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FSH Treated Cells and the Effects on Cell Signaling

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By

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Introduction: The endocrine system is essential to the management of homeostasis (source 1). The endocrine system is such an integral part of human survival and homeostasis because it involves almost every cell, tissue, and organ within the body. The endocrine system functions by the secretion of chemical signals, referred to as hormones, by a collection of cells called glands. These hormones are passed through the blood stream to reach their target, which possesses a receptor specific to a given hormone (source 1). These endocrine glands are not to be confused with exocrine glands, which secretes chemical signals through a gland's duct opening to an epidermal surface instead of the bloodstream (source 2).

Hormones of the endocrine system can be broken down into two general classes (source 2). First, there are the peptide hormones, which are chains of various amino acids and they interact with their target cell through a receptor on the outer membrane of responsive cells. Following the binding to an extracellular receptor, a cascade is initiated to generate a second messenger, inducing a response in the target cell. The other class of hormones is the steroid hormones, which are synthesized from cholesterol precursors. Unlike peptide hormones, steroid hormones are lipid soluble and pass through the extracellular membrane and bind to a specific receptor protein in the cytoplasm or the nucleus. This receptor–hormone complex then binds to the cell's DNA. This binding of the receptor protein to the DNA can either increase or decrease the transcription of specific mRNA (source 3).

One of the many target tissues that is part of the endocrine system are the gonads. Gonads are typically paired organs in animals and they are responsible for producing reproductive cells or gametes. Males possess testes, which produce spermatozoa and the female ovaries produce ova (source 4). The male testes produce the hormone testosterone, which helps maintain the secondary sexual characteristics such as growth of the penis and testes, development of body and facial hair, deepening of the voice, increased muscle strength, and increase in height (source 5). The ovaries produce the hormones estrogen and progesterone, which are also responsible for maintaining the female secondary sexual characteristics as well as controlling the menstrual cycle (source 5). The regulation of the sex hormones is done via the hypothalamic-pituitary-gonadal axis. . The hypothalamus is located in the middle of the base of the brain and it is thought of as the main control center of the endocrine system (source 6). The hypothalamus releases gonadotropin- releasing hormone (GnRH), which affects the anterior pituitary gland. The anterior pituitary has a vascular connection with the hypothalamus called the hypophyseal artery (source 6). The pituitary gland, in response to GnRH releases many hormones, two of which are the gonadotropins, leutinizing hormone (LH) and follicle stimulating hormone (FSH). Both FSH and LH are responsible for the release of the previously mentioned sex hormones (source 7).

The gonadotropin hormones have roles other than stimulating secondary sex characteristics. FSH stimulates Sertoli cells to produce the protein called the androgen binding protein, which promotes the process of spermatogenesis thereby creating sperm. Also, FSH promotes the sertoli cells to produce inhibin which acts as a negative feedback mechanism on the anteritor pituitary gland, which causes a decrease in the secretion of FSH. In males, LH

stimlutes leydig cells to produce testosterone, which also participates in the negative feedback mechanism by acting on the anterior pituitary gland and hypothalamus.

In a female, estrogen acts as a negative feedback mechanism when the estrogen levels are low. Also, estrogen is involved as a positive feedback mechanism because it is in high concentration at the end of the follicular phase and it acts as a positive inducer of the anterior pituitary. This positive feedback causes the anterior pituitary to release more FSH and LH. This in turn causes the ovary to produce more estrogen. This eventual spike in LH causes the ovulation of the ovum. The hormone progesterone is responsible for stimulating vascular activity, which prepares the uterus for the implantation of the embryo. Increasing vasculature of the uterus means the thickening of the uterus lining. After ovulation, the corpus luteum, which is the yellow body left over in the ovary after the secondary oocyte is expelled from ovulation, begins to secrete progesterone. However, the corpus luteum is only a temporary structure and when it regresses, progesterone levels fall and the thick vascularized lining in the endometrium regresses and then sloughs off (Source 8).

Both FSH and LH are responsible for the development of the egg, not just thickening of the endometrium and causing the release of the secondary oocyte. Although there are many factors that are involved in the development of the egg and its eventual fertilization, without the action of FSH, it would not be possible. This goes to show that many women who are unable to become pregnant might possibly have issues regarding the expression or activity of FSH. For example, when women get older and the number of eggs a woman has left is running low, it takes a higher amount of FSH to produce an egg (source a). If a woman is unable to provide enough FSH hormone, then she is unable to produce an egg that could be fertilized.

Infertility is a major issue that many women confront. Unlike men who generate sperm cells from puberty until the day they die, women are born with a set number of eggs. With a set number of eggs, there is a limited number of opportunities for a woman to get pregnant before menopause is reached (**Source b)**. Menopause is the failure of the ovaries to ovulate due to a lack of functional follicles. Following menopause, women can no longer become pregnant and there are many other changes experienced in relation to change in hormone levels (**source c).** Approximately 15% of couples in the United States have difficulty conceiving a child **(Source b).** Also, starting at the age of 30 a woman's probability of conceiving a child decreases 3-5% each subsequent year. The connection between infertility and heightened levels of FSH raises the possibility that understanding FSH action in granulosa cells may help women who face infertility issues.

A hormone secreted from a gland is useless without its receptor. In order for the target tissue to respond to a chemical message, a receptor is necessary to initiate a cascade of events to achieve the desired response from the target cell. FSHR is a member of the G-coupled protein receptor (GPCR) family and it is characterized as a transmembrane protein. FSHR has 7 domains that span the plasma membrane of the cell and the intracellular portion is coupled to a G(s), which starts a signal cascade upon the extracellular portion interacting with FSH (Source 12).

One aspect of the receptor that deserves attention is lipid rafts. Lipid rafts are organized along the membranes that possess highly organized microdomains of lipids that influence the distribution of colocalized proteins. With the phospholipids regions contain lipid rafts that are enriched with sphingolipids and cholesterol the saturated fatty acids can pack tightly. This tight arrangement takes away from the typical fluidity of a phospholipids bilayer

and it is considered to have more order (**Source d)**. In these lipid rafts are proteins, which are either located in the liquid disordered regions or are partitioned within the ordered raft domains. Some examples of such proteins are glycosylphosphatidylinositol (GPI) anchored proteins that are attached to the outer leaflet of the membrane and the Src- family tyrosine kinases that are anchored to the inner leaflet. One particularly identifiable raft structure is caveolae, which appear as flask-shaped invaginations within the plasma membrane. Another component of lipid rafts is the flotillin molecules, which are non-caveolar proteins that organize and bring together the microdomains that compose the actual rafts. In an attempt to reveal different biochemical properties of the lipid raft itself detergent resistant membrane (DRM) components were revealed with the extraction by Triton X-100. Scientists have determined that lipid rafts may only engage in membrane activity if its components are clustered together. These clusters exist on both sides of the plasma membrane and flotillins promote this by acting as clustering agents. In these cluster formations specific sets of cell signaling are accomplished (**Source E)**.

One of the membrane functions that lipid rafts take part is trafficking. In regards to trafficking, caveolae mediate endocytosis into the cell. It has been shown that cross-linked GPI anchored proteins translocate to caveolae and are eventually endocytosed. There have also been cases where non- enveloped viruses such as SV40 utilize the caveolae as a means to enter the cell through endocytosis. When cholesterol depletion of lipid raft components occurs, the endocytosis of objects is redirected to other internalization mechanisms. With the ability to adjust and still be able to endocytize objects, the lipid raft demonstrates plasticity.

Lipid rafts' ability to perform signal transduction is crucial. Lipid rafts form concentrated platforms for receptors that are activated by ligand bonding. For signaling systems like tyrosine kinase signaling, adaptors, enzymes and scaffolds are recruited to the inner part of

the cytoplasmic membrane after ligand activation occurs. Upon receptor activation, the signaling complex that is formed is protected from non- raft enzymes that could affect the signaling pathway. An example of a non- raft enzyme is phosphatase. In the process of raft binding, the recruited proteins are in a new environment where the phosphorylation process can be altered by local kinases and phosphatases. The signaling platforms that lipid rafts form amplifies signaling the formation of raft clusters. Raft clusters form through a network of adaptors, scaffolds, and anchoring proteins coming together (source F). Raft clusters from the liquid- disordered lipid matrix then insulate the signaling complex that forms. This formation leads to an amplification of cell signaling by creating a high concentration of signaling molecules and by excluding undesired modulators. The process of forming these signaling platforms is described as both dynamic and reversible. These raft clusters can be disassembled by negative modulators or by endocytosis, which removes parts of lipid rafts. The movement of these raft clusters is influenced by cytoskeletal infrastructure as well as secondary messengers (source f).

Future research regarding lipid rafts involve further understanding both the structure and function. If the structure of rafts can be more specifically identified, then it can be understood whether more than one type of raft exists on the cell surfaces of other types of cells. In doing so, more raft- associated proteins and lipid composition need to be determined on both the exoplasmic and cytoplasmic sides of a particular cell. The proposed method of further identifying lipid rafts involves isolating a single raft or ligand- activated raft. Although detergent leads to lipid raft aggregation, it is not possible to isolate a lipid raft in its preserved state. A method needs to be developed in order for lipid raft structure to be accurately depicted. Also, processes such as cluster formation during signal transduction is not very well understood. To properly understand this process real- time imaging of the assembly of signaling complexes in

both normal and cholesterol depletion would be most ideal. Another aspect of lipid rafts that needs to be clarified is the caveolae's function during signal transduction. Some questions scientists would like answered include: whether caveoloae enrichment is induced by cluster formation, what protein signals are required for caveolar trapping, and how the internalization of caveolae is regulated (source f).

Lipid rafts are an important topic of research in regards to FSH receptors. FSH accomplishes its actions including the maturation of an egg and estrogen production by inducing gene expression in a target granulose cell via a signaling cascade. There are numerous signaling cascades involved with FSH such as extracellular regulated kinases (ERKs), p38 mitogen-activated protein kinases (MAPKs), and phosphatidylinositol- 3 kinase (PI3K). The upstream kinase that seems to responsible for the initiation of signals that lead to gene expression is protein kinase A (PKA). The activity of this kinase includes direct phosphorylation of transcription factors such as cAMP, it promotes remodeling of chromatin by phosphorylating histone H3, and it enhances the activity of the p38, MAPK, ERK, and PI3K pathways. Both research and accumulating evidence suggests that the initiation of a single pathway is not enough activate target gene expression. Instead, target gene expression is initiated by multiple pathways that rely on eachother through cross- talking (source g).

In our experiments we will analyze the role of lipid rafts in FSH-dependent signal transduction by manipulating the lipid raft structure. MβCD (Methyl-Beta-Cyclodextrin) is used to manipulate the lipid rafts by removing cholesterol, which alters signaling proteins within the lipid raft (Krishnan 2004). Other studies have utilized the method of depleting the lipid rafts of cholesterol with MβCD to test the function of lipid rafts (Draber 2002). In our experiment, we plan on demonstrating that the manipulation of the lipid rafts will reveal varying amounts of

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activation of the MAP kinases in response to FSH (Miyoshi 2007) and (Gonzalex- Robayna 2000).

Methods: Our experiment begins with cultivating 293R cells and placing them in 4 of the 6 wells within each 6-well dish. We then allow for these cells to grow to the point at which they have 80% confluence. This experiment requires two different 6-well dishes because one dish represents cells treated with just Follicle Stimulating Hormone (FSH) (control experiment) and the other 6-well dish will represent cells that are treated with MβCD as well as FSH (MβCD experiment). To prepare the media for each experiment, 10mL of serum free media are placed in two separate conical tubes. The conical tube that will be used for MβCD treatment has 66mg of MβCD added to create a 5mM concentration. Prior to adding this media, we must aspirate and remove the cell media that the cells were previously bathed in. After aspiration, 0.9mL of serum free media is added to each well of our control experiment and 0.9mL of serum free media with MβCD is added to each well of the MβCD experiment. Next these 6- well dishes are placed in a _____incubator for an hour. During the incubation time 5µL of FSH stock is added to 500µL of both serum free media with the MβCD and to the serum free media without the MβCD. Also a lysis buffer is prepared by adding both one tablet a protease inhibitor and one tablet of a phosphatase inhibitor to 10mL of Triton-DOC. Once an hour has passed, the 6 well dishes should be retrieved from the incubator so they may be treated with FSH. The FSH treatment begins as soon as 100µL of each experiment's respective serum free mixed with FSH stock is added. This 100µL FSH solution will only be added to one well at this point in time for each experiment. Following each FSH treatment the 6 well dishes should be placed back in the incubator until another FSH treatment is needed. A timer should be set so that it counts down from 30 minutes. The 6- well dishes will be treated at the following times during the timer's countdown at 15 minutes, 5 minutes, and 0 minutes. At 0 minutes both 6- well dishes should be placed on ice in order to end cell activity in response to FSH. After the wells are be aspirated

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2mL of 1x PBS is added to each well. After aspiration again, 0.5mL of the prepared lysis buffer needs to be added to each well. The 6- well dishes, at this point, should still be placed on ice. After 10 minutes has passed _____ (name of the blue swuegee thingys) are used to scrape the surface of each well. The lysis buffer solution that remains in each well should be pipetted around to gather all the cells in a pool located at the bottom of each well upon tilting the 6- well dishes. The cells and the lysis buffer are gather with a pipette tip and placed in their own respective microfuge tubes. After the contents of each well are extracted the contents of each microfuge tube representing their respective wells should be dounced with approximately 10 strokes and the liquid contents are placed back in their original microfuge tube. After douncing the contents from each well, the douncing equipment should be washed with warm water, dionized water, and then dried with Kim Wipes. The microfuge tubes are then paced in a centrifuge located in a 4°C cold room and are centrifuged at max speed for 10 minutes. The supernatant is then extracted and placed in new microfuge tubes. The remaining pellets should be saved. These samples placed in the microfuge tubes are then used to complete a BCA assay. Next, 100µL of 2x sample buffer is added to each microfuge tube and then it is boiled in using at heat block at 70°C for 5 minutes.

To prepare the samples for a Western Blot, a 12.5% resolving gel needs to be created. In a 50mL conical tube the following is to be added: 5mL of water (DI), 6.25mL acrylamide, 3.75mL 4x running buffer, and 150µL 10%SDS. The final two components are not to be added until the Western Blot apparatus is prepared and ready to have resolving gel injected into it. These two final components include 50µL 10% APS (0.1g of APS and 900µL of water (DI)) and 10µL of TEMED. This resolving gel solution is enough to add the appropriate amount to the necessary two Western Blot apparatuses. After 3.5 mL (how much resolving gel should be added to each

apparatus) is added, isopropanol is added until it reaches the top of the apparatus. The resolving gel should solidify in 45 minutes. After solidification, tilt the apparatus to remove excess isopropanol and use whantman paper to remove any residual remaining isopropanol. The next step is to prepare the stacking gel which requires 6.1mL of DI water, 1.3 mL of acrylamide, 2.5mL of 4x stacking gel, 100µL 10% SDS, 50µL of 10% APS, and 20µL of TEMED. Again, both the 10% APS and TEMED should not be added until you are ready to add the stacking gel to the apparatus. Once the stacking gel is injected on top of the solidified layer of resolving gel, it should take 45 minutes for the stacking gel to solidify. The apparatus should be submerged in the Western Blot tub and filled with 1x electrophoresis buffer. Next your samples should be loaded into the different wells of the gel. Typically a protein marker is used in the first well followed by the rest of the protein samples. Once all of the samples are loaded, a power source runs a current through the gel. The voltage should be set at 100V and once the protein markers pass the stacking gel, the voltage is increased to 150V. Once the protein markers have nearly reached the bottom of the gel, the power is turned off and the gels are removed. The gels and p-membrane are soaked in transfer buffer for 5 minutes. Prior to soaking the p-membrane in transfer buffer, the p-membrane is to be dipped in methanol. Next, the gel, whatman paper, and p-membrane are used to construct a "gel-transfer sandwich," with 3 pieces of whatman paper, 1 p-membrane, gel, and 3 pieces whatman paper starting from the bottom. The whatman paper should be dipped in the transfer buffer prior to constructing the "gel-transfer sandwich." The dimensions of the p-membrane and whatman paper should be 2.5 inches by 3.25 inches. The transfer sandwich is then placed in the transfer apparatus and is set at 15V for 15 minutes. After the transfer is completed, the p- membranes are placed in a sealed freeze-dried food bag with 20mL of a solution with 5 grams of powdered milk and 100mL of TBST. The sealed bags are

incubated in the cold room over night on top of the belly dancer. When resuming the experiment the following day, the sealed bag should be opened to allow for the removal of the milk solution. Next, each blot is probed with its designated primary antibody. The solution inserted in the new freeze-dried bag is 10mL of the TBST and milk mixture and 5μL of its respective primary antibody. The freeze-dried bag containing the probed plots is placed on the belly dancer in the fridge overnight. Prior to placement on the belly dancer, air bubbles should not be present in the sealed bag. The following day, the blots are removed from their respective bags and are washed for three rounds with 50mL of TBST for 5 minutes each round. Next, a secondary antibody is prepared with 20mL of TBST and 4μL of antibody. The solution is mixed and 10mL is placed in each separate freeze-dried bag with its own blot. Once the air bubbles are removed, the blots are placed on the belly dancer for another hour. The blots are then washed with 50mL of TBST again for 3 rounds, each lasting 5 minutes. The blots are removed from their respective freeze-dried bags and residual solution is shaken off. The blots are then layered on top of a sheet of syran wrap. The luminol kit is used and 2mL of both solutions (solution A and solution B) are mixed together in a conicle tube and then are placed evenly on top of each blot. After 5 minutes of the luminal solution interacting with the blots, the blots are ready to be analyzed with the Fuji Film Image Reader.

Results: We finally got results on our Western Blots when looking for activated p44 MAP Kinase. The image provided in Figure 1 is a 5 min exposure of the Western blot of total p44 on the left and phosphorylated p44 on the right. The numbers located above the blots listed as 30, 15, 5, and 0 represent the length of time the cells were treated with FSH. There are bands located at each lane for the time treatments of 30, 15, 5 and 0 for the blot that tests for total p44. This was a expected result, and for the blot that was testing for phosphorylated p44 we were expecting a band to appear in lane representing a 5 minute hormone treatment. This band can be visualized on the blot on Figure 1, but a better image of this band is provided by the 30-minute exposure time as seen in Figure 2.

Discussion: While running gels that tested for phosphorylated p44 in the pathway of FSHR we had difficulty with getting blots that didn't have high background. These blots having high background looked rather unusual and blotchy (Figure 3). With Western Blots that lacked defined bands, we had to figure out what part of Western Blot protocol we needed to change. One of the first things we tried to change was the blocking reagent used. We tried 5% milk, 5% BSA, and 3% gelatin as our blocking reagents and neither of these changes provided normal blots. Then we considered decreasing the primary antibody as well as the secondary antibody and that didn't seem to bring success either. Next, we tried decreasing the secondary antibody and that was not the solution to resolving our problem of high background either. It wasn't until we switched our secondary antibody completely that we finally had success. The secondary antibody we originally were using was called goat-anti-rabbit HRP and so was the new secondary antibody we had success with, but the difference was the company that made it. Jackson Immunoresearch, West Grove, PA, produced the original secondary antibody that provided a blotchy looking blot and the company of the new secondary antibody that we had success with was Invitrogen, Carlsbad, CA. Other research supports our results of activation of the p44 pathway. One research group found that HEK293N cells stimulated the phophorylation of Erk (p44) MAP kinases through β-arrestins (Source 8). After analyzing the signal transduction pathway of GPCR to MAPK/Erk we noticed that there are two different pathways that lead to Erk (Source 9). One pathway leads to Erk through the internalization of the GPCR and the other pathway leads to Erk through camp indirectly activating Erk (Source 9). We would like to question whether FSHRs utilize one or both of these pathways. Research data lends support that activated cAMP, which independently phosphorylates Erk, occurs when the FSHRs have a low plasma membrane density (Source 8). This data has lead us to believe that changing

the signal pathway occurs based on changes in the receptor density within the plasma membrane (Source 8). Despite finding data that might answer our questions about the activation of p44 we have considered future studies to provide us with conclusive results in regards to determining which of the two Erk pathways FSHR takes to eventually activate p44.

In our future studies determining whether p44 activation takes one pathway instead of the other would require us to understand the differences between the two pathways that we are aware of. One pathway to p44 requires the internalization of the GPCR and the other pathway requires cAMP to indirectly activate p44. To test to see if it is just the pathway that uses cAMP to indirectly activate p44 we would have to prevent the possibility of the GPCR becoming internalized. Preventing the internalization of the GPCR would allow us determine whether p44 is activated through cAMP indirectly activating p44 because it is the only pathway that can possibly lead to p44 activation if internalization of the GPCR is inhibited. The method in which we would run this experiment would occur through downregulation of the GPCRs through lysosomal degradation or by removing, blocking, or degrading β-arrestins (Source 10). According to this research, β-arrestins and lysosomal degradation could inhibit GPCR internalization. To test for the other potential p44 activation pathway we would have to block cAMP. To inhibit cAMP production, my suggestion would be to activate GPCRs that activate the Gi class of Ga subunits that are responsible for inhibiting the cAMP that adenylyl cyclase is responsible for producing (Source 11). By successfully blocking each of the two different p44 activation pathways we can determine which pathway FSHR signal transduction utilizes. If there is p44 activation in both of these pathways it is likely that FSHR signal transduction utilizes both pathways to activate p44.

Our future studies also involve making an addition to the methods section of the experiments that I recently carried out. The only change to this section would be treating the cells with MβCD in addition to FSH. FSHRs and their components known as lipid rafts are composed of cholesterol and MβCD has the ability to remove cholesterol. We believe that lipid rafts play a vital role in the method of signal transduction pathway of FSHRs. By destroying the integrity of lipid rafts we hypothesize that p44 activation will not occur in MβCD treated cells.

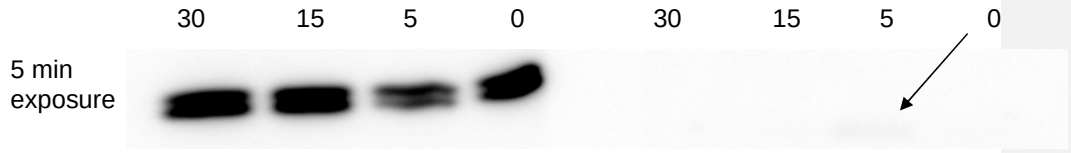
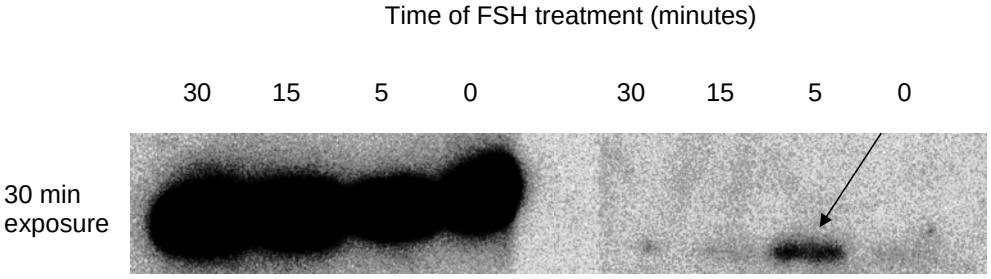


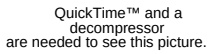
Figure 1



Immunoblot: Primary: anti-total-p44 (Cell Signaling, Beverly, MA). Secondary: goat-anti-rabbit HRP (Invitrogen, Carlsbad, CA)

Immunoblot: Primary: anti-phospho-p44 (Cell Signaling, Beverly, MA). Secondary: goat-anti-rabbit HRP (Invitrogen, Carlsbad, CA)

Figure 2


QuickTime™ and a
decompressor
are needed to see this picture.

(Figure 3).

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    e. e.) Lipid rafts and membrane dynamics Lawrence Rajendran and Kai Simons*

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i. LIPID RAFTS AND SIGNAL TRANSDUCTION Kai Simons*‡and Derek Toomre‡

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