Animal Reproduction Science 181 (2017) 93–102

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

# Heifers express G-protein coupled receptor 61 in anterior pituitary gonadotrophs in stage-dependent manner

Kiran Pandey[a], Onalenna Kereilwe[a], Vitaliano Borromeo[b], Hiroya Kadokawa[a,*]

[a] Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi-shi, Yamaguchi-ken, 1677-1, Japan
[b] Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Italy

ARTICLE INFO

*Keywords:*
Follicle stimulating hormone
Gonadotropin-releasing hormone receptor
Lipid raft
Luteinizing hormone
Orphan receptor
Ruminant

ABSTRACT

Gonadotropin-releasing hormone receptors (GnRHRs) colocalize with insulin and glucocorticoid receptors in lipid rafts of the gonadotroph plasma membrane, where they facilitate downstream signaling. We recently found that orphan G-protein-coupled receptor (GPR)61 is expressed in the anterior pituitary (AP) of heifers, leading us to speculate that GPR61 colocalizes with GnRHR in the plasma membrane of gonadotroph and is expressed at specific times of the reproductive cycle. To test this hypothesis, we examined the coexpression of GnRHR, GPR61, and either luteinizing hormone (LH) β subunit or follicle-stimulating hormone (FSH) β subunit in AP tissue and cultured AP cells by immunofluorescence microscopy. GPR61 was detected in gonadotrophs, with a majority of them being colocalized with GnRHR and the remainder present at other parts of the cell surface or in the cytoplasm. We obtained a strong positive overlap coefficient ($0.71 \pm 0.01$) between GPR61 and GnRHR on the cell-surface of cultured GnRHR-positive AP cells. Real-time PCR and western blot analyses found that expression was lower ($P < 0.05$) in AP tissues during early luteal phase as compared to pre-ovulation or mid- or late luteal phases. Additionally, the 5'-flanking region of the *GPR61* gene contained several sites with response elements similar to those of estrogen or progesterone. These data suggested that GPR61 colocalizes with GnRHR in the plasma membrane of gonadotrophs, and its expression changes stage-dependently in the bovine anterior pituitary gland.

## 1. Introduction

Gonadotrophs in the anterior pituitary (AP) secrete gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) to regulate reproductive functions in animals. These cells are controlled by gonadotropin-releasing hormone (GnRH) via the G protein-coupled, GnRH receptor (GnRHR) at the gonadotroph surface. However, gonadotropin secretion in ruminants is modulated by various factors including nutrition, estrous stage, season, and stress (Kadokawa et al., 1998; Phogat et al., 1999; Stackpole et al., 2003; Kadokawa 2007), although the detailed mechanisms thereof are not fully understood.

GnRHRs are present in gonadotroph plasma membrane lipid rafts (Navratil et al., 2009; Wehmeyer et al., 2014; Kadokawa et al., 2014), which are distinct, relatively insoluble regions that have lower density and are less fluid than surrounding membrane (Simons and Tooter, 2000; Head et al., 2014). Lipid rafts facilitate signaling by allowing colocalization of membrane receptors and their downstream signaling components (Simons and Tooter, 2000; Head et al., 2014); as such, they are important pharmacological targets in human medicine (Jaffrès et al., 2016). Lipid rafts containing GnRHR also harbor insulin receptor (Navratil et al., 2009) and glucocorticoid receptor (Wehmeyer et al., 2014) in the LβT2 clonal murine gonadotroph cell line, thus providing a potential means of

* Corresponding author.
*E-mail address:* hiroya@yamaguchi-u.ac.jp (H. Kadokawa).

http://dx.doi.org/10.1016/j.anireprosci.2017.03.020
Received 1 February 2017; Received in revised form 21 March 2017; Accepted 30 March 2017
Available online 04 April 2017
0378-4320/ 

integrating neuropeptide and energy homeostasis signals to modulate reproductive function. However, gonadotroph lipid rafts containing GnRHR are also likely to contain other types of receptor that have yet to be identified.

G protein-coupled receptor 61 (GPR61) is an orphan receptor that is widely expressed in the brain, including in the hypothalamus and pituitary (Lee et al., 2001; Nambu et al., 2011), although its function and ligand(s) are unknown. GPR61 associates with Gs protein (Takeda et al., 2003; Toyooka et al., 2009) and stimulates extracellular signal-regulated kinase (ERK) signaling in neurons (Hossain et al., 2016). It was also found to inhibit cyclic (c) AMP-dependent reporter (luciferase) gene expression induced by forskolin in Chinese hamster ovary (CHO) cells that are transiently cotransfected with plasmids containing the reporter and GPR61 (Martin et al., 2015). We recently found that GPR61 is expressed in the AP of heifers (our unpublished data). The ERK and cAMP pathways inhibit and stimulate GnRH-induced LH secretion, respectively, in bovine gonadotrophs (Nakamura et al., 2015; Nakamura and Kadokawa 2015; Rudolf and Kadokawa 2016). These findings imply that GPR61 is expressed at the surface of bovine gonadotrophs.

We previously developed a guinea pig polyclonal antibody that recognizes the extracellular region of GnRHR (anti-GnRHR) (Kadokawa et al., 2014) and used this for immunofluorescence detection of GnRHR in bovine gonadotroph lipid rafts (Kadokawa et al., 2014; Pandey et al., 2016). In the present study, we tested the hypothesis that GPR61 colocalizes with GnRHR in the plasma membrane of bovine gonadotrophs and that its expression is dependent on reproductive stage.

## 2. Materials and methods

All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and approved by the Committee on Animal Experiments of Yamaguchi University.

### 2.1. Antibodies

We previously determined using the SOSUI v.1.11 algorithm that bovine GPR61 protein [451 amino acids; accession number AAX31376.1 in National Center for Biotechnology Information (NCBI) reference bovine sequences] contains seven hydrophobic transmembrane domains linked by hydrophilic extracellular and intracellular loops (Hirokawa et al., 1998; http://harrier.nagahama-i-bio.ac.jp/sosui/). Bovine GPR61 has 96% homology to the human protein (451 amino acids; NCBI accession number AAH67464.1). We used a rabbit polyclonal anti-GPR61 antibody against the extracellular region of human GPR61 (ORB183901, Biorbyt, Cambridge, UK) whose peptide antigen corresponds to amino acids 4–18 (SPIPQSSGNSSTLGR) in the N-terminal extracellular domain of human GPR61. This sequence has 100% homology to amino acids 4–18 in the N-terminal extracellular domain of bovine GPR61 but not to other proteins in the bovine genome based on sequences retrieved from DNA Data Bank of Japan/GenBank/European Bioinformatics Institute Data Bank using the protein basic local alignment search tool.

We used a guinea pig polyclonal anti-bovine GnRHR antibody against the N-terminal extracellular domain (corresponding to amino acids 1–29; MANSDSPEQNENHCSAINSSIPLTPGSLP) (Kadokawa et al., 2014) as well as a mouse monoclonal anti-LH β (LHβ) subunit antibody (clone 518-B7; Matteri et al., 1987) for immunohistochemical analysis of AP tissue and cultured AP cells. This antibody does not cross-react with other pituitary hormones (Iqbal et al., 2009).

Finally, we used a mouse monoclonal anti-FSH β (FSHβ) subunit antibody (clone A3C12) that does not cross-react with other pituitary hormones (Borromeo et al., 2004) for immunohistochemical analysis of AP tissue; it was not used in cultured AP cells owing to weak labeling.

### 2.2. Triple immunofluorescence analysis of AP tissue

Nett et al. (1987) reported that the concentrations of LH and GnRHR in AP was greater during the luteal phase in heifers than during the immediate post-estrus period. Therefore, AP tissue was obtained from post-pubertal Japanese Black heifers in the middle luteal phase ($n$ = 3, 26 months of age) at a local abattoir as described previously (Kadokawa et al., 2014), and fixed in 4% paraformaldehyde at 4 °C for 16 h. The methods for immunofluorescence analysis of AP tissue have been described previously (Kadokawa et al., 2014). Briefly, we prepared 15-μm sagittal sections and mounted them on slides Incubation with a cocktail of primary antibodies [guinea pig anti-GnRHR antibody, 1:1000; rabbit polyclonal anti-GPR61 antibody, 1:1000; and either mouse monoclonal anti-LHβ or anti-FSHβ antibody (both at 1:1000)] for 12 h at 4 °C was followed by incubation with a cocktail of fluorochrome-conjugated secondary antibodies (4 μg/mL Alexa Fluor 488 goat anti-rabbit IgG, 4 μg/mL Alexa Fluor 546 goat anti-mouse IgG, and 4 μg/mL Alexa Fluor 647 goat anti-guinea pig IgG [all from Thermo Fisher Scientific, Waltham, MA, US)]) and 1 μg/mL of 4′,6′-diamino-2-phenylindole (DAPI; Wako Pure Chemicals) for 2 h at room temperature. The stained sections on slides were observed by confocal microscopy (LSM710; Carl Zeiss, Göttingen, Germany) equipped with a diode laser 405 nm, argon laser 488 nm, HeNe laser 533 nm, and HeNe laser 633 nm. Images obtained by fluorescence microscopy were scanned with a 40 × or 63 × oil-immersion objective and recorded by a CCD camera system controlled by ZEN2012 black edition software (Carl Zeiss). The optical resolution in the Z-axis (optical slice) was more than 0.6 μm and less than 4.6 μm. GnRHR, GPR61, and LHβ or FSHβ localization was examined in confocal images of triple-immunolabeled specimens. To verify the specificity of the signals, we included several negative controls in which the primary antiserum had been omitted or pre-absorbed with 5 nM antigen peptide synthesized by Scrum Inc. (Tokyo, Japan) or in which normal guinea pig IgG was used instead of the primary antibody. Percentages of single- and double-labeled GPR61- and GnRHR-positive cells were determined from 12 representative confocal images per pituitary gland.