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Characterization of Melanin-concentrating Hormone Receptor Desensitization

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by

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Abstract

Melanin-concentrating hormone (MCH) receptor 1-knockout mice have limited incidence of diet-induced obesity. This makes the MCH signaling pathway a potential pharmacological target to fight human obesity. MCHR1 is a G-protein coupled receptor (GPCR) that activates multiple signaling pathways, including ERK phosphorylation. Overstimulation of GPCR signaling is a hallmark of many diseases. Likewise, inadequate desensitization of MCH signaling could potentiate the obese phenotype. GPCR desensitization typically involves agonist-induced internalization of activated receptors, and subsequent degradation or receptor recycling. The broad aim of this study was to determine the length and intensity of ERK phosphorylation and it's desensitization to MCHR1 activation by MCH. In order to measure this, we maximally stimulated MCHR1-transfected BHK-570 cells with 100 nM MCH for 10 min, then following three washes in serum-free media and a 30 min recovery period, cells were stimulated again. Western blots of lysates for phosphorylated-ERK and total ERK were performed. ImageJ was used to normalize activation levels. MCH was unable to signal a second round of ERK signaling unless we waited 70 minutes, indicating that the MCH signaling pathway is desensitized during this period. We hypothesized that MCHR1 internalization was responsible; however using a cell-based ELISA, we only measured a 15% loss of surface MCHR1 after 30 min of MCH treatment. We tested the hypothesis that G protein-coupled receptor kinases were limiting factors in preventing agonist-mediated endocytosis of MCHR1 however none showed significant gains in internalization. We conclude that MCHR1 can undergo

receptor-mediated endocytosis, but the fraction of available receptors on the plasma membrane does not account for the extensive loss of ERK signaling observed. We also tested the effect that a GRK2 dominant negative would have on MCHR1 desensitization. In a co-transfected BHK-570 model, we did not observe desensitization if GRK2 is not present. This suggests that GRK2 is necessary for MCHR1 desensitization at the plasma membrane. We have also observed similar ERK desensitization following both isoproterenol treatment and MCHR2 activation which could suggest that simply the ERK pathway desensitizing is being observed which could be independent of the agonist. This study suggests that MCH-mediated ERK signaling desensitizes while MCHR1 is at the plasma membrane, rather than via removal of the receptor from the cell surface. Future experiments will be aimed at determining whether this ERK pathway desensitization is homologous or heterologous in addition to observing downstream pathways of MCHR1 activation other than ERK.

Introduction

Obesity

Obesity is now a national pandemic that does not only affect the United States but many other portions of the world. This pandemic has been brought about only in the last century as a result of our modern society developing. For the majority of the last century, food has been relatively inexpensive and easy to come by for most of the world. In addition to easy food access, the rise in high calorie foods and fast food restaurants has increased caloric intake more than what is needed to survive. Add in the fact that technology has removed much of the physical labor that was once needed to survive and all of the pieces of an obesity pandemic are in place (Pi-Sunyer, 2002).

The development of obesity is driven by an abnormal balance between food intake and energy expenditure, when food intake is higher than the latter. Obesity is defined as having a body mass index (BMI) of 30 kg/m^2 or higher which currently affects almost 500 million adults and 40-50 million children worldwide (Kral, 2012). Even more troublesome than the vast number of individuals that are defined as obese is the current trends related to obesity. A 30 year study in Sweden, conducted by Neovius et al. that ended in 2005, found there was a 5-fold increase in obesity among young adult men and estimated that by 2020, 4% of the adult population will be severely obese (Neovius, 2008). The Center for Disease Control and Prevention determined that between 2009 and 2010, 35.7% of the United States adults were obese. Obesity in children and adolescents was recorded at an alarming 16.7% during this time (Pi-Sunyer, 2002).

The increase in obesity has severe consequences both in regards to health and finances. Elevated BMI has been shown to increase the development of many diseases which often have high morbidity and mortality. These diseases include, but are certainly not limited to, insulin resistance, type 2 diabetes, hypertension, coronary heart disease and gallbladder disease (Pi-Sunyer, 2002). If preventing the development of these diseases is not enough motivation to fight obesity, its economic impact may be.

The US health care spending difference from normal weight adults and obese adults increased from 8 to 38% from 1987 to 2007 (Davies, G. et al., 2010). An analysis by the Office of Health Economics in England concluded that 5% of the National Health Service budget was directly related to obesity (O'Neill, 2010). In addition to health care costs, some believe that the severe obese lose a month of productivity for every year compared to normal weight individuals (Finkelstein et al., 2010). Because of the social, economic, and health consequences of obesity, it is not surprising that many organizations are starting to make curbing obesity a top priority.

Many government programs have been put in place to fight this pandemic. New laws even limit the food intake of individuals. These include modifying public school lunch programs so that children are consuming fewer calories (Department of Agriculture 2012). The programs also try to protect adults as seen in New York City's law to limit the size of soft drinks being sold (Grynbaum, 2012).

These government programs have been designed to reduce food intake and increase energy expenditure to curb obesity. In some cases however, changing diet

and exercise is not enough, which means a third factor is involved in the obesity pandemic. This third factor is related to physiology in which genetics can play a role in making individuals more susceptible to obesity.

One of the more well-studied physiological systems is the leptin system. Leptin is a hormone secreted by adipose tissue when fat storage is high and secreted less when fat storage is low. The hormone acts on the hypothalamus where it produces signals to reduce food intake and hunger and to increase energy expenditure. The leptin system helps maintain the lipostatic set point for weight regulation. Alterations in this system can lead to obesity. Mutations that prevent leptin hormone production or mutations that remove or alter the leptin receptor, can prevent individuals from feeling full and thus lead to obesity (Houseknecht, 1998). The leptin receptor is just one type of many G protein-coupled receptors (GPCRs) that have been discovered.

G Protein-coupled Receptors

Since Robert Lefkowitz pioneered the G protein-coupled receptor field in the 1970's, hundreds of GPCRs have been discovered. GPCRs are responsible for several physiological functions as they respond to diverse stimuli such as hormones, neurotransmitters, pheromones, light, and odor (Ferguson, 2001). GPCRs have 7 transmembrane domains at the plasma membrane with an intracellular C-terminus and an extracellular N-terminus end. Spanning the plasma membrane allows GPCRs to convert extracellular signals to intracellular signals through a process called signal transduction.

The structure of GPCRs is highly related to their function. All GPCRs have agonist binding domains that are very specific for a limited number of agonists. The location of these domains differ based on the type of agonist. Smaller ligands can bind to the receptor in the hydrophobic regions created by the transmembrane domains. Larger ligands bind to the extracellular regions of the transmembrane domains and to the N-terminus. They also have specific intracellular domains which allow the attachment of G proteins and subsequent signaling (Ferguson, 2001).

GPCR Activation and Signaling

Over a thousand GPCRs have been discovered and all rely on heterotrimeric G proteins to relay the extracellular signals they receive into intracellular messages. There are many different types of G proteins but they can be grouped into 5 more common groups; G_s, G_i, G_q, G_{12}, and G_{13}. While some GPCRs will interact with only one type of G protein it is also common for a single GPCR to interact with many G proteins which will relay different intracellular signals (Ferguson, 2001).

Heterotrimeric G proteins consist of three subunits; α, β, and γ. G_β and G_γ are essentially always bound to each other and can only be separated by denaturation (Figure 1). G_α is bound to the other subunits when it is also bound to GDP. Some G_α and G_β have sites of lipid modification which suggest they can interact with the plasma membrane. When GDP is exchanged for GTP on the G_α subunit, G_α loses affinity for and dissociates from G_β and G_γ. This exchange process is assisted by GPCRs. When a ligand binds to a GPCR, conformational changes in the intracellular loops encourage heterotrimeric G protein binding. The bound G_α releases its GDP for

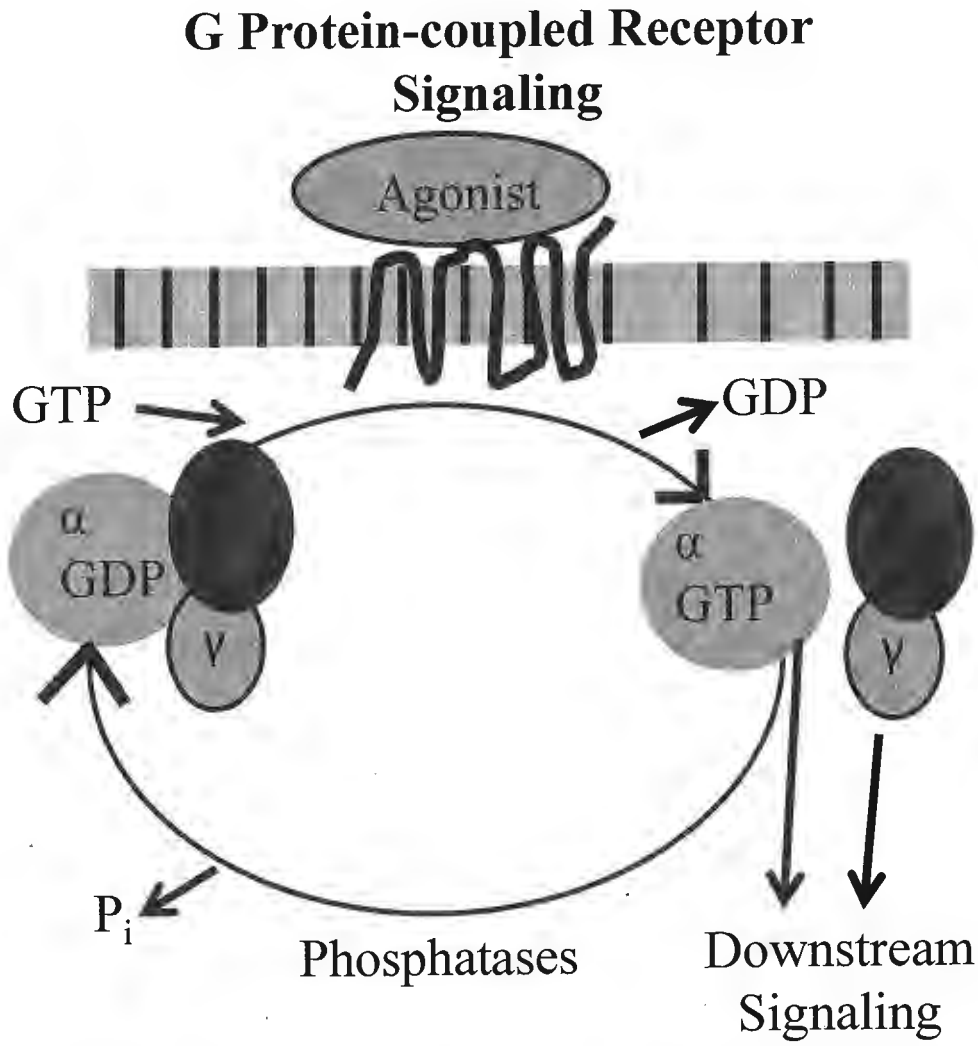


Figure 1: Diagram showing the major signaling steps of GPCRs. Agonist binding to the receptor causes GTP to be added to Gα which causes the separation of it from Gβγ. Gα and Gβγ both activate downstream signaling pathways until phosphatases convert the GTP to GDP on Gα. The G protein subunits then bind back together which ends their signaling.

GTP and the subunits dissociate. G_α is then able to conduct its intracellular signaling. Generally, $G_{\alpha s}$ activates adenylyl cyclase, $G_{\alpha i}$ inhibits adenylyl cyclase, $G_{\alpha q}$ activates phospholipase C, and $G_{\alpha 12}$ and $G_{\alpha 13}$ activate Rho (Buhl, 1995). $G_{\beta\gamma}$ also contribute to intracellular signaling when they are not bound to G_α (Clapham, 1993).

GPCR Desensitization and Resensitization

Activation of GPCRs by specific stimuli sets forth both signal transduction pathways and a process to remove the current signal and to prevent overstimulation. The process of preventing overstimulation is known as desensitization. In order for the cell to respond to a signal properly, it must receive and act on that signal for only a limited amount of time. Without the ability to prevent overstimulation, one signal could unnecessary activate the cell for an extended period of time. For this reason, desensitization plays an enormous role in maintaining normal physiology because many conditions can result from poor receptor desensitization (Hoyer, 2004).

Over the years, more and more factors involved in GPCR desensitization have been discovered but it is unlikely that the entire process has already been described. The process of GPCR desensitization varies greatly from receptor to receptor but it generally includes uncoupling of the heterotrimeric G proteins due to receptor phosphorylation and arrestin binding, the internalization of the receptor to into endosomes, and occasionally a downregulation of the receptor by reducing its mRNA and degradation of existing receptors by lysosomes. The kinetics and magnitude of desensitization varies among receptors. Some range from just seconds to desensitize

completely to stimuli while for other receptors it is simply more difficult to activate the receptor during this period (Ferguson, 2001).

Receptor Phosphorylation

The first step in GPCR desensitization is generally covalent modification in the form of phosphorylation. This process is performed by two families of kinases, second messenger-dependent protein kinases and G-protein coupled receptor kinases (GRKs) (Lefkowitz, 1993). The second messenger-dependent protein kinases include downstream kinases such as cAMP-dependent protein kinases (PKA) and protein kinase C (PKC). These kinases do not discriminate between activated and inactivated receptors so they will phosphorylate both types of receptors (Lohse, 1990). GRKs on the other hand, will only phosphorylate agonist-activated receptors. Phosphorylation of activated receptors promotes the binding of arrestins which sterically prevent the binding of more heterotrimeric G proteins (Lohse, 1990, Ferguson, 2001).

G protein-coupled Receptor Kinases

All GRKs contain 4 similar domains, a catalytic domain to perform receptor phosphorylation, an amino-terminal domain believed to be related to substrate recognition, an Regulator of G-protein signaling (RGS) binding domain, and a carboxyl-terminal domain which helps concentrate GRKs to the plasma membrane. Phosphorylation of receptors by these kinases occurs at both serine and threonine amino acids at either the carboxyl-terminal tail domains or the third intracellular loop (Ferguson, 2001). There are currently seven known types of GRKs and each slightly varies in regards to the receptors they phosphorylate and how they are activated

(Premont, 1995). While GRKs often phosphorylate many sites, it is thought that only the first phosphorylation is necessary for receptor desensitization (Ohguro, 1993).

Kong et al. investigated the role of GRK2 in the desensitization of the β-adrenergic receptor by creating a GRK2-K220L mutant that acted as a dominant negative for GRK2. This mutant lacks the kinase activity of wild type GRK2. Overexpression of GRK2-K220L creates a double mutant because it competes with GRK2. A 10 fold increase in the mutant compared to the wildtype takes away 90% of GRK2's normal function (Kong, 1994).

Jiménez-Sainz et al. has also found a role of GRK2 in shutting down the ERK pathway. The ERK pathway is a popular pathway activated by MCHR1 and it was used as an indicator of receptor activation in this study. Overexpression of GRK2 decreased the amount of ERK phosphorylation while GRK2-K220L caused an increase in ERK phosphorylation. If the GRK2 binding domains to G_{α} and $G_{\beta\gamma}$ are malfunctioned, GRK2 fails to inhibit ERK activation. This suggests that GRK2 is activated downstream of G protein signaling to inactivate the ERK pathway. GRK2 colocalizes with MEK which is the author's hypothesis as to where this interaction with the downstream signaling pathway is taking place (Jiménez-Sainz et al., 2006). Fu et al. found similar effects of GRK2 because GRK2-K220L caused an increase in ERK pathway activation. This was alarming because the GRK2 dominant negative was seen as a potential treatment for heart failure but is causing concerns if it also increases the rate of tumors by increasing the growth promoting ERK pathway (Fu, 2013).

Arrestins

Full receptor desensitization generally requires more than initial phosphorylation. It often requires the binding of an "arresting agent" known as arrestins. The binding of arrestins to receptors both sterically prevents future binding of heterotrimeric G proteins and usually targets the receptor for endocytosis. Thus the binding of arrestins are extremely important to two of the three components of receptor desensitization; separation of the receptor from heterotrimeric G proteins and internalization. Arrestins bind very specifically to the GRK-phosphorylated sites rather than the phosphorylation sites of second messenger receptor kinases which shows that GRK phosphorylation of the receptor may be more important than the phosphorylation of second messenger kinases (Ferguson, 2001).

Internalization

The vast majority of GPCRs use internalization as a way to prevent the receptor from encountering more agonist and to remove the ligand that is already bound to the receptor. Although some GPCRs, such as the D3 dopamine receptor, have shown limited internalization following activation (Kuzhikandathil, 2004). Much of what is known from GPCR internalization was learned through research involving the β-adrenergic receptor (βAR). While βAR endocytosis has been carefully documented, the exact pathway may not be the same for all GPCRs because the kinetics of various receptor internalization differs greatly from receptor to receptor (Ferguson, 2001).

Initially phosphorylation of GPCRs was not considered to be important for internalization, but mutations of sites where GRKs generally act on caused a

decreased level of m2 AChR internalization (Moro, 1993). In addition, GRK2 overexpression promotes some GPCR internalization. It is now accepted that that GRKs play an important role in GPCR internalization through receptor phosphorylation but it is not always required for all GPCRs such as the βAR (Hausdorff et al., 1989). GPCR phosphorylation promotes the binding of β-arrestins which as mentioned earlier both uncouple the receptor from heterotrimeric G proteins and promote internalization. Arrestins target GPCRs for clathrin-coated vesicle mediated internalization through the use of AP-2 heterotetrameric adaptor complex. Arrestins themselves also have clathrin-binding domains which stabilize the receptor in these areas. The clathrin-coated pits cause the internalization of GPCRs. Internalized GPCRs concentrate in vesicles where they are either recycled back to the plasma membrane or degraded by lysosomes.

While internalization is a process of most GPCRs, the actual desensitization of the receptor occurs well before internalization which lessens its importance to desensitization. In addition, blocking GPCR internalization does not change its desensitization profile. However, internalization does seem to have an important role in GPCR resensitization (Ferguson, 2001).

Resensitization

Just as GPCR desensitization is important to prevent overstimulation, resensitization is important to prevent irreversible desensitization. Receptors need to be allowed to signal properly again after they have gone through desensitization to maintain homeostasis. Internalized GPCRs are located in endosomes where they are exposed

to GPCR-specific phosphatases which dephosphorylate the receptor. β-arrestins seem to be excluded from the endosomes which allows the dephosphorylation of GPCRs (J. Zhang 1999). These receptors then follow one of two fates of resensitization; recycling to the plasma membrane or degradation followed by newly transcribed receptor.

This process varies among GPCRs but at the very least takes several minutes (Ferguson, 2001). The process of internalization seems to be necessary for the GPCR dephosphorylation step because internalization-defective mutant GPCRs do desensitize but cannot resensitize (Barak et al., 1994). This solidifies the role of internalization for resensitization of GPCRs.

MCH

Melanin-concentrating hormone (MCH) in mammals is a 19 amino acid cyclic peptide. MCH was originally isolated from teleost fish where it played a role in skin color (Kawauchi, 1983). In mammals MCH plays a part in a variety of roles. It has been documented to play a role in appetite and energy expenditure (Shimada, 1998), resistance to hepatosteatosis despite fat-enriched diets (Wang, 2010), and resistance to aging-associated insulin resistance (Jeon et al., 2006). It is produced in the lateral hypothalamus (Bittencourt et al., 1992). It binds to two known GPCRs, MCHR1 and MCHR2. Many animals, such as humans, have both GPCRs but rodents, which are often used in MCH research only express MCHR1 (Tan et al., 2002).

MCHR1

Melanin-concentrating hormone receptor 1 (MCHR1) is a GPCR that binds MCH and plays a role in energy expenditure and appetite. MCHR1 signals through Gi, Go, and Gq depending on cell type which indicates that the ERK pathway is significantly activated following MCHR1 activation (Saito 1999). MCHR1 is fairly widely expressed in the body but predominantly within the brain (Takahashi 1995). In the brain specifically, MCHR1 is expressed in areas related to motivation, feeding, and energy homeostasis. MCHR1 is also expressed in adipose tissue where is helps to regulate leptin release (Bradley 2000).

ERK Pathway

The ERK pathway is a signaling pathway that has been shown to influence growth and differentiation of cells. The ERK signaling pathway is a cascade of several molecules that essentially activate one another in a specific order. Research is still being conducted on this pathway but much of the pathway has been well characterized. The order of cascade events was discovered by following phosphorylation from the plasma membrane to transcription factors and in reverse (Seger, 1995). The ERK pathway can be activated by a number of means but it seems all activation eventually converges to activate Ras. Ras-GTP can then recruit Raf which is consequently phosphorylated. Activated Raf then phosphorlyates MEK proteins. MEKs phosphorylate and activate ERKs. P-ERKs can be distinguished from inactivated ERKs through the use of antibodies. Activated ERKs can enter the nucleus where they can phosphorylate a number of transcription factors which results

in alteration of gene expression. Activation of the ERK pathway can result in a wide variety of cellular functions including cell proliferation and apoptosis (Kolch, 2000).

MCHR1 and Obesity

One important study linking MCHR1 and obesity was conducted by Marsh et al. (Marsh et al., 2002). It was previously shown that fasting mice developed increased levels of MCH mRNA which suggests that MCH and MCHR1 function is related to appetite. Marsh et al. generated MCHR1-/- mice and found no pathological abnormalities or infertility. The body weights of normal and MCHR1-/- were also similar but the differences lay in fat mass. Both genders of MCHR1-/- mice have 50% less fat and 7% more lean mass than wild type mice. The most significant data was MCHR1-/- mice response to high fat diets. The body weight of wild type mice increases substantially when fed a high fat diet instead of the regular chow. However, MCHR1-/- mice did not display an increase in body fat when they were fed a high fat diet. This suggests that MCHR1 knockout mice are resistant to diet-induced obesity. The authors suggest that this resistance is a result of increased activity and subsequent energy expenditure (Marsh et al., 2002).

The claim that MCH decreases energy expenditure and increases appetite has recently been disputed by a study. Imbernon et al. has injected Sprague-dawley rats with MCH and observed a similar increase in body weight and fat mass that has previously been observed. However, their reasoning for the change is drastically different than what has previously been reported. Imbernon et al. shows that MCH signaling in the lateral hypothalamus causes a change in liver and adipocyte cell

function. In the liver, MCH signaling causes the cells to retain more lipids. While in adipocytes, MCH signaling causes the fat cells to store more lipids. Together, these changes in cell function show how MCH signaling can increase the fat mass of an individual. While the MCH system is no longer expected to affect the appetite or metabolism of an individual as a result of peripheral signaling, it is still a therapeutic target because of its ability to increase fat mass through signaling in the hypothalamus (Imbernon et al., 2013).

Specific Aims

The specific aims of this study focused on the desensitization and resensitization of MCHR1, observed through the ERK pathway. The first aim was to verify that the ERK pathway desensitizes to MCH. We then wanted to characterize how long this desensitization period lasted. We expected it to be around 30 minutes because that has been observed with other GPCRs (Mundell, 2008). Our third aim was to determine if internalization and degradation of MCHR1 or another mechanism could explain the desensitization. And our final aim was to observe ERK pathway desensitization to other stimuli. This study is significant both to the field of GPCRs and to obesity. Characterizing alternative mechanisms of GPCR desensitization is important in studying other GPCRs. Also, determining a mechanism of MCHR1 desensitization will reveal potential therapeutic targets to fight obesity.

Materials and Methods

Tissue Culture

BHK-570 cells (ATCC) were cultured in DMEM$^-$ media (CellGro) with 10% fetal bovine serum (Atlanta Biological) and 1% antibiotic antimycotic solution (Sigma Cell Culture). 3T3-L1 preadipocytes (ATCC) were cultured in DMEM$^-$ with 5% bovine calf serum (Atlanta Biological) and 1% antibiotic antimycotic solution. Cultured cells were fed every three days and BHK 570 cells were split when they were confluent and the 3T3-L1 cells were split at 75% confluency. All cultured cells were kept in an incubator at 37°C, 5% CO_2, and 80% humidity.

Transfection

Transfections were performed when BHK-570 cells reached 75% confluency and 50% confluency for 3T3-L1 cells. Transfections used LipoD293 reagent from SignaGen following their recommended protocols. Transfection time ranged from 4 to 24 hours depending on the experiment. Experiments were run approximately 48 hours after the start of the transfection. Depending on the experiment, cultured cells were transfected with plasmids containing MCHR1, VSVg-MCHR1, PCDNA3, GRK2, GRK3, and GRK2-K220L (GRK plasmids courtesy of Jeff Benovic's lab).

Cell Based ELISA

BHK-570 cells were seeded into 24-well plated and transfected with 2.5 μL 1 mg/ml each of MCHR1-VSVg ± GRK2-K220L or ± GRK3 or ±GRK5. Forty-eight hours post-transfection, culture media was aspirated and labeling buffer (0.02M HEPES and 5% goat serum (MP Biomedicals) in DMEM-) with 1:1000 mouse α VSVg (Sigma)

was added for 2 hours at room temperature. The wells were washed twice with labeling buffer before 100 nM rat MCH (American Peptide) treatments for 0, 15, and 30 minutes time points. The media was aspirated and the wells were washed with ice cold phosphate buffered saline (PBS) (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO_4, and pH to 7.4 in deionized water). Cells were fixed with 3% paraformaldehyde in PBS for 20 minutes at room temperature. The wells were then washed three times with PBS before the addition of 1:5000 goat α-mouse HRP-conjugated secondary antibody (Bio-Rad) with 5% goat serum in PBS for 45 minutes. The wells were washed three times with PBS before the addition of 175 µL soluble POD blue (Roche) for 15 minutes on an orbital shaker. The reaction was stopped using 175 µL 10% sulfuric acid for 2 minutes. To a 96 well plate, 150 µL of each well was transferred and the absorbance was read at 450 nm using a Synergy H1 plate reader (Bio Tek).

Multiple MCH Treatments for ERK Signaling

Cells were serum starved in DMEM$^-$ 2-12 hours prior to MCH treatments. Select dishes of cells were pretreated with 100 nM MCH for 10-15 minutes by carefully removing the media to limit agitation of the cells. All dishes were washed three times with DMEM$^-$ and the cells were incubated for 30 minutes before cells were treated with 100 nM MCH between 0 and 30 minutes. This procedure created two types of cells, ones that were treated twice with MCH and ones that were treated just once with MCH.

ERK Signaling Cell Lysate Harvesting

Following the MCH treatments, dishes of cells were placed on ice and the media was aspirated. One hundred and fifty microliters of 2x sample buffer (4% SDS, 20% Glycerol, 0.12M Tris pH 6.8, and 10% 2-Mercaptoethanol) was added to each dish and the cells were scraped off of the dish into solution. Cell lysates were frozen prior to SDS-PAGE.

MCHR1 Cell Lysate Harvesting

A lysing procedure developed by Danielle Feligno was slightly modified for effectiveness. The culture media was removed from the dishes and 1 mL of trypsin (HyClone) was added. Cells were scraped into a conical with 1mL cold $DMEM^-$. The conical was centrifuged for 20 minutes at 1,000 rpm at 4°C using an International Clinical Centrifuge. The supernatant was removed and the cells were washed in ice-cold PBS. The conical was centrifuged again for 20 minutes at 1,000 rpm at 4°C. The supernatant was removed and the cells were resuspended in a lysing buffer containing 15 mM NaCl, 35 mM HEPES pH 7.4, 12 mM sodium deoxycholate, 35 mM SDS, and 100 mM DTT in distilled water. The samples were rocked for one hour at 4°C. The samples were then boiled for 5 minutes and centrifuged for 20 minutes at 13,000 rpm in a microcentrifuge. The lysates were kept at -20°C prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Cell lysates in 2x sample buffer were boiled for 5 minutes and centrifuged for 5 minutes at 13,000 rpm before being loaded onto a 12% Bis-acrylamide-Tris SDS gel

using EZ-Run protein ladder (Fisher) as a molecular weight standard. Gels were run at 120 volts with 1x Running Buffer (25 mM tris-base, 220 mM glycine, and 3.5 mM SDS in deionized water) for about an hour or until the sample buffer reaches the bottom of the plates.

Semi-dry Transfer

The gel was soaked in Towbins solution (40 mM Tris-base, 35 mM glycine, 1 mM SDS, and 16% methanol in water) while on a nitrocellulose membrane (GE or BioRad) surrounded by blot paper for 10 minutes. The contents were transferred at 15V for 30 minutes using a semi-dry transfer apparatus (BioRad).

Wet Transfer

A wet transfer method (BioRad) was also used to transfer gel contents onto a nitrocellulose membrane. The following contents were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3); grey side of cassette, fiber pad, filter paper, gel, nitrocellulose membrane, filter paper, and fiber pad for 15 minutes. The transfer apparatus (Mini Trans-Blot® Electrophoretic Transfer Cell) was set up according to BioRad instruction and the transfer was run at 350 mA for one hour.

Western Blot

Following transfer, the nitrocellulose membrane was then blocked in 5% non-fat dry powdered milk (Great Value) in TBS-T (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20 in deionized water pH 7.6) for 1 hour at room temperature on an orbital shaker. Primary antibody was added at 1:1000, in 5% milk in TBS-T overnight at

4°C on an orbital shaker. Various primaries were used: goat α-MCHR1 (Santa Cruz Biotechnology), rabbit α-MCHR1 (Santa Cruz Biotechnology), rabbit α VSV g (Sigma), rabbit α-Total ERK (Cell Signaling), and mouse α-Phosphorylated ERK (Cell Signaling). The nitrocellulose paper was washed with TBS-T three times for 10 minutes before the addition of secondary antibody at 1:5000 in 5% milk in TBS-T. Various HRP conjugate secondaries were used: goat α mouse (BioRad) and goat α rabbit (BioRad). Secondary antibody was added for 45 minutes at room temperature on an orbital shaker. The blot was washed three times for 5 minutes with TBS-T. Western Lightning Plus enhanced chemiluminescence substrate (PerkinElmer) was added to the blot according to its instructions and the luminescence was observed using film (Kodak).

Results

Obesity is both an international and domestic pandemic and the problem is only growing. Easy access to food, especially high caloric foods, has combined with general lack of physically labor to affect a larger portion of adults (Pi-Sunyer, 2002). There are nearly 500 million adults worldwide that are currently obese and that number is only expected to grow (Kral, 2012). Obesity has severe financial and health consequences which include diabetes and heart disease (Pi-Sunyer, 2002). Individuals are mostly limited to dietary changes and exercise to fight their conditions but there is hope therapeutic drugs can also contribute to fighting obesity.

The melanin-concentrating hormone (MCH) has been of interest to fight obesity because of its expected relation to appetite and energy expenditure. Recently, the physiological role of MCH has been shifted from appetite and energy expenditure to lipid accumulation in the periphery. MCH triggers lipid accumulation and uptake in the liver while it stimulated lipid storage in adipocytes (Imbernon et al., 2013). This combined effect of MCH will increase the fat storage in an individual which make the MCH system a potential therapeutic target to fight obesity.

MCH is produced and acts upon the lateral hypothalamus in mammals. The hormone binds to two homolog GPCRs, MCHR1 and MCHR2. Previously, Marsh et al. has shown that MCHR1 knockout mice are less susceptible to diet induced obesity because they were leaner than wild-type mice. Marsh et al. attributed the reduction in obesity to an increase in energy expenditure and decrease in appetite but Imbernon et al. has recently countered these claims (Imbernon et al., 2013). Imbernon et al. has

shown that mice receiving injections of MCH are more obese than mice that do not receive the treatment and this is consistent with prior studies. They have also shown however, that the change in weight is not solely related to appetite or energy expenditure but because of changes in the periphery. MCH signaling increases lipid accumulation by the liver which increases the amount of lipids within the body. MCH signaling also increases lipid storage in adipocytes which increases the fat mass of an individual. MCH signaling increases fat mass by altering the fat storage of individuals (Imbernon et al., 2013). Although the perceived physiological mechanism behind MCH has changed, the system is still a potential therapeutic target.

Activation of MCHR1 signals through Gs, Gq, and Gi proteins. These three pathways eventually converge on the ERK pathway which is why ERK phosphorylation was chosen as the readout of MCHR1 activation. This study focused on characterizing the desensitization and resensitization of MCHR1. Most GPCRs desensitize by internalizing the receptor into the cytosol from the plasma membrane. Saito et al. claims that this is the mode of desensitization of MCHR1 as 40-50% of the receptor is internalizes following 30 minutes of MCH treatment (Saito et al., 1999). Aside from this paper, there is little other evidence to determine the exact mode of desensitization.

MCHR1 Stimulation leads to Activation of the ERK Pathway

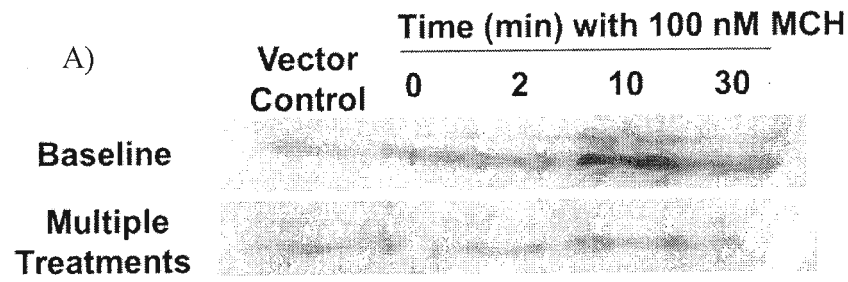
In order to measure the activation of MCHR1, stimulation of the ERK pathway was observed using a Western Blot. BHK-570 cells were transfected with MCHR1 and 48 hours later, these cells were treated with 100nM MCH for 0-30 minutes. Detection of both total and phosphorylated ERK using western hybridization was used as the indicator of MCHR1 activation which is characterized by the phosphorylation of ERK. The blot showed that phosphorylation of ERK was a sufficient indicator of MCHR1 activation because it was time dependent and ERK was not activated in treated cells lacking MCHR1 (Figure 2A, Baseline lanes). This experiment suggests that ERK is an appropriate readout of MCHR1 activation so it was observed in subsequent experiments.

MCHR1 Signaling Desensitizes the ERK Pathway

After determining that the ERK pathway is efficient in observing MCHR1 activation, the next goal was to use it to observe any pathway desensitization. Receptor desensitization is extremely important to their basic function. From a physiological standpoint, an organism does not want one signal to be on forever because it may not always be necessary for survival. To prevent signal overstimulation, receptors desensitize their pathways to the stimulus so that signals can generally be turned on and off in a short period of time.

In order to measure the activation and subsequent desensitization of MCHR1, activation of the ERK pathway was observed using a Western Blot (Figure 2A). In the initial experiments, BHK-570 cells were transfected with MCHR1 and these cells

Limited Restimulation of ERK Following an Initial MCH Treatment



*Total ERK not shown.

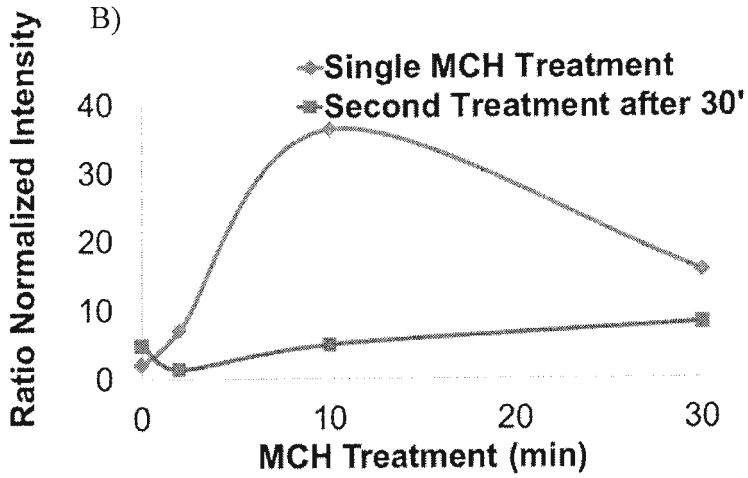


Figure 2: A) Western blot, cells were only treated once with 100 nM MCH (baseline) or cells were treated with MCH for the given time after an initial 15' treatment followed by a 30' washout DMEM- (Multiple Treatments). Desensitization is shown as ERK cannot be phosphorylated again after only a 30' washout. B) Densitometry of the western using ImageJ normalizing the activated ERK to the total ERK.

were treated with 100nM MCH approximately 48 hours later. After an initial 15 minute MCH treatment to half of the dishes, all of the dishes were washed 3 times with DMEM⁻. Following 30 minute incubation, dishes were treated again with MCH for various time points. A Western Blot using both total and phosphorylated ERK was used as the indicator of MCHR1 activation which is characterized by the phosphorylation of ERK. The Western Blot in Figure 2AB shows 7 fold ERK activation of BHK-570 cells with MCHR1 treated only once with MCH for 10 minutes. However, when cells were pretreated with MCH, there is very limited activation of ERK following the 30 minute incubation and second MCH treatment. The amount of total ERK observed in the Western Blot was used to normalize the cell number in the creation of the densitometry of this blot (Figure 2B). This implies that the ERK pathway significantly desensitizes to MCH for at least 30 minutes.

A concern following this experiment is that transfected BHK-570 cells are a nonendogenous model utilizing a cell line that does not naturally express MCHR1. Since nonendogenous models may react differently, it is important to observe any MCHR1 desensitization in an endogenous cell line. 3T3-L1 mouse preadipocytes, which naturally express MCHR1, were used in a very similar experiment as Figure 2. While the treatment and lysate harvesting was identical as before, one major change was that the pretreatment of MCH was changed to 10 minutes instead of 15 for all future experiments. This change was made because Figure 2 showed maximum ERK activation after 10 minutes with MCH so this was chose for the length of the pretreatment. Figure 3 shows the Western Blot of the 3T3-L1 MCH treatments. It

ERK Pathway Desensitization in 3T3-L1 Cells via MCH

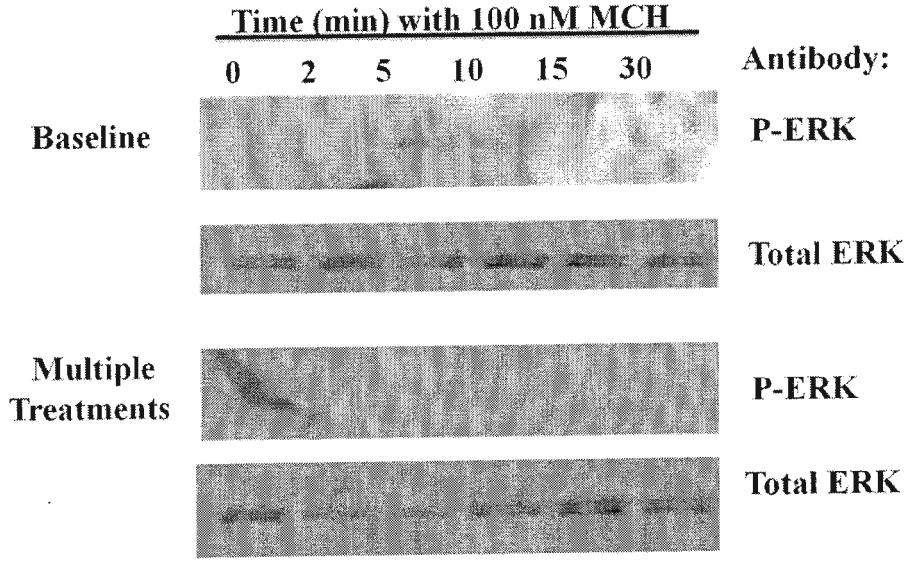


Figure 3: Western blot, 3t3-L1 cells were only treated once with 100 nM MCH (Baseline) or cells were treated with MCH for the given time after an initial 10' treatment followed by a 30' washout (Multiple Treatments). Desensitization is shown as ERK cannot be phosphorylated again after only a 30' washout.

shows ERK activation when cells were treated once with MCH but a reduced ERK activation following the second MCH treatment. The amount of total ERK for each dish is consistent which means that a difference in cell number did not account for the variation in ERK activation. These results are very similar to the nonendogenous model observed in Figure 2 which suggests that using transfected BHK-570 cells with MCHR1 for future experiments is an appropriate cell model.

Resensitization of the ERK Pathway to MCH takes over 70 Minutes

Equally as important as characterizing the desensitization of a receptor is characterizing its resensitization. Determining the length of time that a receptor desensitizes is significant because it sheds light onto the physiology of the hormone. Figures 2 and 3 show that the ERK pathway significantly desensitizes to MCH for at least 30 minutes. The next logical aim is to determine exactly how long MCHR1 mediated ERK pathway desensitization lasts.

In order to determine how long MCHR1 resensitization takes, a Western Blot was used again to observe the phosphorylation of ERK following MCH treatments. BHK-570 cells transfected with MCHR1 were pretreated with 100nM MCH. Unlike in the previous experiments where the incubation period between the two MCH treatments was kept constant, that period is varied in this experiment. By varying the time between MCH treatments and observing ERK activation in a Western Blot, we can determine the length of time needed for the ERK pathway to resensitize to MCH. Figure 4A shows the Western Blot with both total and phosphorylated ERK. The ERK activation in cells treated once with MCH for 10 minutes is used as the level to

MCHR1 Desensitization Lasts At Least 70 Minutes

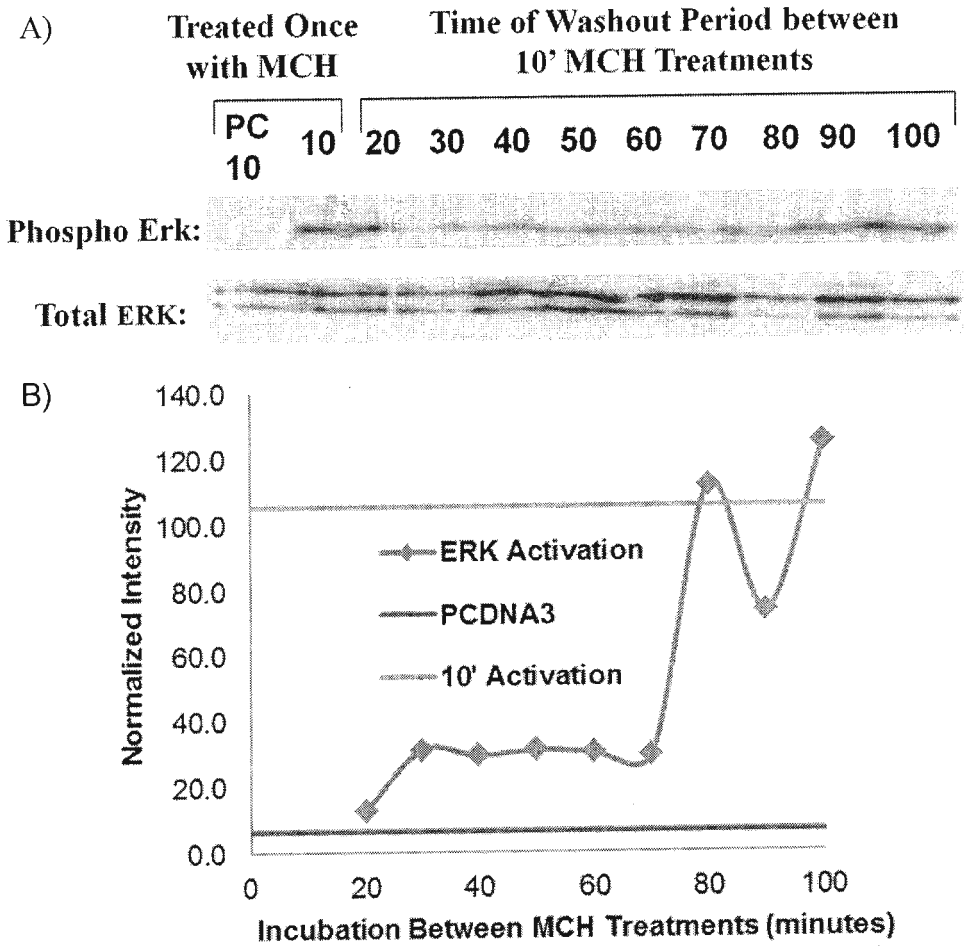


Figure 4: A) Western blot to determine the length of desensitization by varying the incubation period between two 10' 100 nM MCH treatments. Phospho ERK shows activation does not return until after an 80' incubation period. B) Densitometry of the western blot using ImageJ. The phosphorylated ERK was normalized to the amount of total ERK.

determine that ERK is fully resensitized to MCH. The densitometry in Figure 4B shows that ERK activation does not return back to normal for over 70 minutes. This implies that the ERK pathway desensitizes to MCH for over 70 minutes.

Protein Levels of MCHR1 Increase following MCH Treatment

Receptor desensitization is necessary to prevent overstimulation of a cell. There are several potential methods of desensitization that have been observed for GPCRs. The most common method of desensitization of GPCRs is receptor internalization which can be followed by degradation of the receptor.

To test the hypothesis that MCHR1 is degraded following activation, MCHR1 protein levels were observed using a Western Blot following long term MCH treatments. BHK-570 cells were transfected with MCHR1-VSVg and were treated with 100nM MCH for up 24 hours. All of the MCH treatments were lysed at the same time to ensure that all of the cells had equal transfection and time with the receptor plasmid. The Western Blot in Figure 5A shows MCHR1-VSVg protein levels. Figure 5B shows the densitometry of each banding shown in the Western Blot and its relative molecular weight. These figures show an approximate two fold increase in receptor protein levels following an 18 hour MCH treatment which suggests that MCHR1 is not degraded following MCH treatment.

Long-term Treatment of Cells with MCH Increases Receptor Protein Levels

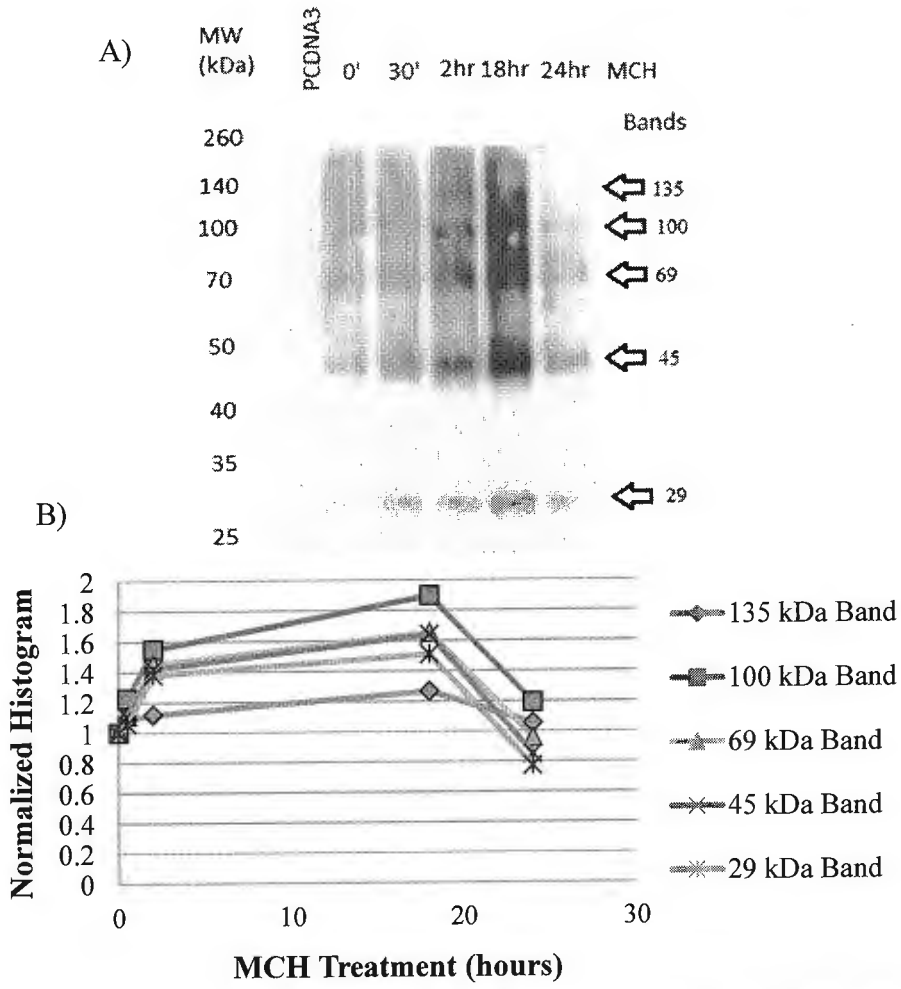


Figure 5: A) Western blot to determine if MCHR1 protein levels decrease following long-term MCH treatment. BHK cells were transfected with VSV-g tagged MCHR1 or pcDNA3 empty plasmid as a negative control. After 24 hours, these cells were then treated with 100nM MCH as noted. The PCDNA3 lane is blank so the darkened areas in the other lanes are all MCHR1. Molecular weights of the bands were determined using the molecular weight standard. B) Densitometry was used to quantify the changes on the blot using ImageJ.

MCHR1 is not Removed from the Plasma Membrane in High Quantities

Internalization is very common for many GPCRs following hormone treatment. Saito et al. has already reported that 40-50% of MCHR1 is internalized following 30 minutes of MCH treatment (Saito et al., 1999). Thus it was a hypothesis that even if MCHR1 does not degrade following MCH treatment, it may still internalize to prevent overstimulation of the cell.

To observe internalization of MCHR1, a modified cell-based ELISA was performed. BHK-570 cells cotransfected with VSVg-MCHR1 and either GRK 2 D/N or ± GRK3 or ±GRK5. GRKs have been shown to help increase GPCR internalization by phosphorylating the receptors. The cell surface was coated with mouse α VSVg prior to 100 nM MCH treatment between 0 and 60 minutes. Cells were fixed and goat α-mouse HRP-conjugated secondary antibody with POD blue. The absorbance was measured which is directly related to the amount of VSVg-MCHR1 at the plasma membrane.

Control cells that were not cotransfected with a GRK showed that ~15% of VSVg-MCHR1 internalized following 30 minutes of MCH treatment which is consistent with what was observed by Jay Moden (Moden, 2012). Internalization was not substantially increased with the cotransfection of GRK5 (Figure 6), or GRK3 (Figure 7). Additionally, GRK2-K220L took away all VSVg-MCHR1 internalization (Figure 7). This suggests that GRK2 is needed to provide any of the MCHR1 internalization that is observed. GRK2 is often needed for GPCR internalization and it has been shown to colocalize with MCHR1 (Kong et al., 1994, Eberle et al., 2004).

Limited MCH-mediated MCHR1 Internalization with GRK5

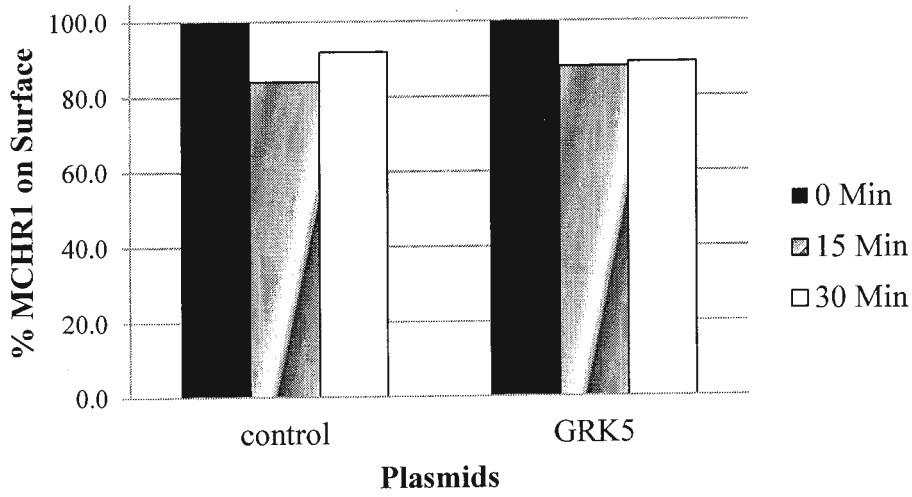


Figure 6: Internalization of MCHR1 was measured using an ELISA protocol. Control BHK-570 cells were transfected with just MCHR1 while other BHKs were cotransfected with MCHR1 and GRK5. Surface levels were determined by comparing the flourescence in the treated wells to the untreated wells.

Limited MCHR1 Internalization with GRK3 and GRK2-K220L

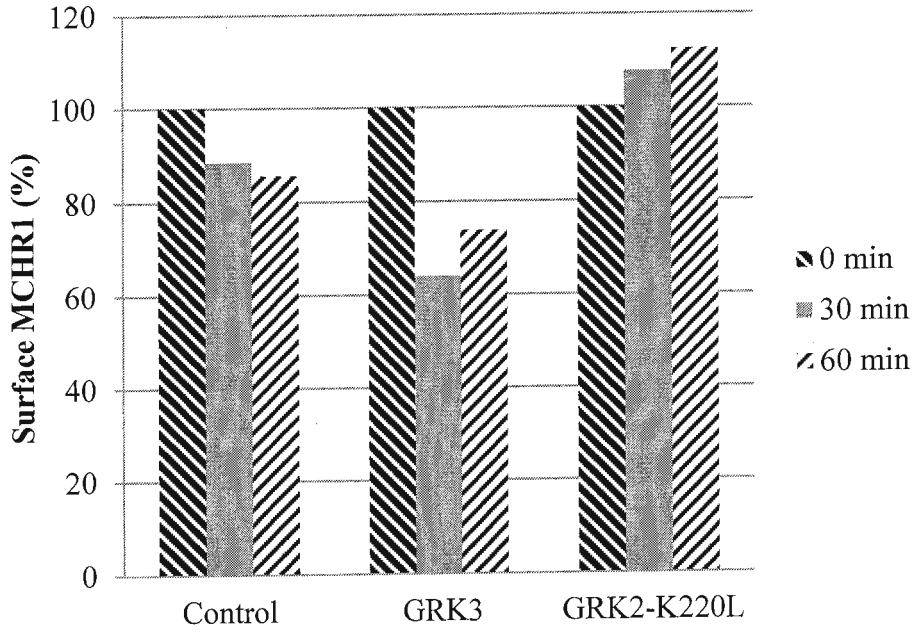


Figure 7: Internalization of MCHR1 was measured using an ELISA protocol. Control BHK-570 cells were transfected with just MCHR1 while other cells were cotransfected with GRK3 and GRK 2 dominant negative.

GRK2 Plays a Role during MCHR1 mediated ERK Desensitization

Since limited internalization of MCHR1 was observed, it was hypothesized that another mode of desensitization is responsible for the limited activation of ERK following repeated MCH treatments. Since this mode would have to occur while MCHR1 is at the plasma membrane because only a limited amount of MCHR1 were internalized, we looked at factors that affect MCHR1 at this location. Previous studies have shown that GRK2 is necessary for β2-adrenergic receptor desensitization which is a GPCR like MCHR1 (Kong et al., 1994). We were drawn to GRK2 because of the affinity it has shown for MCHR1 and we hypothesized that phosphorylation of the receptor could block the binding of G proteins (Eberle et al., 2004). GRKs are known to phosphorylate GPCRs. This phosphorylation step is one of the processes that leads to internalization of GPCRs. Blocking G proteins for an extended period of time could be the method of MCHR1 desensitization.

It was first necessary to determine if MCH could still activate the ERK pathway if GRK2 is nonfunctional. To test this, BHK-570 cells were cotransfected with MCHR1 and GRK2-K220L which is a GRK2 dominant negative and treated with 100nM MCH. ERK activation was observed using a Western Blot. Figure 8 shows that the ERK pathway is still activated by MCH in a similar way to what was seen in prior experiments even if GRK2 is not functional. This means that GRK2 is not necessary for proper activation of the ERK pathway by MCH.

ERK Activation via MCH Treatment with GRK2 Dominant Negative

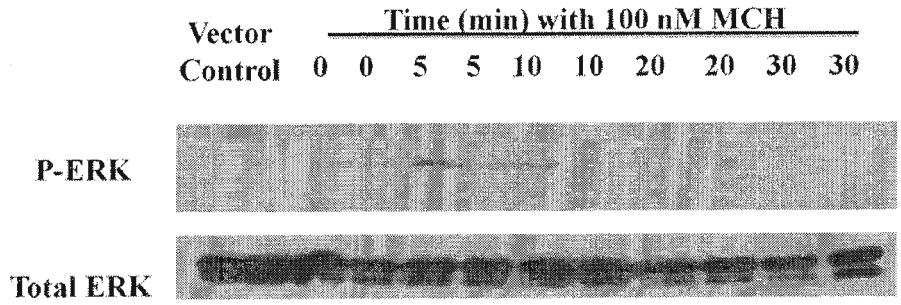


Figure 8: Western blot; BHK-570 cells transiently-expressing MCHR1 in pcDNA3 and GRK2 dominant negative were treated for 0-30 min with 100 nM MCH and harvested. The blot shows that the ERK pathway is still activated by MCH in the presence of a GRK2 dominant negative.

To test the hypothesis that GRK2 has a function in MCHR1 mediated ERK pathway desensitization, ERK activation was observed using a Western Blot in cells without functional GRK2. BHK-570 cells were cotransfected with MCHR1 and GRK2-K220L. This experiment followed a very similar protocol to the earlier desensitization experiments. These cells were pretreated with 100nM MCH for 10 minutes followed by 3 $DMEM^-$ washes. Following a 30 minute incubation period, the cells received a second MCH treatment for various time points. Following lysing, total ERK and phosphorylated ERK were detected using a Western Blot. When the blot in Figure 9 is compared to that in Figure 2A, it is clear that ERK activation is present even after the second treatment of MCH. This suggests that GRK2 is necessary for MCHR1 mediated ERK pathway desensitization. This isn't entirely a surprise because GRK2 has been shown to decrease ERK stimulation (Jiménez-Sainz et al., 2006). However, the complete lack of ERK desensitization is a surprise. In the previous MCHR1 mediated ERK desensitization experiments in Figure 2AB, functional GRK2 is present throughout the experiment and ERK is still stimulated, which suggests that GRK2 may take time to act upon ERK stimulation.

GRK2 Dominant Negative Decreases ERK Pathway Desensitization

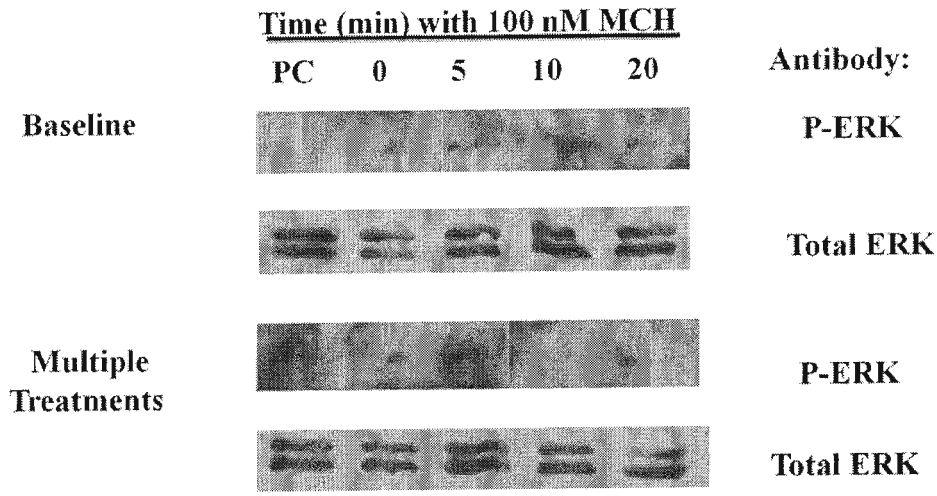


Figure 9: Western blot, BHK-570 cells cotransfected with MCHR1 and GRK2 dominant negative were only treated once with MCH (Baseline) or cells were treated with 100 nM MCH for the given time after an initial 10' treatment followed by a 30' washout (Multiple Treatments). The blot shows that ERK is activated in both MCH treatments.

ERK Desensitization is Similar with other Activators of ERK

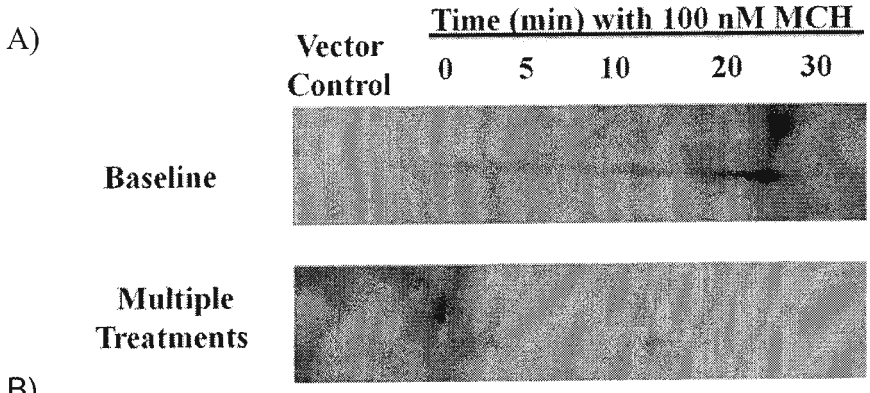
A concern is that during this study, the ERK pathway could be desensitizing itself and that the type of stimulus is irrelevant to the resulting pathway desensitization. To test if the stimulus will affect the type of ERK desensitization that is observed, two other activators of ERK were tested; activation of MCHR2 and isoproterenol treatment. MCHR2 is a homolog of MCHR1 which binds the same hormone and activates the same pathways (An et al., 2001). Isoproterenol, which is an agonist of β-adronergic receptors, activates ERK by signaling through Gs and Gi proteins (Zou et al., 1999).

To test if MCHR2 activation results in the desensitization of the ERK pathway, a Western Blot was used following MCH treatments. BHK-570 cells were transfected with MCHR2. Cell lysates were analyzed with a Western Blot following an MCH treatment protocol identical to the one in Figure 9. The Western Blot in Figure 10A shows ERK activation following a single and multiple MCH treatment for the indicated times. Figure 10B is the densitometry of the Western Blot which uses the amount of total ERK to normalize for cell number. Figure 10 shows that ERK activation is decreased after the second MCH treatment which suggests that the ERK pathway is being desensitized when MCHR2 is activated in a similar manner to the desensitization of the ERK pathway following MCHR1 activation.

Next we wanted to test if the ERK pathway will desensitize in a similar manner when cells were treated with isoproterenol which is another stimulator of this pathway (Zou et al., 1999). BHK-570 cells were treated with isoproterenol in the same manner as the MCH treatments, with one group of cells receiving a single

MCHR2 Desensitization Similar to MCHR1

A)



B)

*Total ERK blots (not shown) revealed equal cell content.

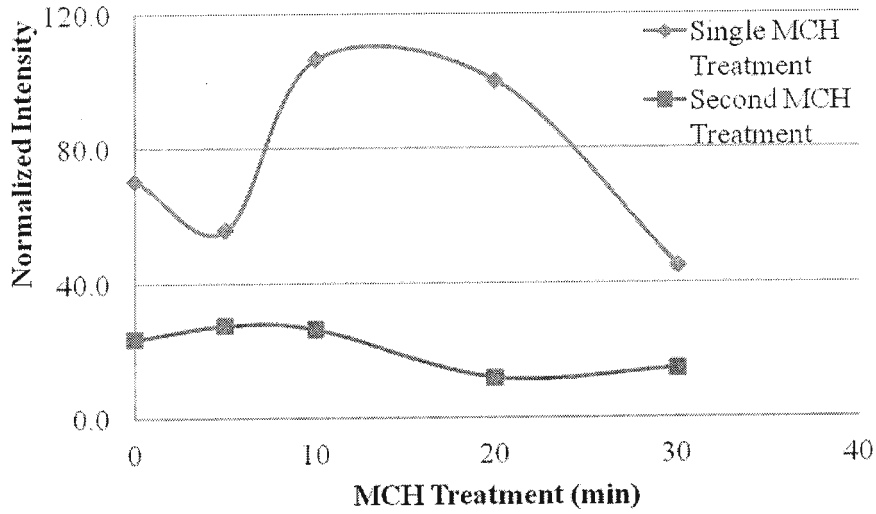


Figure 10: A)Western blot; BHK-570 cells transiently-expressing MCHR2 in pcDNA3 were either treated for 0-30 min with 100 nM MCH and harvested (Baseline), or treated with MCH for 10' followed by a 30' washout and subsequent MCH time course (Multiple Treatments). B) Densitometry of the western blot using ImageJ following normalization with total ERK.

treatment while another received multiple isoproterenol treatments. The activation of the ERK pathway was observed using a Western Blot which is shown in Figure 11. This figure shows that ERK is not activated after the second treatment with isoproterenol. This implies that the ERK pathway may be desensitizing in the same manner when treated with isoproterenol as with the activation of both MCHR1 and MCHR2. This means that it is possible that the ERK pathway is desensitizing itself in the same way regardless of the type of stimulation. If this were the case, different activators of the pathway would result in similar ERK desensitization.

The first aim was to observe MCH-mediated ERK desensitization which is shown in Figures 2 and 3. Resensitization of the ERK pathway to MCH took over 70 minutes. After observing limited internalization of MCHR1, it suggests that resensitization of the receptor occurs at the plasma membrane which is unlike most GPCRs which need internalization to resensitize. When Figures 2, 3, 10, and 11 are compared, they all show a similar desensitization of the ERK pathway which gives support to the argument that the observed desensitization is occurring at the pathway level rather than the receptor level. Figure 9 shows that GRK2 is needed for proper MCH-mediated ERK desensitization. While it was initially thought that phosphorylation of the receptor may induce desensitization, it is possible that GRK2 acts on the ERK pathway to induce desensitization (references from earlier). Since only GPCRs were used to activate the ERK pathway, either scenario is possible because GRK2 could act upon the receptor or/in addition to the ERK pathway.

ERK Pathway Desensitization with Isoproterenol Treatment

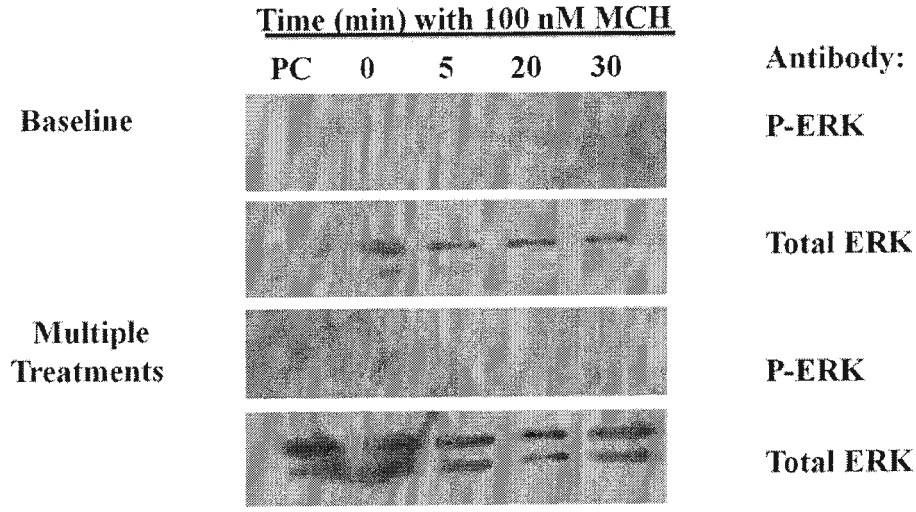


Figure 11: Western blot, BHK cells were only treated once with 10 μM Isoproterenol or cells were treated with Isoproterenol for the given time after an initial 10' treatment followed by a 30' washout. Desensitization is shown as ERK cannot be phosphorylated again after only a 30' washout. Blot is easier to visualize on the film.

Discussion

Obesity is a major issue worldwide in which nearly 500 million adults are obese (Kral et al., 2012). Previous studies have shown that mice lacking MCH and their significant pathways are less susceptible to diet-induced obesity (Marsh et al., 2002). This suggests that targeting MCH or MCHR1 could result in an anti-obesity drug. For this reason, this study chose to focus on the natural desensitization of MCHR1. MCHR1 signals through G_i, G_o, and G_q pathways (Chung, 2009). The ERK pathway is downstream of many G proteins which makes it a good choice to follow MCHR1 activation.

Verifying MCHR1-mediated ERK Desensitization

To determine if the ERK pathway desensitizes to MCH, cultured BHK-570 and 3T3-L1 preadipocytes were treated twice with 100 nM MCH with a washout period in between. The level of phosphorylated ERK was determined using a Western Blot which is a readout of MCHR1 activation since MCH signaling leads to ERK pathway activation (Oh et al., 2010, Cook et al., 2008). We determined that in both cell types, the ERK pathway substantially desensitizes to MCH as there is no phosphorylation of ERK following the second hormone treatment 30 minutes following the first (Figures 2 and 3). We also determined that maximal ERK stimulation occurs following 10 minutes of MCH treatment.

The results in these experiments compared well to literature. Our lab has previously determined that maximally ERK activation occurs following 10 minutes of MCH treatment (Cook 2008). ERK does not remain phosphorylated for an extended

period of time because signals cannot permanently stay on while maintaining proper physiology and homeostasis. The activation likely goes away due to phosphatases that deactivate the activated ERK molecules.

A special aspect of this experiment that should be noted is that we chose to use a fairly high concentration of MCH, 100 nM. The K_d of MCH/MCHR1 binding has been reported to be around 0.1 nM (Drozdz, 1995). In addition, the only reported serum MCH levels were highly debated and questioned (Gavrila et al., 2005, Walters, 2005). Therefore we do not know how our MCH concentration compares to that *in vivo* but we do know that because of the K_d we are flooding MCHR1 with MCH. However, this was our intention. The goal of our MCH treatments was to activate as much MCHR1 as we could at the same time. We did not want only half of the receptors to be activated in the first treatment because if desensitization occurs at the receptor level, half of the receptors are still allowed to activate following the second hormone treatment. If this were the case, we would probably not observe desensitization of the ERK pathway even though it was happening at the receptor level. Our proposed way around this problem was to flood MCHR1 with MCH so that a high percentage of them bound to MCH after the first treatment. This gives us a better idea on if desensitization is occurring or not because we can be confident that most of the receptors were activated after the first MCH treatment.

After observing desensitization of the ERK pathway to MCH, some possible mechanisms were hypothesized. Most GPCRs, like MCHR1, internalize the receptor into the cytosol where the agonist can be removed from the receptor. Occasionally,

GPCR phosphoryaltion leads to desensitization while the receptor is at the plasma membrane. D3 dopamine receptor desensitization is hypothesized to occur because of a conformational change in the receptor due to phosphorylation (Kuzhikandathil et al., 2004). GPCR phosphorylation could also prevent G protein binding which would prevent future signal transduction. Regulators of G protein signaling (RGS) have been shown to accelerate the dephosphorylation of activated G_α which decreases signaling (Hunt, 1996, Watson, 1996). Since expression of RGS increases following some agonist stimulation, increased activated RGS protein could be responsible for desensitization periods (P. Zhang et al., 2011). Another mechanistic explanation for the observed desensitization could be that the pathway desensitizes. Some of these possible mechanisms of MCHR1-mediated ERK desensitization were investigated later in this study.

An alternative explanation for ERK desensitization in Figures 2 and 3 is that the hormone is not being washed off with the three serum-free washes. If MCH remains bound to MCHR1, the receptors may not be reset to future signaling. To increase the chance that MCH was being washed off, we used a common acid/salt buffer to perform the wash instead of serum. However, this method created abnormal and inconsistent ERK activation, possibly because the acidic buffer agitated the cultured cells or altered receptor conformations which could result in the abnormal ERK signaling.

Another way to measure the hormone still in serum following washes was to use fluorescent MCH. Rhodamine-MCH was added to cells and the level of

fluorescence was measured before and after three serum washes. A significant decrease in fluorescence would indicate the hormone is being washed off while consistent fluorescence would suggest it is not being washed off. Neither was the case as consistent fluorescent data was unable to be obtained potentially due to the sensitivity of our plate reader. Even if fluorescence was consistent, the different binding affinity of MCH and Rhodamine-MCH would have to be determined.

In any case, we are fairly confident that the serum washes did remove the majority of MCH from the first treatment. This confidence stems from the fact that changing the media three times should greatly reduce the free concentration of MCH in the serum. When serum MCH concentration is substantially lower that the MCH/MCHR1 K_d of 0.1 nM, bound MCH should be removed from MCHR1 (Drozdz et al., 1995).

Another issue is that a nonendogenous cell model is used for the majority of our experiments. Transfecting a form of MCHR1 into cells was easier to study with for a couple of reasons. Transfection resulted in a substantial amount of MCHR1 which meant it was easier to observe ERK activation. Using VSVg-MCHR1 was easier for some experiments because our antibodies for VSVg were much better than those for MCHR1. We do not believe using a nonendogenous cell model is an issue because ERK activation and desensitization was observed to be similar in an endogenous model (Figure 3). Also, VSVg-MCHR1 has been shown to signal similarly to normal MCHR1 so for the experiments were it was used we don't think that should be a concern either (Cook 2008).

Another concern is that only the ERK pathway was observed to visualize MCHR1 activation, desensitization, and resensitization. MCHR1 activates a number of pathways in addition to ERK so the assumption cannot be made that what is happening to the ERK pathway following MCHR1 activation is also occurring to the many other pathways activated by MCHR1. A future direction is to observe the other pathways activated by this receptor and compare their potential desensitization to that of the ERK pathway which could provide more clues as to how MCHR1 desensitization is occurring. Another future direction of this portion of this study would be to verify that MCH is actually being washed off.

The significance of observing MCHR1-mediated ERK desensitization is substantial. Better understanding of signaling desensitization following MCHR1 activation could lead to new therapeutics that would treat of a variety of disorders. MCH signaling serves roles in many conditions including mood (Gehlert et al., 2009) and obesity (Segal-Lieberman et al., 2003). MCHR1 antagonists have already been shown to limit weight gain in leptin-deficient mice (Segal-Lieberman et al., 2003).

Characterization of ERK Resensitization to MCH

The next aim in this study was to determine the length of time required for the ERK pathway to resensitize to MCH. BHK-570 cells transfected with MCHR1 were treated twice with MCH with the incubation period in between treatments being varied in length. We wanted to determine the minimum amount of time required for ERK phosphorylation to return to the first MCH treatment level. A Western Blot

revealed that it takes at least 70 minutes for the ERK pathway to resensitize to MCH (Figure 4).

Literature has seemed to neglect characterizing the exact length of desensitization of GPCRs. One study using $P2Y_1$ and $P2Y_{12}$ receptors showed that these GPCRs resensitized to agonist following 30 minutes of incubation (Mundell et al., 2008). This is slightly surprising because it takes MCHR1 over twice as long to resensitize but not completely unexpected because they are different receptors and will behave differently.

Resensitization of GPCRs typically requires dephosphorylation of the receptor. It was once believed that receptor endocytosis is required for the dephosphorylation step. The A_{2A} adenosine receptor does not dephosphorylate or resensitize when internalization is blocked by an inhibitor (Mundell et al., 2008). However, not all GPCRs require receptor endocytosis for resensitization. Both IP-prostanoid receptor and thryrotropin-releasing hormone receptor dephosphorylate and resensitize normally if internalization is blocked (Mundell et al., 2008, Jones, 2005). Since we observed very limited MCHR1 internalization (Figures 6 and 7), it suggests that MCHR1 does not require endocytosis to resensitize to stimulus. However, the dephosphorylation and resensitization of the receptor may be skewed or irrelevant if the observed desensitization occurs independent of receptor phosphorylation. For example, if the ERK pathway is desensitizing itself or by a component unrelated to the receptor, receptor phosphorylation and dephosphorylation may be irrelevant to resensitization for MCHR1 and the ERK pathway.

MCHR1 seems to desensitize the ERK pathway in such a way that the desensitization is 'all or nothing.' What this means is that the ERK signal does not not gradually comeback following the second MCH treatment, but rather remains at basal activation for 70 minutes until full activation comes back at the 80 minute interval. This suggests that the desensitization mode is definite and cannot be simply overcome by increasing the stimuli but this will need to be further tested to be confirmed. Additionally, the fact that ERK activation is either basal level or maximal level during the resensitization suggests that most of MCHR1 is being bound by MCH at the same time. We used a higher concentration of MCH so that all of the receptors would become activated at the same time and Figure 4 suggests that this is indeed the case.

In future MCHR1 resensitization experiments, it would be necessary to compare the ERK pathway resensitization to that observed in the other pathways activated by MCHR1. It would also be interesting to see if an increased dosage of MCH for the second treatment could return ERK activation quicker than 70 minutes. This could indicate if the desensitization of the ERK pathway following MCH signaling is absolute or just more difficult to activate during the desensitized period.

Observing the resensitization of the ERK pathway to MCH is significant because literature has shown a role for MCH and the ERK pathway in the hypothalamus. Pereira-de-Silva et al. has shown that MCH signaling results in ERK pathway desensitization in the hypothalamus (Pereira-da-Silva, 2005). Our experiment sheds some light to the time period that takes place during the

resensitization of the ERK pathway to MCH. Pereira-de-Silva et al. has shown evidence that MCH's most important role in the hypothalamus may not be activation of ERK but rather the subsequent desensitization of ERK following MCH signaling (Pereira-da-Silva et al., 2005).

Determining the Mechanism of ERK Desensitization to MCH

MCHR1 does not Degrade Following MCH Treatment

VSVg-MCHR1 protein levels were measured using a Western Blot following MCH treatment of transfected BHK-570 cells for up to 24 hours (Figure 5). VSVg-MCHR1 protein levels increased for 18 hours following a decrease after 24 hours of MCH treatment. The increase in MCHR1 protein suggests that the receptor becomes more stable following MCH binding and not that more of it is transcribed because the promoter is not included in the transfected plasmid. The decrease in MCHR1 protein after 24 hours is an anomaly because the VSV-g tagged MCHR1 plasmid was present in every treatment time for the same amount of time. Therefore, the degradation of the plasmid is not a likely explanation. It is possible that these cells simply were not transfected well. Regardless, the increase in MCHR1 protein following MCH treatment suggests that the receptor is not degraded following hormone treatment as is observed in some GPCRs.

A special aspect of this experiment and in the later ELISA experiments is that VSVg tagged MCHR1 was used rather than normal MCHR1. The reasoning behind this choice is that the antibody to VSVg works much better than the antibodies we had to MCHR1. Since our lab has previously shown that VSVg-MCHR1 signals

through the ERK pathway in a similar manner to normal MCHR1, we still feel this experiment is relevant (Cook 2008).

However, an alternate explanation for the results of this experiment could be that VSVg-MCHR1 is simply more resistant to normal MCHR1 to degradation. One way we tried to ease these concerns was to follow fluorescently labeled MCHR1 to lysosomes. MCHR1-eYFP was used with LysoTracker to visualize any receptor trafficking and localization to lysosomes following MCH treatment. Unfortunately, this experiment was unsuccessful possibly do to insufficient labeling of the cultured cells.

This experiment was significant because it suggests that degradation of MCHR1 is not a likely reason for the observed ERK desensitization immediately following MCH signaling. This encouraged us to look at other desensitization mechanisms. One interesting aspect of this experiment is the suggestion that activated receptors are more stable. Increasing the stability of MCHR1 suggests that upon an initial activation, a second round of hormone could increase the response from the first as long as they are resensitized to the hormone. A way to test this would be to treat cells with MCH and wait 12 hours before a second treatment and compare the activation of ERK to cells that were not pretreated with MCH.

Limited MCHR1 Internalization following MCH Treatment

A modified cell-based ELISA was used to measure the rate of VSVg-MCHR1 internalization in BHK-570 cells following MCH treatment. To encourage internalization, GRKs 3 and 5 were cotransfected into the cultured cells. GRKs have

been shown to help increase GPCR internalization by phosphorylating the receptors. However, none of the GRKs were able to substantially increase MCHR1 internalization and GRK2-K220L removed all MCHR1 internalization (Figures 6 and 7).

About 15% of MCHR1 internalized following 30 minutes of MCH treatment. GRK3 only partially increased MCHR1 internalization to 35% following 60 minutes of MCH treatment. MCHR1 cotransfected with GRK2-K220L did not internalize at all following 60 minutes of MCH treatment. This suggests that MCHR1 does not internalize well enough to account for the level of desensitization that is observed in the ERK pathway.

A concern about this assay is that the antibody losing affinity for the receptors could be confused with internalization of the receptor. This is accounted for by comparing the fluorescence of the experimental cells to cells that were not treated with MCH and therefore should not be internalizing anyway. A way to ease this concern would be to measure MCHR1 internalization in another manner. YFP-MCHR1 was transfected into cultured cells and the fluorescence was measured using a plate reader following MCH treatment. If internalization occurred, fluorescence should decrease. This experiment did not work unfortunately; possibly do to sensitivity of the plate reader.

The results of the cell-based ELISA assay contradict those reported earlier (Saito et al., 1999). Saito et al. found that up to 50% of MCHR1 internalized after 30 minutes of MCH treatment. We were unable to observe the level of MCHR1

internalization observed in their study even with the addition of GRKs. Even if we did observe 50% of MCHR1 internalizing, it does not account for the near 100% level of desensitization we see in the ERK pathway. This suggests that another method of desensitization must be responsible for MCHR1 desensitization.

What makes this portion of this study significant is that the desensitization of MCHR1 seems to be over simplified or poorly understood. The only explanation for desensitization is internalization and it seems that the internalization is not always observed. Again, an alternative mechanism must be responsible for the desensitization of the ERK pathway following MCHR1 activation.

GRK2 is at least partially responsible for MCHR1-mediated ERK Desensitization

It was hypothesized that phosphorylation of MCHR1 by GRK2 may be responsible for the observed desensitization of ERK. Phosphorylation could detach G proteins from the receptor or cause a conformational change that would alter the binding of MCH. GRK2 has already been determined to be necessary for β2-adrengergic receptor desensitization (Kong et al., 1994).

In addition to interacting with GPCRs, GRK2 has recently been shown to act directly onto the ERK pathway to decrease ERK activation. Overexpression of GRK2 has been shown to limit ERK activation in chemokine signaling which occurs through GPCRs. The limited ERK activation is independent of GPCR phosphorylation as mutant GRK2 that has lost its kinase ability still decreases ERK activation. If the GRK2 binding domains to G_{α} and $G_{\beta\gamma}$ are malfunctioned, GRK2

fails to inhibit ERK activation. This suggests that GRK2 is activated downstream of G protein signaling to inactivate the ERK pathway (Jiménez-Sainz et al., 2006).

To determine if GRK2 is responsible for MCHR1-mediated ERK desensitization, BHK-570 cells cotransfected with MCHR1 and GRK2-K220L were treated twice with MCH (Figure 9). Following the second treatment, we observed similar ERK activation as the first. This is unlike our previous experiments without GRK2-K220L because the desensitization of the ERK pathway is not being observed.

Literature supports two different roles of GRK2 in signal desensitization. One is that GRK2 acts at the receptor level to prevent further signaling. The D3 dopamine receptor is suggested to require phosphorylation by GRK2 to cause a conformational change that either decreases hormone binding or disrupts signal transduction (Kuzhikandathil et al., 2004). β-Adrenergic receptor requires GRK2 for proper desensitization of cAMP signaling (Kong et al., 1994). This is significant because it shows how GRK2 can be related in the desensitization in pathways other than ERK.

The role of GRK2 desensitizing the ERK pathway directly is another role of GRK2 in desensitization. Jimenez-Sainz et al. has shown that GRK2 is activated by G proteins and that overexpression of GRK2 is responsible for decreasing ERK activation (Jiménez-Sainz et al., 2006). This shows that GRK2 can act directly on the ERK pathway to desensitize it. Supporting data shows that a GRK2 dominant negative increases ERK signaling in mice (Fu et al., 2013).

These two types of literature regarding GRK2 shows that it is responsible for desensitization of GPCR signaling with other receptors. MCHR1 can reasonably rely

on GRK2 to desensitize the receptors or its downstream pathways. Our results show that the ERK pathway cannot desensitize to MCH without GRK2 so this molecule is at least involved in desensitization. It is significant to find suggestions for an alternative mechanism of MCH desensitization because the previous mode of internalization does not seem to explain the substantial ERK desensitization. A future direction regarding GRK2 would be to determine if it acts directly on MCHR1 or on the ERK pathway to cause the observed desensitization of the pathway following MCH signaling (Figure 12). If a precise mechanism can be determined, we would be at a stage where new therapeutics could be produced to mimic the actions of GRK2 related to MCH signaling and potentially treat some of the conditions related to overactive MCH signaling.

Determining if ERK Desensitization is Homologous or Heterologous

There are two types of pathway desensitization, homologous and heterologous. Homologous desensitization means that the desensitization is agonist specific or that only one type of receptor is prevented from activating the pathway. In heterologous desensitization, multiple types of receptors are prevented from activating a pathway (Chuang, 1996). Observing the desensitization profile of other activators of ERK could indirectly shed light onto whether homologous or heterologous desensitization is being observed following MCHR1 activation. If the profile is similar, it suggests the desensitization of the ERK pathway would be heterologous. This would imply that GRK2 acts on the pathway level rather than at the receptor level. Likewise, if the

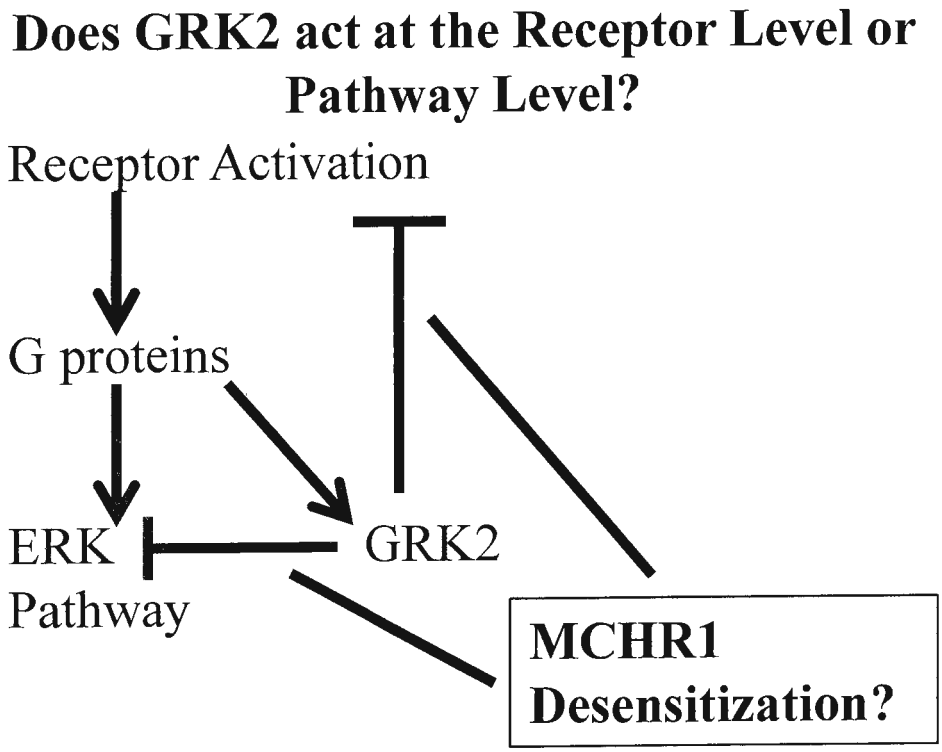


Figure 12: Diagram to show how GRK2 could potential desensitize the ERK signaling pathway to MCH. GRK2 could cause desensitization by acting directly on MCHR1 or by acting at the pathway level within the ERK pathway.

desensitization profile is different, it suggests the pathway is undergoing homologous desensitization and that GRK2 is likely acting at the receptor level.

We decided to activate ERK again with MCH, but this time through MCHR2, and by using isoproterenol (Figures 10 and 11). We determined that in both cases, ERK desensitizes in a similar manner as is observed when MCHR1 is activated. This implies that the pathway is undergoing heterologous desensitization. This goes against some literature as insulin has been shown to induce homologous desensitization of the ERK pathway (Fucini, 1999). However, insulin does not signal through a GPCR as MCH and isoproterenol do so this might account for the difference (Ullrich et al., 1985).

To gain confidence that MCHR1 activation induces heterologous ERK desensitization, several experiments could be performed. One that we tried was to stimulate the pathway with one agonist followed by a second agonist 30 minutes later to observe if the pathway was desensitized to the different agonist. If ERK activation was observed, then the pathway would likely be in homologous desensitization. If the pathway cannot be activated it is likely in heterologous desensitization. When this experiment was conducted, Western Blotting difficulties were encountered.

An alternate explanation for suggesting that the ERK pathway is undergoing heterologous desensitization is that it may just be chance that the other stimuli have a similar desensitization profile. Suggesting heterologous desensitization is largely based on assumptions and probability and cannot be said with much confidence until other experiments are performed. It is possible that MCHR1, MCHR2, and

isoproterenol simply cause similar ERK desensitization profiles by chance and not because the pathway is in heterologous desensitization.

There are several general concerns about this study. The main concern is the lack of repeated experiments. For the most part, each experiment was only conducted once which means any conclusions are just suggestions at best. To further prove any of the conclusions in this study, each experiment needs to be conducted with similar results several more times. The primary future direction of this study will be to increase the number of experiments conducted so that the conclusions can be further supported.

The recent news that MCHR1 is potentially only activated in the neurons of the lateral hypothalamus has limited the physiological importance of using 3T3-L1 preadipocytes as the endogenous cell model. While this preadipocyte cell line does naturally express MCHR1, the existence of the receptor may just be in small basal level amounts or even the remnants of an ancestor whose original function is lost. While a preadipocyte does not appear to be a perfect model anymore, it still could be physiologically relevant if MCHR1 desensitizes in preadipocytes in the same manner as neurons which will need to be tested in the future.

Finally, a major future direction regarding this study is to pinpoint the role of GRK2 in MCHR1 mediated ERK desensitization. It was hypothesized that the kinase activity of GRK2 could phosphorylate MCHR1 which could be enough for desensitization. The phosphate group could prevent G proteins from binding to the receptor or cause a conformational change in the extracellular region of MCHR1 that

could decrease its affinity for MCH. Jimenez-Sainz et al. has shown that the presence of GRK2 decreases the level of ERK activation (Jiménez-Sainz et al., 2006). Therefore, another possibility is that MCHR1 could activate GRK2 by G protein signaling which then shuts down ERK activation. In either case, the most significant conclusion for this study is that GRK2 plays a role in MCHR1-mediated ERK desensitization and with further mechanistic study, this knowledge could lead to new therapeutics.

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