An intracellular VHH targeting the Luteinizing Hormone receptor modulates G protein-dependent signaling and steroidogenesis

Camille Gauthier, Pauline Raynaud, Frédéric Jean-Alphonse, Amandine Vallet, Océane Vaugrente, Vinesh Jugnarain, Thomas Boulo, Christophe Gauthier, Eric Reiter, Gilles Bruneau, et al.

Molecular and Cellular Endocrinology 589 (2024) 112235

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

An intracellular VHH targeting the Luteinizing Hormone receptor modulates G protein-dependent signaling and steroidogenesis

Camille Gauthier [a], Pauline Raynaud [a], Frédéric Jean-Alphonse [a,b], Amandine Vallet [a], Océane Vaugrente [a], Vinesh Jugnarain [a], Thomas Boulo [a], Christophe Gauthier [a], Eric Reiter [a,b], Gilles Bruneau [a], Pascale Crépieux [a,b,*]

[a] INRAE, CNRS, Université de Tours, PRC, 37380, Nouzilly, France
[b] Inria, Inria Saclay-Ile-de-France, 91120, Palaiseau, France

ARTICLE INFO

Keywords:
LHR
Intracellular VHH
G protein-coupled receptor
Reproduction
Cell signaling
Steroidogenesis

ABSTRACT

Luteinizing hormone (LH) is essential for reproduction, controlling ovulation and steroidogenesis. Its receptor (LHR) recruits various transducers leading to the activation of a complex signaling network. We recently identified iPRC1, the first variable fragment from heavy-chain-only antibody (VHH) interacting with intracellular loop 3 (ICL3) of the follicle-stimulating hormone receptor (FSHR). Because of the high sequence similarity of the human FSHR and LHR (LHCGR), here we examined the ability of the iPRC1 intra-VHH to modulate LHCGR activity. In this study, we demonstrated that iPRC1 binds LHCGR, to a greater extent when the receptor was stimulated by the hormone. In addition, it decreased LH-induced cAMP production, cAMP-responsive element-dependent transcription, progesterone and testosterone production. These impairments are not due to Gs nor β-arrestin recruitment to the LHCGR. Consequently, iPRC1 is the first intra-VHH to bind and modulate LHCGR biological activity, including steroidogenesis. It should help further understand signaling mechanisms elicited at this receptor and their outcomes on reproduction.

1. Introduction

G protein-coupled receptors (GPCRs) are the largest family of membrane proteins in humans. They are involved in many physiological responses, hence many pathologies, and as such they are the first class of therapeutic targets. Forty-three percent of FDA-approved drugs target GPCRs, including opioid analgesics, antihistamines, antipsychotics, antimigraine drugs, and drugs against asthma and hypertension (Heukers et al., 2019; Wacker et al., 2017). GPCRs have a common structure with an extracellular domain of highly variable length, 7 transmembrane (TM1-7) domains linked by 3 extracellular loops and 3 intracellular loops, and an intracellular domain. Once it is activated by its ligand, the receptor undergoes multiple conformational changes. Different agonists stabilize various conformations of a single receptor, leading to the activation of the transducing proteins, mainly G proteins and β-arrestins, with various efficacy, a phenomenon known as biased agonism (Wacker et al., 2017).

VHHs, alternatively named as nanobodies, have been used to study GPCR for more than a decade. A VHH is the variable fragment of the heavy chain of single-chain camelid antibodies. It is a small antibody fragment (~15 kDa) that can bear a long complementarity-determining region 3 (CDR3) potentially capable of reaching cryptic epitopes (Manglik et al., 2017; Stijlemans et al., 2004). This feature is particularly advantageous to target GPCRs folded within the cell membrane. Several anti-GPCR VHHs that recognize intracellular epitopes (intra-VHH) have been reported. Most of them have been used to stabilize active receptor conformations, hence facilitating 3D structure determination. This is the case for Nb80, Nb9-8, and Nb39 which served as molecular chaperones to help crystallizing active β2 adrenergic receptor (ADRB2) (Rasmussen et al., 2011), muscarinic acetylcholine receptor (CHRM1) (Kruse et al., 2013) and μ-opioid receptor (OPRM1) respectively (Huang et al., 2015). Intra-VHHs fused to a fluorescent protein have been also used as biosensors to track active GPCRs within various cell compartments. For instance, Nb80 fused to GFP has been used to visualize active β2 adrenergic receptor at the plasma membrane and in the endosome (Irannejad et al., 2013; Lin et al., 2023). Fluorescently labeled Nb39 has been used to study the internalization of mu and delta opioid receptors (OPRM1 and OPRD1) into endosomes as well as in the Golgi apparatus

* Corresponding author. INRAE, CNRS, Université de Tours, PRC, 37380, Nouzilly, France.
E-mail address: pascale.crepieux@inrae.fr (P. Crépieux).

https://doi.org/10.1016/j.mce.2024.112235
Received 11 August 2023; Received in revised form 31 March 2024; Accepted 4 April 2024
Available online 15 April 2024

and to unravel the effect of agonists and antagonists selectively in these sub-cellular compartments (Stoeber et al., 2018). In contrast, only a few intra-VHHs have been described as modulators of GPCR signaling. This is only exemplified by intra-VHHs against the β2 adrenergic receptor (ADRB2), opioid receptor (OPRK1, OPRD1, and OPRM1), and the human cytomegalovirus (HCMV) encoded chemokine receptor (US28) (for review see (Raynaud et al., 2022). These 5 receptors are all small ligand receptors, with short extra-cellular domains.

The luteinizing-hormone receptor (LHCGR in Humans or LHR in all other species), a class A GPCR, is a glycoprotein hormone receptor (GPHR), together with the Follicle-Stimulating Hormone Receptor (FSHR) and Thyroid-Stimulating Hormone Receptor (TSHR) (Jiang et al., 2014). In women, LHCGR is expressed by granulosa cells, thecal cells, and luteal cells, whereas in men, it is expressed by Leydig cells. LHCGR binds not only the Luteinizing Hormone (LH) that controls ovulation and steroid production, but also chorionic gonadotropin (CG) secreted during the first trimester of pregnancy, which enhances the *corpus luteum* steroidogenic activity. In men, the LHCGR promotes Leydig cell differentiation and stimulates testosterone production (Ascoli et al., 2002). The LHCGR structure departs from other class A GPCRs by the presence of a large ectodomain (~350 aa) containing 11 leucine-rich repeats (LRR) shared by the three GPHRs (Jiang et al., 2014). LH or CG binding to the LHCGR ectodomain induces an outward movement of 45° of the ectodomain compared to the inactive state of the receptor where the ectodomain is closer to the cell membrane. LHCGR activation causes multiple conformational changes of the transmembrane domains (TM) (Duan et al., 2021) leading to the preferential coupling of Gαs over Gαq/11 and Gαi, and recruitment of β-arrestins (Ascoli et al., 2002). Gαs activation triggers the cyclic Adenosine Monophosphate (cAMP)/Protein Kinase A (PKA) pathway, that regulates steroidogenesis (Dufau et al., 1977). Among these mechanisms, Gs-mediated ERK1/2 (Extracellular signal-Regulated Kinases) and CREB (cAMP Response Element-binding protein) phosphorylations have been shown to control several steps of steroid production (Manna et al., 2002; Riccetti et al., 2017). For instance, CREB phosphorylation induces the transcription of STARD1 and CYP19 genes that both regulate steroidogenesis (Manna et al., 2002; Payne and Hales, 2004). Overall, LHR triggers complex signaling mechanisms that remain partially understood.

Pharmacological tools to decipher the complexity of the signaling network activated downstream of the LHR are sparse. The iPRC1 intra-VHH was recently isolated by our group in a screen against the human FSHR (hFSHR) intracellular loop 3 (ICL3) and was identified as the first intra-VHH known to bind and modulate this receptor (Raynaud et al., 2024). So far, such a tool does not exist for the LHCGR. However, given the high degree of similarity existing between FSHR and LHCGR ICL3, we hypothesized that iPRC1 could cross-react with LHCGR. Here we demonstrate that iPRC1 binds the LHCGR and negatively modulates the LH-induced cAMP and steroidogenic responses.

2. Material and methods

All reagents, their references and manufacturer used in this article are assembled in Table 1 of the supplementary material and methods.

2.1. Selection of nanobodies

Phage libraries were generated using lymphocytes of llamas immunized with intramuscular injection of cDNA encoding the FSHR and LHCGR (In-Cell-Art, France). The phage library was screened by phage display with the intracellular loop 3 peptide of the hFSHR as described elsewhere (Raynaud et al., 2024).

2.2. Cell culture

Murine Leydig Tumoral cell (mLTC-1) (Rebois, 1982) and Human Embryonic Kidney 293 cell lines (HEK293A) were respectively cultured in RPMI 1640 and in DMEM, both with Glutabio and $NaHCO_3$ and both supplemented with 10% heat-inactivated fetal bovine serum and 1% Penicillin. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2. All reagents were purchased at Eurobio Scientific (Les Ulis, France).

2.3. Flow cytometry

mLTC-1 and HEK293A cells were respectively transfected with 90 ng or with 150 ng of pcDNA3.1 plasmid encoding iPRC1-6xHis or T31-6XHis (negative control) and 10 ng of pcDNA3.1 encoding FLAG-hLHCGR (kindly provided by Pr. Aylin Hanyaloglu, Imperial College London, UK), using Metafectene PRO (Pro Biontex, München, Germany) following the company's protocol. Then, cells were starved for 4 h and detached with Hanks Balanced Salt Solution (HBSS) without phenol red and Ca^{2+}/Mg^{2+} (Eurobio Scientific). Then, cells were resuspended and incubated for 10 min at 4 °C in FcRn Blocking Reagent (Miltenyi Biotech, Bergisch Gladbach, Germany) diluted in PBS with 0.5 % BSA according to the manufacturer's protocol. Cells were incubated for 30 min at 4 °C in a solution of anti-FLAG-phycoerythrin (PE) antibody (DYKDDDK antibody, PE, REAfinity™ Miltenyi Biotech) at 1/1000 and with 1/1000 of LIVE/DEAD™ Fixable Violet Dead Cell Stain (Invitrogen, Waltham, Massachusetts, USA). After 3 washes in PBS with 0.5 % BSA, cells were permeabilized and fixed for 20 min at 4 °C in Cytofix/Cytoperm buffer and washed in 1XBD Perm/Wash™ Buffer at 4 °C. Then cells were stained for 30 min at 4 °C with a solution of 1/1000 of Allophycocyanin (APC) conjugated anti-6xHis antibody (Miltenyi Biotech) diluted in 1X BD Perm/Wash™ Buffer. After 3 washes, cells were resuspended in PBS and examined using a MACSQuant®10 (Miltenyi Biotech) flow cytometer. The data were analyzed and plotted with the FlowJo software (FlowJo, Ashland, OR, USA).

2.4. Bioluminescence resonance energy transfer (BRET) experiments

For binding assays, forty thousand HEK293A cells per well were seeded in 96 well plates and transfected with 30 ng of pcDNA3.1 plasmid encoding FLAG-human-LHCGR-Rluc8 (Renilla luciferase) (kindly provided by Pr. Aylin Hanyaloglu, Imperial College London, UK) or V2R-Rluc8 and different amounts of pcDNA3.1 plasmid encoding iPRC1 or T31 fused to a G4S linker and the fluorescent protein acceptor mVenus, using Metafectene PRO (Biontex) following the company's protocol. After 48 h, cells were incubated with a solution of 5 μM coelenterazine H (Interchim, Montluçon, France) diluted in PBS with or without 3.3 nM of human LH (Luveris, Merck Darmstadt, Germany) or 100 nM Arginine Vasopressin AVP (Tocris Bioscience, Noyal-Châtillon-sur-Seiche, France). The signals were recorded for 20 min in a Mithras LB 943 plate reader (Berthold Technologies GmbH & Co., Wildbad, Germany). The 540 nm/480 nm ratio was calculated and all the values were normalized on T31, a non-relevant intra-VHH, previously described in (Raynaud et al., 2024). The data were analyzed with GraphPad Prism 5.0 using One site – Specific binding with Hill slope with the equation : $Y = \frac{Bmax \times X^h}{X^h + Kd^h}$, the h value, corresponding to the Hill slope, being shared by all data sets.

In Mini Gs protein (mGs, minimal engineered GTPase domain of the Gα subunit) recruitment BRET experiments, 30,000 HEK293A cells per well of 96-well plates were transiently co-transfected with plasmids encoding the human LHCGR-Rluc8 (0.1 μg plasmid DNA/cm^2), the NES-Venus-mGs (65 ng plasmid DNA/cm^2, kindly provided by Pr. Nevin A. Lambert, Augusta University, Augusta, GA, USA) (Wan et al. 2018), and VHH-6xHis (0.2 μg plasmid DNA/cm^2).

β-arrestin recruitment to the LHCGR was measured similarly, with plasmids encoding the human LHCGR-Rluc8 (0.1 μg plasmid DNA/cm^2), β-arrestin 2-YPet (kindly provided by Dr. M.G. Scott, Cochin Institute, Paris, France, 65 ng plasmid DNA/cm^2) (Ayoub et al. 2015), and VHH-6xHis (0.2 μg plasmid DNA/cm^2).

2.5. Peptide competition by HTRF

One hundred and twenty μM of FSHR ICL3 biotinylated peptide (HIYLTVRNPNIVSSSSDTRIAKR) (Genecust, Boynes, France) and 30 nM of bacterially-expressed VHHs in PBS 0.1% Tween 20 (PBS-T) purified as described in (Raynaud et al., 2024), were incubated overnight at 4 °C and 30 rpm, with or without 1 mM of LHCG ICL1 (RYKLTVPR) or ICL3 (YFAVRNPELMATNKDTKIAKK) competing peptides (Genecust, Boynes, France). Background signal was obtained with PBS-T supplemented with equivalent amount of DMSO as the other conditions. The sensors, MAb Anti-6 His-Tb cryptate (Cisbio, Waltham, Massachusetts, USA) and Streptavidin-d2 (Cisbio, Waltham, Massachusetts, USA) were added following manufacturer's protocol, and after a 1-h incubation in the dark, fluorescence measurement was performed with a TriStar^2 LB 942 Multimode Microplate Reader (Berthold Technologies GmbH & Co., Wildbad, Germany).

2.6. Quantification of cAMP and steroids

2.6.1. Total cAMP measurement

One hundred thousand HEK293A cells per well were seeded in 24-well plates, then transfected using Metafectene PRO with 10 ng of pcDNA3.1 plasmid encoding FLAG-hLCGHR and 150 ng of pcDNA3.1 plasmid encoding iPRC1 or T31. After 48 h, cells were starved in DMEM-free for 3 h and in HBSS without Ca^{2+} (Eurobio Scientific). Then cells were detached, counted, and seeded in 384 well plates with 7,500 cells per well. Cells were stimulated with 4 μM Forskolin (Fsk) (MedCheExpress, South Brunswick, NJ, USA), or 0.1 nM LH for 15 min. The total cAMP production was measured with the HTRF (Homogeneous Time-Resolved Fluorescence) cAMP Gs dynamic kit (PerkinElmer Cisbio, Waltham, Massachusetts, USA), following the manufacturer's instructions. After excitation of the donor at 620 nm, emission of the acceptor was read at 665 nm (TriStar 2 LB 942 Berthold Technologies GmbH & Co). The 665 nm/620 nm ratio was calculated, the values were normalized on the signal obtained with Fsk, and the results were expressed as percentage of the maximal response to LH.

2.6.2. Measurements of extracellular cAMP and steroids

Sixty thousand mLTC-1 per well were seeded in 48-well plates and transfected with 90 ng of pcDNA3.1 plasmid encoding iPRC1 or T31, or with empty pcDNA3.1. After 24 h, mLTC-1 were starved using free RPMI medium for 17 h. Then cells were incubated with 0.1 nM LH for 3 h, at 37 °C in 5 % CO_2. Both cAMP and steroids were quantified in the supernatant of mLTC-1 cells after dilution in medium, by HTRF (Progesterone kit and Testosterone kit, PerkinElmer Cisbio). The fluorescence was measured as above, the 665 nm/620 nm ratio was calculated and the results were expressed as percentage of the maximal response to LH, calculated in the absence of VHH (empty pcDNA3.1).

2.7. CRE-dependent trancription

HEK293A cells were seeded in 96-well plates previously coated with poly-L-lysine diluted 1:10 (Sigma-Aldrich, St. Louis, MO, USA) at a density of 50,000 cells/well. Then cells were transfected with 20 ng of pcDNA3.1 plasmid encoding FLAG-hLHCGR, 50 ng of pSOM-Luc plasmid expressing the firefly luciferase reporter gene under the control of the cAMP Responsive Element (CRE) of the somatostatin promoter region, and 150 ng of pcDNA3.1 plasmid encoding intra-VHH, using Metafectene PRO. mLTC-1 cells were seeded in a 96-well plate at a density of 40,000 cells/well, and transfected with 50 ng of pSOM-Luc plasmid and 150 ng of intra-VHH-encoding plasmid. After 48 h, HEK293A and mLTC-1 cells were stimulated with 3.3 nM and 0.1 nM of LH respectively, for 6 h at 37 °C. Then, supernatants were discarded, the Bright-Glo Luciferase assay substrate (Promega, Madison, WI, USA) was added, and the emitted light was measured using a Mithras LB 943 plate reader. Values (RLU) were expressed as percentage of the maximal response obtained in the absence of VHH.

2.8. Data analysis and statistic

All experiments have been done at least four times each time in triplicates. The mean of triplicate is represented by one point ± s. e.m. All data were analyzed with the pairwise Mann-Whitney test done by GraphPad Prism 5.0 (San Diego, CA, USA).

3. Results

3.1. iPRC1 interacts with the intracellular domain of LHCGR

The iPRC1 intra-VHH has been selected by phage display against the human FSHR ICL3. This intra-VHH has been reported to interact with both inactive and active conformations of the hFSHR and to significantly decrease cAMP production in response to FSH (Raynaud et al., 2024) (Fig. 1A). Given the high sequence similarity between the hFSHR and LHCGR ICL3s (~74%) (Fig. 1B), we examined whether iPRC1 could also interact with the human LHCGR and modulate its activity.

To assess a potential interaction in living cells, BRET assay with the LHCGR fused to Rluc8 and iPRC1 fused to mVenus was carried out. It had been previously shown that, despite the reducing condition of the cytosol, unfavourable to disulfide bond formation, the overexpression of either iPRC1 or of the T31 negative control in HEK293A cells did not generate aggregates (Raynaud et al., 2024). Increasing amounts of iPRC1-mVenus encoding plasmid were co-transfected in HEK293 cells with a constant amount of LHCGR-RLuc8 encoding plasmid, pcDNA3.1 plasmid encoding T31-mVenus, an irrelevant intra-VHH-mVenus fusion, and the Arginine Vasopressin receptor type 2 (V2R) C-terminally fused with RLuc8 encoding plasmid. V2R was used as a specificity control because of the high divergence of its ICL3 sequence, despite the presence of a basic motif (Fig. 1B).

In unstimulated conditions, we observed that iPRC1 interacted significantly more with the LHCGR than with the V2R (Fig. 2A, Figs. S1A and S1B), whether the receptors were stimulated or not with their cognate ligand. Importantly, iPRC1 binding to the LHCGR was significantly more important when the receptor was stimulated than with its inactive counterpart, suggesting that this VHH is sensitive to receptor conformations.

3.2. iPRC1 inhibits the LH-induced cAMP-dependent pathway

The impact of iPRC1 on LH-induced cAMP was assessed in HEK293 cells by co-expressing LHCGR and either iPRC1 or T31. In all experiments, we verified by flow cytometry that similar levels of iPRC1 and T31 on the one hand, and of LHCGR on the other hand, were expressed in each condition (Fig. S2). By HTRF, we observed that LH-induced cAMP production was significantly reduced (~31%) in the presence of iPRC1 when compared to T31 (Fig. 3A and S3A). In support of the specificity of iPRC1 effects on gonadotropin receptors, it was previously shown that iPRC1 has no effect on the cAMP production induced by stimulated β2-AR or V2R (Raynaud et al., 2024). Consistently, cAMP-responsive element-dependent transcription in the presence of increasing concentrations of LH was decreased when plasmids encoding LHCGR, a CRE-luciferase reporter gene (Troispoux et al., 1999) and either iPRC1 or T31 were co-expressed (Fig. 3B and S3B). No difference in CRE-dependent activity was observed in the presence of iPRC1 in non-stimulated cells.

HADDOCK modelling suggests that the binding of iPRC1 on the LHCGR ICL3 causes a minimal steric clash with Gαs protein (Fig. S4), that could explain its partial, but not complete, inhibitory effect on the cAMP response. Therefore, we assessed the recruitment of Mini-Gs to the LHCGR in absence or in presence of T31 or iPRC1 by BRET. No difference was visible and the EC_{50} were similar, when comparing the effect of T31 or iPRC1 (Fig. 3C). β-arrestin recruitment remained also unchanged

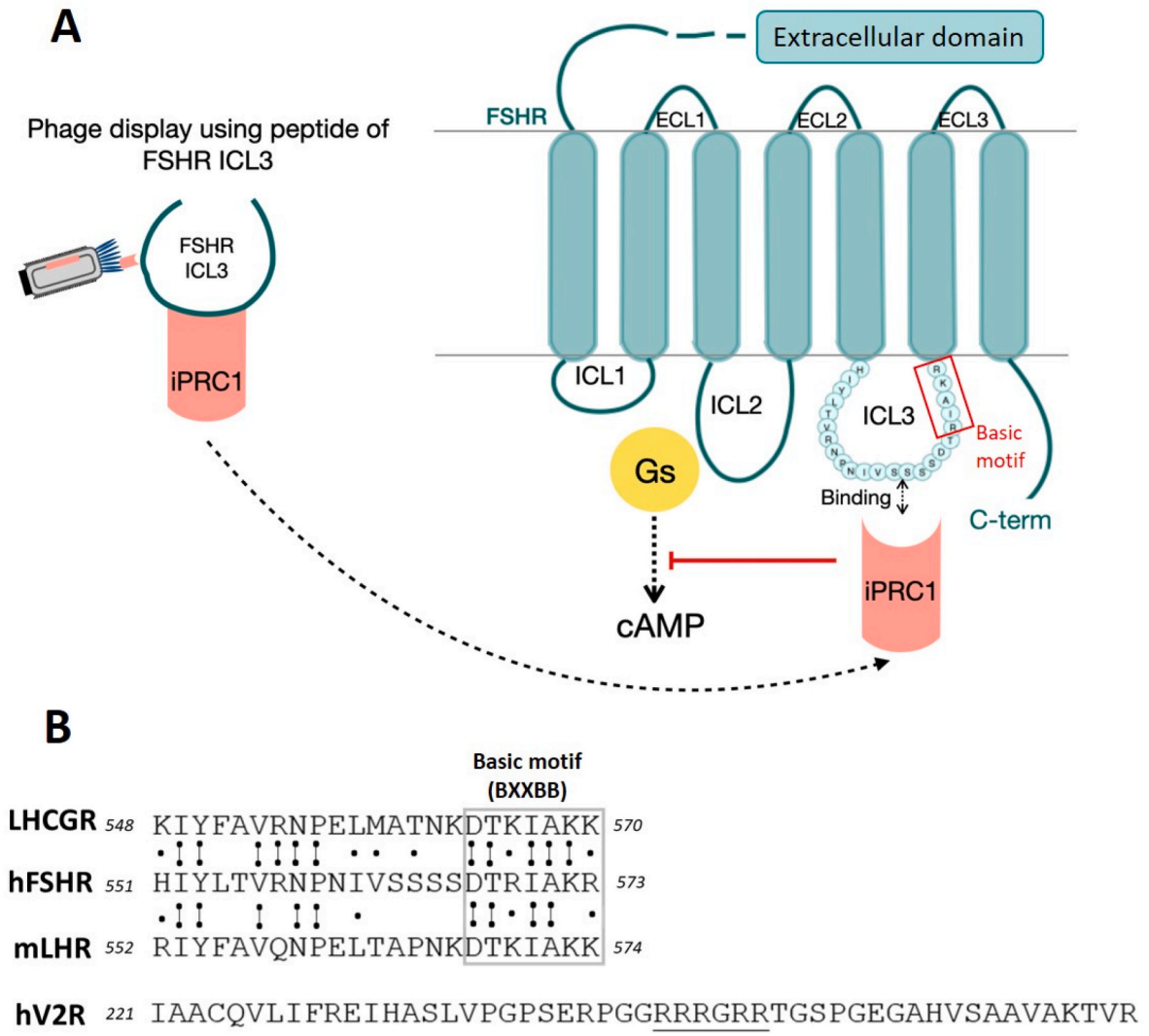


Fig. 1. Principle of selection of the iPRC1 intra-VHH. (A) Schematic diagram of the selection of iPRC1 by phage display and of its effect on the FSHR. iPRC1 binds the hFSHR ICL3 and negatively affects cAMP production in response to FSH (Raynaud et al., 2024). **(B)** Alignment of the ICL3 sequences of hFSHR, LHCGR, and mLHR and hV2R. When compared to the hFSHR sequence, the identity of the sequences is represented by two points and a line, non-conservative substitutions of amino acids are represented by no dot, conservative ones are represented by a dot, the most conserved region (the basic motif) is boxed.

in the presence of iPRC1, which rules out that increased LHCGR desensitization would be causal in decreasing cAMP production, that is observed when the intra-VHH is expressed (Fig. 3D).

3.3. iPRC1 inhibits LH-induced cAMP and steroidogenesis in mLTC-1 cells

Given the effects of iPRC1 on cAMP production and CRE-dependent transcription in HEK293 cells and the similarity of sequence with conservative substitutions between LHCGR and mLHR (~91.3%), and between hFSHR and mLHR (~60.8%) (Fig. 1B), we next examined the effect of iPRC1 on a steroidogenic cell model expressing LHR endogenously, the murine Leydig Tumoral Cells (mLTC-1). In these cells, that endogenously express the mLHR, transfected iPRC1 and T31 were expressed at comparable levels (i.e. 5.95 % and 4.14 % respectively) (Fig. S5). In terms of quantitative ratio, the lower intra-VHH expression level in mLTC-1 when compared to HEK293 was likely compensated by the fact that endogenous LHR is also very low in mLTC-1 (Rebois, 1982). cAMP produced intracellularly can accumulate extracellularly, as previously shown (Godinho and Costa-Jr, 2003). In mLTC-1, cAMP was measured in the culture media, following 3 h of stimulation with 0.1 nM LH. iPRC1 decreased of ~25% the extracellular accumulation of cAMP in response to LH, when compared to T31, which confirmed our observations in HEK293A cells with the LHCGR (Fig. 4A and S3C). Consistently, iPRC1 also significantly decreased CRE-dependent luciferase activity when compared to T31 (Fig. 4B and S3D). Therefore, iPRC1 exerts similar effects on mLHR and LHCGR. To build on these data, we took advantage of the steroidogenic abilities of mLTC-1 cells. Progesterone and testosterone production were measured by HTRF, in mLTC-1 stimulated with 0.1 nM of LH for 3 h. In these conditions iPRC1, but not T31, significantly decreased (~30%) progesterone and testosterone (46%) productions (Fig. 4C–D and S3E-S3F).

4. Discussion

In the present study, we characterized an intra-VHH that binds to LHCGR and mLHR, leading to negative modulation of LH-induced cAMP, CRE-dependent transcription, and ultimately steroidogenesis.

iPRC1 was originally selected against FSHR ICL3, from a library obtained from a llama immunized with FSHR, LHCGR and TSHR (Raynaud et al., 2024), and we show here that it also binds the LHCGR ICL3. The amino acid sequence identity between human FSHR and LHCGR ICL3s is 43.7 %, and reaches 74% when considering conservative substitutions (Fig. 1B). The part of the sequence the most conserved

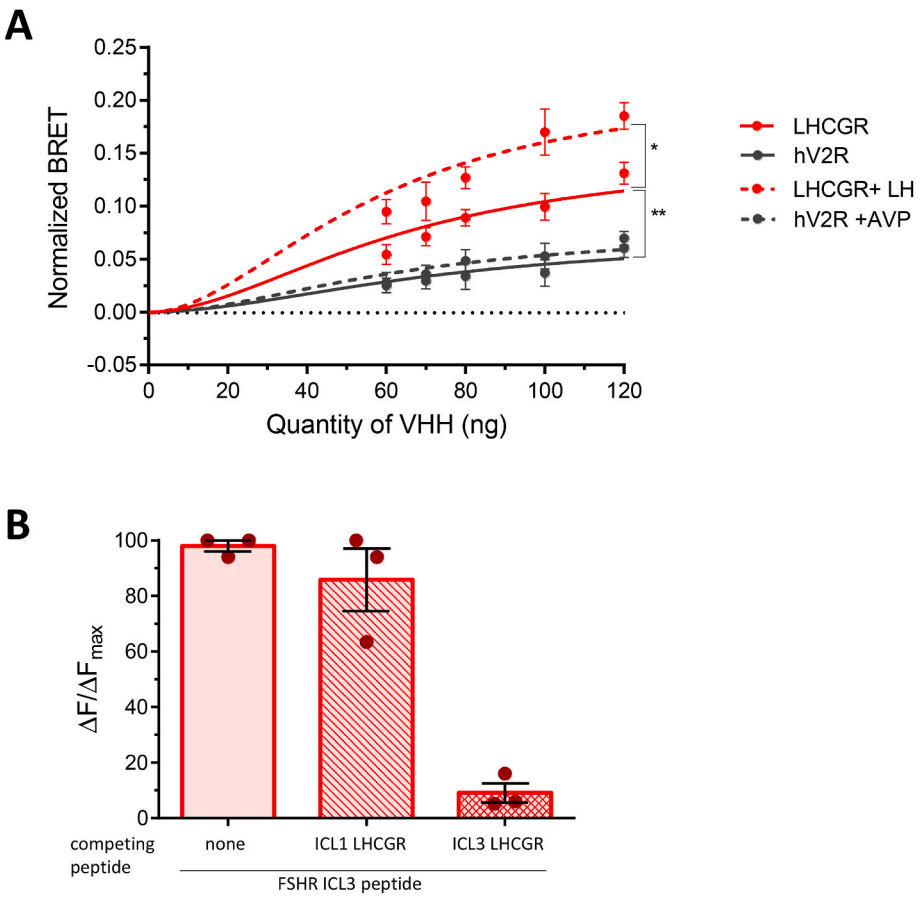


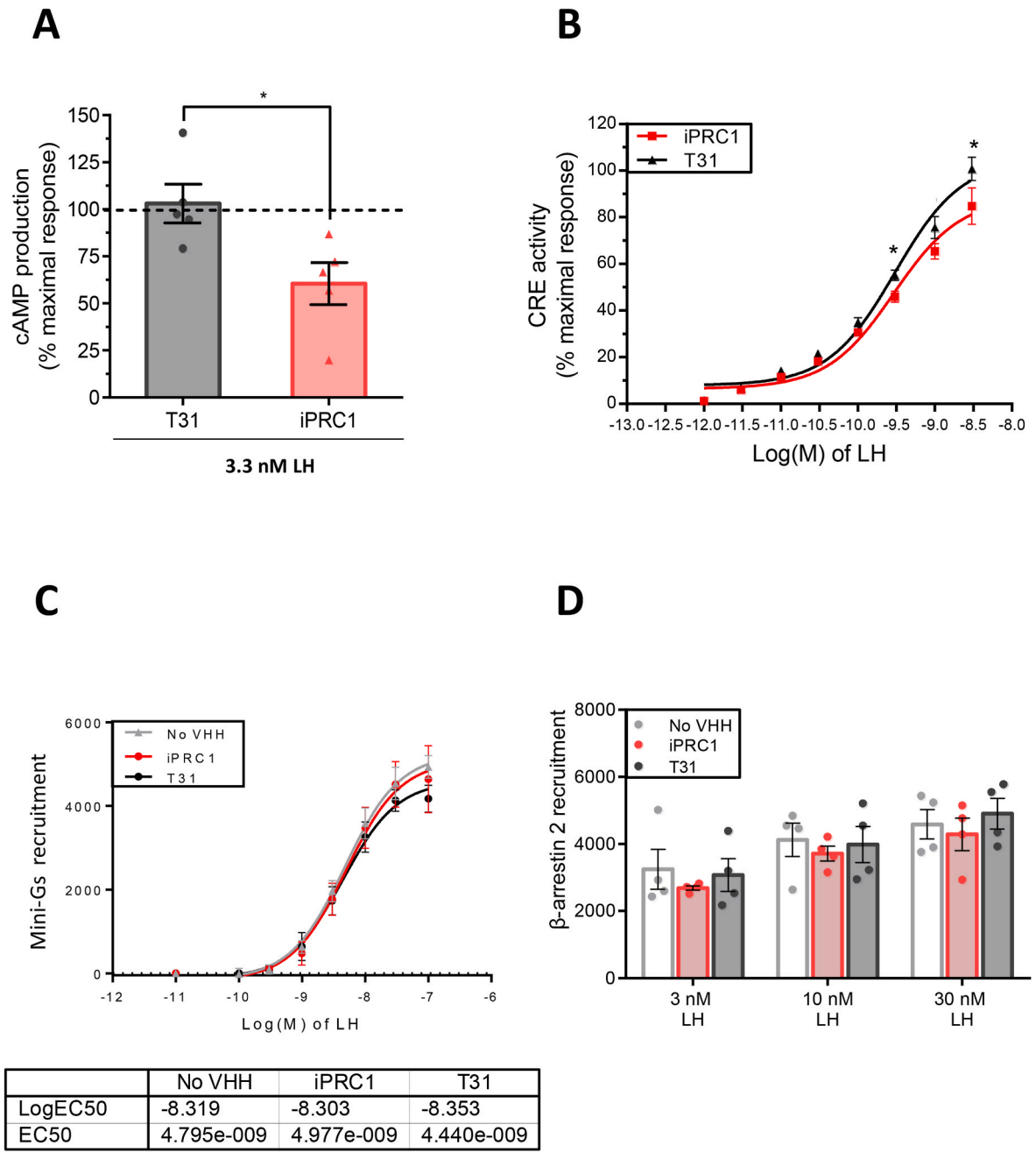
Fig. 2. iPRC1 interacts with LHCGR. (A) HEK293 cells transiently expressing LHCGR-RLuc8 or V2R-RLuc8 and different amounts of plasmid encoding iPRC1-Venus or T31-Venus were mock-stimulated with PBS or were incubated for 15 min with either 3.3 nM LH or 100 nM of Arginine Vasopressin (AVP) (N = 5). Mann and Whitney test was done on the maximum values at each point of the curve and also between each dose of intra-VHH (*p < 0.05; **p < 0.01). **(B)** Ability of LHCGR ICL3 to compete iPRC1 biding to a biotinylated FSHR ICL3 peptide, measured by HTRF (N = 3).

between hFSHR, LHCGR and mLHR is DTR/KIAKR/K (positions 564–570 of hLHCGR). Within this basic motif of GPHRs, it has been shown that mutation of the first or last basic amino acid to an alanine decreases cAMP production (Timossi et al., 2004), which could explain the decrease of cAMP production induced by LH in the presence of iPRC1 (Figs. 3A and 4A). However, careful examination of the putative epitope on the FSHR excludes the basic region (Raynaud et al., 2024), including amino acids NPNIV of this receptor, replaced by the NPELM in the LHCGR. Given the important charge change it provokes, it can be hypothesized that the presence of a glutamic acid in the LHCGR instead of an asparagine in the FSHR might explain the differential sensitivity to receptor conformation of both receptors. However, the absence of iCL3 in the recently solved structure of the LHCGR (Duan et al., 2021) prevents to definitely conclude on this point. In light of this structure, it can be hypothesized that the TM6 shift that causes an outward movement of ICL3 possibly exposes more the iCL3 for interaction with iPRC1 in the active LHCGR than in the inactive one. In contrast, iPRC1 interacts similarly with both inactive and active hFSHR (Raynaud et al., 2024). However, the BRET ratios were higher than the ones recorded with active LHCGR, suggesting that iPRC1 might have a greater affinity for the FSHR than for the LHCGR. Like LHCGR, the binding of FSH to the FSHR causes a 14.96 Å outward shift of TM6 (Duan et al., 2023), as opposed to 12.3 Å in the LHCGR. This difference might expose the ICL3 further out in the FSHR than in the LHCGR, hence facilitating iPRC1 binding. Alternatively, the Venus BRET acceptor fused to iPRC1 may be oriented slightly differently when interacting with each receptor, resulting in different BRET signals.

Since it does not affect Gs recruitment to the LHCGR, iPRC1 likely affects Gs activity, hence compromising activation of the cAMP pathway. Recently, it has been shown that ICL3 is important for the selectivity of G protein recruitment and activation. The inactive state of ICL3 obstructs the cytoplasmic cavity of the β2 adrenergic receptor that is required for signal transducers, such as G proteins and β-arrestins. When the β2 adrenergic receptor is activated ICL3 moves away from its resting position, opening the G protein-binding pocket that leads to the activation of signaling pathways (Sadler et al., 2023).

The inhibition of cAMP and CRE-dependent transcriptional activity by iPRC1 correlates with the decrease in steroidogenesis, which is consistent with the literature: cAMP activates PKA that phosphorylates CREB activity (Auger, 2003; Shaywitz and Greenberg, 1999). Once activated, CREB binds to the CRE sequence present in the promoter regions of many genes encoding steroidogenic enzymes (Martin and Nguyen, 2022; Payne and Hales, 2004). For example, CREB transcriptionally stimulates genes involved in steroidogenesis such as StAR, that is essential for the transport of cholesterol into the mitochondria, where it is converted into progesterone (Manna et al., 2002), among others. Likewise, the use of the H-89 PKA inhibitor decreases CREB phosphorylation and reduces testosterone production (Riccetti et al., 2017). In the future, it would be interesting to examine the LH-induced expression of the StAR gene and protein in the presence of iPRC1.

To conclude, our results suggest that iPRC1 allosterically binds the LHCGR, and partially inhibits LH-induced cAMP-dependent responses. By immobilizing particular LHCGR conformations, iPRC1 may represent a promising tool to investigate in more details the molecular mechanisms of G protein transduction, signaling pathway and to screen new ligands. Additionally, this intra-VHH may also help to investigate the



	No VHH	iPRC1	T31
LogEC50	-8.319	-8.303	-8.353
EC50	4.795e-009	4.977e-009	4.440e-009

Fig. 3. iPRC1 affects the LH-induced cAMP/CREB pathway. (A) Total cAMP production was measured by HTRF in HEK293 cells transiently expressing LHCGR, and iPRC1 or T31. Cells were starved and stimulated for 15 min with 3.3 nM of LH (N = 5). Data were normalized to the forskolin response. **(B)** CRE-dependent transcriptional activity was measured by luminescence in HEK293 cells transiently expressing LHCGR, and iPRC1, T31 or transfected with empty pcDNA3.1, with a pSOM-Luc reporter plasmid. Cells were stimulated for 6 h with increasing concentrations of LH or left untreated (N = 6). Data are expressed as the means of the maximal response to LH in the absence of VHH (Figs. S3A and S3B) ± s. e.m (*$p < 0.05$). **(C)** Comparison of the maximum recruitment of Venus-Mini-Gs to the LHCGR-RLuc8, in the presence of T31 or iPRC1. Data are represented as mean ± s. e.m. of the fold-change (N = 4). **(D)** Comparison of the maximum recruitment of β-arrestin 2- YPet to the LHCGR-RLuc8, in presence of T31 or iPRC1. Data are represented as mean ± s. e.m. of the fold-change (N = 4).

relationships between receptor conformations and may also serve as biosensor to study receptor trafficking.

Funding sources and disclosure of conflicts of interest

This work was funded with the support of Institut National de la Recherche Agronomique et de l'Environnement (INRAE), of the MAbImprove Labex (ANR-10-LABX-53) and of Région Centre Val de Loire ARD2020 Biomédicaments SELMAT grant and APR-IR INTACT grant.

CG was funded by a joint fellowship from the SELMAT grant of Région Centre Val de Loire ARD2020 Biomédicaments Program and by the APR-IR INTACT grant. **PR** was funded by a joint fellowship from Région Centre Val de Loire and INRAE. **OV** was funded by the APR-IR INTACT grant. **VJ** was funded by the SELMAT grant of Région Centre Val de Loire ARD2020 Biomédicaments Program. **TB, GB,** and **ER** were funded by INRAE, **FJ-A,** and **PC** were funded by the Centre National de la Recherche Scientifique (CNRS).

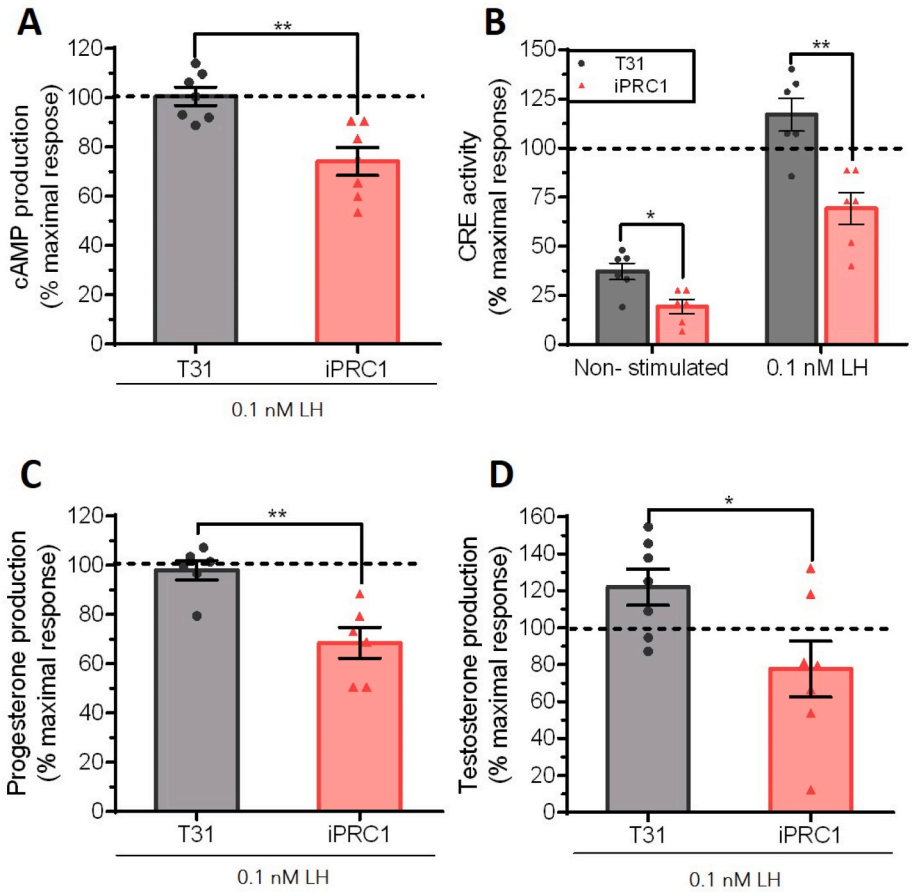


Fig. 4. iPRC1 impairs LH-induced cAMP/CRE pathway and steroidogenesis in mLTC-1 cells. (A) Quantification by HTRF of extracellular cAMP secreted by mLTC-1 cells transfected with iPRC1 or T31, or with the pcDNA3.1 empty vector, and stimulated for 3 h with 0.1 nM of LH (N = 7). **(B)** CRE-luciferase activity of mLTC-1 cells expressing intra-VHH or empty plasmid, stimulated or not with LH (N = 6). Progesterone **(C)** and testosterone **(D)** were measured by HTRF in the supernatant of mLTC-1 cells transfected with intra-VHH and stimulated with LH (N = 6 and N = 7). Data represent the percentage of the mean of the maximal response of LH on cells (No VHH) ± s. e.m (*p < 0.05; **p < 0.01) (Figs. S3C–S3F).

CRediT authorship contribution statement

Camille Gauthier: Writing – original draft, Methodology, Investigation, Formal analysis. **Pauline Raynaud:** Writing – review & editing, Methodology, Investigation. **Frédéric Jean-Alphonse:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Amandine Vallet:** Writing – review & editing, Validation, Methodology. **Océane Vaugrente:** Writing – review & editing, Methodology. **Vinesh Jugnarain:** Writing – original draft, Methodology. **Thomas Boulo:** Writing – review & editing, Methodology. **Christophe Gauthier:** Methodology. **Eric Reiter:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Gilles Bruneau:** Writing – review & editing, Methodology, Conceptualization. **Pascale Crépieux:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to acknowledge the Merck company for kindly providing purified Luveris (LH), Pierre Martineau (Institut de Recherche en Cancérologie de Montpellier, France) for precious technical indications and for kindly providing the pCANTAB 6 phagemid vector and the KM13 helper phage and the help of Yves Combarnous, Danièle Klett and Nicolas Azzopardi for helpful discussions and valuable technical advice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2024.112235.

References

Ascoli, M., Fanelli, F., Segaloff, D.L., 2002. The lutropin/choriogonadotropin receptor, A 2002 perspective. Endocr. Rev. 23 (2), 141–174. https://doi.org/10.1210/edrv.23.2.0462.

Auger, A.P., 2003. Sex differences in the developing brain: crossroads in the phosphorylation of cAMP response element binding protein. J. Neuroendocrinol. 15 (6), 622–627. https://doi.org/10.1046/j.1365-2826.2003.01041.x.

Ayoub, M.A., Landomiel, F., Gallay, N., Jégot, G., Poupon, A., Crépieux, P., Reiter, E., 2015. Assessing gonadotropin receptor function by resonance energy transfer-based assays. Front. Endocrinol. 6, 130. https://doi.org/10.3389/fendo.2015.00130.

Duan, J., Xu, P., Cheng, X., Mao, C., Croll, T., He, X., Shi, J., et al., 2021. Structures of full-length glycoprotein hormone receptor signalling complexes. Nature 598 (7882), 688–692. https://doi.org/10.1038/s41586-021-03924-2.

Duan, J., Xu, P., Zhang, H., Luan, X., Yang, J., He, X., Mao, C., et al., 2023. Mechanism of hormone and allosteric agonist mediated activation of follicle stimulating hormone receptor. Nat. Commun. 14 (1), 519. https://doi.org/10.1038/s41467-023-36170-3.

Dufau, M.L., Tsuruhara, T., Horner, K.A., Podesta, E., Catt, K.J., 1977. Intermediate role of adenosine 3':5'-cyclic monophosphate and protein Kinase during gonadotropin-induced steroidogenesis in testicular interstitial cells. In: Proceedings of the National Academy of Sciences of the United States of America, vol. 74, pp. 3419–3423. https://doi.org/10.1073/pnas.74.8.3419, 8.

Godinho, R.O., Costa Jr., V.L, 2003. Regulation of intracellular cyclic AMP in skeletal muscle cells involves the efflux of cyclic nucleotide to the extracellular compartment. Br. J. Pharmacol. 138 (5), 995–1003. https://doi.org/10.1038/sj.bjp.0705130.

Heukers, R., De Groof, T.W.M., Smit, M.J., 2019. Nanobodies detecting and modulating GPCRs outside in and inside out. Curr. Opin. Cell Biol. 57, 115–122. https://doi.org/10.1016/j.ceb.2019.01.003.

Huang, W., Manglik, A., Venkatakrishnan, A.J., Laeremans, T., Feinberg, E.N., Sanborn, A.L., Kato, H.E., et al., 2015. Structural insights into M-opioid receptor activation. Nature 524 (7565), 315–321. https://doi.org/10.1038/nature14886.

Irannejad, R., Tomshine, J.C., Tomshine, J.R., Chevalier, M., Mahoney, J.P., Steyaert, J., Rasmussen, S.G.F., et al., 2013. Conformational biosensors reveal GPCR signalling from endosomes. Nature 495 (7442), 534–538. https://doi.org/10.1038/nature12000.

Jiang, X., Dias, J.A., He, X., 2014. Structural biology of glycoprotein hormones and their receptors: insights to signaling. Mol. Cell. Endocrinol. 382 (1), 424–451. https://doi.org/10.1016/j.mce.2013.08.021.

Kruse, A.C., Ring, A.M., Manglik, A., Hu, J., Hu, K., Eitel, K., Hübner, H., et al., 2013. Activation and allosteric modulation of a muscarinic acetylcholine receptor. Nature 504 (7478), 101–106. https://doi.org/10.1038/nature12735.

Lin, T.-Y., Mai, Q.N., Zhang, H., Wilson, E., Chien, H.-C., Wah Yee, S., Giacomini, K.M., Olgin, J.E., Irannejad, R., 2023. Cardiac contraction and relaxation are regulated by distinct subcellular cAMP pools. Nat. Chem. Biol. 20 (1), 62–73. https://doi.org/10.1038/s41589-023-01381-8.

Manglik, A., Kobilka, B.K., Steyaert, J., 2017. Nanobodies to study G protein–coupled receptor structure and function. Annu. Rev. Pharmacol. Toxicol. 57 (1), 19–37. https://doi.org/10.1146/annurev-pharmtox-010716-104710.

Manna, P.R., Dyson, M.T., Eubank, D.W., Clark, B.J., Lalli, E., Sassone-Corsi, P., Zeleznik, A.J., Stocco, D.M., 2002. Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. Mol. Endocrinol. 16 (1), 184–199. https://doi.org/10.1210/mend.16.1.0759.

Martin, L.J., Nguyen, H.T., 2022. Basic leucine zipper transcription factors as important regulators of Leydig cells' functions. Int. J. Mol. Sci. 23 (21), 12887 https://doi.org/10.3390/ijms232112887.

Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25 (6), 947–970. https://doi.org/10.1210/er.2003-0030.

Rasmussen, S.G.F., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., Thian, F.S., et al., 2011. Crystal structure of the B2 adrenergic receptor–gs protein complex. Nature 477 (7366), 549–555. https://doi.org/10.1038/nature10361.

Raynaud, P., Gauthier, C., Jugnarain, V., Jean-Alphonse, F., Reiter, E., Bruneau, G., Crépieux, P., 2022. Intracellular VHHs to monitor and modulate GPCR signaling. Front. Endocrinol. 13, 1048601 https://doi.org/10.3389/fendo.2022.1048601.

Raynaud, P., Jugnarain, V., Vaugrente, O., Vallet, A., Boulo, T., Gauthier, C., Inoue, A., et al., 2024. A single-domain intrabody targeting the follicle-stimulating hormone receptor impacts FSH-induced G protein-dependent signalling. FEBS Lett. 598 (2),, 220–232. https://doi.org/10.1002/1873-3468.14765.

Rebois, R.V., 1982. Establishment of gonadotropin-responsive murine Leydig tumor cell line. JCB (J. Cell Biol.) 94 (1), 70–76. https://doi.org/10.1083/jcb.94.1.70.

Riccetti, L., De Pascali, F., Gilioli, L., Potì, F., Giva, L.B., Marino, M., Tagliavini, S., et al., 2017. Human LH and hCG stimulate differently the early signalling pathways but result in equal testosterone synthesis in mouse Leydig cells in vitro. Reprod. Biol. Endocrinol. 15 (1), 2. https://doi.org/10.1186/s12958-016-0224-3.

Sadler, F., Ma, N., Ritt, M., Sharma, Y., Vaidehi, N., Sivaramakrishnan, S., 2023. Autoregulation of GPCR signalling through the third intracellular loop. Nature 615 (7953), 734–741. https://doi.org/10.1038/s41586-023-05789-z.

Shaywitz, A.J., Greenberg, M.E., 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu. Rev. Biochem. 68, 821–861. https://doi.org/10.1146/annurev.biochem.68.1.821.

Stijlemans, B., Conrath, K., Cortez-Retamozo, V., Van Xong, H., Wyns, L., Senter, P., Revets, H., De Baetselier, P., Muyldermans, S., Magez, Stefan, 2004. Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies. J. Biol. Chem. 279 (2), 1256–1261. https://doi.org/10.1074/jbc.M307341200.

Stoeber, M., Jullié, D., Lobingier, B.T., Laeremans, T., Steyaert, J., Schiller, P.W., Manglik, A., von Zastrow, M., 2018. A genetically encoded biosensor reveals location bias of opioid drug action. Neuron 98 (5), 963–976.e5. https://doi.org/10.1016/j.neuron.2018.04.021.

Timossi, C., Ortiz-Elizondo, C., Pineda, D.B., Dias, J.A., Conn, P.M., Ulloa-Aguirre, A., 2004. Functional significance of the BBXXB motif reversed present in the cytoplasmic domains of the human follicle-stimulating hormone receptor. Mol. Cell. Endocrinol. 223 (1), 17–26. https://doi.org/10.1016/j.mce.2004.06.004.

Troispoux, C., Guillou, F., Elalouf, J.M., Firsov, D., Iacovelli, L., De Blasi, A., Combarnous, Y., Reiter, E., 1999. Involvement of G Protein-Coupled receptor Kinases and arrestins in desensitization to follicle-stimulating hormone action. Mol. Endocrinol. 13 (9), 1599–1614. https://doi.org/10.1210/mend.13.9.0342.

Wacker, D., Stevens, R.C., Roth, B.L., 2017. How ligands illuminate GPCR molecular pharmacology. Cell 170 (3), 414–427. https://doi.org/10.1016/j.cell.2017.07.009.

Wan, Q., Okashah, N., Inoue, A., Nehmé, R., Carpenter, B., Tate, C.G., Lambert, N.A., 2018. Mini G protein probes for active G protein-coupled receptors (GPCRs) in live cells. J. Biol. Chem. 293 (19), 7466–7473. https://doi.org/10.1074/jbc.RA118.001975.