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Investigating the Role of the Cholesterol Recognition/interaction Amino-acid Consensus Sequence in Follicle Stimulating Hormone Receptor Function and Structure

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Investigating the Role of the Cholesterol Recognition/interaction Amino-acid Consensus Sequence in Follicle Stimulating Hormone Receptor Function and Structure

By

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Acknowledgments

This research is near and dear to my heart as I have worked on this project for the past 3 years. The success of this project would not have been possible without the support of my Union College community.

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Abstract

Human infertility is a complex disorder that can often be attributed to a dysfunction of the endocrine system. Follicle-stimulating hormone (FSH) is one of many hormones that participate in a complex process in both women and men to regulate normal reproduction. The dysfunction of this hormone and its receptor are some of the many causes of infertility. FSH is secreted by the anterior pituitary and, in women, initiates a cascade of biological events that enable ovulation. FSH carries out its function by binding and activating specific receptors. The FSH receptor (FSHR) is a G protein-coupled receptor (GPCR) that is located in the cell membrane of target cells in the ovaries and testes. GPCRs often interact with a variety of molecules beyond their ligands including, in some cases, cholesterol in the plasma membrane. The Cholesterol Recognition/Interaction Amino Acid Consensus Sequence (CRAC) is one such binding domain and is represented by the sequence LV-XXXX-Y-XXXXX-K/R. The human FSHR contains a sequence consistent with the CRAC motif in the first intracellular loop L-TTLQ-Y-KLTVP-R. We hypothesized that mutating this domain will negatively affect the signaling of the receptor upon ligand activation. To test this hypothesis tyrosine 375 was mutated to phenylalanine (Y375F) which should render the CRAC inactive. After establishing a cell line stably expressing the mutant receptor, activation of the p44/42 (ERK1/2) pathway was measured. Preliminary data suggest that signaling of the hFSHR-Y375F mutant is decreased relative to the wild-type receptor. As we learn more about this receptor, we continue to gain knowledge about conditions such as infertility. This information can facilitate the development of innovative treatments which can help many families who struggle to conceive.

Introduction

Follicle Stimulating Hormone

Infertility affects about 12% of women, aged 15 to 44, nationally; in approximately 35% of infertile couples, it was found that the male also contributed to the inability to reproduce (Aguirre-Ulloa). Conditions involving reduced ovulation, including ovarian failure, account for 1 in 4 cases of infertility (CDC). Furthermore, low sperm levels, formally known as oligospermia, can also contribute to the inability to conceive (Mayo Clinic). Some of these causes of infertility can be attributed to defects in the effects of follicle-stimulating hormone (FSH). FSH is secreted by the anterior pituitary and, in women, initiates a cascade of biological events that enable ovulation (Mayo Clinic). In males, FSH regulates testosterone production; low FSH levels are often a direct cause of oligospermia (Mayo Clinic). Hormones, including FSH, carry out their role as signaling molecules by binding and activating specific receptors.

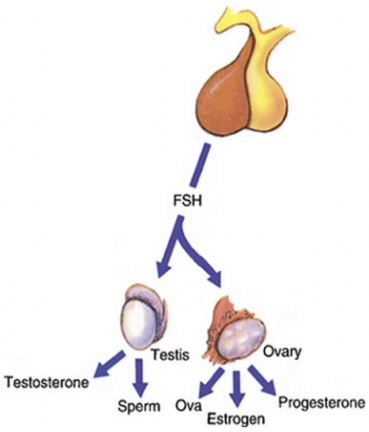


Figure 1. Illustration of downstream endocrine effects of FSH secretion from the anterior pituitary gland.

The FSH receptor (FSHR) is fundamental in the complex process of reproduction. In women, it is crucial in the regulation of menstrual cycles. Studies suggest that women who express homozygosity for a variant FSHR experience higher rates of infertility (Aguirre-Ulloa). Moreover, males who lack proper FSHR function experience decreased sperm production (Tapanainen, Aittomäki, Min, Vaskivuo, & Huhtaniemi, 1997). An understanding of FSHR behavior may lead to a better understanding of infertility.

The FSHR is a G protein-coupled receptor (GPCR) that is located in the cell membrane of target cells in the ovaries and testes. Within the membrane, these receptors are located in

microdomains with high cholesterol concentrations, which are known as lipid rafts (Geoghegan, 2011). The binding of FSH to FSHR causes an increase of intracellular cAMP, eventually leading to activation of PKA and sex steroid production and cell metabolism.

G-Protein Coupled Receptors

In general, G-protein coupled receptors are characterized by initiating a multitude of downstream effects.

These receptors constitute a unique subset of cell surface receptors that cross a membrane a total of 7 times. Ligand binding occurs in the extracellular space whereas G-protein function occurs in the cytosol (Norton 2018). The generic G-protein that is responsible for the function of a receptor has 3 subunits: α, β, and γ (Norton 2018).

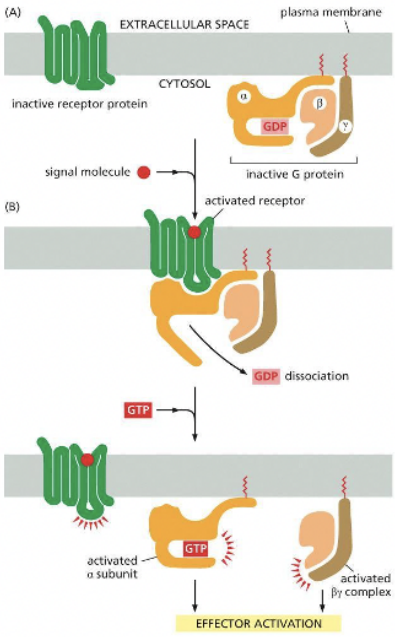


Figure 2. Basic mechanism of GPCR activation after ligand binding.

The receptor remains inactive when the α subunit has a GDP molecule bound; however, once a ligand binds to its respective GPCR, a conformational change occurs which dissociates GDP from the α subunit (Norton 2018). This allows the α subunit to proceed with the binding of GTP which causes activation of the G-proteins in other subunits. In this way, ligand binding leads to the transduction of a signaling cascade by initiating the conformational change of a G-protein. Each activated subunit will then go on to affect additional cellular proteins, this cascade continued until a desired cellular response is achieved (Norton 2018).

The following are three examples of GPCRs that really exemplify the importance of this subset of cell surface receptors. The β_2 adrenergic receptor relies on the binding of adrenaline and noradrenaline for the successful completion of muscle contraction, a highly complex process

(Rosenbaum, Rasmussen, & Kobilka, 2009). In the presence of light, the rhodopsin receptor causes a signaling cascade that terminates in the brain's perception of visual stimuli (Jastrzebska). Lastly, the majority of serotonin receptors are classified as GPCRs and have been of recent interest due to their success as targets for medicinal usage. The successful binding of serotonin is responsible for a myriad of biological functions from appetite, memory, and breathing to peristalsis, blood coagulation, and vasoconstriction (McCorvy). Rosenbaum et. al said it best: "as the receptors for hormones, neurotransmitters, ions, photons, and other stimuli, GPCRs are among the essential nodes of communication between the internal and external environments of cells."

Follicle Stimulating Hormone Receptor

Follicle-stimulating hormone signaling transduction is achieved through interactions with its specific G protein-coupled receptor, termed the follicle-stimulating hormone receptor. The FSH signaling pathway initiates when the beta subunit of FSH interacts with FSHR, though recent studies have suggested that the alpha subunit may also be implicated in this interaction, Figure 3 (Fan & Hendrickson, 2005).

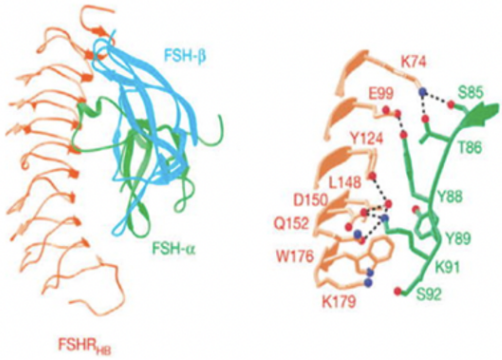


Figure 3. Interaction between α (green) and β (blue) subunits of FSH with its receptor, FSHR (orange). Left-most figure displays specific residue interactions.

This binding stimulates cyclic adenosine monophosphate (cAMP) production. cAMP then binds to the Regulatory (R) and Catalytic (C) subunits of Protein Kinase A (PKA), Figure 4 (Liu, Ke, Zhang, Zhang, & Chen, 2020). The binding of cAMP decreases the affinity of these subunits for each other, which allows for the C subunit to phosphorylate

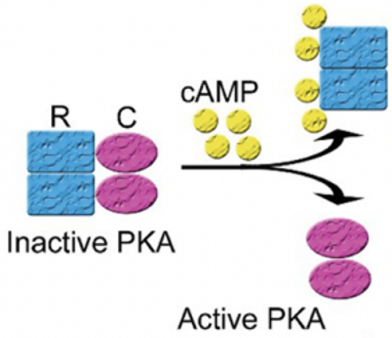


Figure 4. Protein Kinase A (PKA) activation via cAMP binding to regulatory subunit dimer.

downstream targets for signal transduction. PKA dissociation leads to the direct phosphorylation of the cAMP response element-binding protein (CREB) or indirectly by phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK 1/2)(Liu, Ke, Zhang, Zhang, & Chen, 2020).

Binding of the follicle-stimulating hormone to its GPCR results in downstream effects such as mitogenic signals which regulate the cell cycle and steroidogenic signals which are responsible for the production of sex hormones (Casarini & Crépieux, 2019). This signaling cascade has also been implicated in a number of survival signals as well and a summary is provided in Figure 5 (Casarini & Crépieux, 2019).

The important thing to note is these signals only occur with proper receptor function, which is dependent on the success of FSH binding.

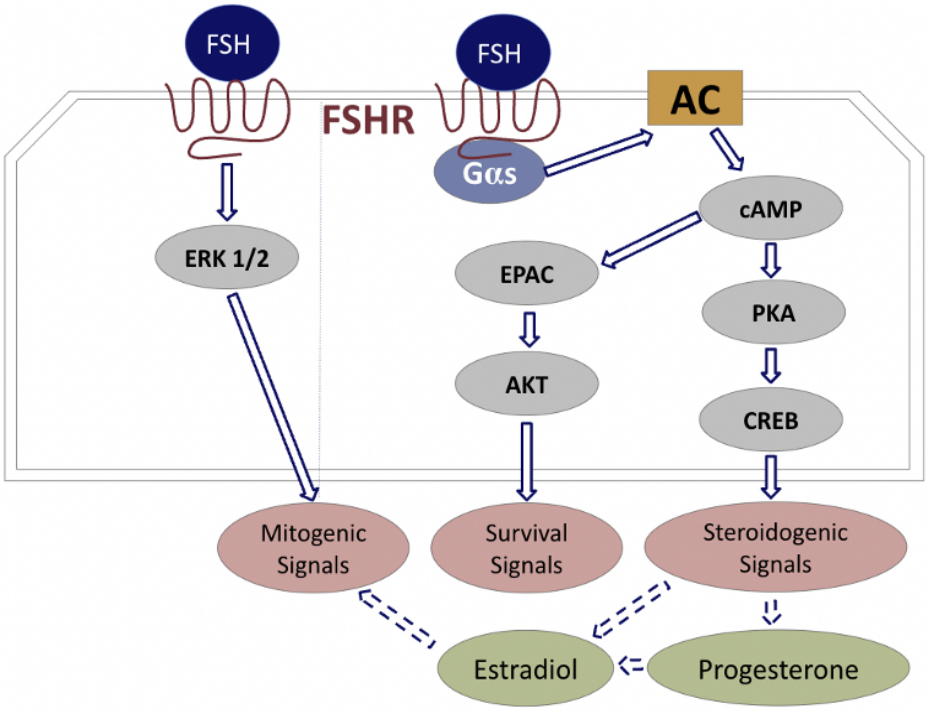


Figure 5. Pathway displaying downstream effects of FSH binding to hFSHR. Research will focus on ERK 1/2, or p44/42 MAP kinase activation.

Cholesterol and G-Protein Coupled Receptors

Though it has been previously established that membrane cholesterol affects GPCR's activity, the specific interaction between them is still being investigated. Cholesterols' amphiphilic nature makes them an important biomolecule. Its polar hydroxyl groups prefer aqueous environments while its nonpolar body exhibits hydrophobic characteristics. Its unique behavior in aqueous environments makes it especially useful for membrane composition. The plasma membrane is also composed of other amphiphilic molecules such as phospholipids and hydrophobic molecules such as glycolipids. Within the membrane, cholesterol functions to increase rigidity at high temperatures and decrease fluidity at low temperatures. Furthermore, GPCRs are likely to be found in lipids rafts, or areas of high cholesterol concentration within the membrane. Geiger et. al has further hypothesized that "cholesterol acts as an allosteric modulator for GPCR function" (Geiger et al., 2021). They also found that while cholesterol affects ligand binding directly through membrane fluidity, it also accounts for the activation equilibrium via membrane curvature.

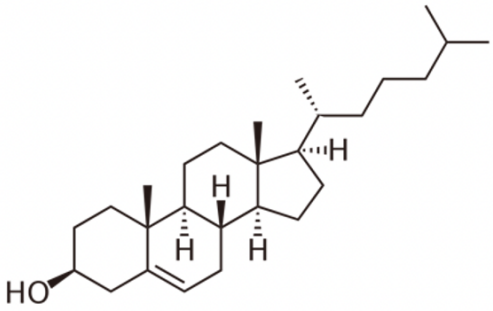


Figure 6. Chemical structure of cholesterol. Cholesterol is amphiphilic due to its polar hydroxyl group and non-polar body.

Cholesterol Recognition/interaction Amino-acid Consensus (CRAC)

Recent studies have held the cholesterol recognition/interaction amino-acid consensus (CRAC) sequence as responsible for cholesterol interaction with the plasma membrane. An L/V near the amino end, a K/R near the carboxyl end, and a highly conserved Y within the middle region of the sequences indicated the presence of this specific cholesterol binding domain. This presents itself as the following sequence of amino acids: L/V – XXXX -Y – XXXXX – K/R.

CRAC is a cholesterol-binding domain that is found in many GPCRs such as Rhodopsin,]Beta-adrenergic, and Serotonin receptors (see Figure 7) (Jafurulla, Tiwari, & Chattopadhyay, 2011).

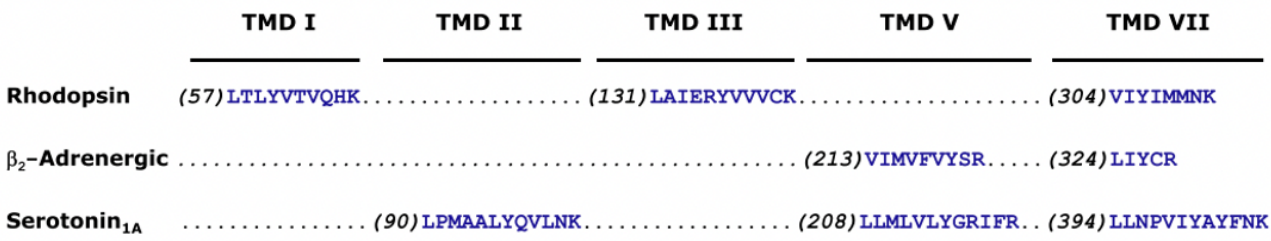


Figure 7. Presence of conserved CRAC sequences in Rhodopsin, β-Adrenergic and Serotonin GPCR. CRAC was present in transmembrane domains (TMD) 1, 3 and 5 in the Rhodopsin receptor, TMD 4 and 5 in the β-Adrenergic receptor and TMD 2, 4 and 5 in the Serotonin receptor.

In a 2011 study by Jafurulla et. al, three conserved CRAC sequences were identified in the serotonin receptor across many species; see yellow amino acids within transmembrane domains II, V, and VII (Figure 8A) (Jafurulla et al., 2011). The high level of conservation seems to suggest that CRAC has presented an evolutionary advantage to the correct function of the serotonin receptor (Figure 8B).

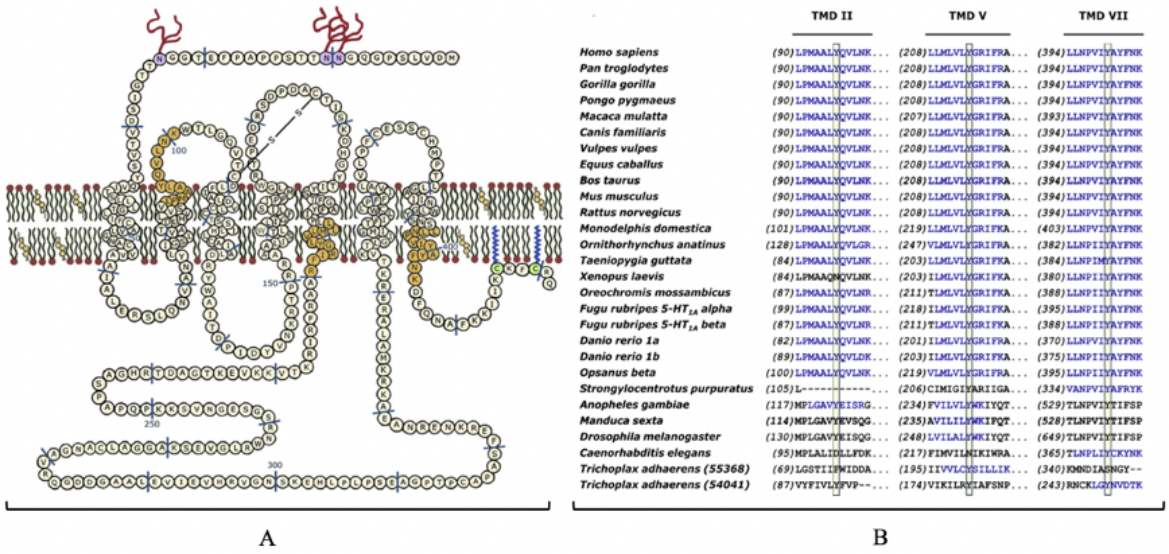


Figure 8. (A) CRAC sequence in transmembrane domains 2, 5 and 7 of serotonin receptor – CRAC residues highlighted in yellow; **(B)** Sequence comparison between conserved CRAC among 28 different species.

Furthermore, Geiger et. al found a CRAC sequence (Figure 9) within the seventh transmembrane domain among 38% of class A GCPRs studied. One of these GCPRs, β_2AR, is known to be sensitive to membrane cholesterol. When tryptophan 326 of β_2AR's CRAC was mutated, expression was abolished. The high level of conservation of CRAC among class A GCPR along with the evident consequence of mutating the CRAC of β_2AR suggests the importance of this cholesterol-binding domain in GCPR function (Geiger et al., 2021).

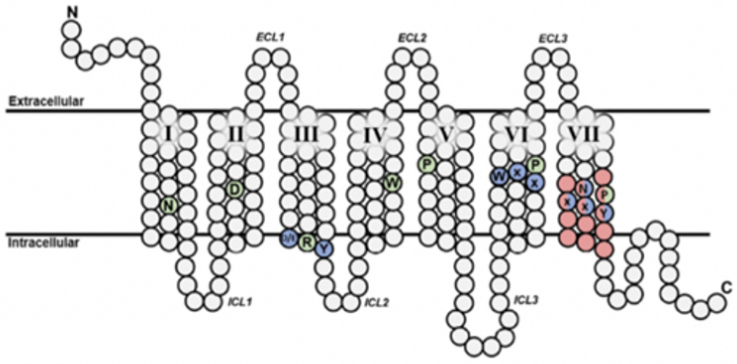


Figure 9. Conserved CRAC sequence (transmembrane domain 7) found within 38% of class A GPCR's studied; reg regions indicate a CRAC sequence while blue regions indicated areas of high conservation.

My Research

CRAC In FSHR

This research examines a CRAC sequence in hFSHR. As shown in Figure 10, the sequence of amino acids for the CRAC motif consensus matches a sequence of amino acids found in the first intracellular loop of the hFSHR. In other GPCR, the presence of CRAC seems to be vital in cholesterol binding (Fantini & Barrantes, 2013) and normal receptor function (Geoghegan, 2011; Oddi et al., 2011). This suggests that in the absence of CRAC, cholesterol-binding will decline, leading to the diminution of lipid rafts, and the eventual loss of FSHR function.

Three amino acids are responsible for the interaction with cholesterol Leucine/Valine, Tyrosine, and Lysine/Arginine. Figure 10A depicts the theoretical interaction between these residues and cholesterol.

Combined with the fact that tyrosine is the only non-variable residue in the consensus and the procedure of previous research, I have decided to mutate this tyrosine to phenylalanine to learn more about this pathway. It was found that while phenylalanine can structurally replace tyrosine, it is impossible for phenylalanine to achieve the hydrogen bonding necessary for proper signal transduction (Fantini & Barrantes, 2013).

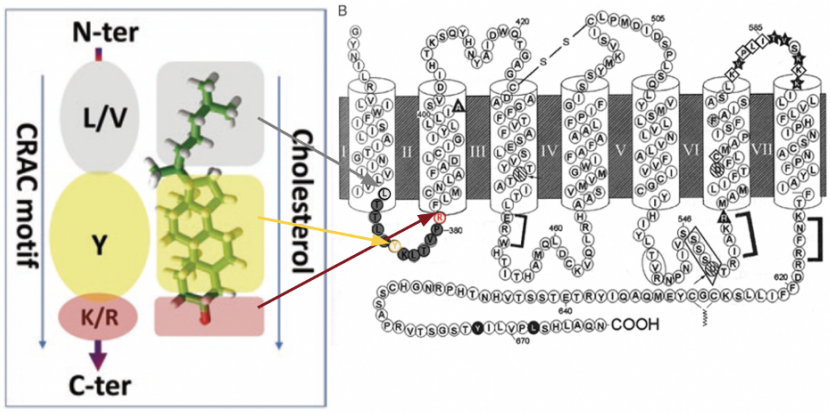


Figure 10. Identification of a Cholesterol Recognition/interaction Amino Acid Motif in the human FSH Receptor. **(A)** Illustration of the transmembrane domain of the hFSHR with shaded circles identifying the location of the putative hFSHR Cholesterol Recognition/interaction Amino Acid Motif; tyrosine 375 circled in yellow **(B)** Alignment of the CRAC consensus sequence with the amino acid sequence of hFSHR

Hypothesis

I believe that the Cholesterol Recognition/Interaction Amino-Acid Consensus (CRAC) Sequence is important for cholesterol binding and proper FSHR structure and function.

Mutagenesis and Transfection

Using a Q5 site-directed mutagenesis kit from New England Biolabs, I mutated the original tat (encoding for tyrosine) of the FSHR to ttt (encoding for phenylalanine).

```
ctaactaccagccaatataaactcacagtccccaggttc
 L  T  T  S  Q  Y  K  L  T  V  P  R  F
ctaactaccagccaatttaaactcacagtccccaggttc
 L  T  T  S  Q  F  K  L  T  V  P  R  F
```

The procedure for this mutagenesis is as follows: Amplification, Treatment, and Transformation. A polymerase chain reaction (PCR) was used to amplify mutated DNA, treatment was achieved using KLD and DPNI enzyme mix, and transformation was performed on competent E.coli cells. See Figure 11 for the NEB-provided procedure summary.

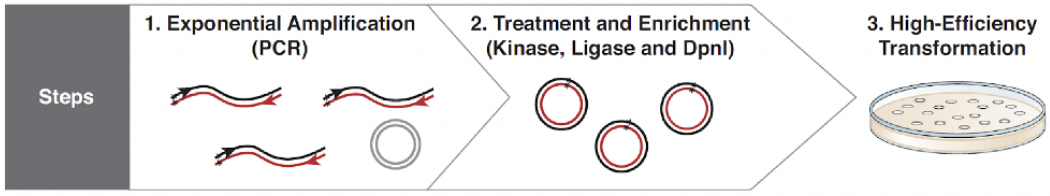


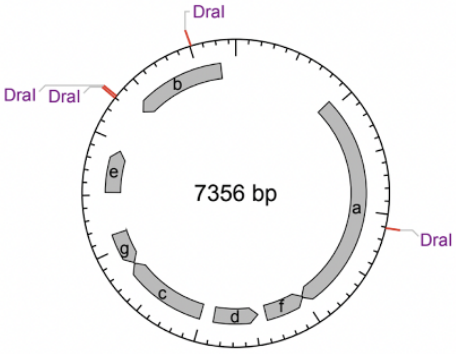
Figure 11. New England Biolabs mutagenesis procedure

In preparation for the annealing step of PCR both forward (red) and reverse (blue) primers were designed using a primer tool provided by New England Bioloabsas displayed below. The Y375F mutation has been bolded and underlined while the adjacent DRAI restriction site has been underlined.

5′ CATAGTGCTAGTGATCCTAACTACCAGCCAA**<u>ttt</u>**<u>aaa</u>CTCACAGTCC 3′

3′ GTATCACGATCACTAGGATTGATGGTCGGTTAAATTTGAGTGTCAGG 5′

In addition to incorporating the mutation into the plasmid, we added a restriction site for the endonuclease DRAI for the confirmation of the mutation. The mutant plasmid should produce fragments of 4,192 bp, 2,453 bp, and 692 bp (Figure 12). The 19bp fragment was determined to be insignificant.



#	Ends	Coordinates	Length (bp)
1	DraI-DraI	2108-6299	4192
2	DraI-DraI	7011-2107	2453
3	DraI-DraI	6319-7010	692
4	DraI-DraI	6300-6318	19

Figure 12. Model of Y375F mutant plasmid with DRAI restriction enzyme site. Predicted fragment of 4,192bp, 2,453bp and 692bp.

Colonies were harvested and their DNA was extracted and digested by the DRAI enzyme mix. Two of the clones selected displayed the correct fragments, as seen in Figure 13.

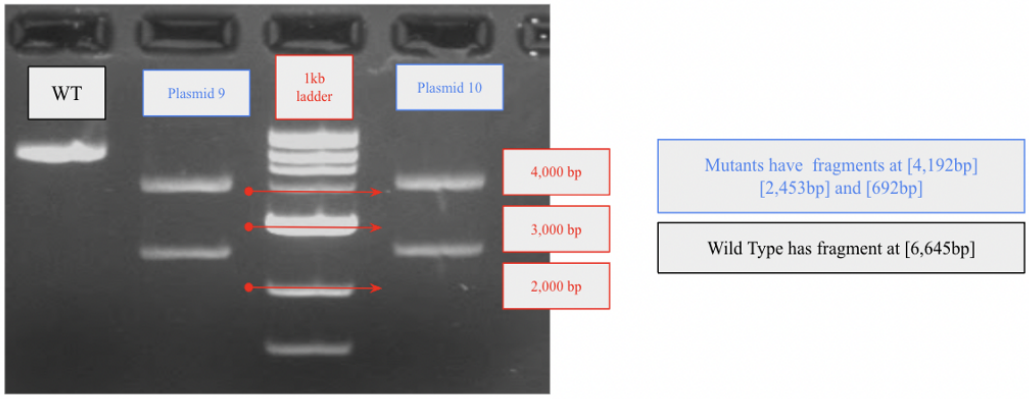


Figure 13. Potential Y375F plasmids were digested with the enzyme DRA1. Mutants have fragments of 4192, 2453, and 692bp (not shown on gel). Wild Type has fragments of 6,645 and 692bp (not shown on gel).

Once it was confirmed that the correct mutation had been incorporated into the plasmid, my next step was to transfect this DNA into human embryonic kidney cells (HEK293) to create a stable cell line for more advanced signaling experiments. The mutant plasmid was combined with a Transit IT transfection reagent. This complex then entered the cell membrane via endocytosis and mutant DNA was taken up by the cell. Transcription and translation then allowed for the formation of mutant Y375F-FSHR-HEK293 cells which will be used moving forward. This process is illustrated in Figure 14 (ibidi.com).

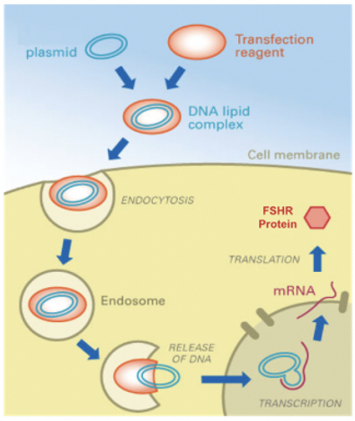


Figure 14. Illustration of transfection reaction. Transit IT transfection reagent was used; Y375F mutant plasmid was used.

Before running any expression experiments, the presence of an intact receptor needed to be confirmed. Without an intact receptor, any change in signaling would be erroneously attributed to Y375F. To achieve this, an anti hFSHR monoclonal antibody was used to detect the presence of the receptor. As seen in Figure 15, signaling present in Y375F was almost identical to that of wildtype HEK293-hFSHR.

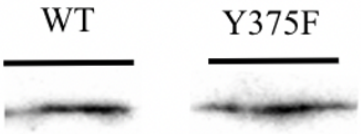


Figure 15. Western blot comparing hFSHR expression into cells stably expressing either wild type (WT) or the Y375F mutant. An anti hFSHR monoclonal antibody was used to detect the presence of the receptor

Experimental Approach

This experimental approach included comparing untreated cells to cells with the wild type hFSHR in which cholesterol was removed with detergent to cells expressing the hFSHR-Y375F mutant receptor. The detergent used was methyl-beta-cyclodextrin or MBCD. Treatment of cells with MBCD is a common tool when attempting cholesterol depletion in plasma membranes. The HEK293 cells were separated into two categories, those implicated in MBCD treatment and those with the Y375F cells. In each case, wildtype HEK293-hFSHR was to serve as a control; those that served as a control in MBCD treatment will be referred to as "untreated" moving forward.

In both groups, respective cells were grown to about 80% confluency, see Figure 16 for example. Both cells were serum-starved for an hour. Cells within the MBCD group were treated with 5mM MBCD in the last 30 minutes of serum starvation. A 30-minute FSH treatment time course was performed; FSH was added at 30 minutes, 15 minutes, and 5 minutes. In summary, the experimental approach remained the same for each group outside of the addition of detergent in the last 30 minutes of serum starvation for MBCD cells.

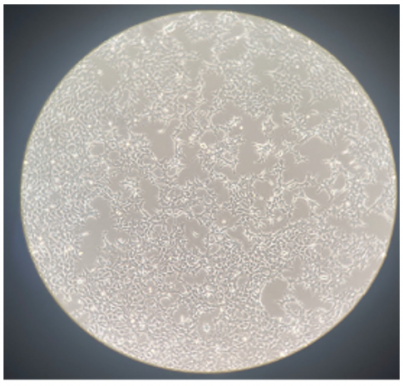


Figure 16. Example of 80% confluency of HEK293 cell line. Once this confluency was achieved, cells were used for FSH time course.

At the completion of the time course, cells were harvested and lysed. A BCA assay was performed to standardize samples in preparation for western blot analysis. Western blot analysis is a tool used, in combination with SDS-PAGE, for detecting the presence of proteins within a sample. Cell samples are lysed, standardized by concentration, and run through an acrylamide gel matrix. Once gel electrophoresis is complete, proteins are transferred to a porous membrane and are treated with specific antibodies to detect the relative abundance of each protein.

Results and Discussion

p44/42 MAP Kinase Activation

This project investigated the activation of ERK 1/2 with an anti-phospho-p44/42 MAP kinase antibody. This pathway was chosen for two reasons: First, it is one of two major downstream pathways activated by the FSH receptor, the other being cAMP (Casarini & Crépieux, 2019). Second, previous research in our lab had demonstrated that MBCD affects p44/42 MAP kinase activation more significantly than cAMP.

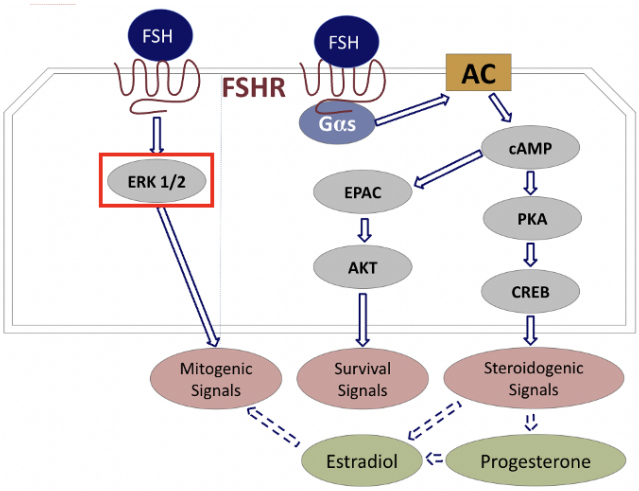


Figure 17. Pathway displaying downstream effects of FSH binding to hFSHR. Research will focus on ERK 1/2, or p44/42 MAP kinase activation (red box).

MBCD treated vs. Untreated

In untreated cells there is minimal activation of the ERK 1/2 pathway at time 0 and then an increase at 5 minutes with stable activation at 15 minutes and 30 minutes of FSH treatment. Treated cells exhibit an increased signal at time 0 and time 5 compared to the negative control.

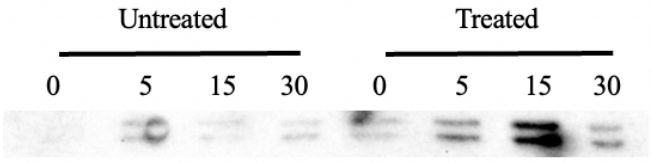


Figure 18. Western blots comparing HEK293 cells treated with methyl-beta-cyclodextrin (MβCD) and untreated cells. Hormone treatment time course was in minutes. An anti-phospho-p44/42 MAP kinase antibody was used to test signaling. Increased p44 MAP kinase activation is seen at basal levels at at 5 minutes.

hFSHR-WT vs hFSHR-Y375F

In wildtype FSHR, there is minimal activation of the pathway at time 0 with increased activation at 5 minutes and sustained activation at 15 minutes and 30 minutes. Y375F cells displayed increased signaling compared to the control at time 0 and time 5.

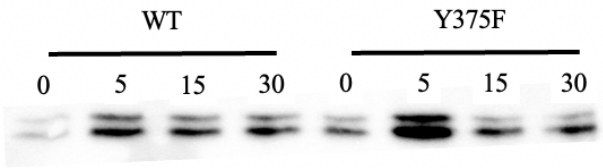


Figure 19. Western blots comparing WT-HEK293 and Y375F-HEK293 signaling Hormone treatment time course was in minutes. An anti-phospho-p44/42 MAP kinase antibody was used to test signaling. Increased p44 MAP kinase activation is seen at basal levels at at 5 minutes.

This indicates that the Y375F mutation produced similar activation patterns in cholesterol-depleted plasma membranes.

We predicted that Y375F should disrupt cholesterol interaction with FSHR, but previously it was unclear whether MBCD signaling was due to lipid raft disruption of cholesterol depletion. The consistency of results between these 2 approaches suggests the effect on the pattern is a result of cholesterol interaction with the receptor, not lipid raft interaction with the receptor. This conclusion can be drawn due to the fact that Y375F is only implicated in cholesterol interactions and not lipid raft interactions.

Future Research

Future research includes investigating how cholesterol interactions affect receptor localization. Previous research within the Cohen lab has found that lipid rafts are important for FSHR localization and that cholesterol is an important component of lipid rafts.

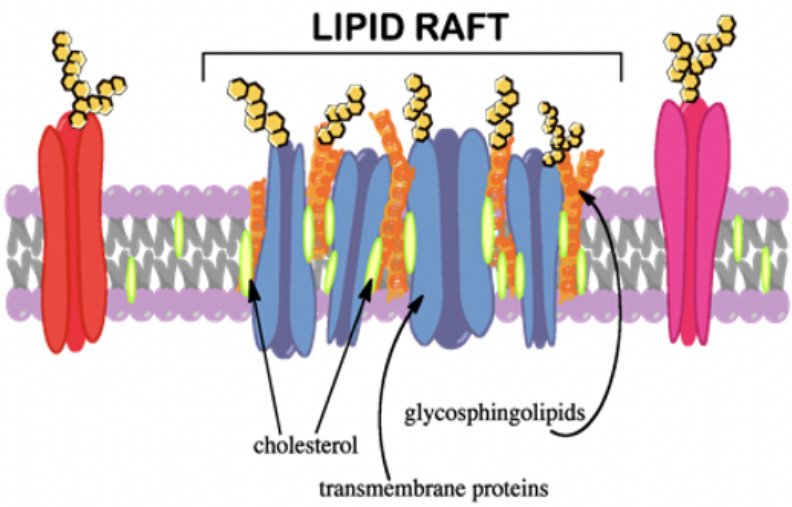


Figure 19. Illustration of a lipid raft in a plasma membrane demonstrating cholesterol interaction with transmembrane proteins and sphingolipids

This project has concluded that Y375F displayed similar signaling to cholesterol-depleted cellular membranes. Future research will thus investigate if Y375F shows the same membrane localization as the wild type receptor to determine if Y375 is involved in targeting hFSHR to lipid rafts, potentially by binding to cholesterol, which is at high concentration in the raft domains.

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