RESEARCH

Role of GPER in the anterior pituitary gland focusing on lactotroph function

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Abstract

Ovarian steroids control a variety of physiological functions. They exert actions through classical nuclear steroid receptors, but rapid non-genomic actions through specific membrane steroid receptors have been also described. In this study, we demonstrate that the G-protein-coupled estrogen receptor (GPER) is expressed in the rat pituitary gland and, at a high level, in the lactotroph population. Our results revealed that ~40% of the anterior pituitary cells are GPER positive and ~35% of the lactotrophs are GPER positive. By immunocytochemical and immuno-electron-microscopy studies, we demonstrated that GPER is localized in the plasmatic membrane but is also associated to the endoplasmic reticulum in rat lactotrophs. Moreover, we found that local *Gper* expression is regulated negatively by 17β-estradiol (E2) and progesterone (P4) and fluctuates during the estrus cycle, being minimal in proestrus. Interestingly, lack of ovarian steroids after an ovariectomy (OVX) significantly increased pituitary GPER expression specifically in the three morphologically different subtypes of lactotrophs. We found a rapid estradiol stimulatory effect on PRL secretion mediated by GPER, both *in vitro* and *ex vivo*, using a GPER agonist G1, and this effect was prevented by the GPER antagonist G36, demonstrating a novel role for this receptor. Then, the increased pituitary GPER expression after OVX could lead to alterations in the pituitary function as all three lactotroph subtypes are target of GPER ligand and could be involved in the PRL secretion mediated by GPER. Therefore, it should be taken into consideration in the response of the gland to an eventual hormone replacement therapy.

Key Words

- pituitary
- lactotrophs
- GPER
- prolactin
- ovariectomy

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Introduction

The involvement of estrogens in the control of pituitary function has been extensively studied (reviewed in Seilicovich 2010). Initially, estradiol was described to induce lactotroph proliferation through ERα; however, apoptotic (Zarate *et al.* 2009) and antiproliferative (Perez *et al.* 2015) actions of estradiol in anterior pituitary cells were also demonstrated. These opposite effects depend on the duration of the stimuli, the receptor subtype involved, and receptor subcellular localization. For example, nuclear ERα may trigger lactotroph proliferation, whereas membrane-associated ERα was described to mediate antimitogenic (Gutierrez *et al.* 2008) and apoptotic effects (Zarate *et al.* 2009). On the other hand, ERβ receptors, expressed in the lactotroph

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population (Mitchner *et al.* 1998), are also able to mediate antiproliferative estradiol actions (Perez *et al.* 2015).

There is substantial evidence that estradiol exerts rapid non-genomic effects initiated at the cell surface through binding to membrane estrogen receptors (Kelly & Levin 2001, Levin & Hammes 2016). Although it was demonstrated that membrane-initiated signaling could be mediated by the classic receptors ERα and ERβ trafficked to the cell membrane (Zhang *et al.* 2011, 2012, Zarate *et al.* 2012, Micevych *et al.* 2017), the involvement of the 7-transmembrane G protein-coupled estrogen receptor (GPER, formerly named GPR30) in estradiol-induced rapid, non-genomic events has been in the spotlight during the last decade (Maggiolini & Picard 2010, Zimmerman *et al.* 2016, De Francesco *et al.* 2017, Thomas 2017, Fredette *et al.* 2018).

It has been proposed that GPER collaborates with membrane ERα signaling (Levin 2009). However, there are numerous studies demonstrating specific-GPER function in estradiol-induced non-genomic events in ER-negative cells (Filardo *et al.* 2000, 2002, Thomas *et al.* 2005), as well as studies performed in GPER KO mice (Martensson *et al.* 2009, Prossnitz & Hathaway 2015) which clearly support the idea that GPER can act as a 'stand-alone' receptor.

GPER, a transmembrane receptor belonging to the GPCR family, was first identified in human breast cancer cells (Filardo *et al.* 2000), but was later found to be expressed ubiquitously, even in the rat brain and pituitary (Brailoiu *et al.* 2007, Hazell *et al.* 2009, Rudolf & Kadokawa 2013). Although previous reports have provided strong evidence of GPER expression in the pituitary gland, most of these studies focused on gonadotroph cells (Brailoiu *et al.* 2007, Hazell *et al.* 2009), meanwhile GPER involvement in the physiology and pathology of the lactotroph population remains to be elucidated.

In the present study, we examined the expression and localization of GPER in the lactotroph population, the local regulation of this receptor by estradiol and progesterone, as well as the local alterations induced in GPER expression in the anterior pituitary gland after ovariectomy. In addition, using pharmacological tools (GPER agonist and antagonist) the involvement of GPER in the regulation of PRL release was studied *in vitro* and *ex vivo*, in the GH3 cell line and in rat pituitaries respectively.

Materials and methods

Animals

Adult Sprague–Dawley (SD) rats (3-month old, 250±30 g) were maintained at 25±2°C and 12 h light–dark cycle, lights 07:00–19:00 h. The animals were provided with food and water *ad libitum*. All the animal procedures were carried out in accordance with the National Institutes of Health guidelines for animal research (8th ed. 2010, NRC, USA) and the European Communities Council Directive of November 2010 (2010/63/UE) and approved by Institute of Biology and Experimental Medicine Animal Care and Use Committee (CICUAL).

SD female rats were ovariectomized (OVX) under anesthesia (Ketamine 50 mg/kg + Xylazine 10 mg, i.p.) as previously described (Ferraris *et al.* 2014). Two weeks after surgery, animals were killed by decapitation and anterior pituitaries were carefully excised and the neurohypophysis was removed. In addition, cycling rats were monitored daily by vaginal smears, during 4–5 day estrous cycles, and killed at diestrus, proestrus or estrus. Control female rats were used at diestrus. Anterior pituitary glands were kept in Dulbecco Eagle's Modified Medium (DMEM) (Sigma-Aldrich) or Trizol Reagent (Ambion, Life Technologies) at −70°C until assays were conducted. For immunogold electron microscopy, anterior pituitaries from female rats in diestrus and OVX rats were collected in a mixture of 4% v/v formaldehyde, 1.5% v/v glutaraldehyde and 0.1 M cacodylate buffer and processed as described below.

In vivo experiments

Adult female SD rats in diestrus were injected with estradiol valerate (0.2 mg/kg sc, Schering, Buenos Aires, Argentina), progesterone (6.5 mg/kg sc, Sigma-Aldrich) or castor oil (vehicle, control group). Animals were killed by decapitation after 1, 2 or 24 h. Anterior pituitaries were collected in Trizol reagent for qRT-PCR studies.

Ex vivo assay

Female SD rats in diestrus were killed by decapitation and anterior pituitaries were collected in 250 µL of Dulbecco Eagle's Modified Medium (DMEM) supplemented with 15% v/v horse serum (Internegocios, Argentina), 2.5% v/v fetal bovine serum (Natocor, Argentina) and 20 µg/mL of gentamicin (Sigma-Aldrich). Anterior pituitaries were washed and cut in pieces with fresh media and incubated for 2 h at 37°C. The GPER receptor antagonist G36 (1 µM) or vehicle (ethanol, 1 µM) were added to pituitary explants and incubated for 30 min at 37°C. At the end of 30-min period, explants (with or without G36) were stimulated with either vehicle, 17β-estradiol (E2, 100 nM) or the GPER receptor agonist G1 (100 nM) for 15 min at 37°C. At the end of the treatment period, secreted medium and

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pituitaries were collected and PRL levels were measured by radioimmunoassay (RIA).

GH3 cell culture

GH3 clone was established in 1965 by A H Tashjian Jr *et al.* from a pituitary tumor carried in a 7-month-old female Wistar–Furth rat (Tashjian Jr *et al.* 1970). GH3 cells (ATCC CCL-82.1, authenticated by STRS analysis) were cultured with DMEM supplemented with 10% v/v fetal bovine serum and 10% v/v horse serum (previously adsorbed), 1 mg/mL MEM amino acids, 1 mg/mL glutamine and 100 mg/mL of gentamicin. Medium was changed every 1–2 days and 0.025% v/v trypsin-EDTA was used to harvest cells.

For experiments, GH3 cells seeded on 24-well culture plates were incubated with DMEM containing vehicle (ethanol, 1 µM) or GPER antagonist (G36, 1 µM) for 30 min. Then, cells were incubated with 17β-estradiol (E2, 10 nM) or GPER agonist (G1, 1 µM) alone or in combination with G36 for additional 15 min. GH3 cells incubated with vehicle (Ethanol, 1 µM) were used as controls. After experimental treatments, medium was collected and stored at −70°C until rat prolactin radioimmunoassays (rPRL RIA) were performed.

Rat prolactin radioimmunoassay (rPRL RIA)

PRL levels were measured by RIA using reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program (NHPP) (Dr A F Parlow, NHPP, Torrance, CA, USA). Results are expressed as ng/mL in terms of referent preparation 3 (RP3). Intra- and inter-assay coefficients of variation were 6.7 and 11.9%, respectively.

Quantitative real-time RT-PCR (qRT-PCR)

Anterior pituitaries from different experimental groups were collected in TRIzol reagent. Total RNA was isolated according to the manufacturer's protocol as described in Faraoni *et al.* (2017). Reverse transcription was performed using 1 µg of total RNA and the resulting cDNA was used for qRT-PCR analysis. A working solution of cDNA was prepared by adding 5 µL of samples diluted 1:20 with RNase-free water to a 5 µL master mix containing 2 µL EVA green qPCR mix (Solis BioDyne, Estonia) and 0.5 µM of specific primers for *Gper*: 5′-ACGCTCAAGGCAGTCATACC-3′ (sense); 5′-CTCCCCTGTCCGTTTTCCTC-3′ (antisense). To determine the appropriate housekeeping gene as an internal control to normalize the differences in the amount of starting template between samples, two reference genes were evaluated: the 60S ribosomal protein L38 (*Rpl38*): 5′-GTTCGGTGCTCGCTCCTGT-3′ (sense) and 5′-CAGATTTGGCATCCTTCCGC-3′ (antisense); and Cyclophilin B (*Cypb*): 5′-GACCCTCCGTGGCCAACGAT-3′ (sense) and 5′-GTCACTCGTCCTACAGGTTCGTCTC-3′ (antisense). qPCR efficiency of each pair of primers was tested using serially diluted samples and was established by means of calibration curves. Amplification efficiency was determined from the slope of the log-linear portion of the calibration curve. Specifically, PCR efficiency was calculated as $10^{(-1/\text{slope})}-1$, when the logarithm of the initial template concentration was plotted on the *x* axis and Ct was plotted on the *y* axis. All primers showed similar efficiencies, approximately 95–100%. *Rpl38* was selected as the most proper housekeeping gene due to the parallelism presented between its slope of the regression line (and consequently on the value of the correlation coefficient) with the *Gper* slope. Table 1 shows average of Ct values obtained in the *in vivo* treatment with estradiol showing stable expression levels of *Rpl38* regardless of the experimental conditions, ensuring a proper normalization within the samples and a robust q-RT-PCR analysis. Relative fold change in target mRNAs was quantified using the $2^{-\Delta\Delta Ct}$ method, where ΔCt was determined by subtracting the average control ΔCt from the ΔCt of the sample. Each ΔCt was calculated as by substracting the Cts of *Rpl38* from the *Gper* Cts. All cDNA samples were assayed in duplicate for each gene and melt curve analysis was performed to ensure specificity of amplification.

Immunostaining by confocal laser scanning microscopy

Pituitaries from 3-month-old female SD rats in diestrus were removed immediately after euthanasia and the pituitary cells were dispersed and seeded on glass coverslips (13 mm) at a density of 2.5×10^4 cells/well. Then, the cells were maintained in DMEM supplemented with 4% v/v fetal calf serum and 8% v/v horse serum (Gibco) in an

Table 1 Average of Ct values showing stable expression of *Rpl38* within samples.

Ct values ($\bar{X}$)	*Rpl38*	*Gper*
Diestrus	24.44	28.43
E2 1 h	24.16	28.31
E2 2 h	24.34	29.43
E2 24 h	24.22	30.07

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incubator with a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 3 days, and finally were fixed in 4% v/v formaldehyde.

For GPER detection, dispersed pituitary cells in coverslips were permeabilized with 0.5% v/v Triton X-100/PBS, blocked for 1 h in 5% PBS–BSA, incubated overnight in primary antibody (anti-rat GPER, ab39742, Abcam, 1:100) and exposed to Alexa 594 anti-rabbit secondary antibody (Invitrogen, 1:1000) for 1 h. Then, the cells were blocked for 1 h in 5% PBS–BSA, incubated with guinea pig antibody directed against rat PRL or rat LH or rat GH (1:1000, Dr A Parlow, NHPP, Torrance, CA, USA) and further incubated with Alexa 488 anti-guinea pig secondary antibody (Invitrogen, 1:1000) for 1 h. The glass coverslips were mounted with fluoromount (Sigma) containing DAPI. Negative controls were carried out incubating the coverslips with the corresponding normal serum, instead of primary antibody or with antibody dilution plus five-fold excess of the control peptide antigen (GPER Peptide, ab41565, Abcam) overnight at 4°C. Images were obtained using the inverted confocal laser scanning microscope FluoView FV 1000 (Olympus). The analysis of confocal microscopy images was performed using the software FV10-ASW 1.6 Viewer. Briefly, the presence of GPER in GH3 cells was evaluated by immunostaining as described above. GH3 cells (2×10^5 cells per well) were seeded onto glass coverslips in 24-well tissue culture plates and fixed with 4% v/v paraformaldehyde (PFA) in PBS for 20 min. After cell permeabilization and 1-h blocking in a humidified chamber, cells were incubated with GPER antibody (anti-rat GPER, ab39742, Abcam, 1:50) and with an Alexa Fluor 488 goat anti-rabbit secondary antibody (1:100) for 1 h at room temperature. Cells were stained with DAPI and mounted with Vectashield. Then, cells were visualized in a fluorescence light microscope (Axiophot, Carl Zeiss, Jena, Germany).

Immunogold electron microscopy

The subcellular localization of GPER in lactotroph cells was determined by applying a labeling post-embedding protocol. Pituitary glands from female rats at diestrus stage or OVX rats were fixed in a mixture of 4% v/v formaldehyde, 1.5% v/v glutaraldehyde and 0.1 M cacodylate buffer, pH 7.3, at room temperature, with osmiun fixation being omitted. After dehydration and embedding in LR White (London Resin, UK), thin sections were cut using a JEOL ultramicrotome with a diamond knife. Then, the grids were labeled for GPER overnight at 4°C (anti-rat GPER, ab39742, Abcam, 1:50), washed and incubated with anti-rabbit secondary antibody conjugated to 15 nm colloidal gold particles (1:18; Electron Microscopy Sciences; Hatfield, USA). To confirm that lactotroph cells expressed GPER, ultrastructural immunocytochemistry for PRL was performed. Thin sections were incubated overnight at 4°C with antisera raised against rat PRL diluted 1:5000 (NIHDDK, Bethesda, MD, USA), washed and incubated with anti-rabbit secondary antibody conjugated to 5 nm colloidal gold particles (1:50; Electron Microscopy Sciences; Hatfield, USA). To validate the specificity of the immunostaining, controls were performed with 1% v/v BSA in PBS instead of primary antiserum. Then, sections were stained with an aqueous uranyl acetate saturate solution, examined in a Zeiss LEO 906-E electron microscope, and photographed with a megaview III camera.

Flow cytometry

Control and OVX female rats were killed by decapitation and anterior pituitary glands were removed within minutes and collected in 1000 µL of DMEM supplemented and processed as previously described in Ferraris *et al.* (2014). Cell viability, as assessed by trypan blue exclusion, was over 95%. Cells were fixed using PFA 0.2% for 15 min at room temperature, washed and resuspended in PBS. Then, after permeabilization of the cells with saponine-PBS 0.2% w/v, washing and centrifuging, immunostaining of GPER-positive cells and of lactotrophs was performed using a rabbit anti-rat GPER (1 µg/mL) and a guinea pig antiserum directed against rat PRL (1:2000) (Dr A Parlow, NHPP, Torrance, CA, USA) for 1 h at 37°C. Cells were then washed in PBS and incubated with goat PE-conjugated anti-rabbit (Chemicon International, Temecula, CA, USA) (1:67) and donkey FITC-conjugated anti-guinea pig antibody (Chemicon International) (1:75) for 40 min at 37°C in slow agitation. Cells incubated with guinea pig serum instead of PRL antiserum and rabbit IgG instead of specific primary antibodies were used as isotype controls. Cells were washed, resuspended in PBS and analyzed by FACS (Zarate *et al.* 2009). Fluorescence intensity of ≥10,000 gated-cells/tube was analyzed using a FACScalibur (BD). Data was analyzed using WinMdi and FlowJo Softwares.

The experiments ($n=6$) were performed using two different GPER antibodies to ensure specificity. Similar results were obtained using either a rabbit antibody against rat GPER (sc-48525, Santa Cruz Biotechnology Inc, 5 µg/mL) or a rabbit anti-rat GPER (ab39742, Abcam, 1 µg/mL).

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Statistical analysis

Results are expressed as mean±S.E.M. and the significance levels were chosen at $P<0.05$. Student's t test was used to compare OVX and control group data. Estradiol and progesterone acute treatments were analyzed by a One-way ANOVA followed by a Tukey's *post hoc* test. *In vitro* and *ex vivo* experiments were repeated three times with at least three replicates, and treatments were compared by a One-way ANOVA followed by a Tukey's *post hoc* test.

Results

GPER is expressed in the lactotroph population

First, in order to establish the localization of GPER in pituitary cells, a double indirect immunofluorescence using confocal microscopy was performed. Our results showed lactotroph cells (immunoreactive to PRL), somatotroph cells (GH) and gonadotroph cells (βLH) with a circumferential staining pattern, evidencing the presence of GPER in plasmatic membrane in addition to punctuated diffuse fluorescence signal distributed in the cytoplasm (Fig. 1).

Immunocytochemical controls evaluated the specificity of the primary antiserum, and no immunolabelling was found after the omission of the primary antibody and pre-absorbing the antibody with purified antigen.

Next, to determine the percentage of GPER-positive cells expressing PRL, dispersed and double-immunostained (GPER, PRL) anterior pituitary cells from female rats were analyzed by flow cytometry. Our results revealed that 38.5±8.4% were GPER positive among the total anterior pituitary cells (Fig. 2A and B). Interestingly, 39.5±9.8% of the GPER-positive cells were PRL positive, and 35.5±3.5% of the PRL-positive cells were GPER positive (Fig. 2C and D).

Estradiol and progesterone negatively regulates pituitary *Gper* mRNA expression

17β-Estradiol (E2) and progesterone (P4) typically upregulate or downregulate the expression of their classical receptors according to the tissue and the physiological situation. Then, in order to study E2 and P4 regulation of pituitary *Gper* expression, we next performed acute *in vivo* assays in adult female rats. *In vivo* treatment with E2 significantly decreased pituitary *Gper* levels after 2 and 24 h compared to control rats in diestrus (CTRL), (Fig. 3A). In addition, *in vivo* treatment with P4 decreased pituitary *Gper* expression after 24 h (Fig. 3B). In accordance, and due to the loss of the control by ovarian steroids, *Gper* expression was significantly increased in the pituitary gland of OVX adult female rats (Fig. 3C).

In order to evaluate the physiological impact of the regulatory effects of gonadal steroid hormones, *Gper* mRNA levels were measured in the anterior pituitary gland of female rats at different stages of the estrous cycle. Interestingly, *Gper* mRNA levels were the lowest on the morning of proestrus, when steroid levels were at their highest (Freeman 1986) (Fig. 3D). Finally, we evaluated putative gender differences, but similar levels of pituitary *Gper* mRNA were found in male pituitaries when compared with those found in females in diestrus (data not shown).

Ovariectomy increases GPER-positive cells among the lactotroph population

To examine whether the increased pituitary *Gper* mRNA expression after OVX (Fig. 3C) was associated to an increase in the protein receptor expression in the lactotroph population, dispersed anterior pituitary cells from controls (diestrus) and OVX rats were double-immunostained (GPER, PRL) and analyzed by flow cytometry. The cytometry analysis shows that the percentage of GPER-positive anterior pituitary cells significantly increased in OVX rats compared to CTRL rats in diestrus (Fig. 4A). Interestingly, this increment was primarily due to an increase in GPER expression in the lactotroph population (GPER-positive lactotrophs, Fig. 4B) since no differences were found, neither the percentage of GPER-positive non-lactotrophs cells (Fig. 4C) nor the percentage of lactotrophs/total pituitary cells among groups (Fig. 4D).

GPER in different morphological subtypes of lactotrophs

It is well known that lactotroph population exhibits morphological and functional heterogeneity (Kukstas *et al.* 1990, De Paul *et al.* 1997, Christian *et al.* 2007). In fact, three subtypes of lactotrophs, defined morphologically by electron microscopy (De Paul *et al.* 1997), could be observed in the anterior pituitary gland from rodents. Interestingly, the proportion of each lactotroph subtype depends, at least partially, on estradiol levels; then it was described that the depletion of estrogen, induced remarkable changes in the lactotroph population.

To the extent of deepening the study of GPER subcellular localization and the lactotroph subtypes expressing GPER, a post-embedding immunolabelling

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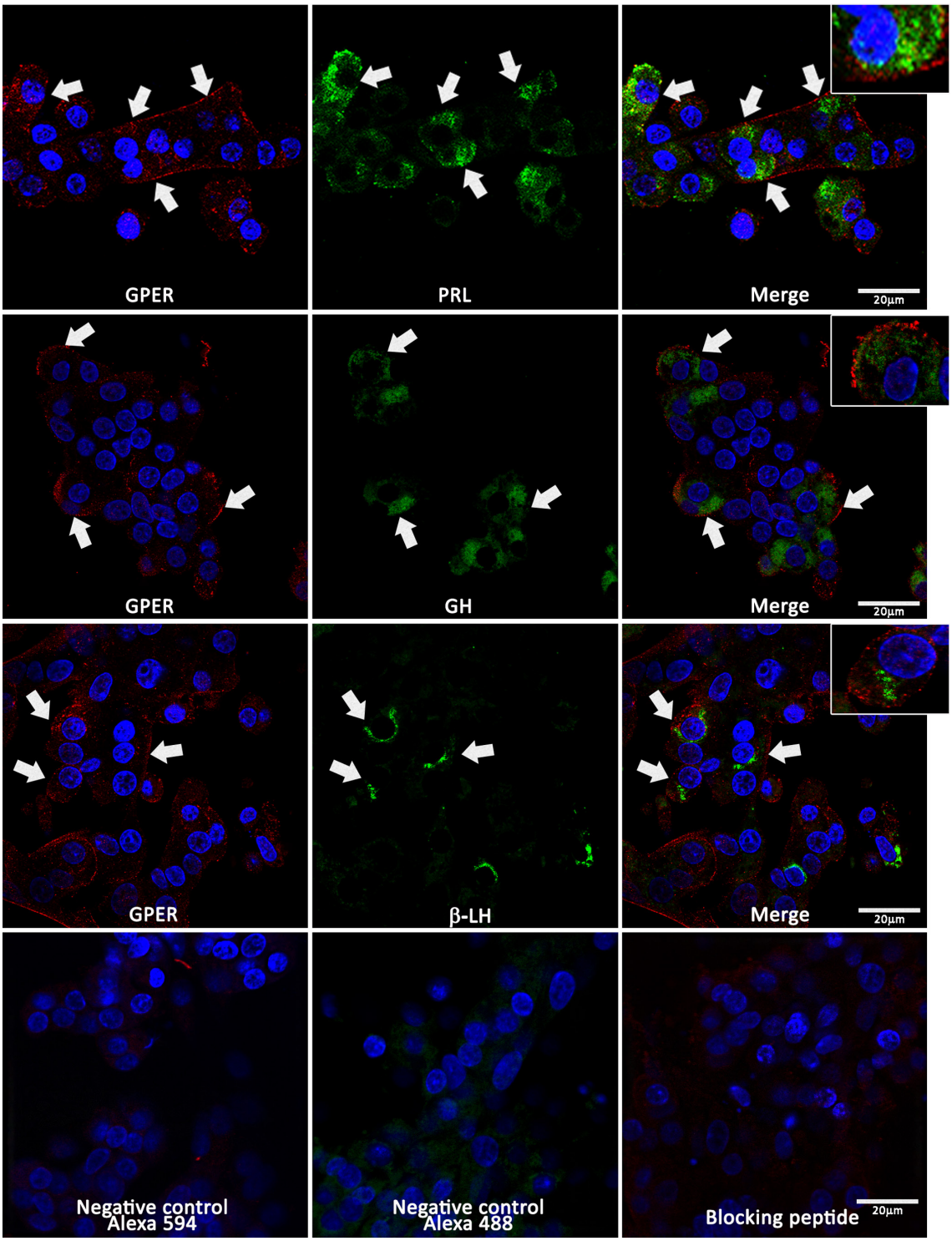

Figure 1
GPER expression in anterior pituitary gland. Anterior pituitary cells from 3-month-old female rats in diestrus were processed for GPER identification. White arrows show lactotrophs (PRL), somatotrophs (GH) and gonadotrophs (β-LH) expressing GPER. Nuclei were stained with DAPI. To validate the specificity of the immunostaining, negative controls were performed using blocking peptide or replacing primary antibody with the corresponding normal serum and then incubated with secondary antibody Alexa 594 or Alexa 488. *Bar* = 20 µm.

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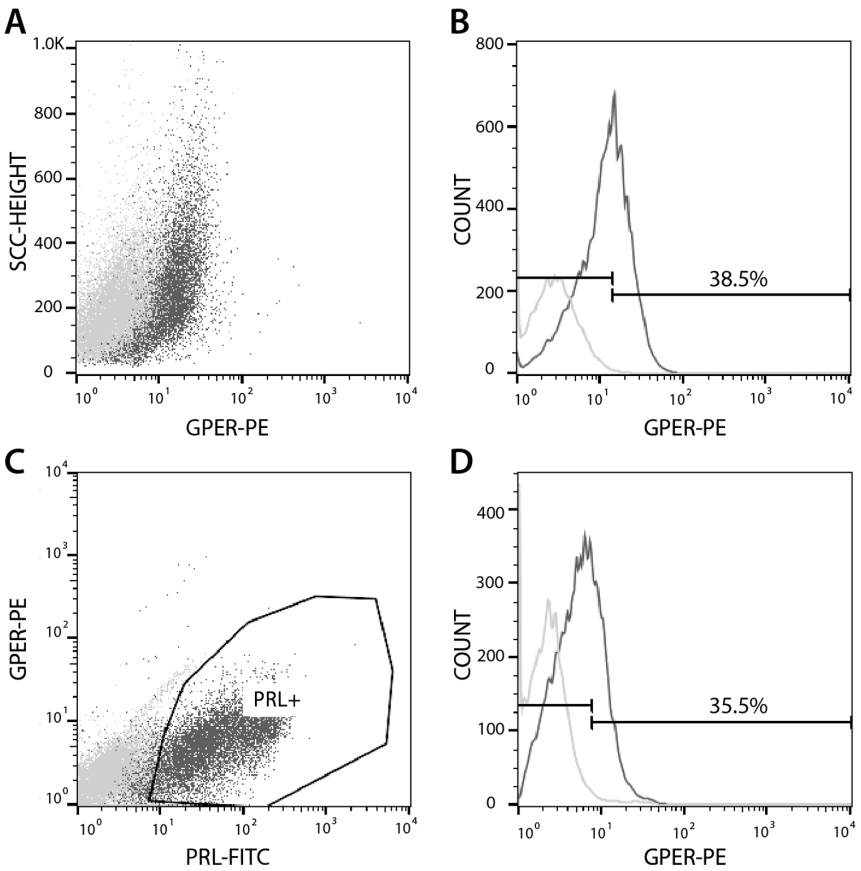

Figure 2
Flow cytometry analysis of GPER-positive cells in dispersed anterior rat pituitary cells. Dispersed anterior pituitary cells were incubated with anti-GPER antibody (ab39742, Abcam) and analyzed by flow cytometry, $n = 8$. Representative dot plots and histograms showing: (A and B) percentage of total anterior pituitary cells GPER-positive (GPER+) and (C and D) lactotrophs GPER-positive. Gray: isotype controls; gate: lactotrophs.

with IgG-colloidal gold for transmission electron microscopy was used. GPER protein was immunolabelled using secondary antibody conjugated to colloidal gold particles of 15 nm and lactotroph cells were identify immunolabelling PRL with a secondary antibody conjugated to colloidal gold particles of 5 nm. Our results show lactotroph cells expressing GPER, being the subcellular localization in plasmatic membrane, with the gold particles appearing to be attached to the inner surface of the plasmalemma, in rough endoplasmic reticulum and with a few colloidal gold particles being observed in the free cytosol (Fig. 5). In female rats in diestrus, the lactotroph cells were recognized by their irregular, large and polymorphic secretory granules of sizes ranging between 300 and 700 nm distributed in the cytoplasm and immunolabelled for PRL, typical characteristics of subtype I lactotrophs (Fig. 5A, B and C). In pituitaries from OVX rats, the three morphological subtypes of lactotrophs (I, II and III), were GPER positive. The subtype I was recognized by the irregular and large granules (Fig. 5E), the subtype II were recognized by the medium-sized spherical granules about diameter 200–250 nm (Fig. 5F), and the subtype III was distinguished by their small spherical granules, between 100 and 200 nm (Fig. 5G).

GPER activation induces PRL release

GH3 cells

In order to investigate the involvement of GPER in rapid estradiol effect on prolactin secretion, we performed *in vitro* assays using the GH3 cell line. First, the GPER protein expression in GH3 cells was demonstrated by ICC (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Then stimulation assays with E2 and GPER agonist and antagonist were performed. After 15 min of stimulation both E2 and G1 increased PRL secretion (**$P = 0.005$), whereas the GPER antagonist G36 prevented the G1 effect, and partially the E2 stimulation (Fig. 6A) without exerting any *per se* effects.

Ex vivo *assay*

Once stimulation of PRL secretion induced by E2 and G1, involving GPER receptors was confirmed *in vitro*, this effect was assayed in female rat pituitary explants. As shown in Fig. 6B, both E2 and G1 increased PRL release after 15-min stimulation. These effects were not observed when tissues were pre-incubated with G36, implying that GPER receptors are involved in rapid E2 and G1 stimulation of PRL release. G36 did not modify PRL secretion *per se*.

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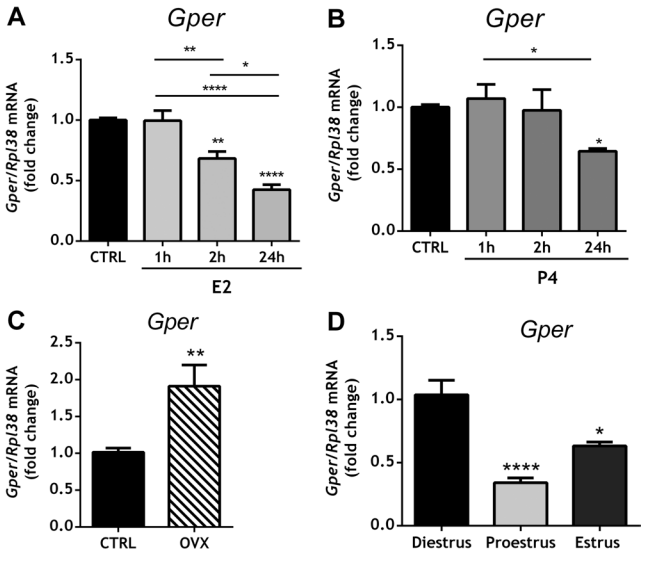

Figure 3
Regulation of *Gper* mRNA expression in the rat pituitary by E2 and P4. Alterations induced by OVX, and during the estrous cycle. (A) E2 regulation of pituitary *Gper* mRNA levels was assessed *in vivo* in female rats in diestrus (E2, 0.2 mg/kg BW, sc) 1, 2 and 24 h or vehicle (CTRL). Pituitary *Gper* expression was analyzed by qRT-PCR. One-way ANOVA followed by Tukey's *post hoc* test, $n = 5$, $^{**}P < 0.0052$ E2 2 h vs CTRL; $^{****}P < 0.0001$ E2 24 h vs CTRL; $^{**}P = 0.0059$ E2 1 h vs 2 h; $^{****}P < 0.0001$ E2 1 h vs 24 h and $^{*}P = 0.0239$ E2 2 h vs 24 h. (B) P4 regulation of *Gper* mRNA levels was studied similarly, *in vivo* (P4 6.5 mg/kg BW, sc, 1, 2 and 24 h) or castor oil (CTRL) in female rats in diestrus. One-way ANOVA followed by Tukey's *post hoc* test, $n = 5$, $^{*}P = 0.0484$ P4 24 h vs CTRL and $^{*}P = 0219$ P4 1 h vs 24 h. (C) The effect of OVX (15 days post-OVX) on pituitary *Gper* mRNA levels, analyzed by qRT-PCR. Student's *t* test, $n = 6$, $^{**}P = 0.0083$ OVX vs control. (D) *Gper* mRNA levels in pituitaries from cycling rats. One-way ANOVA followed by Tukey's test, $n = 5$; $^{***}P < 0.0001$ proestrus vs diestrus; $^{*}P < 0.0139$ estrus vs diestrus.

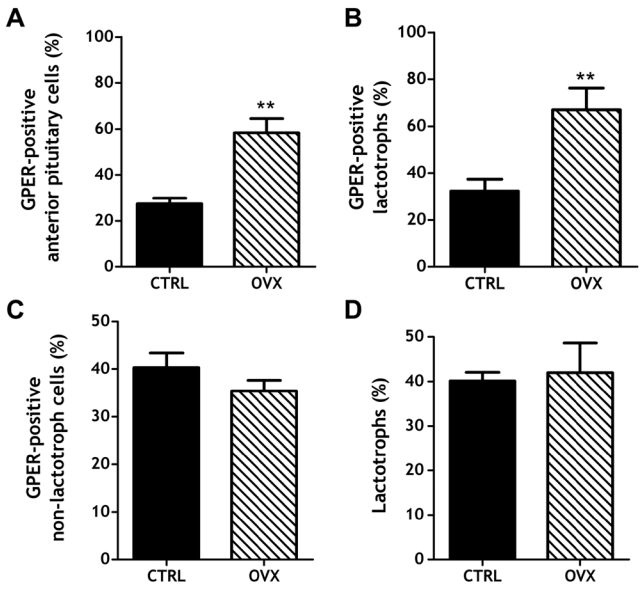

Figure 4
Effect of OVX in GPER expression in lactotrophs. (A) Percentage of GPER+ pituitary cells measured by flow cytometry in OVX rats compared to their control in diestrus: Student's *t* test, $n = 6$, $^{**}P = 0.0022$. (B) Percentage of GPER+ lactotrophs (PRL+) population: Student's *t* test, $n = 6$, $^{**}P = 0.0063$. (C) Percentage of GPER+ non-lactotrophs (PRL–) pituitary cells, Student's *t* test, $n = 6$, $P > 0.05$. (D) Percentage of lactotrophs in both groups. Student's *t* test, $n = 6$, $P > 0.05$.

Discussion

The involvement of estradiol in the control of PRL secretion was widely demonstrated (Mitchner *et al.* 1998, Seilicovich 2010). Although this effect has been long proposed to be mediated by ERα (Yen *et al.* 1974, Ben Jonathan *et al.* 2009), in this study, we provide new evidences that estradiol can rapidly stimulate PRL secretion in a mechanism mediated by GPER in the lactotroph population.

Previous studies have provided strong evidence of GPER expression in the pituitary gland, but focusing on gonadotroph function (Brailoiu *et al.* 2007, Hazell *et al.* 2009). For example, Rudolf *et al.* reported that approximately 50% of GPER-positive cells express LH in bovine anterior pituitaries. This finding supports the idea that GPER is expressed in non-gonadotroph pituitary cells as well (Rudolf & Kadokawa 2013). Our present results are in agreement with previous studies, and showed GPER expression in gonadotrophs, lactotrophs and also in somatotrophs. Moreover, the flow cytometry analysis revealed that about the 40% of the GPER-positive cells are PRL-positive and immunocytochemical and inmuno-electron-microscopy studies strongly demonstrated GPER expression in lactotroph population. The present results, indicating that GPER expression in is primarily localized to the plasma membrane, are consistent with previous findings demonstrating the localization of this receptor in the cell surface of other cell types (Filardo *et al.* 2000, 2008, Kelly & Levin 2001, Thomas 2017). In agreement, and using electronic microscopy, we confirmed the subcellular localization of GPER in plasmatic membrane, with gold particles attached to the inner surface of the plasmalemma. However, GPER was also localized in the cytosol. This is in accordance with previous results describing GPER localization in Golgi membranes and in the endoplasmic reticulum in several cancer cell lines. Interestingly, this intracellular localization seems to have a specific role. Revankar *et al.* demonstrated that activation of intracellular GPER by estradiol induces intracellular calcium mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus. Then, GPER was postulated as a plasmatic membrane and intracellular transmembrane estrogen receptor (Revankar *et al.* 2005, 2007).

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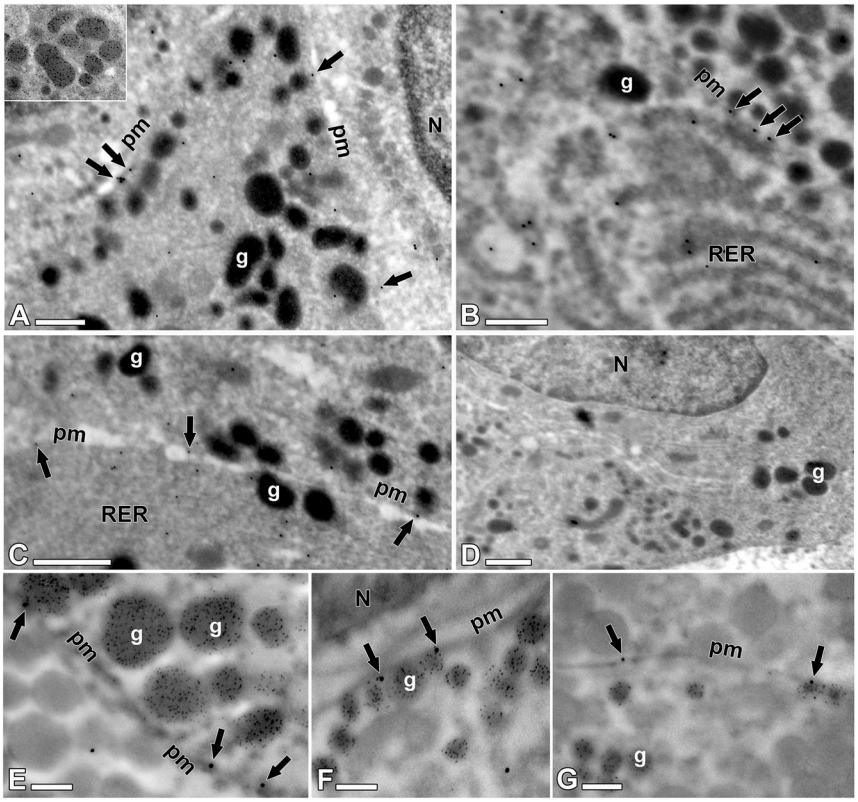

Figure 5
Immuno-electron-microscopy for GPER. (A, B and C) Subtype I lactotroph cells from female rat at diestrus with gold particles of 15 nm indicating the presence of GPER in plasmatic membrane (arrows), rough endoplasmic reticulum (RER) and free cytosol. Inset: Irregular, large and polymorphic secretory granules immunolabelled for PRL (5 nm gold particles). (D) Negative control. Bar = 0.5 µm. (E, F and G) Lactotroph cells from OVX female rat immunolabelled for GPER with gold particles of 15 nm (GPER) in plasmatic membrane (arrows). PRL was immunostained with 5 nm gold particles identifying lactotroph cells with large and irregular secretory granules (E: Subtype I lactotroph), lactotrophs with spherical granules about diameter 200–250 nm (F: Subtype II), and lactotroph cells with small spherical granules, between 100 and 200 nm (G: Subtype III). N, nucleus; pm, plasmatic membrane; g, secretory granules. Bar = 0.2 µm.

The biological significance of GPER being highly expressed in rat lactotrophs, suggested a role for this receptor in this cell type population. Previous studies postulated GPER involvement in prolactin secretion: (i) induced by xenoestrogens in the GH3 cell line (Vinas & Watson 2013) or (ii) an indirect effect activating GPER in hypothalamus (Lebesgue *et al.* 2009). Regarding the latter, this is particularly relevant considering that a high expression of GPER was found in the paraventricular nucleus and supraoptic nucleus in rats (Brailoiu *et al.* 2007). In fact, it has been described that GPER agonist G1 administered *in vivo* into the third ventricle triggers a PRL surge similar in amplitude to the one observed in response to E2 (Lebesgue *et al.* 2009). In the light of the aforementioned results it may be interesting to study the hypothalamic influence of E2-GPER in the neuroendocrine regulation of proestrus surge of PRL secretion. The complex mechanism is poorly understood and appears to be due to a complex mechanism starting in the hypothalamus, more than a direct estradiol-mediated rapid action in the lactotroph population (Szawka *et al.* 2007).

Explicit data regarding the role of GPER in normal pituitary lactotrophs is missing. Our present results demonstrate that GPER activation rapidly increases PRL secretion *in vitro* (in GH3 cell line) and *ex vivo* (female rat pituitary explants). Moreover, this effect was counteracted when cells or tissues were pre-incubated with G36, a GPER antagonist. Taken together, our results provide the first evidences of a specific role of GPER in rat lactotrophs.

In addition, we found that pituitary *Gper* expression is negatively regulated by estradiol and progesterone treatments, and, moreover, it changes during the estrous cycle. In consequence, GPER expression (protein and transcript) was found increased after OVX, likely due to the lack of ovarian steroids. In fact, according to the flow cytometry studies with double immunostaining (PRL, GPER), the increase in pituitary GPER expression after OVX was observed specifically in the lactotroph population, as the proportion of GPER-positive cells, and GPER-positive lactotrophs significantly increased in OVX rats compared to controls in diestrus and no differences were found neither in the proportion of non-lactotroph GPER-positive cells nor in the proportion of lactotrophs among groups.

It was previously described that three subtypes of lactotrophs, defined morphologically by electron microscopy, could be observed in the anterior pituitary gland from rodents (De Paul *et al.* 1997). In our present work, GPER was observed mainly in the subtype I (lactotroph cells), in pituitaries from female rats at diestrus. This is reasonable considering that, in adult

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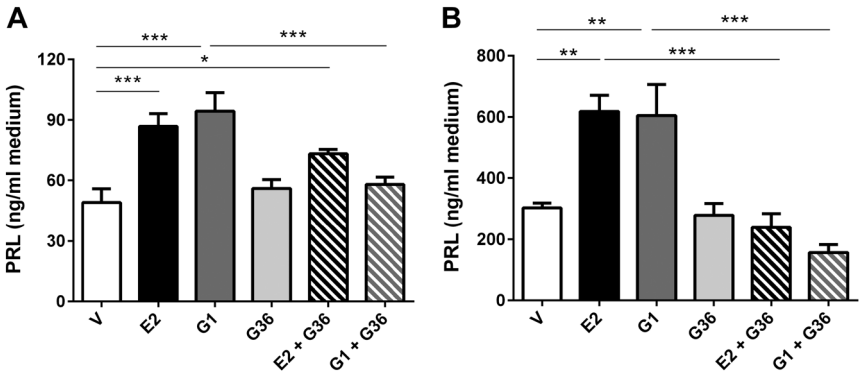

Figure 6
Effect of E2, G1 and/or G36 on PRL levels *in vitro* and *ex vivo*. (A) GH3 cells were incubated with DMEM containing vehicle (V) (ethanol, 1 μM) or G36 (1 μM) for 30 min and then estradiol (100 nM) or G1 (100 nM) were added alone or in combination with G36 for 15 min at 37°C. After treatments, medium was collected and rat PRL levels were measured by RIA. One-way ANOVA followed by Tukey's *post hoc* test, $n = 3$, three replicates in each set of experiments, ***$P < 0.001$ E2 vs V, *$P < 0.05$ E2 + G36 vs V, ***$P < 0.001$ G1 vs V and ***$P < 0.001$ G1 vs G1 + G36. (B) SD rats were sacrificed and anterior pituitaries were collected. Explants were incubated 30 min with G36 (1 μM) or vehicle (ethanol, 1 μM) and then estradiol (100 nM) or G1 (100 nM) were added alone or in combination with G36 for 15 min at 37°C. After treatments, PRL levels were measured by RIA in secreted medium. One-way ANOVA followed by Tukey's *post hoc* test, $n = 3$, three replicates in each set of experiments, **$P < 0.01$ E2 vs V, **$P < 0.01$ G1 vs V, ***$P < 0.001$ E2 vs E2 + G36 and ***$P < 0.001$ G1 vs G1 + G36.

female rats, the subtype I represents about the 90% of the total of lactotroph population, and the subtype II and III account for less than 10% (Kurosumi *et al.* 1987). However, when the electron microscopy was performed in pituitary glands from OVX female rats, the GPER expression was found extended to the three lactotroph subtypes, characteristic of this model (pituitaries from OVX rats) where the 35% of lactotrophs are subtype I, 30% are subtype II and about 36% are subtype III (Maldonado & Aoki 1994).

This result could explain the specific increase of GPER-positive lactotrophs observed in OVX rats compared to controls in diestrus, and moreover, shows that all three lactotroph subtypes are target of GPER ligand and could be involved in the PRL secretion mediated by GPER in OVX rats.

Considering that a rapid estradiol stimulatory effect on PRL secretion mediated by GPER was demonstrated *in vitro* and in pituitary explants, the elevated expression of GPER observed in the lactotroph population after an OVX, should be taken into consideration in: (i) the use of OVX as animal models, (ii) the response of the gland to an eventual hormone replacement therapy after OVX.

Estrogen replacement therapy is frequently suggested in women after bilateral prophylactic oophorectomy to prevent the potential negative effects of losing of natural hormone production (Watson *et al.* 2008, Erekson *et al.* 2013). As the major concern in those patients is the risk of cancer, the impact of the oophorectomy in the pituitary function, with or without hormone replacement therapy, is usually ignored.

Even though our present results do not include the involvement of GPER in lactotroph proliferation, several studies performed in many cancer cell lines and tumors of breast, endometrium, ovaries, thyroid and prostate among others, suggest that high levels of GPER protein expression correlate with increased tumor size and poor outcome, and, moreover, stimulation of GPER with estrogenic compounds such as atrazine, bisphenol A or tamoxifen activates cell proliferation (Prossnitz & Barton 2011).

In the light of these facts and our present results, it is worth facing future studies to investigate the involvement of GPER in physiological and pathological lactotroph proliferation and the significance of increased expression of GPER observed in lactotrophs after OVX in rats.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0402.

Declaration of interest
The authors declare that there is no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M A C, D P, J F, S G and G D T conception and design of research; M A C, A A M, J F, E Y F, P A P and S G performed experiments; M A C, A A M, J F, P A P, S G, D P and G D T analyzed data; M A C, A A M, J F, S G and G D T interpreted results of experiments; M A C, A A M, P A P and S G prepared figures; M A C, S G and G D T drafted manuscript; M A C, A A M, J F, E Y F, S G, D P and G D T edited and revised manuscript; M A C, A A M, J F, D P, P A P, S G and G D T approved final version of manuscript.

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