycoplasma* free, were of mouse origin, and had similar markers as the original cell line aliquot.

Protocols for GnRH stimulation were selected to provide well-characterized response patterns. Hence, early gene responses in LβT2 cells are sensitive to GnRH concentration, while Fshb transcript levels respond to variations in both GnRH concentration and pulse patterns (9, 13). Because GnRH stimulation of LβT2 cells was previously shown to induce an early gene program, whose transcripts are upregulated within an hour of GnRH exposure (12, 13), a 40- to 45-min GnRH treatment was used in this study to analyze the transcriptome response to GnRH. For the analysis of chromatin accessibility by ATAC-seq, a pattern of pulsatile GnRH exposure was employed to capture the chromatin state that accompanies maximal *Fshb* induction (9, 18).

Bulk RNA-seq Assay

The generation of the RNA-seq data set that we analyze in this study was previously described (30). Briefly, LβT2 cells were serum starved overnight and stimulated with either 5 nm GnRH or vehicle for 45 min. Each group consisted of four independent replicates. Total RNA (2.5 μg) from each replicate was sequenced at the Mount Sinai Genomics Core Facility using an Illumina platform (Illumina, Inc., San Diego, CA, USA) and a HiSeq 2000 sequencing system (100-nucleotide length, single read type, multiplexing three samples per lane). The RNA-Seq data are deposited in GEO (GSE42120).

Assay for Transposase-Accessible Chromatin with High-Throughput Sequencing

Assay for transposase-accessible chromatin with high-throughput sequencing was performed as previously described (25) on two replicate samples of LβT2 cells treated with 2 nM GnRH pulses every 2 h for a duration of 6 h and 45 min (4 pulses in total; cells harvested 45 min after last pulse) in a high-throughput GnRH pulse system (9). Briefly, 4,500 cells were washed with cold PBS at 4°C and lysed for 10 min at 4°C. Cell pellets were resuspended in the transposase reaction mix [5 μl 2× TD buffer, 0.5 μl transposase (Illumina) and 4.5 μl nuclease-free water] and incubated at 37°C for 30 min. DNA from the transposase reaction was purified with a DNA Clean & Concentrator-5 Kit (Zymo Research). PCR amplification was performed using Nextera PCR primers. The optimal number of cycles was determined *via* quantitative real-time PCR (qPCR) to stop the amplification before saturation. Libraries were purified with AMPure beads and then quantified using a KAPA Library Quantification Kit (Kapa Biosystems) and High-Sensitivity DNA Bionalyzer kit (Agilent) and sequenced on an Illumina HiSeq 2500 to >164M reads with 50 bp read length, paired-end. The ATAC-seq data are deposited in GEO (GSE102480).

GEM Drop-seq Assay

Gel bead-in-emulsion Drop-seq was performed as described [10× Genomics, Pleasanton, CA, USA (29)]. Briefly, LβT2 cells were seeded at 350,000 cells per well in 12-well plates for 48 h and treated on day 3 with either 2 nM GnRH or vehicle for 40 min. Cells were then trypsinized and resuspended in medium before being washed and resuspended in 1× PBS/0.04% BSA. Following filtration of the cell suspension, cells were counted on a Countess instrument, and viability was assessed to be above 90% using Trypan Blue. Final concentration was set at 1,000 cells/μl in 1× PBS/0.04% BSA. As a starting point, ~8,000 cells from each sample were loaded into the fluidics chip. Reverse transcription was performed in the emulsion, and cDNA was amplified for 12 cycles before library construction. Quality control (QC) and quantification of the amplified cDNA were assessed using the High-Sensitivity DNA Bioanalyzer kit. Library quality control and quantification were evaluated. The SC data set is deposited in GEO (GSE102480).

Quantification and QC of RNA and Libraries

RNA concentrations were determined with Quant-iT RiboGreen RNA reagent (Invitrogen, Carlsbad, CA, USA) using a fluorescence microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA). RNA quality was assessed by determining the RNA Integrity Number using Bioanalyzer.

Library QC and quantification were assessed using Nanodrop, Qubit (fluorometric quantitation, ThermoFisher Scientific), Kapa (quantification, Kapa Biosystems), High-Sensitivity DNA Bioanalyzer kit (Agilent), and qPCR of selected genes.

Quantitative Real-time PCR

Following total RNA isolation, 1 μg of RNA was reverse transcribed with the Affinity Script reverse-transcriptase (Agilent, Santa Clara, CA, USA). Next, samples were diluted 1:20 in molecular biology grade H_2O (Cellgro, Manassas, VA, USA). SYBR Green qPCR assays were performed (40 cycles) in an ABI Prism 7900HT thermal cycler (Applied Biosystems, Foster City, CA, USA) using 5 μl of cDNA template and 5 μl of master mix containing the specific primers for the targeted gene, Platinum®Taq DNA polymerase, and the required qPCR buffer, following the manufacturer's recommendations. Three technical qPCR replicates were run for each biological replicate. Results were exported as cycle threshold (Ct) values, and Ct values of target genes were normalized to that of *Rps11* in subsequent analysis. Data were expressed as arbitrary units by using the formula, $E = 2{,}500 \times 1.93^{(\text{rps11 CT value} - \text{gene of interest CT value})}$, where E is the expression level in arbitrary units. Primer sequences were as previously described (9, 12).

Bulk RNA-seq Data Analysis

The RNA-seq data generated about 36–45 million reads per sample. The RNA-seq reads were aligned using STAR (31) v2.5.1b with the mouse genome (GRCm38 assembly) and gene annotations (release M8, Ensembl version 83) downloaded from the https://www.gencodegenes.org/web site. 91–93% of the reads were uniquely mapped to the mouse transcriptome. The matrix counts of gene expression for all eight samples were computed by featureCounts v1.5.0-p1 (32). Differentially expressed genes (5% FDR and at least 2 log2 fold change) were identified using the

voom method (33) in the Bioconductor (34) package Limma (35). When comparing the bulk RNA-seq analysis with SC RNA-seq or ATAC-seq data, the transcripts per million (TPM) computed by RSEM (36) was used for the comparison.

ATAC-seq Data Analysis

Primary data analysis involved read mapping *via* bowtie2 (37) to GRCm38 (mm10), followed by duplicate read removal and peak calling *via* MACS2 (38) with parameters "-g dm –nomodel –shiftsize 100 extsize 200." For examining correspondence between chromatin accessibility and gene expression (**Figure 4A**), all peaks were first annotated to the nearest gene, and the most significant peak score for each gene was selected.

SC RNA-seq Data Analysis

Single-cell RNA-seq data were processed using the Cell Ranger pipeline v1.3, which provides a data matrix of expression for all genes and all cells. Differentially expressed genes were analyzed using the sSeq method (41), as implemented in the R package cellrangerRkit v1.1. The cell phase computation for the single cells follows the ideas described in the Supplementary Material of the study by Macosko et al. (28) with our own customized R script implementation. A schematic of the cell phase score computation is described in **Figure 6A**. The t-SNE analysis (42, 43) was performed using the implementation from the Cell Ranger pipeline.

RESULTS

Transcriptome Profiling of LβT2 Cells

To characterize the transcriptome response to GnRH in LβT2 cells for comparison with the chromatin accessibility and SC studies described below, we analyzed an RNA-seq experiment in which LβT2 cells were exposed to either GnRH (5 nM) or vehicle for 45 min [(30); n = 4 for each group]. We identified 112 differentially expressed genes relative to the control (>4-fold at FDR < 0.05, see **Figure 2A**; Figure S1 in Supplementary Material and Table S1 in Supplementary Material), including a large number of early gene transcripts that are known to be regulated by GnRH in gonadotropes, such as Egr1, Fos (c-Fos), and Jun (c-Jun) (12, 45, 46). As is evident from the asymmetrical expression change versus significance volcano plot (**Figure 2B**), the majority of regulated transcripts were upregulated at this time point. The plot highlights the most highly upregulated genes, which include Jun, Fos, Fosb, Egr1, Egr2, Egr3, Egr4, Nr4a3, and Nr4a1.

We investigated the functional relationships of the 21 highest fold-change annotated GnRH-regulated genes in the context of a human pituitary gene network using Genome-scale Integrated Analysis of gene Networks in Tissues [GIANT; **Figure 2C**; (39)]. GIANT uses a massive public data compendium to infer tissue-specific data-driven functional gene–gene relationships. The output of a GIANT analysis is a graph indicating the strength of the functional relationship of each pair of the input genes in the pituitary, as well as the inference of additional highly related genes. The edges connecting the nodes indicate the statistical strength of the evidence for that relationship in the human pituitary. Notably, the highest regulated genes form highly interrelated subgroups (e.g., *Jun*/*Fos*/*Ier2*), and many highly related inferred genes are regulated by GnRH (e.g., *Dusp1, Nab2*) or known to be central to key gonadotropin developmental or regulatory processes [e.g., *Pitx2*; Table S2 in Supplementary Material; (12, 14, 47–50)]. Included among the statistically significant gene enrichment set were MAPK signaling and SMAD protein signaling (Table S3 in Supplementary Material). Overall, this analysis reveals a coordinated gene program activated by short-term GnRH exposure.

Genome-Wide Mapping of Chromatin Accessibility in LβT2 Cells

To map open chromatin regions, we carried out ATAC-seq in LβT2 cells treated with GnRH (**Figure 1A**). ATAC-seq uses the hyperactive Tn5 transposase, loaded with adapters for high-throughput DNA sequencing, to integrate into regions of accessible chromatin. The resulting DNA fragments, generated from locations of open chromatin, are amplified, sequenced, and computationally mapped to the genome to obtain a genome-wide accessibility landscape (24, 25). The ATAC-seq libraries and the sequence data showed a characteristic ~200 bp size distribution periodicity (**Figure 3A**), reflecting individual nucleosome occupancy patterns and confirming specific transposase activity and assay accuracy. Primary data analysis (see Materials and Methods) identified more than 58,000 statistically significant regions of open chromatin (peaks). The open chromatin map was found to be reproducible across independent samples and libraries. We note that, while determining whether chromatin accessibility changes with GnRH exposure is an important question, the present study was intended only to provide a baseline analysis of open chromatin structure. Performing a reliable comparative analysis of changes in specific regions with GnRH stimulation would require assaying a large number of samples, which was not feasible for this investigation.

We examined the 2,000 most prominent open chromatin regions (showing the highest peak scores; see Materials and Methods) with respect to their location relative to annotated genomic features. Approximately 75% of these peaks were located in immediate gene promoters or introns, which is consistent with open chromatin in the proximal regions of transcriptionally active genes (**Figure 3B**). Focusing on the sequence fragments in genomic areas flanking the transcription start sites (TSS, −3 to 3 kb), we observed a distribution highly preferential to the regions in close vicinity to the TSS (**Figure 3B**). We next examined chromatin state in the proximal promoter regions of several key genes (**Figure 3C**). While open chromatin peaks were detected at genes that are either constitutively expressed in LβT2 cells (*Gnrhr*) and/or regulated by GnRH (*Lhb*, *Cga*, and *Egr1*), chromatin was closed at the *Tshb* gene that is not expressed in gonadotropes. To gain insight into the overall transcriptional regulatory state of these cells, we used the HOMER tool to determine TF binding motifs showing enrichment among all open chromatin regions (40). The enrichment results show extremely high statistical significance (see Table S4 in Supplementary Material), reflecting the high power of this global analysis. Finding highly enriched global binding motifs for the TFs Smad2 and Six6, which have

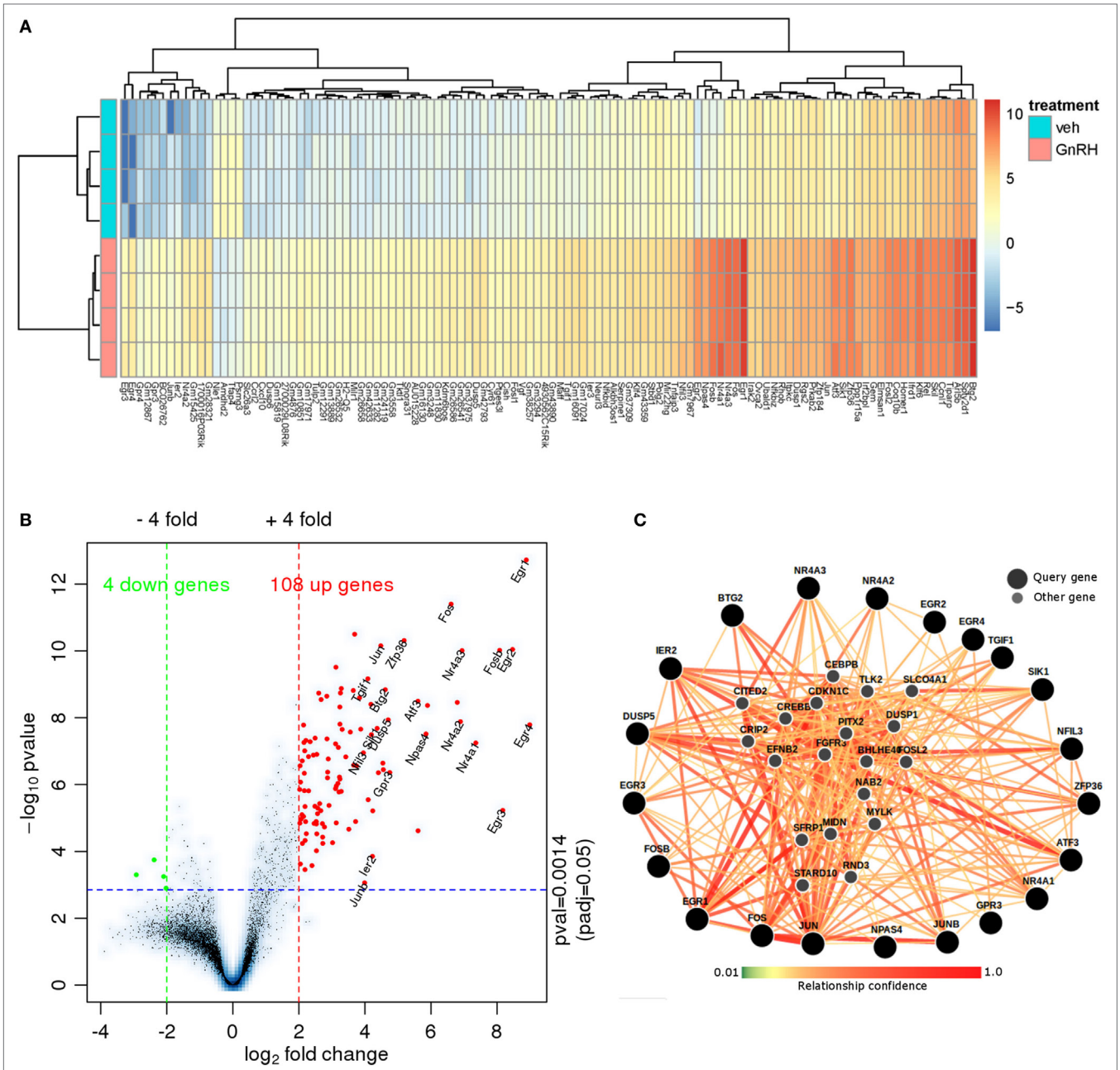

FIGURE 2 | RNA-seq data analysis. **(A)** Heat map of RNA-seq expression data showing the genes that were differentially regulated following treatment with 5 nM gonadotropin-releasing hormone (GnRH) for 45 min. Gene expression is shown in normalized log2 counts per million. Differentially expressed genes were selected based on a fourfold change and FDR < 0.05. **(B)** Volcano plot with the log2 fold changes in gene expression after GnRH treatment on the x-axis and the statistical significance (−log10 *p* value) on the y-axis. The gene symbols of 21 annotated genes with at least a 16-fold expression change are displayed. **(C)** Functional network analysis of the top 21 genes using (Genome-scale Integrated Analysis of gene Networks in Tissues) (39). Nodes related to those 21 genes are shown (smaller markers) in this human hypophysis (pituitary) data-driven Bayesian functional network.

been implicated in *Fshb* gene expression (51, 52), suggests that this analysis is likely to have generated useful leads for further study. In these high-resolution ATAC-seq data, we were also able to observe TF footprints, which create a notch in a region of otherwise open chromatin due to the presence of a bound TF. As an example, **Figure 3D** shows a notch detected in the otherwise open *Jun* promoter that precisely matches an SP1 consensus site. This finding is consistent with the known role of SP1 in mediating induction of this gene (53).

To examine the relationship of the epigenetic landscape and the global pattern of mRNA expression, we compared the ATAC-seq and RNA-seq data. While the GnRH treatment conditions used for ATAC-seq versus RNA-seq experiments differed, they were both compatible with early gene induction. Indeed,

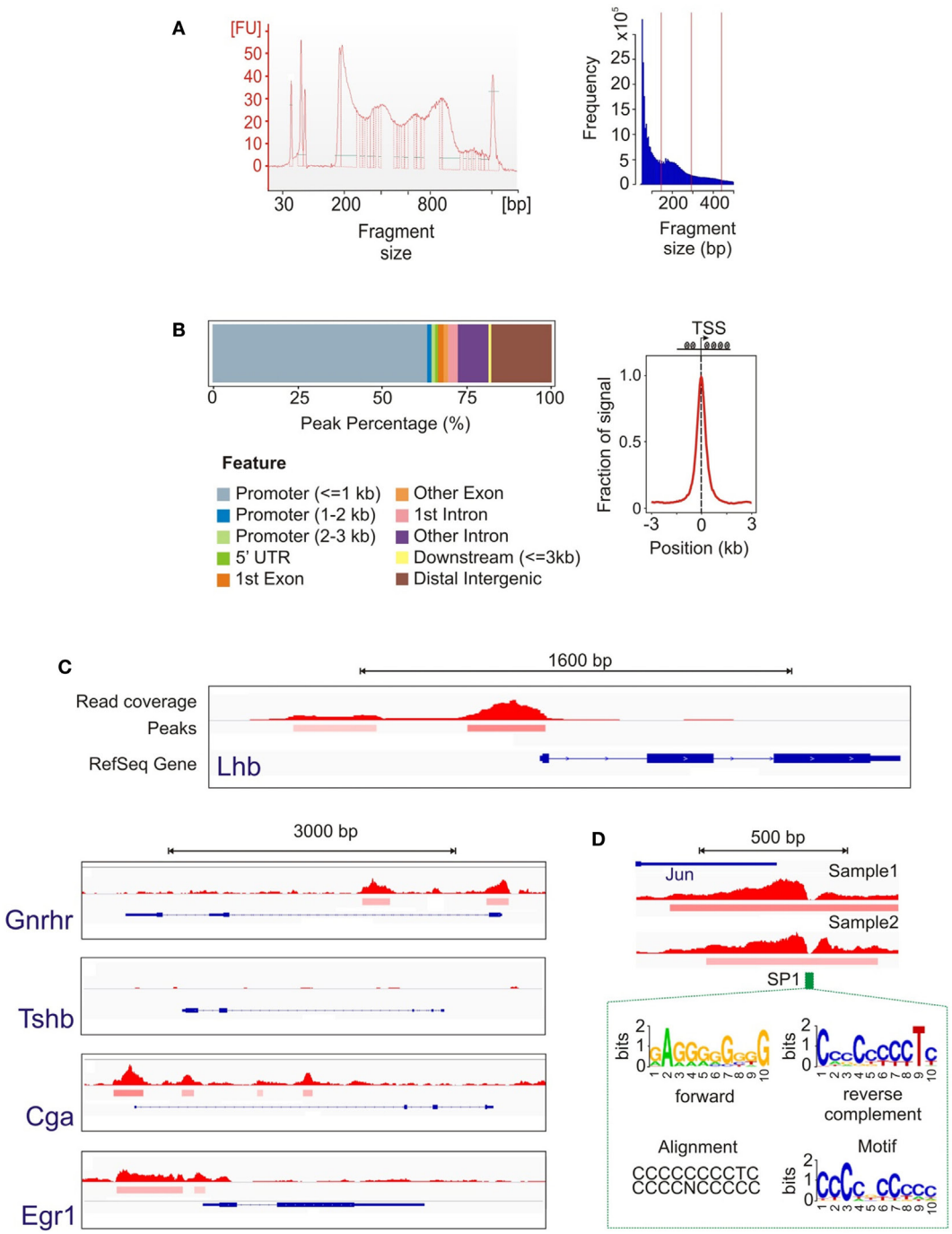

FIGURE 3 | Assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) data analysis. **(A)** Library and sequence fragment size distribution showing a periodicity characteristic of the effect of nucleosome binding. **(B)** Position of open chromatin peaks with respect to genomic features shows their genome-wide distribution are concentrated around the active transcription start sites (TSS), indicating that TSS are enriched for open chromatin. **(C)** Representative loci of genes showing the open chromatin signal. The top track represents the read coverage (density of sequence reads) at each location. The middle peak track indicates genomic regions that achieve statistical significance for chromatin accessibility. The bottom track shows the RefSeq gene annotation (intron/exon locations, transcription direction, etc.). As expected, accessible chromatin segments were detected around the promoter regions for *Lhb*, *Gnrhr*, *Cga*, and *Egr1*, but not for *Tshb*. **(D)** Example of transcription factor binding identification based on the footprint analysis of the open chromatin signal. The notch in the open chromatin region corresponds to the location of a consensus SP1 site. The HOMER analysis suite utilized (40) calculates a data-driven motif model from our ATAC-seq peak data, shown in forward and reverse configurations, and then associates this motif with known motifs. In this example, the known SP1 consensus motif is nearly identical to the data-driven motif.

previous analysis of temporal responses of the early genes *Fos* and *Egr1* in response to GnRH pulse stimulation demonstrated that they are highly expressed within 40 min after the fourth pulse (9). In this analysis, we identified the open chromatin peak showing the highest ATAC-seq peak score associated with each annotated gene. This estimate of chromatin accessibility for each gene was plotted against its level of expression following GnRH treatment using the RNA-seq data (**Figure 4A**). The results show a general pattern of increased chromatin accessibility for expressed genes. Genes found to be highly induced by GnRH (e.g., *Egr1*, *Egr2*, *Egr4*, *Jun*, *Fosb*, *Atf3*) tend to show high chromatin accessibility.

The gonadotropin subunit genes *Lhb* and *Fshb* showed very high levels of chromatin accessibility at specific locations. The *Lhb* area of high accessibility, shown in **Figure 3C**, is centered around the proximal promoter. In contrast, *Fshb* does not show statistically significant open chromatin in the region of the proximal promoter. Notably, several significant open chromatin peaks were found between −40 and −90 kb upstream of the *Fshb* TSS. The segment of highest chromatin accessibility, at −90 kb, contains a binding motif for AP1, a known *Fshb* regulator [**Figure 4B**; (14)]. This pattern raises the possibility that this region may represent a distal *Fshb* enhancer.

SC Transcriptome Analysis of LβT2 Cells and Characterization of Early Gene Response to GnRH

To investigate cell-to-cell variability in gene expression in the gonadotrope response to GnRH, we performed a SC transcriptome analysis of LβT2 cells exposed to either GnRH or vehicle for 40 min using GEM Drop-seq (see **Figure 1B**). This assay measures the entire transcriptome in thousands of individual cells from each sample. Specifically, we sought to assess cell-to-cell heterogeneity with respect to the individual cell response to GnRH.

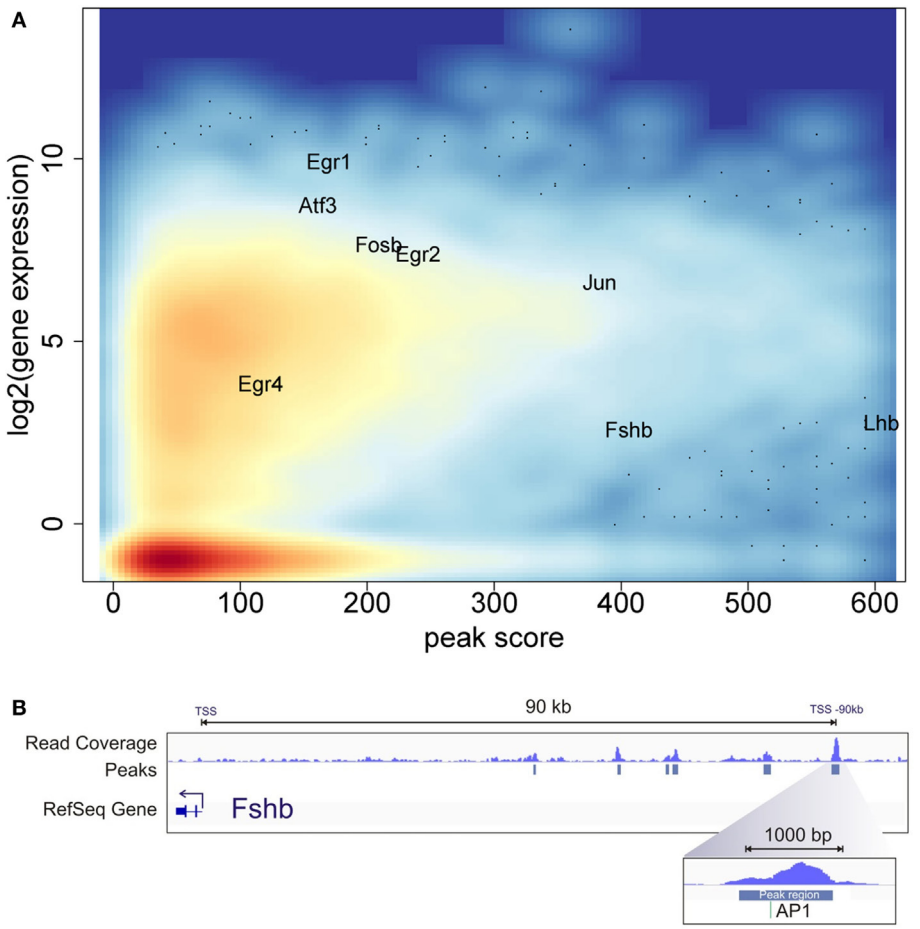

FIGURE 4 | Chromatin accessibility and gene expression landscape. **(A)** Average log2 gene expression for each gene from the RNA-seq experiment compared to the highest assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) peak score associated with the same gene. Color intensity indicates density of gene population, with red representing the highest density and light blue the lowest. **(B)** ATAC-seq and computational analysis of the upstream region of the *Fshb* gene. The top track shows read coverage. The middle track shows the location of significant peak regions. The bottom track indicates the *Fshb* transcription start sites. The direction of *Fshb* transcription is to the left in this diagram. The most distal open chromatin region is the strongest peak detected near *Fshb* and encompasses a consensus AP1 site.

We started with ~8,000 LβT2 cells in each sample. The resulting SC RNA-seq libraries all exhibited the expected electropherogram traces on the Agilent Bioanalyzer High Sensitivity Chip, thus passing the QC assessment. In addition, qPCR assays of several early response genes in the library provided further evidence that our SC RNA-seq libraries were suitable for the detection of individual gene expression and regulation (data not shown). We sequenced to a depth of ~300 million reads in the SC libraries from each sample (vehicle- and GnRH-treated LβT2 cells). We obtained good sequence data for 1,992 vehicle-treated and 1,889 GnRH-treated cells, with ~98,000 mean reads/cell and >4,000 median genes/cell above detection threshold. Analysis of the GEM Drop-seq data demonstrated even coverage, with the slow drop-off of expression/cell in the top nearly 2,000 cells being similar in untreated versus GnRH-treated cells (**Figure 5A**). These features are indicative of a high-quality SC transcriptome data set.

A heat map of selected genes expressed in all 3,881 analyzed cells revealed a global pattern of differential expression between GnRH-treated and vehicle-treated cells (**Figure 5B**). We identified 95 differentially expressed genes that included known GnRH-regulated immediate-early genes such as *Egr1*, *Fos*, *Fosb*, *Jun*, *Btg2*, *Junb*, and *Nr4a1* (Table S5 in Supplementary Material). Notably, we observed high cell-to-cell heterogeneity in response to GnRH, and several GnRH-treated cells even exhibited a gene expression pattern similar to that of untreated cells (**Figure 5B**; Figure S2 in Supplementary Material). Comparison of SC gene expression measurements averaged across two randomly selected subgroups of 996 cells highlighted the high reproducibility and consistency of the data, as the correlation coefficient was >0.99 in either vehicle- or GnRH-treated cells (**Figure 5C**). To further evaluate the performance of the digital transcriptome, we compared Drop-seq gene expression measurements averaged across all SC with the bulk RNA-seq measurements analyzed above, in either vehicle- or GnRH-treated cells. Despite the SC and bulk sequencing data sets coming from different experiments, the aggregated Drop-seq data showed high correlation with the bulk RNA-seq data (correlation coefficient >0.90; **Figure 5D**). The high cell-to-cell variation in the response to GnRH and in gene expression levels results from a combination of technical measurement variation and true cell-to-cell biological variation. To gain a sense of the degree of cell expression measurement resulting from technical noise, we modeled the technical measurement variation by a Poisson distribution [(54); **Figure 5E**]. Notably, at high levels of expression, the contribution of technical noise is relatively small and supports the presence of high levels of true biological cell-to-cell expression variation in these cells.

Analysis of Cell Cycle Dependence of the SC Transcriptome of GnRH-Stimulated LβT2 Cells

One limitation in many studies of response to GnRH performed by our group and others in LβT2 cells is that the effects of cell division on GnRH-induced gene regulation are not controlled. LβT2 cultures comprise asynchronously dividing cells. A SC transcriptome experiment provides the possibility of identifying the cell cycle stage of each individual cell and determining whether cell cycle stage influences the response to GnRH. Previous studies in yeast indicate that cell cycle has global effects on protein and RNA synthesis, thus affecting the transcriptional activity (55–57). RNA levels are controlled by transcriptional bursting that can vary during different cell cycle stages (58).

We examined cell cycle state of LβT2 cells as well as the influence of cell cycle phase on gene expression. Comparison of each cell's average expression with gene sets known to be enriched in one of the five cycle phases [G1/S, S, G2/M, M, and M/G1; Table S6 in Supplementary Material; for reference, see Ref. (44)] enabled alignment of each cell by the cell cycle stage (**Figures 6A,B**). Consistent with this alignment, a gene associated with the M cycle phase (*Cenpf*) exhibited the highest expression at the M phase and high expression at the G2/M phase (**Figure 6C**). In contrast, *Cenpf* was poorly expressed at G1/S and moderately expressed at S and M/G1. Likewise, the expression levels of other genes known to be associated with a specific phase of the cell cycle (e.g., *Pcna* with G1/S and S, *Top2a* with both S and G2/M) were highest at that cell cycle phase (Figure S3 in Supplementary Material). On the other hand, immediate-early genes induced by GnRH (e.g., *Egr1*, *Fos*, *Junb*) and other GnRH-regulated genes (e.g., *Gdf9*) showed no significant expression change with cell cycle phase (**Figure 6C**). The overall cell distribution with respect to cell cycle phase was comparable in GnRH-treated cells versus control (vehicle-treated cells) cells (data not shown). Additional analysis showed that the five cycle phases were represented throughout all analyzed cells, with individual cells partly forming clusters based on their cell cycle phase (**Figure 6D**).

DISCUSSION

In this study, we analyzed the global transcriptional, epigenetic, and single-cell transcriptional landscapes of the LβT2 gonadotrope cell line using RNA-seq data, ATAC-seq data and GEM Drop-seq data, respectively. To our knowledge, this represents the first genome-wide epigenetic characterization and the first SC transcriptome study performed in any gonadotrope experimental system. Our results provide insight into the global transcriptional regulatory processes of these cells and provide data sets and hypotheses to guide further work in this field.

A recent characterization of epigenetics in gonadotrope models reported CpG methylation, DNAse hypersensitivity, and histone modification at regions of several specific genes in several cell lines, including LβT2 cells (59). Their finding of open chromatin correlating with expression at the specific genes investigated corresponds with our genome-wide characterization of this relationship. The global ATAC-seq epigenetic approach that we have pursued opens the avenue to entirely unexpected discovery, such as the putative new *Fshb* enhancer identified as highly open chromatin (see **Figure 4B**). Further study is needed to evaluate the functional role of this novel putative enhancer.

Analysis of SC transcriptome in vehicle- and GnRH-treated cells demonstrates, for the first time, that there is no influence of cell cycle stage on the gene response to GnRH. The SC variability observed at the level of basal gene expression and gene induction by GnRH is high and largely not explained by the technical

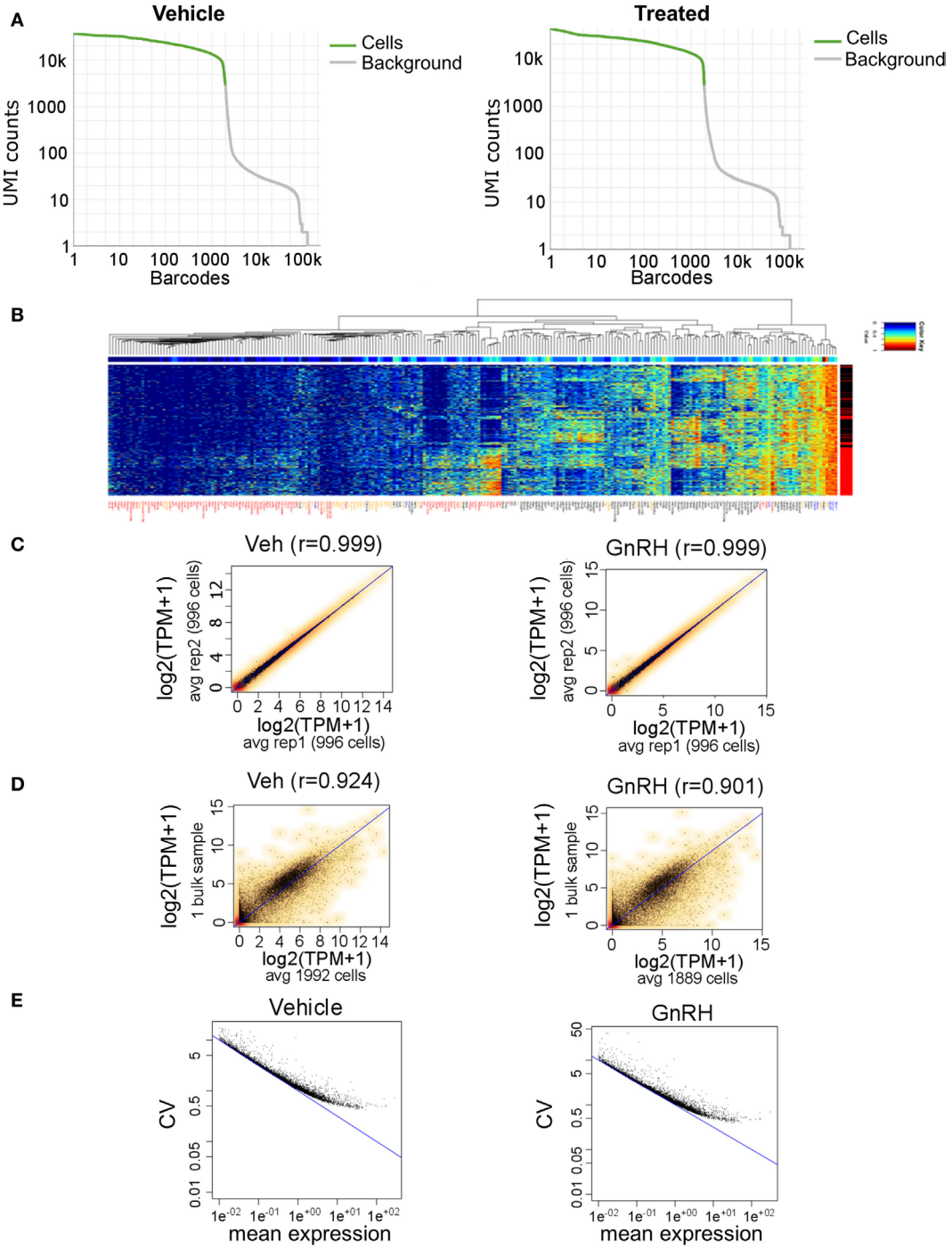

FIGURE 5 | Gel bead-in-emulsion Drop-seq data. Analysis of 3,881 LβT2 cells (1,992 vehicle-treated and 1,889 GnRH-treated cells for 40 min) having a median of 4,027 distinct genes quantified per cell from 380 million reads. **(A)** Drop-off of expression in unique molecular identifiers (UMIs) counts for each cell (barcode), demonstrating even coverage. **(B)** Heat map of selected genes in all 3,881 cells. Each horizontal line is one cell, with red and black on the right side indicating gonadotropin-releasing hormone (GnRH) and vehicle treatment, respectively. Gene symbols are color coded as follows: red, genes that are differentially regulated by GnRH; black, genes that show high cell-to-cell variability in gene expression; yellow, genes that have been involved in gonadotrope gene regulation based on the literature; and blue, housekeeping genes. **(C)** Scatter plots of transcripts per million (TPM) averages obtained for each gene from randomly dividing the population of cells in each sample into two subgroups: vehicle-treated subgroups (left) and GnRH-treated subgroups (right). **(D)** Scatter plots of TPM averaged across all SC and TPM from the bulk RNA-seq data, in either vehicle- or GnRH-treated cells. **(E)** Scatter plots of coefficient of variation versus mean expression level (average number of reads per gene) across all vehicle-treated cells (left) and GnRH-treated cells (right). The blue line denotes the Poisson technical noise, while the black dots signify total variation, which includes cell-to-cell biological variation.

variation to which SC transcriptome analysis is prone (60). High levels of SC expression and gene induction variation in LβT2 cells could be anticipated based on previous studies of response variation and noise scale of selected transcripts (61, 62). The cause of this high level of variation in expression and response to GnRH in LβT2 cells and whether it accurately models the expression and

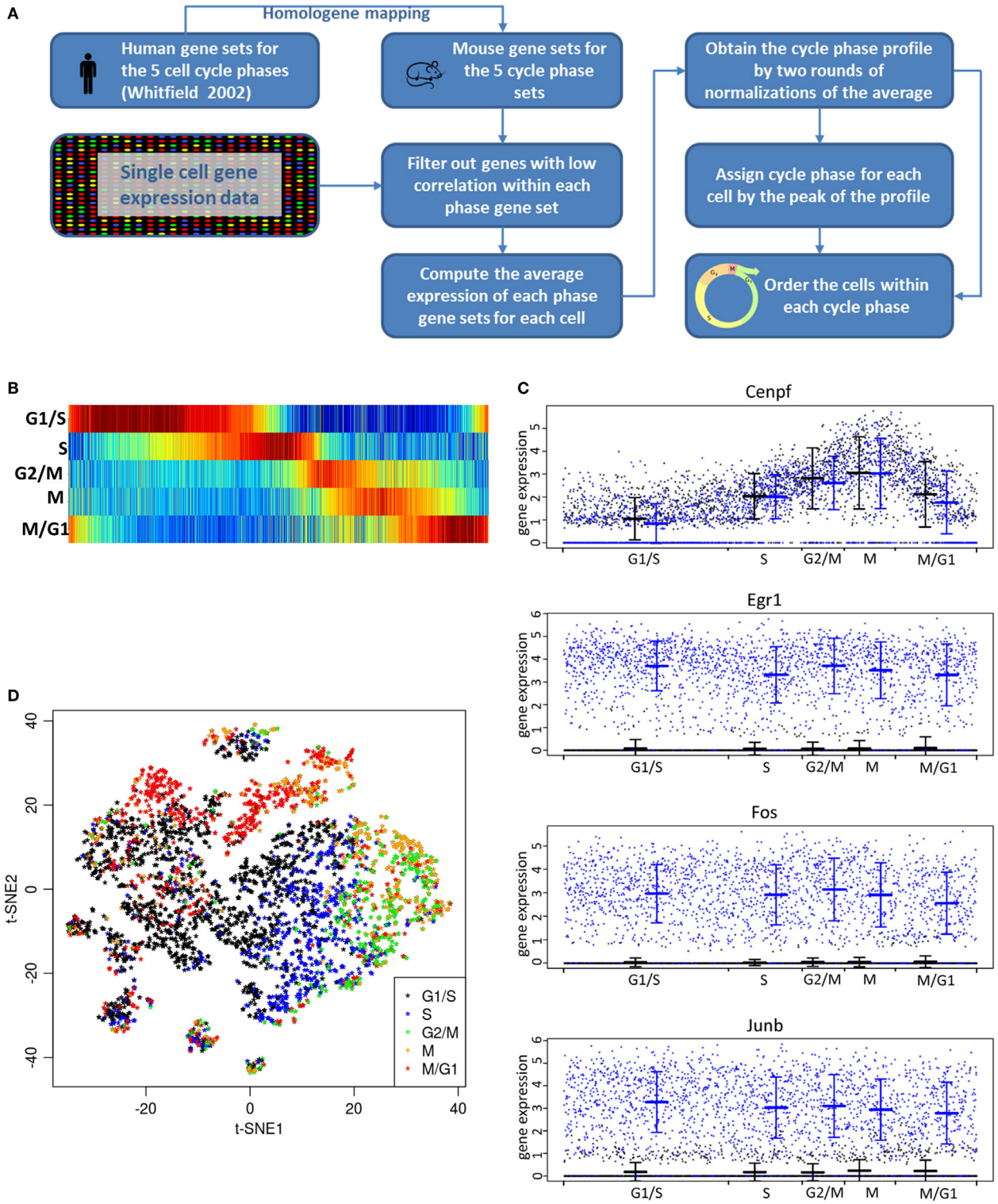

FIGURE 6 | Continued

FIGURE 6 | Analysis of effects of cell cycle. **(A)** Flowchart diagram of the cell cycle analysis method. The score computation is based on the Supplemental Material from the study by Macosko et al. (28) with the cell cycle genes taken from their Table S2 in Supplementary Material mapped to mouse gene using Homologene. The gene sets for the five cell cycle phases (G1/2, S, G2/M, M, and M/G1) were determined by comparing cells that were arrested at different cell cycle phases (44). **(B)** Each of the 3,881 (GnRH- and vehicle-treated) cells on the x-axis is aligned by the cell cycle progression. Each vertical line represents one cell with the color code indicating low (dark blue) to high (dark red) score assignment to the cell cycle phase. All cells are ranked by cell cycle progression according to the steps depicted in **(A)**. The five cell cycle phases are indicated by the labels on the horizontal lines. **(C)** Single-cell expression of individual transcripts in relationship to cell cycle phase. Shown is the expression [log2 (TPM + 1)] of a gene associated with the mitotic (M) phase of cell cycle (*Cenpf*) and of GnRH-regulated genes (*Egr1*, *Fos*, and *Junb*). X-axis indicates cell cycle progression, as derived from **(B)**. The mean and SD for vehicle- (in black) and GnRH-treated cells (in blue) are shown at each cell cycle phase. Note that the expression level of any gene in these cells at any point in the cell cycle can be accurately determined without experimental cell cycle synchronization. **(D)** t-SNE plot of all 3,881 cells based on global expression of all genes (see Materials and Methods). The color of each cell indicates the cell cycle phase as determined in **(B)**. The fact that cells are clustered according to the cell cycle phase indicates that the primary cause of gene expression change is the cell cycle phase change.

response patterns of the intact mouse gonadotrope are unknown. While recapitulating many properties of a mature gonadotrope, LβT2 cells are a transformed cell line generated by tumorigenesis. It is conceivable that the heterogeneity of these cells has been augmented by the process of transformation (63, 64). Cell-to-cell variation in expression and response to stimulation can also result from normal biological variation and stochastic mechanisms (65). Thus, alternatively, this line may faithfully reflect SC expression and response variation and represent an accurate model of the behavior of the primary gonadotrope.

Individual gonadotropes largely function as a SC processor in controlling the reproductive axis and understanding the role of cell-to-cell variation in the engineering of this system is a relevant question to approach through SC biology. Evaluation of chromatin accessibility variation at the SC level [SC ATAC-seq (66, 67)] is also an interesting area for future investigation. The generation of high-quality global ATAC-seq chromatin accessibility and SC GEM Drop-seq transcriptome data should provide a useful resource for the research community.

AUTHOR CONTRIBUTIONS

FR-Z and MF designed and performed research, analyzed, and interpreted data; YG, EZ, GN, ST, and HW contributed analytic tools and analyzed data; VN performed research and analyzed data; HP interpreted data and drafted the work; JT analyzed and interpreted data; SC conceived research, analyzed data, and drafted the work. All authors drafted or revised the work critically and approved the final version to be submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fendo.2018.00034/full#supplementary-material.

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HYPOTHESIS AND THEORY
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Leptin Regulation of Gonadotrope Gonadotropin-Releasing Hormone Receptors As a Metabolic Checkpoint and Gateway to Reproductive Competence

Angela K. Odle, Noor Akhter, Mohsin M. Syed, Melody L. Allensworth-James, Helen Beneš, Andrea I. Melgar Castillo, Melanie C. MacNicol, Angus M. MacNicol and Gwen V. Childs**

Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, Little Rock, AR, United States

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***Correspondence:**
Angela K. Odle
akodle@uams.edu;
Gwen V. Childs
childsgwenv@uams.edu

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The adipokine leptin signals the body's nutritional status to the brain, and particularly, the hypothalamus. However, leptin receptors (LEPRs) can be found all throughout the body and brain, including the pituitary. It is known that leptin is permissive for reproduction, and mice that cannot produce leptin (Lep/Lep) are infertile. Many studies have pinpointed leptin's regulation of reproduction to the hypothalamus. However, LEPRs exist at all levels of the hypothalamic–pituitary–gonadal axis. We have previously shown that deleting the signaling portion of the LEPR specifically in gonadotropes impairs fertility in female mice. Our recent studies have targeted this regulation to the control of gonadotropin releasing hormone receptor (GnRHR) expression. The hypotheses presented here are twofold: (1) cyclic regulation of pituitary GnRHR levels sets up a target metabolic checkpoint for control of the reproductive axis and (2) multiple checkpoints are required for the metabolic signaling that regulates the reproductive axis. Here, we emphasize and explore the relationship between the hypothalamus and the pituitary with regard to the regulation of GnRHR. The original data we present strengthen these hypotheses and build on our previous studies. We show that we can cause infertility in 70% of female mice by deleting all isoforms of LEPR specifically in gonadotropes. Our findings implicate activin subunit (InhBa) mRNA as a potential leptin target in gonadotropes. We further show gonadotrope-specific upregulation of GnRHR protein (but not mRNA levels) following leptin stimulation. In order to try and understand this post-transcriptional regulation, we tested candidate miRNAs (identified with *in silico* analysis) that may be binding the *Gnrhr* mRNA. We show significant upregulation of one of these miRNAs in our gonadotrope-Lepr-null females. The evidence provided here, combined with our previous work, lay the foundation for metabolically regulated post-transcriptional control of the gonadotrope. We discuss possible mechanisms, including miRNA regulation and the involvement of the RNA binding protein, Musashi. We also demonstrate how this regulation may be vital for the dynamic remodeling of

gonadotropes in the cycling female. Finally, we propose that the leptin receptivity of both the hypothalamus and the pituitary are vital for the body's ability to delay or slow reproduction during periods of low nutrition.

Keywords: gonadotropes, Musashi1, miRNAs, infertility, female, gonadotropin-releasing hormone receptor, leptin receptors, post-transcriptional regulation

INTRODUCTION

The Leptin Signal Permits Reproduction

Leptin is a hormone largely produced by adipocytes that regulates appetite and signals levels of adiposity and nutritional status (1–8). When physiological conditions are normal, serum leptin levels correlate well with fat mass and signal optimal nutritional states (9–11). When nutrition is deficient, the resulting reduction in serum leptin becomes a critical metabolic signal for starvation (12–17), stimulating increases in appetite and food-seeking behavior. At the same time, the low leptin signal reduces or prevents the activation of energetically expensive reproductive processes such as pregnancy and lactation (3, 14, 18–33).

Serum leptin levels are a critical link between sufficient nutrition and the function of the hypothalamic–pituitary–gonadal (HPG) axis. The importance of leptin to the HPG axis is emphasized by evidence in humans deficient in leptin receptors (LEPRs) (20) or leptin (34, 35), who are hypogonadal and infertile. Furthermore, low gonadotropin levels and functional hypothalamic amenorrhea occur when leptin is reduced by energy deficits caused by weight loss, excessive exercise, or eating disorders. Women with hypothalamic amenorrhea have low leptin levels and do not express the normal diurnal leptin rhythm (19, 22, 24, 36–40).

Leptin therapy normalizes reproductive hormone levels (2) and restores cycles in women with functional amenorrhea (39, 40). Specifically, leptin increases luteinizing hormone (LH) levels and pulse frequency, ovarian volume, serum estradiol, and numbers of dominant follicles (16, 22, 38–41). Leptin's therapeutic benefit has also been shown in studies of a leptin-deficient prepubertal child (42) and of adult men (43).

Leptin's role in reproduction has also been modeled in lower mammals. Fasting that lowers serum leptin also reduces pulses of LH in rodents or non-human primates (10, 44–48). Leptin antiserum administered into the ventricular system of fed rats disrupts cyclicity and LH secretion (49). Conversely, leptin treatment increases serum prolactin and LH pulse frequency and amplitude in fasted rats (50, 51).

In vitro, leptin treatment of pituitary cells from fasted rats restores LH stores depleted by food deprivation (52). Similarly leptin injections reverse the loss of reproductive function, decrease LH levels, and prolong estrous cycles in mice that are food-deprived for 48 h (2). Exogenous leptin given to leptin-deficient mice also restores fertility (27, 53, 54). Most recently, studies in non-human primates by Sarmento-Cabral et al. have reported that leptin stimulates growth hormone, prolactin, adrenocorticotropin and follicle-stimulating hormone (FSH) secretion from monolayer pituitary cultures derived from two groups of female monkeys (55).

The observation that a threshold level of fat (and, thus, leptin signaling) is required to permit puberty indicates that the leptin signal is vital for the timing of puberty. In fact, early studies showed that leptin accelerates puberty (1, 53, 56), suggesting that it might be a metabolic trigger, although this was disputed by studies that found no correlation between prepubertal serum leptin levels and the timing of puberty in normal rodents (57–59) or primates (60–63). Furthermore, the rise in leptin during development [i.e., during the second trimester in the human fetus (64) or postnatally in rodents (58, 59, 65, 66)] appears to be too early for it to have direct impact as the trigger for puberty (6, 7, 59), although evidence indicates that leptin does play a permissive role in puberty (67).

The Role of Distinct Leptin-Target Cells throughout the Reproductive Axis

Leptin receptors can be found in cells throughout the HPG axis, and much research over the past two decades has focused on the relative importance of each set of target cells. The preponderance of evidence points to target cells in the hypothalamus as being most critical for mediating leptin signaling for fertility. However, the identity of the target cells has been a subject for investigation. Pioneering studies by McMinn et al. (8). reported that loss of LEPR in 50–75% of hypothalamic neurons caused obesity and glucose intolerance, but fertility and cold tolerance remained normal. This suggests a division of labor in the neurons responsive to leptin, and that LEPR deficiency must be seen in all neurons for the full set of deficiencies.

This presentation will discuss evidence for different groups of LEPR-target cells and build the case for including the pituitary gonadotrope. In fact, we will propose that leptin sets up an active partnership between leptin-responsive neurons in the hypothalamus and leptin-responsive gonadotropes in the anterior pituitary. In the later sections focused on the hypotheses, we will propose pathways that may be activated by leptin to permit reproduction. First, we will discuss evidence for a role for each of these leptin-target cells as responders to leptin's permissive actions.

The Case for the Importance of Neuronal Target Cells to Reproduction

Cre-*loxP* deletion of both alleles of the LEPR gene specifically in all neurons resulted in deletion mutant mice that were infertile (8). This important finding supported the original hypothesis that states that the major target cells for leptin's permissive effects on reproduction were neurons. Because GnRH neurons do not have LEPRs, a number of studies were then initiated to identify leptin-responsive neuronal pathways that regulate

GnRH (4, 23, 27, 30, 57, 68–71) and report evidence for leptin interactions with these neurons (2, 4, 14, 31, 72–85). The relative importance of these neuronal pathways was then strengthened by evidence from two laboratories showing that restoration of LEPR in the neurons of LEPR-null mice partially or completely restored fertility (50, 82, 85). Collectively, this led to the view that other leptin-target cells, such as gonadotropes were considered secondary or redundant responders to leptin's metabolic signals (50, 82, 85).

The Case for the Importance of Pituitary Gonadotrope LEPR-Target Cells

Gonadotropes reside within the anterior pituitary, synthesize, store, and secrete LH and FSH in a strict temporal order during the estrous cycle, and are stimulated by GnRH. Evidence supporting gonadotropes as leptin-target cells initially came from studies showing that they express functional LEPR (33, 86–93), and that leptin- or LEPR-deficient mice have reduced numbers of gonadotropes (6, 7, 91, 94). Cytophysiological studies showed that leptin modulates the expression and/or secretion of gonadotropins (27, 30, 33, 95–100). Fasting concomitantly reduced levels of serum leptin and numbers of gonadotropes defined by LH stores or GnRH-binding sites (52). Stores of LH were recovered following a 1-h treatment *in vitro* with leptin, which provides supporting evidence for direct interactions of leptin with pituitary gonadotropes (52). Further evidence stems from our report that pituitary LEPR expression varies with the stage of the estrous cycle with the highest expression before the LH surge (33).

In spite of the evidence for leptin interaction with gonadotropes, questions still remained about their importance as metabolic sensors of leptin signals. A recent study tested the role of LEPR in gonadotrope functions in a recent study that used Cre-*LoxP* technology with a genetically engineered line of mice ubiquitously deficient in LEPR (101). In this study, the recombination event restored LEPR selectively in pituitary gonadotropin releasing hormone receptor (GnRHR) target cells and FSH levels were elevated, although fertility was not restored (101). However, lack of fertility may have been secondary to the fact that the hypothalamic neuronal target cells remained LEPR-null and the mice remained morbidly obese. The GnRH pulse signal, which is vital to the pituitary gonadotrope was still lacking (101).

Thus, restoration of leptin signaling to gonadotropes will not rescue leptin's permissive effects on fertility in a LEPR-null mouse. However, evidence does indicate that gonadotrope LEPR plays a significant role in optimizing fertility. Our studies ablated the signaling domain of LEPR (encoded by exon 17) in gonadotropes *via* Cre-*LoxP* technology and reported a significant impairment of fertility in females (33). Specifically, there was a reduction in the levels of pituitary GnRHR proteins and activin mRNA (in females). Local activin and its downstream pathways are believed to be vital for the synthesis of FSH (102–104). Analysis of fertility showed significant delays in the time to first litter, abnormal estrous cycles, and lower numbers of pups/litter in breeding cages with deletion mutant dams. Gonadotrope LEPR deletion mutant males showed lower GnRHR proteins, but their fertility was unaffected. Thus, loss of the signaling domain of LEPR in gonadotropes appears to cause subfertility selectively in females.

Ablation of All Isoforms of LEPR in Gonadotropes May Result in Complete Infertility

To strengthen the case for gonadotropes as important LEPR-target cells, we recently produced a more severe ablation of LEPR selectively in LH gonadotropes with methods described in previous studies (33). All animals were handled and cared for under an animal use protocol that was reviewed and approved annually by the UAMS Animal Use and Care Committee.

We used a different floxed line of mice in which *Lepr exon 1* is flanked by LoxP, and Cre-recombinase is driven by the bovine *Lh-beta* promoter. The breeding strategy to produce this line is described in more detail in previous studies in which these Cre-bearing mice were also used (33). The resulting Cre-recombinase ablation removes the region encoding the signal peptide and prevents the translation of all isoforms of LEPR (105). We reasoned that ablation of the signal peptide would have a deleterious effect on the LEPR-receptor population as seen in our previous studies of mice in which *Lepr exon 1* was ablated in somatotropes (106).

The method is as follows. We produced deletion mutants in three breeding cages with F2-generation *Lh-cre* positive females bearing one allele of floxed *Lepr exon 1* (heterozygotes) and Cre-negative males bearing two alleles of floxed *Lepr exon 1*. Females always passed down the Cre-recombinase because the *Lh-cre* is known to be expressed in the testes (33). All mice were of the same FVB strain and at least 3 months of age when they entered the breeding cages. The reproductive competence of the homozygous and heterozygous mutant females was compared with that of females in cages containing control mice of the same strain background (FVB.129P), which had delivered during the same time. As in our previous studies (33), we tested the period that normally produced 3–4 litters in the wild type FVB.129P strain (65–85 days). The time was extended, however, for cages with mutants that produced few (or no) pups.

The three breeding cages of F2-generation heterozygous females produced an average of eight pups/litter, with a normal time-span between litters of 21–22 days. Thus, their productivity was not different from that in the FVB.129P wild type females. This group of females produced the test population of 11 F3-generation mutant homozygous females (bearing *Lh-cre* and two alleles of floxed *Lepr exon 1*) and 2 F3-generation mutant heterozygous females. This test population came from five different F2-generation litters.

Data on the breeding study are summarized in **Table 1**. Three of the five F3-generation homozygous deletion mutant females showed total infertility, failing to produce pups after 240–281 days of breeding with a proven Cre-negative male. Two homozygous mutant females were fertile although they produced litters slowly (every 30–45 days) compared with FVB.129P females, which produce at 21–22 day intervals. One of these females produced 6 litters and 42 pups in 199 days, with an average litter size that was nearly normal. The other mutant female produced only 4 litters

TABLE 1 | Deleting all isoforms of leptin receptor (LEPR) in gonadotropes causes infertility.

Female[a], date of birth	Genotype *Lh-cre*	# Days with male	# Litters	Average # pups/litter	Total # pups
F0, 8/19/2015	*Lepr*$^{exon\ 1\ f/wt}$ Heterozygous	65	0	0	0
F6, 8/19/2015	*Lepr*$^{exon\ 1\ f/wt}$ Heterozygous	65	0	0	0
F1, 5/21/2015	*Lepr*$^{exon\ 1\ f/f}$ Homozygous	199	6	7	42
F0, 6/11/2015	*Lepr*$^{exon\ 1\ f/f}$ Homozygous	199	4	5	19
F6, 3/21/2015	*Lepr*$^{exon\ 1\ f/f}$ Homozygous	240	0	0	0
F1, 3/21/2015	*Lepr*$^{exon\ 1\ f/f}$ Homozygous	240	0	0	0
F0, 1/20/2015	*Lepr*$^{exon\ 1\ f/f}$ Homozygous	281	0	0	0

[a]*F3-generation females bearing Lh-cre and one or two floxed alleles of Lepr*$^{exon\ 1}$ *were bred with males bearing only floxed alleles of Lepr*$^{exon\ 1}$*. These seven females came from five different F2-generation litters and three different breeding cages. 71% of these females showed infertility.*

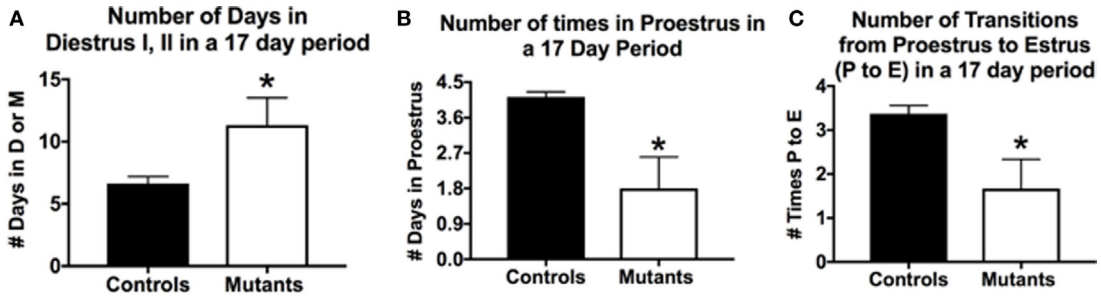

FIGURE 1 | Deletion of *Lepr exon 1* in gonadotropes leads to severe reproductive deficiencies. Deletion mutant mice bearing two floxed alleles of *Lepr* (*Lepr-exon 1*$^{loxP/loxP}$ obtained from Dr. Jeffery Friedman) were bred to female mice bearing the *Cre-recombinase* gene driven by the *bLh*-β promoter (*bLh*β*-cre*) developed by Dr. Sally Camper (108). The *Cre-recombinase* was passed through the female line, because it was reported to be expressed in the testes (108), and all breeding was tested only in females. The resulting offspring carried two floxed *Lepr* alleles and were either positive (one allele) or negative for *Cre-recombinase* in gonadotropes. Breeding studies done as described previously (33) produced seven female homozygous mutants that were evaluated for cyclicity over a 17-day period. **(A)** On average, females spent more days in diestrus I (metestrus) or II in the 17-day period days (compared with control females). **(B)** Mutants spent fewer days in proestrus and **(C)** In the 17-day test period, there were only 1.6 proestrous to estrus transitions in the mutants. Stars over mutant bars indicate significantly different values as compared to controls by the Student's *t*-test.

and 19 pups in 199 days, and one of the litters did not survive. **Table 1** also shows that the two test F3-generation heterozygous females also showed no evidence of pregnancy with a proven Cre-negative male. Therefore, breeding with these females was stopped after 65 days.

The breeding generated six F3-generation homozygous mutant females, which could be used in parallel studies of cyclicity, comparing their cycles with those of eight littermate controls bearing no *Cre-recombinase* (**Figure 1**). We analyzed vaginal smears from these animals daily over a 17-day period with methods described in previous studies (33, 107). Two of the mutant females remained in diestrus during the entire 17-day test period; the remaining 4 showed some degree of cyclicity. The average number of days in diestrus I or II for all mutants was significantly higher compared with controls ($p = 0.03$; Student's *t*-test; **Figure 1A**).

In a 17-day test period, one would expect to see 4–5 proestrous days (assuming a 4- to 5-day cycle). Mutants exhibited on average <2 days in proestrus in this test period, which was significantly lower than control values of 4.1/17 days ($p < 0.03$, Student's *t*-test; **Figure 1B**). We also evaluated the number of times mice exhibited a proestrus to estrus transition (P–E), which would indicate the completion of a cycle and readiness for copulation during early estrous. **Figure 1C** shows that controls had 3.4 P–E transitions in a 17-day test period; however, mutants had less than half of these P–E transitions (1.6), which was significantly lower than controls ($p = 0.01$, Student's *t*-test). Thus, whereas four of the six mutant females cycled, the opportunities for a pregnancy in the 17-day period (seen by the P–E transition) were significantly reduced, which correlates with the low number of litters in the breeding cages of the two subfertile females reported in **Table 1**.

In conclusion, these data showed a more severe infertility phenotype in mice lacking all isoforms of LEPR in gonadotropes. This resulted in unreliable breeding or infertility, which supports our assertions that gonadotrope LEPR is important to the HPG axis. Analysis of serum levels of gonadotropins and other pituitary and ovarian hormones in ongoing studies will identify the full mechanism behind the loss of fertility in these gonadotrope-*Lepr exon 1* deletion mutants. In spite of the hypothalamic *Lepr*

gene remaining intact, gonadotropes having all LEPR isoforms deleted were unable to function normally in most (71%) of the F3-generation female mice tested and preformed sub-optimally in the remaining two mice. The infertile group also included a subset of F3-generation heterozygous mice (which lacked only one allele of *Lepr exon 1* in gonadotropes). Thus, the phenotype could become more severe with the next generation and we may be limited to F2-generation litters for future analyses.

To summarize, in this introductory section, we presented evidence that leptin is vital to the reproductive system. We also presented evidence suggesting that gonadotrope LEPR may be vital for optimal fertility. This evidence sets the stage for our two hypotheses in which we integrate findings from studies of neuronal and pituitary leptin-target cells. The first hypothesis will focus on leptin's regulation of fertility *via* the gonadotrope, specifically regarding how the cyclic production of GnRHR proteins might provide a critical checkpoint for metabolic signaling. The second hypothesis will integrate the findings in the literature with those from our studies. In this hypothesis, we propose that leptin's metabolic signaling involves multiple molecular gateways and checkpoints that can permit, delay, or stop reproduction.

HYPOTHESIS 1: CYCLIC REGULATION OF PITUITARY GnRHR LEVELS SETS UP A TARGET METABOLIC CHECKPOINT FOR CONTROL OF THE REPRODUCTIVE AXIS

Pituitary gonadotropes in females are a fascinating subset of pituitary cells that must be remodeled every cycle to support a preovulatory LH surge and a postovulatory rise in FSH (109). Depending on the gonadotrope gene marker being detected and the stage of the cycle, these heterogeneous cells represent at least 15% of the pituitary population. Our studies over the past 42 years have shown that precise accounting of the gonadotrope population is complicated by their dynamic remodeling, such that they can be difficult to identify or detect when a marker gene product has been downregulated or secreted. At least two gonadotrope markers must be detected to identify the entire population, especially during periods of low gonadotropin storage (estrus or metestrus).

Identification of the structural and molecular mechanisms behind the remodeling of gonadotropes and the regulators that drive these changes has been the subject of decades of investigative studies (109–116). **Figure 2** shows a cartoon depicting some of the molecular and cytological changes that occur during the remodeling process that produces an actively secreting gonadotrope. Included in this population would presumably be any progenitor cell that contributes to the secreting, GnRH-responsive gonadotrope population, such as the somatogonadotrope (117).

Thanks to cytoskeletal remodeling, gonadotropes become more structurally elongated and stellate during diestrus and proestrus before the LH surge (109, 114, 118, 119), sending processes to blood vessels to facilitate surge-level secretion. Because they have actively secreted their stores during the LH surge and FSH rise, gonadotropin storage is significantly reduced on the morning of estrus (after the surge), reducing numbers of detectable gonadotropes (109, 110, 114, 120). Gonadotropes also increase their content of LEPRs during proestrus (33).

Figure 2 shows that, early in the cycle (metestrus), the cells destined to support the estrous rise in FSH and proestrous LH surge begin to produce gonadotropin and *Gnrhr* mRNA, which is followed by translation of these proteins during diestrus (121–123). The transcription of *Gnrhr* mRNA is under the control of GnRH

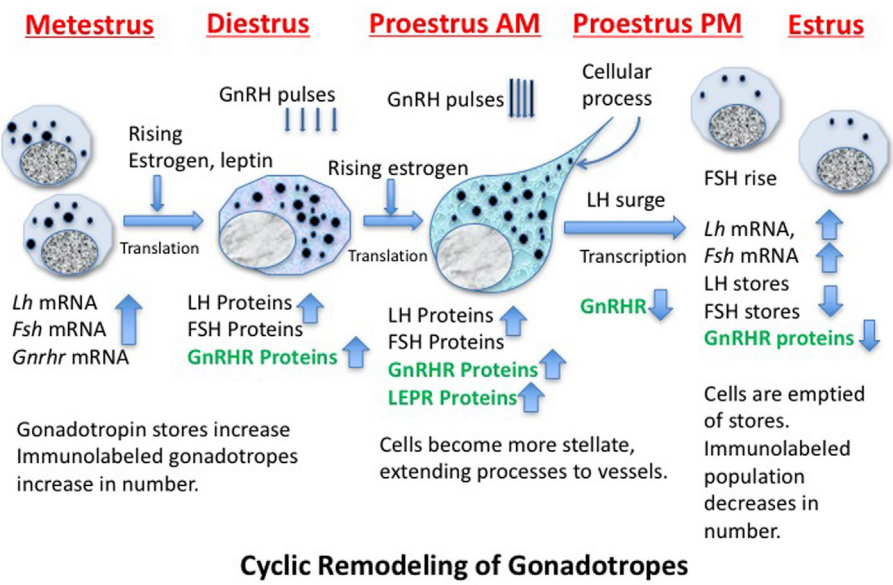

FIGURE 2 | Cartoon showing stages of remodeling in the population of gonadotropes destined to support the luteinizing hormone (LH) surge secretory activity during proestrous and early estrus. The activity is driven by GnRH pulses, which increase in frequency and amplitude before the LH surge. The timing of the production of critical gene products is noted and those in green are known to be leptin targets.

pulses and rising levels of estrogen from the ovarian follicles (which had been stimulated by FSH early in estrus).More rapid pulses of GnRH in proestrus will facilitate the actual LH surge.

A critical step in this gonadotrope remodeling is the increase in GnRHR proteins. The changes in GnRHR depicted in **Figure 2** were first reported by early radioreceptor assays, which detected the timing of the cyclic increase in GnRHR (121, 123) in rodents. The reports showed that gonadotropes undergo an increase in numbers of GnRHR early in diestrus I (metestrus) to reach a peak in late diestrus or on the morning of proestrus. Just before the LH surge, GnRHR numbers fall precipitously to remain low throughout the remaining stages of the cycle. This renders the gonadotrope population relatively quiescent during the postovulatory period of the cycle. There are LH and FSH pulses during this quiescent period, but they are of lower frequency and amplitude than those seen during mid-cycle surge activity.

The complex mechanisms controlling the increase in GnRHR clearly precede the remodeling needed to increase stores of gonadotropins needed for the surge activity, and it is not surprising that this initial process is regulated by pulses of GnRH itself (109). We have observed that the increase in GnRHR reflects an increase in the percentages of living gonadotropes that bind a biotinylated analog of GnRH (111). Collectively, these changes culminate in an increased population of responsive gonadotropes, which could then respond in synchrony to the higher GnRH pulse amplitude and frequency seen at mid-cycle.

The foregoing review of gonadotrope remodeling sets the stage for Hypothesis 1, which states that the cyclic changes in pituitary GnRHR expression create a mechanism by which the gonadotropes are activated only when environmental conditions are optimal. This mechanism would constitute an ideal checkpoint for metabolic regulation by leptin.

This hypothesis originated when we discovered that GnRHR proteins and activin subunit mRNA levels were reduced in pituitaries lacking LEPRs in gonadotropes. Our studies of mice with LEPR ablated in gonadotropes discovered that both of these gene products were reduced (33). More recent studies determined if GnRHR and activin were direct targets of leptin. We assayed mRNA extracts from pituitary pieces that were stimulated for 3 h with 10 nM leptin. Methods describing our approach to leptin stimulation are detailed in previous studies (33, 52, 124, 125). Pituitary pieces or cells in 24-h culture are exposed to leptin for 3 h at 37°C, and then extracts of proteins and mRNA are produced, as described (126). Methods describing our RT-PCR assays are found in the legend to **Figure 3** and in Ref. (33). **Figure 3A** shows leptin stimulation of pituitary activin (but not inhibin) subunit mRNA levels (see reference (33) for information on primer sets). Similarly, leptin stimulation for 3 h does not affect *Gnrhr* mRNA levels (**Figure 3B**). This correlates well with our previous study showing that lack of LEPR in gonadotropes does not affect *Gnrhr* mRNA (33).

Our *in vitro* studies also show that leptin stimulates GnRHR proteins in a dose-dependent manner, with 10 nM resulting in the highest levels of GnRHR proteins or percentages of cells that bind biotinylated analogs of GnRH (127). This study was recently expanded to determine if the gonadotropes were the target cells. We used our established fluorescence-activated cell sorting-purification protocol (126) to separate gonadotropes by their eGFP fluorescence (with mice bearing *Lh*-cre and floxed tdTomato-eGFP). Freshly purified gonadotrope fractions (66,000 cells) were split. Half of the population was stimulated for 3 h with 10 nM leptin, and the remaining half received vehicle. Immunolabeling showed that the eGFP fractions were 98% gonadotropes. EIAs showed that the fraction contained most of the GnRHR, LH, and FSH, with other hormones assayed in the non-eGFP fraction. Protein extracts from the leptin- or vehicle-treated gonadotropes were assayed for GnRHR as described (33). **Figure 3C** shows that the leptin stimulation resulted in an increase in GnRHR protein

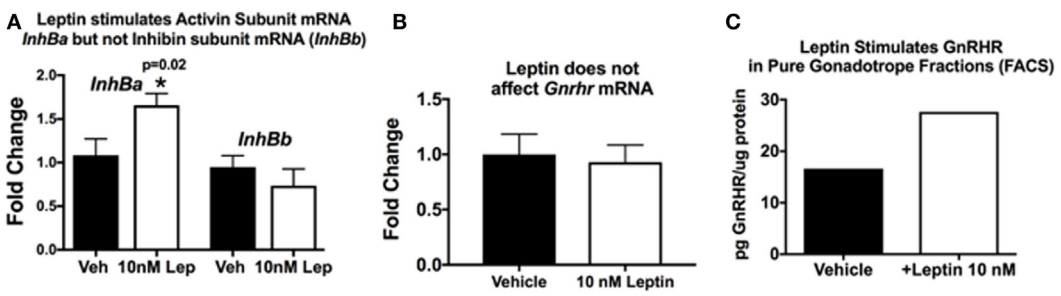

FIGURE 3 | Pituitary responsiveness to leptin: activin and gonadotropin releasing hormone receptor (GnRHR). **(A,B)** Individual pituitaries were placed in 400 µL DMEM with protease inhibitor cocktail and ITS supplement (Sigma), with or without 10 nM recombinant mouse leptin (Sigma). The pituitaries were triturated two times with a 26 G needle and syringe. The pieces incubated for 3 h at 37°C, followed by mRNA extraction as previously described [(33)]. cDNAs were assayed by real-time PCR for the levels of activin/inhibin subunit **(A)** and *Gnrhr* **(B)** mRNA levels. Leptin stimulates an increase in pituitary activin subunit mRNA (*InhBa*) level and does not have an effect on inhibin (*InhBb*) or *Gnrhr* mRNA levels. **(C)** In a separate set of experiments, we used fluorescence-activated cell sorting (FACS) to purify gonadotropes from control females bearing *Lh-cre* and floxed alleles of *tdTomato-eGFP* as described previously (126). The Cre-recombinase ablates the *tdTomato* leaving *eGFP* to be expressed in membranes of gonadotropes. Our analysis showed that the eGFP fraction was 98–99% gonadotropes by immunolabeling for LH and follicle-stimulating hormone (FSH). In this experiment, a female gonadotrope fraction containing 66,830 cells was split and half of the cells were stimulated for 3 h with 10 nM leptin. Protein extracts of these cells were assayed by EIA for GnRHR (127). **(C)** GnRHR proteins were higher in the gonadotropes stimulated with leptin (from 16.67 µg GnRHR/mg protein to 27.6 µg/mg protein). The amount of protein in the extracts did not allow for multiple samples and, therefore, there are no error bars. More details about the GnRHR assays are reported in Akhter et al., (33). Details about the FACS protocol can be found in Odle et al. (126, 127).

levels in this population of pure gonadotropes, which agrees with recently published evidence (127). This is the first evidence for leptin's direct stimulation of gonadotropes.

Thus, collectively, our studies of gonadotrope-*Lepr*-null mice and *in vitro* responses to leptin highlight the importance of gonadotrope leptin-target cells to the HPG axis and support our hypothesis that gonadotrope GnRHR represents a metabolic checkpoint. However, we have expanded this hypothesis to include a novel leptin-mediated post-transcriptional pathway to control translation of the *Gnrhr* mRNA. This expansion is based on the following evidence: (1) GnRHR protein levels are reduced in gonadotrope *Lepr*-null mutants, but *Gnrhr* mRNA levels are unchanged (33); (2) leptin does not directly stimulate *Gnrhr* mRNA levels (**Figure 3B**), and (3) leptin directly stimulates GnRHR proteins in a population of purified gonadotropes (**Figure 3C**) or mixed pituitary cultures (127).

We propose that the levels of *Gnrhr* mRNA are normal in our gonadotrope-*Lepr* exon 17-null mutants likely because LEPR was not ablated in the hypothalamus, allowing GnRH secretion and the regulation of transcription of *Gnrhr*, *Lh*, and *Fsh* mRNA (33, 128, 129). LH and FSH stores are also normal in these mutant gonadotropes (33). However, the diestrous gonadotropes did not appear to secrete normally, as reported by low serum LH and FSH levels (33). Whereas we can explain the fact that *Gnrhr* mRNA is normal, the mechanism underlying leptin's permissive modulation of GnRHR protein synthesis is unknown. As a first hypothesis, we, therefore, propose that leptin may stimulate translation by alleviating repression of *Gnrhr* mRNA by mRNA regulatory proteins and/or miRNA.

miRNAs are small (~22 nucleotides), single-stranded RNAs that interact with target sequences within cellular mRNAs and exert translational repression. A significant role for leptin signaling in regulation of miRNA-mediated translational control has been observed in adipocytes and hepatic cells (130). In *ob/ob* mice, *miR-103* and *miR-107* levels are increased in the absence of leptin, contributing directly to insulin resistance (130). Furthermore, leptin signaling involves JAK–STAT pathways and precedent for pSTAT3-dependent downregulation of target miRNAs has been reported in breast cancer (131).

For our study, we initially wanted to determine which miRNAs might target the *Gnrhr* mRNA 3′-untranslated region (UTR). Our *in silico* analysis [Targetscan 7.1 (132)] revealed that the *Gnrhr* mRNA 3′-UTR (ENSMUST00000031172.8) contained 16 potential miRNA binding sites, including two that are also conserved in humans: *miR-581/669d* and *miR-3061-3p*. We began assays to detect differences, if any, in expression of candidate miRNAs. RT-PCR assays of whole pituitaries from control and gonadotrope *Lepr exon 17*–null diestrous females ($n = 4$–5 mice/group) determined that *miR-581/669d* was increased in the absence of leptin signals to gonadotropes, consistent with increased repression of *Gnrhr* mRNA translation in the mutants (**Figure 4B**). Detailed methods of our RT-PCR assays for miRNA are in the Figure legend of **Figure 4**. The specific role of *miR-581/669d* and the remaining 14 candidate miRNAs are currently being investigated. Complementary to this candidate approach, ongoing miRNA sequence analyses will provide an unbiased global analysis of pituitary miRNA expression related to loss of LEPR.

We also identified three consensus binding elements for the translational regulatory protein Musashi (MSI) (MBEs) in the 3′-UTR of murine *Gnrhr* mRNA (**Figure 4A**). The two vertebrate members of the MSI family, Musashi1 (MSI1) and

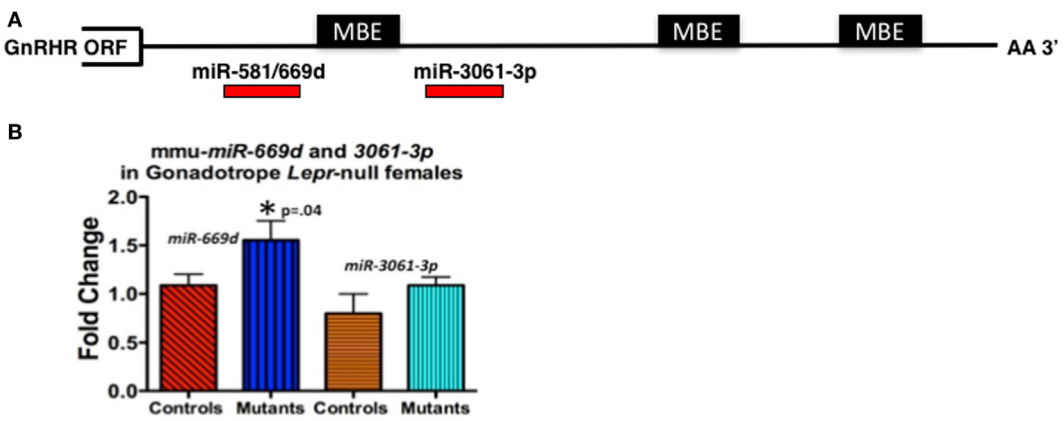

FIGURE 4 | **(A)** Candidate regulatory elements within the murine *Gnrhr* 3′-untranslated region (UTR) include MBEs and at least two miRNA target sites. *In silico* analyses (TargetScan 7.1, ENSMUST00000031172.8) indicate three consensus MSI binding sites or elements (MBEs) and 16 miRNAs target sites (only 2 shown) within the murine *GnRHR* 3′UTR (182 nucleotides). The relative positions of the three MBEs and two miRNAs (*miR-581/669d* and *miR-3061-3p*) are shown schematically. The MBE closest to the open-reading frame (ORF) is flanked by sequences encoding *miR-581/669d* and *miR-3061-3p*. **(B)** *miR-581/669d* and *miR-3061-3p* levels in *Lepr*-null gonadotropes. Total RNA enriched for miRNA was isolated from whole pituitaries of control and gonadotrope-*LeprEx17*-null females using the Maxwell miRNA tissue kit (Promega, AS1470). We used the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, 4366596) with TaqMan small RNA assays to amplify our miRNAs of interest. We performed qRT-PCR using the TaqMan small RNA qPCR primers with the TaqMan Universal PCR Master Mix II (Applied Biosystems, 4440038) in triplicate. We used the protocol provided with the master mix and performed the experiment using a QuantStudio 12k Flex (Applied Biosystems) and the protocol provided with the master mix. Real-time PCR showed *miR-581/669d* elevated in the absence of leptin signaling, which is consistent with a role for this miRNA as a repressor of *Gnrhr* mRNA translation. *Significantly different from controls by Student's *t*-test.

Musashi2 (MSI2) are highly related, sequence-specific RNA binding proteins. MSI typically functions as a repressor of target mRNA translation and is specifically implicated in promoting stem/progenitor cell self-renewal where it functions to oppose translation of mRNAs encoding pro-differentiation factors and inhibitors of cell cycle progression under both physiological and pathological conditions (133). While the mouse pituitary is reported to express *Msi* mRNA (134), the function of MSI in the pituitary has not been determined. Ongoing studies show promise as they demonstrate binding by MSI to the *Gnrhr* mRNA 3′UTR and MSI repression of reporter expression driven by *Gnrhr* 3′UTR. We also have evidence that leptin reduces *Msi* mRNA (127).

Therefore, at this point, the evidence points to the hypothesis that leptin may play an important role in de-repressing *Gnrhr* mRNA during the critical period of cyclic upregulation of these vital receptors. We propose that MSI1 as well as at least one miRNA may be candidate *Gnrhr* mRNA regulators. Specifically, we propose that if nutrition and energy stores are normal, the mid-cycle leptin surge opposes MSI1-dependent mRNA repression, allowing the continued translation of *Gnrhr* during diestrus to reach optimal levels needed for a full LH surge. We propose that our mice that lack all isoforms of LEPR in gonadotropes (**Table 1**; **Figure 1**) may have not been able to produce sufficient GnRHR to mount an effective LH surge. Also, based on previous data (33) and **Figure 3A**, we propose that activin levels might be reduced, which would compromise FSH secretion and the development of the follicles. This first hypothesis will now be integrated into our second hypothesis about the overall mechanisms by which leptin signals metabolic information to the HPG axis.

HYPOTHESIS 2: MULTIPLE CHECKPOINTS ARE REQUIRED FOR METABOLIC SIGNALING THAT REGULATES THE REPRODUCTIVE AXIS

As stated in the introduction, early studies of leptin regulation of reproduction have emphasized the hypothalamus as a primary target site for leptin and suggested that other sites might be less important or even redundant. The pioneering studies by McMinn et al. were the first to note the diversity in the LEPR-responsive neurons and the fact that all must be receptor deficient to cause infertility (8). Two studies selectively restored LEPR in the hypothalamus. The first of these studies reported that obesity, diabetes, and infertility in *Lepr*-null db/db mice could be rescued completely by re-introducing neuron-specific LEPR-B transgenes (82) to restore LEPR function selectively in the neurons. One of the drivers that introduced LEPR into the LEPR-null neurons was Synapsin (SYN-1). The selectivity of the SYN-1 driver was shown by expression in the brain; however, weak expression was also reported in the pituitary. This pituitary expression of SYN-1 was recently confirmed in Lβ-T2 gonadotropes and pituitary explants (135). Thus, based on the most recent evidence, we hypothesize that the Syn-1 driver may have introduced LEPR-B transgenes into both neuronal and anterior pituitary cells. Specifically, the expression of Synapsin in Lβ-T2 gonadotropes suggests that gonadotropes or their progenitor cells would have been among the restored leptin-target cells. Thus, fertility in these mice may have been restored by leptin-target neurons regulating GnRH and by leptin-target gonadotropes expressing GnRHR.

The second study by Donato et al. used Flp/FRT recombination approaches and a strain of mice carrying a neomycin cassette flanked by FRT sites targeted to the *Lepr* locus (50), which rendered the mutant mice globally LEPR-null. They selectively restored LEPR in the ventral premammillary (PMV) neurons of these mice by injecting an adeno-associated virus vector expressing Flp recombinase. The virus-restored mutant female mice showed evidence of pubertal development and cyclicity. In addition, five of the six females became pregnant although fertility was not optimal as four of these females did not carry the pups to term and the pups from the one female who delivered did not survive and died with no milk spots evident. These responses may also be due to the fact that the females remained morbidly obese. Thus, whereas the restoration of LEPR in the PMV clearly and selectively confirmed the importance of these neurons in the regulation of GnRH and the production of young, it appears that other leptin-target cells are vital to ensure that the progeny survive.

Based on our recent studies of *Lepr*-null gonadotropes (33), we hypothesize that the LEPR-null pituitaries in the study by Donato et al. expressed sufficient GnRHR on gonadotropes to go through puberty, cycle, and become pregnant. Because GnRH is an important stimulator of *Gnrhr* mRNA transcription [(128, 129) and **Figure 2**], restoration of LEPR in the PMV may have resulted in sufficient GnRH secretion to induce functional levels of GnRHR in gonadotropes. The observation that none of the litters survived, however, indicates that extra-PMV, pituitary, and ovarian LEPR-target cells are needed to support full reproductive competence. Also, the morbid obesity is a confounding factor. Detecting levels of gonadotropins, growth hormone, prolactin, estrogen, and progesterone may determine elements of the HPG axis that might have been most affected.

The importance of the working partnership between the hypothalamus and the pituitary is further elucidated in a recent study in which Cre-*LoxP* technology was used to restore only pituitary gonadotrope LEPR (101). As stated in the introduction, fertility was not restored in these animals presumably because LEPR-target neurons stimulating GnRH secretion remained deficient and unable to induce functional GnRHR signaling in gonadotropes (128, 129). This study provides another important clue to a role for leptin in gonadotropes, as they reported that FSH was elevated in this gonadotrope-specific LEPR model (101). As reported in our previous study (33), female mice bearing *Lepr*-null gonadotropes have reduced activin mRNA in the absence of leptin signals. We also reported reduced *Fsh* mRNA in these mutant animals. As activin stimulates FSH synthesis, we suggest that when LEPR was restored in pituitary gonadotropes, activin production may have been rescued (33). In the present report, we add evidence that leptin directly stimulates levels of activin mRNA (**Figure 3A**), which further supports this hypothesis. Also, recent studies of leptin actions in monkey pituitary cells

show that 4 h of leptin stimulation *in vitro* results in elevated FSH secretion (55). It is interesting to note that leptin did not stimulate LH secretion *in vitro* in these female monkeys, which were reported to be of mixed cycles. We have shown that LEPRs in LH cells are maximal during the preovulatory period (33), and perhaps leptin's effects on LH secretion are dependent on the stage of the menstrual cycle.

Based on these findings and the studies described above, we hypothesize that leptin's role in the permissive regulation of the reproductive cycle depends on timed events that involve multiple interactive target cells in the HPG axis. **Figure 5** proposes a set of integrating pathways by which changing energy stores could allow leptin to signal metabolic information and permit, delay, or stop the next cycle. As shown in this figure, nutritional and fat level sufficiency will result in optimal leptin levels that in turn will signal target cells in the hypothalamus and pituitary gonadotropes. We hypothesize that leptin acts on hypothalamic and pituitary target cells to signal changing energy stores. The pathway designated in green shows how leptin may activate gonadotropes directly to effect transcription of activin subunits

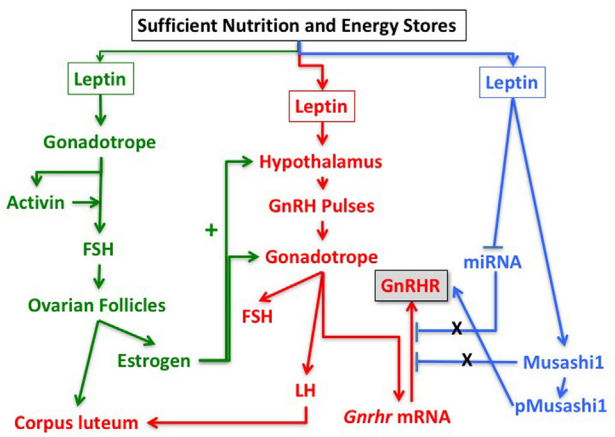

FIGURE 5 | Pathways in the reproductive axis and the critical leptin targets that must be activated for reproductive competence. The pathway in green is proposed based on the evidence that leptin stimulates activin mRNA (**Figure 2**) and that activin mRNA is reduced in leptin receptor (LEPR)-null gonadotropes (33). It is also based on evidence that restoration of LEPR in LEPR-null gonadotropes results in elevated follicle-stimulating hormone (FSH) (101) as activin is important for FSH synthesis. We propose that this well-established FSH-driven pathway provides the stimulation to the follicle that results in a rise in estradiol needed early in the cycle to promote responses from GnRH neurons and gonadotropes. The red pathway is focused on the well-established circuitry in which leptin regulates LEPR-target neurons in the hypothalamus to stimulate GnRH neurons. It is based on evidence from key studies showing that restoration of LEPR in the PMV improves fertility (50, 82). The pathway shows that GnRH pulses are needed for transcription of *Gnrhr* mRNA as well as transcription and translation of LH and FSH. The blue pathway is based on our recent studies showing that mice lacking LEPR in gonadotropes are subfertile (33) or infertile (**Figure 1**; **Table 1**) and have significantly reduced gonadotropin releasing hormone receptor (GnRHR) proteins, but not mRNA levels (33). We hypothesize that leptin plays a direct post-transcriptional role in de-repressing mRNA translation by inhibiting miRNAs and/or MSI1, which then allows activation of *Gnrhr* mRNA translation.

to raise local activin levels and stimulate synthesis of FSH. This would support the early estrous rise in FSH, which stimulates ovarian follicles to develop and secrete estradiol, which then exerts positive feedback on the hypothalamus and the pituitary. Estrogen-sensitive neuronal pathways stimulate GnRH neurons to increase secretion and pulse frequency. The pathway in red highlights the important role of leptin in stimulating the LEPR-sensitive neurons in the hypothalamus to ultimately regulate GnRH neurons. The red pathway also shows that GnRH pulses stimulate *Gnrhr* mRNA, as well as LH and FSH secretion. Most of the elements in the green and red pathways are well established, although the role of leptin in stimulating activin in the green pathway is relatively novel.

What is most novel is the hypothetical blue pathway. Based on our studies of gonadotrope *Lepr*-null mice, we propose that leptin sends a third signal directly to gonadotropes that de-represses the translation of *Gnrhr* mRNA. The timing of this gateway signal could be during the metestrous to diestrous increase in GnRHR proteins. Our studies of females that lack all isoforms of gonadotrope LEPR (**Table 1**; **Figure 1**) strongly emphasize the importance of this blue pathway for optimal reproductive success. As discussed for Hypothesis 1, we propose that *Gnrhr* mRNA translation may normally be inhibited by MSI1 and possibly miRNAs. Consequently, leptin signaling acts to de-repress the *Gnrhr* mRNA by blocking the inhibitory action of MSI1 and/or miRNA repressive activity. This ultimately would activate translation of *Gnrhr* mRNA and provide the full complement of receptors needed for a fully responsive gonadotrope population ready for the LH surge and estrous rise in FSH.

CONCLUSION

Our two hypotheses reconcile and integrate findings from several studies of leptin-target cells. First, with the use of the Syn-1-driver, de Luca et al. (82) restored LEPR in both the brain and pituitary of *db/db* mice, which allowed multiple target cells seen in **Figure 5** to function in partnership. Donato et al. (83) restored LEPR in the PMV of global LEPR-null mice, which stimulated GnRH to produce sufficient GnRHR and improve gonadotrope functions, although LEPR-target cells in the pituitary were still deficient and full reproduction (defined by the production of living pups) was not successful. As shown by Allen et al. (101) and Donato et al. (83), the system diagrammed in red and green pathways in **Figure 5** will function only if leptin signaling to the brain is normal and only if there are GnRH pulses to stimulate the gonadotropes to make *Gnrhr* mRNA. However, as shown by our studies [**Figure 1**; **Table 1**; Ref. (33)], there must also be leptin input to gonadotropes for optimal levels of GnRHR proteins as well as responses to GnRHR for successful reproduction. Without that input, gonadotrope *Lepr exon 1*-null females failed to reproduce or had impaired fertility (**Table 1**).

Thus, the collective findings from the selective ablation or restoration of LEPR have highlighted the importance of leptin and LEPR to regulate function of the reproductive axis. Most importantly, they show that leptin's permissive actions are operating in both the brain and the pituitary. These studies

have further identified important pituitary signaling molecules activated directly or indirectly by leptin. Our hypotheses are integrated into the model in **Figure 5** to indicate where each signal is needed and to highlight the fact that they act in partnership to optimize gonadotrope function. We also include a novel regulatory pathway that may involve control of MSI1 and/or miRNAs. Leptin regulation of these post-transcriptional pathways mediates the rapid de-repression and translation of *Gnrhr* mRNA, allowing for sufficient GnRHR to respond in synchrony and produce the LH surge. Subsequently, MSI1 and/or miRs would re-repress the *Gnrhr* mRNA late in the cycle, resulting in lower GnRHR levels and rendering the gonadotropes less responsive to GnRH. Continued studies are clearly needed to fully elucidate the targets and molecular pathways for leptin control of the HPG axis.

ETHICS STATEMENT

This study was carried out under the guidelines of the Department of Lab Animal Medicine and the protocols were approved by the UAMS Animal Use and Care Committee.

AUTHOR NOTE

These authors MM, AM and GC are all designated as Senior Authors on this manuscript as they supervised different elements of the studies that led to the manuscript.

AUTHOR CONTRIBUTIONS

AO performed the analysis of the estrous cycles in the gonadotrope LEPR-null females, all experiments involving leptin stimulation of gonadotropes and FACS separation of gonadotropes, all qPCR assays for mRNA and miRNAs. She also helped with the literature review and the development of the hypotheses. NA and MS performed the cytochemical labeling studies that are cited in this work showing leptin stimulation of GnRHR. MA-J helped with cyclicity studies and cytochemistry cited in the paper. HB, MC, and MM worked on studies of MSI binding that are cited in this paper. AM did the *in silico* analysis of the GnRHR 3′-UTR and, with MM, designed experiments to test MSI binding. GC set up and monitored all breeding studies and wrote the initial drafts of the manuscript. All authors helped with the development of the hypotheses and the editing. MM, AM and GC are Co-Senior authors on this study working as equal partners in the development of the hypotheses and monitoring the final writing and editing.

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Reactive Oxygen Species Link Gonadotropin-Releasing Hormone Receptor Signaling Cascades in the Gonadotrope

*Tomohiro Terasaka[1], Mary E. Adakama[1], Song Li[1,2], Taeshin Kim[1], Eri Terasaka[1], Danmei Li[1] and Mark A. Lawson[1]**

[1] Department of Reproductive Medicine, University of California, San Diego, La Jolla, CA, United States, [2] Neonatal Intensive Care Unit, Dongguan Eighth People's Hospital Dongguan City, Dongguan, China

Biological rhythms lie at the center of regulatory schemes that control many aspects of living systems. At the cellular level, meaningful responses to external stimuli depend on propagation and quenching of a signal to maintain vigilance for subsequent stimulation or changes that serve to shape and modulate the response. The hypothalamus–pituitary–gonad endocrine axis that controls reproductive development and function relies on control through rhythmic stimulation. Central to this axis is the pulsatile stimulation of the gonadotropes by hypothalamic neurons through episodic release of the neuropeptide gonadotropin-releasing hormone. Alterations in pulsatile stimulation of the gonadotropes result in differential synthesis and secretion of the gonadotropins LH and FSH and changes in the expression of their respective hormone subunit genes. The requirement to amplify signals arising from activation of the gonadotropin-releasing hormone (GnRH) receptor and to rapidly quench the resultant signal to preserve an adaptive response suggests the need for rapid activation and feedback control operating at the level of intracellular signaling. Emerging data suggest that reactive oxygen species (ROS) can fulfill this role in the GnRH receptor signaling through activation of MAP kinase signaling cascades, control of negative feedback, and participation in the secretory process. Results obtained in gonadotrope cell lines or other cell models indicate that ROS can participate in each of these regulatory cascades. We discuss the potential advantage of reactive oxygen signaling for modulating the gonadotrope response to GnRH stimulation and the potential mechanisms for this action. These observations suggest further targets of study for regulation in the gonadotrope.

Keywords: reactive oxygen species, gonadotropin-releasing hormone, pulsatility, DUSP1, gonadotropins, mitogen-activated protein kinase, ERK, pituitary, metabolism

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****Correspondence:***
Mark A. Lawson
mlawson@ucsd.edu

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Abbreviations: DAG, diacylglycerol; DPI, diphenyleneiodonium; DUOX, dual oxidase; DUSP, dual-specificity protein phosphatase; EGFR, epidermal growth factor receptor; FFA, free fatty acids; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; GSH, glutathione; HPG, hypothalamic–pituitary–gonad; IP_3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; MEKK, MAPK kinase; NAC, N-acetyl cysteine; NOX, NADPH oxidase; OLA, oleate; PRDX, peroxiredoxin; ROS, reactive oxygen species; SRXN1, sulfiredoxin 1; TRX, thioredoxin; UPR, unfolded protein response.

THE CHALLENGE OF PULSATILE GONADOTROPIN-RELEASING HORMONE (GnRH) SIGNALING IN GONADOTROPES

The fundamental role of pulsatile stimulation of gonadotropes by the hypothalamic neuropeptide GnRH, or GnRH-I in maintaining function of the hypothalamic–pituitary–gonad (HPG) axis is one of the earliest principal findings after discovery of the hormone (1, 2). Studies in nonhuman primates demonstrated the requirement for pulsatile stimulation of the pituitary to maintain the reproductive axis (3). The identity of the signaling molecules and mechanisms that contribute to pulse interpretation has been the subject of extensive study since many models developed to explain signaling control of gene expression (4–13). But questions remain concerning the mechanism of pulse interpretation and the signaling factors responsible. The hypothalamic neuropeptide GnRH and its receptor GnRHR are the prototypic members of a superfamily that has evolutionary roots reaching to the emergence of the bilateria (14). In vertebrates, a feature of this pair is its central role in regulating the anterior pituitary gonadotropes (15, 16). The hypothalamic GnRH neurons release hormone into the adenohypophyseal portal circulation in an episodic manner that is central to the development and operation of the HPG axis and fertility. In this system, the GnRHR governs the release of the gonadotropins LH and FSH and regulates expression of their subunit genes. The mammalian GnRHR is unique in structure, lacking a cytoplasmic tail that is normally associated with β-arrestin-mediated downregulation of receptor signaling. Thus, GnRHR itself faces unique challenges in transmitting an episodic signal in which alterations in amplitude and frequency are meaningful, yet, receptor homologous desensitization is not an accessible regulatory scheme. It is likely that pulse interpretation is accomplished by the operation of the signaling cascades themselves rather than desensitization or receptor availability at the membrane.

In mouse LβT2 cells, the switch between LH and FSH preference occurs at the 60-min pulse interval (17). A general switching mechanism is achieved by the expression and decay of activating and repressing transcription factors that create high- or low-pass filters to govern gene expression. For *Lhb*, this is the pairing of the immediate-early *Egr1* family of transcriptional activators with the Nab1/2 family of repressors. Transient frequency and amplitude-dependent stimulation of *Egr1* expression is countered by pulse-insensitive expression of *Nab*1/2, establishing a high-pass filter that requires sustained stimulation to overcome suppression (17). Features of this model have been confirmed by *in vivo* studies and mixed primary pituitary culture in rats and in αT3-1 cells that do not express gonadotropin β-subunit genes (18). On the other hand, *Fshb* prefers low frequencies for promoter activator (*c-Fos and c-Jun*) upregulation, and high frequencies lead to upregulation of *Fshb* promoter inhibitors, such as *Skil* and *Tgif1* (19). A feature of pulse decoding in the GnRH system is the occurrence of maximal responses at submaximal stimulation, creating a bell-curve frequency response that requires complex regulation but imparts true frequency decoding (20, 21). Components of the signaling network may exhibit digital tracking in which each response is resolved between pulses and acts dependently, or in the case of slower, incomplete resolution, exhibits integrative tracking in which the cumulative stimulation creates a maximal response (10). Transcriptional regulation of gonadotropin subunit genes is modest overall but exhibits integrative interpretation (22). GnRH also regulates protein synthesis and the distribution of mRNA in polyribosomes (23–26). Each of these may utilize different interpretative mechanisms.

MAP KINASE SIGNALING IN RESPONSE TO GnRH

GnRHR is a G protein-coupled receptor that signals primarily *via* the $G_{\alpha q/11}$ G protein subfamily, although interaction with other G proteins is also documented *in vivo* (27, 28). Stimulation of gonadotropes or gonadotrope-derived cell lines causes activation of phospholipase C, resulting in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) production. IP_3 mobilizes Ca^{2+} from intracellular stores and influx *via* L-type voltage-gated Ca^{2+} channels. The mobilization of Ca^{2+} is associated with initiation of the secretory response and fusion of secretory granules with the extracellular membrane. In a related signaling branch, DAG along with Ca^{2+} activates multiple PKC isozymes, including the conventional isoforms PKCα, PKCβII, the novel isoforms PKCδ and PKCε, and the atypical PKCζ in αT3-1 and LβT2 cells (29, 30). These activated signals link to downstream induction of mitogen-activated protein kinases (MAPK) (18, 31–33). The role of MAPK1/3 (ERK1/2) is sexually dimorphic and essential in female reproduction (34). Phosphorylation of MAPK1/3 is highly stimulated within a few minutes and rapidly resolved such that MAPK1/3 activation is restored to prestimulation levels well within the 60-min interval switch point of differential gene expression (**Figure 1**) (35). The connection between PKC and MAPK1/3 activation is well appreciated, but the intervening sequence of Ras/Raf/MAPK kinase (MEKK) signaling is not well described (29, 30). MAPK1/3 activation can occur through the c-SRC-mediated RAS activation (30, 36) and, in other cells, RAS activation occurs through DAG-dependent GRP1/2. However, recent evidence has shown that GnRH-stimulated MAPK1/3 activation in gonadotropes depends on reactive oxygen species (ROS) production by the NADPH oxidases (37). This suggests that multiple pathways contribute to MAPK1/3 activation and examination may shed light on their contribution to pulse interpretation.

ROS INTEGRATION INTO PATHWAYS PROMOTING MAP KINASE SIGNALING

Reactive oxygen species are partially reduced metabolites of oxygen produced through intracellular mechanisms or encountered in extracellular environments. Mitochondrial ROS are produced by aerobic respiration and incomplete oxidation of fatty acids and can indicate mitochondrial and endoplasmic reticulum stress. ROS are also employed as rapid signaling molecules through production by the NADPH/Dual Oxidase (NOX/DUOX) family, which are targets of activation by intracellular kinases or elevated intracellular Ca^{2+} (38). Exposure to ROS can cause oxidative

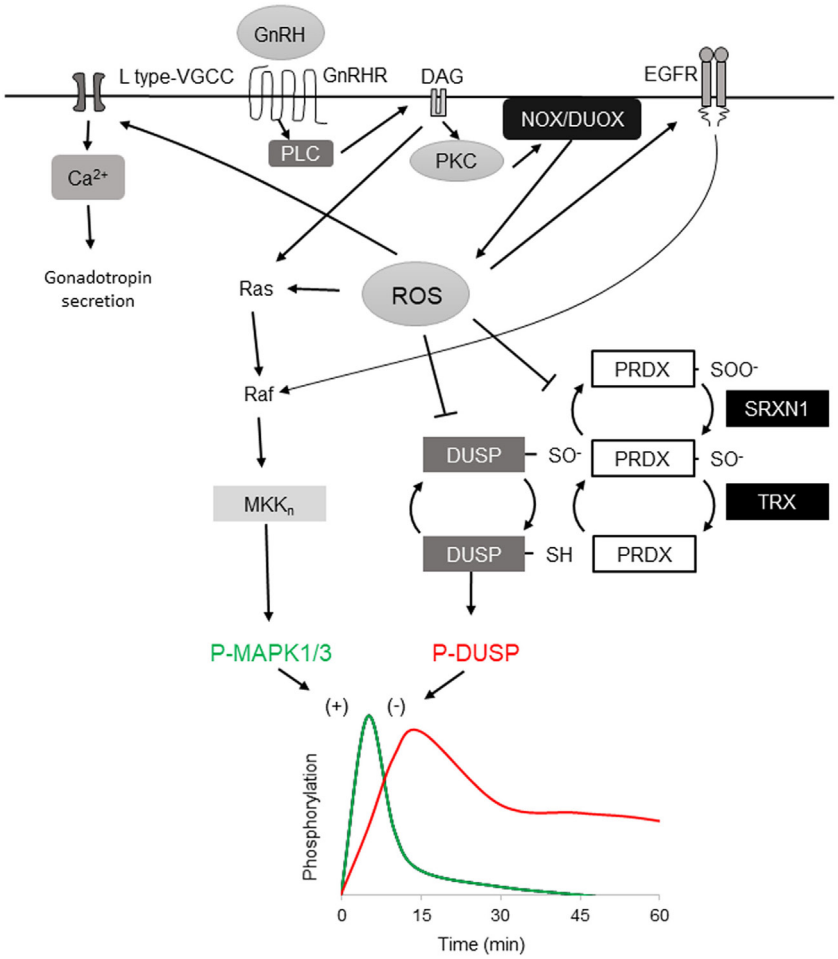

FIGURE 1 | Reactive oxygen species (ROS) involvement in mitogen-activated protein kinases (MAPK) 1/3 activation by gonadotropin-releasing hormone (GnRH) and resolution in gonadotrope cells. Activation profiles of MAPK1/3 and DUSP1 as determined by phosphorylation in response to a single GnRH pulse [adapted from Ref. (35)]. GnRH receptor-signaling *via* $G_{aq/11}$ activates phospholipase C, leading to diacylglycerol (DAG) and IP_3 production. The DAG and IP_3-induced rise in intracellular Ca^{2+} activate both NOX and DUOX family members, resulting in increased ROS production. ROS stimulates MAPK1/3 activation by promoting Ras and Raf activation of the MEKn cascade ultimately targeting MAPK1/3. Oxidative activation of epidermal growth factor receptor (EGFR) contributes to MAPK1/3 activation through Raf. ROS may also transiently inactivate negative feedback through reversible oxidation of the DUSP active-site cysteine. ROS is normally reduced by peroxiredoxin (PRDX) by conversion of active reducing site cysteine thiol C–SH to sulfenic C–SOH. Sulfenic cysteine is recycled by thioredoxin (TRX) reduction. Excess ROS contributes to PRDX hyperoxidation that further oxidizes the sulfenic C–SOH to the sulfinic C–SOOH, which is reduced by the ATP-dependent reductase activity of sulfiredoxin 1 (SRXN1), preserving PRDX capacity but allowing transient DUSP inactivation. DUSP activity is resumed after ROS level declines, permitting feedback control of MAPK1/3. ROS activation of L-type VGCC promotes intracellular Ca^{2+} that supports exocytosis and activation of DUOX.

damage to many biomolecules, resulting in nucleic acid damage or mutation, enzymatic dysfunction, or cell death. Therefore, management of ROS is a focus of cellular homeostasis. Reducing systems operating through peroxiredoxins (PRDX1-6), thioredoxin (TRX), and glutathione (GSH) exchange of free radical oxygen are present in all cells and PRDX isoforms partition into subcellular regions for specialized action. Each of the six mammalian PRDX isoforms is represented in to the top 5% of cellular protein content (39), collectively constituting a high proportion of cellular protein and a significant investment in localizing ROS action and limiting oxidative damage.

Elevated ROS is associated with MAPK activation in multiple cell types (40). Insulin-like growth factor I activation of MAPK1/3 increases ROS production and antioxidants inhibit activation of the MAPK1/3 pathway, showing dependence on ROS (38–41). Similarly, MAPK8/9 (JNK) and MAPK14 (p38 MAPK) phosphorylation is associated with ROS generation (42–44). The MAP3K-related kinase ASK1 associates with TRX and is released upon TRX oxidation, permitting activation of MAPK8/9/14 (44). In other professional secretory cells, ROS is central to secretion and activation of biosynthesis. In the endocrine pancreas, NOX enzymes are involved in stimulated insulin secretion and excess ROS production increases oxidative stress and loss of function (45–47). NOX/DUOX participate in the signaling response activating thyroid hormone biosynthesis (48–50). ROS mediates enhanced MAP kinase activation in activated eosinophils, contributing to IL-5-mediated cell death (51). In contrast, NOX is a target of MAP kinase activation in neutrophils and ROS signaling

is utilized in formation of neutrophil extracellular traps (52). NOX proteins are, therefore, both upstream activators of MAP kinase signaling and targets of MAP kinase action, suggesting plasticity in how NOX/DUOX-derived ROS is deployed.

Gonadotropin-releasing hormone stimulation of mouse primary pituitary and LβT2 cells that endogenously express all gonadotropin subunit genes (53, 54), results in ROS production that is blocked by pharmacological inhibition of NOX/DUOX enzymes with diphenyleneiodonium (DPI). N-acetyl cysteine (NAC), which is general ROS scavenger, also attenuates MAPK1/3 and MAPK8/9 activation. Both DPI and NAC attenuate activation of *Lhb* and *Fshb* transcription (37). Further, GnRH-mediated activation of ROS depends on PKC and Ca^{2+} availability. This places ROS between PKC and MAPK1/3 and supports an intermediate role in activation similar to ROS-mediated activation of Ras through kinase and regulatory subunit regulation (55, 56). The rapid and transient activation of MAPK1/3 by GnRH is similar to that reported by direct activation by H_2O_2 *via* epidermal growth factor receptor (EGFR) (57). The cysteine-rich motifs of growth factor receptors including EGFR are proposed targets of activation by oxidation (42). But demonstration using suramin and its known broad actions that include inhibition of G-protein receptor signaling suggests that revisiting this may be warranted. GnRH signaling is also associated with EGFR activation possibly through matrix metalloprotease liberation of extracellular ligand (58–60). An alternative ligand-independent activation pathway through oxidative activation of EGFR could support the rapid elevation of MAPK 1/3 activation after GnRH stimulation (41).

In addition to activation of MAP kinase pathways through positive regulation of signaling cascades, ROS may also play a central role in promoting MAP kinase phospho-activation through inactivation of negative feedback. Dual-specificity protein phosphatases (DUSP's, also MKP's) serve a primary role as negative feedback regulators of MAP kinase signaling through dephosphorylation of activated MAP kinases (**Figure 1**) (61). DUSP's and other protein tyrosine phosphatase superfamily members share a common catalytic site motif of [I/V]HCXXGXXR[S/T] in which the invariant cysteine residue serves as a catalytic nucleophile that is susceptible to reversible inactivating oxidation (62). Oxidative suppression of DUSP's can support sustained activation of MAPK 8/9 and drive TNF-α-mediated cell death (63) and oxidative control of DUSP and MAPK signaling has been observed in pancreatic β-cells (46, 64), supporting this mechanism in professional secretory cells. Oxidation of DUSP's also promotes their proteosomal degradation, limiting their availability to inhibit MAPK kinase (65). In LβT2 gonadotropes, high amplitude GnRH stimulation causes sustained activation of MAPK1/3 similar to that observed with ROS-mediated suppression of DUSP feedback (35). Chronic stimulation with GnRH also results in ROS production (37) but the status of DUSP after prolonged exposure to ROS or chronic GnRH stimulation has not been directly examined. The participation of ROS in rapid activation of MAP kinase signaling in gonadotropes through positive control of signaling cascades and negative control of feedback suggests that ROS contributes to the rapid activation of MAP kinases that is observed in response to GnRH stimulation. Involvement in both MAPK 1/3 and MAPK 8/9 activation suggests that both *Fshb* and *Lhb* transcription can be regulated through ROS.

RESOLUTION OF GnRH-STIMULATED MAPK SIGNALING

Feedback control of MAPK activation by DUSP family members is central to the control of MAP kinase signaling networks. For cells to remain vigilant for change in GnRH pulses, sensitivity to a subsequent pulse is maintained and interpreted in context, which implies a capacity for hysteresis. In either digital or integrative pulse tracking, some balance between activation, negative feedback, and response decay must be achieved. Signaling networks may switch between modes by changing this relationship. Hysteresis in cell signaling was initially proposed and tested in the model of bistable MAPK1/3 activation by platelet-derived growth factor receptor, which showed that MAPK1/3 response amplitude is dictated by the degree of DUSP1 feedback activated by a previous signaling response (66). The role of DUSP's in negative feedback control of MAPK1/3 activation has been examined extensively in the context of GnRHR signaling. In LβT2 cells, activation of MAPK1/3 by physiological levels of GnRH is resolved within 30 min (35). Overexpression or knockdown of nuclear-resident DUSP1 suppresses or increases activation of MAPK1/3 in response to GnRH, respectively (35). But studies in cells that do not natively express GnRHR or gonadotropin genes or using reporters of translocation have questioned this observation (67). LβT2 gonadotropes show elevated DUSP1 in unstimulated cells, suggesting that they are primed for suppression of MAP kinase signaling activation. This available phosphatase activity is subject to rapid inactivation by ROS but the reversibility of inactivation suggests some capacity is maintained or quickly recovered, contributing to the rapid resolution of MAPK1/3 activation. Another possibility is the involvement of cellular mechanisms limiting ROS through reduction by PRDX and TRX. These proteins are part of a larger network of factors controlling oxidative stress that includes GSH, catalase, superoxide dismutase, and the ATP-dependent redox factor sulfiredoxin 1 (SRXN1, also NPN3). These factors contribute to the maintenance of reductive capacity through resolution of oxidized or hyperoxidized PRDX, returning it to the pool of available reductase. Although the 2-cysteine PRDX1-4 family members are efficient ROS scavengers, they can be hyperoxidized by conversion of their nucleophilic thiol to sulfinic acid (**Figure 1**). Hyperoxidized PRDX is recycled through ATP-dependent reduction by SRXN1 (68, 69). In LβT2 cells, *Srxn1* gene expression is proportionally induced by increasing pulsatile and tonic GnRH stimulation (17), implying a role in resolution of oxidative stress.

The restoration of feedback control can also be achieved through increased DUSP synthesis in response to GnRH stimulation. In LβT2 cells, GnRH stimulation causes transient activation of the unfolded protein response (UPR) (24). Translation is largely inhibited by the UPR but *Dusp1* and *Dusp8* mRNA escape translation inhibition and DUSP1 is increased during the time the UPR is active (23, 35). Translational control of *Dusp1* mRNA is MAPK1/3 dependent and is attributed to the 3′UTR ELAVL1 binding site known to contribute to mRNA stability (23, 70). Pulsatile GnRH increases *Dusp1*, *Dusp8*, and *Dusp16* expression, all of which target MAP kinases (17). Although DUSPs are subject to rapid inactivation by ROS, the reversibility of

oxidative inhibition, the rapid translational response of the UPR, and the long-term transcriptional response to GnRH stimulation provide mechanisms for preserving feedback regulation while permitting short-term activation of MAP kinase signaling.

On a broad time scale, ROS may regulate gonadotropins through regulation of gene expression and sensitivity to GnRH through microRNA modulation of gonadotropin and $G_{\alpha q/11}$ signaling component gene expression. MiR132/212 regulates *Fshb* mRNA expression and secretion *via* SIRT1 deacetylation in gonadotropes (71). MiR-7a2 or miR-200b and miR-429 participate in maintenance of gonadotropin gene expression (72, 73). Further, miR125b contributes to desensitization of sustained GnRH stimulation by targeting components of the $G_{\alpha q/11}$ pathway (74). MicroRNA regulation occurs through oxidation-sensitive transcription factors such as CEBPB and ZEB1 and microRNA also directly affects MAPK signaling (75). The ROS-sensitive regulation of microRNA may further link GnRH signaling to ROS directly through GnRH receptor stimulation of ROS production or to ROS derived from other sources.

ROS IN GnRH-INDUCED GONADOTROPIN SECRETION

Hormone secretion by exocytosis in endocrine cells is triggered by Ca^{2+} released from intracellular stores. A rise of intracellular Ca^{2+} regulates several steps of exocytosis; including, vesicle priming and fusion to the plasma membrane (76). Localized Ca^{2+} increase with IP_3 stimulation is necessary for gonadotropin exocytosis (77). Increased Ca^{2+} also induces ROS production by DUOX activation (37) and both voltage-gated and L-type calcium channels are activated by ROS (78, 79). This may tie localized Ca^{2+} to enzymatic ROS generation by DUOX. Thrombin promotes Ca^{2+} influx in smooth muscle cells by NOX-derived ROS activation of L-type calcium channels (80). Also, insulin-induced NOX increases IP_3 receptor activity and Ca^{2+} release in skeletal muscle (81). In gonadotrope cells, DPI blocks GnRH-induced gonadotropin secretion, indicating dependence on ROS for secretion (37) and suggesting integration of Ca^{2+} and ROS signaling in exocytosis.

Interestingly, ROS in the form of nitric oxide may play an important role in maturation and regulation of the hypothalamus in concert with pituitary ROS. Nitric oxide production in the hypothalamus elicits GnRH secretion and expression of GnRH mRNA is modulated through miR-200 and miR-155 expression before puberty by controlling the nitric oxide-sensitive regulators CEBPB and ZEB1 (82, 83). These phenomena indicate that microRNA and ROS are tightly linked to rapid and long-term control of the HPG axis.

ROS AS A METABOLIC REPORTER IN THE GONADOTROPE

Energy balance has a profound influence on reproductive fitness and operation of the HPG axis. The critical fat hypothesis suggests that an optimal level of body fat is permissive to menarche and may be necessary for optimal operation of the HPG axis (84, 85). Adipose-associated changes in gonadotropin levels imply the presence of a sensing mechanism that reports energy status to the reproductive endocrine axis (86–91). Reproductive disorders such as polycystic ovary syndrome and hypogonadotropic hypogonadism are associated with metabolic dysfunction and obesity. However, not all metabolic signals associated with obesity explain the inverse relationship between adiposity and gonadotropin levels observed in men and women (92–95). Adipose-derived endocrine signals such as leptin play a role in modulating hypothalamic or pituitary function (96). Another potential modulator of the HPG axis are free fatty acids (FFA). Data suggest that FFA have a direct impact on gonadotropes in ruminants, and FFA suppresses gonadotropin secretion in cultured primary pituitary (97, 98). Unsaturated FFA can induce mitochondrial ROS production and activation of the UPR (99). We examined the ability of the monounsaturated fatty acid oleate (OLA) to induce mitochondrial ROS in LβT2 cells (**Figure 2**). We found that moderate physiological OLA, 500 μM, can induce mitochondrial. Unlike OLA, GnRH does not impact mitochondrial ROS production as

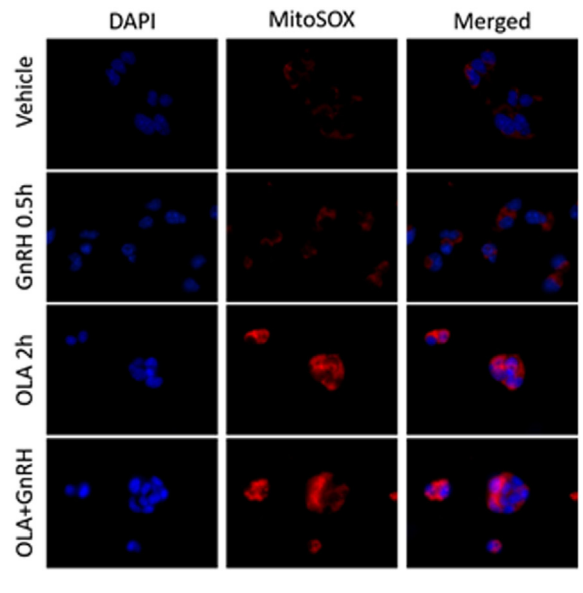

FIGURE 2 | Oleate (OLA), but not gonadotropin-releasing hormone (GnRH), induces mitochondrial superoxide production. LβT2 cells (RRID:CVCL_0398) cultured on Poly-L-Lysine (Sigma-Aldrich Inc.) coated Nunc Lab-Tek II chamber slides (Thermo Fisher Scientific) for 24 h were serum starved overnight, then exposed to either vehicle or 500 μM oleate for 2 h, then with vehicle or 10 nM GnRH for 30 min. Cells were subsequently treated with 5 μM red-fluorescent MitoSOX probe (Thermo Fisher Scientific) for 5 min. Afterward, cells were directly fixed with 2% paraformaldehyde solution for 15 min, washed, and cover slipped with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs) to visualize nuclei in blue. Blue DAPI and Red MitoSOX fluorescence was captured by wide-field fluorescent microscopy using Nikon TE2000-U microscope (Nikon America Inc., Melville, NY, USA) equipped with an X-Cite 120PC collimated light source (Lumen Dynamics Group Inc.) and a DAPI-1160A or mCherry-C000 filter set (Semrock, Inc.) using a CoolSNAP DYNO CCD camera (Photometrics Inc.). In LβT2 cells, GnRH alone does not enhance mitochondrial ROS production as determined by changes in red fluorescence, whereas OLA-treated cells showed a highly elevated signal. Co-treatment with GnRH and Oleic acid did not appear to alter overall staining intensity.

measured by conversion of the indicator dye MitoSOX nor does there appear to be any synergistic action, although GnRH induces ROS production in the same cells *via* NOX/DUOX (37). This supports the enzymatic ROS production with GnRH stimulation, and casts us the question that mitochondria produced ROS needs to be further examined in the aspect of both gonadotropin secretion and GnRH-induced gene expressions.

CONCLUSION

The emerging evidence that ROS are integrated into multiple cell signaling cascades has led to appreciation as an important signaling molecule. In gonadotropes, it appears that enzymatic ROS plays a role in GnRH receptor signaling. The rapid and transient nature of ROS signaling is well-suited for the episodic pattern of GnRH stimulation and ROS signaling can contribute to both rapid activation and rapid resolution of activated signaling cascades. Further studies are needed to confirm or disprove the role of PRDX and SRXN1 in resolution of MAPK activation. It is also necessary to examine the impact of FFA-induced ROS on cell stress and to confirm the potential role of enzymatic ROS on L-type calcium stimulation in gonadotrope cells. The integration of ROS in GnRH receptor signaling provides an opportunity for other ROS sources to impact gonadotrope function at the cellular level. Incorporating ROS into our view of GnRH signaling is likely to yield useful insight into the mechanism of pulse interpretation and integration of stress signaling into the reproductive axis.

AUTHOR CONTRIBUTIONS

TT authored the manuscript and prepared figures. MA produced new data presented in **Figure 2** under direction and technical support of TT and DL. TK and SL developed the original concept, experimental approaches, and produced pilot data not reported here. ET participated in editing and writing the manuscript. ML edited the manuscript and secured support for all co-authors.

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REVIEW
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Expression and Role of Gonadotropin-Releasing Hormone 2 and Its Receptor in Mammals

*Amy T. Desaulniers[1], Rebecca A. Cederberg[1], Clay A. Lents[2] and Brett R. White[1]**

[1] Department of Animal Science, University of Nebraska-Lincoln, Lincoln, NE, United States, [2] USDA, ARS, USMARC, Clay Center, NE, United States

Gonadotropin-releasing hormone 1 (GnRH1) and its receptor (GnRHR1) drive mammalian reproduction *via* regulation of the gonadotropins. Yet, a second form of GnRH (GnRH2) and its receptor (GnRHR2) also exist in mammals. GnRH2 has been completely conserved throughout 500 million years of evolution, signifying high selection pressure and a critical biological role. However, the *GnRH2* gene is absent (e.g., rat) or inactivated (e.g., cow and sheep) in some species but retained in others (e.g., human, horse, and pig). Likewise, many species (e.g., human, chimpanzee, cow, and sheep) retain the *GnRHR2* gene but lack the appropriate coding sequence to produce a full-length protein due to gene coding errors; although production of GnRHR2 in humans remains controversial. Certain mammals lack the *GnRHR2* gene (e.g., mouse) or most exons entirely (e.g., rat). In contrast, old world monkeys, musk shrews, and pigs maintain the coding sequence required to produce a functional GnRHR2. Like GnRHR1, GnRHR2 is a 7-transmembrane, G protein-coupled receptor that interacts with $G_{\alpha q/11}$ to mediate cell signaling. However, GnRHR2 retains a cytoplasmic tail and is only 40% homologous to GnRHR1. A role for GnRH2 and its receptor in mammals has been elusive, likely because common laboratory models lack both the ligand and receptor. Uniquely, both GnRH2 and GnRHR2 are ubiquitously expressed; transcript levels are abundant in peripheral tissues and scarcely found in regions of the brain associated with gonadotropin secretion, suggesting a divergent role from GnRH1/GnRHR1. Indeed, GnRH2 and its receptor are not physiological modulators of gonadotropin secretion in mammals. Instead, GnRH2 and GnRHR2 coordinate the interaction between nutritional status and sexual behavior in the female brain. Within peripheral tissues, GnRH2 and its receptor are novel regulators of reproductive organs. GnRH2 and GnRHR2 directly stimulate steroidogenesis within the porcine testis. In the female, GnRH2 and its receptor may help mediate placental function, implantation, and ovarian steroidogenesis. Furthermore, both the *GnRH2* and *GnRHR2* genes are expressed in human reproductive tumors and represent emerging targets for cancer treatment. Thus, GnRH2 and GnRHR2 have diverse functions in mammals which remain largely unexplored.

Keywords: GnRH2, GnRH2 receptor, reproductive function, G protein-coupled receptor, G protein-coupled receptor signal transduction, autocrine/paracrine mechanisms, testis, cancer

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****Correspondence:***
Brett R. White
bwhite2@unl.edu

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BACKGROUND

The Classical Form of Mammalian Gonadotropin-Releasing Hormone (GnRH1)

The classical, hypophysiotropic GnRH1 is hailed as the master regulator of reproduction in mammals. GnRH1 is a decapeptide (pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2) produced by hypothalamic neurons and secreted in a pulsatile manner into hypophyseal portal capillaries where it travels to the anterior pituitary gland. GnRH1 then binds to its receptor (GnRHR1) on gonadotrope cells, promoting the synthesis and secretion of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), into peripheral circulation where they act on their target organs, the gonads. In females, FSH stimulates follicular development, whereas LH promotes ovulation and maintenance of the corpus luteum. Within the testes, FSH regulates spermatogenesis and LH elicits secretion of testosterone. Ultimately, the gonads cease to function and reproduction is halted in the absence of GnRH1 (1–3).

GnRH Variants in Mammals

Gonadotropin-releasing hormone 1 was first identified in the hypothalami of pigs and sheep (4–6) and was originally thought to be a novel peptide. However, 23 other forms of GnRH have since been discovered (7), all with 10 amino acids and at least a 50% sequence identity (8). Within these forms, the sequences of both the N-terminus (pGlu–His–Trp–Ser) and C-terminus (Pro–Gly–NH_2) are conserved (7, 9). The amino acid substitutions only occur between residues 5 and 8 (7, 9). In vertebrates, three forms of GnRH (GnRH1, GnRH2, and GnRH3) are the most common. The third form of GnRH (GnRH3; pGlu–His–Trp–Ser–His–Asp–Trp–Lys–Pro–Gly–NH_2) was first discovered in lamprey (10) but the *GnRH3* gene has only been confirmed in fish and amphibians to date (7, 11). Therefore, only GnRH1 and GnRH2 are produced in mammals (7).

GONADOTROPIN-RELEASING HORMONE 2

The Second Form of Mammalian GnRH (GnRH2)

A second structural variant of GnRH, GnRH2, has been identified in mammals. Like GnRH1, GnRH2 is a decapeptide but it was first isolated from the hypothalami of 10,000 chickens and therefore named "chicken GnRH2" (12). It was later discovered in mammals, the first being marsupials (13), and renamed simply "GnRH2" to prevent confusion (14). Since then, GnRH2 has been found in animals of every vertebrate class including primitive orders (e.g., bony fish) as well as complex mammals (15). GnRH2 is absent only in jawless fish (16). Notably, the sequence of GnRH2 remains entirely conserved throughout evolution, indicating high selection pressure and a critical function (17). Absolute conservation of GnRH2 has persisted despite 500 million years of evolution (15), indicating that it may be the most ancient form of GnRH (18). In contrast, GnRH1 evolved 350 million years ago and its sequence varies greatly among vertebrates (19).

The Gene for GnRH2

GnRH2 is not merely a splice variant of the *GnRH1* gene; instead, it is produced from its own gene that encodes the peptide, prepro-GnRH2 (20). The *GnRH2* gene is located on chromosome 20 in humans, chimpanzees, and orangutans, chromosome 13 in the cow, chromosome 22 in the horse, chromosome 10 in the rhesus macaque, and chromosome 17 in the pig (21). The genomic orientation of the *GnRH2* gene is highly conserved across species (21, 22). It is flanked by the *PTPRA* and *MRPS26* genes in all mammalian and non-mammalian vertebrates examined to date (21, 22). The *PTPRA* gene resides about 5–6 kb upstream of the *GnRH2* gene (21) and encodes the enzyme, receptor-type tyrosine-protein phosphatase α, which is critical for neural development (23). The *MRPS26* gene resides about 300 bp downstream of the *GnRH2* gene (21), encoding mitochondrial ribosome protein S26, which assists in protein synthesis (24). A graphical representation of the porcine *GnRH2* gene is depicted in **Figure 1A**.

The human *GnRH2* gene has three coding exons like the *GnRH1* gene; however, the *GnRH2* gene is notably shorter (2.1 versus 5.1 kb), primarily due to differences in intron length (20). Otherwise, organization of the *GnRH1* and *GnRH2* genes remain similar (25). The first coding exon in humans encodes the signal sequence, mature decapeptide, and a portion of the GnRH-associated peptide (GAP). The second and third exons encode the remaining GAP (20). Likewise, porcine prepro-GnRH2 is encoded by 3 exons and yields a 110 amino acid product (**Figure 1B**) that must undergo post-translational proteolytic processing for functionality (20).

Presence of the GnRH2 Gene in Mammals

Although the *GnRH2* gene was first identified in humans (20), Stewart et al. (21) examined the genomes of mammals encompassing 10 orders for the presence of the *GnRH2* gene. The *GnRH2* gene was positively identified in 21 animals. Using bioinformatics, the authors concluded that gene coding errors likely prevent the successful production of GnRH2 in many species (21). A summary of the coding errors present in the *GnRH2* gene of mammals is available in **Table 1**. A premature stop codon truncates the mature decapeptide in the chimpanzee, orangutan, mouse lemur, sheep, and cat (21, 26), whereas the rabbit, pika, cow, dog, cat, and dolphin *GnRH2* genes encode an inactive peptide (21, 26). Early evidence implied that the rat and mouse also maintain a *GnRH2* gene as immunoreactive GnRH2 was detected in the rodent brain (27–29). Although it was later determined that the mouse genome only maintains a fragment of the *GnRH2* gene (exon 1) on chromosome 2 and it is completely deleted from chromosome 3 in the rat (21, 22, 30). Together, these data reveal that the *GnRH2* gene is absent or functionally inactivated in many mammals. In contrast, 10 species (human, macaque, marmoset, tarsier, tree shrew, guinea pig, musk shrew, common shrew, horse, and pig) maintain the appropriate genomic sequence to produce a biologically active

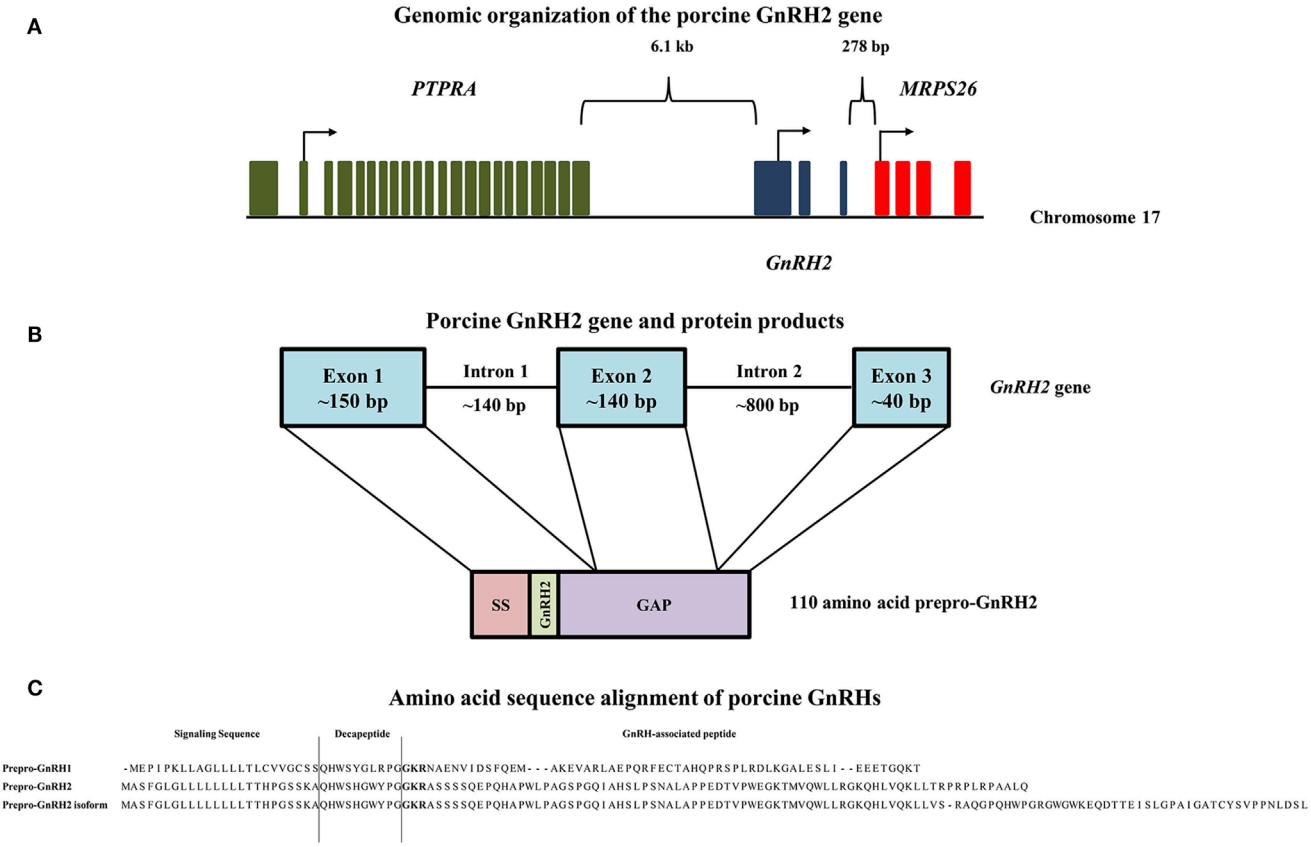


FIGURE 1 | Genomic organization of the porcine GnRH2 gene and its products. **(A)** The *PTPRA* gene (green) is located 6.1 kb upstream, whereas the *MRPS26* gene (red) is positioned 278 bp downstream of the porcine *GnRH2* gene (blue) on chromosome 17. Arrows indicate start codons for each gene. **(B)** The porcine *GnRH2* gene contains three coding exons and two introns. Exon 1 (~150 bp) of the porcine *GnRH2* gene encodes the signaling sequence (SS), mature GnRH2 decapeptide, and a portion of the GnRH-associated peptide (GAP). Exon 2 (~140 bp) and exon 3 (~40 bp) encode the remaining GAP sequence. Note that introns and exons are not drawn to scale. **(C)** Amino acid sequence alignments of predicted porcine prepro-GnRH2 isoforms (NCBI accession numbers XP_005672842 and XP_013840618) with porcine prepro-GnRH1 (NCBI accession number NP_999439). Prepro-GnRH1 is 91 amino acids in length compared with prepro-GnRH2, which has 110 residues. An isoform of prepro-GnRH2 (143 amino acids) is also predicted to be produced from the porcine *GnRH2* gene due to alternative splicing. The amino acids that correspond to the SS, the mature decapeptide, and the GAP are indicated. The proteolytic cleavage sites are highlighted in bold.

decapeptide (21). We re-evaluated the presence of the *GnRH2* gene in mammals by surveying updated NCBI gene databases and found that an additional 68 mammals, comprising 9 additional orders, possess the *GnRH2* gene to date (**Table 2**). Thus, 89 mammals from 19 orders maintain the *GnRH2* gene, further suggesting that the *GnRH2* gene was present in a common mammalian ancestor (21). However, whether the *GnRH2* gene encodes a functional peptide in all of these animals remains unknown.

GnRH2 Is Ubiquitously Expressed within Mammals

Like GnRH1, GnRH2 has been identified in the pre-optic and medio-basal hypothalamic areas (7), albeit scarcely (33). Likewise, our group detected low levels of GnRH2 within the hypothalamus of the pig, implying that GnRH2 is not a prominent regulator of gonadotropin secretion (34, 35). GnRH2 has also been discovered in the midbrain and limbic structures, suggesting a role in the modulation of reproductive behavior (14, 29). In primates and humans, GnRH2 is prevalent in the caudate nucleus, hippocampus, and amygdala (36, 37) and has also been detected in the midbrain and hindbrain (7). White et al. (20) quantified expression of the *GnRH2* gene in 50 different human tissues, including numerous regions of the brain (20). Surprisingly, *GnRH2* mRNA was identified in all tissues examined and levels were highest in peripheral tissues; the converse was true for GnRH1.

In the periphery, transcript levels for *GnRH2* were 30-fold higher in the kidney and 4-fold higher in bone marrow and the prostate compared with the brain. GnRH2 is also produced in organs of the thoracic (e.g., heart, lung, and aorta), digestive (e.g., salivary gland, stomach, and intestine), endocrine (e.g., adrenal, pancreas, and thyroid), and immune (e.g., tonsil, leukocyte, and lymph node) systems (**Table 3**) (11, 29). Moreover, GnRH2 has been identified in numerous female (e.g., ovary, uterus, endometrium, and myometrium) and male (e.g., testis, epididymis, seminal vesicles, and prostate) reproductive organs (**Table 3**) (11, 29). The ubiquitous nature of this decapeptide is also

TABLE 1 | Presence and potential functionality of the GnRH2 and GnRHR2 genes within mammals.[a]

Order	Mammal (genus species)	GnRH2			GnRHR2		
		Gene	Coding disruption[b]	Functional protein	Gene	Coding disruption[b]	Functional protein
Artiodactyla	Alpaca (*Vicugna pacos*)	+	?	?	+	?	?
	Cow (*Bos taurus*)	+	AAA	–	+	PSC	–
	Pig (*Sus scrofa*)	+	–	+	+	–	+
	Sheep (*Ovis aries*)	+	PSC	–	+	PSC	–
Carnivora	Cat (*Felis catus*)	+	PSC; FM; AAA	–	+	PSC	–
	Dog (*Canis familiaris*)	+	PSC; AAA	–	+	PSC; FM	–
Cetacea	Bottle-nosed dolphin (*Tursiops truncatus*)	+	AAA	–	+	FM; AAA; PSC	–
Lagomorpha	Pika (*Ochotona princeps*)	+	AAA; BI	–	?	?	?
	Rabbit (*Oryctolagus cuniculus*)	+	MS; PSC	–	+	MS; FM; PSC	–
Perissodactyla	Horse (*Equus caballus*)	+	–	+	+	FM; PSC	–
Primates	African green monkey (*Cercopithecus aethiops*)	?	?	?	+	–	+
	Chimpanzee (*Pan troglodytes*)	+	PSC	–	+	FM; PSC	–
	Common marmoset (*Callithrix jacchus*)	+	–	+	+	–	+
	Human (*Homo sapien*)	+	–	+	+	FM; PSC	–
	Mouse lemur (*Microcebus murinus*)	+	PSC	–	+	?	?
	Orangutan (*Pongo pygmaeus*)	+	PSC	–	+	–	+
	Rhesus macaque (*Macaca mulatta*)	+	–	+	+	–	+
	Tarsier (*Tarsius syrichta*)	+	–	+	+	?	?
Proboscidea	African elephant (*Loxodonta africana*)	+	?	?	+	–	+
Rodentia	Ground squirrel (*Spermophilus tridecemlineatus*)	+	?	?	+	PSC; AAA	–
	Guinea pig (*Cavia porcellus*)	+	–	+	+	PSC; FM	–
	Kangaroo rat (*Dipodomys ordii*)	+	?	?	+	AAA	?
	Mouse (*Mus musculus*)	+	GR	–	–	–	–
	Rat (*Rattus norvegicus*)	–	–	–	+	GR	–
Scandentia	Tree shrew (*Tupaia belangeri*)	+	–	+	+	–	+
Soricomorpha	Common shrew (*Sorex araneus*)	+	–	+	+	BD; FM; PSC	–
	Musk shrew (*Suncus murinus*)[c]	+	–	+	?	?	+

[a]*Data are based on bioinformatics. Absence of the gene or functional protein is denoted by a (–), whereas presence is indicated by a (+). Species in which there was not enough genomic information available (or the functionality has not yet been assessed) will be noted as a (?). Adapted from Stewart et al. (21).*

[b]*In many species, the gene for GnRH2 and/or GnRHR2 is present but presumed non-functional due to gene coding errors. If the gene is affected, the nature of the disruption is indicated. Absence of a gene disruption is indicated by a minus symbol (–).*

[c]*The genome of the musk shrew has not been fully annotated. Therefore, the presence of GnRHR2 in the musk shrew is putative based on the work of Temple et al. (31) and Kauffman et al. (32) establishing a functional GnRHR2.*

Abbreviations: PSC, premature stop codon; FM, frameshift mutation; AAA, amino acid alteration; GR, gene remnant; BI, base insertion; BD, base deletion; MS, missing sequence.

evident in the many immortalized cell lines in which GnRH2 has been isolated, including cells derived from breast tissue (38), lymphocytes (39), ovaries (40, 41), and neural tissue (42). A summary of the mammalian cell lines that produce GnRH2 is available in **Table 4**. Ultimately, these data demonstrate that GnRH2 is ubiquitously expressed, indicating a divergent function from GnRH1.

Transcriptional Regulation of GnRH2 Gene Expression

The expression of *GnRH2* is regulated by several different reproductive hormones including androgens, 17β-estradiol, progesterone, the gonadotropins, and GnRH2 itself. Darby et al. (54) demonstrated in tumors from prostate cancer patients that androgens enhance *GnRH2* expression. This effect was likely mediated at the transcriptional level because sequence analysis of the 5′ flanking region for the human *GnRH2* gene revealed the presence of a putative androgen response element and direct interaction with the androgen receptor was confirmed *via* chromatin immunoprecipitation assays (54). In females, *GnRH2* expression in the hypothalamus of rhesus macaques is stimulated by 17β-estradiol treatment (55). Similarly, treatment of human granulosa-luteal cells with 17β-estradiol resulted in a dose-dependent increase in *GnRH2* mRNA expression (56) and 17β-estradiol exerted a stimulatory effect on *GnRH2* expression in human neuronal cells (42). The effect of progesterone has also been examined in humans. Primary cultures of human granulosa-luteal cells treated with RU486 (progesterone receptor antagonist) increased *GnRH2* expression in a time- and dose-dependent fashion (56). However, neither progesterone nor RU486 affected *GnRH2* expression in human neuronal cells (57).

There is also evidence that protein hormones modulate GnRH2 production. Treatment with FSH or human chorionic gonadotropin (hCG) upregulated *GnRH2* gene transcription

TABLE 2 | Identification of the GnRH2 gene within 68 additional mammals *via* NCBI gene database queries.

Order	Common name	Genus species	NCBI gene ID
Afrosoricida	Cape golden mole	*Chrysochloris asiatica*	102818594
Artiodactyla	Alpaca	*Vicugna pacos*	102537645
	Arabian camel	*Camelus dromedarius*	105096036
	Bactrian camel	*Camelus bactrianus*	105083071
	Bison	*Bison bison bison*	104986175
	Goat	*Capra hircus*	102171992
	Texas white-tailed deer	*Odocoileus virginianus texanus*	110145856
	Tibetan antelope	*Pantholops hodgsonii*	102339298
	Water buffalo	*Bubalus bubalis*	102408995
	Wild bactrian camel	*Camelus ferus*	102511575
	Wild yak	*Bos mutus*	102280393
	Zebu cattle	*Bos indicus*	109567205
Carnivora	Amur tiger	*Panthera tigris altaica*	102950818
	Cheetah	*Acinonyx jubatus*	106980160
	Ferret	*Mustela putorius furo*	101672225
	Giant panda	*Ailuropoda melanoleuca*	100469838
	Leopard	*Panthera pardus*	109274781
	Pacific walrus	*Odobenus rosmarus divergens*	101378339
	Polar bear	*Ursus maritimus*	103674386
	Weddell seal	*Leptonychotes weddellii*	102734468
Cetacea	Killer whale	*Orcinus orca*	101276573
	Minke whale	*Balaenoptera acutorostrata scammoni*	103020677
	Sperm whale	*Physeter catodon*	102986533
	Yangtze river dolphin	*Lipotes vexillifer*	103085292
Chiroptera	Big brown bat	*Eptesicus fuscus*	103285836
	Brandt's bat	*Myotis brandtii*	102246670
	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	109448424
	Little brown bat	*Myotis lucifugus*	102433482
	Myotis davii bat	*Myotis davii*	102763285
Dasyuromorphia	Tasmanian devil	*Sarcophilus harrisii*	100916503
Didelphimorphia	Gray short-tailed opossum	*Monodelphis domestica*	103098126
Diprotodontia	Koala	*Phascolarctos cinereus*	110221117
Erinaceomorpha	Western European hedgehog	*Erinaceus europaeus*	103128681
Eulipotyphla	Star-nosed mole	*Condylura cristata*	101633945
Macroscelidea	Cape elephant shrew	*Elephantulus edwardii*	102862182
Perissodactyla	Donkey	*Equus asinus*	106844450
	Przewalski's horse	*Equus przewalskii*	103555821
	Southern white rhinoceros	*Ceratotherium simum simum*	106802382
Primate	Angolan colobus	*Colobus angolensis*	105512752
	Black snub-nosed monkey	*Rhinopithecus bieti*	108529998
	Bolivian squirrel monkey	*Saimiri boliviensis*	101028145
	Coquerel's sifaka monkey	*Propithecus coquereli*	105827546
	Crab-eating macaque	*Macaca fascicularis*	102124425
	Drill	*Mandrillus leucophaeus*	105535577
	Golden snub-nosed monkey	*Rhinopithecus roxellana*	104674617
	Green monkey	*Chlorocebus sabaeus*	103247081
	Ma's night monkey	*Aotus nancymaae*	105711412
	Northern white-cheeked gibbon	*Nomascus leucogenys*	100594202
	Olive baboon	*Papio anubis*	100997952
	Pig-tailed macaque	*Macaca nemestrina*	105481609
	Pygmy chimpanzee	*Pan paniscus*	100971926
	Sooty mangabey monkey	*Cercocebus atys*	105582156
	Sumatran orangutan	*Pongo abelii*	100441160
	Sunda flying lemur	*Galeopterus variegatus*	103588840
	Western gorilla	*Gorilla gorilla*	101151325
	White-headed capuchin monkey	*Cebus capucinus imitator*	108286755
	White-tufted-ear marmoset	*Callithrix jacchus*	103792807
Proboscidea	African elephant	*Loxodonta africana*	100668639
Rodentia	Alpine marmot	*Marmota marmota marmota*	107143918
	American beaver	*Castor canadensis*	109686520
	Damara mole-rat	*Fukomys damarensis*	104853177
	Degu	*Octodon degus*	101565240
	Kangaroo rat	*Dipodomys ordii*	105981455
	Long-tailed chinchilla	*Chinchilla lanigera*	102005135
	Naked mole-rat	*Heterocephalus glaber*	101717034
	Thirteen-lined ground squirrel	*Spermophilus tridecemlineatus*	101971577
Scandentia	Chinese tree shrew	*Tupaia chinensis*	102500815
Tubulidentata	Aardvark	*Orycteropus afer afer*	103191804

in granulosa-luteal cells of humans (58). Likewise, *GnRH2* mRNA and protein levels increased in human neuronal cells in response to cAMP treatment, a downstream messenger of the gonadotropins (59). This effect probably occurs at the transcriptional level given that mutation of a putative cAMP-responsive element in the 5′ flanking sequence of the human *GnRH2* gene suppressed activity of the *GnRH2* promoter (59). However, when normal and cancerous ovarian cells were treated with LH and FSH, *GnRH2* expression was reduced in the majority of cell lines tested; only CaOV-3 and SKOV-3 cells were unaffected by treatment, despite expression of receptors for the gonadotropins (47). GnRH2 may mediate its own expression in an autocrine/paracrine manner. For example, granulosa cells secrete GnRH2 (60) and culture of human granulosa-luteal cells for 10 days increased *GnRH2* mRNA expression (56). A different study demonstrated that treatment of luteinized granulosa cells with GnRH2 downregulated *GnRH2* expression (58).

Prepro-GnRH2

The porcine prepro-GnRH2 is only 56% homologous to prepro-GnRH1 (NCBI accession numbers XP_005672842 and NP_999439, respectively) but contains the same components: a signal sequence, the decapeptide, a conserved cleavage site, and GAP (20). As with all peptide hormones, the signal

TABLE 3 | Production of GnRH2 and GnRHR2 in mammalian tissues.[a]

Tissue or cell type	GnRH2		GnRHR2		Reference
	Identified[b]	Species	Identified[b]	Species	
Central nervous system					
Brain (whole)	+	h	+	h	(11, 20, 29)
Forebrain[c]	+	h, r	+	h, r, m	(16, 20, 29)
Midbrain[c]	+	h, r	+	h, r, m	(16, 20, 29)
Hindbrain[c]	+	r	+	h, r, m	(16, 20, 29)
Spinal cord	+	h	+	h, m	(20, 29)
Endocrine					
Hypothalamus	+	p	+	m	(29, 35)
Pituitary (whole)	+	h	+	m, h	(11, 20, 29)
Anterior pituitary	+	p	+	p	(35)
Adrenal gland	+	h	+	h, m	(11, 20, 29)
Pancreas	+	h	+	h, m	(16, 20, 29)
Thyroid	+	h	+	h, m	(11, 20, 29)
Thoracic					
Heart	+	h	+	h, m	(11, 16, 20, 29)
Aorta	+	h			(20)
Lung	+	h	+	h, m	(11, 20, 29)
Thymus gland	+	h	+	h, m	(11, 20, 29)
Trachea	+	h			(20)
Digestion and metabolism					
Salivary gland	+	h			(20)
Stomach	+	h	+	h, m	(11, 20, 29)
Small intestine	+	h	+	h	(11, 20)
Duodenum	–	h			(43)
Jejunum	+	h			(43)
Ileum	+	h			(43)
Large intestine	+	h	+	h	(11, 20)
Cecum	+	h			(43)
Colon	–	h			(43)
Rectum	–	h			(43)
Liver	+	h	+	h, m	(11, 16, 20, 29)
Skeletal muscle	+	h	+	m, h	(11, 16, 20, 29)
Renal					
Bladder	+	h	+	m	(20, 29)
Kidney	+	h	+	h, m	(11, 16, 20, 29)
Immune					
Peripheral leukocyte	+	h			(20)
T lymphocyte	+	h			(39)
Lymph node	+	h			(20)
Tonsil	+	h			(20)
Bone marrow	+	h			(20)
Spleen	+	h	+	h, m	(11, 20, 29)
Female reproductive					
Ovary	+	h	+	h, m	(11, 20, 29)
Ovarian surface epithelial cells	+	h			(40, 41)
Granulosa cells	+	h			(41)
Oviduct			+	m	(29)
Uterus	+	h	+	h, m	(11, 20, 29)
Endometrium	+	h			(20)
Myometrium	+	h	+	h	(44)
Breast/mammary	+	h	+	h, m	(11, 20, 29, 38)
Placenta	+	h	+	h	(11, 20)
Male reproductive					
Testis	+	h, p	+	h, m, p	(11, 20, 29, 35, 45)
Leydig cells	+	p	+	p	(35, 45)
Sertoli cells	+	p	+	p	(35, 45)
Germ cells	+	p	+	h, p	(35, 45, 46)
Spermatozoa			+	h, p	(34, 45, 46)
Epididymis	+	p	+	m, p	(29, 34)
Seminal vesicles	+	p	+	m, p	(29, 34)
Bulbourethral	+	p	+	p	(34)
Prostate	+	h, p	+	h, m, p	(11, 20, 29, 34)

[a]*Adapted from Millar (16). Blank cells indicate tissues that have not yet been examined.*

[b]*Either mRNA or protein was discovered.*

[c]*See Millar (16) for specific regions of the brain that produce GnRH2 and/or GnRHR2.*

Abbreviations: +, positive; –, negative; p, pig; h, human; m, marmoset; r, rhesus macaque.

sequence directs the hormone to the secretory pathway (61). Interestingly, the signal sequence of prepro-GnRH1 and prepro-GnRH2 are dissimilar in the pig (**Figure 1C**). This distinction could be important because composition of the signal sequence has been reported to influence the efficiency of secretion (61). The cleavage site (Gly–Lys–Arg) is conserved between prepro-GnRH2 and prepro-GnRH1 (**Figure 1C**), indicating that carboxypeptidase E, the enzyme responsible for cleaving GnRH1 from GAP in mice (62), likely processes prepro-GnRH2 as well.

To date, the GAP of prepro-GnRH2 has not been studied directly. However, the GAP of prepro-GnRH1 is secreted with the mature decapeptide (63) and has been associated with prolactin and gonadotropin secretion (64, 65). Prepro-GnRH1 and prepro-GnRH2 in the human have similar lengths except the GAP, which is 50% longer in prepro-GnRH2 (20). Porcine prepro-GnRH2 (110 amino acids) is also longer than prepro-GnRH1 (91 amino acids), primarily due to a longer GAP (73 versus 55 amino acids, respectively; **Figure 1C**). A similar result was also reported for the tree shrew (66), indicating that a longer GAP in prepro-GnRH2 may be common in mammals (20) and could have functional relevance.

Notably, White et al. (20) reported the presence of two GnRH2-GAP variants in humans. Certain tissues (e.g., fetal brain and thalamus) expressed a longer GAP variant than others (e.g., kidney) (20). Likewise, Cheon et al. (67) discovered two transcript variants of *GnRH2* with differing GAP lengths in the endometrium of women. The porcine *GnRH2* gene is also predicted to produce two forms of prepro-GnRH2. The classical product is 110 amino acids (NCBI accession number XP_005672842), whereas the splice variant encodes a 143 amino acid isoform (NCBI accession number XP_013840618), due to the retention of intron 2. The only differences between the two products were detected in the GAP region (**Figure 1C**). Although, the biological significance of these variants of GAP for prepro-GnRH2 has not yet been elucidated.

TABLE 4 | Production of GnRH2 and GnRHR2 in mammalian cell lines.

Cell line	Origin	Species	GnRH2[a]	GnRHR2[a]	Reference
Nervous					
TE671	Neuronal medulloblastoma	Human	+		(41)
Lung					
A549	Alveolar adenocarcinoma	Human		+	(11)
Digestive					
SW480	Colorectal adenocarcinoma	Human		+	(11)
IPEC-J2	Intestinal epithelial cells	Porcine		+	(48)
Immune					
HL-60	Promyelocytic leukemia	Human		+	(11)
Jukat	T cell leukemia	Human	+	+	(39, 49)
Mammary					
MDAMB-231	Breast adenocarcinoma	Human	+		(38)
MCF-7	Breast adenocarcinoma	Human	+		(38)
MCF-10A	Breast epithelium	Human	+		(38)
Female reproductive					
HeLa	Cervical adenocarcinoma	Human		+	(11, 50)
Hec-1A	Endometrial adenocarcinoma	Human		+	(51)
Hec-1B	Endometrial adenocarcinoma	Human			(52)
Ishikawa	Endometrial adenocarcinoma	Human		+	(51)
HHUA	Endometrial adenocarcinoma	Human		+	(49)
EFO-21	Ovarian cystadenocarcinoma	Human		+	(51)
EFO-27	Ovarian adenocarcinoma	Human		–	(52)
OVCAR-3	Ovarian adenocarcinoma	Human	+	+	(40, 51)
SK-OV-3	Ovarian adenocarcinoma	Human	+	+	(40, 51)
CaOV-3	Ovarian adenocarcinoma	Human	+		(40)
BG-1	Ovarian adenocarcinoma	Human	+	+	(47, 52)
IOSE-29	Ovarian surface epithelia	Human	+		(40)
SVOG-4O	Granulosa-luteal cells	Human	+		(41)
SVOG-4m	Granulosa-luteal cells	Human	+		(41)
Male reproductive					
ST	Fetal testis	Porcine		+	(53)
ALVA-41	Prostate adenocarcinoma	Human		+	(50)
PPC-1	Prostate adenocarcinoma	Human		+	(50)
DU-145	Prostate carcinoma	Human		+	(49, 50)
Urinary					
TSU-Pr1	Bladder carcinoma	Human		+	(49, 50)
COS-1[b]	Kidney	African green monkey		+	(11)
HEK293	Embryonic kidney	Human		+	(50)

[a]*The presence of a (+) indicates that either mRNA or protein has been identified, whereas a (–) specifies that the tissue was negative. Blanks designate cell lines that have not yet been examined.*

[b]*The presence of GnRHR2 protein is putative as GnRH2 treatment of COS-1 cells yielded IP accumulation.*

The Structure of GnRH2

GnRH2 (pGlu–His–Trp–Ser–His–Gly–Trp–Tyr–Pro–Gly–NH_2) differs from GnRH1 by three amino acids [His^5, Trp^7, and Tyr^8; (12)], resulting in 70% sequence identity (20). Amino acids His^5, Trp^7, and Tyr^8 help stabilize GnRH2, whereas the N-terminal ($pGlu^1$, His^2, Trp^3, and Ser^4) and C-terminal (Pro^9, $Gly–NH_2{}^{10}$) residues are essential for receptor binding and activation (68). Structurally, GnRH2 is more negatively charged and slightly bulkier than GnRH1 (30). GnRH2 has a β-turn conformation that is similar to GnRH1; however, GnRH2 exists in a preconfigured conformation. Thus, GnRH2 does not require extensive conformational changes for receptor activation (68). The conformation of GnRH2 may render it less sensitive to peptidases (69), likely increasing (6-fold) its stability (70) and half-life (71, 72) compared with GnRH1, which is rapidly degraded (73).

GONADOTROPIN-RELEASING HORMONE 2 RECEPTOR

Identification of GnRHR2 in Mammals

Originally cloned in African catfish (74), a 7-transmembrane (TM) G protein-coupled receptor (GPCR) specific to GnRH2 (GnRHR2) has also been discovered in mammals (11, 29). The *GnRHR2* gene was first identified in mammals by surveying the human genome for genes with high homology to *GnRHR1* (11, 29). A gene with 40% homology to *GnRHR1* was identified

but it was more similar to *GnRHR2* in fish (65% identity) and amphibians (55% identity), suggesting the identification of a human *GnRHR2* gene (11). Mammals only maintain genes for *GnRHR1* and *GnRHR2*, although a *GnRHR3* gene has been discovered in other vertebrates (16).

The Gene for GnRHR2

The human, chimpanzee, and rhesus macaque *GnRHR2* gene is located on chromosome 1 (21). In other species, however, the gene for *GnRHR2* is located on chromosome 17 (dog), chromosome 5 (horse), chromosome 3 [cow; (21)], and chromosome 4 [pig; (21, 75)]. The *GnRHR2* gene is closely associated in the antisense orientation with the *RBM8A* and *PEX11B* genes in all species (21, 22, 76). In humans, the promoter regions of *GnRHR2* and *PEX11B* overlap and the 3′ untranslated region (UTR) of *RBM8A* overlaps with two exons of the *GnRHR2* gene (76, 77). The *RBM8A* gene encodes RNA-binding motif protein 8A, which helps modulate the post-translational regulation of gene expression (78) and the *PEX11B* gene codes for peroxisomal membrane protein 11β, which is involved in the regulation of peroxisome abundance (79). In the pig, the *RBM8A* gene is located 423 bp downstream of the *GnRHR2* gene (**Figure 2A**). The 3′ UTRs of these two genes overlap, in opposite orientation on complementary strands. Upstream of the *GnRHR2* gene, the *PEX11B* gene is also located on the antisense strand in the opposite orientation (**Figure 2A**). The proximal promoters of these two genes overlap, with start codons 616 bp apart (75).

The porcine *GnRHR2* gene has three exons and two introns (**Figure 2B**). Exon 1 (530 bp) of the porcine *GnRHR2* gene encodes the extracellular N-terminus, TM domains 1–3, part of TM domain 4, intracellular loop (ICL) 2, and extracellular loop (ECL) 1. Exon 2 (215 bp) encodes the remainder of TM4 as well as TM5, ECL2, and part of ICL3. Exon 3 (450 bp) encodes the remainder of ICL3, TM6–7, ECL3, and the C-terminal tail (**Figure 2B**). Intron 1 of the porcine *GnRHR2* gene is relatively large (2.8 kb) compared with intron 2 (470 bp; **Figure 2B**), similar to reports from other species (21).

An additional truncated *GnRHR2* gene has also been discovered on chromosome 14 in humans (80), containing only exons 2 and 3 (46). Interestingly, exons 2 and 3 of this apparent pseudogene are 100% identical to the corresponding exons of the complete *GnRHR2* gene on chromosome 1 (80). This distinction may be especially critical when designing primers/probes for analysis of *GnRHR2* gene expression in human tissues. The truncated *GnRHR2* gene (chromosome 14) is more transcriptionally active and widely expressed than the full-length gene [chromosome 1; (46)]. This could explain discrepancies in *GnRHR2* mRNA levels of human tissues across studies performed prior to this finding; Neill et al. (11) utilized a riboprobe specific to exon 3, whereas Millar et al. (29) used a DNA probe specific to exon 1 (46). Consistent with the full-length gene on chromosome 1, this truncated *GnRHR2* gene is flanked by *RBM8A*, indicating it was duplicated from the chromosome 1 locus *via* retrotransposition (77). In fact, transcripts

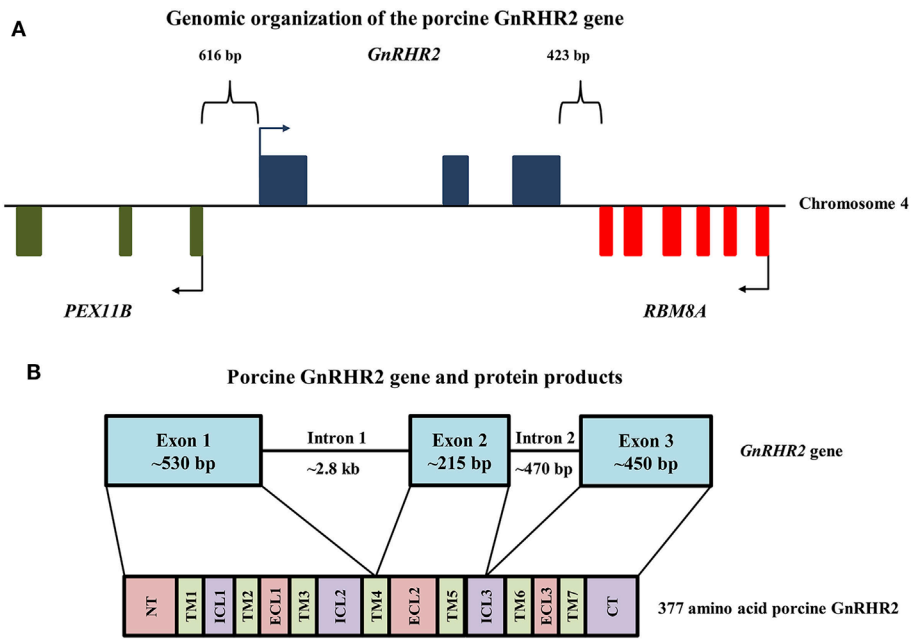


FIGURE 2 | Genomic organization of the porcine GnRHR2 gene on chromosome 4. **(A)** The *PEX11B* gene (green) is located 616 bp upstream, whereas the *RBM8A* gene (red) is positioned 423 bp downstream of the *GnRHR2* gene (blue). Arrows indicate start codons for each gene. Reprinted from Brauer et al. (75) with permission from Elsevier. **(B)** The *GnRHR2* gene contains 3 coding exons and 2 introns, yielding a 377 amino acid product. Exon 1 of the porcine *GnRHR2* gene encodes the extracellular N-terminus (NT), transmembrane (TM) domains 1–3, part of TM4, intracellular loops (ICL) 1–2 and extracellular loop (ECL) 1. Exon 2 encodes the remainder of TM4 as well as TM5, ECL2, and part of ICL3. Exon 3 encodes the remainder of ICL3, TM domains 6–7, ECL3, and the C-terminal tail (CT). Note that introns and exons are not drawn to scale.

of this truncated gene contain exon 2 and 3, retain intron 2, and also include the 3′ UTR of *RBM8A*, except in the antisense orientation (46). Likewise, the genome of the African elephant contains a *GnRHR2* pseudogene with several point mutations/deletions and lacking exon 1 (21).

Presence of the GnRHR2 Gene in Mammals

Using bioinformatics, the *GnRHR2* gene was identified in the genomes of 22 mammalian species, although sequence analysis revealed that gene disruptions occurred in 12 of these species [**Table 1**; (21)]. Early evidence implied that the *GnRHR2* gene was functional in the human, sheep, and mouse as immunostaining for GnRHR2 was detected in brain tissue of these species (29). However, the ovine *GnRHR2* gene contains a premature stop codon in exon 1 and a 51 bp deletion in exon 2, preventing translation of a full-length protein (26, 81). In humans (and chimpanzees), a frameshift mutation occurs due to a missing nucleotide (compared with the marmoset) in the 5′ flanking sequence. A premature stop codon is also present due to a single base change in an arginine codon in exon 2 (26, 81). Furthermore, the *GnRHR2* gene was subsequently determined to be absent from chromosome 3 in the mouse genome (21, 22) and only a remnant of exon 1 remains on chromosome 2 in the rat (22, 30). In other species, the *GnRHR2* gene likely encodes a non-functional protein as well. The bovine *GnRHR2* gene is disrupted by frameshift mutations in all three exons, in addition to premature stop codons in exons 2 and 3 (26). The squirrel *GnRHR2* gene contains a premature stop codon and an amino acid substitution, whereas the guinea pig has frameshift mutations in all three exons and two premature stop codons in exon 3 (21). The rabbit gene has a 14 bp deletion that results in a frame shift and premature stop codon, and the cat gene has a premature stop codon (21). In addition, the *GnRHR2* genes in the common shrew, dolphin, horse, and dog harbor several frameshifts and premature stop codons (21). In fact, only eight species (orangutan, African green monkey, rhesus macaque, marmoset, tree shrew, kangaroo rat, pig, and elephant) possess the appropriate gene sequence to produce a functional GnRHR2 [**Table 1**; (21)].

Our laboratory surveyed the updated NCBI gene databases to re-evaluate which mammals maintain the *GnRHR2* gene. The *GnRHR2* gene was confirmed in the genomes of 61 additional species representing 11 more orders (**Table 5**). Based on this report and Stewart et al. (21), the gene for *GnRHR2* is present in the genomes of 83 species to date, encompassing 22 different mammalian orders. However, it is unclear if the gene is functional or silenced in these animals. Therefore, future bioinformatics work is needed to examine the characteristics of the *GnRHR2* gene within these species.

Coupled with the aforementioned *GnRH2* gene distribution data, it is clear that few mammalian species have a functional GnRH2–GnRHR2 system. The maintenance of only part of the system in some species (e.g., ligand or receptor only) assumes interaction with the GnRH1–GnRHR1 system to maintain functionality. Millar et al. (18) proposed that the presence of GnRH2, but not a functional GnRHR2, in some species

TABLE 5 | Identification of the GnRHR2 gene within 61 additional mammals *via* NCBI gene database queries.

Order	Common name	Genus species	NCBI gene ID
Afrosoricida	Cape golden mole	*Chrysochloris asiatica*	102817241
	Lesser hedgehog tenrec	*Echinops telfairi*	101659402
Artiodactyla	Alpaca	*Vicugna pacos*	102526131
	Arabian camel	*Camelus dromedaries*	10510588
	Bactrian camel	*Camelus bactrianus*	105079482
	Bison	*Bison bison bison*	104983739
	Goat	*Capra hircus*	102184212
	Texas white-tailed deer	*Odocoileus virginianus texanus*	110144360
	Wild bactrian camel	*Camelus ferus*	102518691
	Wild yak	*Bos mutus*	102266467
	Zebu cattle	*Bos indicus*	109555832
Carnivora	Amur tiger	*Panthera tigris altaica*	102969203
	European domestic ferret	*Mustela putorius furo*	101687337
	Giant panda	*Ailuropoda melanoleuca*	100484454
	Pacific walrus	*Odobenus rosmarus*	101379215
	Polar bear	*Ursus maritimus*	103678407
	Weddell seal	*Leptonychotes weddellii*	102742574
Cetacea	Killer whale	*Orcinus orca*	101271242
	Minke whale	*Balaenoptera acutorostrata scammoni*	103006588
	Sperm whale	*Physeter catodon*	102982977
	Yangtze river dolphin	*Lipotes vexillifer*	103074337
Cingulata	Nine-banded armadillo	*Dasypus novemcinctus*	101438616
Dasyuromorphia	Tasmanian devil	*Sarcophilus harrisii*	100925975
Didelphimorphia	Gray short-tailed opossum	*Monodelphis domestica*	100014969
Diprotodontia	Koala	*Phascolarctos cinereus*	110219988
Erinaceomorpha	Western European hedgehog	*Erinaceus europaeus*	103127802
Eulipotyphla	Star-nosed mole	*Condylura cristata*	101623053
Macroscelidea	Cape elephant shrew	*Elephantulus edwardii*	102866982
Perissodactyla	Donkey	*Equus asinus*	106847655
	Przewalski's horse	*Equus przewalskii*	103556230
	Southern white rhinoceros	*Ceratotherium simum simum*	101400748
Pholidota	Malayan pangolin	*Manis javanica*	108400376
Primates	Angolan colobus	*Colobus angolensis*	105518331
	Black snub-nosed monkey	*Rhinopithecus bieti*	108531432
	Bolivian squirrel monkey	*Saimiri boliviensis*	101045352
	Coquerel's sifaka monkey	*Propithecus coquereli*	105809593
	Crab-eating macaque	*Macaca fascicularis*	102142398
	Drill	*Mandrillus leucophaeus*	105550280
	Golden snub-nosed monkey	*Rhinopithecus roxellana*	104671044
	Green monkey	*Chlorocebus sabaeus*	103225877
	Ma's night monkey	*Aotus nancymaae*	105730634
	Mouse lemur	*Microcebus murinus*	109730387

(*Continued*)

TABLE 5 | Continued

Order	Common name	Genus species	NCBI gene ID
	Northern white-cheeked gibbon	*Nomascus leucogenys*	100593225
	Olive baboon	*Papio anubis*	101008793
	Pig-tailed macaque	*Macaca nemestrina*	105479042
	Pygmy chimpanzee	*Pan paniscus*	100995783
	Small-eared galago	*Otolemur garnettii*	100963950
	Sooty mangabey monkey	*Cercocebus atys*	105592861
	Sumatran orangutan	*Pongo abelii*	100460080
	Sunda flying lemur	*Galeopterus variegatus*	103595864
	Tarsier	*Tarsius syrichta*	103268450
	Western gorilla	*Gorilla gorilla*	101137503
	White-headed capuchin monkey	*Cebus capucinus imitator*	108293668
	White-tufted-ear marmoset	*Callithrix jacchus*	100399755
Rodentia	Alpine marmot	*Marmota marmota marmot*	107160395
	American beaver	*Castor canadensis*	109682308
	Damaraland mole rat	*Fukomys damarensis*	104865794
	Degu	*Octodon degus*	101580342
	Naked mole-rat	*Heterocephalus glaber*	101703276
Scandentia	Chinese tree shrew	*Tupaia chinensis*	102498810
Sirenia	Florida manatee	*Trichechus manatus latirostris*	101357387
Tubulidentata	Aardvark	*Orycteropus afer afer*	103203244

signifies that GnRHR1 has adopted a dual role for the actions of GnRH1 and GnRH2 through alternative ligand conformations and downstream signaling events. In species that produce both GnRH2 and its receptor, however, this system was likely critical to survival to have avoided gene inactivation throughout evolution.

Does the Human Produce the GnRHR2?

When GnRHR2 was first discovered in mammals, GnRHR2-specific immunostaining was detected in the human brain (29), implying that humans produce a full-length GnRHR2. However, it was later discovered that coding errors likely interrupt successful translation of human *GnRHR2* mRNA (21). Indeed, Neill et al. (82) reported the inability to identify translatable *GnRHR2* transcripts that would yield a full-length receptor in humans, at least *via* conventional mechanisms. Recall, the human *GnRHR2* gene contains a frameshift mutation in exon 1 and a premature stop codon in exon 2 (76, 82). Yet, the gene remains transcriptionally active and produces transcript variants due to alternative splicing, suggesting functionality as most pseudogenes are promoterless (76). These conflicting results have been the subject of much research and debate.

Despite the apparent gene coding errors, there is evidence for functionality of the GnRHR2 in humans (82). For example, use of a GnRHR1 antagonist mitigated the effects of GnRH1, but not GnRH2, in human decidual stromal (83) and trophoblast (84) cells. GnRH2, and not GnRH1, effectively suppressed proliferation of SK-OV-3 cells containing only *GnRHR2* mRNA (51). In human cancer cells with reduced GnRHR1 levels, GnRH2 (not GnRH1) retained the ability to inhibit cell proliferation (52). Small interfering RNA targeting *GnRHR1* inhibited the actions of GnRH1 on trophoblast invasion, but GnRH2-mediated effects persisted (84). GnRH1 and GnRH2 also have differing effects in primary cultures of human decidual stromal cells; GnRH1 increased whereas GnRH2 suppressed mRNA and protein levels (83). Although the results of the latter study could be related to divergent signaling of GnRH2 at the GnRHR1, these data ultimately support the presence of a functional GnRHR2 in humans.

Many hypotheses have arisen for how the disrupted *GnRHR2* gene may retain functionality including: (1) counteractive shifts in the reading frame, (2) recoding of stop codons, (3) alternative splicing, (4) alternative protein translation, or (5) production and functionality of GnRHR2 fragments (82). The mechanisms that could potentially yield a functional receptor from a seemingly non-translatable mRNA sequence were reviewed by Neill et al. (82). First, a corrective shift in the reading frame, albeit rare, has been demonstrated in eukaryotes, allowing for the production of a full-length protein (85). Indeed, Millar et al. (18) isolated human *GnRHR2* transcripts missing the stop codon. Second, the use of an alternative start codon (e.g., GUG instead of AUG) has also been considered. In humans, a GUG codon is downstream of the frameshift and would yield a 5-TM receptor with a truncated (22 amino acids) N-terminus. Interestingly, a similar phenomenon occurs in the translation of African green monkey *GnRHR2* mRNA (11, 21, 82). Furthermore, the GUG codon within the human transcript meets the two Kozak criteria necessary for an alternative start codon (86, 87). Third, it has been proposed that the premature stop codon may be recoded (82), which occurs in mammals (85, 88, 89). In the human *GnRHR2* mRNA transcript, the premature stop codon is UGA but this codon can also encode for the amino acid, selenocysteine, potentially preventing the termination of translation (16, 46, 76, 80). However, attempts to identify selenocysteine incorporation thus far have been fruitless (76). Moreover, alternative splicing in exon 1 would yield a 5-TM *GnRHR2* transcript without the reading frame disruption near the N-terminus of the 7-TM isoform (82). The premature stop codon, however, would still be present. Therefore, translation of 5-TM receptor mRNA would also require a stop codon read-through. A fourth hypothesis is that fragments of both the 5- and 7-TM isoforms are produced and reassociate non-covalently after translation, a characteristic that has been shown with other GPCRs, including GnRHR1 (90–92). Data from Grosse et al. (92) showed that two coexpressed GnRHR1 fragments (corresponding to TM domains 1–5 and 6–7) reassociated to produce a full-length, functional receptor. If protein fragments of the 7-TM GnRHR2 isoform (containing TM domains 1–4 and 6–7) reassociated, they would form a 6-TM isoform. However, it is unclear if a 6-TM receptor would be functional (82).

The final theory is that GnRHR2 fragments are successfully produced and modulate GnRHR1 activity (82). This hypothesis is seemingly supported by the work of Pawson et al. (93) who demonstrated that a GnRHR2 protein fragment (termed the GnRHR2 reliquium) inhibits post-translational GnRHR1 protein

abundance *via* interactions with GnRHR1 inside the nucleus, endoplasmic reticulum, and/or Golgi apparatus (93). This fragment spans the cytoplasmic end of the 5-TM domain to the carboxyl terminus of the full-length receptor and would be produced by the aforementioned alternative start site (GUG) downstream of the premature stop codon (93). Interestingly, others have reported that GnRHR1 fragments with similar domains (TM6–7) suppress GnRH-mediated signaling when coexpressed with the full-length GnRHR1 (92). This theory is supported by early work revealing GnRHR2 immunostaining in human tissues with an antibody (ZGRH-II-5) directed against ECL3 (29), which is retained in the GnRHR2 reliquium (93). Likewise, the ovine *GnRHR2* gene may produce a GnRHR2 reliquium as well, given that ZGRH-II-5 antiserum also revealed GnRHR2 immunostaining in the sheep brain (93). In contrast, a different antibody directed against ECL2 (absent in the GnRHR2 reliquium) failed to indicate GnRHR2 immunostaining in sheep (93). Notably, mRNA that encodes for the GnRHR2 reliquium has been successfully identified in many human organs and cell lines, suggesting that this protein fragment may be physiologically relevant (76). In addition, when the full-length cDNA sequence for human *GnRHR2* (including frame shift and premature stop codon) is transiently transfected into COS cells, expression of *GnRHR1* is enhanced (18). Thus, it is plausible that a functional (albeit unconventional) GnRHR2 is produced in humans. To date, however, the controversy remains unresolved and actions of GnRH2 in humans are predominantly ascribed to GnRHR1 signaling.

Characterization of the Mammalian GnRHR2 Gene Promoter

While transcriptional regulation of the *GnRHR1* gene has been evaluated in mice (94), rats (95), humans (96), sheep (97), and pigs (98), much less is known regarding the regulation of *GnRHR2* gene expression. The *GnRHR2* gene is transcriptionally active in several mammals, including humans, sheep, monkeys, marmosets, musk shrews, and pigs (11, 32, 46, 81, 82, 99). However, the regulatory elements governing the expression of this gene have only been studied in the marmoset and pig (75, 99). Utilizing luciferase reporter constructs containing either the *GnRHR1* or *GnRHR2* pig promoter in transient transfection assays with cell lines from several tissues, the *GnRHR2* promoter was active in all cell types examined, whereas activity of the *GnRHR1* promoter only exceeded the promoterless control in gonadotrope-derived αT3-1 cells [**Figure 3**; (75)]. Initial studies in immortalized swine testis (ST) cells revealed that activity of the porcine *GnRHR2* promoter was partially conferred by nuclear factor-κB, specificity protein 1 and 3 (SP1/3), and overlapping early growth response 1/SP1/3 (EGR1/SP1/3)-binding sites (75). The EGR1 and SP1/3-binding sites are located in a region of the 5′ UTR that is highly conserved compared with the marmoset *GnRHR2* promoter and previously shown to enhance promoter

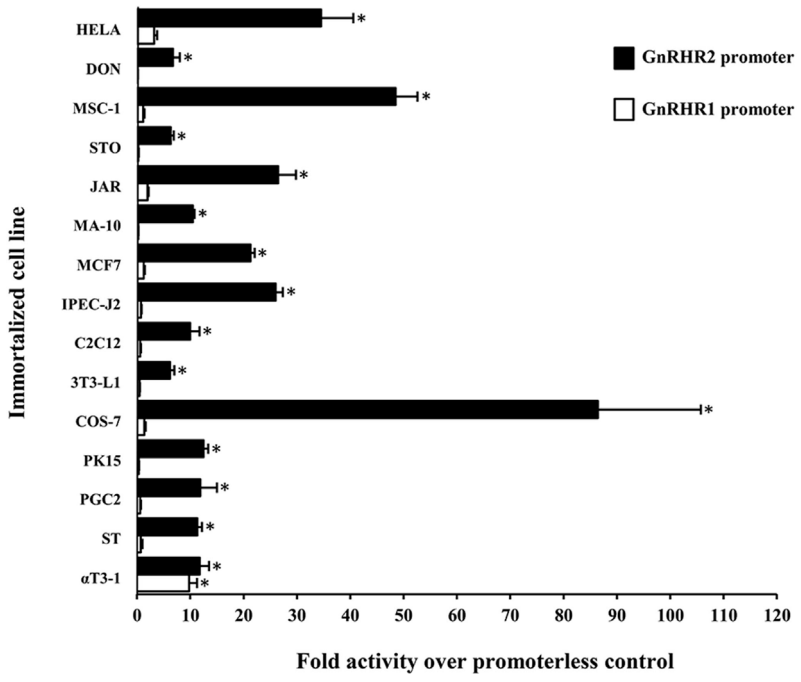


FIGURE 3 | Activity of the porcine GnRHR1 and GnRHR2 gene promoters within different mammalian cell lines. Cells were transiently transfected with either the porcine *GnRHR1* (white) or *GnRHR2* (black) gene promoter in a luciferase reporter vector and expressed as fold activity over the promoterless control. Abbreviations: HELA, human cervical cancer; DON, Chinese hamster lung; MSC-1, mouse Sertoli cell; STO, mouse embryonic fibroblast; JAR, human placenta; MA-10, mouse Leydig cell; MCF7, human breast cancer; IPEC-J2, piglet intestinal epithelium; C2C12, mouse muscle myoblast; 3T3-L1, mouse adipocyte fibroblast; COS-7, green monkey kidney; PK15, porcine kidney; PGC-2, porcine granulosa cell; ST, swine testis; and αT3-1, murine gonadotrope. An asterisk indicates Least Squares Means that are significantly greater than the promoterless control ($P < 0.05$). Reprinted from Brauer et al. (75) with permission from Elsevier.

activity (99). Given the ubiquitous expression of *GnRHR2*, it was not surprising that a transcription factor such as SP1, also considered to be widely produced, would be involved in regulation. This contrasts greatly from the three steroidogenic factor 1-binding elements required for basal expression of the porcine *GnRHR1* gene in the gonadotrope-derived αT3-1 cell line (98). So, in accord with divergent expression patterns of the *GnRHR1* and *GnRHR2* genes, their transcription is differentially regulated as well.

The GnRHR2 Gene Is Ubiquitously Expressed in Mammals

Similar to GnRH2, the *GnRHR2* gene is widely expressed throughout the body (11, 29). Within the brain, the GnRHR2 was found in the forebrain, midbrain, and hindbrain (16). Expression was pronounced in areas that regulate sexual behavior, such as the putamen, occipital lobe, cerebellum, and caudate nucleus, but reduced within the anterior pituitary gland (29). In addition, *GnRHR2* mRNA was also found in peripheral tissues including the heart, stomach, intestine, kidney, spleen, skeletal muscle, thymus, lung, liver, pancreas, adrenal, thyroid, placenta, uterus, ovary, breast, seminal vesicles, epididymis, prostate, and testis [**Table 3**; (11, 29)]. Relative to *GnRHR2* mRNA amounts in the pituitary, expression levels were lowest in the marmoset bladder and highest in the testis (29). Likewise, van Biljon et al. (46) detected strong *GnRHR2* signal in the human testis *via in situ* hybridization and our laboratory reported abundant GnRHR2 protein levels in the testis compared with the anterior pituitary gland of the pig (35). The receptor has been found in reproductive cancer cell lines derived from the prostate, cervix, endometrium and ovary, as well as cell lines produced from other organ systems (e.g., respiratory, digestive, mammary, immune, and urinary; **Table 4**). GnRHR2 was also identified by our laboratory in cell lines derived from porcine intestine and testis [**Table 4**; (48)]. Our group has also detected *GnRHR2* mRNA in various porcine tissues (e.g., testis, anterior pituitary, spleen, liver, large intestine, small intestine, and stomach) using conventional PCR (48).

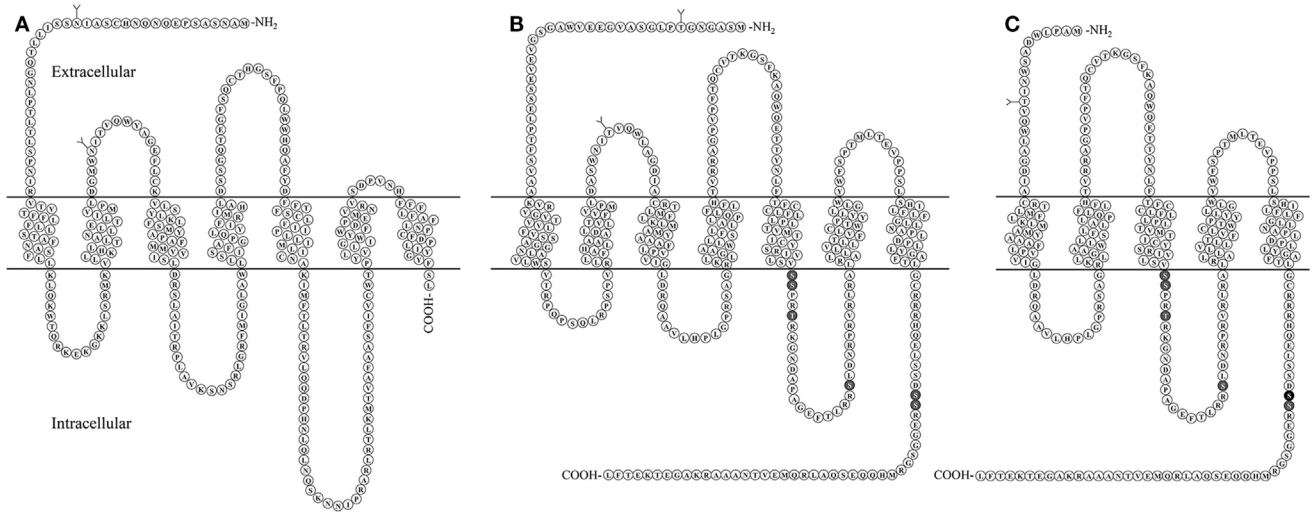
The Structure of GnRHR2

The full-length porcine GnRHR2 is 377 amino acids (NCBI accession number AAS68622.1) and has 42% homology to GnRHR1, which is 328 amino acids in length (**Table 6**; **Figures 4A,B**; NCBI accession number NP_999438.1). Like GnRHR1, the GnRHR2 is a member of the rhodopsin superfamily of GPCRs containing an extracellular N-terminus as well as seven TM α-helical domains connected *via* three ECLs and three ICLs [**Figures 4A,B**; (74)]. Differences in amino acid number within the domains of GnRHR1 and GnRHR2 are depicted in **Figure 4D**. Strikingly, GnRHR2 maintains a 52 amino acid C-terminal tail that is uniquely absent in GnRHR1 but similar to non-mammalian GnRHRs [**Figures 4A,B**; (74)]. Cytoplasmic tails are common among GPCRs and promote rapid receptor internalization and desensitization (100, 101). For example, the monkey GnRHR2 fully desensitized to GnRH2 treatment after 60 min whereas the tail-less human GnRHR1 failed to desensitize to a GnRHR1 agonist (Triptorelin) during the entire sampling period [90 min; (80)]. The cytoplasmic tail contributes to internalization differences between GnRHR1 and GnRHR2 as well. GnRHR1 is internalized without interacting with β-arrestin, whereas GnRHR2 can utilize β-arrestin for internalization (102), although it is not absolutely required (**Table 6**) (103). The phosphorylation of serine residues 338 and 339 in the C-terminus by GPCR kinases is critical for β-arrestin-independent internalization of GnRHR2 (103), whereas other regions within the C-terminal tail or ICL3

TABLE 6 | Structural and functional characteristics of GnRHR1 and GnRHR2 in mammals.[a]

Characteristic	GnRHR2	GnRHR1	Reference
Structure			
Number of amino acids	377–380	327–328	(16, 74, 104)
5-transmembrane isoform	+	–	(80)
C-terminal tail	+	–	(16, 74, 104)
Amino acid conferring receptor activation in TM2 and TM7	Asp/Asp	Asn/Asp	(74, 105)
Amino acids conferring ligand selectivity in ECL3	Val–Pro–Pro–Ser	Leu–Ser–Asp/Glu–Pro	(106)
Relative-binding affinities of native peptides[b]			
GnRH1	1	15	(16)
GnRH2	24	1	(16)
Relative activities of native peptides[c]			
GnRH1	1	12	(16, 80)
GnRH2	90–440	1	(11, 16, 80)
Relative activities of GnRHR agonists[c]			
[D-Ala^6] GnRH1	1	309	(80)
[D-Ala^6] GnRH2	4	1	(104)
Buserilin	1	548	(80)
Triptorelin	1	395	(80)
Relative activities of GnRHR antagonists[c]			
Cetrorelix (SB-75)	1	>5,050	(80)
Trptorelix-1	1,660	1	(104)
Antide	1	>9,523	(80)
Antagonist 135-18	Full agonist	Full antagonist	(16)
Coupling and signaling			
$G_{\alpha q/11}$	+	+	(16)
Inositol phosphate (IP)	+	+	
Ca^{2+}	+	+	(16, 50)
Protein kinase C	+	+	(16)
ERK 1/2	+ (sustained)	+ (transient)	(16)
p38 MAPK	+	–	(16)
c-Jun N-terminal kinase	–	–	(16)
Mammalian homolog r-Src of Rous sarcoma virus (c-Src)	–	+	(16)
Receptor internalization			
Rapid desensitization	+	–	(16)
Internalization rate	Rapid	Slow	(16)
β-Arrestin-dependent[d]	–	–	(102, 103, 107)
Dynamin dependent[e]	+	–/+	(103, 107)
Clathrin mediated	+	+	(103, 107)

[a]*Adapted from Millar (16) and Cheng and Leung (108).*
[b]*Relative fold increase in affinity compared with the non-cognate ligand.*
[c]*Relative fold increase in activity compared with the non-cognate ligand or analog.*
[d]*Internalization of GnRHR2 can be mediated via β-arrestin but it is not required.*
[e]*GnRHR1 internalization is dependent on dynamin in the rat but not human.*
Abbreviations: TM, transmembrane; ECL, extracellular loop; ERK 1/2, extracellular signal-regulated kinase 1 and 2; MAPK, mitogen-activated protein kinase.



D

	NT	TM1	ICL1	TM2	ECL1	TM3	ICL2	TM4	ECL2	TM5	ICL3	TM6	ECL3	TM7	CT	Total
GnRHR1	38	20	19	20	18	22	27	20	28	20	49	19	6	20	2	328
GnRHR2	37	24	14	21	16	23	17	23	31	24	32	24	15	24	52	377
5-TM GnRHR2	21	-	-	-	-	23	17	23	31	24	32	24	15	24	52	286

FIGURE 4 | Structural comparison of GnRHRs in the pig. **(A)** GnRHR1 **(B)** GnRHR2 and **(C)** predicted 5-transmembrane (TM) GnRHR2 in the pig. GnRHR1 and GnRHR2 are both 7-TM G-protein coupled receptors; however, GnRHR1 lacks a C-terminal tail present in GnRHR2. The 5-TM GnRHR2 isoform lacks TM domains 1 and 2 due to alternative splicing in exon 1 of the *GnRHR2* gene, resulting in coupling of a truncated extracellular N-terminus directly to TM3. Glycosylation sites are represented by branched structures (Y) and the darkened residues in the GnRHR2 isoforms represent predicted sites of phosphorylation for internalization based on the work of Madziva et al. (102) and Ronacher et al. (103). Structures are predicted according to NCBI database records (GnRHR1, accession number NP_999438.1; GnRHR2, accession number AAS68622.1; 5-TM GnRHR2, accession number AAS68623.1). Adapted from Neill (80) and Neill et al. (82) with permission from Oxford University Press and Elsevier. **(D)** Comparison of residue number within each domain of GnRHR1, GnRHR2, and the predicted 5-TM GnRHR2 in the pig. Abbreviations: NT, extracellular N-terminus; TM, transmembrane; ICL, intracellular loop; ECL, extracellular loop; CT, C-terminal tail.

are sufficient for β-arrestin-dependent internalization (102). Therefore, two distinct pathways coordinate internalization of GnRHR2 (103). In addition, GnRHR2 internalization is dependent upon dynamin and likely mediated by both clathrin-coated pits and caveolae (103).

Other notable differences between the structure of GnRHR1 and GnRHR2 include alterations in residues that are highly conserved among GPCRs. GnRHR2 has an Asp/Asp microdomain in TM2 and 7, which is prevalent among GPCRs (74), whereas the GnRHR1 has a distinct Asn/Asp domain (**Table 6**). This region is thought to be important for receptor configuration and activation (105). Likewise, GnRHR2 has a divergent ligand-binding site (Val–Pro–Pro–Ser) within ECL3 compared with GnRHR1 (Leu–Ser–Asp/Glu–Pro; **Table 6**). This sequence probably determines the selectivity of GnRHR2 for GnRH2, as all other known ligand-binding sites are conserved between GnRHR1 and GnRHR2 (106). GnRHR2 and GnRHR1 also differ in the charge distributions of their extracellular domains, which may affect ligand binding (30).

A 5-Transmembrane GnRHR2 Isoform

A 5-TM GnRHR2 isoform has been identified in pigs [**Figure 4C**; (109)]. Transcripts from this isoform are produced *via* alternative splicing in exon 1 and a different start codon. Therefore, TM1 and 2 are absent and the truncated extracellular N-terminus, comprised primarily of residues from ECL1, couples directly to TM3 [**Figures 4C,D**; (82)]. Transcripts for the 5-TM isoform were originally isolated from porcine pituitaries (109) but we have also found the 5-TM *GnRHR2* transcript in various tissues of the boar (e.g., testis, spleen, liver, large intestine, small intestine, and stomach) *via* conventional RT-PCR (48). The 5-TM transcript possesses all the characteristics of a translatable protein (109, 110) and would yield a 286 amino acid receptor (**Figures 4C,D**). Functionality of the 5-TM GnRHR2 has not been characterized thus far, but there is precedence for biologically active 5-TM GPCRs (111–114). As noted previously, the human may also produce a 5-TM *GnRHR2* transcript; however, it retains an in-frame premature stop codon (82).

THE INTERACTION BETWEEN GnRH2 AND GnRHR2

Functionality and Ligand Selectivity of GnRHR2

Receptor binding (29) and inositol phosphate (IP) accumulation (11, 29) assays established the selectivity of GnRH2 for GnRHR2. The binding affinity of GnRH2 for GnRHR2 is 24-fold greater than for GnRHR1 [**Table 6**; (29)]. When COS-1 cells overexpressing porcine *GnRHR2* cDNA were treated with GnRH2 or

GnRH1, production of IP was stimulated with an EC_{50} of 0.5 nM for GnRH2 and 220 nM for GnRH1 (109). Conversely, the EC_{50} for GnRH2 binding the human GnRHR1 was 7.41 nM compared with 0.63 nM for GnRH1. The African green monkey (11) and marmoset GnRHR2 (29) were found to be functional and selective for GnRH2 as well. Thus, GnRH2 is 100- to 400-fold more active at the GnRHR2 than is GnRH1. In contrast, GnRH1 is only approximately 10-fold more active at GnRHR1 than is GnRH2 (**Table 6**).

Since GnRH1 and GnRH2 activate alternative GnRHRs, it became prudent to assess whether GnRH analogs are actually specific to their cognate receptors. However, it appears that GnRH1 and GnRH2 analogs, including GnRHR1 superagonists (Triptorelin and Buserilin), remain specific (**Table 6**) (80). At the GnRHR2, the native peptide (GnRH2) had an EC_{50} of 0.58 nM compared with 7.5 and 48 nM for Triptorelin and Buserilin, respectively. Therefore, GnRH2 is about 13- to 83-fold more potent at the GnRHR2 than are GnRHR1 superagonists (80). Non-native GnRH peptides (e.g., GnRH3) were considerably less potent (0.02%) than GnRH2 at eliciting IP production (80). Receptor specificity was also verified for GnRHR antagonists. Trptorelix-1 was identified as a GnRHR2-specific antagonist (104, 115), whereas Cetrorelix (also known as SB-75) and Antide are reported to be selective for GnRHR1 (80). For example, Antide mitigates both GnRH1- and GnRH2-induced IP production in cells overexpressing GnRHR1 but failed to ablate GnRH2-induced IP production in COS-7 cells overexpressing GnRHR2 (69). At elevated concentrations, however, Cetrorelix (SB-75) can non-specifically bind the GnRHR2 (50, 115). In addition, an established GnRHR1 antagonist (antagonist 135–18) was found to have agonistic properties at GnRHR2 (29). These data demonstrate the functionality and selectivity of the mammalian GnRHR2, but also indicate that both GnRHs can bind both mammalian receptors. However, GnRHR2 is highly selective for GnRH2, whereas GnRHR1 binds both ligands relatively well (16). Therefore, early studies examining the function of GnRH2 may be limited given that receptor binding was not addressed, and the actions of GnRH2 may have been inappropriately ascribed to GnRH1 (82).

Cell Signaling of GnRHR2

Like GnRHR1, GnRHR2 couples to $G_{\alpha q/11}$ to initiate the production of IP, calcium mobilization, and the activation of protein kinase C (PKC; **Table 6**) (16, 84, 116). After the activation of PKC, however, GnRH1 and GnRH2 differentially stimulate mitogen-activated protein kinases (MAPKs) (29). GnRH1 transiently activated extracellular signal-regulated kinases 1/2 (ERK 1/2) and the proto-oncogene tyrosine-protein kinase, Src (c-Src) in COS-7 cells overexpressing GnRHR1 [**Table 6**; (29)]. Conversely, GnRH2 did not activate c-Src; instead, the interaction between GnRH2 and GnRHR2 activated ERK 1/2 in a prolonged manner as well as p38 MAPK in COS-7 cells overexpressing GnRHR2 (**Table 6**) (16, 29). Neither GnRH1 nor GnRH2 activated c-Jun N-terminal kinase (JNK) *via* their cognate receptors [**Table 6**; (29)]. Therefore, GnRHR1 and GnRHR2 exhibit differential signaling upon binding to their respective ligands. Moreover, activation of GnRHR1 by GnRH2 initiates different signaling pathways than GnRH1 (18). The seminal research uncovering the divergent signaling of GnRHR2 was conducted in transiently transfected cell lines (COS-7) by Millar et al. (29). More recent work utilizing immortalized human cancer cell lines has further explored GnRH2-induced signaling (50, 117–120). Although it is often unclear which receptor (GnRHR1 or GnRHR2) mediates the signal, because the presence of the GnRHR2 in humans is controversial (82). Another limitation is the use of cancer cells, which are inherently abnormal. Thus, there is a gap in our knowledge regarding the signaling cascades of GnRHR2 under normal physiologic conditions.

DIVERGENT PHYSIOLOGICAL EFFECTS OF GnRH2 ACTIVATING GnRHR1 AND GnRHR2

Gonadotropin Secretion

It was originally hypothesized that GnRH2 might function similarly to GnRH1 and elicit gonadotropin release from the anterior pituitary gland (12) or could be the much sought after FSH-releasing factor (16, 121–123). In support of these hypotheses, GnRH2 is present within regions of the brain (e.g., pre-optic and medio-basal hypothalamic areas) associated with the regulation of gonadotropin secretion (16). However, GnRH2 production in hypothalamic regions is scarce (33) and GnRH2 does not coexpress with GnRH1 in the hypothalamus (124). In contrast, GnRH2 is more highly abundant in other regions of the brain, such as the midbrain (28, 36, 37). Immunopositive GnRHR2 was detected on 69% of mammalian gonadotrope cells (29) but GnRH2 has never been isolated from hypothalamic portal blood (69, 125). Our laboratory detected immunoreactive GnRH2 in the hypothalamus of pigs, but abundance was low compared with the testis (34, 35). Millar et al. (29) identified GnRHR2 in the pituitary of the marmoset, although it was expressed at similar levels in numerous tissues unrelated to reproduction. It is now well established that GnRH2 and GnRHR2 are more highly expressed in peripheral tissues than the hypothalamus and anterior pituitary gland, respectively (20, 29, 33, 35), suggesting little role in gonadotropin secretion.

Upon its discovery, Miyamoto et al. (12) demonstrated that GnRH2 was less effective than GnRH1 at eliciting release of LH (68% less) and FSH (59% less) from pituitary cell cultures derived from rats. Other investigators confirmed these results in the rat and sheep through *in vitro* studies (125–127). GnRH2 was 92% less effective than GnRH1 at stimulating gonadotropin secretion from primary cultures of ovine pituitary cells (126). *In vivo*, a bolus (10 μg) of GnRH2-stimulated LH and FSH release in rams, although less robustly (40-fold) than GnRH1. There was a modest preference (2-fold) for FSH over LH secretion in response to treatment with GnRH2 (29). In the rat (121) and rhesus macaque (128), however, GnRH2 did not preferentially stimulate FSH release compared with GnRH1.

The effects of GnRH2 on gonadotropin secretion in rats and sheep are likely mediated through the GnRHR1, because both species lack GnRHR2 (21) and GnRHR1 can be activated by GnRH2, although with 10-fold less activity than GnRH1 (29).

Indeed, a GnRHR1-specific antagonist completely blocked both chronic and acute GnRH2-stimulated secretion of gonadotropins in sheep (69) as well as in pituitary cell cultures that were derived from rats (127). These data provide strong evidence that GnRH2 is a weak stimulator of gonadotropin secretion in mammals *via* interaction with GnRHR1, although it remains plausible that this interaction, albeit minimal, may still be physiologically relevant (80). For example, it has been suggested that GnRH2 primes activity and/or production of GnRHR1. However, cotreatment of monkey pituitary cells (125, 128) and rams (69) with GnRH2 and GnRH1 did not enhance LH or FSH secretion above GnRH1 treatment alone. Alternatively, Urbanski (129) proposed that GnRH2 activates GnRHR1 to mediate the preovulatory LH surge. However, this hypothesis has not yet been evaluated *in vivo*.

The effect of GnRH2 on secretion of LH and FSH in species that produce a functional GnRHR2 has also been examined. Treating musk shrews with GnRH2-stimulated ovulation, with 10-fold less potency than GnRH1, but this effect could be blocked with a GnRHR1 antagonist (33). Treating rhesus macaques with GnRH2 elicited increased secretion of gonadotropins *in vivo* during the follicular and luteal phase of the menstrual cycle, but the response was not compared with GnRH1 (37). Others showed that a high dose (1 μg/kg of body weight) of GnRH1 and GnRH2 were equipotent at stimulating release of LH and FSH in the female rhesus macaque (128). However, in cultures of pituitary cells derived from male rhesus macaques, GnRH2 was a less effective stimulator of gonadotropin secretion than GnRH1 (125). GnRH2 stimulated secretion of LH with an EC_{50} of 0.37 nM (compared with 0.10 nM for GnRH1) and FSH with an EC_{50} of 0.59 nM (versus 0.10 nM for GnRH1) (125). Receptor antagonism was used to clarify if GnRHR1 or GnRHR2 mediated these effects. Interestingly, GnRH2-induced gonadotropin secretion was completely blocked by treatment with Antide (125, 128), a GnRHR1-specific antagonist that has minimal activity (EC_{50} of 10,000 nM) at the GnRHR2 (80). Thus, the stimulatory effects of GnRH2 on gonadotropin secretion were attributed to its interaction with GnRHR1.

Similar results were observed in the pig; Cetrorelix (GnRHR1 antagonist) mitigated GnRH2-induced LH and FSH secretion from porcine gonadotrope cell cultures (109). Data from our laboratory support these results. Treatment of boars with [D-Ala^6] GnRH2 weakly stimulated secretion of LH compared with [D-Ala^6] GnRH1 (35). In addition, treatment of males with a GnRHR2-specific antagonist (Trptorelix-1) failed to suppress LH secretion (35). Likewise, immunization of boars against GnRH2 did not affect gonadotropin secretion compared with control males (130). Finally, LH secretion was unaffected in transgenic swine with ubiquitous knockdown of GnRHR2 (53). Collectively, these data demonstrate that only high doses of GnRH2 can elicit weak gonadotropin release *via* the GnRHR1. Therefore, GnRH2 and its receptor do not appear to be physiological stimulators of gonadotropin secretion in mammals.

Reproductive Behavior and Energy Balance

Given the neuroanatomical location of GnRH2 (e.g., midbrain and limbic structures), investigators hypothesized that it may be important in sexual behavior (14, 29, 131). The first evidence demonstrating a role for GnRH2 in female reproductive behavior occurred when GnRH2, but not GnRH1, infusions into the brain of female sparrows increased receptivity to songs of male sparrows (132). In mammals, the effects of GnRH2 on sexual behavior may be dependent on metabolic state. When feed was restricted by 60%, female musk shrews displayed fewer sexual behaviors (31) and had reduced *GnRH2* mRNA expression (midbrain GnRH2 cells) and protein abundance (ventromedial nucleus, medial habenula, GnRH2 cells, and midbrain central gray) compared with *ad libitum* fed animals (133). Sexual behaviors as well as *GnRH2* mRNA expression (midbrain) and protein levels in some regions (ventromedial nucleus and medial habenula) returned to normal after only 90 min of *ad libitum* feeding after restriction (133). The lordosis response in mice during nutrient restriction was enhanced by GnRH2, but not GnRH1 (134); no effect of GnRH2 on lordosis was observed when female mice were fed *ad libitum*. Mice lack GnRH2 and GnRHR2 (30), therefore the biological significance of these results is unclear. In male musk shrews, GnRH2 abundance was examined in all regions of the midbrain prior to and after feed restriction. Unlike females, GnRH2 abundance and sexual behaviors were not affected by feed restriction in male musk shrews. This is potentially due to sexually dimorphic expression patterns of GnRH2 between male and female musk shrews or the level of feed restriction (133, 135).

The enhancement of sexual behaviors in female musk shrews treated with GnRH2 during feed restriction were not attenuated by the addition of a GnRHR1 antagonist (Antide), suggesting the effect of GnRH2 on sexual behavior is mediated *via* the GnRHR2 (32). Similarly, reproductive behaviors of female musk shrews during food restriction were rescued by treatment with antagonist 135–18 (32), which simultaneously acts as an antagonist of GnRHR1 and an agonist of GnRHR2 (29). This agrees with results in female marmoset monkeys showing that GnRH2 and antagonist 135–18, but not GnRH1, increased proceptive (sexual solicitation) behaviors (136). In contrast, other studies showed that high doses (25 μg) of GnRH1 do elicit sexual proceptivity of female marmosets (137), but these effects are likely mediated by GnRHR2 (136).

The interaction between metabolic state and the effects of GnRH2 on sexual behavior may be related to the potential effects of GnRH2 on the mechanism of food intake. Intracerebroventricular infusions of GnRH2 reduced feed intake (33%) in female musk shrews that were underfed (138). A similar reduction (28%) in food intake of musk shrews was also apparent with GnRH2 treatment during *ad libitum* feeding (138). The ability of GnRH2 to reduce food intake was acute, beginning 90 min after GnRH2 infusion and persisting for 3 h, regardless of nutritional plane (138). The effect of GnRH2 on food intake is probably mediated through the GnRHR2 as treatment with Antide (GnRHR1 antagonist) did not prevent a GnRH2-induced reduction in feed intake (32).

The results of these studies demonstrate that GnRH2 influences female reproductive behavior through the GnRHR2, not GnRHR1. GnRH2 may be acting as a permissive neuropeptide that links reproductive behavior with nutritional status in some species (32, 136). If energy balance is low, GnRH2 production is

decreased, inhibiting reproductive behaviors and increasing feed intake. If energy is abundant, increased GnRH2 expression will promote mating behaviors (32).

Testicular Function

For 35 years, evidence has been accumulating that GnRH1 and GnRHR1 have extrapituitary functions. Both GnRH1 and GnRHR1 are expressed within the testes of some mammals (e.g., humans, rats, and mice) (139–144) and their interaction directly elicits testosterone secretion from Leydig cells (145–148). The contribution of GnRH1 and its receptor to localized control of gonadal function is not well understood and therefore often overlooked (149). Localized regulation of the gonads by GnRHs may have existed early in evolution to control reproduction before the formation of an organized pituitary gland (7). For instance, GnRH peptides control reproductive function of invertebrates (e.g., mollusks, echinoderms, and prochordates) that lack an anterior pituitary gland (150–152). *Ciona intestinalis* synthesizes GnRH1 and GnRH2 directly within its gonads and treating gonadal cultures with GnRH1 and GnRH2 stimulates secretion of sex steroids (153). Moreover, seven novel tunicate GnRHs stimulated the release of gametes in *C. intestinalis*, indicative of direct action on the gonads (152). Local control of gonadal function may have been retained in mammals for a specific purpose not met by the gonadotropins. McGuire and Bentley (149) proposed that function might be a rapid, transient responsiveness to environmental stimuli.

Of the 31 organs examined in the marmoset monkey, *GnRHR2* transcript levels were most abundant in the testis (29). In fact, several laboratories have now reported the presence of GnRH2 and/or its receptor within mammalian testes (11, 20, 29, 35, 46, 154), suggesting an autocrine/paracrine role in testicular function. Abundance of GnRH2 in the testis of the pig was 7-fold greater than levels within the anterior pituitary gland or hypothalamus (**Figure 5A**) (35). This corresponded to 6-fold more GnRHR2 protein in the testis than in the anterior pituitary gland

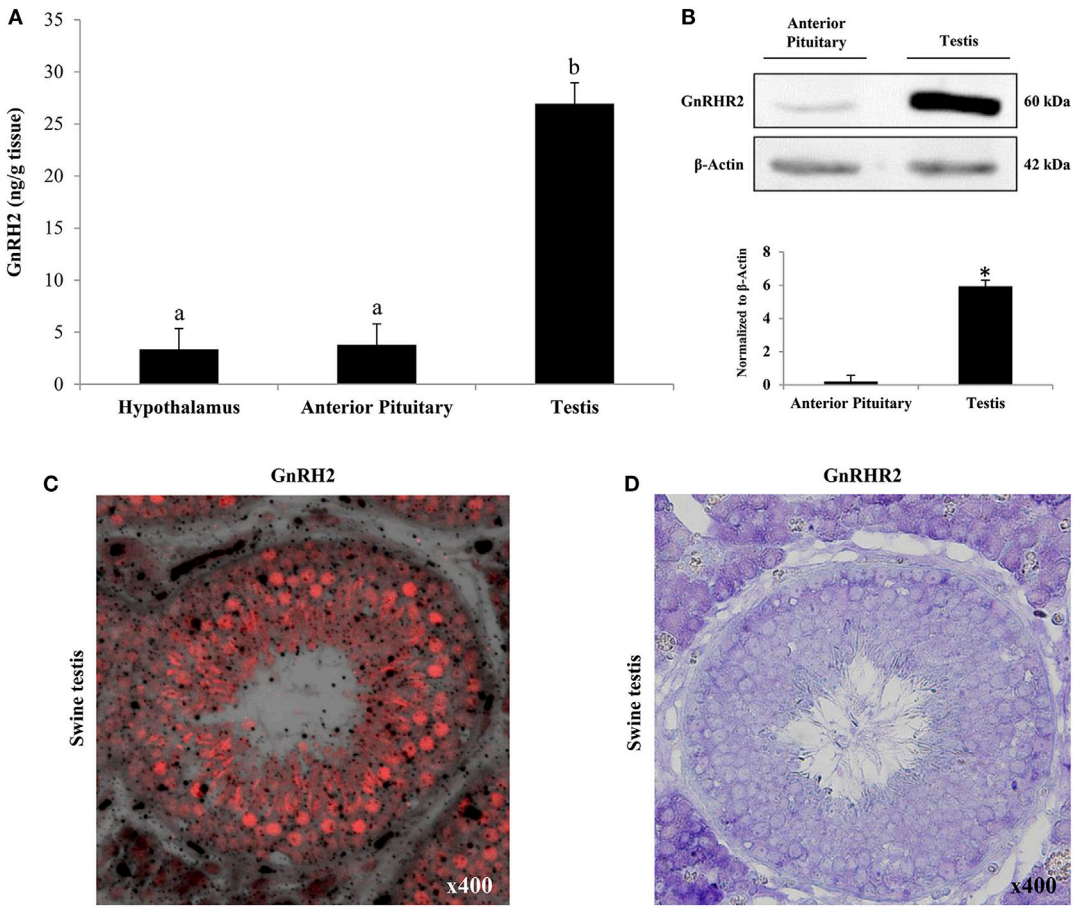


FIGURE 5 | Both GnRH2 and GnRHR2 are abundantly produced within the porcine testis. **(A)** GnRH2 levels were determined *via* ELISA of homogenates from the hypothalamus, anterior pituitary gland, and testis of males ($n = 6$). [a,b]Bars with alternate letters differ ($P < 0.0001$). **(B)** Representative western blot of anterior pituitary gland and testicular tissue from boars ($n = 5$) using an antibody directed against GnRHR2 (upper panel). Immunoblot quantification revealed a difference in GnRHR2 protein levels between tissue types (*$P < 0.0001$; lower panel). **(C)** GnRH2 is primarily localized to the tubular compartment of the porcine testis. Representative immunofluorescence image (400×; merge of fluorescence and transmitted light) of porcine testes ($n = 5$) using an antibody directed against GnRH2 (red). **(D)** GnRHR2 localizes to both the tubular and interstitial compartments of the porcine testis. Representative immunohistochemistry image (400×) of porcine testes ($n = 7$) using an antibody directed against GnRHR2 (purple). Reprinted from Desaulniers et al. (35) with permission from Oxford University Press.

(**Figure 5B**). We also observed the most intense GnRH2 immunostaining within the seminiferous tubules of the boar, primarily localizing to germ and Sertoli cells (**Figure 5C**) (35), although some signal was present within the interstitium. Likewise, we have detected immunoreactive GnRHR2 on germ and Sertoli cells as well as the plasma membrane of porcine Leydig cells (**Figure 5D**). Subcellular localization of GnRHR2 in immortalized ST-derived cells, recently shown to exhibit Sertoli cell-like properties (155), revealed plasma membrane as well as perinuclear immunostaining (**Figure 6**) (48). These data establish that GnRH2 and its receptor are abundantly produced in the porcine testis, indicating an important autocrine/paracrine role in testis biology.

The first association of GnRH2 with testicular function was reported in humans. Although the levels of *GnRHR2* mRNA were not evaluated, testicular *GnRH1*, *GnRH2*, and *GnRHR1* transcript abundance was increased in infertile (azoospermic) men, corresponding with elevated intra-testicular testosterone levels and increased expression of genes encoding steroidogenic enzymes (*CYP11A1* and *HSD3B*) (154). Notably, *GnRH1* and *GnRH2* mRNA levels were positively correlated with expression of *HSD3B*, intra-testicular testosterone levels, and concentrations of FSH in serum, indicating that the testicular GnRH system helps regulate spermatogenesis and steroidogenesis in humans (154). Consistent with this, *GnRHR2* transcripts have been detected in post-meiotic germ cells and human sperm (46). Our laboratory has immunolocalized GnRHR2 to ejaculated porcine spermatozoa, implying a role for GnRH2 in sperm function of boars (34, 45). In contrast, it was concluded that GnRH2 did not impact spermatogenesis in mice (156); however, mice lack both GnRH2 and its receptor (21), which limits the interpretation of these results.

Based upon the aforementioned discovery of GnRHR2 on porcine Leydig cells (35), our laboratory became interested in whether GnRH2 and its receptor are autocrine/paracrine regulators of steroidogenesis in the pig. Previous research has indirectly revealed a role for GnRH2 and its receptor within the testes of mature swine. For example, testosterone secretion was reduced in males immunized against GnRH2, but concentrations of LH in serum remained unchanged (130). Primary cultures of Leydig cells from boars immunized against GnRH2 demonstrated impaired secretion of testosterone basally and when stimulated with LH (130). In a different study, treatment of males with a GnRHR1 antagonist (SB-75; Cetrorelix) blunted hCG-induced secretion of testosterone (157). In a subsequent trial, release of testosterone in boars was continuously reduced during chronic administration of SB-75, yet secretion of LH was only transiently suppressed (158). In addition, SB-75 attenuated hCG-stimulated secretion of testosterone from porcine testicular explants (158). These data imply that a testicular GnRHR was directly regulating steroidogenesis locally within the swine testis. Given that GnRHR1 is not expressed within the porcine testis (158), the results of these studies may be ascribed to GnRHR2. Therefore, we hypothesized that GnRH2, produced locally in the testis, binds to the GnRHR2 on porcine Leydig cells to stimulate LH-independent secretion of testosterone.

To test this hypothesis, we treated porcine testicular explants with hCG in the presence or absence of GnRH2. Secretion of testosterone was significantly stimulated after treatment with GnRH2 or hCG but there was no synergistic effect of treating with hCG and GnRH2 (35). These results established that GnRH2 was a stimulator of acute testosterone secretion *ex vivo*. We next tested the effect of GnRH2 *in vivo*. White crossbred boars were fit with indwelling jugular cannulae to perform serial bleeding trials following treatment with GnRH analogs. GnRH2 infusion robustly elicited testosterone secretion, similar to GnRH1 treatment, despite minimal LH secretion when compared with GnRH1-stimulated males [**Figure 7**; (35)]. Furthermore, GnRH2-induced secretion of testosterone was blunted by pretreatment with the GnRHR1 antagonist, SB-75 (35), which can

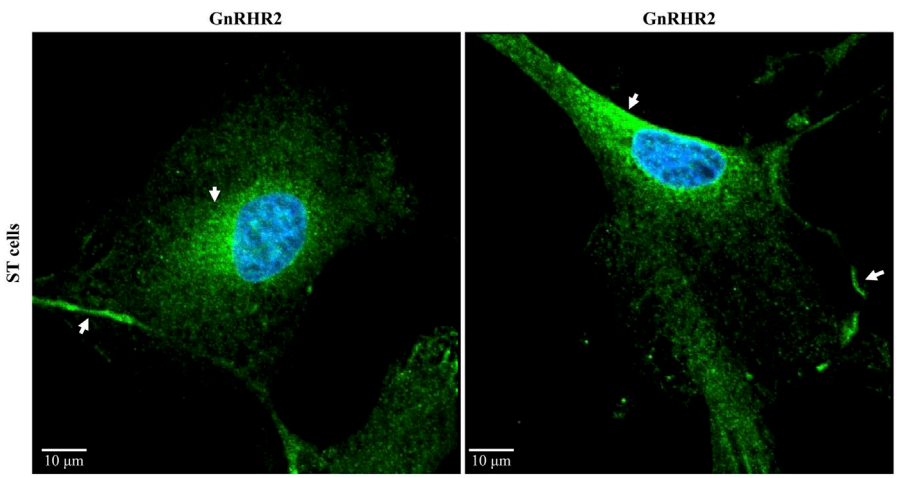


FIGURE 6 | Subcellular localization of the GnRHR2 in a swine testis (ST)-derived cell line. Immunocytochemistry was performed on ST cells (CRL-1746; American Type Culture Collection, Rockville, MD, USA) with an antibody directed against GnRHR2 (1:100–1:200; sc-162889; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and labeled with an Alexa Fluor 488 secondary antibody (green); nuclei were stained with DAPI (blue). The experiment was replicated three times. Two representative confocal microscopy images are shown. Note the plasma membrane and perinuclear staining (arrows). The scale bar represents 10 μm. Adapted from Cederberg et al. (48).

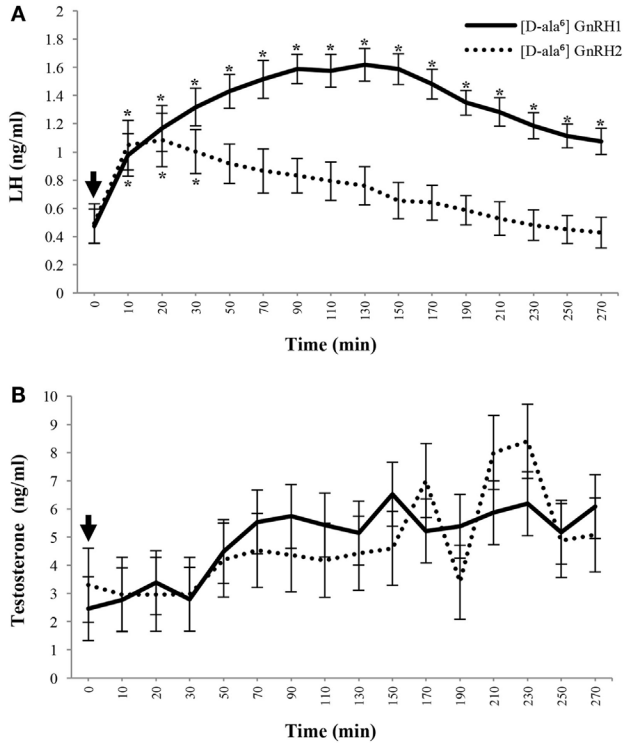


FIGURE 7 | GnRH2 stimulates secretion of testosterone similar to GnRH1 despite reduced luteinizing hormone (LH) release in mature male pigs. Plasma concentrations of LH **(A)** and testosterone **(B)** after intravenous administration of [D-ala^6] GnRH1 (solid line; $n = 8$) or [D-ala^6] GnRH2 (dotted line; $n = 6$) to cannulated adult males. After pretreatment sampling, treatments were administered (arrow; 0 min) and blood was serially collected. Data are presented as the Least Squares Means (±SEM). For LH, Treatment, $P < 0.001$; Time, $P < 0.0001$; Treatment × Time, $P < 0.03$. For testosterone, Treatment, $P = 0.1929$; Time, $P < 0.002$; Treatment × Time, $P = 0.7190$. *Within treatment, concentrations differ from pre-injection levels ($P < 0.05$). GnRH1 differences are indicated above error bars and GnRH2 differences are labeled below error bars. Reprinted from Desaulniers et al. (35) with permission from Oxford University Press.

antagonize GnRHR2 (50, 115). We hypothesize that SB-75 reduced GnRH2-induced testosterone secretion by antagonizing GnRHR2 directly in the testis because the secretory pattern of LH was unaffected compared to trials where only GnRH2 treatments were administered (35). Finally, intratesticular injections of either GnRH1 or GnRH2 stimulated secretion of testosterone compared with saline-treated controls; however, GnRH2 did so without eliciting the release of LH, unlike GnRH1 (159). Together, these data support our hypothesis that GnRH2 is stimulating testosterone production directly at the testis in the absence of the classical androgen regulator, LH.

To further study the role of GnRH2 and its receptor in pigs, our laboratory generated a GnRHR2 knockdown swine line (53). These animals ubiquitously express short hairpin RNA targeting the porcine GnRHR2. Consequently, testicular *GnRHR2* mRNA levels were reduced by 70% in adult males compared with littermate control animals (53). During pubertal development, GnRHR2 knockdown boars had smaller testes (**Figure 8A**) despite a normal body weight (**Figure 8B**), implying impaired testicular function. Moreover, testosterone concentrations tended to be lower (**Figure 8C**) in transgenic versus littermate control males, yet LH concentrations were unaffected [**Figure 8D**; (53)]. These results support our hypothesis that activation of GnRHR2 on porcine Leydig cells stimulates LH-independent testosterone secretion. These swine represent the first genetically engineered animal model to study the function of GnRH2 and its receptor in mammals and are currently being utilized to identify the molecular mechanisms linking GnRHR2 and steroidogenesis in male pigs. Given that testosterone and its metabolites govern male fertility (e.g., sex differentiation, reproductive tract maintenance, libido, spermatogenesis, and accessory sex gland function), GnRH2 and its receptor are novel molecular targets to enhance reproductive efficiency in swine.

Female Reproduction

Although GnRH2 and its receptor are expressed within female reproductive tissues (**Table 3**), few studies have directly examined the role of the GnRH2/GnRHR2 system in the female. Several lines of evidence suggest that GnRH2 and its receptor are novel regulators of placental function, implantation, and ovarian steroidogenesis. GnRH2 is produced by the human placenta in a pulsatile manner and is six times more stable than GnRH1 when exposed to placental enzymes (70). GnRH2 stimulated the production of hCG in human placental explants (70, 160–162), whereas GnRH1 treatment did not (70). Moreover, a high affinity receptor specific for GnRH2 (70), as well as immunoreactive GnRHR2 (163), have been detected in the human placenta.

When compared with GnRH1, GnRH2 potently enhanced invasion of human trophoblasts (84) through extracellular matrix remodeling (164). Notably, antagonizing or knocking down GnRHR1 abrogated GnRH1-mediated effects on trophoblast cells but did not influence GnRH2-stimulated invasion (84, 165, 166). In addition, GnRH2 (mRNA and protein) was found in the uterine endometrium (stromal and glandular epithelial cells) of women during all phases of the menstrual cycle, although GnRH2 production increased during the secretory phase, indicative of a role in implantation (67). The GnRHR2 may be produced in human endometrium as well given that both high and low affinity-binding sites for GnRH1 were identified in human endometrial cancer cells (167). Given that GnRH1 binds GnRHR2 with 15-fold less affinity (29), the low affinity-binding site for GnRH1 detected in this study may be the GnRHR2. Indeed, others have demonstrated that immunoreactive GnRHR2 is present in human endometrial adenocarcinomas (163). Treatment of rhesus macaques with a GnRHR2 agonist near ovulation conferred contraceptive actions in 100% of females; this effect was not mediated by the inhibition of progesterone secretion (168). In a subsequent study, pregnancy was prevented in all females receiving GnRH2 treatment, whereas 62.5% of saline-treated controls became pregnant. Interestingly, the low doses of GnRH2 (2–8 μg/day) inhibited secretion of progesterone, whereas high doses (16–32 μg/day) had no effect (169). Nevertheless, pregnancy was prevented in all treatment groups, indicating that the contraceptive activity of GnRH2 is not confined to the suppression of progesterone production alone.

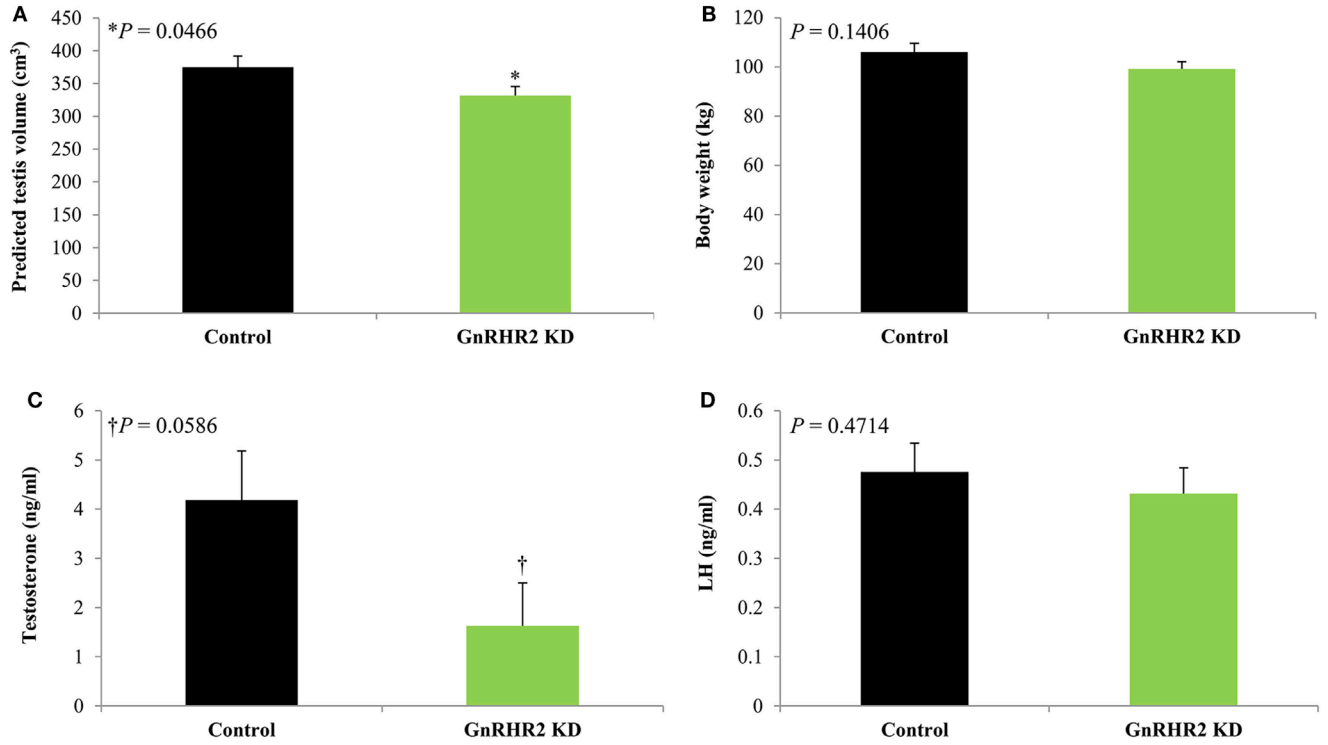


FIGURE 8 | GnRHR2 knockdown (KD) swine have smaller testes and reduced serum testosterone concentrations but similar body weights and circulating luteinizing hormone (LH) levels compared with littermate control animals during pubertal development. At 40, 100, 150, 190, 225, and 300 days of age, blood was collected from GnRHR2 knockdown (n = 10) and littermate control (n = 7) males; at the same time points, body weight and predicted testis volume were measured. Serum testosterone and LH concentrations were determined by radioimmunoassay. Data are presented as the Least Squares Means (±SEM). The P-value for the main effect of line is indicated. **(A)** Predicted testis volume, **(B)** body weight, **(C)** serum testosterone concentrations, and **(D)** serum LH concentrations. Adapted from Desaulniers et al. (53); no permission is required from the copyright holder.

In other species, GnRH2 appears to modulate secretion of progesterone as well. Kang et al. (58) reported that GnRH2 inhibited basal and hCG-stimulated progesterone secretion from human granulosa-luteal cells. In the baboon, GnRH2 is present in the ovary and released from granulosa cells *in vitro*. Exogenous GnRH2 administration suppressed production of progesterone from cultured granulosa cells by 75% (60), whereas GnRH1 failed to suppress progesterone release. Binding kinetics indicated two binding sites for GnRH2, a high and a low affinity site, compared with only one binding site for GnRH1, suggesting the presence of a GnRH2-specific receptor in the baboon ovary (60). Collectively, these data are compelling; however, more studies are needed to determine how GnRH2 and its receptor impact female reproduction in mammals. Toward this end, research in our laboratory is currently underway to define the role of GnRH2 and its receptor in reproductive function of the female pig.

Cancer

It is well established that GnRH1 and its receptor are expressed in cancer cells derived from reproductive tissues and administration of GnRH1 analogs inhibits their proliferation (51, 170, 171). GnRH2 and its receptor may also influence the progression of reproductive cancers, given that both are expressed in cancer cells (**Table 4**) and tumors derived from reproductive tissues (38, 44, 51, 170, 171). Growing evidence indicates that treatment of cancer cells with GnRH2 analogs inhibits their proliferation. This has been demonstrated in prostate (54, 172), ovarian, breast, and endometrial cancer cells (38, 40, 170, 171). Interestingly, GnRH2 appears to have a more potent anti-proliferative effect than GnRH1 (51). The cellular mechanisms by which GnRH2 mediates this effect have been the subject of recent research efforts. Treatment of breast cancer cells with either GnRH1 or GnRH2 inhibited ribosomal phosphoproteins, which are needed for proper protein translation and cell proliferation (38). GnRH2 also increased metalloproteinase production, key regulators of tumor invasion, in ovarian cancer cells (173). In addition, treatment with a GnRH2 agonist reduced cell proliferation and inhibited the mitogenic effects of epidermal growth factor in human endometrial and ovarian cancer cells (120). In addition, GnRH2 and its receptor may have emerging roles in the modulation of cell proliferation *via* extracellular vesicles. For example, glioblastoma-derived microvesicles increased proliferation of tumor cells *in vitro*; the same microvesicles were also found to carry *GnRH2*, *GnRH1*, *GnRHR2*, and *GnRHR1* mRNA (174). Thus, GnRH/GnRHR transcripts packaged in extracellular vesicles could be an unexploited mechanism to affect tumor progression in humans.

In addition to anti-proliferative actions, GnRH2 analogs may also exert pro-apoptotic effects on cancer cells. Treatment

with several GnRHR2 antagonists inhibited growth of human endometrial and ovarian cancer cells *in vitro* and *in vivo via* caspase 3-dependent mechanisms (175); a different GnRHR2 antagonist (SN09-2) induced apoptosis in prostate cancer cells (172). Likewise, GnRH2 can increase apoptosis *via* the caspase-dependent pathway in human granulosa cells (176). In breast cancer cells, yet another GnRHR2 antagonist induced apoptotic cell death *in vitro* and *in vivo* (177). In addition to anti-proliferative and pro-apoptotic effects, GnRH2 might also regulate cellular autophagy. Human prostate cancer cells treated with a GnRHR2 antagonist (Trptorelix-1) displayed increased mitochondrial dysfunction as well as autophagosome formation (178). These cells had decreased Akt phosphorylation and increased c-Jun phosphorylation, additional hallmarks of cell autophagy. In numerous cases, the effects of GnRH2 on cancer cells were not mediated through the GnRHR1, implying a role for human GnRHR2 (50, 52, 120, 177). Thus, GnRH2 and possibly its receptor, regulate cancer cell proliferation/survival and represent emerging targets for the development of new cancer therapies.

CONCLUSION

GnRHs are ancient peptides which may have first functioned to directly regulate the gonads before evolving into specialized modulators of gonadotropin secretion. GnRH2 is the most ancient of the GnRHs and has been completely conserved from boney fish to man, signifying a critical biological role. Moreover, GnRH2 is structurally unique from GnRH1, which promotes its stability and half-life. A highly selective receptor specific for GnRH2 is also produced in mammals. The GnRHR2 is dissimilar from GnRHR1, containing an intracytoplasmic tail and eliciting divergent cell signaling cascades. The genes for *GnRH2* and/or *GnRHR2* have been deleted or inactivated in many species, but both are functional in old world monkeys, musk shrews and pigs, implying an essential role in these animals. Moreover, evidence continues to suggest the presence of GnRHR2 in humans despite apparent coding errors in the gene. Data from numerous species (including humans) demonstrates that GnRH2 and its receptor are ubiquitously expressed. Notably, both are produced in low abundance in regions of the brain associated with gonadotropin secretion and highly expressed in peripheral reproductive organs. Thus, GnRH2 and its receptor are both structurally and functionally distinct from their classical counterparts.

Contrary to their name, GnRH2 and GnRHR2 are not physiological stimulators of gonadotropin secretion in mammals. Instead, GnRH2 and its receptor have been implicated in various functions, mostly pertaining to mammalian reproduction. The first defined function of GnRH2 and its receptor was the modulation of sexual behavior, based on nutritional status, in females. Within peripheral tissues, GnRH2 and its receptor are important regulators of reproductive organs in both males and females. For example, GnRH2 and its receptor directly regulate steroidogenesis within the porcine testis. In the female, GnRH2 and its receptor may mediate placental function, implantation, and ovarian steroidogenesis. Furthermore, both *GnRH2* and *GnRHR2* are expressed in human reproductive tumors and are emerging targets for cancer treatment. Therefore, GnRH2 and its receptor are critical modulators of reproductive function in mammals, albeit *via* a divergent mechanism from the classical GnRH1 and GnRHR1 interaction. More work is needed to better understand the importance of localized regulation by the GnRH2/GnRHR2 system, but its contribution to mammalian reproduction is unequivocal.

Despite these data, the ubiquitous nature of GnRH2 and GnRHR2 suggests that many more biological functions remain undefined. The deletion of the *GnRH2* and *GnRHR2* genes from the rat and mouse has undoubtedly inhibited the study of this system in a widely available and economical laboratory animal. However, the recent development of a GnRHR2 knockdown swine line provides an essential animal model to explore the functions of GnRH2 and its receptor in mammals. Given the recent discovery of the *GnRH2* and *GnRHR2* genes in numerous mammalian species, this system may be physiologically relevant and unexploited in a wide range of species.

AUTHOR CONTRIBUTIONS

AD and BW determined the structure and content of this review. AD performed the literature review and composed the manuscript with input and assistance from RC, CL, and BW. RC and AD developed the figures/tables. AD, RC, CL, and BW edited and revised the manuscript.

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REVIEW
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Gonadotropin-Releasing Hormone and Its Role in the Enteric Nervous System

Bodil Ohlsson[1,2]*

[1] Lund University, Lund, Sweden, [2] Division of Internal Medicine, Skåne University Hospital, Lund, Sweden

Gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone, and luteinizing hormone orchestrate the reproduction cycle and regulate the sex steroid secretion from the gonads. In mammals, GnRH1 is secreted as a hormone from the hypothalamus, whereas both GnRH1 and GnRH2 are present as neurotransmitters/peptides in various tissues, where the peptides exert many different effects. mRNA coding for GnRH1 and GnRH2 have been described in the human gastrointestinal tract, and GnRH has been found in both submucosal and myenteric neurons. mRNA coding for GnRH and the fully expressed peptide have been found in rat enteric neurons by some researchers but not by others. mRNA coding for GnRH receptors, but not the fully expressed receptor, has been found in one rat study. GnRH influences gastrointestinal motility and secretion. GnRH analogs are clinically used in the treatment of sex hormone-dependent diseases, i.e., endometriosis and malignancies, and as pretreatment for *in vitro* fertilization. Reduced numbers of enteric neurons and IgM antibodies against GnRH and progonadoliberin-2 (precursor of GnRH2) have been observed after such treatment, with the clinical picture of gastrointestinal dysmotility. Similarly, a rat model of enteric neurodegeneration has been developed after administration of the GnRH analog buserelin. Serum IgM antibodies against GnRH1, progonadoliberin-2, and GnRH receptors have been described in patients with signs and symptoms of gastrointestinal dysmotility and/or autonomic dysfunction, such as irritable bowel syndrome, enteric dysmotility, diabetes mellitus, and primary Sjögren's syndrome. Thus, apart from regulation of reproduction and sex hormone secretion, GnRH also constitutes a part of enteric nervous system (ENS) and its functions during physiological and pathological conditions. This review aimed to describe the role of GnRH in the ENS.

Keywords: enteric nervous system, enteric neurodegeneration, gonadotropin-releasing hormone, gonadotropin-releasing hormone receptor, gonadotropin-releasing hormone antibodies

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***Correspondence:**
Bodil Ohlsson
bodil.ohlsson@med.lu.se

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile fashion from hypothalamic neurons into the portal circulation, where GnRH receptors on the anterior pituitary are activated with subsequent secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (1, 2). FSH and LH target the gonads and regulate the secretion of steroid hormones (3). Since GnRH

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; IBS, irritable bowel syndrome; IVF, *in vitro* fertilization; LH, luteinizing hormone; MMC, migrating myoelectric complex; PCR, polymerase chain reaction.

is secreted into the portal circulation and has a half-life of a few minutes, the hormone levels cannot be analyzed in peripheral blood (2). Instead, measurements of FSH and LH levels in blood are used to estimate the hypothalamic–pituitary function. In vertebrates, 23 native decapeptides of GnRH exist. Changes of amino acids in molecular positions 5–8 differ the decapeptides from each other (4). In mammals, two types of GnRH have been found: GnRH1 and GnRH2. GnRH1 is secreted from the hypothalamus, whereas both types are present in several organs and tissues of the body, e.g., neural tissue, where they exert neuroendocrine, paracrine, and autocrine functions in the central and peripheral nervous system (4). The GnRH receptor is a G-protein-coupled receptor with seven transmembrane domains (5). Although several different receptors are described, only the GnRH1 receptor is expressed in mammals (3). Both GnRH1 and GnRH2 act through the GnRH1 receptor (4).

ORGANIZATION OF THE ENTERIC NERVOUS SYSTEM (ENS)

The autonomic nervous system is divided into three parts called the sympathetic nervous system, the parasympathetic nervous system, and the ENS (6). The ENS consists of more than 100 million neurons, which are as many neurons as in the spinal cord. The ENS has the ability to control gastrointestinal function independent of brain and spinal cord (7). It is organized in microcircuits, with interneurons and intrinsic afferent neurons, which can initiate reflexes. All kinds of neurotransmitters in the central nervous system (CNS) can be detected in the ENS (7). Nevertheless, 90% of vagal neurons are afferent, suggesting that the brain is mostly a receiver of information (8). The greatest efferent traffic from CNS to the gastrointestinal tract is to the most proximal and most distal parts of the tract, e.g., regulating functions such as mastication and swallowing (7). There is a great evidence that pathophysiological mechanisms in the CNS could also affect the ENS in a similar manner (9).

The ENS consists of two plexus: the myenteric nervous plexus, situated in-between the longitudinal and circular muscle layers, and the submucosal plexus, situated deep in the submucosa. The submucosal plexus mainly regulates the sensory and secretory functions of the gut, whereas the myenteric plexus mainly regulates the motility (7). Both plexus contain excitatory and inhibitory neurotransmitters (7). The submucosal plexus is by unknown reasons less often affected by neurological diseases (9).

EXPRESSION OF GnRH AND GnRH RECEPTORS IN THE ENS

Gonadotropin-releasing hormone receptor mRNA was initially described in rat myenteric neurons (10). Later, mRNAs and the fully expressed peptide of GnRH were found in both submucosal and myenteric nerve plexus, whereas GnRH receptors only were found in parasympathetic ganglion cells in rat digestive tract (11). However, neither GnRH nor GnRH receptor could be detected by immunocytochemistry in rat gastrointestinal tract *in vivo* by another research group (12, 13). mRNA for both GnRH1 and GnRH2 could be detected by polymerase chain reaction (PCR) in the human gastrointestinal tract, whereas the GnRH receptor could neither be detected by PCR nor by immunocytochemistry (12) (**Table 1**). The cellular localization of GnRH has been described in about half of the submucosal and myenteric neurons along the entire human gastrointestinal tract (14, 15).

EFFECTS OF GnRH OR GnRH ANALOGS ON THE FUNCTION OF THE ENS

Gonadotropin-releasing hormone and its analog alarelin have been shown to inhibit gastric secretion and gastrin release in rat and dog (**Table 2**) (16, 17). The mechanisms behind the inhibition seems to be mediated both through direct actions on the parietal cells and by inhibition of the vagus nerve (16, 17). When studying jejunal motility in rats, migrating myoelectric complexes (MMCs) were frequently found during fasted state, albeit more seldom postprandially. After ovariectomy, low-dose treatment of the GnRH agonist leuprolide rendered typical fed-state patterns without MMCs. High-dose treatment of leuprolide inhibited the fed-state pattern and MMCs occurred at a frequency similar to fasted control rats (**Table 2**). Thus, reproductive hormones have significant effects on gastrointestinal motility (18). However, another GnRH analog could not inhibit the substance P-induced contractions of isolated guinea pig ileum, as it could inhibit substance P-induced elevation of arterial blood pressure (19).

TABLE 1 | The expression of gonadotropin-releasing hormone (GnRH), GnRH receptor, and luteinizing hormone (LH) receptor in the gastrointestinal tract in rat and humans.

	GnRH1	GnRH2	GnRH		GnRH receptor	LH receptor
	mRNA	mRNA	mRNA	Immunocytochemistry	mRNA	Immunocytochemistry
Rat						
Neuron			Huang et al. (11)	Huang et al. (11)	Ho et al. (10)	Sand et al. (12) Sand et al. (13) Sand et al. (28)
Human						
Neuron	Sand et al. (12)	Sand et al. (12)		Ohlsson et al. (14) Hammar et al. (15)		Sand et al. (12) Hammar et al. (47)

Numbers in brackets are the related references that have described the expression of the mRNA and/or protein.

TABLE 2 | The function of gonadotropin-releasing hormone in the gastrointestinal tract in rat and humans.

	Rat	Dog	Humans
Gastric secretion	Inhibited (16)	Inhibited (17)	
Gastrin release		Inhibited (17)	
Gastric motility	Modification (18)		Increased (37) Reduced (14, 15, 20)
Enteric neuron survival	Reduced *in vivo* and *in vitro* (13, 25–28)		Reduced (14, 15, 20)
Abdominal pain			Increased (21, 22) Decreased (29–32)

Numbers in brackets are the related references.

Thus, the analog may act as a substance P receptor antagonist in CNS which can inhibit the sympathetic vasomotor outflow, but without effect on peripheral substance P receptors (19).

GnRH ANALOG-INDUCED ENTERIC NEURODEGENERATION IN HUMANS

Pharmacologic treatment with GnRH analogs of endometriosis and pretreatment of *in vitro* fertilization (IVF) has induced severe, gastrointestinal dysmotility in some women (**Table 2**) (14, 15, 20). Histopathological examination of the patients have revealed a reduced total amount of enteric neurons and a reduced percentage of GnRH-expressing enteric neurons, along with serum IgM antibodies against GnRH1 and/or progonadoliberin-2 (14, 15, 20). Polymorphism in the LH receptor was common in the women who developed severe dysmotility after GnRH treatment (20).

When examining consecutive patients at an infertility clinic, treatment with buserelin led to significantly more symptoms of constipation, nausea and vomiting, impaired psychological well-being, and negative influence of intestinal symptoms on daily life, and a tendency to increased abdominal pain and bloating, compared with prior treatment (21). Five years after the start of the treatment, the patients had increased abdominal pain and better psychological well-being compared with prior IVF treatment. Fifteen percent had developed irritable bowel syndrome (IBS), or had exacerbated symptoms, but none had developed severe dysmotility (21).

In a cohort of women with endometriosis ($n = 109$), patients with a history of GnRH treatment had more severe abdominal pain than patients who had never been treated with GnRH analogs (22). Antibody development seems not to be obligate after GnRH treatment and occurred only in patients developing complications to the treatment (23, 24).

BUSERELIN-INDUCED ENTERIC NEURODEGENERATION IN RAT

In rat, GnRH-induced enteric neuropathy has been developed after four repeated treatment sessions of buserelin, one session consisting of 5 days of 20 μg daily subcutaneous injections with 3 weeks of recovery (**Table 2**). This rendered a 50% reduction of both submucosal and myenteric neurons throughout the gastrointestinal tract, although most pronounced in myenteric neurons, and more pronounced distally than proximally in the gastrointestinal tract (13). Signs of ganglionitis were observed (25). Raised serum levels of estradiol, synchronization of the hormonal cycle, and thickened uterine muscle layer point to elevated FSH and LH secretions behind the neurotoxicity (13, 26). Furthermore, a reduced relative number of LH receptor-containing neurons were observed, preceded by increased expression of activated caspase-3 (13). Subclassification of neuron populations in colon showed increased relative numbers of neurons expressing cortiocotropin-releasing factor (CRF) in submucosal neurons and an absolute increased amount of CRF-containing myenteric neurons (27), whereas the relative numbers of neurons expressing calcitonin gene-related peptide, cocaine- and amphetamine-related transcript, galanin, gastrin-releasing peptide, neuropeptide Y, nitric oxide synthase, substance P, vasoactive intestinal peptide, and vesicular acetylcholine transporter were unaffected (26).

An *in vitro* study failed to show any effects on rat enteric neuron survival by the GnRH analog buserelin or by continuous LH stimulation. Instead, intermittent stimulation by a LH analog (lutrotroptin alpha) led to reduced neuronal survival (**Table 2**) (28).

EFFECTS OF GnRH ON ABDOMINAL SYMPTOMS

In a randomized, double-blind, placebo-controlled study of patients with moderate to severe functional bowel disease, continuous treatment with leuprolide during 12 weeks improved symptoms of nausea, vomiting, bloating, abdominal pain, early satiety, and overall gastrointestinal symptoms (**Table 2**) (29). Continued treatment for 1 year led to even more significant improvements of the symptoms (30). A multicenter study could confirm a significant and persistent improvement in nausea and abdominal pain (31). Leuprolide treatment also improved all gastrointestinal symptoms and quality of life in women with menstrual cycle-related IBS (32).

Two hypotheses to the improved effect by leuprolide on gastrointestinal symptoms in functional bowel disorders have been described (29–32). First, GnRH binds to specific GnRH receptors on the pituitary and controls the secretion of gonadotropins (1). Both LH and ovarian products, such as progesterone and human chorionic gonadotropin (hCG), are neural antagonists of gastrointestinal motility (33, 34). By continuous stimulation of leuprolide, the hypothalamic–pituitary–gonadal axis is downmodulated and the secretion of gonadotropins and gonadal products are inhibited (3, 35). Second, by acting on GnRH receptors on myenteric neurons (10), leuprolide is an effective neural modulator through regulating the voltage-gated calcium channels and the endoplasmic reticulum calcium pump, resulting in the movement and control of intracellular and extracellular calcium (36). However, this assumption is dependent on the presence of fully expressed GnRH receptors in the ENS, which has never been demonstrated at the moment in rat or humans

(10–12). Still, peripheral leuprolide restored gastrointestinal motor function both in a transplanted woman who developed chronic intestinal pseudo-obstruction after a virus infection (37) and in female ovariectomized rats (18), whereas administration of the same drug into the intraventricular system of the rat brain had no effect (38).

ANTIBODY FORMATION AGAINST GnRH AND GONADOTROPINS

An enzyme-linked immunosorbent assay has been developed to measure GnRH antibodies in serum (14, 21, 39–41). IgM antibodies against GnRH1 have been found in patients with diabetes mellitus, gastrointestinal dysmotility, IBS, posterior laryngitis, and primary Sjögren's syndrome, independent of treatment with GnRH analogs, in contrast to patients with celiac disease, inflammatory bowel disease, microscopic colitis, and sclerodermia, who express antibodies to the same extent as controls (39–45). IgM antibodies against GnRH receptors have been found in patients with dysmotility, IBS, and primary Sjögren's syndrome (44, 45), and IgM antibodies against progonadoliberin-2, the precursor of GnRH2 (46), have been found in patients with diabetes mellitus, dysmotility, and IBS (45). Measurements over time showed that the antibody titer in serum was high after each buserelin administration, and the titer was then lowered after some time (14). All patients with reduced number of GnRH-containing enteric neurons displayed IgM antibodies against GnRH1 in serum, independent of GnRH treatment (15).

DISCUSSION

Gonadotropin-releasing hormone has been found in enteric neurons in both rat and humans by several scientists (11–15). The expression of GnRH receptor is more uncertain, since only one article has described the presence of mRNA for the receptor in rat ENS (10), and no one has demonstrated the fully expressed receptor in submucosal or myenteric plexus of ENS. On the contrary, GnRH receptors have been found in parasympathetic ganglion cells outside the ENS in rat gastrointestinal tract (11), sites not examined in humans. The described effects of GnRH on the ENS are modulation of gastrointestinal motility and secretion (16–19). GnRH treatment has led to enteric neuron death in both rat *in vivo* and *in vitro* trials and in human *in vivo* trials (13–15, 20, 26–28). IgM antibodies against GnRH1, progonadoliberin-2, and GnRH receptors may occur in a subgroup of patients with functional bowel disorders and dysmotility, both in idiopathic forms and when associated with diabetes mellitus, posterior laryngitis, primary Sjögren's syndrome, or GnRH treatment (39, 40, 42–45).

The GnRH analogs stimulate the anterior pituitary rendering elevated LH secretion with stimulation of the LH receptors and ensuing elevated steroidal sex hormone secretion (1–3, 13, 26). Since the GnRH receptor has not been found in human gastrointestinal tract, the harmful effects evoked on the gastrointestinal tract could be mediated by LH receptors, which are found in the gastrointestinal tract in humans and rat (12, 13, 28, 47), and are downregulated after GnRH stimulation (13). Both LH and hCG exert their effects through LH receptors. As well, LH, hCG, and progesterone are known to reduce gastrointestinal motility (33, 34), which could explain the reduced symptom burden after continuous GnRH stimulation due to downregulated secretion of gonadotropins and sex steroids (1, 2, 29–32). The effects evoked by LH receptor stimulation seem to be mediated through cAMP/protein kinase A. Furthermore, LH stimulation leads to a change in gene transcripts coding for steroidogenic enzymes, cytoskeletal proteins, in addition to signaling molecules coding for pro- and antiapoptotic processes (48). A downregulation of LH receptors is therefore accompanied by decreased apoptosis (49), which was reflected by the increased relative number of activated caspase-3 immunoreactive enteric neurons prior to the neuronal loss in the GnRH-induced rat model of neuropathy (13). *In vitro* trials on enteric rat neurons confirmed this theory, with a reduced neuron survival only after intermittent stimulation with the LH analog lutrotropin alpha, and not with GnRH analogs (28).

In vitro fertilization treatments leads to repeated unphysiological LH stimulation, which may be the cause of severe dysmotility observed in some women with a polymorphism in the LH receptor (20). The LH receptor is present in both genital organs and the gastrointestinal tract (12, 13, 28, 47, 50) and could be a plausible explanation to the observed association in women between dysfunction of the digestive tract and diseases of genital organs (18, 51, 52). As much as 50% loss of enteric neurons were accompanied with mostly normal gastrointestinal function (26, 27), suggesting a huge reserve capacity of the ENS. Thus, full-thickness biopsies are mandatory to examine the effects of GnRH treatment on the ENS (13–15, 26, 27) and to differ functional bowel symptoms from enteric dysmotility.

The fact that some research groups have been able to demonstrate GnRH and its receptor mRNA in the rat ENS (10, 11), while not found by others (12, 13), may have several reasons. The native GnRH receptor could not be found in adult rat neurons from the superior cervical ganglion (53). However, after microinjection of cRNA coding for the human GnRH receptor, the expression of the protein could be demonstrated (53). Thus, the expression of GnRH receptors in the neural tissue may vary. Although the GnRH receptor has not been able to demonstrate, it can still be present in the ENS. In addition to a central stimulation by GnRH administration, GnRH may thus also exert peripheral effects, direct on the ENS. In rat hypothalamus, a cross talk between *N*-methyl-D-aspartate and adrenergic neurotransmission has been demonstrated in the regulation of the hypothalamic GnRH gene expression (54). Due to release of nitric oxide from endothelial cells, the vascular endothelium is involved in the release of neurohormones from the median eminence (55). Theoretically, similar cross talks and mediators from the epithelial cells may be present in the ENS, which has not been examined at all regarding the release and regulation of GnRH and LH and their receptors. Thus, we are in the beginning of this research field, and experimental *in vivo* and *in vitro* trials are necessary to further determine the expression and function of these peptides in the ENS and digestive tract. If GnRH receptors

are present in the ENS, the same mechanisms may be involved in the GnRH-induced response in ENS as in the gonadotrophs, e.g., activation of cAMP, cGMP, phospholipases, and calcium channels (3). It has been found that subjects with autonomic dysfunction had an abnormal hypothalamic gonadotrophin secretion (56). The mechanisms involved in the regulation of GnRH, LH, and their receptors in subjects with dysfunction of the ENS have not been determined to date.

Although chronic GnRH treatment may improve abdominal symptoms, these analogs are not used in the clinical setting, due to the risk of menopausal symptoms and development of osteoporosis in long-term treatment (57). During the last years, stimulation with GnRH agonists has been replaced by administration of GnRH antagonists in the IVF setting, to prevent the LH surges and thereby to reduce the side effects (2). The observation of more abdominal pain in endometriosis patients with GnRH treatment may reflect that GnRH analogs in this treatment group induce enteric neuropathy with ensuing abdominal pain, apart from endometriosis pain (22). Even if the GnRH analog-treated endometriosis patients are the patients with most severe disease and pain, and the elevated pain in this group could reflect more severe disease, GnRH treatment seems not to be efficient to reduce pain.

Antibodies against neuronal tissue have previously been described secondary to gut dysmotility (58). GnRH antibodies may represent neuronal damage in a subgroup of patients and may not be causal, since GnRH analogs *per se* did not induce serum antibody expression in humans or rat (13, 21, 26), and antibodies were present also in patients without previous GnRH treatment (14, 15, 20, 39, 40, 42–45). The absence of GnRH antibodies in rats may depend on lack of GnRH expression in rat enteric neurons (13, 26), or very small amounts of the peptide (10, 11). GnRH1 and GnRH2 are present in both the central and peripheral nervous system (3, 4, 12, 46). Autonomic neuropathy and gastrointestinal complaints are common in patients with diabetes mellitus and primary Sjögren's syndrome (42, 44) and autonomic neuropathy, depression, and affective disorders are common in patients with functional bowel diseases and gastrointestinal dysmotility (58–61). Theoretically, antibodies against GnRH may be secondary to either a central neuronal damage or a peripheral neuronal damage (39, 40, 42, 44, 45).

CONCLUSION

Gonadotropin-releasing hormone has been found in the human ENS in repeated examinations. GnRH has been found in rat ENS in some studies, although not reproducible by others. Fully expressed GnRH receptors have never been found in rat or human ENS. GnRH modulates gastrointestinal motility and secretion. Treatment with GnRH analogs may induce enteric neurodegeneration in both rat and human ENS. LH receptor activation is the postulated target of the effects observed, since LH receptors are described on enteric neurons and enteric rat neuronal survival was decreased after intermittent *in vitro* stimulation of the LH receptor. Autoantibodies against GnRH and its receptor are found in a subgroup of patients with disturbances from the gastrointestinal tract and/or autonomic nervous system, independent of treatment with GnRH analogs.

Altogether, the knowledge about GnRH expression and function in the gastrointestinal tract suggests a role for GnRH on the ENS, but the field is rudimentary studied. The key point in this research field is to study the local effect of GnRH in the gastrointestinal tract and the communication between GnRH and LH receptors. Future research should include a search for fully expressed GnRH receptors in rat and human ENS. Further, a controlled study comparing the effect of GnRH analogs compared with GnRH antagonists on neuron survival should be performed *in vitro* and *in vivo*. Another alternative is to compare the effects on the ENS by sole GnRH analogs and GnRH analogs in combination with LH receptor antagonists. Both cell culture trials with enteric neurons and organ bath experiments are needed to characterize the route of effects by GnRH on the ENS, and the effects evoked.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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The Role of Gonadotropin-Releasing Hormone in Cancer Cell Proliferation and Metastasis

Carsten Gründker and Günter Emons*

Department of Gynecology and Obstetrics, Georg-August-University, Göttingen, Germany

In several human malignant tumors of the urogenital tract, including cancers of the endometrium, ovary, urinary bladder, and prostate, it has been possible to identify expression of gonadotropin-releasing hormone (GnRH) and its receptor as part of an autocrine system, which regulates cell proliferation. The expression of GnRH receptor has also been identified in breast cancers and non-reproductive cancers such as pancreatic cancers and glioblastoma. Various investigators have observed dose- and time-dependent growth inhibitory effects of GnRH agonists in cell lines derived from these cancers. GnRH antagonists have also shown marked growth inhibitory effects on most cancer cell lines. This indicates that in the GnRH system in cancer cells, there may not be a dichotomy between GnRH agonists and antagonists. The well-known signaling mechanisms of the GnRH receptor, which are present in pituitary gonadotrophs, are not involved in forwarding the antiproliferative effects of GnRH analogs in cancer cells. Instead, the GnRH receptor activates a phosphotyrosine phosphatase (PTP) and counteracts with the mitogenic signal transduction of growth factor receptors, which results in a reduction of cancer cell proliferation. The PTP activation, which is induced by GnRH, also inhibits G-protein-coupled estrogen receptor 1 (GPER), which is a membrane-bound receptor for estrogens. GPER plays an important role in breast cancers, which do not express the estrogen receptor α (ERα). In metastatic breast, ovarian, and endometrial cancer cells, GnRH reduces cell invasion *in vitro,* metastasis *in vivo,* and the increased expression of S100A4 and CYR61. All of these factors play important roles in epithelial–mesenchymal transition. This review will summarize the present state of knowledge about the GnRH receptor and its signaling in human cancers.

Keywords: gonadotropin-releasing hormone, cancer, proliferation, metastasis, signal transduction

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Università di Genova, Italy

****Correspondence:***
Carsten Gründker
grundker@med.uni-goettingen.de

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EXPRESSION OF GONADOTROPIN-RELEASING HORMONE (GnRH) AND ITS RECEPTOR IN HUMAN CANCERS

In several earlier studies, it has been demonstrated that cancers of the breast, ovary, and endometrium have receptors for GnRH (1). Receptor-binding abilities are different between pituitary gonadotrophs and cancer cells. In cancer cells are two types of GnRH-binding sites, one with low affinity and high capacity and a further one with high affinity and low capacity. The second is similar to the GnRH receptor found in pituitary gonadrotrophs (1–3). The low-affinity binding site is similar to that found in human placenta and corpus luteum and is unable to discriminate between GnRH agonists and superactive GnRH agonists (4). In addition, the low-affinity GnRH receptor is only

activated at high concentrations of GnRH agonists, whereas the high-affinity GnRH receptor is fully activated at low levels of GnRH agonists.

Expression and sequence analysis of the GnRH receptor found in human pituitary gonadotrophs were first demonstrated in 1992 (5). Due to these findings, intensive research was carried out, which lead to the demonstration of high-affinity GnRH receptors in ovarian and endometrial cancer cell lines and in about 80% of their respective primary tumors (5–8). High-affinity/low-capacity-binding sites, strongly related to the pituitary GnRH receptor, were found in specimens of ovarian and endometrial cancers and cell lines, which express mRNA for the GnRH receptor known from pituitary gonadotrophs (6, 7, 9–13). Kakar et al. (14) confirmed that the DNA sequence of GnRH receptors in human breast and ovarian cancers is identical to that within the pituitary. Harris et al. (15) reported on GnRH mRNA expression in two human breast cancer cell lines. About 50–64% of human breast cancers have high-affinity GnRH receptors, according to various studies (16–19). A more recent study reported that GnRH receptor expression was detected in 67% of hyperplasia cases (4 out of 6), in 100%of benign fibroadenoma cases (3 out of 3), in 100% of carcinoma *in situ* cases (4 out of 4), and in 71% cases of malignant breast cancers (22 out of 31) (20). The therapeutic options today are incredibly limited in particular for triple-negative breast cancers (TNBCs), which do not exhibit either the estrogen receptor α (ERα) or the progesterone receptor and do not overexpress the HER2-neu gene. It has been shown that 74% of TNBCs ($n = 42$) have GnRH receptor expression (21). In another study, GnRH receptors were found in all analyzed TNBCs ($n = 16$) (22). Since breast, ovarian, and endometrial cancers express both GnRH and its receptor, it appears plausible to consider that there may be a regulative system locally based on GnRH in many of these tumors. This also applies to prostate cancer cells (23–25). In addition, expression of GnRH receptor has also been found in some cancers of non-reproductive tissues, such as cancers of the urinary bladder, pancreatic cancers, and glioblastoma in addition to that found in breast cancers (26–29).

Besides GnRH, another structural version of GnRH is present in mammals. GnRH-II is completely conserved in its structure from fish to mammals and is different from GnRH in three amino acids. A specific functional receptor for GnRH-II was identified in different species including non-human primates (30–33). The existence of a GnRH-II receptor in humans is, however, controversial (34). The full-length human GnRH-II receptor is known to be a 7 transmembrane receptor. It has not yet been possible to successfully clone or sequence this receptor (31, 35–37). A functional GnRH-II receptor is likely to be expressed in a variety of splice variants (32). Assuming that a functional GnRH-II receptor is secreted by human tissues, it might be a 5 transmembrane domain receptor, which lacks the transmembrane regions 1 and 2 (32). It was possible to identify mutations of chemokine receptors which are functional 5 transmembrane G-protein-coupled receptors where the N-terminus is linked right to transmembrane domain 3 due to deletion of transmembrane domains 1 and 2 (38). Morgan et al. learned that the human GnRH-II receptor is also present in a number of splice variants (39). It is suspected that the GnRH-II receptor is non-functional due to a stop codon within exon 2 (35, 39). A GnRH-II receptor, composed of the three exons required for a complete receptor protein, has recently been cloned from human sperm by Van Biljon et al. (40). This transcript also has a stop codon and a frame shift mutation. While this would suggest that this gene is a transcribed pseudogene, the authors speculate that the GnRH-II receptor in human sperm and testis may have a functional role (40). Evidence for the existence of a functional GnRH-II receptor in human cancers was demonstrated in earlier studies carried out in our laboratory (35, 41, 42). A GnRH-II receptor-like protein could be detected in cancers of human reproductive organs using an antiserum to the putative human GnRH-II receptor (41). In membrane preparations of these cancer cell lines, a band at approximately 43 kDa was detectable whereas in ovaries obtained from marmoset monkey (*Callithrix jacchus*) a band at approximately 54 kDa was shown (41). To identify the GnRH-II receptor-like antigen, the photo-affinity-labeling technique was used. Photo chemical reaction of ^{125}I-labeled (4-Azidobenzoyl)-N-Hydroxysuccinimide-[D-Lys^6]-GnRH-II with membrane preparations of human endometrial and ovarian cancer cells yielded a band at approximately 43 kDa. Western blot analysis of the same gel using the anti-human GnRH-II receptor antiserum identified this band as GnRH-II receptor-like antigen (41). In competition experiments, GnRH-II agonist [D-Lys^6]-GnRH-II showed a strong decrease of ^{125}I-labeled (4-Azidobenzoyl)-N-Hydroxysuccinimide-[D-Lys^6]-GnRH-II binding to its binding site (41). Kim et al., however, has shown that the effects of GnRH and GnRH-II can be reversed by the transfection of short-interfering RNA to nullify the GnRH receptor gene expression (43). These findings of Kim et al. suggest that the effects of GnRH and GnRH-II are produced by utilizing the GnRH receptor. Our recent work shows that GnRH-II antagonists bind with the GnRH receptor in a similar way to how they bind with the GnRH antagonist cetrorelix (19). We were also able to demonstrate that, although GnRH-II antagonists are clearly antagonists at the GnRH receptor, [D-Lys^6]GnRH-II is an agonist at the GnRH receptor (44). Similar results were found for prostate cancer. The GnRH receptor mediates the effects of GnRH-II on prostate cancer cells (45).

ANTIPROLIFERATIVE ACTION OF GnRH IN HUMAN CANCERS

Dependent upon dose and time, GnRH agonists were found to reduce proliferation of human endometrial, ovarian, and breast cancer cell lines (1, 46). Comparable results were found for prostate cancer cell lines (23–25). When tested on most tumor cell lines, GnRH antagonists act like agonists, which indicate that the dichotomy of GnRH agonist/GnRH antagonist, as described in gonadotrophic cells of the pituitary, is not valid for the GnRH system in tumors of the human being. GnRH antagonists also caused a time- and dose-dependent reduction in cell growth (1, 46). In tumor cells, GnRH receptors may be mainly coupling with Gi proteins, which, according to cell lineage, may result in the production of different receptor conformation and signaling

complexes (47–49). This may help to explain how tumor GnRH receptors have different actions compared with pituitary cells. A reduction in proliferation of human endometrial, ovarian, and breast cancer cells can also be demonstrated with GnRH-II agonists. These effects are significantly greater than those produced by GnRH agonists (35). The reduction in cancer cell growth caused by GnRH or GnRH-II agonists does not appear to be due to induced apoptosis (1). Instead, GnRH and GnRH-II agonists counteract the signaling of growth-factor receptors through activation of a phosphotyrosine phosphatase (PTP). This results in a reduction in cancer cell growth (47, 50, 51). This is discussed in Section "GnRH Receptor Signal Transduction in Human Cancers."

Antagonistic analogs of GnRH and GnRH-II, in contrast to GnRH and GnRH-II agonists, however, do induce apoptotic cell death in several human cancer cells (44, 52, 53). In human endometrial and ovarian cancer cells, this occurs due to a dose-dependent loss of mitochondrial membrane potential and induction of caspase-3 (44, 52). It was possible to confirm these effects in nude mice. The progress of human endometrial and ovarian tumors grown in mice was significantly inhibited by GnRH-II antagonists without causing any apparent side effects (44, 52). Apoptotic cell death induced by antagonists of GnRH-II is permitted *via* the intrinsic cascade through stress-activated mitogen-activated protein kinases (MAPKs) p38- and JNK-induced stimulation of the proapoptotic factor Bax, together with the loss of mitochondrial membrane potential, cytochrome c release, and caspase-3 activation (44, 52).

ANTIMETASTATIC ACTION OF GnRH IN HUMAN CANCERS

By using coculture to mimic tumor cell invasion, we have forced non-invasive MCF-7 breast cancer cells to behave in an invasive manner resulting in a marked increase in the number of cells undergoing epithelial–mesenchymal transition (EMT) (54–57). By prolonged mammosphere culture, we have made a mesenchymal transformed MCF-7 cell line (MCF-7-EMT), which as opposed to wild-type MCF-7 cells, exhibits a significant increase in invasive behavior both *in vitro* and *in vivo* as well as increased expression of EMT-related genes (55). When non-invasive wild-type MCF-7 breast cancer cells were cocultured with human primary osteoblasts or osteoblast-like cell line MG63, the invasion of tumor cells through an artificial basement membrane was dramatically increased (54). Treatment with GnRH analogs significantly reduced the capability to invade through the basement membrane and to migrate in response to the cellular stimulus (54). GnRH analogs exhibited comparable antimetastatic effects in prostate cancer cells (58).

Approximately 10–15% of breast cancers are TNBCs, which do not have estrogen receptor α and progesterone receptors and show not an overexpression of HER2-neu (59–61). TNBCs are believed very aggressive and have a poor prognosis. The most frequent site for metastasis formation in breast cancers is bone, followed by the lungs and liver (62). Development of bone metastasis by MDA-MB-435 TNBC cells grown in the mammary glands of nude mice was significantly inhibited by treatment with GnRH analogs. GnRH analogs also significantly inhibited bone metastasis formation from circulating MDA-MB-231 TNBC cells, which were injected intracardially (63). This indicates that GnRH analogs may have an influence on the biology of circulating breast cancer cells as well as influencing the first steps of breast cancer metastasis including EMT, migration, and invasion as was already known from *in vitro* data (54).

The S100 calcium-binding protein A4 (S100A4) and the cysteine-rich angiogenic inducer 61 (CYR61, CCN1) promote cancer cell motility and thus play important roles in EMT, invasion, and metastasis (64–68). Highly invasive MDA-MB-231 breast cancer cells exhibit high expression of both genes (20). An increased CYR61 level correlates with a poor prognosis, poor lymph node status, and metastatic propagation (69, 70). Jenkinson et al. showed that S100A4 has a clear influence on the invasiveness of breast cancer cells (71). Breast cancer cells with S100A4 overexpression were shown to be markedly more invasive than the non-transfected controls. High levels of S100A4 and CYR61 were found in biopsy specimens of malignant human breast cancers, whereas in carcinoma, *in situ,* the expression levels were much lower. No expression of S100A4 and CYR61 was detectable in normal breast tissues and benign fibroadenoma (20). MCF-7 cells are non-invasive and show very low levels of S100A4 and CYR61 expression (20). Invasion of cells and levels of S100A4 and CYR61 expression in MCF-7 cells was markedly increased after mesenchymal transition (MCF-7-EMT) (20). The increase in invasive behavior could be reduced by anti-S100A4 and anti-CYR61 antibodies (20). The use of anti-S100A4 and anti-CYR61 antibodies also reduced invasive behavior in naturally aggressive MDA-MB-231 cells (20). Treatment of mesenchymal transformed MCF-7-EMT and naturally highly invasive MDA-MB-231 cells with a GnRH agonist resulted not only in a significant decrease of invasion but also a reduced expression of S100A4 and CYR61 (20). The neutralization of CYR61 resulted in inhibition of breast cancer metastasis *in vivo* (72). The precise mechanisms remain unclear and are part of our current research. However, the use of GnRH agonists or similar treatments to block S100A4 and CYR61 should be further explored as they may have new antimetastatic therapeutic potential.

GnRH RECEPTOR SIGNAL TRANSDUCTION IN HUMAN CANCERS

Interaction of GnRH Receptor and Growth Factor Receptor Signaling

Over the last two decades, the signal transduction mechanisms affecting the growth inhibiting actions of GnRH analogs in cancer cells of the breast, ovary, and endometrium have been discussed (**Figure 1**). The GnRH receptor signal transduction in human malignant tumors is different from that found in gonadotrophic cells in the pituitary, where GnRH receptors bind to G-protein αq and induce activation of phospholipase C (PLC), protein kinase C (PKC), and adenylyl cyclase (AC) (1). The signal transduction mechanisms activated by GnRH in gonadotrophic

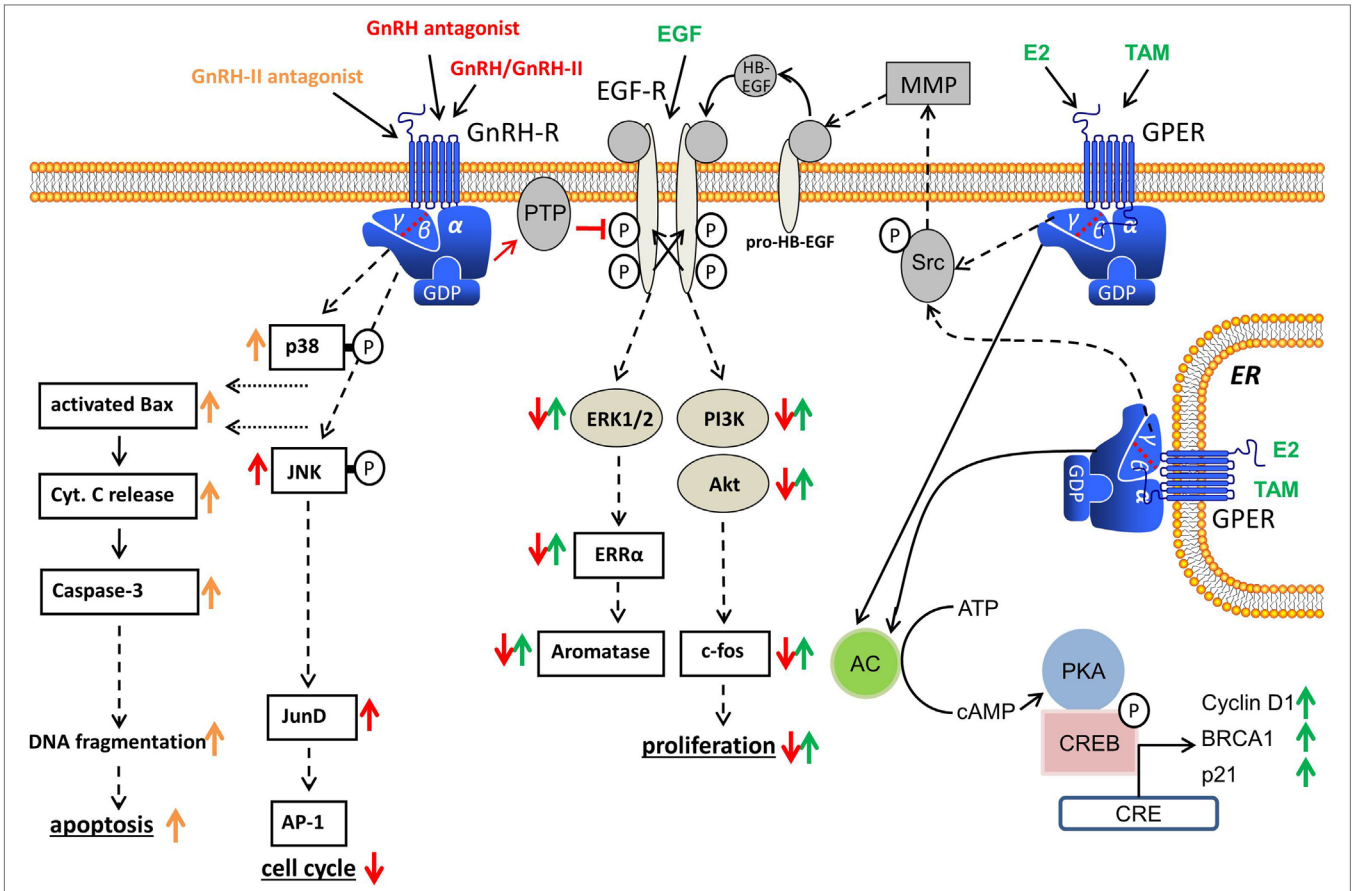

FIGURE 1 | Gonadotropin-releasing hormone (GnRH) receptor signal transduction in human cancers. Binding of GnRH or GnRH-II agonists to GnRH receptor causes G-protein αi-mediated activation of phosphotyrosine phosphatase (PTP), resulting in dephosphorylation of activated EGF receptor (EGF-R) and inhibition of EGF-R signal transduction. GnRH antagonists also show GnRH receptor-induced PTP activation. GnRH-induced activation of PTP also inhibits G-protein βγ subunit-mediated Src/MMP/HB-EGF signaling cascade of GPER and inhibits E2-induced proliferation in ERα-negative breast cancer cells. In addition, GnRH agonists activate the JNK/activator protein-1 (AP-1) pathway independent of known AP-1 activators, protein kinase C, or mitogen-activated protein kinase, resulting in an increased $G_{0/1}$ phase of cell cycle and decreased DNA synthesis. GnRH-II antagonists induce apoptosis in human breast, endometrial, and ovarian cancer cells through activation of the intrinsic apoptotic pathway.

cells of the pituitary were not turned on by GnRH agonists in cancers of the ovary, endometrium, and breast even though activation of PLC, PKC, and AC in cells of these cancers by pharmacological stimulation was clearly shown (23, 47). The cancer GnRH receptor binds to G-protein αi after ligand binding and induces activation of a PTP (23, 47, 73–76). The EGF receptors (EGF-Rs) are dephosphorylated by the PTP (47). Because of this, mitogenic signal transduction, caused by EGF-R activation, is prevented, which leads to the downregulation of EGF-permitted activation of MAPK (23), *c-fos* expression (51), and EGF-induced proliferation (77). These findings agree with other reports of GnRH analogs reducing the expression of growth factor receptors (78–80) and/or growth factor-induced tyrosine kinase activity (23, 73, 74, 76, 79, 81–83). The explanation for the dissimilarities of GnRH receptor signal transduction between gonadotrophic cells of the pituitary and cancer cells is still unclear, as we were unable to identify mutations or splice variations in the cancer cell GnRH receptor, which can have explained the phenomenon (47).

The effects of GnRH are not confined to mitogenic signal transduction of growth factor receptors. GnRH agonists stimulate activator protein-1 (AP-1) activity *via* G-protein αi in human ovarian and endometrial cancer cells. In addition, GnRH agonists also activate JNK, which is a known trigger of AP-1 (84). In earlier research, it was demonstrated that GnRH agonists do not induce PLC and PKC in endometrial and ovarian cancer cells (23). GnRH agonists have also been found to inhibit mitogen-activated protein kinase (MAPK, ERK) activity caused by growth factors (23). Activation of the JNK/AP-1 signaling caused by GnRH in endometrial cancer cells is, therefore, independent of the AP-1 activators, PKC, or MAPK (ERK). Yamauchi et al. demonstrated that JNK is involved in the downregulation of cell proliferation, which is caused by the α1B-adrenergic receptor in human embryonic kidney cells (85). In an analysis in rats, it was suggested that *c-jun* mRNA suppression and endometrial epithelial cell growth may be linked (86). Cytokines show inhibitory action on cell growth in UT-OC-3 ovarian cancer cells and activate AP-1 and NFκB (87). As the JNK/*c-jun* signaling is activated by

antiproliferative GnRH agonists and JNK/*c-jun* was also found to be integrated in reducing cell growth in distinct systems, it seems plausible to consider whether the JNK/*c-jun* signaling is involved in the inhibitory effect of the GnRH agonists. We have also shown that GnRH agonists cause JunD-DNA binding, which results in decreased cell proliferation shown by an increased $G_{0/1}$ phase of cell cycle and reduced DNA synthesis (88).

Interaction of GnRH Receptor and Estrogen Receptor Signaling

Different studies have shown that estrogen receptor α (ERα) mediates 17β-estradiol (E2)-activated expression of c-*fos*, which is induced as an immediate early response gene in ERα-positive breast cancer cell lines (89–96). ERα activates the serum response element (SRE) in MCF-7 breast cancer cells *via* MAPK-dependent Elk-1 phosphorylation (97, 98). Duan et al. have shown that SRE in breast cancer cells is activated through the Ras/MAPK cascade by both E2 (ERα-dependent) and growth factors (ERα-independent) (97).

Because GnRH agonists antagonize EGF-induced cell growth and c-*fos* gene expression through the Ras/MAPK pathway, we have analyzed whether E2-induced activation of SRE and expression of c-*fos* in ERα-positive human breast, endometrial, and ovarian tumor cells is also inhibited by GnRH agonists and whether GnRH reduces E2-induced cell proliferation (1). Dormant ERα-positive/ERβ-positive breast, endometrial, and ovarian tumor cell lines were stimulated to multiply by treatment with E2 but ERα-negative/ERβ-positive cell lines were unaffected. This action was time- and dose-dependent inhibited by co-treatment with GnRH agonists (99). We were also able to show that in ERα-positive/ERβ-positive cell lines, E2 activates the SRE and the expression of c-*fos*. These effects were antagonized by GnRH agonists (99). GnRH agonists did not affect the activation of the estrogen response element caused by E2. Transcriptional SRE activation by E2 is due to activation, by ERα, of the MAPK pathway. GnRH blocks this pathway, which results in a decrease of activated SRE caused by E2 and, in consequence, a decrease in E2-mediated expression of c-*fos*. This causes a reduction in the cancer cell proliferation caused by E2 (99). PTP activation caused by GnRH also inhibits G-protein βγ subunit-mediated Src/MMP/HB-EGF signaling cascade of G-protein-coupled estrogen receptor 1 (GPER, GPR-30), which is a membrane-bound receptor for estrogens, which plays an important role in breast cancers, which do not show expression of estrogen receptor α (ERα) (100–103). Because of the inhibition of GPER signaling, cancer cell proliferation, due to E2, in ERα-negative breast cancer cells was prevented (100–102).

Recently, we demonstrated that human breast cancer cells are resensitized by GnRH analogs to the estrogen antagonist 4OH-Tamoxifen (104). We have developed sublines of 4OH-Tamoxifen resistant cell lines and compared the expression levels of ER, Her-2, EGF-R, and GnRH receptor in the wild-type and the resistant cell lines. We identified slightly decreased expression of GnRH receptors and increased levels of EGF-R in the developed sublines (104). Apoptotic cell death induced by 4OH-Tamoxifen in wild-type MCF-7 and T47D cells was unaffected by GnRH analogs, but, when the resistant sublines were pretreated with analogs of GnRH, sensitivity for 4OH-Tamoxifen was completely restored in these cells (99). Analogs of GnRH counteract EGF-dependent growth and probably interrupt the change in growth regulation, from being estrogen dependent to being EGF dependent, which ocurrs after acquiring secondary resistance to 4OH-Tamoxifen. This interruption of EGF-R signaling resensitized the resistant cell lines for a therapy using 4OH-Tamoxifen (104).

GnRH RECEPTOR AS TARGET FOR CANCER THERAPY

Apart from pituitary cells and reproductive organs, most other tissues and hematopoietic stem cells do not show expression of the GnRH receptor (**Figure 2**). The reproductive organs, ovaries, fallopian tubes, and uterus are regularly eliminated during surgery of ovarian or endometrial cancer (105). These receptors could, therefore, be used to deliver a targeted therapy with improved antitumor effects and reduced side effects. Cytotoxic GnRH agonists, in which a cytotoxic substance is covalently coupled to a GnRH agonist, have been developed (106). These GnRH analogs, which are covalently bound to a cytotoxic agent couple specifically to GnRH receptors with their peptide fraction and operate as chemotherapeutic drug after internalization of the receptor–ligand complex (106). Thus, these cytotoxic GnRH analogs selectively attack only cells that have membrane GnRH receptors and cause fewer side effects than not conjugated cytotoxic substances (106). We demonstrated that such a cytotoxic GnRH agonist, Zoptarelin Doxorubicin (AEZS-108, AN-152), in which doxorubicin is covalently coupled to the GnRH analog [D-Lys^6]GnRH, is selectively accumulated in the nucleus of human GnRH receptor-positive breast, ovarian, and endometrial cancer cell lines. The uptake of Zoptarelin Doxorubicin could be competitively blocked by an excess of another GnRH agonist. No intracellular Zoptarelin Doxorubicin could be found in tumor cell lines that do not have membrane GnRH receptors (107). Zoptarelin Doxorubicin was more potent than doxorubicin in inhibition of cell growth, *in vitro,* in most GnRH receptor-positive cancer cell lines. These results indicated that Zoptarelin Doxorubicin had a selective receptor-mediated effect on GnRH receptor-positive cancer cell lines and inspired us to analyze the effectiveness of Zoptarelin Doxorubicin *in vivo* (105). In testing on experimental cancers in nude mice, Zoptarelin Doxorubicin was less toxic than unbound Doxorobicin and more effective in decreasing the growth of GnRH receptor-positive tumors (105, 108). This is thought to be due to the receptor-mediated admission of Zoptarelin Doxorubicin and the reduced causation of multidrug resistance (109, 110). Clinical trials of Zoptarelin Doxorubicin were planned as it appears that the drug allows a more effective and less toxic targeted chemotherapy for GnRH receptor-positive cancers. In a dose escalation and pharmacokinetic trial, Zoptarelin Doxorubicin was used by women with GnRH receptor-positive cancers. The maximum tolerated dose in the absence of supportive medication was found to be 267 mg/m^2. This dose was recommended as the starting dose for therapeutic

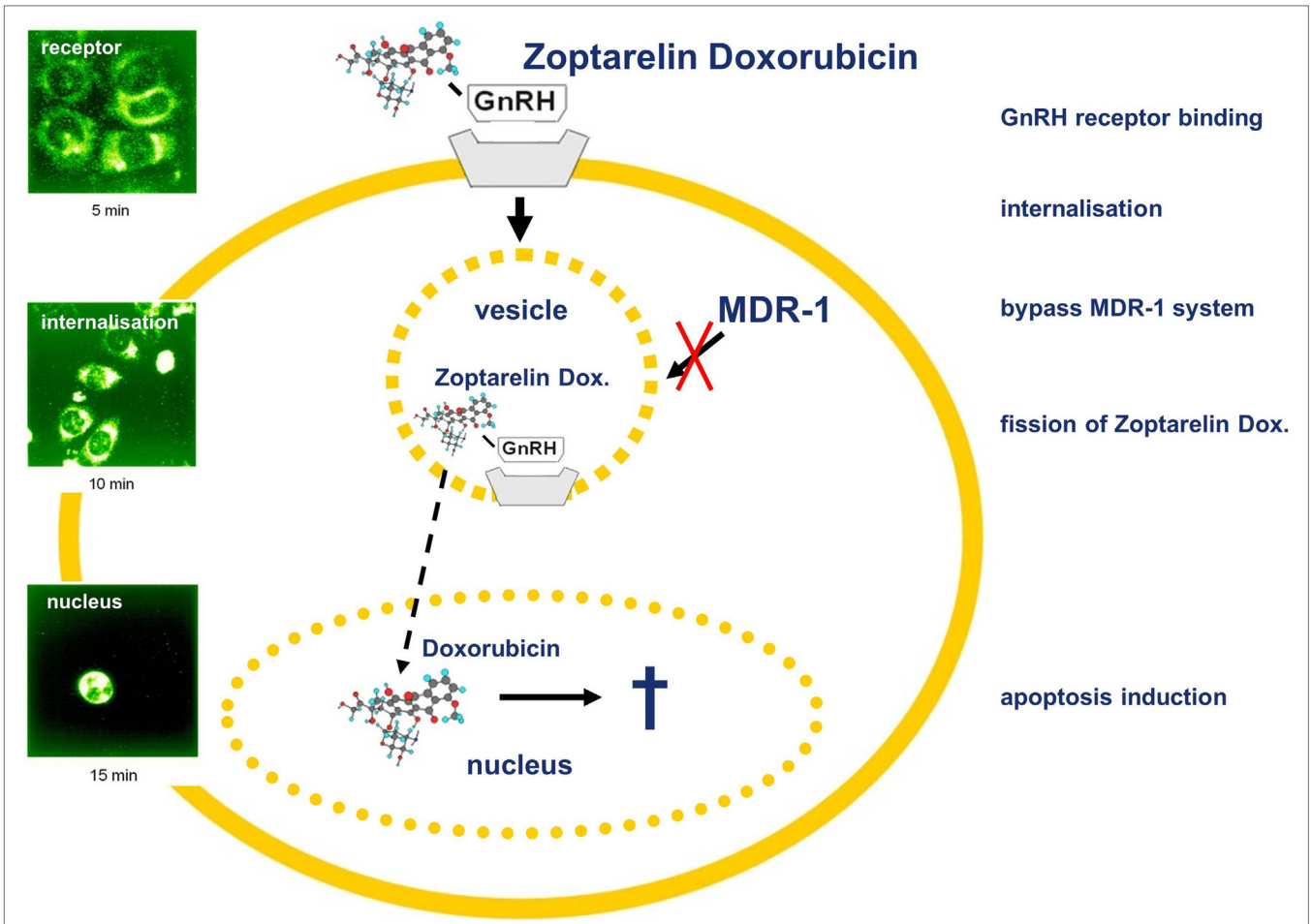

FIGURE 2 | Gonadotropin-releasing hormone (GnRH) receptor-targeted chemotherapy using Zoptarelin Doxorubicin. Internalization of cytotoxic GnRH analog Zoptarelin Doxorubicin induces multidrug resistance gene (MDR-1)-independent apoptosis. After receptor binding, the Zoptarelin Doxorubicin/GnRH receptor complex is internalized *via* coated vesicles bypassing the MDR-1 system. Thereafter, Zoptarelin Doxorubicin is split and free doxorubicin is accumulated within the nucleus, inducing apoptosis. Detection of Zoptarelin Doxorubicin and doxorubicin was performed using laser scanning microscopy (102).

phase II trials (111). It has also been shown, *in vitro*, that Zoptarelin Doxorubicin is an effective therapeutic option in TNBC where there is a high percentage of GnRH receptor-positive cancers (21). Other types of tumors were found to be suitable for treatment with Zoptarelin Doxorubicin. Thirty-two percent of pancreatic cancers express GnRH receptors (28). We demonstrated that treatment of GnRH receptor-positive MiaPaCa-2 and Panc-1 human pancreatic cancer cells with Zoptarelin Doxorubicin resulted in apoptosis *in vitro*. The antitumor effects could be also demonstrated in nude mice (28). In 2014, the first data from a multicenter phase II trial were published demonstrating that Zoptarelin Doxorubicin proved to be effective and of low toxicity in women with advanced or recurrent GnRH receptor-positive endometrial cancer (112). A second multicenter phase II trial confirmed that Zoptarelin Doxorubicin is an effective and safe compound for the treatment of women with platinum refractory or resistant ovarian cancers (113). Zoptarelin Doxorubicin is currently in a phase III clinical trial on patients with ovarian or endometrial cancer.

CONCLUSION

Gonadotropin-releasing hormone plays an important role in the control of mammalian reproduction. In addition to this well-documented classic hypophysiotropic action, GnRH might have a role as a modulator of cell growth and metastasis in a number of human malignant tumors, including cancers of the breast, ovary, endometrium, and prostate. In addition, GnRH receptors expressed in many tumor types provide suitable targets for the therapy with GnRH analogs.

AUTHOR CONTRIBUTIONS

Both authors participated in drafting the article.

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REVIEW
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Invertebrate Gonadotropin-Releasing Hormone-Related Peptides and Their Receptors: An Update

*Tsubasa Sakai[1], Akira Shiraishi[1], Tsuyoshi Kawada[1], Shin Matsubara[1], Masato Aoyama[2] and Honoo Satake[1]**

[1] Bioorganic Research Institute, Suntory Foundation for Life Sciences, Kyoto, Japan, [2] Faculty of Science, Department of Biological Sciences, Nara Women's University, Nara, Japan

Gonadotropin-releasing hormones (GnRHs) play pivotal roles in reproductive functions via the hypothalamus, pituitary, and gonad axis, namely, HPG axis in vertebrates. GnRHs and their receptors (GnRHRs) are likely to be conserved in invertebrate deuterostomes and lophotrochozoans. All vertebrate and urochordate GnRHs are composed of 10 amino acids, whereas protostome, echinoderm, and amphioxus GnRH-like peptides are 11- or 12-residue peptide containing two amino acids after an N-terminal pyro-Glu. In urochordates, *Halocynthia roretzi* GnRH gene encodes two GnRH peptide sequences, whereas two GnRH genes encode three different GnRH peptides in *Ciona intestinalis*. These findings indicate the species-specific diversification of GnRHs. Intriguingly, the major signaling pathway for GnRHRs is intracellular Ca^{2+} mobilization in chordates, echinoderms, and protostomes, whereas *Ciona* GnRHRs (Ci-GnRHRs) are endowed with multiple GnRHergic cAMP production pathways in a ligand-selective manner. Moreover, the ligand-specific modulation of signal transduction via heterodimerization among Ci-GnRHR paralogs suggests the species-specific development of fine-tuning of gonadal functions in ascidians. Echinoderm GnRH-like peptides show high sequence differences compared to those of protostome counterparts, leading to the difficulty in classification of peptides and receptors. These findings also show both the diversity and conservation of GnRH signaling systems in invertebrates. The lack of the HPG axis in invertebrates indicates that biological functions of GnRHs are not release of gonadotropins in current invertebrates and common ancestors of vertebrates and invertebrates. To date, authentic or putative GnRHRs have been characterized from various echinoderms and protostomes as well as chordates and the mRNAs have been found to be distributed not only reproductive organs but also other tissues. Collectively, these findings further support the notion that invertebrate GnRHs have biological roles other than the regulation of reproductive functions. Moreover, recent molecular phylogenetic analysis suggests that adipokinetic hormone (AKH), corazonin (CRZ), and AKH/CRZ-related peptide (ACP) belong to the GnRH superfamily but has led to the different classifications of these peptides and receptors using different datasets including the number of sequences and structural domains. In this review, we provide current knowledge of, and perspectives in, molecular basis and evolutionary aspects of the GnRH, AKH, CRZ, and ACP.

Keywords: gonadotropin-releasing hormone, adipokinetic hormone, corazoin, receptor, invertebrate

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****Correspondence:***
Honoo Satake
satake@sunbor.or.jp

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INTRODUCTION

Discovery of gonadotropin-releasing hormones (GnRHs) as a hypothalamic releasing factor for luteinizing hormone (LH) by Andrew V. Schally and Roger Guillemin in 1971 paved the way for investigation of basal endocrine reproductive systems (1, 2). This is also the origin of long and wide exploration of the GnRH kingdom. Over the past 20 years, GnRH and its related peptides have been identified in the central nervous system of not only non-mammalian vertebrates but also invertebrates such as ascidians, amphioxus, echinoderms, annelids, and mollusks (3–6). Invertebrates lack orthologs of gonadotropin hormones and pituitary glands, indicating that invertebrate GnRHs cannot serve as "gonadotropin-releasing hormones" in the hypothalamus, pituitary, and gonad axis (HPG axis) but rather function as neuropeptides that directly regulate target tissues. The expression of GnRH receptors (GnRHRs) in various tissues also supports non-hypothalamic functions of invertebrate GnRHs.

Various neuropeptides structurally related to GnRHs (**Figure 1**), such as adipokinetic hormone (AKH), corazonin (CRZ), and AKH/CRZ-related peptide (ACP), have also been identified in diverse invertebrates (3–11). As shown in **Figure 1**, these peptides share the N-terminal pyro-Glu residue and C-terminal amide and conserve Phe, Trp, or Tyr residue in position 3, Ser or Thr in position 4, and Trp or Tyr in position 7 with a vertebrate GnRH2 (pQHWSHGWYPGa). Furthermore, molecular phylogenetic and phylogenomic analyses of peptide genes have led to the presumption that GnRH, AKH, CRZ, and ACP originated from common ancestors of the Bilateria (3, 7–9), whereas the four peptides have been shown to exhibit distinct physiological functions including activation of lipid-mobilization by AKHs, stimulation of heart rate by CRZs (10), and down-regulation of oocyte proliferation and elevation of total hemolymph lipids by ACP (11) in arthropods. The cognate receptors for these peptides have also been identified in a wide invertebrate species, revealing that all of these receptors belong to the Class A G protein-coupled receptor (GPCR) family (5, 8, 12). Furthermore, sequence comparison and molecular phylogenetic analysis of these receptors have proposed several evolutionary scenarios for hundred million years (**Figure 2**), leading to the presumption that GnRH, AKH, CRZ, and ACP and their receptors constitute a superfamily (3, 5, 9, 13).

In this review, we provide basic and the latest knowledge regarding primary sequences, signal transductions, biological activities of GnRH, AKH, CRZ, and ACP and their receptors, and an overview of molecular evolution of these peptides and receptors.

Gonadotropin-Releasing Hormones

Vertebrate GnRHs

Gonadotropin-releasing hormones are composed of 10 amino acids with consensus sequences of pyro-Glu^{1}-His^{2}-Trp^{3}-Ser^{4} and Pro^{9}-Gly^{10}-amide and play pivotal roles in reproduction as releasing factor of gonadotropins in vertebrates (4). As shown in **Table 1**, two types of GnRHs (GnRH1 or Type 1 GnRH and GnRH2 or Type 2 GnRH) have been characterized in most vertebrates, whereas the third subtype was found in teleost and lamprey (14–16). Molecular phylogenetic tree and phylogenetic genomic analyses suggest that these subtypes have been generated by gene duplications within the species (15, 16). In other words, teleost GnRH3 and lamprey GnRH-III are specific paralogs to the respective species.

Urochordate GnRHs

To date, 12 GnRH peptides have been identified in ascidians (**Table 1**). t-GnRH-1 and -2 were originally identified within the neural extract of an ascidian, *Chelyosoma productum* (17). Subsequently, ascidian GnRHs were isolated from other ascidians, *Ciona intestinalis* and *Ciona savignyi* (18). The former ascidian produces t-GnRH-3 to -8, and the latter generates t-GnRH-5 to -9 (18). In *Halocynthia roretzi*, t-GnRH-10 and -11 were characterized (19). All of these ascidian GnRHs conserve the consensus sequences of pyro-Glu^{1}-His^{2}-Trp^{3}-Ser^{4} and Pro^{9}-Gly^{10}-amide of vertebrate GnRHs. Furthermore, a unique

Peptides	Phylum	Species name	1 2 3 4 5 6 7 8 9 10 11 12
GnRH2	Vertebrata	*Homo sapiens*	pQ - - H W S H G W Y P Ga
lGnRH-II	Vertebrata	*Petromyzon marinus*	pQ - - H W S H G W F P Ga
t-GnRH-6	Urochordata	*Ciona intestinalis*	pQ - - H W S K G Y S P Ga
Oct-GnRH	Mollusca	*Octopus vulgaris*	pQ N Y H F S N G W H P Ga
AKH	Arthoropoda	*Locusta migratoria*	pQ - L N F T P N W G Ta
CRZ	Artholopoda	*Periplaneta americana*	pQ T F Q Y S R G W T Na
ACP	Arthlopoda	*Anophels gambiae*	pQ - V T F S R D W N Aa

FIGURE 1 | Amino acid sequence alignment of vertebrate gonadotropin-releasing hormones (GnRHs), urochordate GnRH, molluscan GnRH, adipokinetic hormone (AKH), corazonin (CRZ), and AKH/CRZ-related peptide (ACP).

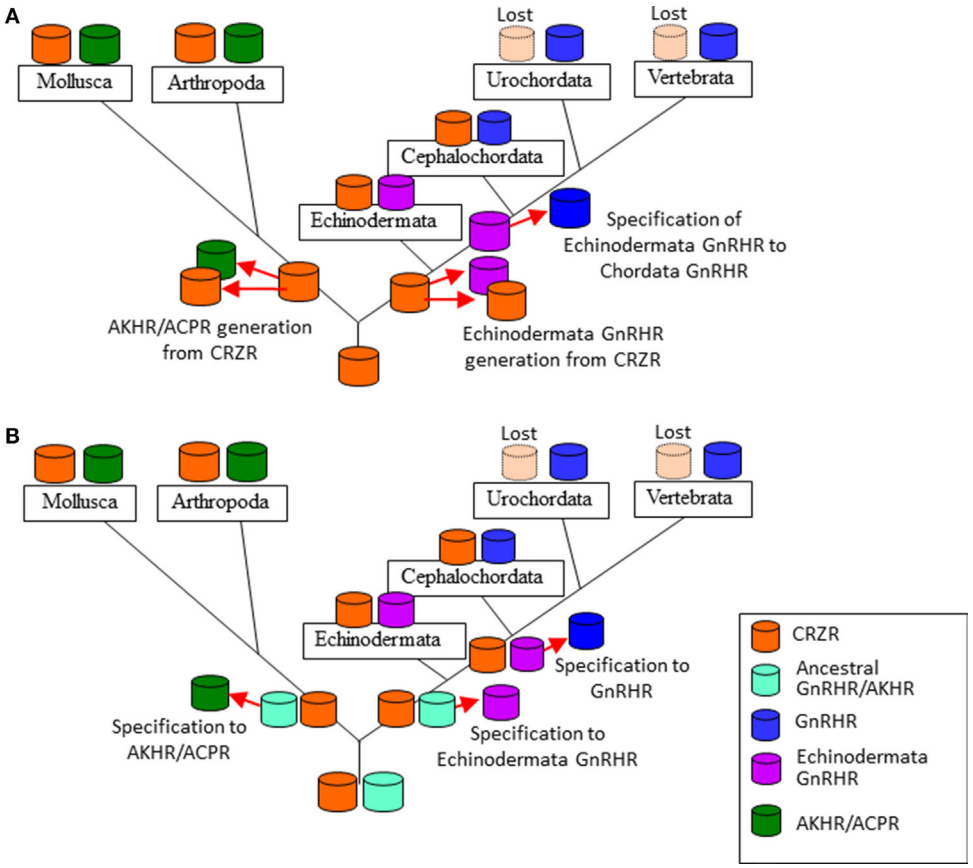

FIGURE 2 | Two evolutionary scenarios of the formation of the gonadotropin-releasing hormone receptor (GnRHR) superfamily. **(A)** an ancestral corazonin receptor (CRZR), which has been conserved in the Echinodermata and Hemichordata, and the Annelida and the Mollusca, generated two lineages; (1) leading to CRZR and adipokinetic hormone receptor (AKHR), subsequently AKHR generated artholopod ACPR, (2) leading to GnRHR in the Chordata. CRZR was lost after evolution of the Urochordata in deuterostomes. **(B)** GnRHR and CRZR might have been arisen from an ancestral peptide receptor by gene duplication in a common ancestor of the Bilateria and a second gene duplication of GnRHR gave rise to the AKHR and ACPR in the Protostomia. CRZR has been preserved in all phyla except the Urochortada and the Vertebrata.

GnRH-related peptide, Ci-GnRH-X, was isolated from the neural tissue of *C. intestinalis* and was found to be composed of 16 amino acids harboring the consensus sequence of pyro-Glu^{1}-His^{2}-Trp^{3}-Ser^{4} and Pro^{9}-Gly^{10} and C-terminal Gly-amide (4, 20). The striking feature of ascidian GnRHs is multicopies of GnRH sequences in a single precursor, unlike vertebrate and non-ascidian invertebrate GnRH genes that encode a single GnRH sequence (4, 21). For instance, *ci-gnrh-1* encodes t-GnRH-3, -5, and -6, whereas t-GnRH-4, -7, and -8 sequences are found in another gene, *ci-gnrh-2* (18). Likewise, the *H. roretzi* GnRH gene encodes t-GnRH-10 and -11 (19). These findings indicate conservation and species-specific diversification of GnRHs in urochordates.

Cephalochordate and Echinoderm GnRH-Like Peptides

In the cephalochordate (amphioxus), *Branchiostoma floridae*, a GnRH-like peptide, Amph.GnRHv (pQEHWQYGHWYa, **Table 1**) was identified (12). Recently, GnRH-like peptides, SpGnRHP and ArGnRH (**Table 1**), were identified in the echinoderms, the sea urchin, *Strongylocentrotus purpuratus* (22) and the starfish, *Asterias rubens* (8), respectively. Unlike vertebrate and ascidian GnRHs, SpGnRHP and ArGnRH are 12-residue peptides containing a Val^{2}-His^{3} or Ile^{2}-His^{3} sequence, respectively (**Table 1**). These peptides share several amino acids with urochordate and vertebrate GnRHs and protostome GnRH-like peptides, including the N-terminal pGlu, His^{4} (corresponding His^{2} in chordate GnRHs), Gly^{8} (corresponding Gly^{6} in vertebrate GnRHs), Trp^{9} (corresponding Trp^{7} in vertebrate GnRHs), and C-terminal Pro-Gly-amide, whereas the GnRH N-terminal consensus motif displays quite low sequence homology (**Table 1**). Thus, categorization of the echinoderm peptides as the authentic GnRH family may remain to be concluded.

Protostome GnRH-Like Peptides

Over the past 15 years, GnRH-like peptides have been identified in protostomes including mollusks and annelids (4) (**Table 1**): an octopus, *Octopus vulgaris*; a cuttlefish, *Sepia officinalis*; a pacific oyster, *Crassostrea gigas*; a sea hare, *Aplysia californica*; a marine

TABLE 1 | Amino acid sequences of gonadotropin-releasing hormones (GnRHs).

GnRH			
Deuterostome			
Vertebrate			
Human	*Homo sapiens*	GnRH1	pQ--HWSYGLRPGa
		GnRH2	pQ--HWSHGWYPGa
Guinea pig	*Cavia porcellus*	GnRH1	pQ--HWSYGVRPGa
Trout	*Oncorhynchus mykiss*	GnRH3	pQ--HWSYGWLPGa
Lamprey	*Petromyzon marinus*	l-GnRH-I	pQ--HYSLEWKPGa
		l-GnRH-II	pQ--HWSHGWFPGa
		l-GnRH-III	pQ--HWSHDWKPGa
Invertebrates chordate			
Urochodate			
Tunicate	*Chelyosoma productum*	t-GnRH-1	pQ--HWSYGLRPGa
		t-GnRH-2	pQ--HWSLCHAPGa
	Ciona intestinalis	t-GnRH-3	pQ--HWSYEFMPGa
		t-GnRH-4	pQ--HWSNQLTPGa
		t-GnRH-5	pQ--HWSYEYMPGa
		t-GnRH-6	pQ--HWSKGYSPGa
		t-GnRH-7	pQ--HWSYALSPGa
		t-GnRH-8	pQ--HWSLALSPGa
	Ciona savignyi	t-GnRH-9	pQ--HWSNKLAPGa
	Ciona intestinalis	Ci-GnRH-X	pQ--HWSNWWIPGAPGYNGa
	Halocynthia roretzi	t-GnRH-10	pQ--HWSYGFSPGa
		t-GnRH-11	pQ--HWSYGFLPGa
Cephalochordate			
Amphioxus	*Branchiostoma floridae*	Amph. GnRHv	pQE-HWQYGHWYa
		Amph. GnRH	pQILCARAFTYTHTWa
Echinodermata			
Sea urchin	*Strongylocentrotus purpuratus*	Sp-GnRHP	pQVHHRFSGWRPGa
Starfish	*Asterias rubens*	Ar-GnRH	pQIHYKNPGWGPGa
Protostomes			
Mollusks and annelid			
Octopus	*Octopus vulgaris*	Oct-GnRH	pQNYHFSNGWHPGa
Cuttlefish	*Sepia officinalis*	Oct-GnRH	pQNYHFSNGWHPGa
Swordtip squid	*Loligo edulis*	Oct-GnRH	pQNYHFSNGWHPGa
Oyster	*Crassostrea gigas*	Cg-GnRH	pQNYHFSNGWQPa
Yesso scallop	*Patinopecten yessoensis*	Py-GnRH	pQNFHYSNGWQPa
Sea hare	*Aplysia californica*	Ap-GnRH	pQNYHFSNGWYAa
Owl limpet	*Lottia gigantean*	Lg-GnRH	pQHYHFSNGWKSa
Marine worm	*Capitella teleta*	Ca-GnRH	pQAYHFSHGWFPa
Leech	*Helobdella robusta*	Hr-GnRH	pQSIHFSRSWQPa

The N-terminal pyroglutamic acid and C-terminal amide are shown by "pQ" and "a," respectively.

worm, *Capitella teleta*; a leech, *Helobdella robusta*; a scallop, *Patinopecten yessoensis*. Noteworthily, two-amino acid insertion after position 1 is found in all protostome GnRH-like peptides (**Table 1**). Collectively, these GnRH sequences indicate that 10-amino acid sequence length is conserved within ascidians and vertebrates, whereas protostome and non-chordate invertebrate GnRHs are featured by 2-amino acid insertion. In other words, ancestral GnRHs might have harbored such two amino acids after pyro-Glu, which might have been lost during the chordate evolutionary process.

The C-terminal Pro-Gly-amide of ascidian and vertebrate GnRHs is found in oct-GnRH of cephalopods and echinoderms but not in GnRH-like peptides of gastropods, bivalves, and annelids (**Table 1**), suggesting that cephalopods and echinoderms might have conserved the C-terminal Pro-Gly during their evolutionary processes. Furthermore, all known protostome GnRH-like peptides and SP-GnRHP share the Ser in position 6 or 7, while the Gly^8-Trp^9 sequence is conserved in cephalopod GnRH, echinoderm GnRHs, and l-GnRH-II but not in ascidian GnRHs and other vertebrate GnRHs except l-GnRH-II (**Table 1**). Additionally, substitution of Trp^3 in the N-terminal consensus motif with Phe was found in most protostome GnRHs (**Table 1**). Altogether, these sequences led to the presumption that the ancestral GnRHs might have been composed of pQ-H(F/W)S-GW-PGa or pQ-H(F/W)S-GW-a, and thereafter, chordate GnRHs might have diverged via various substitution and deletion of the two N-terminal amino acids in the evolutionary process of each species.

Vertebrate GnRHRs

Gonadotropin-releasing hormone receptors belong to the Class A GPCR family (4, 14). In most vertebrates, two or three molecular forms of GnRHRs are present (14). Molecular phylogenetic analyses have provided evidence that vertebrate GnRHRs are classified into three groups, type-I, -II, and -III. The type-I GnRHRs were characterized from a wide range of vertebrate species such as teleost, amphibians, reptiles, birds, and mammals (23). Mammalian type-I GnRHRs completely lack the C-terminal tail region, which is present in its non-mammalian receptors (14). The type-II *gnrhr* gene is found in the genome of amphibians, reptiles, aves, and mammals (23). Most mammalian type-II *gnrhr* is non-functional due to the deletion of functional domains or interruption of full-length translation by the presence of a stop codon. In contrast, type-II GnRHRs of several monkeys, pigs, and other non-mammalian vertebrates were shown to be functional (23). Type-I GnRHRs show high affinity for both GnRH1 and 2, whereas type-II GnRHRs are specifically responsive to GnRH2 (14). Type-III GnRHRs were identified in non-mammalian vertebrates (24). In chicken, type-III GnRHR exhibits a 35-fold higher affinity for GnRH2 than for GnRH1 (24). GnRHRs are in general coupled with Gq protein and activate a typical phospholipase C (PLC)–inositol triphosphate (IP)–intracellular calcium mobilization signaling cascade, occasionally leading to phosphorylation of mitogen-activated protein kinase (MAPK) including ERK1/2 (4, 25, 26), while some GnRHRs are also found to trigger or suppress cAMP production via coupling with Gs or Gi protein (21, 25–28).

Invertebrate GnRHRs

Ascidian GnRHRs

In *C. intestinalis*, four GnRHRs, *Ciona* GnRHR (Ci-GnRHR)-1 to -4, have been identified and shown to regulate exceptionally complicated signaling pathways involving ligand-receptor selectivity,

TABLE 2 | Characteristics of ascidian gonadotropin-releasing hormone (GnRH) receptors.

Receptor	Preferable ligands	G proteins	Signaling pathway	Effect by *Ciona* GnRHR (Ci-GnRH)-X	Effect by heterodimerization with R-4
Ci-GnRHR-1	t-GnRH-6	Gq, Gs	Ca^{2+}, cAMP	Moderate inhibition	Potentiation of Ca^{2+} signaling
Ci-GnRHR-2	t-GnRH-7, -8, -6	Gs	cAMP	No effect	Decreasing cAMP production
Ci-GnRHR-3	t-GnRH-3, -5	Gs	cAMP	Moderate inhibition	None
Ci-GnRHR-4	No ligand	None	None	None	–

coupling with multiple G-protein subtypes, and receptor heterodimerization (**Table 2**).

Ciona GnRHR-1, -2, and -3 sequences were found to harbor a long C-terminal tail, whereas a short tail is present in the C-terminus of Ci-GnRHR-4 (27). Ci-GnRHR mRNAs are distributed in the neural complex, heart, intestine, endostyle, branchia sac, and ovary, although biological roles of GnRHs largely remain unclear (26–28). Notably, the elevation of intracellular calcium, which is a typical response of GnRHR activation, was observed only in the t-GnRH-6 and Ci-GnRHR-1 pair (27). t-GnRH-6 also induces cAMP production via Ci-GnRHR-1 (27). Ci-GnRHR-2 exclusively stimulates cAMP production in response to t-GnRH-7, -8, and -6 in this order of potency (27). Ci-GnRHR-3 triggers cAMP production in the presence of t-GnRH-3 and -5 to a similar extent in a ligand-specific fashion. Ci-GnRHR-4 exhibited neither elevation of intracellular calcium nor production of cAMP (27). Induction of intracellular mobilization only by t-GnRH-6 and Ci-GnRHR-1 pair is attributed to the conservation of Gly^6 essential for adoption of the tertiary structure for coupling with Gq (14) exclusively in t-GnRH-6 (**Table 1**). Such signaling profiles indicate that a major *Ciona* GnRH signaling is a cAMP production. Additionally, *Ciona* 16-amino acid GnRH-structurally related peptide, Ci-GnRH-X, was shown to exhibit moderately inhibit activation of Ci-GnRHR-1 and -3 (20). Also of particular interest in Ci-GnRHR signaling is that Ci-GnRHR-4 heterodimerizes with Ci-GnRHR-1 and then potentiates the elevation of intracellular calcium via both calcium-dependent and -independent protein kinase C subtypes and ERK phosphorylation in a ligand-selective fashion (26). Ci-GnRHR-4 was also found to heterodimerize with Ci-GnRHR-2 (28). The Ci-GnRHR-2/-4 heterodimer decreased cAMP production by 50% in a non-ligand selective manner by shifting of activation from Gs protein to Gi protein by Ci-GnRHR-2, compared to the Ci-GnRHR-2 monomer/homodimer (28). These findings verify that Ci-GnRHR-4 serves as a protomer of GPCR heterodimers rather than a ligand-binding receptor (4, 21, 29). In addition, molecular phylogenetic analysis demonstrated that Ci-GnRHRs are included in vertebrate GnRHR clades but form an independent cluster in chordate GnRHRs, suggesting that these receptors have evolved within the *Ciona* species (4, 21, 27, 29, 30). Collectively, these findings indicate ascidian-specific molecular and functional diversity of ascidian GnRH signaling systems.

Amphioxus GnRHRs

Four amphioxus receptors have been identified in the amphioxus, *B. floridae*. Amphioxus GnRHR-1 and -2 were activated only by vertebrate GnRHs but not by Amph.GnRHv, a putative *B. floridae* endogenous GnRH-like peptide that displays the highest sequence similarity to other species GnRHs (**Table 1**), whereas GnRHR-3 was activated exclusively by another amphioxus GnRH- and CRZ-like peptide (**Table 2**), oct-GnRH, and AKH at physiological concentrations (12, 31), indicating that amphioxus GnRHR-3 exhibits extensive ligand selectivity for GnRH superfamily peptides. Unlike Ci-GnRHRs, *B. floridae* GnRHR-1 to -3 were shown to stimulate only intracellular IP accumulation (12). In contrast, no ligands induced IP accumulation or cAMP stimulation via amphioxus GnRHR-4 (12, 31). It should be noted that the Amph.GnRHv failed to activate any of the four GnRHRs (12). Molecular phylogenetic analysis demonstrated that amphioxus GnRHR-1 and -2 are included in the vertebrate GnRHR clade, while amphioxus GnRHR-3 and -4 are likely to belong to the CRZR/GnRHR clade, as described later. Consequently, the authors presumed that the sequence of the neuropeptide might reflect ancestral sequence of CRZ/GnRH or the transition state between CRZ and GnRH (12). Moreover, of keen interest is the identification of authentic (endogenous) ligands for amphioxus GnRHR-1 and -2. Thus, the elucidation of authentic amphioxus GnRH–receptor pairs requires further investigation. Such difficulty may be attributed to some mismatch between amphioxus GnRHRs and cultured cells employed for heterologous functional analysis because of unsuccessful translation of the receptor mRNA or degradation of the receptor protein in heterologous expression systems (32).

Echinoderm GnRHRs

Tian et al. (8) demonstrated that Ar-GnRH (**Table 1**) specifically activated intracellular Ca^{2+} mobilization of a cognate receptor, ArGnRHR in the starfish, *A. rubens*. Four GnRH/CRZ-type receptors have also been identified in the sea urchin, *S. purpuratus* using *in silico* screening (22). However, no functional analysis of these receptors has been reported. Additionally, these echinoderm receptors are included in the invertebrate CRZ/GnRHR clade (12, 31).

Protostome GnRHRs

The first protostome GnRHR was identified in an octopus, *O. vulgaris*. The octopus GnRHR, oct-GnRHR, activates intracellular Ca^{2+} mobilization by oct-GnRH but not vertebrate GnRHs (33). Notably, an oct-GnRH synthetic analog with Asn^2-Tyr^3 deletion abolished the ability to activate the Ca^{2+} pathway via oct-GnRHR, whereas a chicken GnRH-II synthetic analog with an Asn-Tyr insertion after position 1 exhibited weak activation (33). These findings verify that Asn^2-Tyr^3 is required for the activation of oct-GnRHR, suggesting that the two amino acids after position 1 in non-chordate GnRHs are responsible for activating the protostomian GnRHR. *Oct-gnrhr* is expressed in the central nervous system, digestive tissues, aorta, heart,

salivary gland, branchia, radula retractor muscle, egg, and genital organs in the common octopus (33). In another mollusk, gastropod (a sea hare) *A. californica* GnRHR, ap-GnRHR, was also cloned and was found to be expressed in the abdominal, cerebral, and buccal ganglia of the central nervous system and a few peripheral tissues including the chemosensory organ, small hermaphroditic duct, and ovotestis (13). ap-GnRH was shown to increase the IP accumulation but not cAMP production in ap-GnRHR-expressing *Drosophila* S2 cells in a ligand-specific manner (13). Phylogenetic analysis suggests that ap-GnRHR is clustered with several molluscan GnRHRs including oct-GnRHR, amphioxus GnRHR-3 and -4, and multiple insect CRZRs (13).

Biological Functions

In vertebrates, GnRH is synthesized in the hypothalamus, transported to the pituitary and triggers release of follicle-stimulating hormone (FSH) and LH from the pituitary, eventually regulating reproductive functions via the HPG axis. GnRH also serves as a peripheral bioactive peptide including induction of the synthesis and release of sex steroids in vertebrate reproductive tissues (14). The HPG axis-directed endocrine systems were acquired during the vertebrate evolutionary process, and thus, invertebrate GnRHs are likely to have prototypic or species-specific biological roles.

In ascidians, GnRHs were found to increase water flow and then induce the release of eggs and sperm by injection into the gonaducts, ovary, stomach, and posterior body cavity of *C. intestinalis* (18, 34). All four *Ci-gnrhr* genes were shown to be expressed in the brain of the larva of *C. intestinalis* (30). *Ci-gnrhr-1* and *-2* genes are expressed in muscle cells, while *Ci-gnrhr-3* gene is expressed in notochord cells in the larval tail, which is rapidly resorbed during metamorphosis (30). Intriguingly, Kamiya et al. (35) demonstrated that tGnRH-3 and -5 suppressed the growth of adult organs by arresting cell cycle progression and the promotion of tail absorption. These results indicate that t-GnRHs play a pivotal role in the development and/or metamorphosis.

oct-GnRH induced contraction of the oviduct (36) and releases sex steroids, including testosterone-, progesterone-, and 17β-Estradiol-like steroids from the follicle and spermatozoa in octopus (33). In another mollusk, the yesso scallop (*Patinopecten yessoesis*), py-GnRH induces testicular cell proliferation (37). These findings suggest that molluscan GnRHs directly activate the gonadal organs as a bioactive peptide. In contrast, injection of the cognate ap-GnRH into sexually mature and immature sea hares exhibited no effects on ovotestis mass, reproductive tract mass, egg-laying, or penile eversion, altering oocyte growth and egg-laying hormone accumulation and secretion (38). Instead, ap-GnRH exerted stimulation of the parapodial opening, inhibition of feeding, and promotion of substrate attachment (38). These findings, combined with distribution of GnRHR mRNAs in various tissues, suggest that invertebrate GnRHs regulate not only reproductive responses but also other various biological behaviors. Indeed, oct-GnRH induced contraction of the radula retractor muscle expressing *oct-gnrhr* (33).

Adipokinetic Hormones

Adipokinetic hormone was originally identified in the migratory locust, *Locusta migratoria* as a lipid mobilizing factor (39). To date, AKHs have been isolated from insects, mollusks, and nematode (**Figure 1**; **Table 3**). AKHs are composed of 8–10 residues, harboring pGlu in position 1, an aliphatic or aromatic amino acid residue at position 2, Phe-Ser, Phe-Thr, or Tyr-Ser residues at positions 4 and 5, Trp at position 8, and Trp-amide, Trp-Gly-amide, or Trp-Gly-X-amide (where "X" is variable) at the C terminus (5). Li et al. have proposed to classify these peptides in the Protostomia as follows: authentic AKHs that fulfill the above hallmarks, AKH-like peptides that 10 amino acid residues have Trp-X-Gly-amide or Trp-X-Pro-amide at the C terminus, and proto-AKHs that are longer than 10 amino acid residues but have only 2–4 of the AKH hallmarks (40).

AKH Receptors

Adipokinetic hormone receptors belong to the Class A GPCR family identified in protostomes. Zhu et al. demonstrated that AKH activates both cAMP accumulation and Ca^{2+} mobilization via AKHR of the silkworm moth, *Bombix moli* (41). Recently, Li et al. demonstrated that AKHR of the oyster *Crassostrea gigas* was activated by oyster AKH at physiological concentrations (40). Moreover, Nagasawa et al. detected expression of *Py-AKHR* mRNAs in the nerve ganglia, lip, foot, CPG, mantle, testis, and ovary in Yesso scallop, *Patinopecten yessoensis*. The differential expression profile of *Py-AKHR* mRNA in the gonad during gonadal maturation stages suggests their reproductive function (42).

Biological Functions

Adipokinetic hormones have so far been shown to stimulate the fat body, resulting in lipid and carbohydrate mobilization into the hemolymph in insects and crustaceans. Furthermore, a homolog of AKH in the northern shrimp, *Paudalus borealis*, red pigment concentrating hormone, influenced the concentration of pigment chromatophore, causing its body color change

TABLE 3 | Amino acid sequences of adipokinetic hormones (AKHs).

AKHs			
Protostome			
Mollusks			
Oyster	*Crassostrea gigas*	Cg-AKH	pQ-VSFSTNWGSa
Owl limpet	*Lottia gigantea*	Lg-AKH	pQ-IHFSPTWGSa
Sea hare	*Aplysia californica*	Ap-AKH	pQ-IHFSPDWGTa
Arthropod			
Centipede	*Strigmaia maritima*	Smar-AKH	pQ-INFSPGWGQa
Fruit fly	*Drosophila melanogaster*	Dm-AKH	pQ-LTFSPDWa
Silk worm	*Bombix mori*	Bm-AKH1	pQ-LTFTSSWGa
Locust	*Locusta migratoria*	Lm-AKH3	pQ-LNFTPWWa
Nematode			
Nematode	*Caenorhabditis elegans*	Ce-AKH	pQ-MTFTDQWT

The N-terminal pyroglutamic acid and C-terminal amide are shown by "pQ" and "a," respectively.

(43). Notably, AKHs also showed a reduction in oocyte protein and carbohydrate content in the crickets, *Gryllus bimaculatus*, and a reduction in vitellogenin of oocytes in *L. migratoria* (44), indicating a regulatory role for AKHs in insect reproduction. AKH-deficient flies displayed the opposite phenotype in which hemolymph trehalose levels decreased and storage lipid in the fat body accumulated (45). An AKH receptor-deficient strain showed a similar phenotype to AKH-deficient flies (46). In the cricket *G. bimaculatus*, AKH receptor knockdown by RNAi increased feeding frequency and reduced locomotor activity (47).

Corazonins

Corazonins were originally characterized as 11-amino acid arthropod neuropeptides from the cockroach, *Periplaneta americana* (10). A striking feature is the highest conservation of sequence similarity of CRZs in the Arthropoda regardless of diverse functions throughout a variety of species (**Table 4**). [Arg^7]-CRZ (pQTFQYSRGWTN-amide) is the most typical CRZ peptide, and only a few homologs such as [His^7]-CRZ, [Gln^{10}]-CRZ, and [His^4-Gln^7]-CRZ have been found in several insects (5). Recently, however, neuropeptides weakly similar to CRZ have been identified in starfish (HNTFTMGGQNRWKAG-amide), sea urchin (HNTFSFKGRSRYFP – amide), and acorn worm (pQPHFSLKDRYRWK-amide) (**Table 4**), and the starfish peptide was shown to be responsive to the cognate CRZ-type receptor, leading to the presumption that these peptides are invertebrate deuterostome CRZs as putative CRZ-type receptor ligands (8).

CRZ Receptors (CRZRs)

Corazonin receptors are class A family GPCRs. The first CRZR was identified in *Drosophila melanogaster*, and then orthologous receptors have been cloned from moths, mosquitoes, honey bee, and other insects (48) (**Figure 3**). CRZR of the silkworm moth *Bombix mori* induces cAMP accumulation, Ca^{2+} mobilization, and ERK1/2 phosphorylation via the Gq- and Gs-coupled signaling pathways in response to CRZ (49). Various molecular phylogenetic analyses indicate that the annelid and molluscan GnRHRs are clustered with the CRZRs and the annelid and molluscan GnRHRs have been recognized as the members of CRZR/GnRHR clade (5, 12, 13). In the starfish, *Asterias rubens*, CRZ-like peptide (HNTFTMGGQNRWKAG-amide) was identified and also found to activate the cognate receptor (8). Likewise, GnRHR-type receptor was identified and found to be activated specifically by the cognate GnRH-like peptide (pQIHYKNPGWGPG-amide)

TABLE 4 | Amino acid sequences of corazonins (CRZs).

CRZ			
Hemichordate			
Acorn worm	*Saccoglossus kowalevskii*	Sk-CRZ-like	pQPHFSLKDRYRWKPa
Echinoderm			
Sea urchin	*Strongylocentrotus purpuratus*	Sp-CRZ-like	HNTFSFKGRSRYFPa
Starfish	*Asterias rubens*	Ar-CRZ-like	HNTFTMGGQNRWKAGa
Arthropod			
Most arthropods	–	CRZs	pQTFQYSRGWTNa
Centipede	*Strigmaia maritima*	Smar-CRZ	pQTFQYSKGWEPa
Locust	*Locusta migratoria*	Lm-CRZ	pQTFQYSHGWTNa

The N-terminal pyroglutamic acid and C-terminal amide are shown by "pQ" and "a," respectively.

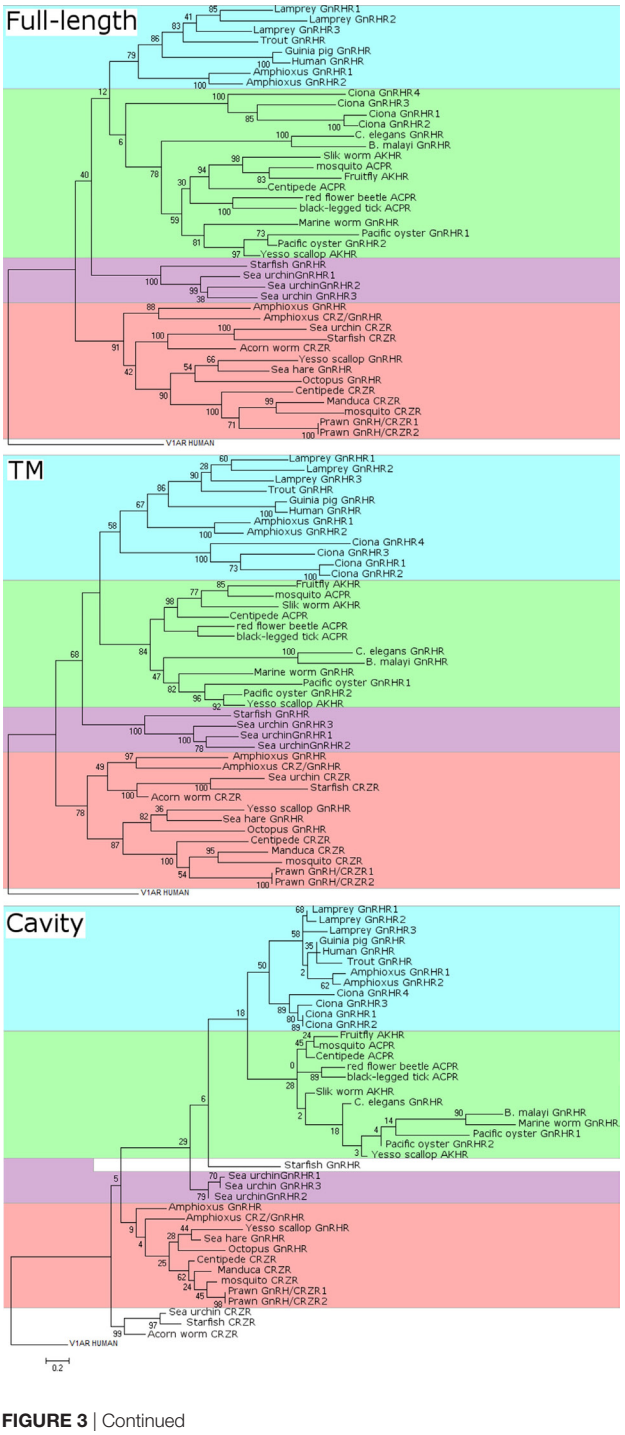

FIGURE 3 | Continued

FIGURE 3 | Continued
Molecular phylogenetic analysis of full-length (top), transmembrane (TM) domain (middle), and cavity (bottom) sequences of gonadotropin-releasing hormone receptors (GnRHRs), AKHRs, corazonin receptors (CRZRs), and ACPRs. The sequence alignments were constructed using MUSCLE in MEGA version 7 and GPCRalign (61) for full-length alignments and TM alignments, respectively. GPCRalign is a PSSM-based alignment algorism and output total 201-length gapless alignments corresponding to TM region. The output TM sequences are listed in Supplementary Material 1–7. The cavity amino acid positions in TM alignment were extracted according to previous report (60). The cavity sequences are listed in Supplementary Material 8. A phylogenic tree of GnRHRs was constructed by the maximum likelihood method based on the JTT matrix-based model. For full-length phylogenetic tree, all positions containing gaps and missing data were eliminated. The scale bar indicates the evolutionary distance of 0.2 amino acid substitutions per protein. The number at each branch node represents percentage given by 100 bootstrap replicates. Evolutionary analyses were conducted in MEGA version 7. The sequences used were as follows: human GnRHR (GNRHR_HUMAN); guinea pig GnRHR (GNRHR_CAVPO); marine worm GnRHR (R7U4C9_CAPTE); sea urchin GnRHR-1 (B2BF80_STRPU); sea urchin GnRHR-2 (B2BF81_STRPU); sea urchin GnRHR-3 (B2BF82_STRPU); tunicate GnRHR-1 (Q869J2_CIOIN); tunicate GnRHR-2 (Q869J1_CIOIN); tunicate GnRHR-3 (D2KZ68_CIOIN); tunicate GnRHR-4 (D2KZ69_CIOIN); trout GnRHR (Q9I986_ONCMY); lamprey GnRHR-1 (A9XCD3_PETMA); lamprey GnRHR-2 (A9XCD4_PETMA); lamprey GnRHR-3 (A9XCD5_PETMA); octopus GnRHR (GNRHR_OCTVU); amphioxus GnRHR-1b (A9XCD1_BRAFL); amphioxus GnRHR-2b (A9XCD2_BRAFL); amphioxus GnRHR-3 (C0IP22_BRAFL); amphioxus GnRHR-4 (C4N9P5_BRAFL); pacific oyster GnRHR-2 (B1GVI7_CRAGI); nematode GnRHR (O44731_CAEEL); sea hare GnRHR (Refseqid:AHE78444); filarial nematode worm GnRHR (A8PVQ9_BRUMA); starfish GnRHR (A0A1B0YGS0_ASTRU); yesso scallop GnRHR (Refseqid: BAX08608); pacific oyster AKHr1b (B1GVI4_CRAGI); fruit fly AKHR (Q71EB3_DROME); silk worm AKHR (Q8T6U9_BOMMO); yesso scallop AKHR (Refseqid: BAX08609); centipede ACPR (Refseqid: AFFK01020326); red flour beetle ACPR (D5FFV2_TRICA); black-legged tick ACPR (A0A0 × 7YC79_IXOSC); honeybee CRZR (B7ZKE3_APIME); tobacco hawk moth CRZR (Q6UJG5_MANSE); sea urchin CRZR (Refseqid: XP_011680711); starfish CRZR (A0A1B0YGT7_ASTRU); centipede CRZR (Refseqid: AFFK01019957); and acorn worm CRZR (Refseqid: XP_006820827).

in a ligand-specific manner (8). Collectively, these results suggest that echinoderms, at least *A. rubens*, may be endowed with the GnRH- and CRZ-directed signaling systems (8).

Notably, as stated earlier, *B. floridae* (amphioxus) GnRHR-3 and -4 are highly homologous to the protostome CRZR/GnRHR receptor family and GnRHR-3 was activated by the amphioxus GnRH-like peptide (pQILCARAFTYTHTW-amide), oct-GnRH (pQNYHFSNGWHPG-amide), and AKH (pQLTFTSSW-amide) at physiological concentrations, indicating that *B. floridae* GnRHR-3 exhibits extensive ligand selectivity for GnRH superfamily peptides. The CRZ/CRZR signaling system has been lost in urochordates, vertebrates, nematodes, and some insects (50).

Biological Functions

Corazonins have a number of physiological roles associated with control of heartbeat, ecdysis behavior initiation, and cuticle coloration in the Artholopoda (5, 48). Recently, its regulatory functions on insulin producing cells in the brain of *D. melanogaster* (51) and on larval–pupal transition and pupariation behavior have been found in the fruit fly, *Bactrocera dorsalis* (52). Intriguingly, CRZs also show reproductive activities in invertebrates. In male flies, CRZs act on its receptor in a small cluster of posterior serotoninergic neurons to control activity of the accessory glands and sperm ejaculation during mating (53). In the giant freshwater prawn *Macrobrachium rosenbergii*, CRZs inhibit testicular development and spermatogenesis and androgenic gland secretion (54). Ablation of CRZ-GAL4 neurons increased locomotion and dopamine level in male files, *D. melanogaster*. Furthermore, silencing of CRZR-GAL4 neurons in male flies elicits infertility and blocks sperm and seminal fluid ejaculation (53). In *B. mori*, dsRNA-mediated knockdown of BmCrzR indicated a role of CRZ signaling in the regulation of silkworm growth and silk production (49).

TABLE 5 | Amino acid sequences of adipokinetic hormone/corazonin-related peptides (ACPs).

ACP			
Protostome			
Arthropod			
Mosquito	*Anopheles gambiae*	Agam-ACP	pQ-VTFSRDWNAa
Kissing bug	*Rhodnius prolixus*	Rhopr-ACP	pQ-VTFSRDWNAa
Flour beetle	*Tribolium castaneum*	Tc-ACP	pQ-VTFSRDWNPa
Centipede	*Strigmaia maritima*	Smar-ACP	pQ-VTFSRDWTPAs
Prawn	*Macrobrachium rosenbergii*	Mro-ACP	pQ-ITFSRSWVPQa

The N-terminal pyroglutamic acid and C-terminal amide are shown by "pQ" and "a," respectively.

AKH/CRZ-Related Peptides

Adipokinetic hormone/CRZ-related peptide is a10–11-amino acid arthropod peptide originally identified from the malaria mosquito, *Anopheles gambiae* (55). In contrast to AKHs, sequences of ACPs, in particular, the N-terminal sequence "QXTFSRXW" (where "X" is variable) and C-terminal amidation, are well conserved in arthropods (**Table 5**), which is reminiscent of an intermediate between AKHs and CRZs (5).

ACP Receptors

Adipokinetic hormone/CRZ-related peptide receptors are Class A family GPCRs identified only in insects (**Figure 3**). Hansen et al. showed that the *A. gambiae* ACP receptor transfected into mammalian cells stably expressing the human G-protein G16, a universal G protein adapter, was activated specifically by the cognate ligand (55). Zandawala et al. characterized three splice variants encoding ACP receptors in the kissing bug *Rhodnius prolixus*; Rhopr-ACPR-A has only five transmembrane (TM) domains, and Rhopr-ACPR-B and C have seven TM domains. All Rhopr-ACPR-A, -B, and -C were activated by Rhopr-ACP but neither Rhopr-AKH nor Rhopr-CRZ with different sensitivities on mammalian cells stably expressing the G-protein G16, whereas Rhopr-ACPR-B and -C indicated coupling with Gq when expressed in CHO-K1-aeq cells (56).

Biological Functions

To date, the ACP signaling system has been found only in arthropods and its major biological roles are still unclear. However,

recently, ACP was shown to decrease germ cell proliferation and increases in total hemolymph lipids were found by administration of the peptide in female prawn, *M. rosenbergii* (11). The expression of *MroACP* mRNA in the eyestalk, central nervous system, thoracic ganglia, and *MroACPR* mRNA in the neural tissues and the ovary throughout different stages of ovarian maturation indicated a neuronal regulation of ACP signaling in reproduction (11).

PROPOSED EVOLUTIONARY SCENARIOS OF GnRH, AKH, CRZ, ACP, AND THEIR RECEPTORS

Based on the aforementioned sequence homology and molecular phylogeny, several studies suggested that GnRH, AKH, CRZ, and ACP constitute a superfamily and originated from a common ancestor (57, 58). However, marked sequence diversity in GnRH, AKH, CRZ, and ACP has led to difficulty in accurate or conclusive classification. For example, Lindemans et al. suggested that GnRH signaling might have been arisen before the divergence of protostomes and deuterostomes on the basis of the presence of the AKH-GnRH signaling system in the nematode *Caenorhabditis elegans* and its biological function in the egg-laying behavior (59). However, molecular phylogenetic analysis led to another presumption that the *C. elegans* AKH-GnRH-like peptide and its receptor belong to the authentic AKH system (5, 50, 59).

As described early, authentic GnRHRs are conserved at least in the Cephalochordata, Urochordata, and the Vertebrata. AKHRs have been identified in the Mollusca, Annelida, and Arthropoda, while ACPRs have been found only in the Arthropoda. Authentic or putative CRZRs are present in all invertebrates except the Urochordata. Molecular phylogenetic analysis (3, 5, 8, 13, 50) has thus far provided two scenarios of their evolutionary processes. The first one is that an ancestral CRZR, which has been conserved in the Ambulacraria (the Echinodermata and the Hemichordata) and the Lophotrochozoa (the Annelida and the Mollusca), generated two lineages: (1) leading to CRZR and AKHR, subsequently AKHR generated artholopod ACPR in the Ecdysozoa and (2) leading to GnRH in the Chordata. CRZR was lost during the evolution of the Urochordata and Vertebrata in deuterostomes (**Figure 2A**). The second one is that GnRHR and CRZR might have been arisen via gene duplication in a common ancestor of the Bilateria, and a second gene duplication of GnRHR might have generated AKHR and ACPR during the divergence of the Lophotrochozoa and Ecdysozoa (the Arthropoda and the Nematoda). CRZR has been conserved in all phyla except the Urochordata and Vertebrata (**Figure 2B**). Notably, these receptors were categorized as different clusters by respective research groups, e.g., GnRHR/AKHR/ACPR and CRZ (8); GnRHR, AKHR/ACPR, and CRZR (13); GnRHR, AKHR/ACPR, and CRZR/protostome GnRHR (3); and GnRHR, AKHR, ACPR, CRZR/protostome GnRHR, and CRZR (5). Furthermore, based on the results of phylogenomic analyses with 36 whole genome sequences and no functional connection of protostome GnRH signaling system to the releasing of gonadotropins because of the lack of the HPG axis in protostomes, Plachetzki et al. classified protostome GnRHs as CRZ-like (or ACP/AKH-like) peptides and categorized the receptors of GnRH superfamily as GnRHRs and CRZR (or ACPR/AKHR) (9).

Such data are mainly attributed to difference in the number, length, and domain of sequences employed for molecular phylogenetic analysis. **Figure 3** shows our reanalysis of the molecular phylogeny of full-length, TM domain, and ligand-binding cavity (60) sequences of 42 receptors including GnRHRs, AKHRs, CRZRs, and ACPRs. Full-length sequences of these receptors were aligned with CLUSTALW using BLOSUM62 substitution matrix. Amino acid sequences of the TM and the cavity were individually aligned with GPCRalign (61). All of molecular phylogenetic tree analyses of the full-length sequences (**Figure 3**) demonstrate that these receptors are classified into the following four major clusters: (1) vertebrate GnRHRs and amphioxus GnRHR-1 and -2 (highlighted in blue), (2) invertebrate GnRHRs/AKHRs/ACPRs including the urochordate GnRHRs, Ci-GnRHRs (highlighted in green), (3) echinoderm GnRHRs (highlighted in purple), and (4) protostome GnRHRs/amphioxus GnRHR-3 and 4/CRZRs (highlighted in orange). Of note, molecular phylogenetic analyses of the TM (**Figure 3**, middle) and cavity (**Figure 3**, bottom) sequences of these receptors indicate that Ci-GnRHRs and amphioxus GnRHR-1 and -2 are included in the clade of vertebrate GnRHRs, although many bootstraps in the molecular phylogenetic tree of the cavity are very low due to much smaller information of cavity sequences (30–40 amino acids) than that of full-length and TM, suggesting extremely weak evolutionary correlations. In contrast, Ci-GnRHRs are included in the AKHR/ACPR cluster in a molecular phylogenetic tree of the full-length sequences (**Figure 3**, top). Moreover, the molecular phylogenetic trees of the TM and the cavity regions indicate that echinoderm "GnRHRs" form a monophyletic clade and display closer homology to the CRZR family than the GnRHR family. This molecular phylogenetic tree is consistent with species-specific sequences of echinoderm GnRH-like peptides (**Table 1**), suggesting species-specific diversification of echinoderm GnRH and GnRHR lineages. In combination, conservation of partial consensus motifs and molecular phylogenetic analyses are not sufficient for substantiating the evolutionary process of the "GnRH/AKH/CRZ/ACP superfamily," which may mislead us to an incorrect conclusion.

CONCLUSION AND PERSPETIVES

In the Vertebrata, GnRHs play pivotal roles in reproductive function as a releasing factor for gonadotropin in the HPG axis and a neuropeptide that directly regulate target tissues. In contrast, reproductive functions of invertebrate GnRHs have not been demonstrated. Instead, there has been a growing body of reports of reproductive functions of invertebrate GnRH-related peptides, AKH, CRZ, and ACP. These findings suggest that, if GnRH, AKH, CRZ, and ACP constitute a superfamily, the superfamily peptides might have been endowed with both common and species-specific reproductive functions as well as other physiological functions. In this regard, of particular interest are biological roles of GnRHs or GnRH-like peptides

in protostomes, echinoderms, cephalochordates, and urochordates, which lack the HPG axis.

Gonadotropin-releasing hormone, AKH, CRZ, and ACP bear approximately 10 amino acids, and the respective "consensus motifs" are frequently diverged among species. Furthermore, the nested clusters (**Figure 3**) within GnRHRs, AKHRs, and CRZRs in molecular phylogenetic trees of the TM and the cavity imply that a small number of amino acid substitutions in these regions can change their ligand selectivity. Therefore, only standard homology-based analysis may lead to insufficient data for understanding the evolutionary process of these peptides and receptors. Consequently, integration of multiple molecular phylogenetic analyses of much more sequence information of these peptides and receptors in other invertebrates with biological roles of these signaling systems in various invertebrate species will enable us to elucidate their biological significance and true evolutionary processes.

AUTHOR CONTRIBUTIONS

TS and HS conducted manuscript preparation. TS, TK, SM, MA and HS investigated background literatures. TS, AS and HS wrote manuscripts. AS and HS analyzed data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2017.00217/full#supplementary-material.

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