LE STUDIUM
Loire Valley
Institute for Advanced Studies

LE STUDIUM Multidisciplinary Journal
ISSN 2669-7807

www.lestudium-ias.com

*FELLOWSHIP FINAL REPORT*

# Investigating the Effects of Steroid Hormones on G Protein-Coupled Receptors *in Vitro*

Mohammed Akli Ayoub[1,3], Eric Reiter[2], Frédéric Jean-Alphonse[2]

[1] Biology Department, College of Science, Khalifa University, PO Box 127788, Abu Dhabi, UAE.

[2] Physiologie de la Reproduction et des Comportements, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, Centre National de la Recherche Scientifique, Institut Français du Cheval et de l'Equitation, Université de Tours, 37380 Nouzilly, France.

[3] LE STUDIUM Institute for Advanced Studies, 45000 Orléans, France

REPORT INFO

*Fellow: Dr. Mohammed Akli Ayoub*
*From Khalifa University (UAE)*
*Host laboratory in region Centre-Val de Loire: PRC, INRAe. Nouzilly*
*Host scientist: Frédéric Jean-Alphonse*
*Period of residence in region Centre-Val de Loire: June – August 2023*

**Keywords :**
Gonadotropins, FSHR, LHR, GPCR, steroids, G protein, arrestin, BRET

ABSTRACT

*In this project, we investigated the pharmacological effects of steroid hormones on gonadotropin receptors, FSHR and LHR, transiently expressed in HEK293 cells. For this, we used BRET technology to assess receptor-Gα protein activation as well as β-arrestin recruitment upon treatment of cells with gonadotropins, FSH or hCG, in the absence or presence of increasing doses of 3 different steroid hormones (SH), estradiol, testosterone, and progesterone. We observed that SH to different extent significantly modulate FSH/hCG-promoted BRET signals between receptor/Gα protein as well as receptor/β-arrestin pairs. Tis indicate an allosteric mode of action of SH on FSHR and LHR. Moreover, we observed differential effects of SH on receptor/β-arrestin pair when comparing FSHR and LHR by decreasing (for FSHR) or increasing (for LHR) the BRET responses. Interestingly, we also observed significant BRET increase with vasopressin (V2R) and angiotensin II (AT1R) receptors. This suggests a more general feature of SH allosterically targeting GPCRs with potential implications in physiology and pathophysiology.*

## 1- Introduction

Steroid hormones (SH) are one of the most important classes of hormones in the human and animal endocrine systems modulating various physiological and pathophysiological processes. According to the classical paradigm, SH promote genomic effects through binding to their intracytoplasmic receptors and translocation of the hormone-receptor complexes into the nucleus where they regulate gene expression [1, 2]. Such delayed genomic effects require few hours or even days due to the different molecular processes that are required to be activated and accomplished. Moreover, SH are known to induce rapid non-genomic responses such as estrogen-mediated blood vessel dilation, progesterone-induced sperm acrosomal action and glucocorticoid-triggered tracheal relaxation [3-8]. These effects are supposed to be independent on their intracellular/nuclear receptors and many receptor families have been proposed as candidates including G protein-coupled receptors (GPCRs). Indeed, solid evidence has been accumulated for the implication of GPCR/G proteins-mediated signaling in the non-genomic responses of SH and for the pharmacological modulation of GPCR activity by steroids such as cholesterol [9-14]. Among GPCRs that may be the targets of SH, FSHR (for follicle-stimulating hormone receptor) and LHR (for luteinizing hormone receptor) represent the interesting candidates due to the relationship that exists between SH and these

two receptors. In fact, FSHR and LHR are expressed in specific cells in the gonads (ovaries and testis) where they play a key role in the endocrine control of human and animal reproduction [18-20]. FSHR and LHR are the molecular targets of pituitary gonadotropins, FSH and LH (along with human placental chorionic gonadotropin or hCG), respectively, that control in an endocrine manner steroidogenesis and gametogenesis in the ovary and testis [18-20]. Thus, these two receptors constitute the interesting pharmacological targets in medicine, especially concerning infertility, contraception, estrogen-dependent diseases and other disorders of the reproductive system. Consequently, substantial advances in the pharmacological targeting of these receptors have been reached and many small molecules acting on FSHR and LHR have been developed and proposed to have potential therapeutic applications (for more references see [21-23]. At the molecular level, upon gonadotropin binding both FSHR and LHR are activated triggering the canonical intracellular cyclic adenosine mono-phosphate (cAMP)-dependent signaling pathway through the activation of the heterotrimeric Gαs protein [18-23]. This leads to the activation of protein kinase A (PKA) and the exchange protein directly activated by cAMP (EPAC), which in turn triggers the activation of multiple downstream kinases that modulate the activity of cAMP response element-binding protein (CREB). CREB is the principal transcription factor controlling the expression of the different genes that are involved in the initiation and regulation of steroidogenesis and gametogenesis [18-23]. Steroidogenesis is the process of chemical synthesis from cholesterol of various SH, such as testosterone, progesterone, and estrogen, involving many key enzymes that are under the control of FSHR and LHR. Once synthesized, SH are then released and act on their target tissues in endocrine and paracrine ways via their classical intracellular/nuclear receptors. Importantly, the increase of SH levels in blood is also sensed by the organisms as a regulatory signal where SH act via short and long negative feedback loops at the pituitary as well as the hypothalamus in order to terminate the initial stimulatory signals leading to the production of the gonadotropins, FSH and LH. Thus, the relationship between FSHR/LHR and SH is more than obvious when considering the physiology of the reproductive system. Indeed, FSHR and LHR promote the production and the secretion of the key SH (progesterone, estrogen, testosterone), and once secreted these SH in turn may exert a negative or positive feedback on the system to modulate the production of FSH and LH and thereby modulating the activity of their respective receptors (FSHR and LHR). Therefore, such system makes that FSHR and LHR are surrounded by SH, which constitutes a solid rationale to consider a possible impact of SH on receptor activity. Therefore, one would argue that SH might also have negative feedback effects at the level of the gonads by directly binding to FSHR and LHR in order to modulate their function as shown for other GPCRs [9-14]. This constitutes the fundamental hypothesis of our project using strong scientific evidence from previous studies on FSHR and LHR as well as on other GPCRs. Indeed, as solid arguments for such a hypothesis, we emphasized above the studies showing that SH mediate non-genomic responses by acting on GPCRs independently on their intracellular/nuclear receptors [9-14]. Moreover, previous studies from the host laboratory on small molecules targeting FSHR and LHR reported many steroid- and cholesterol-related molecules with interesting pharmacological properties on these two receptors [21-25]. Also, our recent studies on the famous endocrine disrupter, p,p'-DDT (1-chloro-4-[2,2,2-trichloro-1-(4 chlorophenyl)ethyl]benzene), revealed a differential allosteric effect of this molecule on FSHR (positive) [24] and LHR (negative) in vitro [25] with potential implication in its endocrine disruptive effects.

All together, these studies clearly demonstrated pharmacological effects of various steroid- and cholesterol-related small molecules supported by the existence of an allosteric binding pocket in FSHR and LHR as showed by molecular docking [24]. This suggests that FSHR and LHR may also be sensitive to SH during the

endocrine regulation of steroidogenesis and gametogenesis with potential implication in physiology and pathophysiology.

## 2- Experimental details

***Cell culture***: Human embryonic kidney (HEK293) cells were used as a model for protein expression and BRET analysis. For this, cells were cultured and maintained at 37°C, 5% CO2 in complete medium (Dulbecco's modified Eagle's medium containing 0.3 mg/ml glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin) supplemented with 10% fetal calf serum.

***Cell transfection***: HEK293 cells were transiently transfected with the plasmids coding for the different BRET partners using Metafectene according to manufacturer's instructions. For BRET assay between GPCRs and Gα or β-arrestin 2, 90 ng of receptor-Rluc plasmid and 30 ng of Venus-Gα or yPET-β-arrestin 2 plasmid were used. For BRET-based cAMP assay, 40 ng of GPCR plasmid and 60 ng of CAMYEL sensor plasmid were used. The plasmid mix were mixed with 0.3 ul of metafectene in 50 ul of serum-free DMEM and incubated 20 minutes at room temperature before adding $10^5$ cells per 96-well resuspended in 150 ul of media. All assays were carried out in 96-well plates 48 hours post transfection upon cell starvation overnight in serum-free media.

***BRET assay***: After media removal, cells were washed once with PBS and incubated 20 minutes with 30 ul/well of increasing doses (0.0001, 0.001, 0.01, 0.1, 1, 10, 25, 50, 100 uM) of either estradiol, testosterone, or progesterone, in PBS. Then, cells were stimulated 30 minutes with 10 ul/well of a sub-saturating dose of GPCR-selective agonist, 3 nM for BRET receptor/Gα protein, 25 nM for BRET receptor/yPET-β-arrestin, and 1 nM for BRET with CAMYEL sensor. After incubation, 10 ul of Coelenterazine h was added to the cells and BRET signals were measured.

## 3- Results and discussion

***Effect of SH on receptor-G protein coupling***: First, we examined the potential effect of SH on receptor-G protein coupling in HEK293 cells using BRET. As shown in **Fig.1**, for FSHR (**Fig.1A**) and LHR (**Fig.1B**), the three SH, estradiol, testosterone, and progesterone had only mild effects on gonadotropin-promoted BRET signals. A very weak decrease in BRET signals was observed between FSHR-Rluc and Venus-mGαs mostly at the higher doses of SH (25, 50 and 100 µM) (**Fig.1A**).

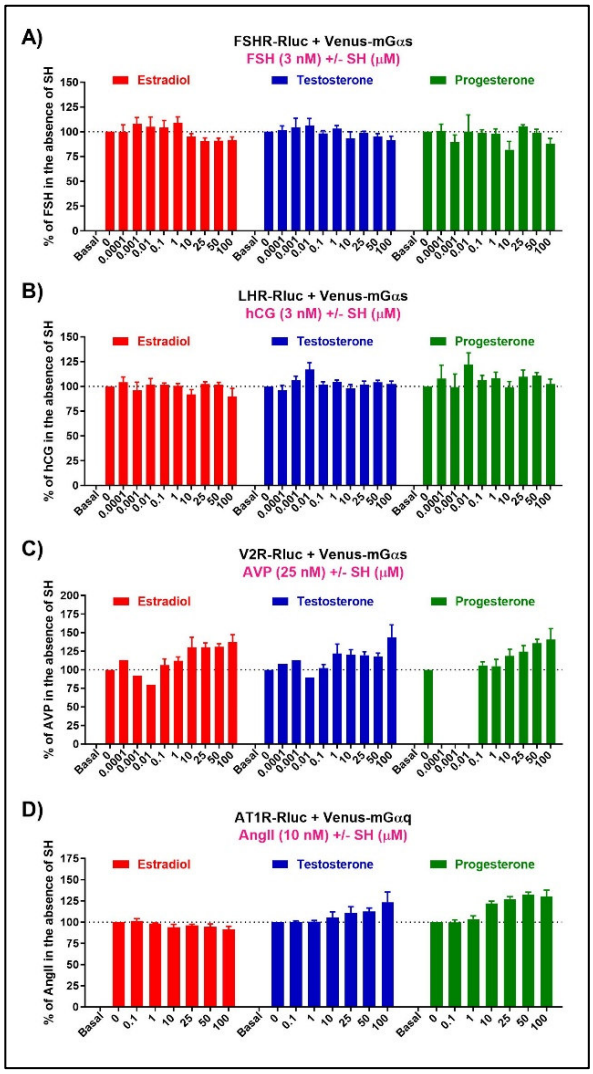


**Figure 1**: *Effect of SH on ligand-promoted BRET signals between various Rluc-fused GPCRs including FSHR (**A**), LHR (**B**), V2R (**C**), and AT1R (**D**), and their cognate mini-G proteins fused to Venus (Venus-mGαs and Venus-mGαq), upon treatment with the different combinations of receptor agonist and SH at the indicated doses, as indicated. The data are SEM of 4-9 independent experiments performed in triplicate.*

Interestingly, we observed that SH significantly and in a dose-dependent manner potentiated BRET signals between V2R-Rluc and Venus-

mGαs (**Fig.1C**) and between AT1R-Rluc and Venus-mGαq (**Fig.1D**), promoted by AVP and AngII, respectively. This is true for the three SH expect estradiol which had no effect on BRET between AT1R-Rluc and Venus-mGαq (**Fig.1D**) suggesting a specific action of estradiol on V2R but not AT1R. The strongest effect (up to 150% of response) was observed with progesterone and then testosterone (**Fig.1C** and **D**). Together, these BRET observations on receptor-G protein activation indicate a positive allosteric action in SH- as well as receptor-dependent manner with an interesting structure-function relationship when comparing the three SH and the four GPCRs (FSHR, LHR, V2R, and AT1R) together.

***Effect of SH on cAMP production*:** Next, we wanted to link our BRET data on receptor-Gαs protein coupling with the downstream cAMP signaling pathway. For this, we used the BRET sensor CAMYEL to quantify the intracellular cAMP production co-expressed with untagged FSHR (**Fig.2A**), LHR (**Fig.2B**), and V2R (**Fig.2C**). As results, we observed that the three SH significantly potentiated hormone-induced cAMP production in a dose-dependent manner and this with all the receptors (**Fig.2**). The effect was stronger on V2R-mediated cAMP (**Fig.2C**) which is consistent with the data on BRET between V2R-Rluc and Venus-mGαs (**Fig.1C**). For FSHR and LHR, the potentiation of cAMP production by SH was observed despite we did not observe significant changes in BRET between Rluc-tagged receptors and Venus-mGαs upon treatment with SH. This may simply due to unfavorable conformational changes within FSHR/LHR-Gs complexes that could not be detected by BRET. These data on cAMP further confirm the positive allosteric action of SH on the tested GPCRs.

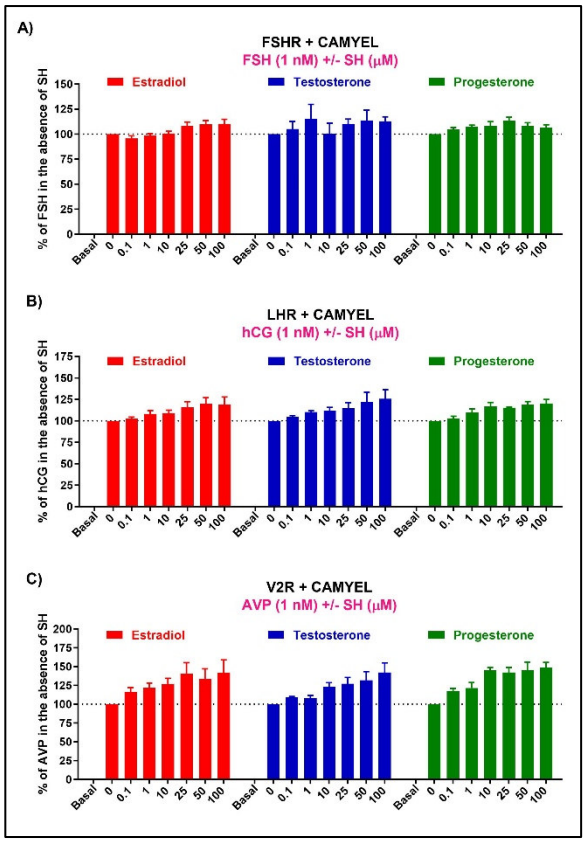


**Figure 2**: *Effect of SH on ligand-promoted BRET signals between various Rluc-fused GPCRs including FSHR (**A**), LHR (**B**), V2R (**C**), and AT1R (**D**), and yPET-β-arrestin 2, upon treatment with the different combinations of receptor agonist and SH at the indicated doses, as indicated. The data are SEM of 4 independent experiments performed in triplicate.*

***Effect of SH on receptor-arrestin interaction*:** Next, we tested the effect of SH on β-arrestin recruitment upon receptor activation using BRET assay. In this assay, we observed different profiles of the three SH on the four GPCRs tested (**Fig.3**). Indeed, all SH tended to decrease by ~40% FSH-promoted BRET signal between FSHR-Rluc and yPET-β-arrestin 2 in a dose-dependent manner (**Fig.3A**), while they nicely potentiated the hormone-promoted BRET signals with LHR (**Fig.3B**), V2R (**Fig.3C**) and AT1R (**Fig.3D**). Again, progesterone looks stronger than testosterone and then estradiol with almost all the receptors except for LHR where estradiol did not seem to have any significant effect. Thus, for FSHR our data suggest that SH have an opposite action by increasing cAMP production and decreasing β-arrestin recruitment. This indicates a differential allosteric modulation of FSHR by SH. By contrast, for other GPCRs, SH

potentiated both cAMP and β-arrestin recruitment indicating a more conventional positive allosteric action. Moreover, such differences between FSHR and the other GPCRs suggest an interesting structure-function relationship when these receptors.

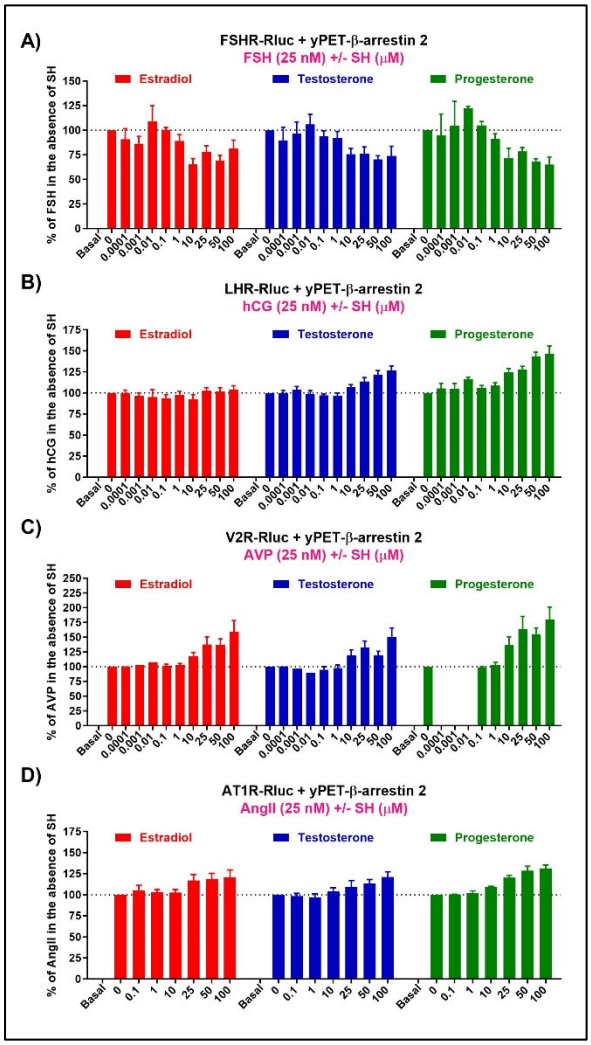


**Figure 3**: *Effect of SH on ligand-promoted BRET signals between various Rluc-fused GPCRs including FSHR (**A**), LHR (**B**), V2R (**C**), and AT1R (**D**), and yPET-β-arrestin 2, upon treatment with the different combinations of receptor agonist and SH at the indicated doses, as indicated. The data are SEM of 4-9 independent experiments performed in triplicate.*

## 4- Conclusion

In this 2 month-project, we investigated the pharmacological effects of three SH (estradiol, testosterone, and progesterone) on various GPCRs including the gonadotropin receptors, FSHR and LHR, and two other receptors, V2R and AT1R, known for key role in renal and vascular physiology. As a readout, we used BRET assay in HEK293 cells to monitor receptor/G protein coupling, receptor/β-arrestin interaction and intracellular cAMP production. Overall, our data highlight for the first time an allosteric action of the three SH on the tested GPCRs. Indeed, we found that SH positively modulate hormone-promoted G protein, cAMP production, and arrestin recruitment. However, for FSHR, we observed that SH had a negative effect on FSH-induced FSHR/β-arrestin interaction although this receptor presents many similarities with LHR in terms of structure and activation mode. Moreover, this study reveals a general feature of SH targeting not only gonadotropin receptors but also other GPCRs. This may have implication in physiology especially during the high pic of steroidogenesis in women when many physiological responses may be affected due to high levels of SH. The impact on pathophysiology may also be there if many non-reproductive GPCRs can be targeted by SH.

## 5- Perspectives of future collaborations with the host laboratory

These interesting preliminary data constitute a solid basis for further collaborations with the host laboratory. Overall, our data suggest a more general feature of SH targeting not only gonadotropin receptors (FSHR and LHR) but also other GPCRs. Thus, we are very excited by this first set of promising data and we already planned to continue working on this project to complete the set of the experiments. There is an exciting structure-function followed by experimental characterization that need to be done.

## 6- Articles published in the framework of the fellowship

None.


## 7- Acknowledgements

I would like to warmly thank LE STUDIUM and its wonderful team for the strong support and all the facilities and benefits provided to me during my stay in Tours. May warm thanks to Dr. Frédéric Jean-Alphonse and Dr. Eric Reiter, for hosting me in their laboratory at INRAe and

for providing me all the chemicals/reagents required for the experimental part of the project. I really appreciated the scientific discussions about the project and the data that we had together along with Dr. Livio Casarini (LE STUDIUM fellow).

## 8- References

1- Tata, J.R. Signalling through nuclear receptors. Nat. Rev. Mol. Cell Biol. 2002, 3, 702-710.
2- Selye, H. Stress and the general adaptation syndrome. Br. Med. J. 1950, 1, 1383-1392.
3- Wang, C. et al. G Protein-Coupled Receptors: Extranuclear Mediators for the Non-Genomic Actions of Steroids. Int. J. Mol. Sci. 2014, 15, 15412-15425.
4- Lösel et al. Non-genomic actions of steroid hormones. Nat. Rev. Mol. Cell Biol. 2003, 4, 46-56.
5- Losel et al. Non-genomic steroid action: Controversies, questions, and answers. Physiol. Rev. 2003, 83, 965-1016.
6- Hammes and Levin. Extranuclear steroid receptors: Nature and actions. Endocr. Rev. 2007, 28, 726-741.
7- Wang et al. Glucocorticoid decreases airway tone via a non-genomic pathway. Respir. Physiol. Neurobiol. 2012, 183, 10-14.
8- Wang et al. Extra-neuronal monoamine transporter mediates the permissive action of cortisol in the guinea pig trachea: Possible involvement of tracheal chondrocytes. PLoS One 2013, 8, e76193.
9- Wang et al. G-protein-coupled receptor controls steroid hormone signaling in cell membrane. Sci Rep. 2015, 2;5:8675.
10- Manna et al. Mechanism of allosteric regulation of β2-adrenergic receptor by cholesterol. Elife. 2016, 29;5. pii: e18432.
11- Wang et al. Specificity out of clutter: A hypothetical role of G protein-coupled receptors in the non-genomic effect of steroids. FEBS Letters. 2013, 587, 823-825.
12- Legler et al. Modulation of Chemokine Receptor Function by Cholesterol: New Prospects for Pharmacological Intervention. Mol Pharmacol. 2017, 91(4):331-338.
13- Gimpl et al. Cholesterol as modulator of receptor function. Biochemistry. 1997, 36(36):10959-74.
14- Guixà-González et al. Membrane cholesterol access into a G-protein-coupled receptor. Nat Commun. 2017, 21;8:14505.
15- Lieberherr et al. Cell signaling and estrogens in female rat osteoblasts: A possible involvement of unconventional non-nuclear receptors. J. Bone Miner. Res. 1993, 8, 1365-1376.
16- Gekle et al. Nongenomic action of the mineralocorticoid aldosterone on cytosolic sodium in cultured kidney cells. J. Physiol. 1998, 511, 255-263.
17- Qiu et al. Non-genomic mechanisms of glucocorticoid inhibition of nicotine-induced calcium influx in PC12 cells: Involvement of protein kinase C. Endocrinology 1998, 139, 5103-5108.
18- Misrahi et al. Gonadotropin receptors and the control of gonadal steroidogenesis: physiology and pathology. Baillieres Clin Endocrinol Metab. 1998, 12(1):35-66.
19- Hai et al. Gonadotropin receptors. Ann Endocrinol (Paris). 1999, 60(2):89-92.
20- Themmen and Huhtaniemi. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. Endocr Rev. 2000, 21(5):551-83.
21- Ayoub et al. Profiling of FSHR negative allosteric modulators on LH/CGR reveals biased antagonism with implications in steroidogenesis. Mol Cell Endocrinol. 2016, 15;436:10-22.
22- De Pascali et al. Pharmacological Characterization of Low Molecular Weight Biased Agonists at the Follicle Stimulating Hormone Receptor. Int J Mol Sci. 2021 Sep 12;22(18):9850.
23- Ayoub et al. Assessing Gonadotropin Receptor Function by Resonance Energy Transfer-Based Assays. Front Endocrinol. 2015, 27;6:130.
24- Munier et al. In Vitro Effects of the Endocrine Disruptor p,p'-DDT on Human Follitropin Receptor. Environ Health Perspect. 2016, 124(7):991-9.

25- Munier et al. In vitro effects of the endocrine disruptor p,p'DDT on human choriogonadotropin/luteinizing hormone receptor signalling. Arch Toxicol. 2021 May;95(5):1671-1681.
26- Ayoub, M.A. Resonance Energy Transfer-Based Approaches to Study GPCRs. Methods Cell Biol. 2016, 132:255-92.