6-2014

Localization of the Follicle-Stimulating Hormone Receptor in Lipid Raft Domains

Tyler Esposito
Union College - Schenectady, NY

Localization of the Follicle-Stimulating Hormone Receptor in Lipid Raft Domains

By

Tyler Esposito

****

Submitted in partial fulfillment

of the requirements for

Honors in the Department of Biological Sciences

Union College

June 2014

Abstract

ESPOSITO, TYLER Localization of the follicle-stimulating hormone receptor in lipid raft domains. Department of Biological Sciences, June 2014.

ADVISOR: Professor Brian Cohen

Lipid raft domains have been shown to be important in receptor signaling, especially for G Protein Coupled Receptors (GPCR). Follicle stimulating hormone is a vital reproductive signal that relies on effective signaling of its GPCR. The goal of this study was to test whether or not the follicle stimulating hormone receptor (FSHR) utilizes lipid raft domains when signaling.

The main methods used were fluorescent antibodies and various forms of microscopy, including confocal microscopy. A fluorescent antibody for the FSHR (mAb 106.105) allowed us to perform these studies.

In the first experiment, cells treated with FSH and stained with the receptor antibody showed aggregation of receptors. This supported the belief that FSHR are clustering in lipid raft domains when signaling. Next nystatin, a lipid raft disrupting chemical, was used to show that if the lipid rafts were disturbed, this clustering of receptor would not be observed. In these trials the cells treated with FSH looked similar to the cells that received no treatment. Finally we used the Cholera Toxin B subunit to fluorescently tag lipid raft domains, while still staining for receptor. This showed colocalization of the receptor and lipid rafts in cells treated with FSH and those who did not receive treatment (but at a lower intensity).

These results support the hypothesis that FSHR utilizes lipid rafts when signaling, most likely to improve signaling efficiency.

Tyler Esposito

Professor Cohen

2014

Localization of the Follicle-Stimulating Hormone Receptor in Lipid Raft Domains

Introduction:

Any animal's most basic evolutionary need is to survive and reproduce. Fertility, the ability of an organism to reproduce, is vital to the continuation of a species. Experiments and studies concerned with fertility range from hoping to cure infertility to working on birth control. In a world where over-population of certain animals and massive extinction of others has become a serious issue for society, finding new ways to affect fertility has become necessary to conserve natural resources at a viable level. There is no denying the importance of studying reproduction and the systems that regulate this process.

The endocrine system plays a major role in regulating the ability of mammals to reproduce. Hormones are regulatory biochemicals that are transported through the bloodstream to a specific part of the body and produce a result (Hanley & Holt, 2007). Most hormone signaling involves a signal traveling from the hypothalamus to the anterior pituitary, where it signals for the release of another molecule, which travels to the target tissue where it has an effect. The anterior pituitary is made up of distinct types of cells that are involved in different signaling pathways. Ten to fifteen percent of the cells in the anterior pituitary are gonadotrophs. The gonadotrophs are basophils, named for their ability to take up basic stains. These cells secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are gonadotrophins (Hanley & Holt, 2007).

Follicle-stimulating hormone (or follitropin) is released from the anterior pituitary in response to gonadotropin releasing hormone (GnRH) signaling from the hypothalamus (Hanley & Holt, 2007). FSH is a 32 kDa heterodimeric glycoprotein (Dias et al., 2002) consisting of an alpha and a beta subunit (figure 1). Release of this dimeric glycoprotein is negatively regulated by stress, prolactin, as well as inhibin (Holt & Hanley, 2007). FSH travels to the testes in men or the ovaries in women (Holt & Hanley 2007). Follicle-stimulating hormone and its receptor are necessary for proper and normal gamete maturation in both males and females (Dias et al., 2002). In males, FSH stimulates spermatogenesis and gonadal maturation by binding to its receptor on Sertoli cells in the testis (Holt & Hanley, 2007). In females, FSH binds to its receptor on granulosa cells and signals for the development of ovarian follicles and eggs (Dias, et al., 2002). It is used as a supplement in females to help with the chance of pregnancy and in males to increase sperm count (Dias et al., 2002). Issues with FSH or its receptor can lead to delayed or precocious (accelerated) puberty, hypogonadism, fertility issues, and many other reproductive problems (Holt & Hanley, 2007).

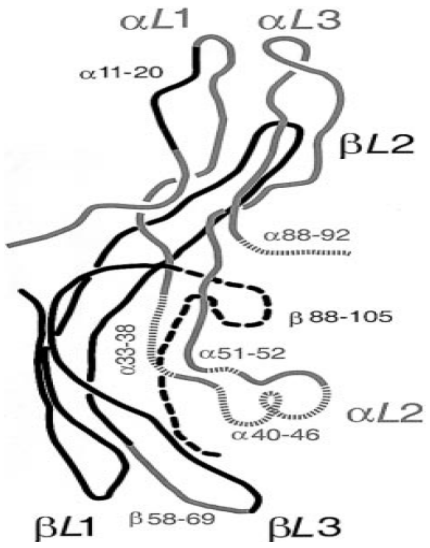

Figure 1. Ribbon diagram of follicle stimulating hormone, which shows the α and β subunits.

The follicle stimulating hormone receptor (FSHR) is a vital part of this system. If the receptor is not functioning properly, this signaling pathway will be incapable of producing any change in the target cells. The FSHR is a G protein coupled (GPCR) which is found on the surface of Sertoli cells of the testis (males) and granulosa cells of the ovaries (females) (Dias, et al., 2002). FSHR have a seven alpha-helical transmembrane domain and contain a large extracellular domain which is used to bind the glycoprotein ligand, FSH (Dias, et al., 2002). The FSHR is highly conserved among species. FSH binds to the large extracellular domain of the receptor in a hand clasp fashion as seen in figure 2 (Fan & Hendrickson, 2005). When bound, FSH undergoes a conformational change which plays a part in receptor activation. The ligand activated receptors utilize G proteins as secondary signaling molecules, and initiates downstream signaling cascades which eventually changes gene expression in these cells, which results in a specific effect (i.e. granulosa cell differentiation in females).

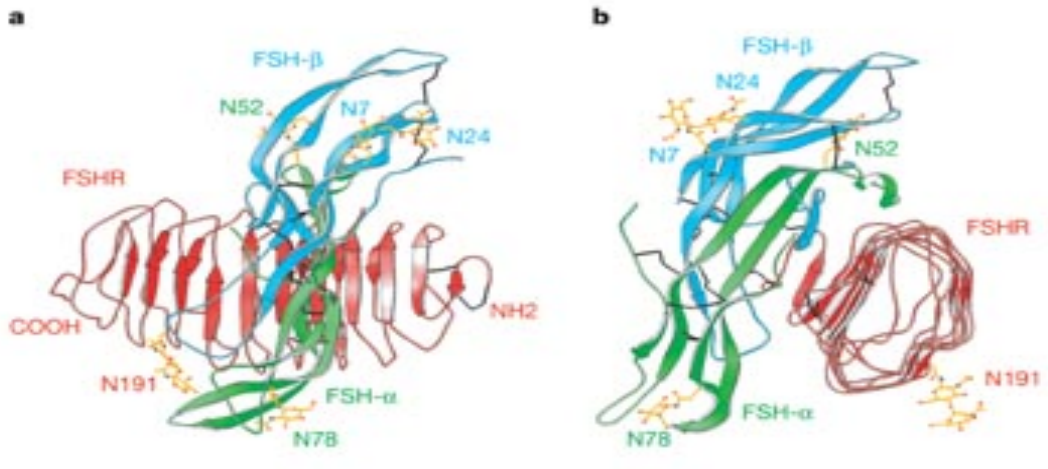

Figure 2. Ribbon diagram of FSH bound to the extracellular domain of the FSHR. The FSH α chain is shown in green and the FSH β chain is in blue. The FSHR is red (Fan & Hendrickson, 2005).

When FSH binds to its receptor it has the eventual consequence of "sustaining ovarian follicle growth in females and spermatogenesis in males" (Telikicherla et al., 2011). In order

accomplish this result; FSH binding must initiate multiple downstream signaling pathways. The main downstream pathway begins, after hormone binding with, the activation of the heterodimeric G-protein (Gs). This protein converts GTP into GDP over a span of time which allows the response of the hormone binding is controlled. This activation causes an increase in adenylate cyclase activity, and production of cyclic AMP (cAMP). The increase in cAMP causes activation of protein kinases, which phosphorylate multiple transcription factors. These transcription factors regulate expression of FSH-target genes. FSH binding to its receptor also activates other secondary messengers, in addition to cAMP, including calcium and IP3. This process causes a total of 265 genes to be regulated (Telikicherla et al., 2011).

Luteinizing hormone, FSH and their respective receptors have many similarities. Luteinizing hormone is also a glycoprotein and a gonadotropin which binds to an extracellular receptor. Like FSH, LH is secreted by the anterior pituitary and is the other hormone mainly responsible for proper reproductive maturity of gametes. Both hormones rely on G protein coupled receptors. These receptors are similar in that they both have the seven trans-membrane region and a large extracellular domain, with multiple motifs in common in these structures (Simoni et al. 1997) (figure 3).

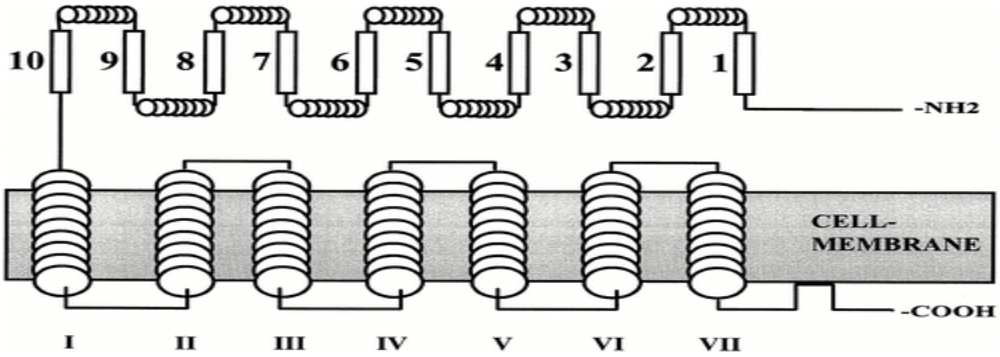

Image 3. Structure of the FSH receptor. Displays the seven transmembrane region and the large extracellular domain. LH receptor is very similar.

A study by Roess et al. showed convincing evidence that the LHR aggregates in lipid raft domains when signaling. When viewing electron micrographs of rat granulosa cells this group noticed that when LH was present and binding to its receptor, the receptors would aggregate quickly in pockets on the plasma membrane. The ability of these receptors to go from being diffuse across the plasma membrane to aggregating in pockets after minutes caused this group to question whether these receptors were grouped in arbitrary areas or in specific microdomains like lipid rafts. Also, it was known that many G protein coupled receptors have been shown to utilize lipid raft domains for signaling; including the Gonadotropin Releasing Hormone, Somatostatin, and Adenosine receptors (Chini & Parenti, 2004). This led to the hypothesis that the LHR is aggregating in lipid raft domains when signaling. Roess et al. showed convincing evidence that their hypothesis is correct using sucrose gradients. The receptor was found in high density portions of the gradient, representative of bulk membrane fractions, before ligand was added. Following the addition of a ligand the receptor was found in low density fractions, representative of lipid rafts. The results of this study and the many similarities between FSH and LH were important in the formation of our hypothesis.

All mammalian cells contain lipid raft microdomains as a part of their plasma membrane, so these components must be significant (Insel & Ostrom, 2004). Lipid rafts are enriched with lipid and proteins. More specifically, lipid rafts are characterized by high levels of sphingolipids and cholesterol. This makes them more ordered than the normal phospholipid membrane (Chini & Parenti, 2004). These domains are important for the compartmentation of certain receptors; especially G protein coupled receptors. In a typical signaling system there is a low concentration of signaling molecules and receptors but a rapid response to hormones is observed. One hypothesis to explain this is that the cell concentrates its signaling molecules in these lipid raft

microdomains (Isnel & Ostrom, 2004). Lipid rafts have been shown to be important for the fine-tuning of the cell signaling machinery located on the cell surface (Chini & Parenti, 2004). The fact that activation of FSHR specifically activates the Gs protein is important. Certain studies have shown that Gs and Gi proteins localize in lipid rafts (Insel and Ostrom, 2004). These G-proteins have an affinity for an ordered lipid environment (Moffet, et al., 2000). As previously discussed, the ability of the FSHR to signal relies on this protein to begin the downstream signaling cascade, and to do so in a controlled manner. The high concentration of G proteins could increase the efficiency of signaling. This is one explanation as to why this receptor may move into these microdomains when signaling. Another explanation is that these domains increase the efficiency of GPCR internalization (Insel, et al., 2005).

Some G protein coupled receptors are always found in lipid rafts (Bradykinin and Dopamine). Some move out of lipid rafts when an agonist is present (Oxytocin). Others have the ability to move in and out of lipid raft domains upon activation (GnRH and Growth Hormone Releasing Hormone) (Insel, et al., 2005). Therefore, there must be some determinant which causes this movement. Some have hypothesized that it involves the extracellular domain of the receptor, others believe it involves the transmembrane domain and its interaction with cholesterol, finally some think this process involves the intracellular loops and carboxylic by protein-protein interaction and fatty acylation (Chini & Parenti, 2004). These different hypotheses have been shown to be important for receptor localization in lipid rafts/caveolae, in different circumstances.

Studies have shown that the relationship between GPCRs and lipid raft/caveolae is variable between cell types, the type of receptor, the metabolic state of the cell and other factors. This fact clouds our understanding of this relationship and makes it difficult to make any

generalizations concerning it. It is clear, however, that when researching a GPCR, the role of lipid rafts in the process of signaling, endocytosis, etc is an important question to understand. As scientists continue to investigate individual receptors and how they utilize raft domains, our knowledge of lipid rafts will inherently grow substantially. Our hypothesis is that these signals should be colocalized and overlap if the receptor is in a lipid raft domain. The knowledge of when FSHR resides in lipid rafts could have medical implications, as well as add to our recognition of the importance of lipid rafts in GPCR signaling.

Methods:

Most current techniques to study lipid rafts involve cell lysates and fractionation studies which destroy the morphology of the cell and rely on biochemical assays and reagents. While these types of studies have a valuable purpose, studying compartmentation of receptors on the plasma membrane of a cell is best studied using microscopy because it is a morphological phenomena (Insel & Ostrom, 2004). Using a technique where the cell can be observed directly without affecting its morphology is desired. This is difficult because even the use of fluorescent markers have a slight effect on cell morphology (Insel & Ostrom, 2004). Despite this fact, using microscopy is preferred over the other techniques which involve lysing the cells. Recently the use of fluorescent antibodies to tag proteins (i.e. receptors) and putative raft markers have been utilized to image colocalization between proteins and rafts (Conn, 2013). This technique has had great success. This effectively avoids affecting the cells' morphology, but is not with some pitfalls. Any technique "requiring labeling, protein or raft/nonraft marker membrane localization/organization might be influenced by their labels" (Conn, 2013). This technique will be used to add more evidence to answer if the FSHR is found in lipid raft domains of human granulosa cells, and not as a lone method to prove anything on its own. This is the technique that we used to study the colocalization of the FSHR in lipid rafts. The use of the confocal microscope was vital for the proper imaging of the cells.

Knowledge of the contents of lipid rafts allowed researchers to develop techniques to create putative markers for lipid raft domains. The most common tag used is the Cholera toxin B (CTxB) subunit which binds to the ganglioside G_{M1} found only in lipid rafts in cells. Groups have used this technique to test for colocalization of receptors in lipid rafts and if lipid rafts are important for the internalization and intracellular trafficking of the signal (Drake, et al., 2003).

The ganglioside G_{M1} can be observed with a fluorescently modified CTxB subunit (image 4). This allows for visualization of lipid raft domains in live cells which are usually too small to be seen using microscopy. The FSHR can also be tagged with a specific monoclonal antibody and a fluorescent antibody to visualize the receptor's location on the same cell. This technique will be used to test for localization of the FSHR in lipid rafts domains, with or without treatments of FSH.

Using fluorescently labeled CTxB is a relatively new technique that has been used with success. It avoids using detergent lysates and fractionation techniques that involve destroying the cell. It is not a perfect system however. The binding of the CTxB subunit to the lipid rafts will inherently alter the location of these domains on the membrane. The fact that we will be comparing the cells with no treatment with cells that have been treated with FSH helps to limit this disadvantage. Any differences between these conditions will presumably be because of the treatment and not the CTxB. Also further studies will be carried out utilizing completely different techniques (co-immunoprecipitation) to support or disprove the results of these experiments.

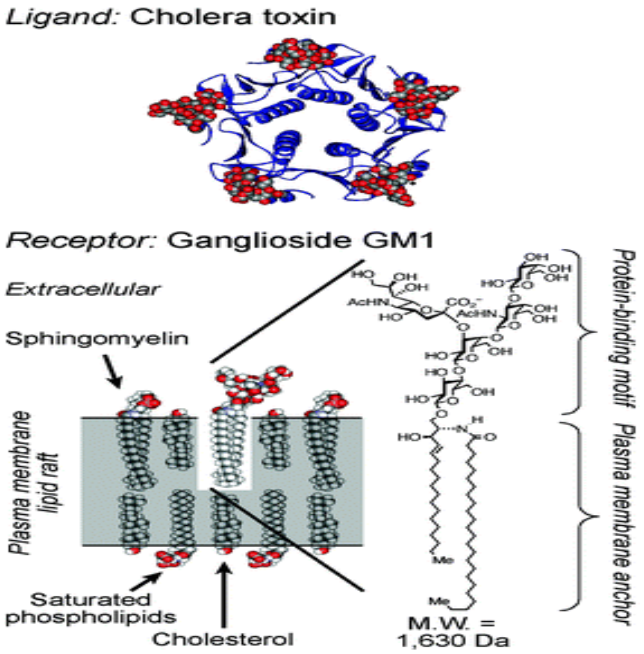

Image 4. Ribbon structure of the cholera toxin b subunit (above). Below that is a depiction of a lipid raft domain of a plasma membrane with the ganglioside GM1 highlighted. The structure of the ganglioside GM1 including the area where CTxB binds and the membrane anchor labeled is shown on the right (Peterson, 2005).

The early testing of this hypothesis required the ability to disrupt lipid rafts. Nystatin is a polyene antibiotic and also a cholesterol sequestering agent. This is an effective research tool because, "cholesterol sequestration alters composition of the plasma membrane micro-organization (lipid rafts)" (Baek, et al., 2013). Using treatments of nystatin allowed us to test whether the loss of stable, normal, lipid raft domains would cause significant changes in the staining of receptor when bound by its ligand (actively signaling).

Being able to culture and use immortalized human granulosa cells was imperative for this study. HGrC1 cells were used for the staining procedures. These cells are representative of the granulosa cells in the ovaries. They express the FSHR in a physiologically relevant level, and therefore signal in response to FSH treatments.

The hope of this research is to test whether FSHR utilizes lipid raft domains when signaling. The use of antibodies for the receptor (mAb 106.105) and the lipid raft marker antibody (Cholera Toxin B kit) allow us to check for colocalization using microscopy. The antibody was provided by Professor James Dias of SUNY Albany. By using hormone treatments of variable lengths we hoped to learn more concerning the mobility of the receptor. The use of specific antibodies makes this research possible and allows for straightforward visualization of the result. It avoids many pitfalls of other lab procedures that typically involve lysing the cell.

Cell Culture

HGrC1 cell line was cultured and used for all experiments (Bayasula, et al., 2012). This cell line is human granulosa cells and express a physiologically relevant number of follicle-stimulating hormone receptor (FSHR). We choose this cell line so that we could effectively stain for FSHR. This avoided an over-expression of receptor, or the need to transfect a receptor gene into a different cell line.

Growing Cells on Cover Slips

Coverslips were sterilized and then placed into a well of a Falcon 6 well plate. Added 0.5 ml of 0.1 mg/ml Poly-D-lysine to each well and let sit for 10 minutes, to promote cell adhesion. Next the wells were washed with sterile water and then allowed to dry for 1 hour. At this point the cells were split and added to the wells.

Treatments

The newly split cells were allowed to grow and become confluent. When confluent, the cells could be stained for FSHR using fluorescent antibodies. First, one hour prior to staining, if there was any nystatin treatment it was carried out (5µg nystatin/1ml of serum free medium/well). If there was any FSH treatment needed it would be performed directly before the fluorescent staining (either 30, 15 or 5 minutes, before staining). The cells would be washed with 2 ml of serum free medium and then treated with FSH dissolved in serum free medium (40ng FSH/2ml of serum free medium/well) for the desired amount of time.

Staining Cells

Next, the cells were washed with PBS. Cells were fixed in 400 µl of paraformaldehyde (PFA) for 30 minutes and then the wells were washed with PBS. Next, they were blocked with 1 ml of 1% BSA in 1x PBS for one hour. Then 250 µl of the primary antibody (mAb 106.105) was added to each well at a dilution of 1:200 with 1% BSA and 1x PBS. This was left to incubate overnight at 4°C. The following day the wells were washed with PBS and then stained with secondary antibody (Goat-anti-mouse-alexa 594) at a dilution of 1:500 with 1% BSA and 1x PBS. This incubated for 1 hour, before the wells were washed a final time with PBS. A drop of ProLong antifade reagent or fluorogel was added to microscope slides. Flourogel was used in the first experiment and allowed for DAPI staining. DAPI stains the nucleus with a blue stain. The later experiments were carried out with ProLong, which effectively seals the slide and prevents fading of the fluorescence, while still staining for DAPI. Each coverslip was placed face down on the drop of ProLong/fluorogel and the bubbles were removed. The slides were allowed to dry at room temperature in a dark area for 24 hours.

Cholera Toxin B

The V-34403 Vybrant Alexa Flour 488 Lipid Raft Labeling Kit was purchased for this project. This kit allowed us to stain for lipid raft domains using the protocol provided with the kit. It was performed after any FSH treatment but prior to staining for the FSHR. The cholera toxin B (CTxB) subunit is added first with a fluorescent marker added with it, while the wells are on ice. Next the CtxB conjugate is added which cross-links the labeled lipid rafts into distinct patches on the plasma membrane (Vybrant-protocol). At this point the staining for the FSHR would be done as described above.

Imaging

Results of the fluorescent staining were imaged using an immuno-fluorescent microscope or a confocal microscope. Both microscopes allowed for imaging of both fluorescent signals and allowed for a "merge" of these images to show if the signals were colocalized. The confocal allowed for "series" imaging. This showed cross-sectional cuts through the cell, which gave images of both signals from one side of the cell all the way to the other side. This was an advantage for comparing the images and being able to visualize what is happening throughout the plasma membrane, not just on one side. The Leica confocal imaging microscope and program was used to complete this imaging.

Results:

Fluorescent Staining of FSHR on HGrC1 cells

Initially, the staining for FSHR with treatments of FSH showed that our antibodies worked effectively to image the location and distribution of the FSHR. The receptor was imaged using the red fluorescent antibody and the nucleus was blue (due to DAPI staining). The staining showed that FSHR was diffuse across the membrane when no ligand was present; when no signaling is occurring, no aggregation of receptor was observed. When exposed to FSH for 30 minutes the cells showed clear aggregation of receptor on the plasma membrane. Not all cells showed signs of strong aggregation. Those that did display grouping showed variable levels of aggregation. The 15 minute treatment and 5 minute treatment showed subtler grouping of receptor along the plasma membrane. The images displayed below are representative of the patterns observed of each treatment (Figure 5). These projects relied on a large number of images being compiled in order to fairly compare each treatment. These "typical" pictures show the trend that was observed. Extreme cases were also observed. A cell with receptors that aggregated at a high level is shown below, as an example of how these treatments varied, and also because it emphasizes the fact that this aggregation is occurring (Figure 6).

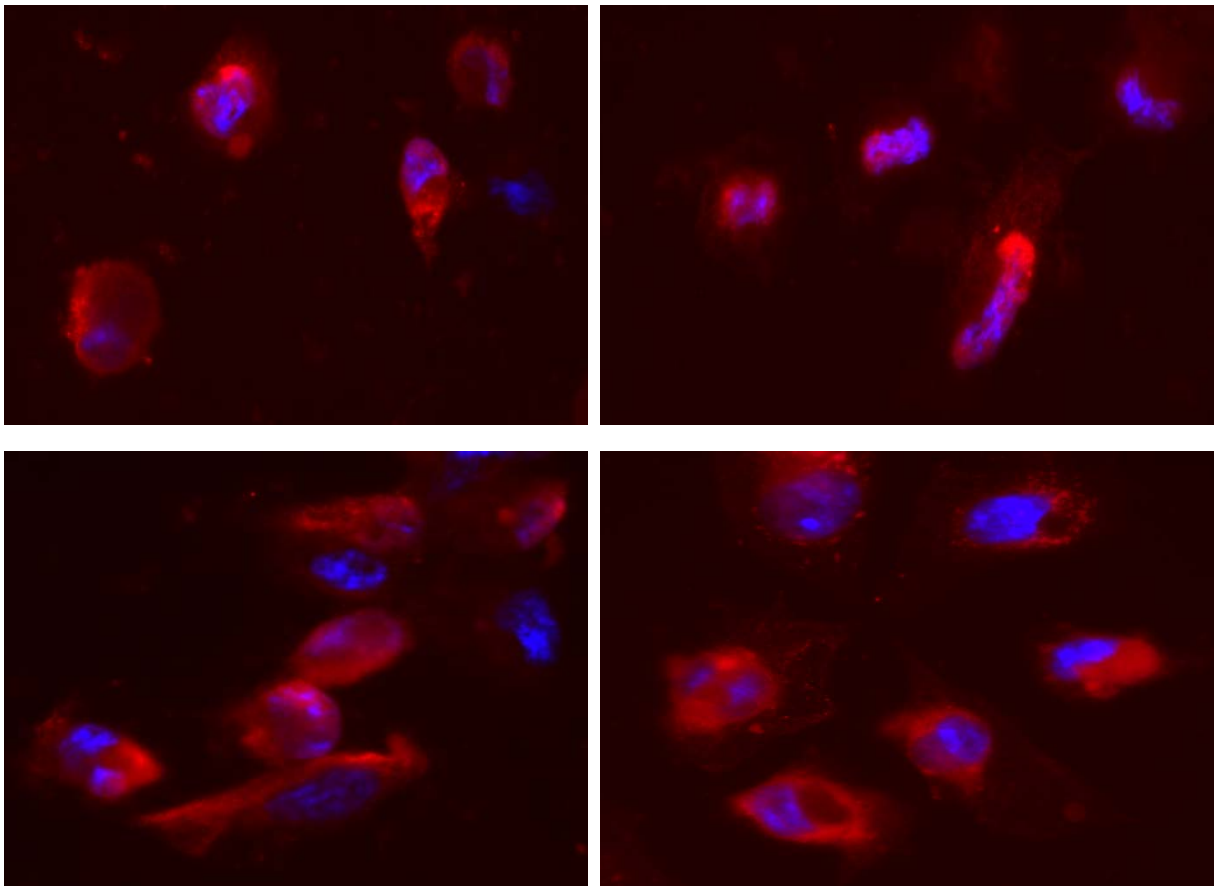

Figure 1. Top Left. No FSH treatment. Top Right. 5 minute FSH treatment. Bottom Left. 15 minute FSH treatment. Bottom Right. 30 minute FSH treatment.

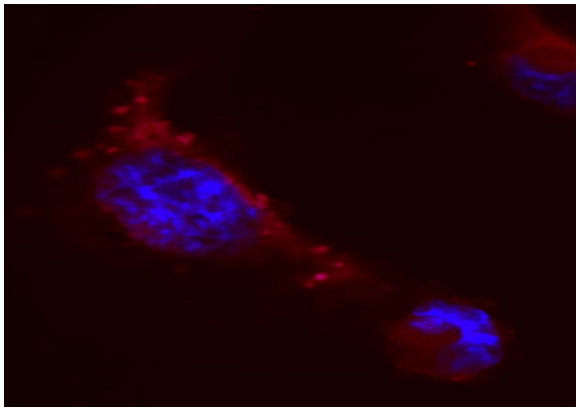

Figure 2. Cell treated with FSH for 30 minutes and then stained for FSHR in red and DAPI staining in blue. This shows extreme aggregation of receptor.

Nystatin Treatments

Following that result, we carried out the same staining with treatments of FSH, but prior to staining we treated the cells with nystatin. Again FSHR was stained for using a red fluorescent antibody. Nystatin was effective in disrupting the formation of these aggregations in the 30 minute FSH treated cells. The staining of these cells shows receptor is spread over the plasma membrane in a diffuse fashion. These cells looked very similar to the cells that were not treated with FSH. The loss of aggregation of the FSHR in the nystatin treated cells was consistent and repeated. As in the previous experiment, the images below are representative of the many images taken and trials completed of this experiment (Figure 3).

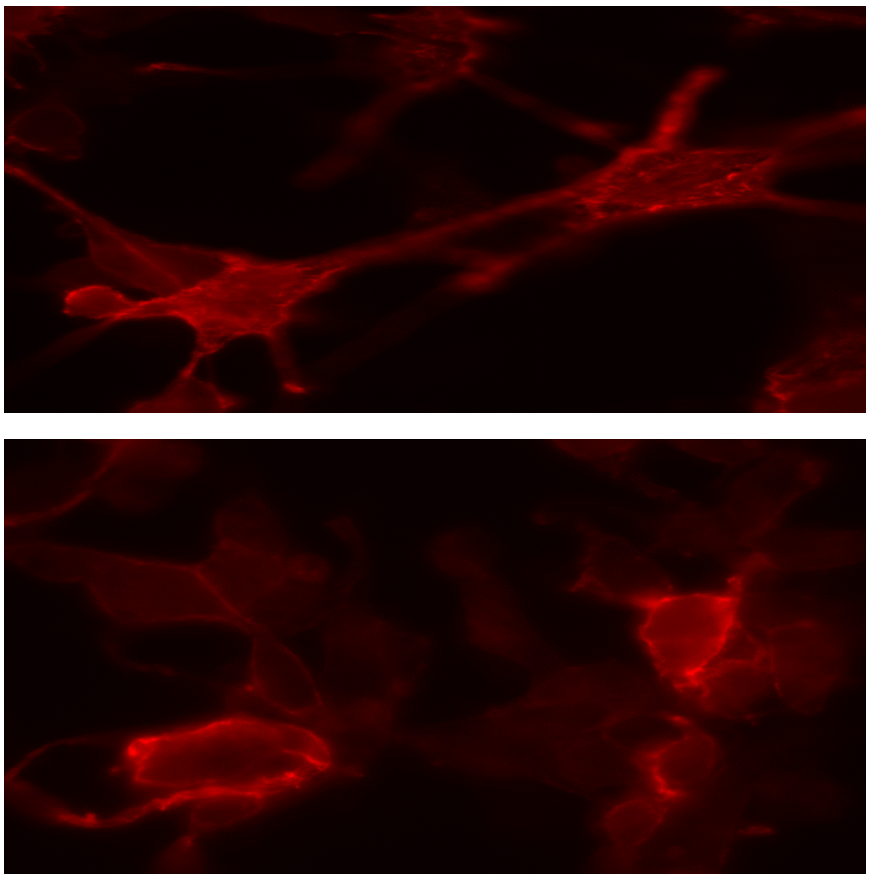

Figure 3. Top. HGrC1 cells treated for 30 minutes with FSH and stained for FSHR. Bottom. HGrC1 cells treated with nystatin, then FSH for 30 minutes, and stained for FSHR.

Cholera Toxin B Staining

Using the confocal microscope images and "series" type videos were taken of the HGrC1 cells stained for FSHR and the lipid raft marker (using CTxB). These images showed undeniable colocalization of FSHR and lipid rafts in all of the FSH treated cells. The receptor was stained using a red fluorescent antibody, and the lipid raft antibody (CTxB) had a green fluorescent marker. Colocalization of these two signals resulted in areas of yellow. Any red or green observed was a sign of these two signals not being colocalized. All three treatments of FSH showed colocalization of the two signals throughout the plasma membrane. The cells treated for 30 minutes had the most consistent colocalization of these two signals, but it varied from cell to cell. The cells treated for 5 minutes and 15 minutes also displayed strong colocalization. The results of the no FSH treatment cells were not as clean. Two trials were completed. In the first trial the cells that did not receive the FSH treatment showed clearly less colocalization than the treated cells. In the second trial more colocalization was observed, and these cells appeared similar to the cells who received treatment. These results show colocalization of the FSHR and the lipid raft when signaling and when no ligand is present (Figure 4). No quantification or statistics could be carried out on this visual data, because of software limitations. Despite the lack of quantification, there was an observable increase in colocalization after hormone was added.

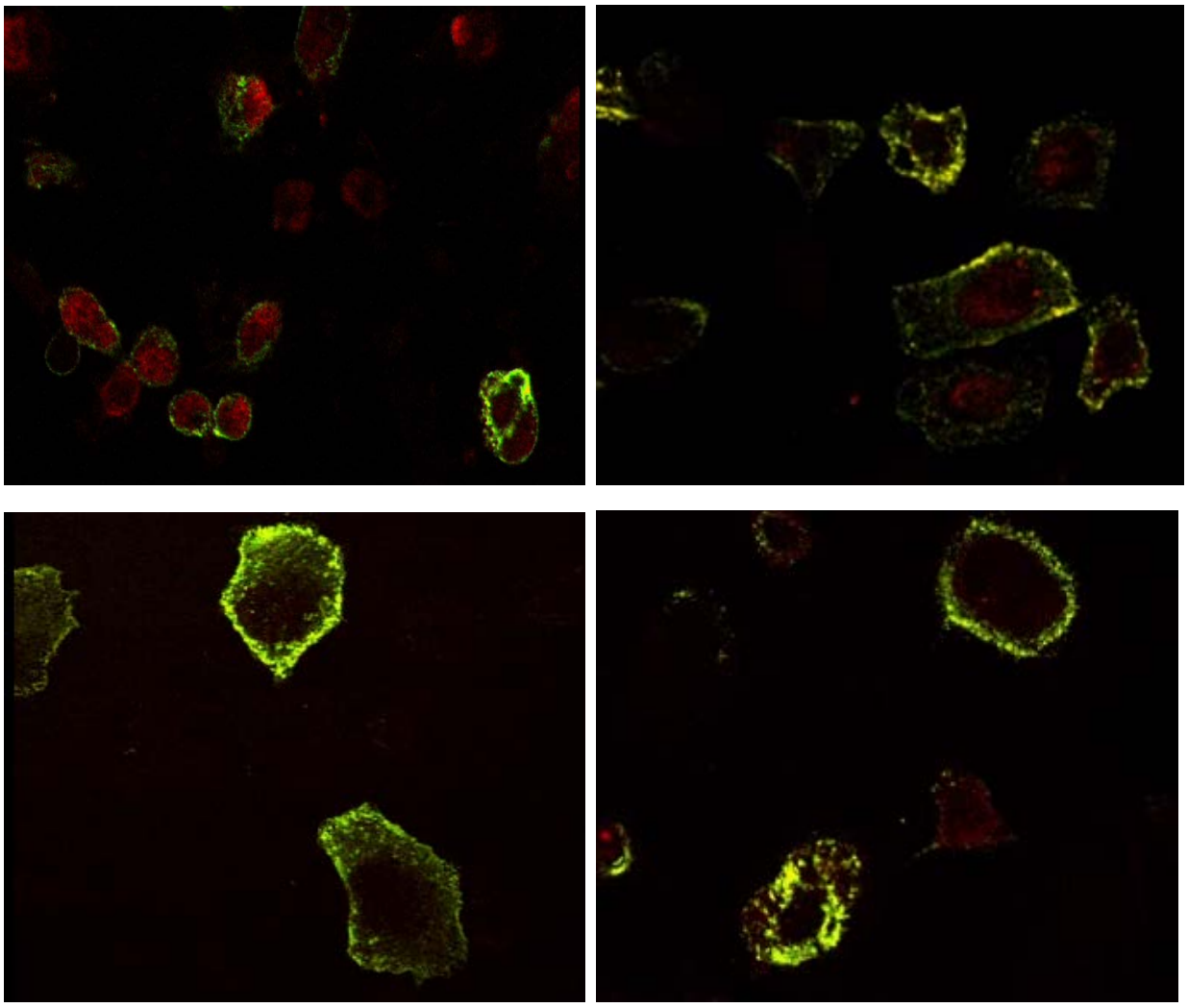

Figure 4. Top Left. No FSH treatment, marginal colocalization. Top Right. Treated with FSH for 5 minutes, shows colocalization. Bottom Left. Treated with FSH for 15 minutes, shows colocalization. Bottom Right. Treated with FSH for 30 minutes, shows strong colocalization.

Discussion

The goal of this project was to address was whether FSHR utilizes lipid raft domains when signaling. The results discussed above show convincing visual evidence of colocalization of the FSHR in lipid rafts. The early experiments show the rationale for the use of cholera toxin B, which had been shown to be very effective in other studies. Initial staining for FSHR and treating with FSH show very subtle and unremarkable differences in staining in most cases. There was however certain cells that showed clear aggregation and this, similar to the Roess, et al. study, lead us to believe that our receptor may be aggregating in lipid raft domains when signaling (Roess, et al., 2006). This is more likely than the receptors aggregating in random areas because of the knowledge that LHR and other G protein coupled receptors group in lipid raft domains when actively signaling (Roess, et al., 2006, Chini & Parenti, 2004).

The cells which were treated with nystatin and FSH resulted in receptors that displayed diffuse staining. This loss of aggregation is evidence that the lipid raft disrupting drug is effectively hindering the aggregation of FSHR. This was the first evidence that the FSHR is in fact grouping in lipid raft domains and not arbitrarily on the membrane. While the evidence shown is convincing, it is difficult to compare these pictures and come to definite conclusions because the differences between the images are very minute and vary slightly from cell to cell. The dissatisfaction with these results caused us to look for a different technique to effectively test our hypothesis.

The benefits of using cholera toxin B have been discussed and this was our next strategy. This staining effectively showed colocalization of the FSHR and lipid rafts during signaling. In the cells not treated with FSH there was some colocalization observed but there was a pattern

that the treated cells generally had more colocalization. This is visual data which relies on a comparison based on slight differences in staining. These antibodies work effectively, so the signals are fairly strong. This makes it even more difficult to make significant conclusions as to the movement of the receptor when signaling, compared to when not. However, these results show evidence that the FSHR utilizes lipid raft domains. Whether the FSHR moves into lipid raft domains at a significant amount, when signaling, is not supported or refuted by these results statistically. If the colocalization of these signals could be calculated and quantified, and statistics could be carried out, it would allow us to make more conclusions concerning the kinetics of the receptor's interaction with lipid rafts. These results do so strong localization with and without treatment which strongly supports the fact that lipid rafts are important for FSHR signaling.

This study shows a line of evidence supporting the hypothesis that FSHR aggregates in lipid raft domains when signaling. Using various microscopy techniques we were able to stain for receptor and compile evidence that supported the initial hypothesis. Further work concerning the quantification of the cholera toxin b staining results could lead to even more interesting conclusions.

Further studies with cholera toxin B would also be useful. We have shown colocalization after treatment with FSH when the cells are stained and fixed after 30, 15 or 5 minutes. It would be interesting to see if the FSHR was given time to signal, the FSH had dissipated, and the cell was back in normal conditions, if this colocalization is lost. Perhaps if we tested FSH treatments for 60 minutes or 120 minutes and then stained using both the FSHR antibody and the CTxB kit, we could learn how long it takes the receptor to move out of the lipid raft domains after signaling has ended. Although I focused on microscopy and fluorescent antibodies, other members of my

research team used very different techniques including co-immunoprecipitation, and transfection of a GFP-labeled lipid raft marker. These projects showed similar results, which support the results of this study.

If the cells treated with nystatin are unable to effectively signal when FSH is present it would further support the importance of lipid rafts in the downstream signaling of this GPCR. We have shown that nystatin is effective in disrupting the aggregation of FSHR, but from this study it cannot be concluded as to whether this loss of aggregation affects the FSH signaling. However this would be a logical assumption to make, because this receptor would not aggregate when signaling unless there was some advantage to doing so.

The approach of using fluorescent antibodies and microscopy allowed us to view the location of the receptor without disturbing or destroying the cells. Also the relatively short amount of time needed for each experiment allowed us to compile a large number of images. This library of images can continue to grow in future experiments and be interpreted further using new software, and compared with new research. This was effective for testing our hypothesis, but the lack of quantification leaves the study with a certain amount of unmet potential. Despite this downfall, this study has shown considerable empirical evidence that FSHR aggregates in lipid raft domains when signaling.

If the FSHR relies on lipid rafts to effectively signal, which this research supports, it would allow for new therapeutic strategies. Medications that affect the lipid raft domains in the gonads specifically could be used to affect fertility. The composition of these microdomains is known; therefore cholesterol sequestering drugs or similar medications could be modified to fit this purpose. It could also be a new developmental explanation for infertility. Some people may

have ample amounts of functioning receptors, but if these receptors rely on lipid rafts, and their cells lack lipid rafts in sufficient numbers they may not have mature gametes. These would be interesting consequences along with further evidence of the importance of lipid raft domains in cell signaling.

References

Bayasula, Iwase, A., Kiyono, T., Takikawa, S., Goto, M., Nakamura, T., Nagatomo, Y., Nakahara, T., Kotani, T., Kobayashi, H., Kondo, M., Manabe, S., and Kikkawa, F. Establishment of a human nonluteinized granulosa cell line that transitions from the gonadotropin-independent to the gonadotropin-dependent status. *Endocrinology* (2012) 153:6;2851-2860.

Chini, B. and Parenti, M. "G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there" *Journal of Molecular Endocrinology.* 32 (2004) 325-338.

Conn, P. Michael. *Receptor-Receptor Interactions.* Academic Press; 1 ed. Vol. 117. (2013).

Dias, J.A., Cohen, B. D., Lindau-Shepard, B., Nechamen, C. A., Peterson, A. J., and Schmidt A. "Molecular, structural, cellular biology of follitropin and follitropin receptor" *Vitamins and Hormones*. 64 (2002) 249-322.

Drake, J., Putnam, M., Moquin, A., Merrihew, M., Outcalt, C., Sorge, E., Caballero, A., and Gondre-Lewis, T. "Lipid raft independent B cell receptor mediated antigen internalization and intracellular trafficking". *The Journal of Immunology.* 170 (2003) 905-912.

Fan, Q. R., and Hendrickson, W. A. "Structure of human follicle-stimulating hormone in complex with its receptor" *Nature.* 433 (2005) 269-277.

Holt, Richard I. G., and Neil A. Hanley. *Essential Endocrinology and Diabetes*. Malden, MA: Blackwell Pub., (2007).

Insel, P.A., Head, B.P., Patel, H.H., Roth, D.M., Bundey, R.A., Swaney, J.S. "Compartmentation of G protein coupled receptors and their signaling components in lipid rafts and caveolae" *Biochemical Society Transactions* 33 -5 (2005) 1131-1134.

Ostrom, R.S., and Insel, P.A. "The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology". *British Journal of Pharmacology.* 143, (2004) 235–245.

Peterson, B. R., "Synthetic mimics of mammalian cell surface receptors: prosthetic molecules that augment living cells" *Organic Biomolecular Chemistry* 3 (2005) 3607-3612.

Roess, D. A., Smith, S. M. L., Lei, Y., Liu J., Cahill, M. E., Hagen, G. M., and Barisas, B. G. "Luteinizing hormone receptors translocate to plasma membrane microdomains after binding of human chorionic gonadotropin" *Endocrinology* 147-4 (2006) 1789-1795.

Simoni, M., Gromoll, J., and Nieschlag, E. " The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology" *Endocrinology Reviews.*18-6. (1997) 739-773.

Telikicherla, D., Ambekar, A., Palapetta, S. M., Dwivedi, S. B., Raju, R., Sharma, J., Prasad, TS. K., Ramachandra YL., Mohan, S. S., Maharudraiah J., Mukherjee, S. and Pandey, A. "A comprehensive curated resource for follicle stimulating hormone signaling" *Bio Medical Central*. 4 (2011) 408-414.