THESIS

EVALUATING LEVELS OF LUTEINIZING HORMONE RECEPTOR DIMERS AND OLIGOMERS

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Duaa A. Althumairy

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Master's Committee:

Advisor: Deborah A. Roess

Charles W. Miller
B. George Barisas

ABSTRACT

EVALUATING LUTEINIZING HORMONE RECEPTOR DIMERS AND OLIGMERS

Luteinizing hormone (LH) receptors are found in the female reproductive organs as well as in male reproductive organs where they play important roles in ovulation and sperm maturation, respectively. LH receptors are members of the G protein-coupled receptor (GPCR) superfamily and serve as drug targets. The role of GPCR oligomerization in receptor function is of considerable interest. Understanding the size of LH receptor clusters formed on the plasma membrane after hormone binding will lead to a better understanding of female and male reproductive functions and enhance the ability to develop better therapeutic approaches to treat diseases related to LH receptor-mediated events.

Here we evaluated the oligomerization state of LH receptors using the method called polarized homo-transfer fluorescence resonance energy transfer (homo-transfer FRET) which has the ability to detect changes in the cluster size of LH receptors found in the plasma membrane. By observing increases in fluorescence anisotropy upon photobleaching of a fluorophore and analyzing the difference between the extrapolated final anisotropy and the predicted anisotropy for an immobile receptor monomer, changes in the distribution of receptor monomers, dimers and higher oligomers can be assessed. In this study different concentrations of human chorionic gonadotropin (hCG) were used to observe the effects of hormone on the oligomerization state of LH receptors. Treatment of CHO cells expressing hLHR-eYFP with increasing concentrations of hCG led to a rapid increase in formation LH receptor oligomers. In addition, this study establishes that the LH receptors may exist constitutively as dimer in control cells. These results demonstrate

that polarization homo-transfer FRET is useful in evaluating the relative numbers of receptor monomers, dimers and oligomers.

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BACKGROUND

BIOLOGICAL ROLE OF LUTEINIZING HORMONE RECEPTORS IN REPRODUCTION

Luteinizing hormone (LH) receptors play an essential role in reproductive function in both mammalian sexes. In females, LH receptors are found in the follicle and corpus luteum (Figure 1A). In the follicle, LH receptors are expressed in the theca cells (outside layer of follicle), interstitial cells (intermediate layer of follicle), and differentiated granulosa cells (inside layer of follicle) (Figure 1B). Signaling by LH receptors leads to steroid production and follicle maturation needed for ovulation. In the corpus luteum, LH receptors are expressed in luteal cells and the signaling by these LH receptors is needed to maintain circulating progesterone levels (Stocco et all., 2007). In males, the LH receptors are found in Leydig cells that form the seminiferous tubule in the testis (Figure 1C) and signaling by LH receptor in these cells leads to sperm maturation and steroid synthesis (Dufau, 1995).

LH receptors in granulosa cells of the ovary regulate the development of the follicle and mediate ovulation. LH receptor is activated by binding of luteinizing hormone (LH) which is secreted regularly from the anterior lobe of the pituitary gland (Dufau, 1995) (Figure 2). Increasing estrogen levels in the blood lead to pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus in the brain. In the anterior lobe of the pituitary gland, GnRH binds to its G protein-coupled receptor on gonadotrope cells (Conn et al., 1987). In response to activation of the GnRH receptor, LH is secreted into the peripheral circulation and affects target cells in the ovary where LH receptors are expressed.

It is recognized that LH receptors are essential for successful reproduction by mammals. If the LH receptor does not function correctly in the female, ovulation does not occur. If the LH receptor does not function correctly in males, sperm maturation does not occur properly (Ascoli., 2002).

STRUCTURE LUTEINIZING HORMONE RECEPTORS AND ITS LIGAND

LH receptors are approximately 80 KDa in both rats and humans (Tsaimorris et al., 1991; Koo et al., 1991; Tsai-Morris et al., 1991). LH receptors are a single polypeptide chain comprised of 699 amino acids that can be divided into three domains (Figure 3) (Dufau, 1995). The extracellular domain is the glycosylated N-terminal domain with 340 amino acid residues and sites for ligand binding (Segaloff & Ascoli, 1993). The second domain is highly conserved and contains seven membrane-spanning sequences. These hydrophobic alpha-helical segments have about 25 to 35 amino acids for each alpha-helical segment. The segments are connected by three extracellular loops and three intracellular loops (McFarland et al., 1989). The third domain is an intracellular C-terminal cytoplasmic tail with 70 amino acid used in signal transduction. This portion of the LH receptor together with intracellular loops interacts with G proteins when the LH receptor is activated by hormone (Sanchezyague et al., 1992).

Two ligands have the ability to bind the LH receptor, luteinizing hormone (LH) and human chorionic gonadotropin (hCG). Both hormones share similar function and structure. Human chorionic gonadotropin (hCG) is approximately 75 KDa and is comprised of an alpha subunit with 92 amino acids and a beta subunit with 145 amino acids. These subunits are attached to each other via non-covalent interactions. The alpha subunit has five disulfide bridges and two N-linked

carbohydrate sites while the beta subunit has six disulfide bridges, two N-linked carbohydrate sites and four O-linked carbohydrate sites that play a role in receptor activation (Wu et al., 1994) (Figure 4). The alpha subunit of LH has 92 amino acids as is the case for hCG, but the beta subunit of LH has 121 amino acids, less than the beta subunit of hCG (Segaloff & Ascoli, 1993). The extra amino acids on hCG can associate with cell proteins and this leads to slower rotational diffusion rates for hCG compared to LH (Roess et al., 2000). LH bound to wild type LH receptor has larger mobile fraction and less energy transfer between receptors than hCG bound to wild type LH receptor (Roess et al., 2000).

LUTEINIZING HORMONE RECEPTOR SIGNALING

LH receptors have the ability to interact with G proteins after receptor activation by binding of hormone. G proteins are heterotrimeric proteins consisting of α, γ and β subunits. After G proteins are activated, adenylate cyclase is activated and this enzyme converts adenosine triphosphate (ATP) to the secondary messenger, cyclic adenosine monophosphate (cAMP).

LH receptors are activated first by high affinity binding of hormone, either LH or hCG. Ligand binding to the LH receptor causes a conformational change in the ligand binding site that leads to a rotation in the sixth transmembrane domain (Abell & Segaloff, 1997). Receptors can be activated through two distinct mechanisms cis-activation and trans-activation (Ganguly et al., 2011; Ji et al., 2002) (Figure 5). In cis-activation, the ligand binds to an extracellular domain of the LH receptor and causes activation of the same LH receptor that originally bound hormone. In trans-activation, the ligand binds to the extracellular domain of one LH receptor and then causes trans-activation of an adjoining receptor that has not bound ligand (Ji et al., 2004; Ji et al., 2002).

Both trans-activation and cis-activation are believed to lead to receptor clustering and formation of receptor dimers or higher order oligomers in the plasma membrane of the cell (Ji et al., 2002). The active LH receptor activates G proteins which displace GDP (guanosine diphosphate) with GTP (guanosine triphosphate) on the α subunit of the G protein. Once GTP is bound, the α subunit dissociates from the G protein γ and β subunits (Figure 6B and 6C) (Hunzicker et al., 2002) and activates the transmembrane adenylate cyclase (AC) enzyme that converts ATP to cAMP (Figure 6D) (Stryer and Bourne,1976; Dufau,1998; Stryer & Bourne, 1986; Dufau, 1995). Hydrolysis of GTP to GDP by an endogenous GTPase in the α subunit leads to inactivation of the G protein. Re-association of the α, γ and β subunits then occurs (Yen & Jaffe, 1991) (Figure 6A and 6E).

Fluorescent photobleaching recovery (FPR) studies suggest that active LH receptors become self-associated to each other (D. A. Roess et al., 2000; Luborsky et al., 1984). Also, FRET methods, whether via spectroscopic methods or flow cytometry techniques, show that ligand binding to LH receptors causes significant increases in energy transfer that reflect increase receptor self-association (Roess et al., 2000).

REASONS TO STUDY THE OLIGMERZATION OF LUTEINIZING HORMONE RECEPTORS

Since the LH receptors have important roles in both sexes in fertility, a better understanding of receptor action including LH receptor function, signaling and the receptor's oligmerization state will provide to better understanding many of diseases that are related to LH receptor. For instance, a constitutively activating mutation of the LH receptor leads to familial male-limited precocious puberty (FMPP), which is early puberty in male children caused by high levels of testosterone (Levi & Gratton, 2007; Wu et al., 2000). Increased levels of LH are related also to cognitive

impairment and Alzheimer's disease (Dufau, 1995). Several studies have shown that receptor oligomers or dimers form early after receptor biosynthesis, suggesting that oligomerization has a primary role in receptor maturation (Terrillon & Bouvier, 2004).

It is important to study and detect oligomerization state of LH receptor because the oligomerization of LH receptor may affect receptor binding and activity in different ways. The dimerization of G protein-coupled receptors (GPCR) influences the downstream signaling and regulates matters such as internalization (Terrillon & Bouvier, 2004). Moreover, oligomerization of LH receptors may also impact the activation of receptors and downstream signaling.

In addition, the oligomerization state of LH receptor has properly added a new dimension to rational drug design and the search for new therapeutics. GPCR, a superfamily that include LH receptors, represents 50% of targets for new drug development (Li et al., 2010; Ganguly et al., 2011). Mounting evidence suggests that the dimerization and oligmerization of GPCR could be important in aspects of regulated pharmacological function for GPCRs (Maggio et al., 2005; Rios et al., 2001).

POLARIZATION HOMO-TRANSFER FLUORESCENCE RESONANCE ENERGY TRANSFER (HOMO-TRANSFER FRET)

Fluorescence resonance energy transfer (FRET) is a popular and useful tool used to detect oligomerization of G protein-coupled receptors (GPCR). FRET can be measured between proteins on the surface of cells and has been used to study LH receptors, Serotonin1A receptors, as well as other GPCR proteins tagged with fluorophores. FRET occurs when two fluorophores are within distances between 2-10 nm (Piston & Kremers, 2007). The Förster distance (R_0) is the distance

between the donor and acceptor at which 50% energy transfer efficiency occurs (Figure 7). If the fluorophores are far away from one other at distances of more than 10 nm, FRET does not occur (Figure 7). FRET efficiency (E) depends on inverse sixth power distance (r) between the donor and acceptor (Chan et al., 2011) where

$$E=1/(1+r/R_0^6) \quad (1)$$

When two fluorophores are close to each other (within 2-10 nm) and when there is overlap between the donor emission and acceptor excitation spectra, there is non-radiative energy transfer from donor to the acceptor and the acceptor can emit fluorescence. To evaluate FRET fluorescence microscope, confocal microscope, or fluorescence wide-field microscope can be used together with an arc lamp or laser source with appropriate filters as an excitation source.

There are technical considerations when evaluating FRET data. The overexpression of fluorescently-tagged receptors affects FRET efficiency. Meyer et al. (2006) addressed the overexpression of neurokinin-1 receptor, which is a member of the GPCR superfamily. When there were ~ 63,00 receptors/cell, the FRET efficiency increased. No FRET was detected when the same receptor was expressed at ~25,000 receptors/cell. This result indicates that the receptor is found as a monomer at normal expression levels and, at higher expression levels, receptors are dimers or oligomers and FRET occurs. The overexpression of membrane proteins such as LH receptor may cause aggregation of receptors in the absence of hormone, resulting in increased FRET efficiency (Crenshaw, 2012). In addition, FRET may occur as a result of the highly crowded nature of the cell membrane if membrane proteins are within the required distance for FRET. This is "bystander FRET" which is FRET that arises from membrane proteins that do not interact with each other but do give FRET signal due to their close proximity to each other (Piston & Kremers, 2007) (Figure 8). Thus, doing FRET experiments under physiological conditions with low levels of protein

expression is essential since the overexpression of receptors or other membrane proteins may affect the efficiency of FRET and lead to misleading results (Meyer et al., 2006; Niswender et al., 1985).

Homo-transfer FRET is a polarization- based type of FRET that is based on energy transfer between identical fluorescence molecules within 2- 10 nm of each other. In homo-transfer FRET, just a single fluorophore is used. As an example, enhanced yellow fluorescent protein (eYFP), which has small Stokes shift between absorption and emission spectra peak, can be used. Homo-transfer FRET is a powerful tool that is used to determine the oligomerization state of plasma membrane proteins and it used in this research to detect the oligomerization state of LH receptor.

Different approaches can be applied to detect homo-transfer FRET. For instance, fluorescence polarization anisotropy, i.e., steady-state anisotropy, can be used to detect homo-transfer FRET where increases in the depolarization state of emission fluorescence due to FRET is measured (Chakraborty & Chattopadhyay, 2015). Equation 2 from Chan et al. (2011) is used to calculate the fluorescence polarization anisotropy.

$$r = (I_{vv}-GI_{vh})/ (I_{vv}+2GI_{vh}) \quad 2$$

The anisotropy r is calculated from the intensity of emission that is vertically oriented, I_{vv}, and the intensity of emission that is horizontally oriented, I_{vh}, where excitation polarization is vertical. I_{vv} and I_{vh} are obtained in our lab from two detectors in the Zeiss Axiovert 200M microscope after appropriate background subtraction

In homo-transfer FRET, a vertically polarized filter is used to pass only vertically polarized light and to excite an anisotropic distribution (Figure 9B) of fluorophores which are oriented parallel to the vertical polarized excitation. This contrasts with an isotropic distribution (Figure 9A), which occurs when excited fluorophores have randomly-oriented transition dipoles relative

to vertically polarized light. So, the vertically polarized light that passes through the filter excites the fluorophores that are oriented parallel to polarized light. When excited fluorophores transfer energy to nearby fluorophores using FRET, the polarization of re-emitted fluorescence is randomized. Subsequently, the emission light will be partly depolarized because the emission dipole is oriented differently than polarized excitation light (Figure C)

In this study, polarized homo-transfer FRET was utilized to monitor the oligomerization of LH receptors. The polarized homo-transfer FRET was quantitated from an increase in fluorescence anisotropy upon progressive fluorescence photobleaching (Ganguly et al., 2011) of the eYFP-tagged LH receptors. Fluorescence photobleaching reduces FRET emission due to photobleaching of fluorophore acceptors and this causes increased anisotropy upon fluorescence photobleaching. Previous studies showed that there was a positive relationship between the increase in fluorescence anisotropy and an increase in the concentration of hCG hormone upon fluorescence photobleaching indicating that at high concentrations of hormone, the receptors tend to be oligmerized (Wolf-Ringwall et al., 2011). Other studies on serotonin 1A receptors, another GPCR, used polarized homo-transfer FRET methods to monitor the oligmerization state of receptors. These studies showed that the serotonin 1A receptor exists as dimers constitutively in absence of hormone and exist as higher order oligomers in present of hormone. These results were obtained by analyzing the differences between the extrapolated anisotropy and predicted anisotropy of an immobile monomer (Chakraborty & Chattopadhyay, 2015; Ganguly et al., 2011), the approach we have used in this project.

RESEARCH GOALS

Previous studies in our laboratory on LH receptors have suggested that LH receptors exist as clustered receptors in the plasma membrane of cells after hormone binding (Roess and Smith, 2003; Wolf-Ringwall et al., 2011). Furthermore, other studies have suggested that LH receptors with a specific mutation at position 578, appear to be both aggregated and constitutively active in the absence of hormone. (Crenshaw, 2012; Ji et al., 2002; Rivero-Muller et al., 2010). Overexpression of the LH receptor by transient transfection may induce molecular crowding in cell membrane and these receptors seem to be also constitutively active in the absence of hormone (Crenshaw, 2012). Moreover, work using hetero-transfer FRET methods has suggested that LH receptor stably expressed in Chinese hamster ovary (CHO) cells exist as monomers in the absence of hormone (Lei et al., 2007), while other studies suggest that LH receptors in 293 cells stably transfected and transiently transfected could exist as monomers and dimers in the absence of hormone and the receptor cluster size increases upon hormone binding using co-immunoprecipitation (Tao et al., 2004a). This result was in agreement with another study using BRET method (Urizar et al., 2005).

One of the previous studies in our laboratory group using polarized homo-transfer FRET method showed that increasing concentration of hormone (hCG) caused an increase in the cluster formation of LH receptors stably expressed in CHO cells This was reflected by increasing anisotropy upon fluorescence photo-bleaching (Wolf-Ringwall et al., 2011) as shown in Figure 10. However, this previous study did not rigorously evaluate whether the LH receptors were present in clusters containing only receptor dimers or oligomers or mixtures of cluster sizes. Here in this

study, changes in size of LH receptor clusters formed on the plasma membrane were detected using polarization homo-transfer FRET on CHO cells stably expressing the eYFP-tagged LH receptor.

MATERIALS AND METHODS

MATERIALS.

Chinese Hamster Ovary (CHO) cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in high glucose Dulbecco's Modified Eagle medium (DMEM) that was purchased from Corning Cellgro (Visalia, CA) and supplemented with geneticin (G418 sulfate) that was purchased from Cellgro. Penicillin/streptomycin and L-glutamine solution were purchased from Gemini Bio-Products (West Sacramento, CA). Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO), and 100X MEM non-essential amino acid solution were purchased from Sigma Aldrich (St. Louis, MO). All cells were grown in 5% CO_2 at 37℃ in humidified environment in a 25cm^2 culture flask. Human chorionic gonadotropin (hCG) was purchased from Fitzgerald Industries (Acton, MA) and was prepared in PBS at pH 7.0. Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma Aldrich (St. Louis, MO). WillCO glass bottom cell culture dishes with a 35 mm diameter and 14 mm diameter glass bottoms were purchased from In Vitro Scientific (Sunnyvale, CA). To increase florescence intensity in experiments, enhanced yellow fluorescent (eYFP) were created from wild type YFP that obtained from Clontech

CELL LINES

CHO stably expressing enhanced yellow fluorescent protein (eYFP) tagged to the C-terminus of the LH receptor as shown in Figure 11 were prepared using a YFP vector from Clontech. Stable clones expressing eYFP were selected based on the acquisition of geneticin (G418) resistance and identified using fluorescence microscopy. CHO cells stably expressed eYFP

were maintained in CHO cell medium supplemented with geneticin (G418), fetal bovine serum (FBS), and penicillin/streptomycin and L-glutamine solution. The number of receptors per cell was evaluated by flow cytometry and was estimated to be approximately 47,000 LH receptors/cell.

SAMPLE PREPARATION FOR POLARIZED HOMO-TRANSFER FRET

CHO cells that stably expressed eYFP tagged to LH receptor were incubated with 5mM EDTA for 3-5 minutes in a 25cm^2 culture flask, diluted in fresh medium and plated in a 35mm glass bottom petri dish. Cells were incubated until cells grew to approximately 80%-90% confluence, a process that took one to two days. After that, cells were washed twice with 1X phosphate buffered saline (PBS) pH 7.0. For untreated cells, the cells pellet was resuspended in 600 ml of PBS alone, while treated cells were incubated with different concentrations of hCG. Cells were incubated at 37℃ for one hr., enough time to activate LH receptors and for formation of LH receptor clusters. Clustering of rat LH receptors occurred within minutes and these clusters do not dissociate for several hours (Horvat, Nelson, Clay, Barisas, & Roess, 1999) (Horvat, Barisas, & Roess, 2001). After taking the dishes from the incubator, cells were washed once, resuspended in PBS and imaged with a Zeiss Axiovert 200M inverted microscope.

EXPERIMENTAL DETERMINATION OF THE G FACTOR

The G factor is the correction factor for different detection sensitivities of vertical and horizontal polarized fluorescence in our instrumental setup. It acts as a normalization factor to compensate for optical properties such as reflections from mirrors that may change the polarization angle of excitation light. The G factor can be measured using aqueous solutions that have anisotropy values near zero such as Rhodamine 6G, which has an anisotropy value of about 0.012

(Dix & Verkman, 1990; Clayton, Hanley, Arndt-Jovin, Subramaniam, & Jovin, 2002). The G factor is then calculated by the following equations as in Dix & Verkman (1990)

$$G = (1/F)\,(1-R)/(1+2R) \quad (3)$$

where the F is the ratio of sensitivities of the detection system from the calculation of emission fluorescence intensity of parallel and perpendicular polarized fluorescence of Rhodamine 6G subtracted from H_2O as in equation 4. R is the literature value for Rhodamine 6G anisotropy.

$$F = (I_{vv}\ R6G - I_{vv}\ H_2O) / (I_{vh}\ R6G - I_{vh}\ H_2O) \quad (4)$$

Where I_{vv} is fluorescence intensity that has parallel direction to vertical polarization light for Rhodamine 6G and H_2O, and I_{vh} is emission fluorescence intensity that has perpendicular direction to vertical polarization light for Rhodamine 6G and H_2O (Dix & Verkman, 1990). The G factor is then used to calculate the fluorescence anisotropy r. The values of the G factor were measured for each sample and they were ranged from 1.5 to 1.7. These values indicate that the sensitivity of detection in our Zeiss Axiovert 200M inverted microscope setup is higher for vertical polarization compared to horizontal polarization.

POLARIZED HOMO-TRANSFER FRET MEASUREMENTS

Energy transfer between two or more eYFP molecules occurs only when donor eYFP and acceptor eYFP are within 2 -10 nm distance to each other (Piston & Kremers, 2007). Homo-transfer FRET data is collected from two detectors in a Zeiss Axiovert 200M inverted microscope using an Andor Du897E EMCCD camera. Images were acquired using an arc lamp for fluorescence excitation together with a polarized excitation filter, a YFP emission filter and a 63x water objective for observing cells. The two detectors collect parallel and perpendicular emission

images (Figure 12) that represent fluorescence parallel to polarization of excitation light on the bottom and fluorescence perpendicular to excitation light in the top. The cells were photobleached for 5 minutes and fluorescence emission was evaluated using MetaMorph software. The G factor was calculated as described above using Rhodamine 6G dye to correct for efficiency differences in instrument optics.

Image J software was used to obtain the parallel and perpendicular fluorescence intensities from cell measurements and for calculation of the G factor. These intensities were used to calculate anisotropy using Equation 2. The fundamental anisotropy (r_0) of eYFP fluorophore is 0.38 (Borst et al., 2005; Ganguly et al., 2011) and we assumed that the fundamental anisotropy of LH receptors tagged with eYFP was close to ~0.38, which represents an immobile monomer receptor. For each treatment, 10 cells were analyzed. The mean, standard deviations, and standard error of the mean reported were calculated for 10 cells for each condition. Anisotropies were corrected so that the final anisotropy after completed bleaching was 0.38 (Chakraborty & Chattopadhyay, 2015).

RESULTS AND DISCUTION

The effect of different hormone concentrations on the oligomerization state of LH receptor was monitored using polarized homo-transfer FRET measurments. Homo-transfer FRET appeared assessed as increased anisotropy upon fluorescence photobleaching. The difference between measured anisotropy under different conditions and the predicted anisotropy of an immobile monomer 0.38 was calculated. CHO cells stably expressing the YFP-LHR were exposed to 100 nM hCG, 30 nM hGC, 10nM hGC, 1nM hGC, and 0.1 nM hGC, respectively, and results for hormone-treated cells were compared with those from untreated cells. Homo-transfer FRET was estimated by calculating the anisotropy for each cell while being photo-bleached to ~10% of initial fluorescence intensity. Photo-bleaching to ~10% of initial fluorescence intensity took 5 minutes and resulted in 6 sequential images per cell. Anisotropy was corrected so that final anisotropy after complete bleaching was 0.38. Standard deviations and standard errors were calculated for each condition and p values are indicated ($p < 0.05$).

The fluorescence anisotropy upon fluorescence photobleaching in control samples where no hormone was present is shown in Figure 13. The initial fluorescence anisotropy was significantly lower, ~0.215, compared to predicted monomer anisotropy of 0.38. This difference between control cells anisotropy values and predicted monomer anisotropy indicates that LH receptors are likely to exist minimally as dimers in untreated cells. This result was in agreement with results suggesting that the LH receptor is a constitutive dimer (Tao et al., 2004; Urizar et al., 2005).

The average anisotropies for different hormone concentrations upon fluorescence-photobeaching are illustrated in Figures 13 and 14 which compare the anisotropies in control cells

and cells treated with 100 nM hCG , 30 nM hCG, 10 nM hCG, 1 nM hCG, and 0.1 nM hCG with the predicted anisotropy of a receptor monomer which is 0.38. The farther the measured anisotropy deviates from predicted anisotropy for a receptor monomer (0.38), the more likely it is that the LH receptors exist as dimers or higher oligomers. In Figure 13 it can be seen that a high concentration of hCG hormone (100 nM) increased the cluster size compared to the predicted anisotropy for a receptor monomer. This suggests that LH receptors may be in high order oligomers when they were treated with 100 nM hGC. A decrease in the concentration of hGC to 30 nM caused a slight reduction in the LH receptor cluster size as indicated by an increase in final anisotropy values. At concentrations of 10 nM, 1 nM, and 0.1 nM hCG, there were no significant difference between anisotropy values. However, there was a slight decrease in fluorescence anisotropy that was not significant when cells were treated with of 0.1 nM comparing to 10 nM hCG and 1 nM hCG. This suggests that, following treatment with 0.1 nM hCG, there may be more receptors that exist as dimers. Thus, more high-ordered oligomers are formed with increased hormone concentrations.

Comparisons of initial and final measured anisotropy with the predicted monomer anisotropy as shown in Figure 13 give an idea as to the size of receptor clusters. A greater difference in monomer anisotropy and extrapolated anisotropy values suggests that cluster sizes are larger. Tables 1- 6 show the fluorescence anisotropy values for each individual cell. Additionally, Figure 14 shows the initial and final anisotropy under each condition. The fluorescence anisotropy decreases the most in cells treated with 30 nm or 100 nM hCG which reflects an increase in the number of high-ordered oligomers.

Finally, when the extrapolated anisotropy value is subtracted from predicted value for receptor monomers, the changes in oligomerziation state of LH receptor can be inferred (Figure 15). The subtracted anisotropy for cells treated with 100 nM hCG is ~0.31, with 30 nM hCG is

~0.27, with 10 nM hCG is ~ 0.22, with 1 nM hCG is ~0.20, with 0.1 nm hCG is ~ 0.17. In control cells the difference is ~ 0.16. This suggests that there is an increased contribution from higher order oligomers in cells treated with 30 nM and 100 nM hCG.

In conclusion, polarized homo-transfer FRET permits us to monitor the oligomerization state of LH receptors under different conditions. Previous techniques have been used to evaluate the effect of different treatments on oligomerization state of serotonin 1A receptor (Chakraborty & Chattopadhyay, 2015). By comparing values for treated, untreated and predicted anisotropies for receptor monomers (0.38) (Ganguly et al., 2011), it appears that more higher-order oligomers are formed with increased hormone concentrations. In agreement with our result, Segaloff and co-workers suggest that the cluster size increases with increasing hCG concentration of hormone using co-immunoprecipitation methods (Tao et al., 2004a). Additionally, our results suggest that LH receptor may exist constitutively as dimers in absence of hormone as it is evident in Figure 13 and 14. The presence of dimeric LH receptors in the absence of hormone has been proposed previously in studies using co-immunoprecipitation (Tao el al., 2004a) and the BRET method (Urizar et al., 2005; Guan et al., 2009). Since the cells used in our polarization homo-transfer FRET studies were stably transfected cells and expressed approximate 47,000 LH receptors/cell, the formation of LH receptor dimers does not arise from molecular crowding or high receptor surface densities which could affect the homo-transfer FRET signal (Crenshaw, 2012).

CONCLUSION AND FUTURE DIRECTIONS

In summary, this study demonstrates the effect of hCG concentrations on CHO cells stably transfected with LH receptor tagged to YFP using polarized homo-transfer FRET methods. All homo-transfer FRET measurements were performed on cells expressing approximately 47,000 LH receptors/cell. Fluorescence anisotropy values have been calculated which evaluate the homo-transfer FRET between LH receptors. This study suggests that there are more higher-order oligomers formed on cells treated with high concentrations of hCG. Also, this study establishes that the LH receptor may exists as dimers constitutively under basal conditions. It seem that, in stably transfected cells, the molecular crowding or receptor surface densities is very low and is not likely to contribute to the homo-transfer FRET signal.

The existence of LH receptor dimers under basal condition in the absence of hormone needs further confirmation. The dimerization of LH receptor in control cells could be confirmed by comparing the fluorescence anisotropy of untreated cells (control cells) with cells that are treated with deglycosylated hCG, an hCG antagonist that blocks interactions between LH receptors. If the LH receptor exist as dimers in control cells, the fluorescence anisotropy of cells treated with deglycosylated hCG should be higher than the fluorescence anisotropy of control cells which is ~ 0.22 (Figure 15).

In addition, co-expression of a cAMP reporter molecule, ICUE3 (Gorshkov & Zhang, 2014), could be used to figure out the effect of the oligomerization state of LH receptor on signaling cascade. This cAMP reporter molecule can detect increases in intracellular cAMP which may be affected by oligomer state of LH receptor.

In conclusion, polarized homo-transfer FRET is a powerful approach to monitor the oligomerization state of LH receptors. Knowing the oligomerzation state of LH receptor enables researchers to assess the impact of oligomer formation on receptor activation and downstream signaling. This could enhance researchers' ability to design improved therapeutic strategies.

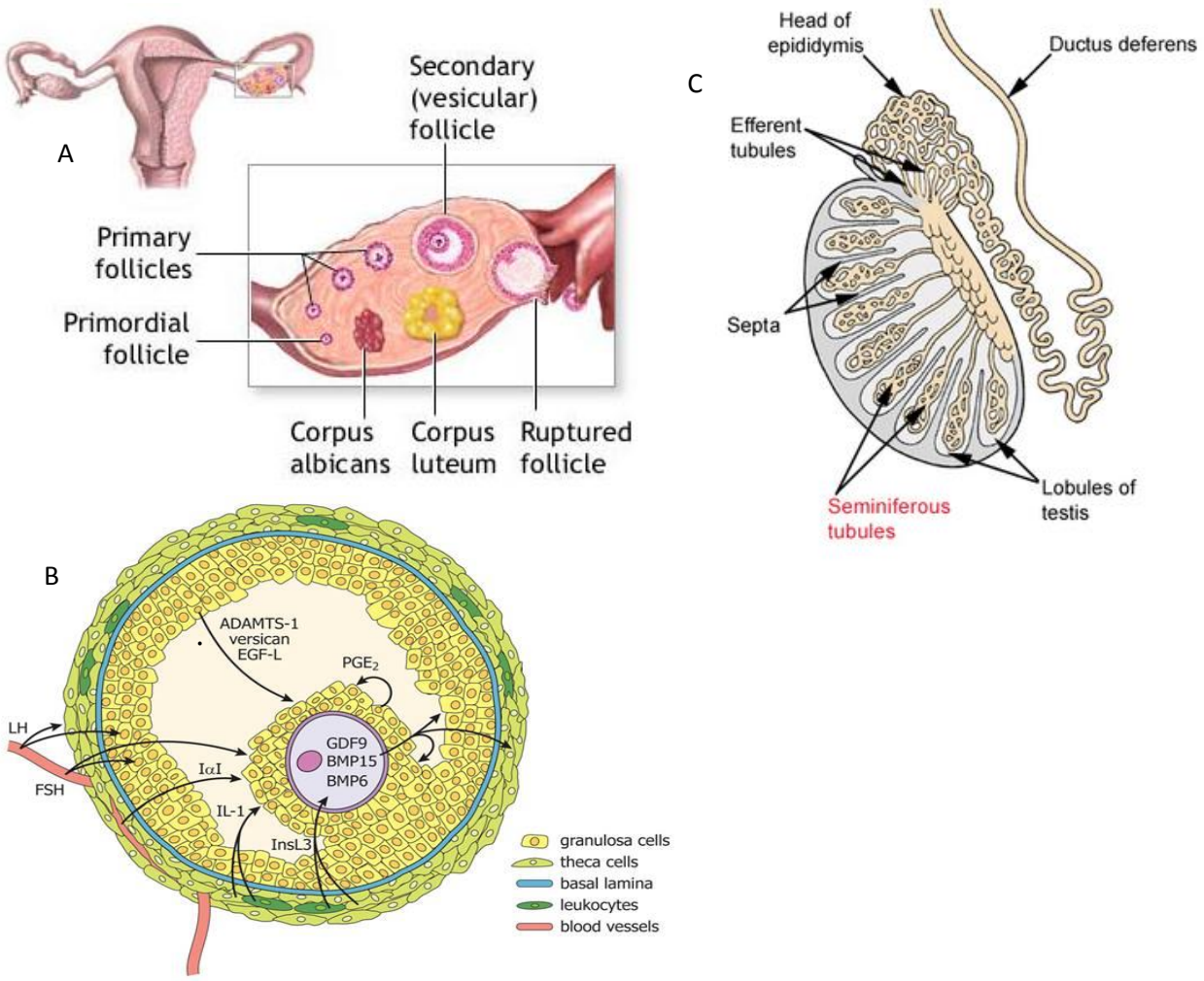

Figure 1: The location of LH receptors in the female and male reproduction systems. Panel A is showing the follicle and corpus luteum of ovary where the LH receptors are expressed. Panel B is a cross section through the follicle and shows that LH receptors are expressed in theca cells, granlusa cells, and interstitial cells. Also LH receptors are found in luteal cells that form the corpus lutem (panel A). Panel C shows the ledig cells that form seminiferous tubules where LH receptors are expressed. From (Tortora, 2001) and (Williams & Erickson, 2012).

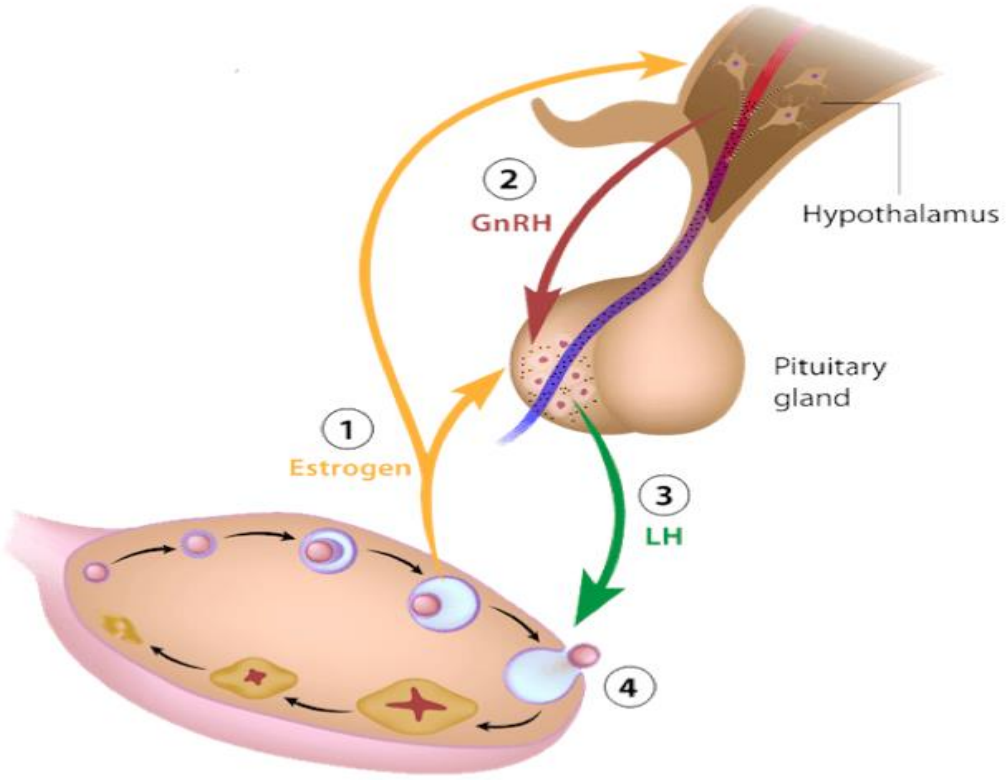

Figure 2: The biological role for the LH receptor in the ovary. Luteinizing hormone (LH) is secreted from the anterior lobe of pituitary gland in response to an increase in estrogen levels in blood. LH receptors regulate the development of follicle, rupture of the follicle to release an oocyte and maintain formation of corpus luteum and progesterone levels. From (Sabil, 2013).

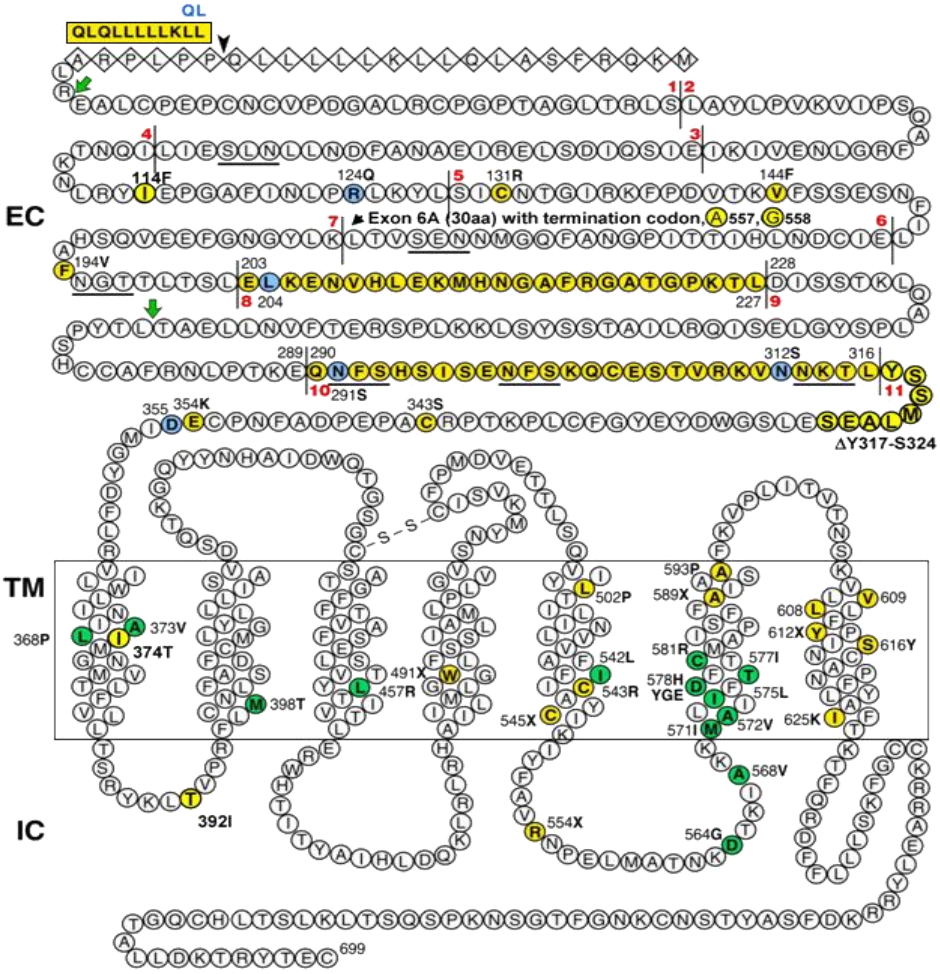

Figure 3: Amino acid sequence of LH receptor. From (Dufau, 1995). LH receptors can be divided to three domains EC (extracellular domain), TM (transmembrane domain) and IC (intracellular domain).

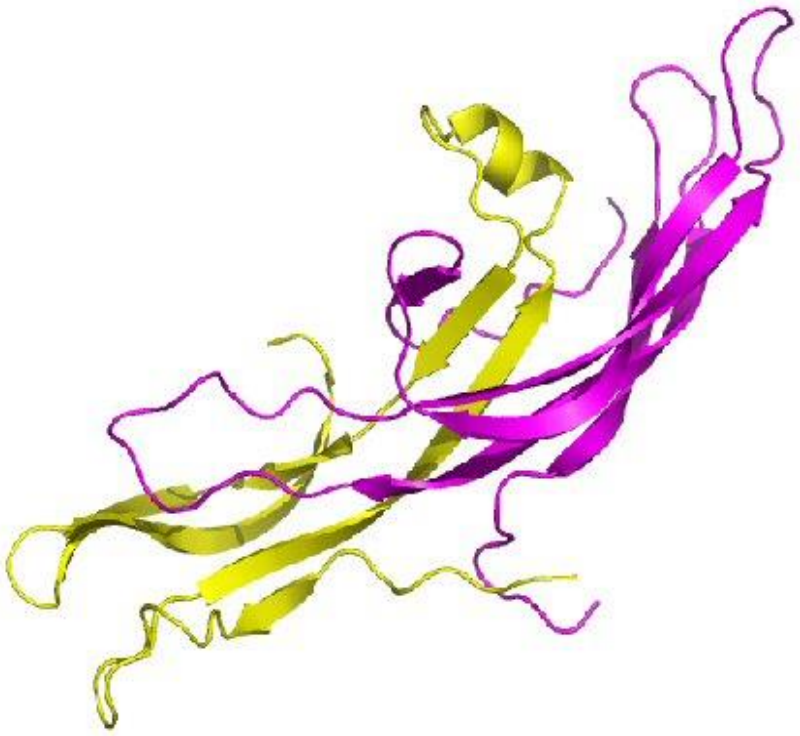

Figure 4: Structure of human chronic gonadotropin (hCG). hCG is comprised of an alpha subunit with 92 amino acids and a beta subunit with 145 amino acids. These chains are attached to each other by non-covalent interactions. alpha and beta domains are colored yellow and purple respectively From (H. Wu et al., 1994).

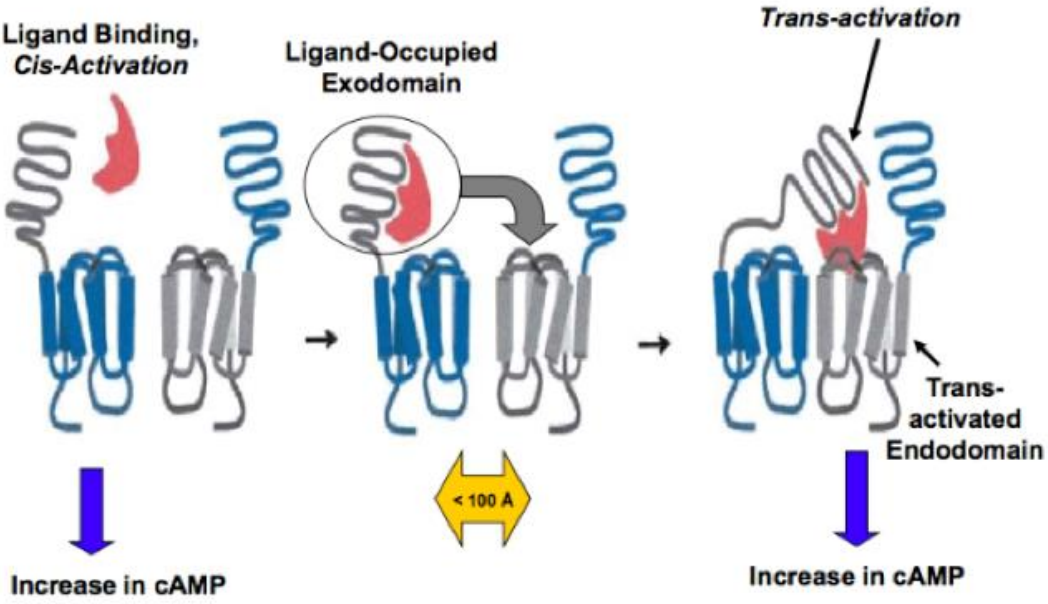

Figure 5: Diagramic representation of cis- activation and trans-activation of LH receptor. Both trans-activation and cis-activation lead to receptors clustering to form dimers/ oligomers in the plasma membrane of cell in distant less than 100 Angstrom. From (Ji et al., 2002).

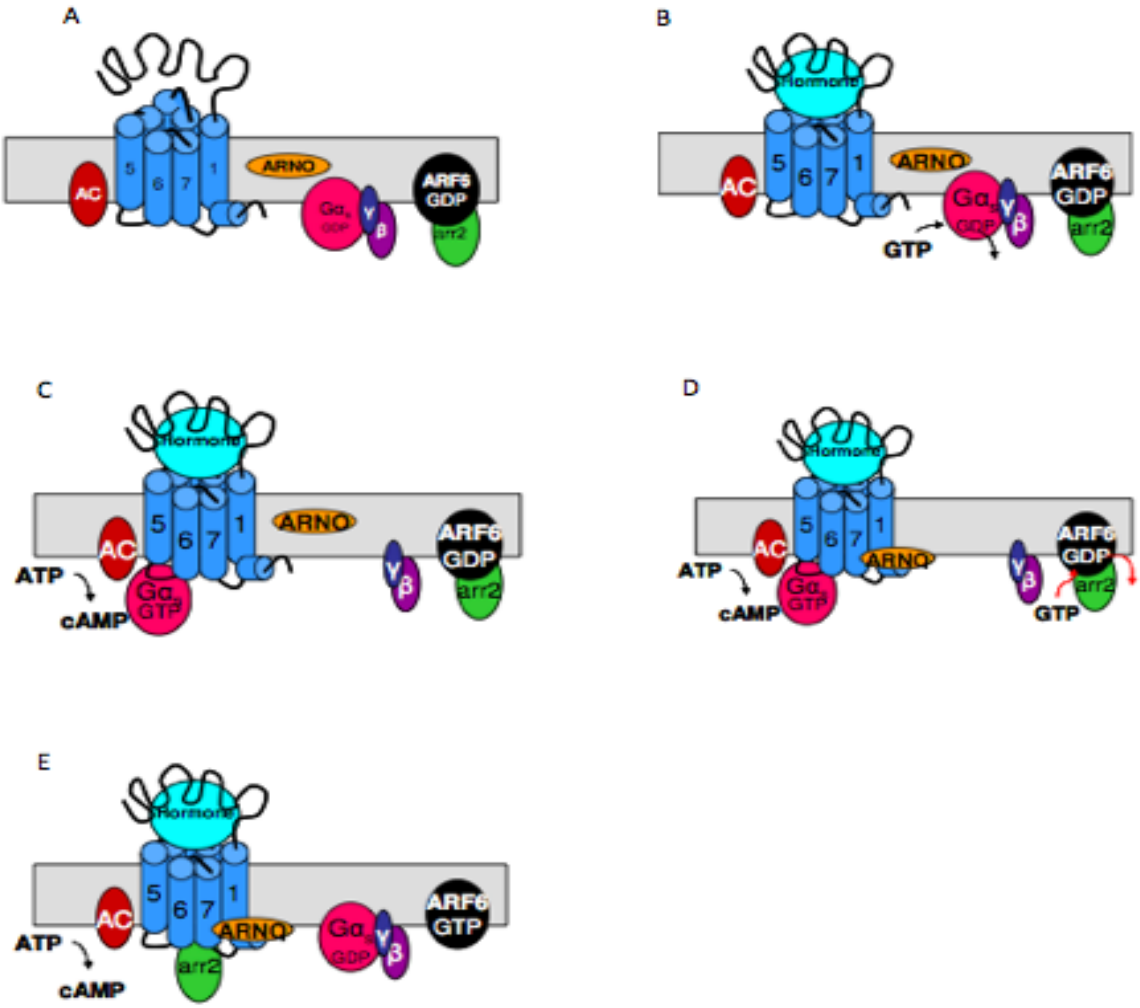

Figure 6: Signal transduction pathway used by LH receptor. (A) Inactive LH receptor when there is no ligand bound. (B) Active LH receptor when ligand binds to the receptor. (C) The α subunit of G protein undergoes an allosteric change by displacing GDP with GTP. The α subunit dissociates from γ and β subunits of the G protein and activates AC enzymes that convert ATP to CAMP. (D and C). There is a negative feedback mechanism to stop signaling and cause hydrolysis of GTP to GDP that leads to inactivation of the G protein as in (A). From (Hunzicker-Dunnet al., 2002)

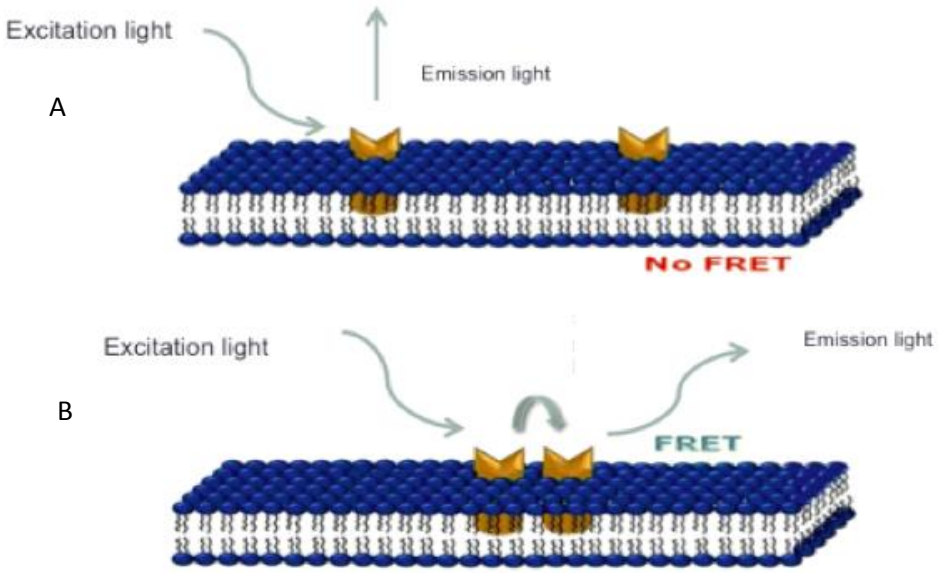

Figure 7: Homo-transfer FRET concept. A is showing when two or more receptors are separated at distance more than 10nm. In this scenario (no FRET), when the first fluorophore is excited with appropriate wavelength, light is emitted right away without transfer of energy because there is no receptor in close proximity to it. B is showing when two or more receptors are close to each other at distances of 2-10 nm. In this scenario FRET occurs. When we excite the first fluorophore with appropriate wavelength, the excited fluorophore transfers energy to nearby receptors which emitted light with less energy than excitation light.

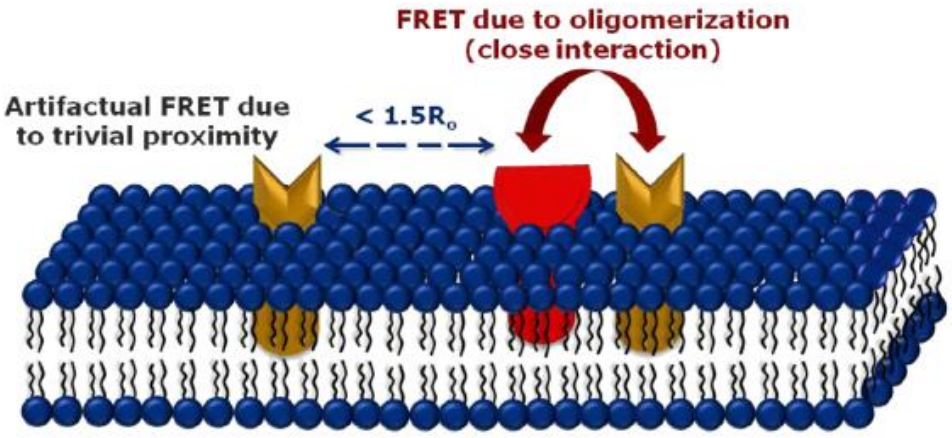

Figure 8: Bystander FRET. When highly crowded nature, the cell membrane gives rise to a FRET signal in the absence of specific protein-protein interactions. From (Chakraborty & Chattopadhyay, 2015).

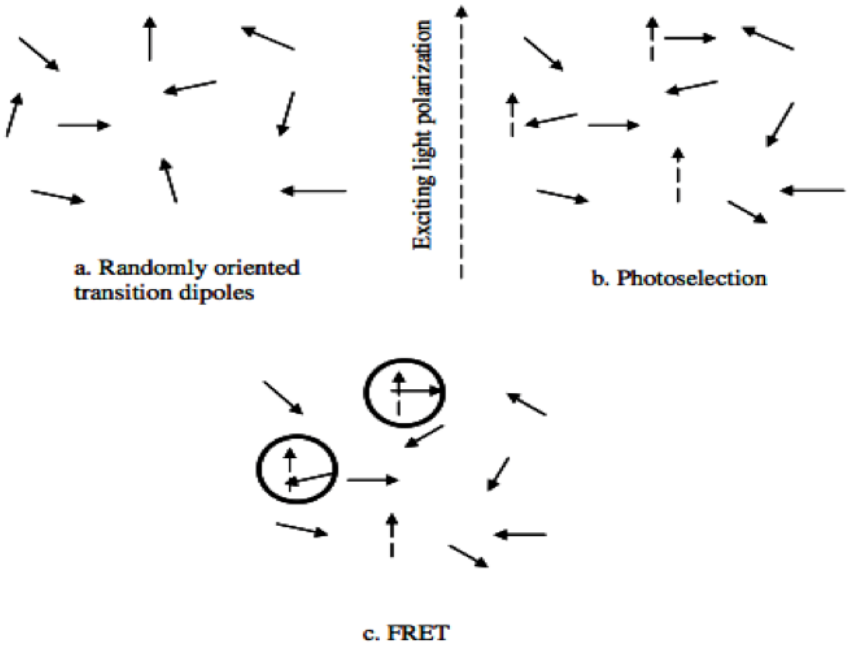

Figure 9: Polarization of fluorescence. (A) Fluorophore with randomly-oriented transition dipoles form an isotropic distributions. (B) When the isotropic distribution of fluorophors are excited with vertically polarized light, the fluorophores that oriented in parallel direction to vertical polarized light are excited. (C) The excited fluorophores transfer energy to adjoining fluorophores that result in randomly oriented emitted fluorescence. From (Crenshaw, 2012)

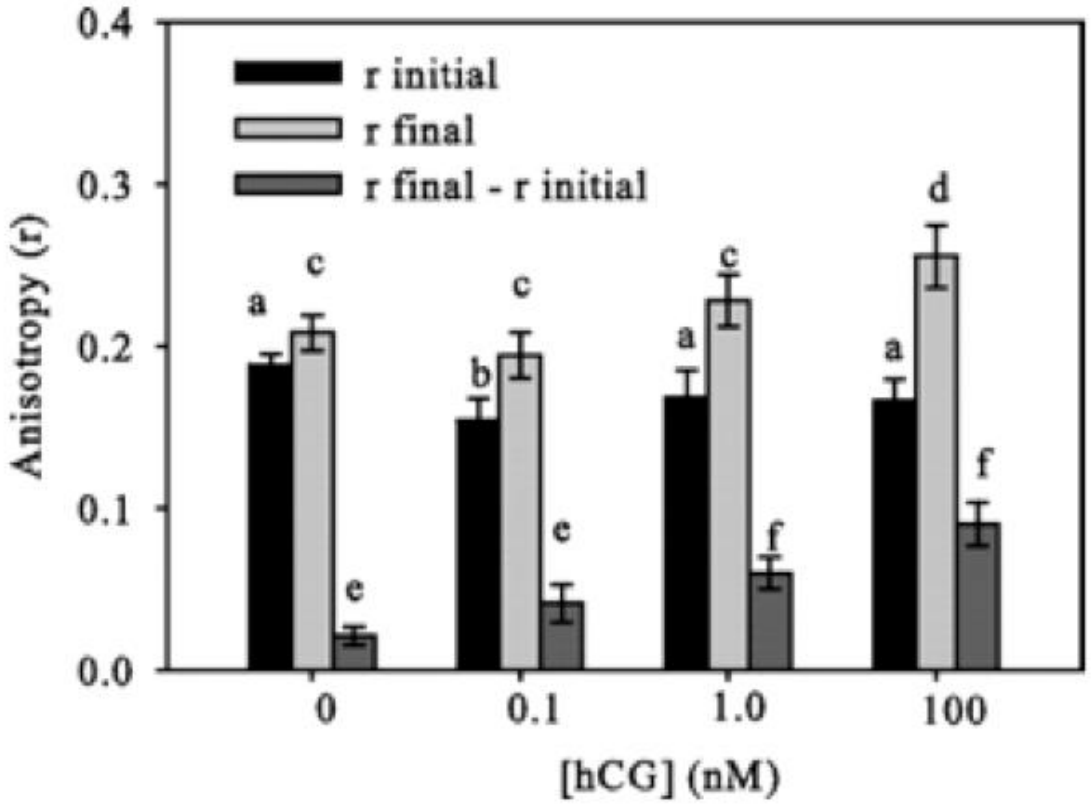

Figure 10: Relationship between fluorescence anisotropy and different concentrations of hCG upon fluorescence photobleaching. Increasing concentration of hormone (hCG) leaded to increases in the formation of LH receptor clusters, this was reflected by increasing anisotropy upon fluorescence photo-bleaching. From (Wolf-Ringwall et al., 2011).

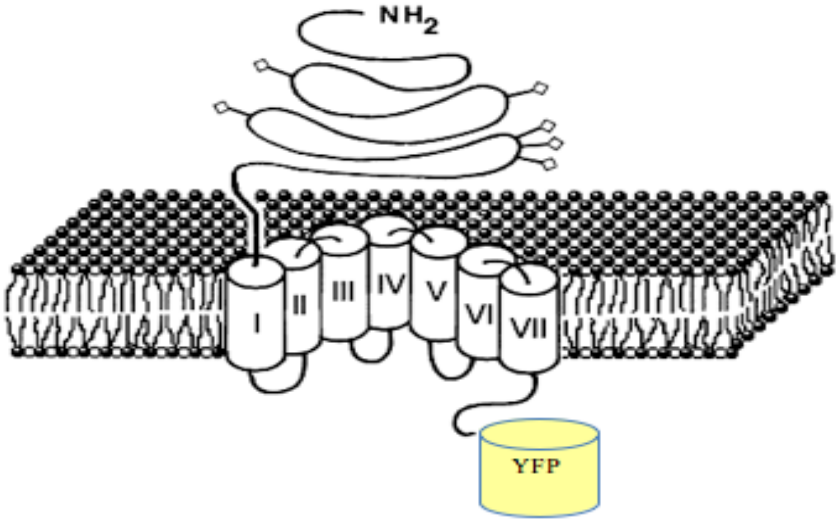

Figure 11: CHO cell line used. CHO cells stably express yellow fluorescence protein (YFP) tagged to the C- terminal cytoplasmic tail of LH receptors.

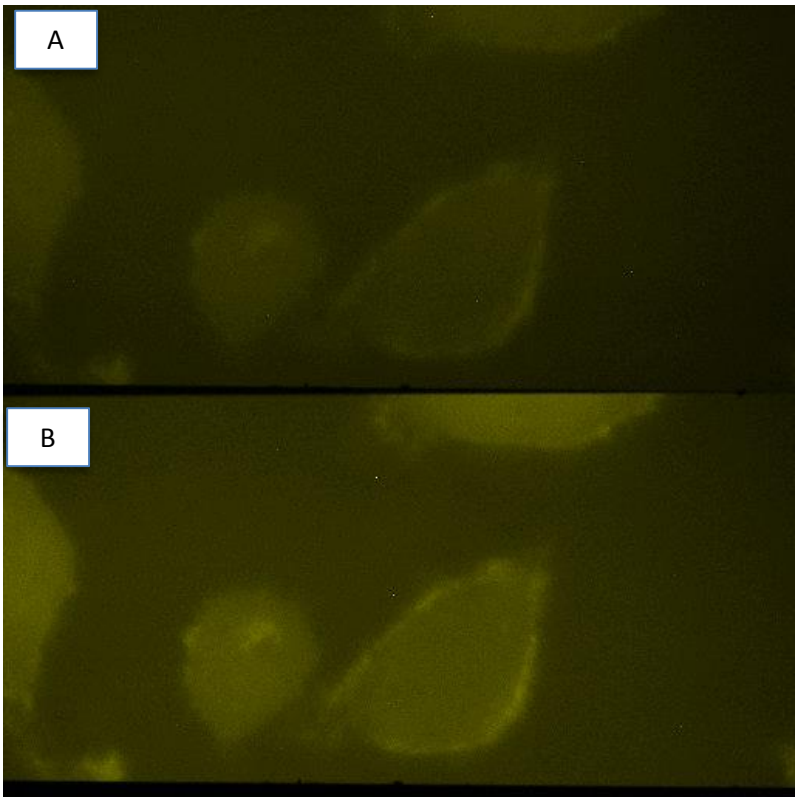

Figure 12: Duel view image of CHO cell. The two detectors in the Zeiss Axiovert 200M inverted microscope gave parallel and perpendicular fluorescence emission images. (A) Illustrates the emission of fluorescence perpendicular to polarized light and (B) illustrates the emission of parallel fluorescence to polarization light.

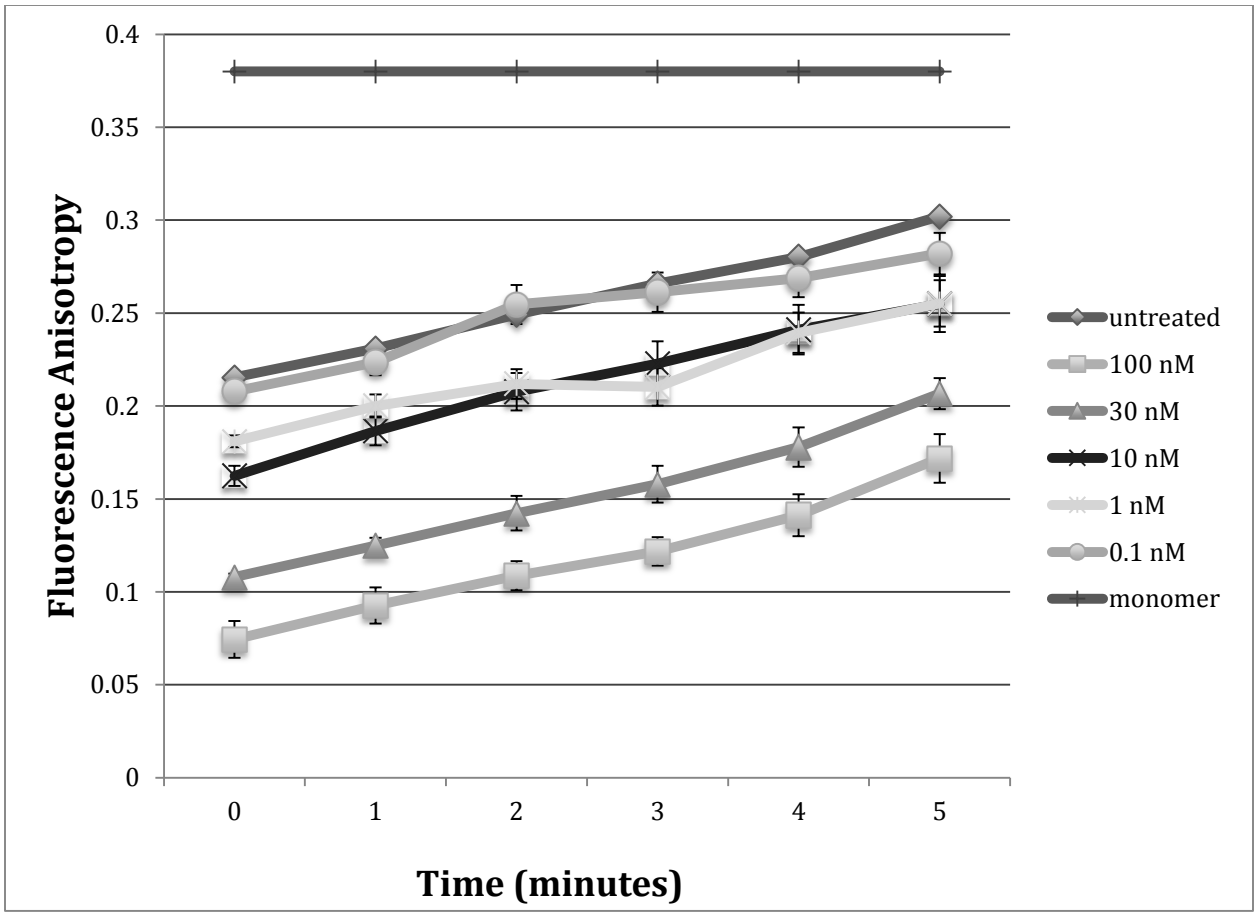

Figure 13: Fluorescence anisotropy upon fluorescence photobleaching for 5 minutes at at vary in concentration of hCG. According to (Chakraborty & Chattopadhyay, 2015), the difference between the monomer anisotropy and extrapolated anisotropy for each condition provides information on the cluster size of LH receptors in plasma membrane. Data shown in tables from tables 1-6

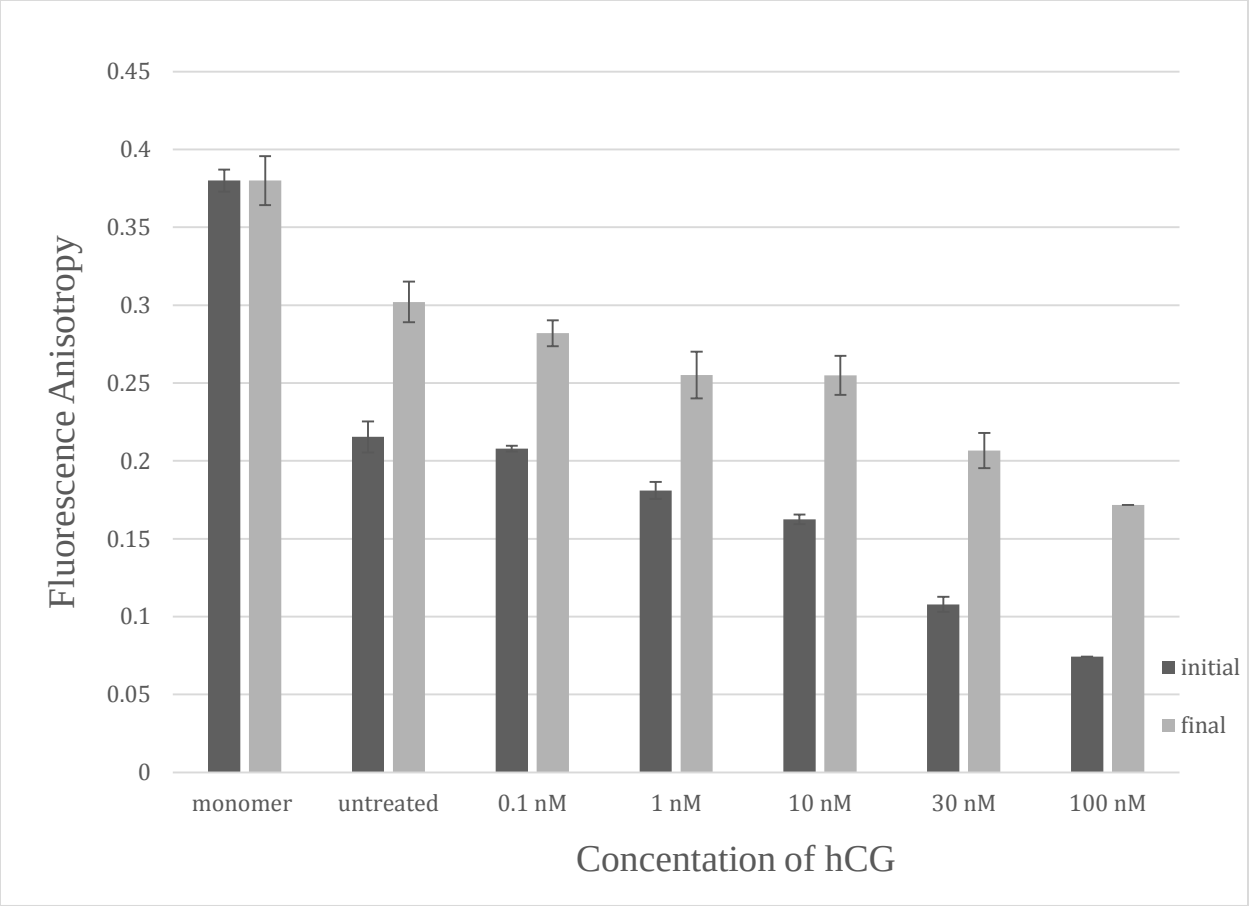

Figure 14: Initial and final fluorescence anisotropies upon fluorescence photobleaching for difference concentration of hCG and as predicted for a monomer anisotropy. Black represents initial fluorescence anisotropy and gray represents final fluorescence anisotropy. Results are the mean ± s.e.m of 10 measurements.

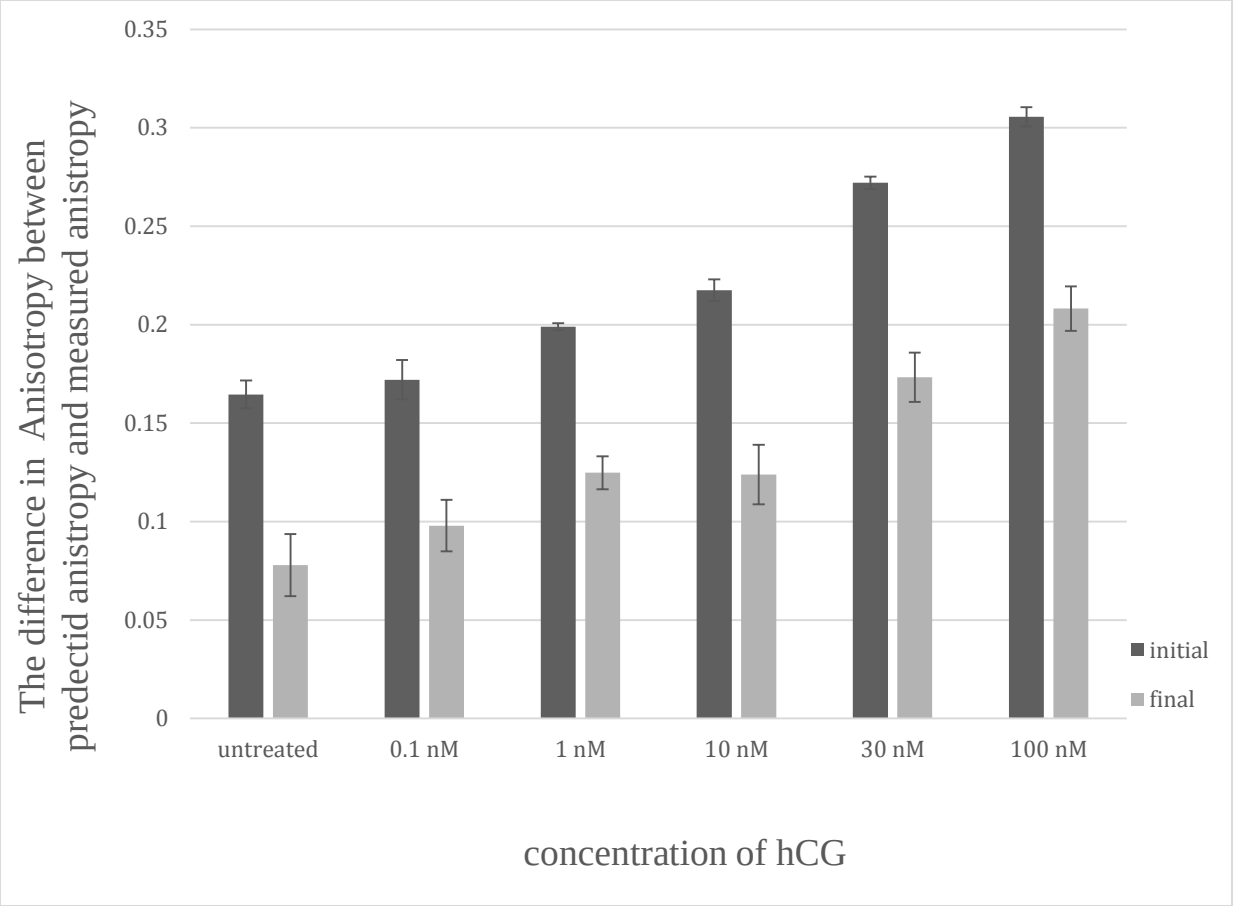

Figure 15: Differences in predicted anisotropy for LH receptor monomers and measured anisotropy values. Black represents initial fluorescence anisotropy and gray represents final fluorescence anisotropy. The resulting values of anisotropy reflect the oligomerization state of LH receptors. Lower values means more dimers in the receptor population while higher values mean more high-order oligomers are present.

Table 1: Fluorescence anisotropy in untreated (control) cells

cell	Fluorescence Anisotropy over time					
	t=0 min	t= 1 min	t=2 min	t= 3 min	t=0 min	t= 5 min
1	0.208	0.224	0.237	0.251	0.262	0.307
2	0.184	0.185	0.196	0.203	0.215	0.219
3	0.206	0.226	0.242	0.245	0.266	0.329
4	0.221	0.231	0.255	0.318	0.33	0.34
5	0.258	0.315	0.326	0.348	0.373	0.38
6	0.197	0.202	0.213	0.227	0.233	0.243
7	0.196	0.201	0.211	0.221	0.234	0.244
8	0.219	0.224	0.277	0.29	0.301	0.305
9	0.24	0.255	0.274	0.282	0.305	0.343
10	0.194	0.203	0.21	0.218	0.23	0.26
MEAN ± SEM	0.212 ±0.0071	0.227 ±0.011	0.244 ±0.012	0.26 ±0.015	0.275 ±0.016	0.300 ±0.016

CHO cells were stably transfected with LH receptor coupled to YFP and imaged. The fluorescence anisotropy calculated every a minute for five minutes. Ten cells are recorded as control cells (untreated). The final row shows the mean ± s.e.m for ten cells.

Table 2: Fluorescence anisotropy for cells treated with 100 nM hCG

cell	Fluorescence Anisotropy over time					
	t=0 min	t= 1 min	t=2 min	t= 3 min	t=0 min	t= 5 min
1	0.088	0.104	0.112	0.13	0.133	0.16
2	0.001	0.033	0.07	0.092	0.099	0.116
3	0.091	0.106	0.123	0.142	0.158	0.204
4	0.104	0.107	0.122	0.136	0.185	0.212
5	0.043	0.056	0.073	0.082	0.095	0.114
6	0.105	0.147	0.152	0.16	0.192	0.22
7	0.078	0.089	0.096	0.103	0.108	0.133
8	0.088	0.108	0.127	0.137	0.177	0.214
9	0.071	0.088	0.103	0.113	0.131	0.159
10	0.075	0.088	0.108	0.123	0.133	0.188
MEAN ± SEM	0.074 ±0.009	0.093 ±0.009	0.109 ±0.008	0.122 ±0.008	0.141 ±0.011	0.172 ±0.008

CHO cells were stably transfected with LH receptor coupled to YFP and treated with 100 nM hCG hormone then imaged after incubated for an hour at 37℃. The fluorescence anisotropy was calculated every one minute for five minutes. Ten cells are recorded. The final row shows the mean ± s.e.m for 10 cells.

Table 3: Fluorescence anisotropy for cells treated with 30 nM hCG

cell	Fluorescence Anisotropy over time					
	t=0 min	t= 1 min	t=2 min	t= 3 min	t=0 min	t= 5 min
1	0.106	0.11	0.131	0.137	0.16	0.211
2	0.105	0.13	0.136	0.151	0.174	0.211
3	0.11	0.143	0.216	0.244	0.27	0.274
4	0.099	0.107	0.122	0.14	0.166	0.202
5	0.103	0.12	0.128	0.144	0.156	0.19
6	0.114	0.13	0.136	0.143	0.181	0.198
7	0.112	0.124	0.1259	0.144	0.154	0.178
8	0.108	0.132	0.154	0.161	0.176	0.208
9	0.118	0.146	0.16	0.163	0.174	0.211
10	0.104	0.11	0.114	0.151	0.169	0.1842
MEAN ± SEM	0.108 ±0.002	0.125 ±0.004	0.142 ±0.009	0.158 ±0.009	0.178 ±0.011	0.207 ±0.008

CHO cells were stably transfected with LH receptor coupled to YFP and treated with 30 nM hCG hormone then imaged after incubated for an hour at 37℃. The fluorescence anisotropy was calculated every one minute for five minutes. Ten cells are recorded. The final row shows the mean ± s.e.m for 10 cells.

Table 4: Fluorescence anisotropy for cells treated with 10 nM hCG

cell	Fluorescence Anisotropy over time					
	t=0 min	t= 1 min	t=2 min	t= 3 min	t=0 min	t= 5 min
1	0.141	0.145	0.15	0.164	0.184	0.201
2	0.129	0.164	0.248	0.274	0.316	0.333
3	0.16	0.19	0.198	0.203	0.234	0.237
4	0.166	0.212	0.22	0.231	0.244	0.259
5	0.164	0.205	0.218	0.22	0.258	0.262
6	0.188	0.222	0.259	0.298	0.302	0.344
7	0.18	0.204	0.208	0.239	0.245	0.251
8	0.168	0.177	0.207	0.214	0.217	0.23
9	0.16	0.169	0.177	0.199	0.201	0.212
10	0.171	0.179	0.192	0.21	0.212	0.232
MEAN ± SEM	0.162 ±0.005	0.187 ±0.008	0.208 ±0.01	0.225 ±0.012	0.241 ±0.013	0.256 ±0.015

CHO cells were stably transfected with LH receptor coupled to YFP and treated with 10 nM hCG hormone then imaged after incubated for an hour at 37℃. The fluorescence anisotropy was calculated every one minute for five minutes. Ten cells are recorded. The final row shows the mean ± s.e.m for 10 cells.

Table 5: Fluorescence anisotropy for cells treated with 1 nM hCG

cell	Fluorescence Anisotropy over time					
	t=0 min	t= 1 min	t=2 min	t= 3 min	t=4 min	t= 5 min
1	0.176	0.198	0.202	0.235	0.237	0.294
2	0.184	0.23	0.239	0.252	0.2566	0.259
3	0.197	0.198	0.199	0.215	0.216	0.217
4	0.183	0.192	0.196	0.218	0.219	0.222
5	0.164	0.177	0.196	0.2	0.2	0.232
6	0.182	0.185	0.19	0.214	0.247	0.255
7	0.192	0.206	0.233	0.29	0.3	0.321
8	0.184	0.233	0.261	0.263	0.279	0.292
9	0.168	0.176	0.182	0.185	0.19	0.195
10	0.181	0.204	0.221	0.249	0.252	0.265
MEAN ± SEM	0.181 ±0.003	0.2 ±0.006	0.212 ±0.008	0.23 ±0.001	0.24 ±0.011	0.255 ±0.013

CHO cells were stably transfected with LH receptor coupled to YFP and treated with 1 nM hCG hormone then imaged after incubated for an hour at 37℃. The fluorescence anisotropy was calculated every one minute for five minutes. Ten cells are recorded. The final row shows the mean ± s.e.m for 10 cells.

Table 6: Fluorescence anisotropy for cells treated with 0.1 nM hCG

cell	Fluorescence Anisotropy over time					
	t=0 min	t= 1 min	t=2 min	t= 3 min	t=0 min	t= 5 min
1	0.24	0.257	0.309	0.316	0.324	0.334
2	0.208	0.228	0.264	0.297	0.298	0.319
3	0.22	0.227	0.294	0.276	0.287	0.293
4	0.214	0.251	0.275	0.278	0.279	0.289
5	0.212	0.244	0.247	0.247	0.255	0.264
6	0.197	0.205	0.217	0.225	0.231	0.24
7	0.204	0.208	0.27	0.282	0.29	0.316
8	0.207	0.224	0.228	0.234	0.246	0.249
9	0.193	0.196	0.234	0.248	0.261	0.286
10	0.185	0.195	0.209	0.211	0.218	0.23
MEAN ± SEM	0.208 ±0.005	0.224 ±0.007	0.255 ±0.011	0.261 ±0.011	0.269 ±0.01	0.282 ±0.011

CHO cells were stably transfected with LH receptor coupled to YFP and treated with 0.1 nM hCG hormone then imaged after incubated for an hour at 37℃. The fluorescence anisotropy was calculated every one minute for five minutes. Ten cells are recorded. The final row shows the mean ± s.e.m for 10 cells.

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LIST OF ABBREVIATIONS

AC:	adenylyl cyclase
α	alpha
γ	gamma
β	beta
CL:	corpus luteum
cAMP:	cyclic adenosine monophosphate
CHO:	Chinese hamster ovary
GnRH:	gonadotropin releasing hormone
Homo-FRET:	homotransfer fluorescent resonance energy transfer
DMEM:	Dulbecco's modified minimum essential medium
EDTA:	ethylenediamineetetraacetic acid
FBS:	fetal bovine serum
FRET:	fluorescent resonance energy transfer
G418:	genticin
GDP:	guanosine diphosphate
GPCR:	G protein coupled receptor
GTP:	guanosine triphosphate
hCG:	human chorionic gonadotropin
ICUE3:	indicator of cAMP using modified EPAC
LH:	luteinizing hormone
LHR	luteinizing hormone receptor

PBS: phosphate buffered saline

PKA: protein kinase A

YFP: yellow fluorescent protein

e YFP: enhanced yellow fluorescent protein