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Discovery of new receptors regulating luteinizing hormone and follicle-stimulating hormone secretion by bovine gonadotrophs to explore a new paradigm for mechanisms regulating reproduction

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Abstract. Previous studies in the 1960s and 1970s have reported that both gonadotropin-releasing hormone (GnRH) and estradiol-activated nuclear estrogen receptors regulate gonadotropin secretion in women. However, I had previously reported that gonadotroph function is regulated by complex crosstalk between several membrane receptors. RNA-seq had previously revealed 259 different receptor genes expressed in the anterior pituitary of heifers. However, the biological roles of most of these receptors remain unknown. I identified four new receptors of interest: G protein-coupled receptor 30 (GPR30), anti-Mullerian hormone (AMH) receptor type 2 (AMHR2), and G protein-coupled receptors 61 and 153 (GPR61 and GPR153). GPR30 rapidly (within a few minutes) mediates picomolar, but not nanomolar, levels of estradiol to suppress GnRH-induced luteinizing hormone (LH) secretion from bovine gonadotrophs, without decreasing mRNA expressions of the LHα, LHβ, or follicle-stimulating hormone (FSH) β subunits. GPR30 is activated by other endogenous estrogens, estrone and estriol. Moreover, GPR30 activation by zearalenone, a nonsteroidal mycoestrogen, suppresses LH secretion. AMHR2, activated by AMH, stimulates LH and FSH secretion, thus regulating gonadotrophs, where other TGF-β family members, including inhibin and activin, potentially affect FSH secretion. I also show that GPR61, activated by its ligand (recently discovered) significantly alters LH and FSH secretion. GPR61, GPR153, and AMHR2 co-localize with the GnRH receptor in unevenly dispersed areas of the bovine gonadotroph cell surface, probably lipid rafts. The findings summarized in this review reveal a new paradigm regarding the mechanisms regulating reproduction via novel receptors expressed on bovine gonadotrophs.

Key words: Anterior pituitary, Anti-Mullerian hormone receptor type 2, Gonadotropin-releasing hormone receptor, G protein-coupled estrogen receptor-1, Lipid raft

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Introduction

Gonadotrophs are important cells located in the anterior pituitaries (APs), which secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to regulate reproduction in animals (Fig. 1). Previous studies in the 1960s and 1970s [1–3] have reported that both gonadotropin-releasing hormone (GnRH) and estradiol-activated nuclear estrogen receptors regulate gonadotropin secretion in women.

However, I speculated whether these canonical concepts adequately explain the complex phenomena observed in various animals under various conditions. For example, Holstein heifers intramuscularly administered with a GnRH analogue displayed a more rapid surge in LH levels in the summer than in the spring [4]. Furthermore, postpartum Holstein dairy cows intramuscularly administered a GnRH analogue displayed a smaller surge in LH levels at 10 days rather than at 30 or 60 days postpartum in high-producing dairy cows [5]. Estradiol rapidly suppresses GnRH-stimulated LH release from the bovine pituitary cells within an hour, which is a very short window to affect mRNA and protein expression of the LHα and LHβ subunits [6, 7]. The two aforementioned canonical mechanisms do not explain these observations.

Therefore, I attempted to further the current understanding of the mechanisms underlying the regulation of LH and FSH secretion in bovine gonadotrophs. Specifically, I aimed to address the following three aspects:

(1) The mechanism through which estradiol rapidly suppresses LH secretion

(2) The structure of the GnRH receptor (GnRHR) expressed on the surface of gonadotrophs

(3) The existence of other receptors on the surface of gonadotrophs, which regulate LH and FSH secretion.

This review discusses mainly rapid (within 30 min) pathways to alter LH and FSH secretion from gonadotrophs [4, 5, 8–10], without alterations in mRNA expression of the LHα, LHβ, or FSHβ subunits in gonadotrophs. Any mechanism that inhibits the rapid stimulation of LH and FSH by GnRH may potentially function more rapidly (within few minutes), suggesting the importance of unidentified cell membrane receptors rather than nuclear receptors.

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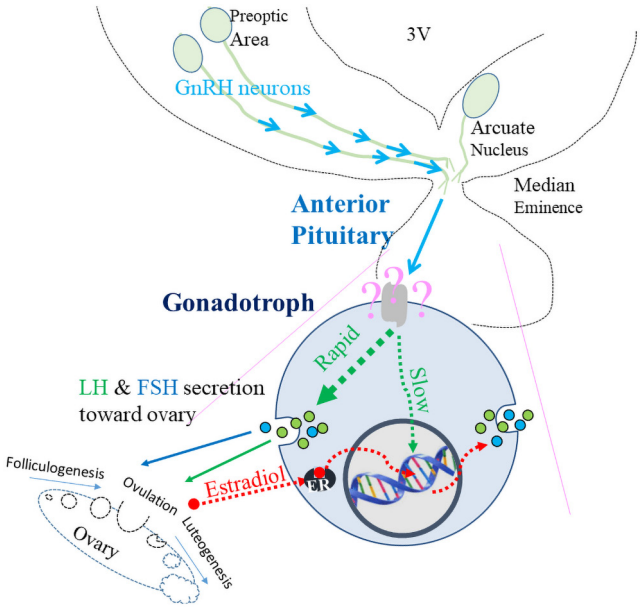

Fig. 1. Gonadotrophs in the anterior pituitary (AP) secrete essential luteinizing hormone (LH; green circle) and follicle-stimulating hormone (FSH; blue circle) to regulate reproduction. The canonical mechanisms regulating gonadotropin secretion in women are as follows: 1) stimulation by gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus [via either a rapid non-genomic (non-transcriptional regulation) pathway (as shown by green, bold, dotted lines), or via a slow genomic (transcriptional regulation) pathway (as shown by green, thin, dotted lines)], and 2) genomic suppression by nuclear-localized estrogen receptors (ERs) (as shown by red, dotted lines) activated by estradiol.

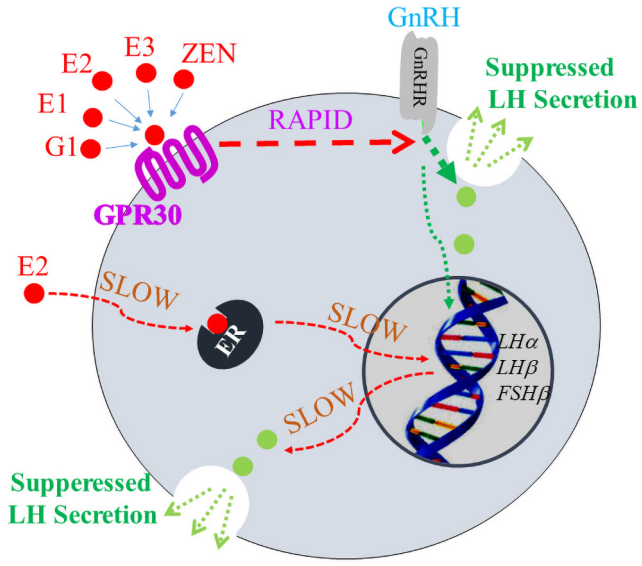

Fig. 2. The membrane estrogen receptor, G-protein-coupled receptor 30 (GPR30), is likely to be expressed on the surface of bovine gonadotrophs to bind endogenous estrogens [estrone (E1), estradiol (E2), and estriol (E3)], GPR30-specific agonist (G1), and nonsteroidal mycoestrogen zearalenone (ZEN) produced by Fusarium. Ligand-activated GPR30 rapidly (within a few minutes) suppresses gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone (LH) secretion (shown as a green, bold, dotted line) via a rapid pathway (shown as a red, bold, dotted line) to regulate LH secretion without affecting LHα, LHβ, and FSHβ mRNA expression, unlike the slow pathway (shown as red thin dotted line) via estradiol-activated estrogen receptor (ER).

Discovery of G-protein-coupled Receptor 30 (GPR30) for Rapid Estradiol-mediated Suppression of LH Secretion from Gonadotrophs

Estradiol is a potent ovarian feedback regulator, which regulates both the GnRH secretion from hypothalamus and the gonadotrophic LH secretion. Blood estradiol concentrations fluctuate in heifers and cows between 0.004 nM (1 pg/ml) and 0.030 nM (8 pg/ml) during the luteal phase of the estrous cycle [11, 12]. Low estradiol concentrations exert a negative feedback effect on hypothalamic GnRH secretion [13–15] and suppress LH mRNA levels in the AP [16].

Estradiol modulates LH and FSH mRNA expression in the gonadotrophs by interacting with nuclear estrogen receptors α or β (ERα or ERβ). However, estradiol rapidly suppresses LH-secretion within a few minutes, without alterations in mRNA expression of the LHα, LHβ, or FSHβ subunits in ruminants [7, 17–20]. Candidate membrane receptors for this rapid regulatory pathway include ERα, ERβ, and GPR30 (*syn*. G protein-coupled estrogen receptor 1). I discovered mRNA and protein of GPR30 in the bovine AP [17]. Furthermore, I clarified that GPR30 is the primary receptor that regulates the rapid estradiol suppression of LH secretion from bovine AP using a GPR30-specific agonist G1, a GPR30-specific antagonist, and an ER antagonist [17, 18] (Fig. 2). Moreover, I clarified that decreased cytoplasmic cAMP levels [18–20], activated protein kinase A (PKA), and extracellular signal-regulated kinase (ERK) [21] are the intracellular mediators downstream of GPR30, which are involved in inducing rapid suppression of GnRH-induced LH secretion from bovine AP cells via estradiol or G1.

Interestingly, neither estradiol nor G1 suppresses FSH secretion from bovine AP cells [17]. Arreguin-Arevalo and Nett [6] reported that estradiol infusion for less than 4 h does not suppress FSH secretion in ewes. Therefore, estradiol may not suppress FSH secretion from gonadotrophs through any rapid mechanism.

Endogenous estrogens, estrone and estriol, are weaker agonists for ERα and ERβ than estradiol and have therefore been designated "weak" estrogens [22]. However, both estrone and estriol are potent, rapid regulators in rat lactotroph-like tumor cells [22]. Hence, estrone and estriol may also modulate GnRH-induced LH secretion from bovine AP via GPR30. Indeed, intramuscular estriol administration reportedly induces an LH surge earlier than that induced via an estradiol injection in cows and ewes [23, 24]. I had previously reported that GnRH-induced LH secretion from primary cultured bovine AP cells was inhibited by estrone, estradiol, and estriol at picomolar levels, but not at femtomolar or nanomolar levels [18].

Furthermore, I had reported that nonsteroidal mycoestrogen zearalenone (ZEN), produced by Fusarium, and the five known ZEN metabolites rapidly suppress GnRH-induced LH secretion [19, 20].

Some of these compounds, even those exhibiting weak suppressive effects through ERα and ERβ signaling, potentially induce rapid and potent suppression of GnRH-induced LH secretion via GPR30 [20]. Moreover, ZEN has been detected in cereal grains, animal feed [25], and cattle urine worldwide [26], and it is retained in ruminant bodies fed contaminated feed for long periods in enterohepatic circulation.

Considering the discovery of GPR30, I hypothesized that bovine gonadotrophs express additional unidentified receptors that can regulate gonadotropin secretion.

GnRH Receptors Localization in the Limited Area of Plasma Membrane of Gonadotrophs

GnRH receptor is a G protein-coupled receptor (GPCR), located in the plasma membrane of gonadotrophs that responds to GnRH, thereby regulating reproduction in mammals [27]. The plasma membrane is not a random sea of lipids. Lipid rafts are common, distinct relatively insoluble regions with lower density and are lower fluidity than the surrounding membrane [28, 29]. They are liquid-ordered phase islands dispersed throughout the lipid bilayer matrix [28, 29].

Lipid rafts are thought to facilitate signaling by promoting the colocalization of membrane receptors and their downstream signaling components [28, 29]. Thus, lipid rafts serve as platforms and hubs for hormone receptors and cytoplasmic signal pathways. Indeed, lipid rafts have been recognized as important pharmacological targets in human medicine [30, 31]. To further the current understanding of GnRHR, I developed an antibody targeting the N-terminus of the extracellular domain of GnRHR for immunohistochemistry, fluorescence-activated cell sorting, and immunocytochemistry assays [32]. Bovine GnRHRs are aggregated and restricted to a limited area of the cell surface (rather than evenly dispersed) of LH-monohormonal, FSH-monohormonal, and bihormonal gonadotrophs [32–35] (Fig. 3). Therefore, I hypothesized that GnRHRs are located in the lipid raft of gonadotroph. Furthermore, I established a method to visualize colocalized novel receptors and GnRHRs on the plasma membrane of gonadotrophs, using the anti-GnRHR antibody and immunofluorescence microscopy.

It is impossible to directly visualize lipid raft location, because they are comprised of lipids of small molecular weight, which cannot be stained. However, GnRHRs can be detected in a low-density fraction of a plasma membrane preparation from mouse AP recovered through a sucrose gradient [36]. I also observed that GnRHRs colocalize with the important lipid raft marker protein, flotillin 1 [37], in limited areas of the plasma membrane of bovine gonadotrophs (Fig. 4). The positive overlap coefficient between GnRHR and flotillin 1 calculated by confocal data analysis software was very strong (0.70). Taking all these results into account, I concluded that GnRHRs are localized to lipid rafts on the cell surface of gonadotrophs (Fig. 5). This conclusion is supported by another research group utilizing the LβT2 clonal murine gonadotroph cell line [38]. In my published study, I include photos demonstrating GnRHRs restricted to discrete areas of the cell surface of bovine gonadotrophs in AP tissues and culture, including Z-stacks [32].

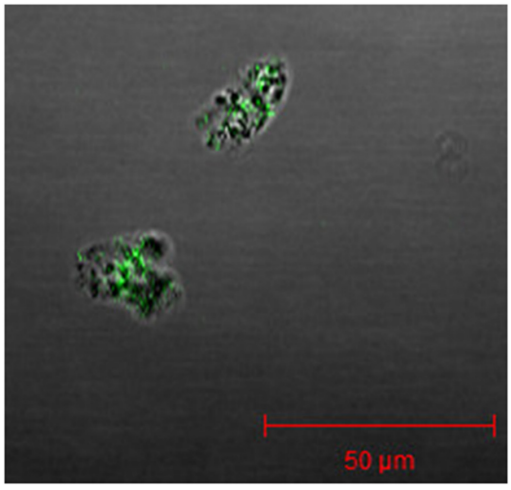

Fig. 3. Representative confocal microscopy images of gonadotrophs purified via fluorescence-activated cell sorting with the antibody targeting the extracellular N-terminal domain of the gonadotropin-releasing hormone (GnRH) receptor (GnRHR). Stained GnRHR (shown as green dots) was observed in the limited, unevenly dispersed area of the plasma membranes of unfixed, floating gonadotrophs.

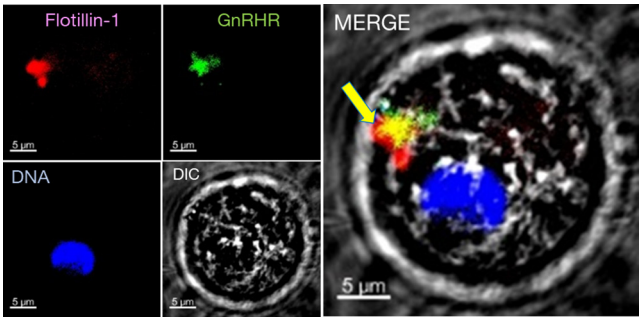

Fig. 4. Immunofluorescence showing gonadotropin-releasing hormone (GnRH) receptor (GnRHR) and lipid raft marker, Flotillin-1, aggregation at the surface of cultured bovine AP cells fixed by CellCover solution (Anacyte aboratories UG, Kuhreder, Hamburg) without Triton X-100 treatment. Images of Flotillin-1 (red) and GnRHR (green) captured using laser-scanning confocal microscopy; nuclei were counterstained with DAPI (dark blue). Cell contours observed using differential interference contrast (DIC) microscopy. Rabbit polyclonal anti-Flotillin-1 (Proteintech, Rosemount, IL, USA) and our original guinea pig anti-GnRHR antibodies [5] bound to Flotillin-1 and GnRHR, respectively, on the gonadotroph surface. Flotillin-1 and GnRHR appeared to aggregate, and GnRHR colocalized with Flotillin-1 in the limited area of plasma membrane of gonadotrophs (Yellow arrows).

Deep Transcriptome Sequencing of the AP of Heifers Before and After Ovulation

In an attempt to discover new receptors expressed in gonadotrophs, I developed two new methods to prepare pure bovine gonadotrophs from the heterogeneous AP cell mixture, using the anti-GnRHR

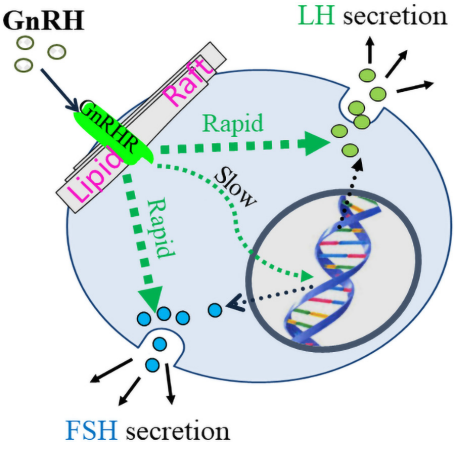

Fig. 5. A schematic representation of gonadotropin-releasing hormone (GnRH) receptor (GnRHR) in lipid rafts of the plasma membrane of gonadotrophs and rapid and slow downstream cytoplasmic signaling pathways regulating luteinizing hormone (LH) (shown as green circles) and follicle-stimulating hormone (FSH) (shown as blue circles) secretion.

antibody: either through a method based on fluorescence-activated cell sorting [32], through a method based on magnetic separation [39]. While both methods can purify bovine gonadotrophs from the heterogeneous AP cell mixture up to 100% purity, I found that the resulting RNA quality was insufficient for efforts to identify new genes via deep transcriptome sequencing (RNA-seq).

Therefore, I assessed gene expression patterns via RNA-seq in the AP, but not in the purified gonadotrophs of heifers before and after ovulation [40]. Consequently, 396 differentially expressed genes were identified in the bovine AP ($P < 0.05$) in pre- and post-ovulation samples, which included two GPCR genes (*GPR61* and *GPR153*), and nine receptors for previously identified ligands. The AP expressed 259 receptor genes, including Anti-Mullerian hormone (AMH) receptor type 2 (AMHR2). Moreover, Ingenuity pathway analysis of the 396 genes revealed a canonical pathway linking GPCR to cytoskeletal reorganization, actin polymerization, microtubule growth, and gene expression.

Colocalization of GPR61, GPR153, and AMHR2 with GnRHR in the Limited Area of Plasma Membrane of Gonadotorophs

The lipid raft containing GnRHR may serve as platforms and hubs for other hormone receptors and cytoplasmic signal pathways by promoting colocalization of membrane receptors. Therefore, I speculated that GPR61, GPR153, and AMHR2 might colocalize with GnRHR in the limited area of the plasma membrane of gonadotrophs. To test this hypothesis, I examined the colocalization of GnRHR with each one of these receptors (and with either the LHβ subunit or FSHβ subunit) in both AP tissue and cultured AP cells using immunofluorescence microscopy [33–35]. I found that GPR61 (Fig. 6a), GPR153 (Fig. 6b), and AMHR2 (Fig. 6c) colocalize with GnRHRs in the limited area of the plasma membrane in bovine

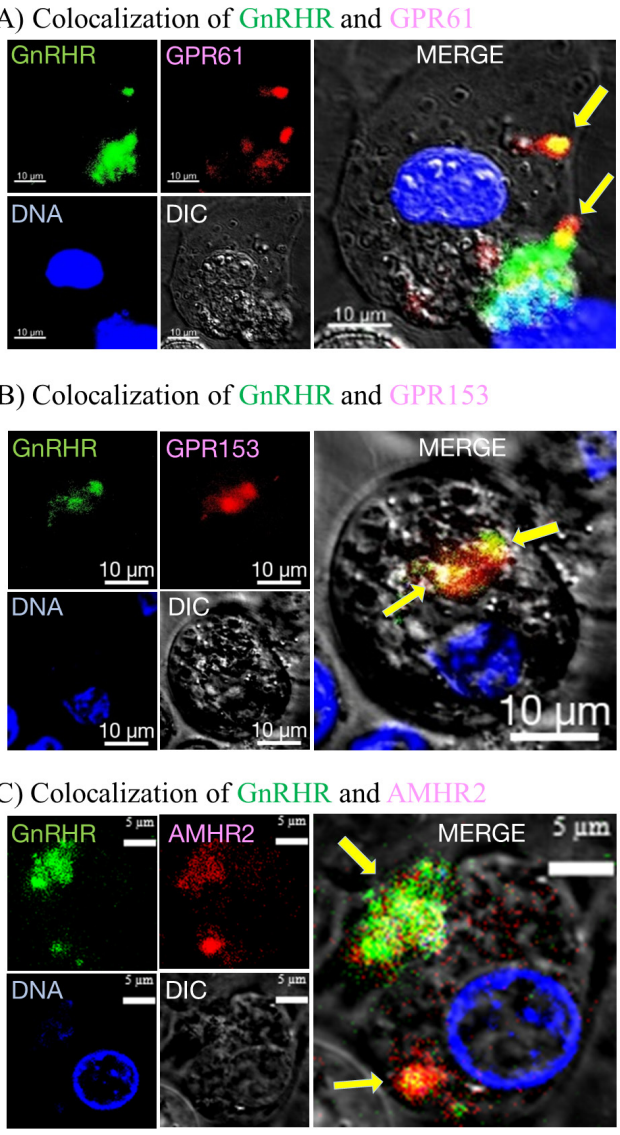

Fig. 6. Immunofluorescence of gonadotropin-releasing hormone (GnRH) receptor (GnRHR) (Green) and immunofluorescence (Red) of G protein-coupled receptors 61 (GPR61) (A), GPR153 (B), or anti-Mullerian hormone (AMH) receptor type 2 (AMHR2) (C), captured on the plasma membrane of bovine gonadotrophs using laser-scanning confocal microscopy. Nuclei were counterstained with DAPI (blue). Cell contours were observed using differential interference contrast (DIC) microscopy. Each primary antibody bound its respective receptor at the surface of gonadotroph. The receptors appeared aggregated and colocalized with GnRHR in the limited area of plasma membrane (Yellow arrows).

gonadotrophs. The positive overlap coefficient calculated by confocal data analysis software was very strong (more than 0.70) between GnRHR and GPR61, between GnRHR and GPR153, and between GnRHR and AMHR2 on the cell-surface of gonadotrophs [33–35].

Bovine Gonadotrophs Express GPR61, Whose Ligand Potentially Contributes to Age-related Infertility

GPR61 is widely expressed in the brain, including the hypothalamus and the AP [41, 42]. However, its function remains unknown. I discovered that GPR61 expression in bovine AP tissues was down-regulated during the early luteal phase as compared to pre-ovulation or mid- or late luteal phases [33]. GPR61 associates with Gs protein [43, 44], and stimulates ERK signaling in neurons [45]. The ERK signaling pathways play an important role in the control of LH secretion in bovine gonadotrophs [21]. These results suggest that the gonadotroph GPR61 has important role in LH and FSH secretion.

A recent study suggested that ethanolamine plasmalogen (EPl) is a ligand for GPR61 in mouse neuroblastoma [45]. Therefore, I tested the hypothesis that EPl extracted from bovine brain stimulate LH and FSH secretion from cultured bovine AP cells [46]. I treated the cultured AP cells derived from postpubertal heifers with increasing concentrations (0, 5, 50, 500, 5,000, 50,000, or 500,000 pg/ml) of commercially available EPl extracted from cattle brains (of ages unidentified by the company), for 5 min before either no treatment or GnRH stimulation. EPl (50–500 pg/ml) stimulated ($P < 0.05$) the basal secretion of FSH but not of LH. EPl at 50 pg/ml also enhanced ($P < 0.05$) GnRH-induced FSH secretion. In addition, my results suggested that ERK, Sma, and Mad pathways are the cytoplasmic pathways responsible for these effects [46].

EPls are a unique class of glycerophospholipids containing a fatty alcohol with a vinyl-ether bond at the sn-1 position, and fatty acids at the sn-2 position of the glycerol backbone. My data suggest the importance of the side chain at the sn-2 position for the observed effect on LH and FSH secretion [46]. Most likely, the organ that secretes EP1 is the brain, and reduction in EP1 levels in aging brains induces age-related diseases including Alzheimer's disease [45]. Molecular species of EPl may differ after aging. Therefore, I am currently investigating the association between bovine brain EPl and infertility after aging, utilizing EPl extracted by myself from young and old bovine brains.

Bovine Gonadotrophs Express GPR153

To date, information about GPR153 is limited; specifically, no role has been previously reported for this orphan receptor. However, GPR153 is widely expressed in the brain, including in the hypothalamic arcuate nucleus and the pituitary [47]. The GPR153 gene is conserved in chimpanzees, Rhesus monkeys, dogs, cows, mice, rats, chicken, frogs, and zebrafish (https://www.ncbi.nlm.nih.gov/homologene/18662). I evaluated whether GPR153 expression in AP is dependent on the reproductive stage. Real-time PCR and western blot analyses found that GPR153 expression was significantly lower in AP tissues during the early luteal phase as compared to pre-ovulation or the late luteal phases [34]. The 5'-flanking region of the GPR153 gene contained a consensus response element sequence for estrogen, but not for progesterone [34]. None of the few reports published describe the function of GPR153 or identify its ligand(s). However, GPCRs function not only as a monomer or homodimer, but also as a heterodimer with another GPCR [48]. Heterodimerization among paralogs of GnRHRs of a protochordate results in the modulation of ligand-binding affinity, signal transduction, and internalization [48]. It is possible that GPR153 forms a heterodimer, affecting ligand-binding affinity, signal transduction, and internalization of GnRHR. Internalization of GnRHR in ovine and rodent gonadotrophs has been reported [49], and such internalization is important for desensitization of GnRH-stimulated gonadotropin secretion [50]. Therefore, GPR153 colocalizing with GnRHR on the cell surface of gonadotrophs might play an important role.

Bovine Gonadotrophs Express AMHR2 and AMH to Regulate LH and FSH Secretion

My previous deep transcriptome sequencing studies in heifer AP before and after ovulation did not reveal significant differences in the expression of AMHR2, the receptor for AMH [40]. AMH is a dimeric glycoprotein belonging to the transforming growth factor (TGF)-β family and it is primarily produced by the granulosa cells of the preantral and small antral follicles in humans and animals [51]. AMHR2 contains a single hydrophobic transmembrane domain linked to hydrophilic extracellular and intracellular regions [35]. Although the role of AMH in the ovaries is well known [52, 53], AMH secreted from preantral and small antral follicles into the blood may function in other organs as well. Indeed, the APs of adult rats express AMHR2 [54], AMH activates LHβ and FSHβ gene expression in a murine gonadotroph-derived cell line [54], and plasma AMH concentrations are positively associated with pregnancy rates in dairy cows [55]. Furthermore, I confirmed that AMH stimulates LH and FSH secretion from bovine gonadotrophs [35]. Therefore, preantral and small antral follicles potentially contribute to the regulation of gonadotropin secretion from the AP.

Other important endocrine hormones for gonadotroph regulation (e.g., GnRH, inhibin, and activin) have paracrine and autocrine effects. Thus, I evaluated AMH expression in bovine gonadotrophs [35] and confirmed that bovine gonadotrophs express AMH.

Interestingly, I recently reported that most cell bodies or fibers of GnRH-secreting neurons in the preoptic area, arcuate nucleus, and internal and external zones of the median eminence in the heifer hypothalamus express AMH [56]. Therefore, my recent data provide a new hypothesis (Fig. 7), that is small follicles and the hypothalamus secrete AMH to regulate gonadotrophs in an endocrine manner, and gonadotrophs secrete AMH to regulate themselves in an autocrine and paracrine manner. Further studies are required to investigate how AMH and AMHR2 function within the hypothalamic, pituitary, and ovarian axis to regulate reproduction.

Conclusions and Unanswered Questions

Membrane receptors can form functionally active homomers and heteromers with different receptors [57]. I reported that GPR61, GPR153, AMHR2, and GnRHR colocalize in the limited area of surface, probably lipid raft, of bovine gonadotrophs. Schneider *et al.* have previously reported that GPR30 localizes to lipid rafts of murine lymphocytes [58]. However, due to the lack of an appropriate antibody raised against the extracellular domain of bovine GPR30, I could not obtain any evidence to show colocalization of GPR30 with GnRHR on the cell surface of bovine gonadotrophs. Heterodimerization among

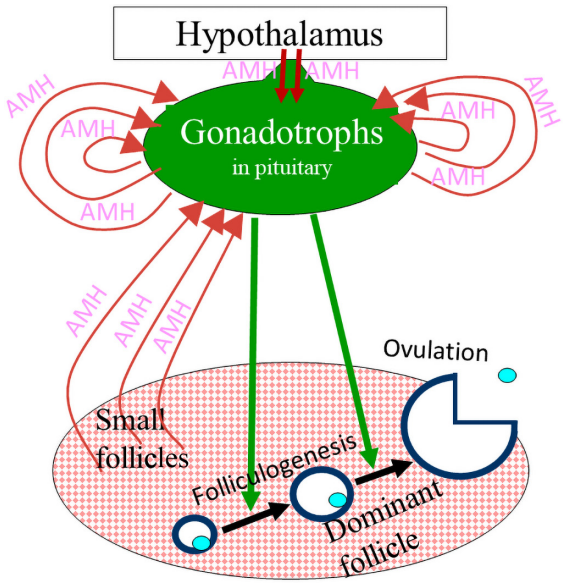

Fig. 7. My recent data suggest a new hypothesis: numerous small ovarian follicles (small brown circles) and the hypothalamus secrete the anti-Mullerian hormone (AMH; shown as brown arrows) to regulate gonadotrophs in an endocrine manner, while gonadotrophs secrete AMH to regulate themselves in an autocrine and paracrine manner.

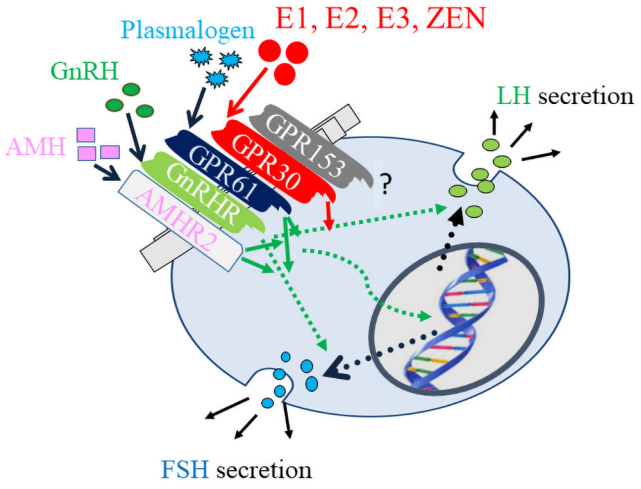

Fig. 8. The receptors may potentially form a heteromer with gonadotropin-releasing hormone (GnRH) receptor (GnRHR) to regulate luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion in bovine gonadotrophs.

paralogs of GnRHRs in protochordates modulates ligand-binding affinity, signal transduction, and internalization [59]. A recent study suggested that GPR61 forms heteromers with other receptors [45]. Therefore, I speculate that the receptors reported in this discovery may potentially form heteromers with GnRHR to regulate the secretion of LH and FSH in gonadotrophs (Fig. 8). Although care must be taken in concluding that the receptors largely contribute to the regulation of LH and FSH secretion from gonadotrophs *in vivo*, my studies revealed that bovine gonadotrophs express the new receptors, GPR30, GPR61, GPR153, and AMRH2 to regulate LH and FSH secretion.

Acknowledgments

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