An Investigation of Melanin-concentrating Hormone Receptor Internalization – Or Lack Thereof

by

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ABSTRACT

Melanin-concentrating hormone (MCH), a cyclic peptide hormone involved in energy homeostasis, is known to bind to two G protein-coupled receptors (GPCRs) in mammals. These receptors, melanin-concentrating hormone receptor 1 (MCHR1) and melanin-concentrating hormone receptor 2 (MCHR2), have been a popular target for MCH antagonists in an effort to fight the ongoing epidemic of obesity. In the presence of prolonged stimulus it is common for GPCRs to undergo rapid desensitization. However, the desensitization mechanisms of MCHR1 and MCHR2 are as yet poorly understood. This study aims to create epitope-tagged expression vectors to allow for the expression of MCHR1 and MCHR2 in a tissue model. Utilizing a modified cell-based ELISA and fluorescence microscopy, the sequestration of MCHR1 and MCHR2 would be measured after agonist stimulus. Receptor interactions with GRK2 and β-arrestins would also be measured. The over expression of both MCHR1 and MCHR2 proved to be cytotoxic to BHK570 cells. The overexpression of GRK2, β-arrestin 1, and β-arrestin 2 showed a relatively small but statistically significant increase in receptor internalization. Fluorescence microscopy suggests that the interaction between MCHR1 and β-arrestins were transient in nature. These finding suggest that MCHR1 can be internalized via the clathrin-mediated pathway. It is likely MCH signaling is mediated in a cell specific manner based on the cellular expression levels of GRKs and β-arrestins.

INTRODUCTION

Obesity

Obesity is the accumulation of excess adipose tissue resulting in the impairment of one's health. Obesity has reached epidemic levels in the United States, with more than 61 million Americans being obese [1]. Between 1985 and 2009, there has been a marked increase in the prevalence of obesity in all 50 states with 12 states currently reporting obesity rates either equal to or greater than 30 percent [2]. In order to be considered obese, a person generally has to have a body mass index (BMI) greater than 30. A person's body mass index is calculated by dividing their weight by the square of their height (kg/m^2) [3].

The underlying cause of obesity lies in the imbalance of energy intake and energy expenditure and can be attributed to a plethora of technological, social, and economic advances experienced during the latter half of the 20th century as well as genetic predisposition. Industrial automation and mechanization has led to a reduction in physical exertion while the increased availability of home electronics, coupled with low cost processed foods with high caloric densities, tend to exacerbate already sedentary lifestyles and account for between 60 and 70 percent of obesity cases [4]. The remaining 30 to 40 percent of obesity cases can be attributed to genetic predisposition where it is believed that environmental factors such as a high fat diet and sedentary lifestyle contribute to the expression of an obese genotype [5].

Obesity has been associated with increased rates of morbidity and mortality. Diseases and disorders showing higher rates of prevalence in obese individuals include coronary heart disease, stroke, hypertension, sleep apnea, osteoarthritis, diabetes mellitus, insulin resistance, and certain forms of cancer including colorectal and prostate [4,6]. The increased morbidity rate amongst the obese directly correlates with a decrease in life expectancy. Caucasian males with a BMI of greater than 35 had a decrease in life expectancy of approximately 3 to 4 years while those with a BMI of 45 or greater experienced an average decrease of 9 to 12 years [7]. The annual obesity-related economic burden in the United States is expected to increase by 66 billion dollars by 2030 [8].

Regulation of Energy Homeostasis

The mammalian brain regulates energy homeostasis and food intake through a complex system involving many neurotransmitters, signaling peptides, and feedback loops allowing for the maintenance of body weight to within 0.5 to 1 percent of an individual's ideal. Through the integration of multiple short term signals from peripheral tissues such as the liver, gastrointestinal tract, pancreas, and adipose tissue, the brain is able to interpret the body's nutritional state and respond to changes in feeding patterns, physical exertion, and environmental variables such as temperature. These short term signals regulating energy homeostasis emanate from stretch receptors of the GI tract and travel to specific regions of the brain through the afferent

pathway of the vagus nerve while hormonal signals such as leptin and ghrelin influence the brain through the endocrine system [9,10].

The regulation of energy homeostasis is achieved through the interaction of multiple regions of the brain ranging from the brain stem to the cortex. This is evident by how the visual appeal and aroma of food and an individual's companionship or lack thereof influences their feeding behavior. Signals from the various higher brain centers appear to be centrally processed by the hypothalamus. The hypothalamus can be divided into several anatomically distinct regions that play key roles in energy homeostasis. These regions include the arcuate region (ARC), paraventricular nucleus, and ventromedial nucleus [10].

The ARC of the hypothalamus acts as a central control center for feeding and energy homeostasis by integrating signals regarding energy homeostasis from other regions of the brain and body through the presence of localized semi-permeable capillaries. Neurons that populate the arcuate region express pro-opiomelanocortin (POMC), neuropeptide Y (NPY), and agouti-related peptide. The paraventricular nucleus is involved in the secretion of thyrotropin-releasing hormone and corticotrophin-releasing hormone. It also plays an important role in the integration of feeding signals with the hypothalamic-pituitary axis and thyroid through the convergence of several neuronal pathways including NPY, POMC, and galanin: an appetite-stimulating hormone. The ventromedial nucleus of the hypothalamus is involved in receiving satiety signals and is a key target for the hormone leptin: one of many peripheral peptides that have anorexigenic properties [9,10].

Recently, several peptides or hormones have been shown to influence energy homeostasis resulting in a negative feedback loop. Although exact signaling cascades and hormonal interactions have yet to be elucidated, peripheral signals from leptin are known to inhibit NPY-releasing neurons in the medial hypothalamus. The decrease in NPY secretion results in decreasing signals to melanin-concentrating hormone (MCH)-secreting neurons in the lateral hypothalamus. MCH is an appetite-stimulating hormone and a decrease in its expression results in deminished feeding behavior [11]. As leptin signaling decreases, MCH signaling increases and vice versa resulting in cyclical feeding patterns.

In cases of severe or morbid obesity, it is difficult to correlate the increase of adipose mass solely to the strength of differential feeding signals from the brain. Several genetic studies of morbidly obese individuals have led to the discovery of mutations affecting the function of hormones or receptors involved in energy homeostasis. Homozygous mutations in the leptin receptor gene can produce leptin receptors lacking a transmembrane domain and/or intracellular C-terminal tail inhibiting cell signaling. Mutations to the leptin peptide itself have also been characterized and produce a physiologically, nonfunctional hormone incapable of modulating MCH secretion from the hypothalamus [12].

GPCR

Many hormones involved in energy homeostasis, such as ghrelin, NPY, orexin, galanin, and MCH trigger cellular and systemic changes through the binding

of G protein-coupled receptors (GPCRs) [9]. While GPCRs show relatively little sequence homology amongst themselves, being classified into over 100 subfamilies, their general structure is highly conserved [13]. The core of a GPCR is composed of 7 hydrophobic transmembrane domains, 3 extracellular loops, 3 to 4 intracellular loops, an extracellular amino terminus and in many cases an intracellular carboxyl terminal tail. The extracellular loops are important in ligand binding and the N-terminus plays an important role in stabilizing the binding of larger signaling peptides and glycoproteins. Amino acid residues on the intracellular loops and the C-terminal tail are important for the binding of heterotrimeric G proteins and for the interaction of proteins involved in desensitization and internalization [13].

GPCR signaling is initiated by the binding of its ligand to the extracellular binding pocket through Vanderwaal's interactions. The binding of the ligand causes either a conformational shift within the GPCR or acts to stabilize the GPCR conformation. The stabilization or conformational shift allows interactions between the second and third intracellular loops as well as domains near the distal end of the C-terminal tail to interact with the G protein causing its activation [14,15].

G Proteins

GPCR's bind and activate G proteins. Heterotrimeric G proteins are made up of three subunits, alpha (α), beta (β), and gamma (γ) and generally act to regulate gene transcription. To date, no less than 20 G_α subunits, 6 G_β subunits and 11 G_γ subunits have been identified [16]. In the inactive state, all three subunits are

associated in the trimeric form with guanosine diphosphate (GDP) bound to the G_α subunit. Once activated through interaction with GPCRs, the G protein conformation is altered allowing for the release of GDP and the binding of guanosine triphosphate (GTP), activating the G protein and causing the dissociation of the G_α and $G_{\beta\gamma}$ subunits [16].

Some of the most extensively characterized G proteins include $G\alpha_s$, $G\alpha_{i/o}$, and $G\alpha_q$. The G_q protein can activate phospholipase C (PLC) resulting in phosphotidylinositol-4,- bisphosphate (PIP2) being cleaved into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). The IP3 induces the release of intracellular calcium stores from the endoplasmic reticulum while the DAG functions to activate protein kinase C (PKC) which phosphorylates Ras resulting in the activation of the mitogen-activated protein kinase (MAPK) pathway. The G_s protein activates protein kinase A (PKA) through the stimulation of adenylyl cyclase, which subsequeny increases cyclic AMP concentrations. The G_o protein activates the MAPK pathway in a similar manner to the G_q protein while the G_i protein inhibits the activation of adenylyl cyclase [16,17].

Desensitization

The desensitization of GPCR signaling helps to modulate receptor activity. There are several different types of receptor desensitization but the two most common are homologous desensitization, which is dependent on the GPCR being activated, and heterologous desensitization, which is a receptor activation-independent pathway

commonly mediated through a second messenger regulated kinase [18]. The general mechanism for desensitization includes the uncoupling of the G-protein from the GPCR which is usually due to receptor phosphorylation. The phosphorylation event is then followed by receptor endocytosis. Once internalized, the receptors can either be degraded or recycled back the cell membrane [19]. Specialized enzymes and proteins, such as GPCR kinases, β-arrestins, and clathrin are commonly implicated in playing important roles in the rapid desensitization of GPCR's [18]. Other methods of GPCR desensitization have also been characterized or proposed including internalization of receptors through lipid rafts or by changes in receptor conformation that terminate signaling in an endocytosis independent manner [20,21].

G Protein-Coupled Receptor Kinases

G-protein coupled receptor kinases (GRK's) are a family of kinases that phosphorylate serine and threonine residues located on the 3rd intracellular loops and C-terminal tails of GPCR's [22]. There are seven known members of the GRK family with all members showing a significant level of sequence homology in the substrate recognizing N-terminal domain, membrane targeting C-terminal domain, and centrally located catalytic domain [23]. The GRKs can be broken down based on functional similarity and tissue specificity with GRK 1 and 7 expressed primarily in the visual tract, GRK 4 generally expressed in the testes, and GRK 2,3,5 and 6 ubiquitously expressed throughout the body [23]. GRK 2 has been widely characterized through the study of the phosphorylization of the β_2 adrenergic receptor

and it has been shown to phosphorylate a number of different GPCR's in an agonist-dependent manner [24].

β -arrestins

β-arrestins are a family of proteins that recognize activated GPCR's through their conformation and phosphorylated serine and threonine residues [25]. There are currently four known arrestins divided into two families: visual arrestins and β-arrestins. The visual arrestin family is almost exclusively expressed in the retina and consists of arrestin 1 and arrestin 4 [18]. The β-arrestin family is ubiquitously expressed at varying levels throughout the body and consists of arrestin 2 (β -arrestin 1) and -arrestin 3 (β -arrestin 2) [26]. At least two splice variants of β -arrestin 1 and β -arrestin 2 have been identified including a variant of β -arrestin 1 with insertions of 8 amino acids and a variant of β -arrestin 2 with an insertion of 11 amino acids and it is hypothesized that these variants may bind to different families of GPCR's [27,28].

The β -arrestins are involved in GPCR desensitization in two distinct ways; through the inhibition of G-protein activation by binding to the activated GPCR and by the internalization of the activated GPCR through the targeting and recruitment of clathrin. It has been shown that arrestins will preferentially bind GRK phosphorylated GPCR's compared to native receptor with the β2 adrenergic receptor showing a 10 to 30 fold increase in affinity [29]. G protein-coupled receptors can be classified into either class A or class B receptors based on their affinity for binding

either β -arrestin 1 or β -arrestin 2. This affinity is conferred through differential sequence on the receptor's C-terminal tail [30].

Class A receptors include the β_2 adrenergic receptor, μ-opioid receptor, and the Dopamine D_{1A} receptor. Receptors that belong to this class show a higher affinity for binding β -arrestin 2 rather than β -arrestin 1. In the class A model of receptors, the interaction between the receptor and β-arrestin 2 is transient in nature with the β -arrestin 2 dissociating from the receptor immediately after receptor localization in clathrin-coated pits [31]. Class B receptors include thyrotropin-releasing hormone receptor, neurokinin NK1 receptor, and the neurotensin 1 receptor. These receptors have been shown to have equal binding affinity for both β -arrestin 2 and β-arrestin 1 and the ability to bind visual arrestin [26]. Class B receptors exhibited stable interactions with arrestins with both the receptor and the β -arrestins co-localizing to endosomes. Class B receptors also resensitize and recycle back to the plasma membrane less rapidly than Class A receptors [31].

Melanin-concentrating Hormone

Melanin-concentrating hormone (MCH) was originally discovered in teleost fish where it functions to aggregate melanosomes in the fish's scales resulting in a lightening of skin color [32]. In mammals, MCH is a highly conserved neuropeptide consisting of a cyclic 19 amino acid chain with a single disulfide bond between amino acids 7 and 16 [33]. MCH has been shown to act as an orexigenic peptide, inducing energy conservation as well as increasing one's hunger sensations [33]. Recent

studies have identified several additional physiological effects of MCH; these include the regulation of sleep cycles, voiding behaviors, and possibly involvement in the regulation of depression[34-36]. Neurons which express MCH are solely expressed in the areas of the central nervous system associated with energy homeostasis: the lateral hypothalamus and zona incerta [37].

Melanin-concentrating Hormone Receptor

Melanin-concentrating hormone binds and activates two known receptors: melanin-concentrating hormone receptor 1 (MCHR1) and melanin-concentrating hormone receptor 2 (MCHR2). MCHR1 was originally identified as the orphan G protein-coupled receptor SLC-1 through the isolation and characterization of its ligand MCH [38]. MCHR1 consists of 353 amino acid residues with its encoding gene located on human chromosome 22 at 22q13.3 and is highly conserved across humans, mice, and rats [33]. MCHR1 mRNA has been identified in multiple regions of the brain including the olfactory tubercle, hippocampus, amygdala, thalamus, ventromedial and dorsalmedial nuclei of the hypothalamus, which is consistent with it being involved in energy regulation. MCHR1 is capable of binding several different heterotrimeric G proteins including $G_{i/o}$ and G_q (Figure 1) allowing it diverse signaling pathways [39]. The second MCH receptor, MCHR2 consists of 340 amino acid residues and exhibits a 38 percent sequence homology with MCHR1. The gene encoding for MCHR2 is found on human chromosome 6 at 6q21 [40,41]. MCHR2

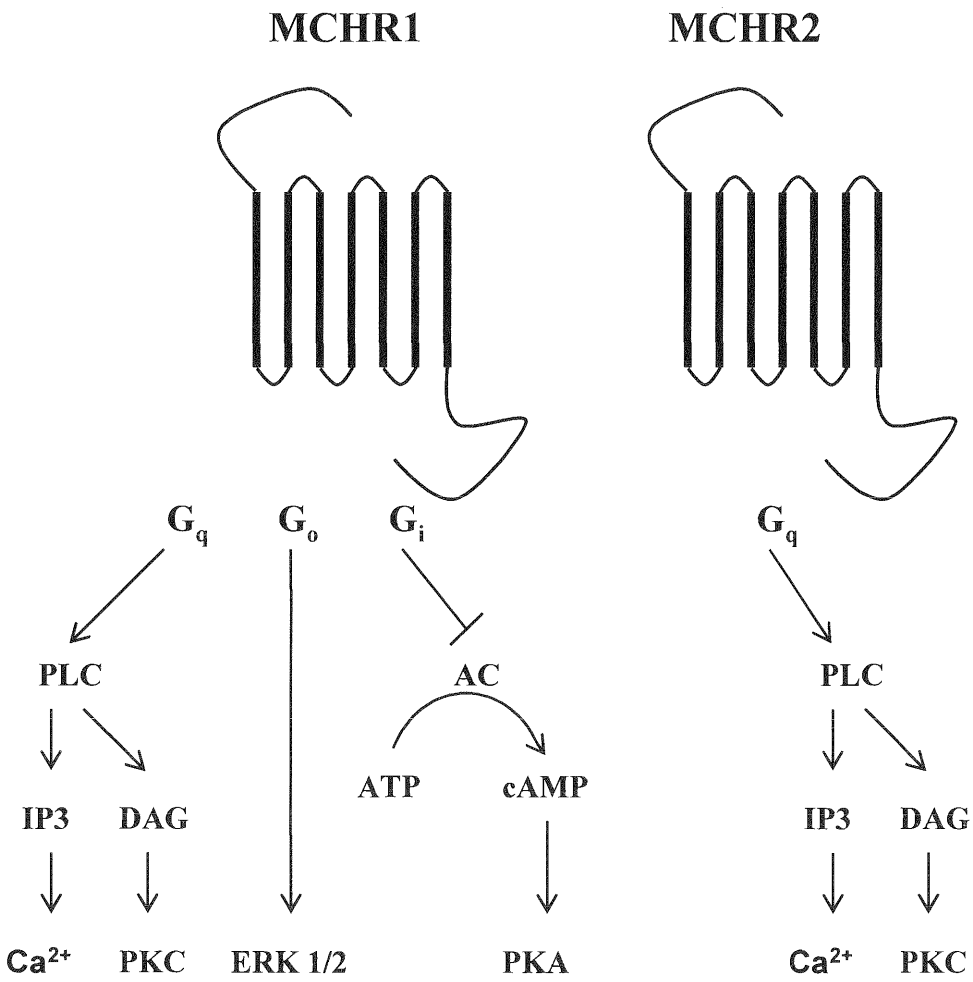

Figure 1: MCHR1 and MCHR2 Signaling Pathways. MCHR1 signals through G_q , G_o , and G_i pathways resulting in an increase in intracellular calcium, PKC, ERK ½, and inhibition of PKA respectively. MCHR2 signals only through the G_q pathway.

mRNA is also widely expressed in the brain including the precentral gyrus, hippocampus, amygdala, putamen, and the caudate nucleus, thalamus, corpus callosum, and medulla oblongata, and hypothalamus. MCHR2 has been shown to primarily couple to only G_q heterotrimeric G proteins most likely due to significant sequence divergence of the 2nd and 3rd intracellular loops compared to MCHR1 [41].

Leptin

Leptin is a peptide hormone involved in modulating energy homeostasis. Leptin was discovered in 1994 by Friedman and colleagues and indirectly plays an important role in the regulation of adipose tissue mass [42]. Leptin mRNA, which is transcribed by adipose cells, is 4.5 kb long and encodes a peptide consisting of 167 amino acids. The hormone targets leptin receptors in the hypothalamus where it functions to regulate energy homeostasis. Homozygous mutations of leptin or its receptor have been shown to reduce energy expenditure and produce severe obese phenotypes in mice [42,43]. Genetic screening of families exhibiting early onset morbid obesity has also identified mutations in the human leptin receptor gene of afflicted family members [44].

MCH Models

The role of MCH in energy homeostasis has been examined using several model systems. A study by Qu et al. identified a significant up-regulation of MCH mRNA expression in the hypothalamus of leptin-knockout mice compared to controls

indicating a potential role for MCH in mediating energy homeostasis [45].

Additional studies involving the injection of MCH into the hypothalamus of rats further supported this hypothesis by showing a marked increase in feeding activity of the satiated rats [46]. In further studies utilizing a transgenic mouse model (MCH-OE) over expressing MCH in the hypothalamus, Ludwig et al. observed that MCH-OE mice were hyperphagic and exhibited an increase of adipose tissue 31% greater than that of control mice when fed a high fat diet while homozygous MCH knockout mice were shown to be hypophagic and lean compared to controls and exhibited increased energy expenditure at rest [47,48].

Chen et al. created a line of MCHR1 knockout mice where the MCHR1 gene was excised through homologous recombination. Studies utilizing this line of mice have shown an increase in resistance to diet-induced obesity and hyperphagia compared to wild-type mice fed a similar diet [49]. Mutations of the MCHR1 gene have also been examined in European populations. These epidemiological studies of German children and young adults exhibiting early-onset obesity have identified several single nucleotide polymorphisms (SNPs) in the MCHR1 gene that are not present in the control population [50]. Compared to MCHR1, there is relatively little known about MCHR2 due to the fact that MCHR2 isn't natively expressed in rodents [51]. However, Ghoussaini et al. performed a genetic study on French children exhibiting an obese phenotype, comparing the genetic sequence of their MCHR2 gene to the sequence of average weight controls. While the study couldn't directly link any of the SNPs identified as a direct cause of obesity, it did identify one that could

potentially mediate overeating behavior in children [52]. Recently, MCH receptors have been targeted by small molecule antagonists as a treatment of obesity and as of late 2007, there were no less than 97 patent applications under review [53]. Animal models utilizing antagonists generally resulted in reduced food intake coupled with a decrease in body mass which was usually due to a decrease in adipose tissue however human trials had yet to be conducted [53].

Significance

Obesity has reached epidemic levels in the United States. More than 61 million Americans are considered obese and this number is expected to grow into the coming decades. Recent studies have implicated MCH signaling through MCHR1 as a target to potentially treat obesity with several MCHR antagonists currently under development. However, relatively little is known about the desensitization mechanisms of MCHR1 and even less about MCHR2 since it's not natively expressed in rodents. The first aim was to clone both MCHR1 and MCHR2 into N-terminal and C-terminal mammalian expression vectors. This was accomplished utilizing genetic cloning techniques and could potentially allow for the measurement of agonist-induced internalization of MCHR1 and MCHR2 in a cultured cell model. Both the second aim was to determine the interaction between MCHR1, β -arrestin 1, and β -arrestin 2 while the third aim was to determine what role GRK2 had in the regulation of receptor desensitization and internalization. The second and third aims were accomplished by cotransfection of MCHR1 and either β -arrestins or GRK2 and

quantifying internalization utilizing a modified cell-based ELISA to measure receptor desensitization and fluorescence microscopy to measure receptor colocalization. Enhancing the understanding of MCHR desensitization and internalization will hopefully allow for better treatment options to fight the growing obesity epidemic.

MATERIALS AND METHODS

PCR

Polymerase chain reaction was performed using the following primers: MCHR1 up primer, MCHR2 up primer, MCHR1&2 down primer. One hundred microliter reactions were utilized to facilitate PCR purification. MCHR1/2 template was prepared and 10ng was pipetted into a sterile PCR tube. A PCR master mix was prepared by pipetting 3µl each of 10µM up and down primers into a microfuge tube along with 20µl of 5X GC buffer, 6µl of 2.5mM dNTP's, 2µl of HIFI Taq polymerase and 64µl of sterile water on ice. The master mix was added to the template in the PCR tube and amplified. Products were verified by running 5 to 10µl on a 1% agarose gel.

PCR Purification

Prior to enzymatic manipulation, PCR products were purified using either a Qiagen or Bio Basic PCR purification kit following the manufacturers recommended protocol. DNA was resuspended in 30µl of elution buffer (2mM Tris-HCL pH 8.0~8.5) and stored at -20°C.

Restriction Enzyme Digestion

Restriction enzyme (RE) digests were performed with a 40µl total volume. A volume of 5µl of DNA was added to a sterile microfuge tube. A master mix was prepared by pipetting 4µl of 10X RE buffer into a microfuge tube along with 1.5µls

of the appropriate enzyme(s) (Promega, New England Biolabs). Sterile water was added to a final volume of 35µls. The master mix was added to the DNA and the digest was incubated for a minimum of 5 hours at 37°C. The digests were then frozen at -20°C. DNA loading buffer was added just prior to gel loading to a final concentration of 1X.

Agarose Gel Purification

Plasmid samples with DNA loading buffer were run on a 1% TAE agarose gel along with a DNA molecular weight standard (Fisher) until DNA bands were well separated. A small metal spatula was used to perforate the gel. DNA filter paper, pre wet with 1X TAE, was inserted into the perforations and the DNA was run on to the filter paper under low voltage. Ultraviolet light was used to verify DNA was present on the filter paper. The filter paper was then removed from the gel and spun down in a microfuge tube to remove the DNA. The presence of DNA was verified by placing the microfuge tubes into an ultraviolet light box.

Ligation

Ligations were performed by adding 3µl of RE digested and gel purified plasmid and 6µl of RE digested and gel purified receptor PCR product to sterile microfuge tubes. To the tubes, a master mix containing 2µl of T_4 ligase buffer, 1µl of T_4 ligase (Fisher), and 11µl of sterile water was added. The ligation mixture was then incubated at 13°C overnight.

Preparation of Calcium Competent *E. coli*

A 100ml culture of DH5α *E. coli* was incubated in LB in an orbital incubator at 37˚C until cells were in mid-log phase (0.5 – 0.7 OD @ 600nm). The culture was then chilled on ice and centrifuged in a SS-34 rotor at 7000RPM for 7 minutes at 4˚C prior to decanting the supernatant. The *E. coli* were resuspended in 10ml of sterile 0.1M $CaCl_2$ solution and incubated for 20 minutes on ice. The cells were then centrifuged and decanted as above prior to being resuspended in 5ml of 0.1M $CaCl_2$/15% glycerol solution. Microfuge tubes with 200µl aliquots were frozen and stored at -80˚C.

Preparation of Electrocompetent *E. coli*

A 100ml culture of DH5α *E. coli* in LB was incubated in an orbital incubator at 37˚C until cells were in mid-log phase (0.5 – 0.7 OD @ 600nm). The culture was then chilled on ice and centrifuged at 7000 rpm for 7 minutes at 4˚C in an SS-24 rotor prior to decanting the supernatant. The *E. coli* was washed with 1 volume of sterile dH_2O, 1/2 volume of sterile dH_2O and $1/20^{th}$ volume sterile 10% glycerol respectively, centrifuging and decanting as previously described between each step. The *E. coli* was then resuspended in $1/500^{th}$ volume sterile 10% glycerol solution before 40µl volumes were aliquoted into microfuge tubes. The aliquots were then frozen and stored at -80˚C. (Note: all wash steps were performed on ice.)

Calcium Competent *E. coli* Transformation

Two hundred microliter aliquots of calcium competent *E. coli* were thawed on ice (as prepared or courtesy of Dr. Rey Sia). Five microliters of plasmid ligation was then added. The aliquots were incubated on ice for 30 minutes, heat shocked for 90 seconds at 42°C water bath and then incubated on ice for 2 minutes. Nine hundred microliters of LB was then added to the aliquots prior to incubation in an orbital incubator at 37°C for 1 hour. One hundred microliter, 10 microliter, and 1 microliter volumes of *E. coli* were plated on LB plus 100μg/ml ampicillin agar plates and incubated overnight at 37°C.

Electroporation of *E. coli*

Electrocompetent *E. coli* were thawed on ice. One microliter of plasmid was added and the aliquots were incubated on ice for 2 minutes. The *E. coli* were transferred to an ice-cold Electroporation cuvette and electroporated at 1.52kv for 6 milliseconds. The *E. coli* was resuspended in 1ml of LB broth, transferred to a sterile microfuge tube and incubated in an orbital incubator at 37°C for 1 hour. One hundred microliter, 10 microliter, and 1 microliter volumes of E. coli were plated on LB plus 100μg/ml ampicillin (Fisher) agar plates and incubated overnight at 37°C.

Plasmid Miniprep

Culture tubes containing 5 ml of LB broth with 100μg per ml ampicillin was inoculated with a single *E. coli* colony from LB amp plates. The cultures were

incubated in an orbital shaking incubator overnight at 37°C. Plasmid DNA was extracted following the recommended Qiagen or Bio Basic Miniprep protocol.

Plasmid Maxiprep

Culture tubes containing 5 ml of LB broth with 100μg per ml ampicillin was inoculated with a single E. coli colony from LB amp plates. The cultures were incubated in an orbital shaking incubator for 6 to 8 hours at 37°C. The cultures were then used to inoculate 500ml LB cultures with 100μg per ml ampicillin which were incubated overnight in an orbital shaking incubated at 37°C. Plasmid DNA was extracted following the recommended Qiagen Maxiprep protocol.

Tissue Culture

BHK-570 cells (ATCC) were cultured as a tissue monolayer using DMEM media (CellGro) containing 10% fetal bovine serum (FBS) (Atlanta Biological). Cells were fed every four days and passaged when they were confluent. Culture conditions were set at 37°C, 5% CO2, and 80% humidity.

Transfection

Cell lines were transfected at 90% confluency for ELISA experiments and 50% confluency for fluorescence microscopy experiments. Transfections were carried out following the recommended protocol from SignaGen utilizing their

LipoD293 reagent. Media was changed 5 hours post- transfection and experiments were run approximately 48 hours post transfection.

Fixed Cell Fluorescence Microscopy

Coverslips seeded with BHK-570 cells were transfected with MCHR1-VSVg (courtesy of G. Milligan) ± β-arrestin 1 or 2 (courtesy of R. Lefkowitz) or ± GRK 2 or GRK2 K220L (courtesy of G. Milligan). Coverslips seeded with BHK-570 cells were transfected with MCHR1-VSVg ± β-arrestin 1 or 2. Cells were then incubated for approximately 24 hours prior to MCH treatment, then treated ± 1μM MCH (American Peptide) for time points of 0, 2, 5, 10, 20, and 30 minutes. The coverslips were then rinsed 2 times with ice-cold PBS before fixing for 10 minutes in 4% paraformaldehyde at room temperature. The coverslips were then rinsed 3 times with PBS before being transferred to a humidification chamber and incubated in blocking buffer for 1 hour. The coverslips were then incubated for 1 hour with rabbit anti-VSVg polyclonal antibodies (Sigma) at 1:50 in blocking buffer. The coverslips were then washed 3 times with PBS before being treated with DAPI (Roche) at 1:500 and AlexaFluor goat anti rabbit secondary antibody (Invitrogen) at 1:250 in blocking buffer for 1 hour. The coverslips were then washed 3 times in PBS before being mounted on slides with Prolong Gold (Invitrogen). The edges of the coverslips were sealed with clear nail polish. Fluorescence micrographs were obtained with a Zeiss Axioskop fluorescence microscope with a Zeiss Axiocam camera and Zeiss Axiovision software.

Live Cell Fluorescence Microscopy

Coverslips seeded with BHK-570 cells were transfected with MCHR1-VSVg ± β-arrestin 1 or 2 or ± GRK 2 or GRK2 K220L. Cells were then incubated for approximately 24 hours prior to MCH treatment. Coverslips were rinsed twice with HBSS preheated to 37˚C. Coverslips were then mounted in a Sykes-Moore chamber. MCH was added at 1μM in HBSS (CellGro) and fluorescence micrographs were taken from time point 0 to 30 minutes using a Nikon inverted fluorescence microscope utilizing a SPOT camera and software.

Cell Based ELISA

Twenty-four well plates were seeded with BHK-570 cells and transfected with MCHR1-VSVg ± β--arrestin 1 or 2 or ± GRK 2 or GRK2 K220L. Twenty-four hours post-transfection, the plates were aspirated and incubated at room temperature in labeling buffer with 1:1000 mouse anti VSVg (Sigma) for 2 hours. The plates were then washed 2 times in labeling buffer before being treated with MCH at 0, 15, and 30 minute time points. The plates were then washed 1 time with ice cold PBS before being fixed in 3% paraformaldehyde for 20 minutes at room temp. The plates were then washed 2 times with PBS for 5 minutes before being incubated with goat anti-mouse HRP-conjugated secondary antibody (Bio-Rad) in PBS plus 5% goat serum for 45 minutes. The plates were then washed 3 times with PBS for 5 minutes prior to added 175μl of POD blue (Roche). The plates were incubated were incubated for 3 minutes on an orbital shaker before being neutralized with 175μl of 10% sulfuric acid

solution. One hundred fifty microliters from each well was transferred to a 96 well plate and the absorbance was read at 450nm using a Synergy 1 or ELx800 plate reader (BioTek).

Statistical Analysis

The averages of the data were reported ± the standard error of the mean (SEM). Student's t-test was performed to determine statistical significance with data scoring equal to or greater than the 95^{th} percentile considered significant.

RESULTS

Designing a Strategy to Epitope Tag MCHR1 and MCHR2

Melanin-concentrating hormone (MCH), a cyclic polypeptide containing 19 amino acid residues, has been shown to play an integral role in regulating energy homeostasis. Previous studies utilizing knockout mice lacking MCH have produced mice with a lean phenotype, which also exhibit increased level of activity and reduced stores of adipose tissue. In these same studies, mice overexpressing MCH tend to be resistant to insulin and express an obese phenotype. In upper mammals, such as primates and humans, MCH can signal through two different GPCR's: MCHR1 and MCHR2, which share 38% sequence homology. MCHR1 has been shown to activate multiple signaling pathways through the activation of G_q, G_i, and G_o proteins while MCHR2 is only known to activate the G_q pathway. While MCHR1 has been widely studied, far less is understood about MCHR2, in part because it is not natively expressed in rodent model systems such as mice or rats.

To further the understanding of the function of MCHR2, both individually and in coordination with MCHR1 in the same cell, we decided to clone both MCHR1 and MCHR2 into tagged mammalian expression vectors. The use of epitope-tagged expression vectors allowed for the ability to utilize several different experimental techniques, such as western blots, immunostaining, and cell-based ELISA's to study the receptor's behavior. The transient transfection nature of the plasmids also allowed for the study of the receptors behavior in multiple types of cells. Different

types of cells can behave differently based on the expression of endogenous proteins involved in receptor regulation and internalization [54].

To create the vectors, N-terminal MYC- and HA-tagged mammalian expression plasmids were obtained from Clontech (Figure 2) and human MCHR1 or human MCHR2 cDNA were cloned into their multiple cloning sites. Direct excision of the MCHR1 pcDNA3 and MCHR2 pcDNA3 cDNA and subsequent ligation into the MYC and HA plasmids proved not feasible due to a reading frame shift between plasmids. In order to modify the MCHR1 and MCHR2 cDNA to correct for this reading frame shift, PCR up primers were designed with an EcoR1 restriction enzyme site upstream of the MCHR1 and MCHR2 coding regions and PCR down primers containing a Xho1 restriction enzyme site downstream of the cDNA. The PCR primers (Appendix 1) were designed to delete the stop codons and have similar annealing temperatures while still being specific to their targets and contained restriction enzyme digestion sequences in the correct reading frame. The PCR was carried out following the program listed in the materials and methods section.

Following the completion reaction, a small sample of the products were run on a 1 percent TAE agarose gel with ethidium bromide for approximately 30 minutes at 100 volts to verify the product. The DNA would be intercalated with ethidium bromide and therefore glow under ultraviolet light. A band of 1294 bp was expected for MCHR1 while a band of 1044 bp was expected for MCHR2. As illustrated in Figure 3, MCHR1, located in lane 2, produced a band of 1300 bp which was approximately what was expected. An additional band was located

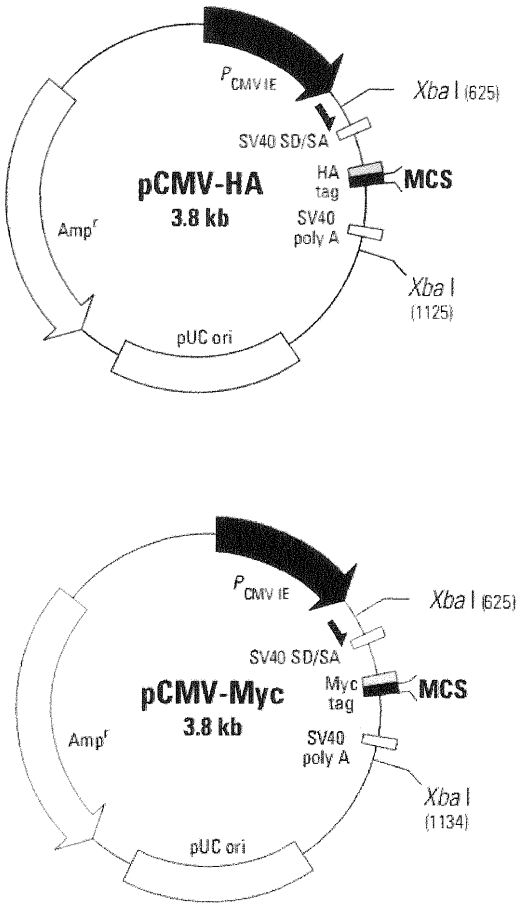

Figure 2: Graphical representation of the N-terminal Myc and HA mammalian expression Vectors. Clontech expression vectors were utilized due to highly reactive HA and Myc epitope tags and high expression levels through a cytomegalovirus promoter. Image courtesy of Clontech.

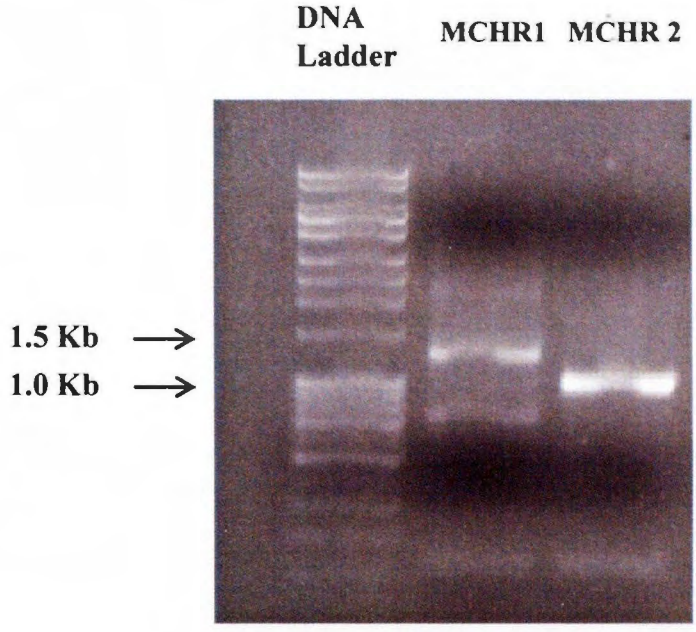

Figure 3: Agarose gel electrophoresis of MCHR1 & MCHR2 PCR Product. MCHR1 and MCHR2 cDNA with missense mutated start codons were generated through PCR, digesting using EcoR1 and Xho1 restriction enzymes and gel purified to prepare for ligation. DNA was stained with ethidium bromide and visualized under UV light.

near 800 base pairs and is most likely due to non specific primer binding due to sub-optimal annealing temperatures. The MCHR2 sample located in lane 3 produced a band of 1025 bp which was consistent with what was expected; the receptor cDNA's were now in the appropriate reading frame for the intended target plasmids.

To facilitate the insertion of the reading frame-modified MCHR1and MCHR2 cDNA into the pCMV MYC and pCMV HA vectors, a restriction enzyme digest was performed followed by gel purification. MCHR1 and MCHR2 PCR cDNA along with pCMV MYC and pCMV HA maxiprep DNA (Figure 4) were digested with EcoR1 and Xho1 restriction enzymes for 5 hours at 37°C. The digested samples were then separated by agarose gel electrophoresis to allow for purification according to the gel purification procedure in the methods section. Five microliter aliquots of the plasmid restriction enzyme digests were separated by agarose gel electrophoresis to verify the bands were approximately the correct length. Single digests containing either EcoR1 or Xho1 enzymes were expected to produce DNA bands at 3782 base pairs for the pCMV HA plasmid and 3791 base pairs for the pCMV MYC plasmid. Double digests containing both EcoR1 and Xho1 enzymes were expected to cut out a 21 base pair section of the polylinker and produce DNA bands of 3761 base pairs for the pCMV HA plasmid and 3770 base pairs for the pCMV MYC plasmid.

As illustrated in Figure 4, undigested pCMV HA and pCMV MYC (lanes 2 and 6 respectively) show bands near 2 kilobases which was expected because the plasmids were in a supercoiled conformation and would therefore travel further on the gel. Single enzyme digests of pCMV HA (lanes 7 and 8) as well as pCMV MYC

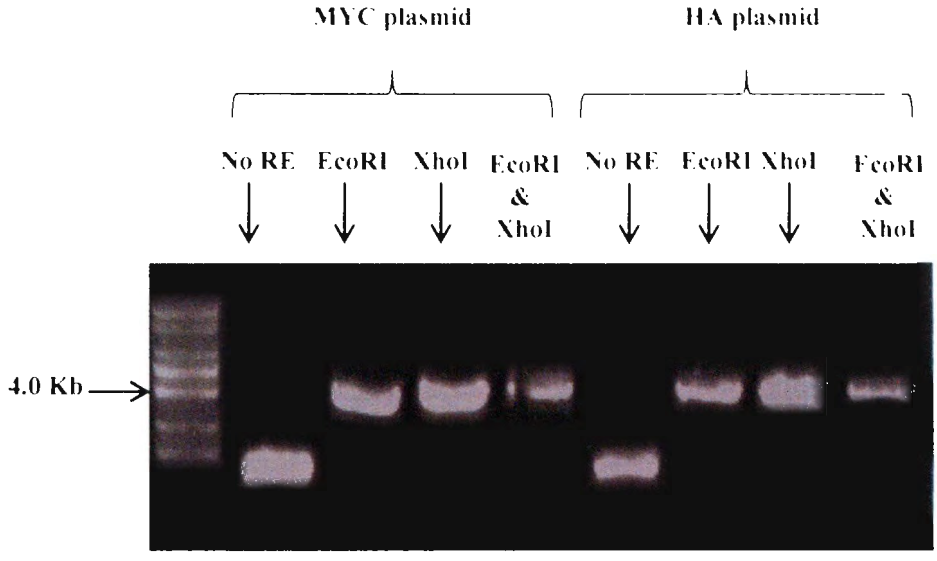

Figure 4: Agarose gel electrophoresis of pCMV Myc and pCMV HA plasmids. pCMV Myc and pCMV HA plasmids were digested using EcoRI and XhoI restriction enzymes and gel purified to prepare for ligation with MCHR1 and MCHR2. DNA was stained with ethidium bromide and visualized under UV light.

(lanes 2 and 3) show bands just under 4 kilobases which was expected while the double digests (lane 5 MYC and lane 9 HA) also show bands just smaller than 4 kilobases which was also expected since a 31 base pair excision would be insufficient to cause a visible shift on the gel. Gel purification was verified by illuminating the purified DNA via ethidium bromide and long wave ultraviolet light.

The gel purified MCHR1 and MCHR2 cDNA was then ligated into the gel-purified pCMV HA and pCMV MYC plasmids as described in the materials and methods section (Figure 5). Receptor cDNA was added to plasmid DNA along with T4 DNA ligase and incubated overnight at 13°C to generate 4 expression vectors: pCMV HA MCHR1, pCMV MYC MCHR1, pCMV HA MCHR2, and pCMV MYC MCHR2. The T_4 DNA ligase was heat-deactivated at 70°C for 10 minutes before aliquots of ligation mixture were used to transform DH5α *E. coli*. Transformed E. *coli* were selected for on LB plus ampicillin agar plates before being miniprepped. The miniprep DNA was then subjected to RE digestion with EcoR1 and Xho1 to verify successful insert ligation through separation by agarose gel electrophoresis. Two DNA bands were expected for each digest. As illustrated with Figure 6, double digestion of pCMV HA MCHR1(bottom gel, lanes 3 and 6) produced bands of just under 4 kilobases and between 1.5 and 1.0 kilobases in length while double digestion of pCMV HA MCHR2 (bottom gel, lanes 9 and 12) produced bands of just under 4 kilobases and just larger than 1.0 kilobases in length which is what was expected if ligation was successful. Results for pCMV MYC MCHR1 and pCMV MYC MCHR2 were similar (top gel, lanes 3, 6, 9, and 12 respectively).

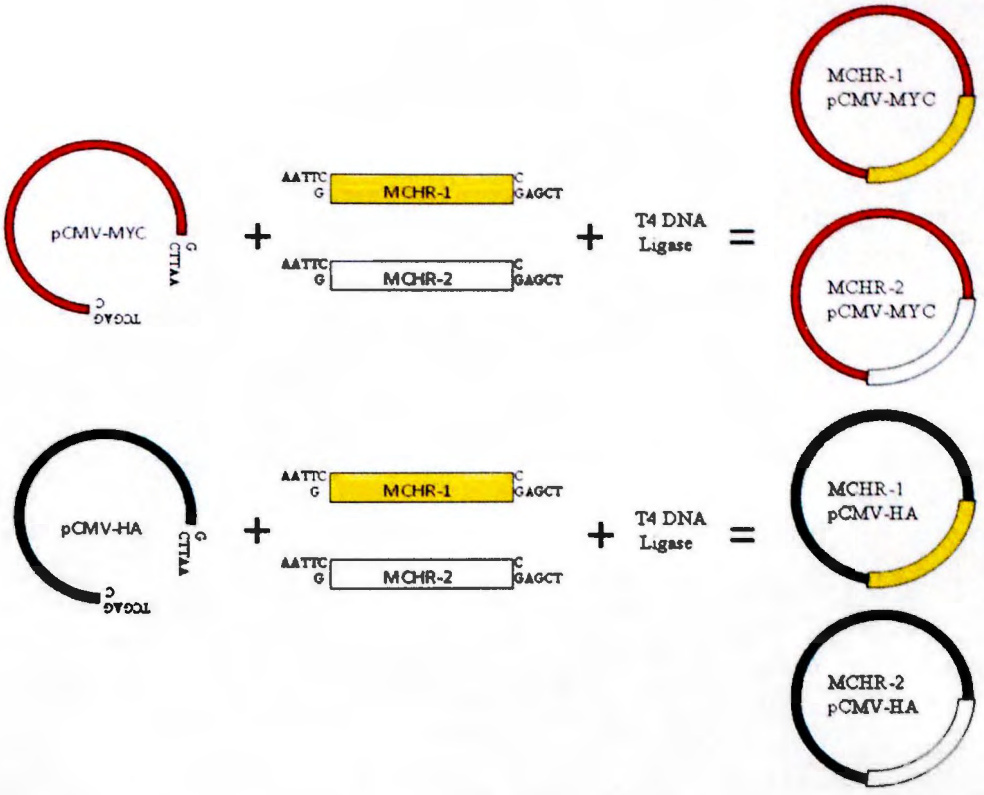

Figure 5: Graphical representation of the ligation process of MCHR1 & MCHR2 into pCMV Myc & pCMV HA plasmids. pCMV Myc and HA vectors were incubated with MCHR1 and 2 cDNA in the presence of DNA ligase. Recombinant clones were selected for and amplified as described in the materials and methods.

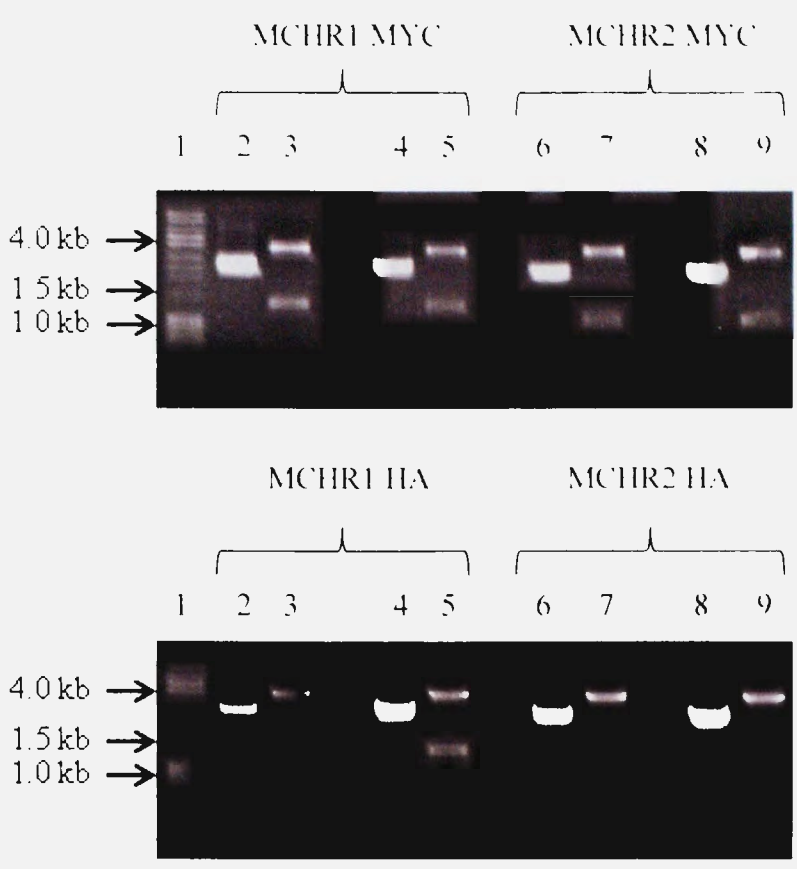

Figure 6: Agarose Gel Electrophoresis of MCHR1 & MCHR2 ligated in pCMV Myc & pCMV HA. EcoR1 and Xho1 double digests of the recombinant clones were run out on an agarose gel along with undigested plasmid to verify the clones contained receptor. DNA was stained with ethidium bromide and visualized under UV light. Gels 1 and 2: lanes 2, 4, 6, and 8 undigested. Lanes 3, 5, 7, and 9 digested.

While restriction enzyme digestion verified MCHR1 and MCHR2 ligation into pCMV HA and pCMV MYC; it could not confirm that the DNA sequence remained intact. So in order to verify that a small point mutation or insertion or deletion had not occurred that could alter experimental results, genetic sequencing was performed. Clone DNA plasmid preps were subjected to DNA sequencing using sequencing primers (Appendix 1) to verify the sequences for MCHR1 and MCHR2 were intact and in frame. Sequencing results indicated that the cDNA for all 4 clones, pCMV HA MCHR1, pCMV MYC MCHR1, pCMV HA MCHR2, and pCMV MYC MCHR2 was correct.

In order to test for the plasmids surface protein expression, BHK cells were transiently transfected with pCMV HA MCHR1, pCMV MYC MCHR1, pCMV HA MCHR2, and pCMV MYC MCHR2. Cells were then fixed and immunostained using MYC- and HA-tag specific primary antibodies and fluorescent secondary antibodies and DAPI before being imaged with a fluorescent microscope. Observation of the cells 24 hours post transfection showed they exhibited a spherical cell morphology in contrast to the nontransfected cell's spiky morphology. These observations indicated the cells were not in a homeostatic state. Fluorescent micrographs indicated limited-to-no surface expression of both MCHR1 or MCHR2 with either tag. The lack of expression coupled with the change in cell morphology indicates that the expression vectors may have been cytotoxic to the cells. The cytotoxicity could be due to several factors including protein folding, tag size, and or tag sequence which will be expanded upon in the discussion section.

Designing a Strategy to Tag MCHR1 and MCHR2 with Fluorescent Proteins.

Since the N-terminal epitope tagging of both MCHR1 and MCHR2 proved unsuccessful, we next decided to utilize C-terminal monomeric red and green fluorescent protein expression vectors to answer questions about co-localization and internalization through fluorescence microscopy. The use of the C-terminal fluorescent tags would help prevent any possible interference of the tag during protein folding. An enhanced yellow fluorescent protein C-terminal expression vector containing MCHR1 cDNA, obtained from Graeme Milligan's lab, had been shown to be successfully expressed post-transfection in both BHK and CHO cells based on experiments performed in our lab.

The cloning procedure was almost identical to the cloning of the MYC- and HA-tagged plasmids. To create the vectors, C-terminal GFP- and monomeric RFP-tagged mammalian expression plasmids were obtained from Clontech (Figure 7) and Human MCHR1and MCHR2 cDNA was cloned into a multiple cloning site. In order to modify the MCHR1 and MCHR2 cDNA to be in the correct reading frame to ligate into the vectors, PCR up primers were designed with a NheI restriction enzyme site upstream of the MCHR1 and MCHR2 coding regions and PCR down primers containing a XhoI restriction enzyme site downstream of the cDNA. The PCR primers (Appendix 1) were designed to delete the stop codons and have similar annealing temperatures while still being specific to their targets and contained restriction enzyme digestion sequences in the correct reading frame. The PCR was carried out following the program listed in the materials and methods section.

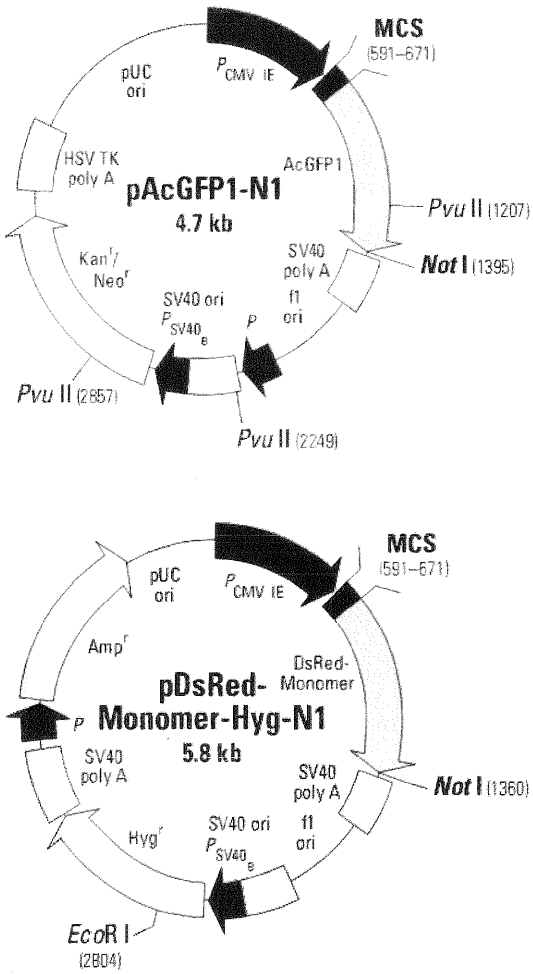

Figure 7: Graphical representation of the C-terminal GFP and RFP mammalian expression Vectors. Fluorescent C-terminal tagged Clontech expression vectors were utilized due to and high expression levels of fluorescently tagged cDNA in the hope of characterizing MCHR1 and MCHR2 simultaneously in the same cell. Image courtesy of Clontech.

After completion of the PCR cycle, small samples of the products were analyzed via agarose gel electrophoresis to verify the band sizes. A band of 1294 base pairs was expected for MCHR1 while a band of 1044 base pairs was expected for MCHR2. As illustrated in Figure 8, MCHR1, located in lane 2, produced a band 1330 bp which was approximately what was expected. The MCHR2 sample loaded in lane 3 produced a band near 1065bp which was consistent with what was expected; the receptor cDNAs were now in the appropriate reading frame for the intended target plasmids.

To facilitate the insertion of the reading frame modified MCHR1and MCHR2 cDNA into the pAcGFP1-N1 and pDsRed-Monomer-Hyg-N1 vectors, a restriction enzyme digest was performed followed by gel purification. MCHR1 and MCHR2 PCR cDNA along with the pDsRed-Monomer-Hyg-N1 maxiprep DNA were digested with Nhe1 and Xho1 restriction enzymes for 5 hours at 37˚C. The digested samples were then separated by agarose gel electrophoresis to allow for purification according to the gel purification procedure in the methods section. Five microliter aliquots of the plasmid restriction enzyme digests were separated by agarose gel electrophoresis to verify the bands were approximately the correct length. Double digests containing both Nhe1 and Xho1 enzymes were expected cut out a 22 base pair section of the polylinker and produce a DNA band of 5726 base pairs for the pDsRed-Monomer-Hyg-N1 plasmid. As illustrated in Figure 7, the double digests of 5 clones of pDsRed-Mnomer-Hyg-N1showed bands just smaller than 6 kb which was expected since a 22 base pair excision would be insufficient to cause a visible shift on the gel

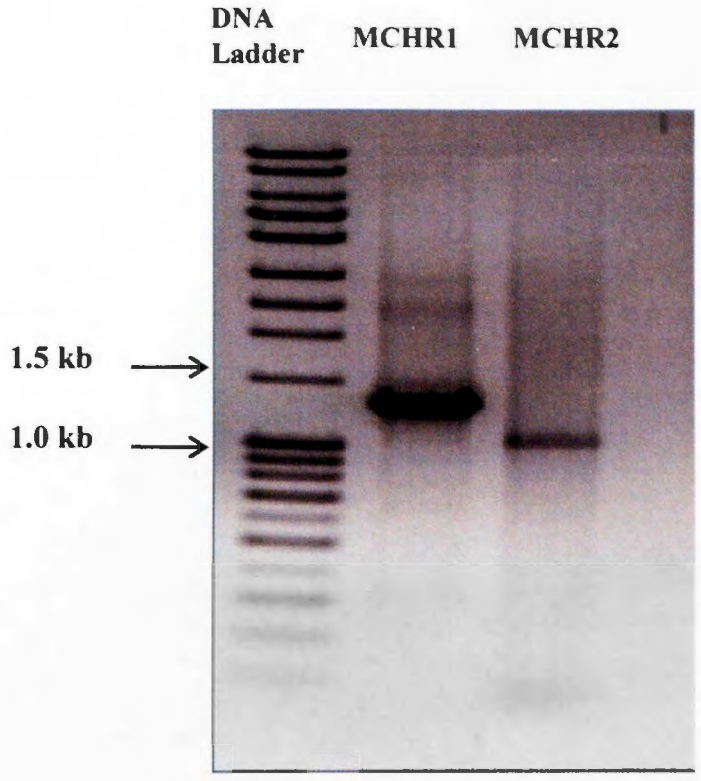

Figure 8: Agarose gel electrophoresis of MCHR1 & MCHR2 PCR product. MCHR1 and MCHR2 cDNA with missense mutations of the stop codon were generated through PCR. The cDNA was digested with EcoR1 and Xho1 restriction enzymes and gel purified to prepare for ligation. DNA was stained with ethidium bromide and visualized under UV light to verify the products.

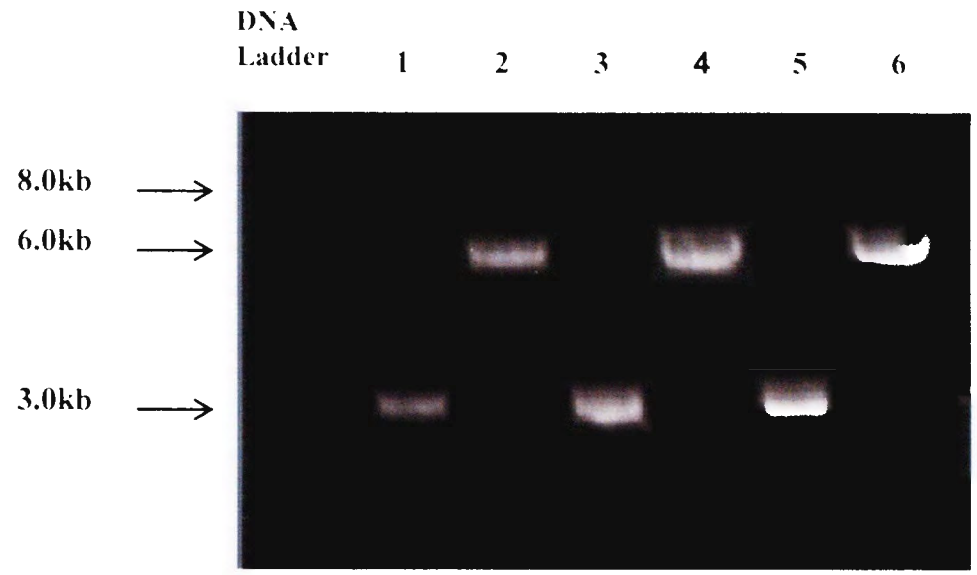

Figure 9: Agarose gel electrophoresis of pDsRed-Monomer-Hyg-N1 plasmid. The pDsRed-Monomer-Hyg-N1 plasmid was digested with Nhe1 and Xho1 restriction enzymes and gel purified to prepare its ligation with MCHR1 and MCHR2. The digests of three clones were run out on an agarose gel for verification, stained with ethidium bromide, and visualized under UV light. Lanes 1,3, and 5 represent undigested plasmid. Lanes 2, 4, and 6 are digested plasmid.

(Figure 9). Gel purification was verified by illuminating the purified DNA via ethidium bromide and long wave ultraviolet light.

The gel purified MCHR1 and MCHR2 cDNA was then ligated into the gel-purified pAcGFP1-N1 and pDsRed-Monomer-Hyg-N1 vectors as described previously to generate 4 expression vectors: pAcGFP1-N1 MCHR1, pDsRed-Monomer-Hyg-N1 MCHR1, pAcGFP1-N1 MCHR2, and pDsRed-Mnomer-Hyg-N1 MCHR2. Aliquots of the ligation mixtures were used to transform calcium competent DH5α *E. coli*. Transformed *E. coli* were selected for on LB + amp agar plates at 37°C overnight. Examination of the plates after incubation revealed no colonies indicating the possibility of incomplete RE digestion prior to ligation.

ELISA Optimization

Little is currently known about the desensitization of MCHR1 in response to agonist treatment. However, a paper published by N. Evans, et.al, in 2001, measured recruitment and internalization of stably-expressing MCHR1 HEK293 cells cotransfected with GRK2, and β -arrestin 1 or 2. Their results indicated that MCHR1 recruited β -arrestin 2 at a preferentially higher rate than β -arrestin 1. Internalization assays showed that neither the overexpression of β -arrestin 1 and β -arrestin 2 resulted in internalization of either β-arrestin. They hypothesized this was a result of the β -arrestin dissociating from the receptor during early stages of receptor internalization [55]. While this paper showed that β -arrestin 2 co-localized with MCHR1, it didn't explicitly measure changes in the rate of MCHR1 internalization

from the cell surface. In another paper by Y. Saito, et.al, published in 2004 and which utilized MCHR1-Flag stably expressing HEK293 cells, over expression of wild type β -arrestin 2 had no effect on the internalization of MCHR1, but transfection of dominant negative β -arrestin 2 (284-409) reduced receptor internalization by nearly 40 percent. Similar to the Evans et.al paper, β -arrestin 1 transfection showed little effect on the internalization of MCHR1 [56]. The inhibition of MCHR1 internalization with cotransfection of dominant negative β -arrestin 2 seems to point towards a clathrin-mediated endocytosis event. However, cotransfection with wild type β-arrestin 2 did not increase receptor internalization as expected if β -arrestin 2 played a function in desensitization, thus contradicting the papers earlier statement.

In order to measure the percent of MCHR1 internalized from the cell surface, a modified Cell-based ELISA was developed. In an experiment performed by Scott Portwood, CHO cells stably expressing VSVg-MCHR-1 were grown in a 12 well plate and incubated with rabbit anti-VSVg antibody in HBSS on ice for 2 hours. One μM MCH in F12k- media was added over a 30 minute time course prior to fixation with 4% paraformaldehyde for 10 minutes. The plate was then blocked with RIPA buffer with 5% goat serum and for 20 minutes at room temperature. Goat anti-rabbit HRP secondary antibody was added in RIPA buffer with 5% goat serum for 1 hour prior to the addition of POD blue. The reaction was stopped by the addition of 10% sulfuric acid and the absorbance was read at 405nm. The results indicated that 100 percent of MCHR1 was internalized from the cell surface after approximately 40 minutes of MCH treatment. To verify the results, a control experiment was

performed minus MCH and the same results were observed [57]. The percent surface receptor measured without MCH treatment fell to zero after 40 minutes which indicated that the assay didn't function as it should and wasn't measuring receptor internalization but rather the primary antibody falling off the receptors on the surface of the cells.

In order to effectively and accurately measure VSVg-MCHR1 internalization following agonist treatment, several different modifications to the ELISA methods were explored. Initially, BHK570 cells transfected with VSVg were plated in 24 well plates. Cells were treated with 1μM MCH for either 0, 15, or 30 minutes and then incubated with rabbit anti VSVg antibody in a 5% milk PBS solution for 20 minutes on ice before fixing with 2% paraformaldehyde for 10 minutes on ice. Cells were then treated with goat anti rabbit HRP secondary antibody in PBS for 40 minutes prior to the addition of POD blue substrate and reading the absorbance at 450 nm using a 96 well plate reader. The results revealed that the signal from the BHK cells expressing VSVg was nearly equal to that of the background signal of the control BHK-VSVg tagged cells not treated with primary antibody.

In order to try and boost the signal to background ratio several alterations to these methods were performed. Multiple fixation methods were performed by changing the concentration of paraformaldehyde from 2% to 4% as well as increasing the fixation time from 10 minutes to 20 minutes which resulted in no significant increase in the assay signal. Fixation followed by primary antibody incubation was also attempted utilizing blocking buffer consisting of 5% milk in RIPA buffer for 20

minutes followed by VSVg antibody in blocking buffer for 90 minutes. As before, there was no appreciable signal present above background. Species of primary antibody was also tested by utilizing both mouse anti-VSVg and rabbit anti-VSVg along with the appropriate HRP conjugated goat secondary antibodies. Experimental results for both species were consistent with one another, but again showed no appreciable signal over background.

A modified cell based ELISA technique developed by the Hinkle lab at the University of Rochester was also tested [58]. This technique involved incubating the cells with mouse anti-VSVg primary antibody at room temperature prior to half the cells being treated with MCH while the other half was treated with just vehicle at 0, 15, and 30 minute time points. The cells were then fixed with 3% paraformaldehyde prior to the addition of secondary antibody (Figure 10). A total of 6 experiments were performed using BHK570 cells cotransfected with VSVg-tagged MCHR1 and a GFP plasmid. Each experiment had a triplicate set of wells for each time point ± MCH resulting in 18 individual samples. The results from all six experiments were averaged and normalized prior to plotting. Results from the experiments indicated BHK570 cells cotransfected with MCHR1 and GFP had constant levels of receptor on the plasma membrane in the absence of MCH with percent receptor at the 0 minute time point 100% ± 2.3%, the 15 minute time point 103% ± 2.3%, and the 30 minute time point 96% ± 2.7%. Cellular exposure to MCH resulted in small but marked decrease in the concentration of surface receptor with percent

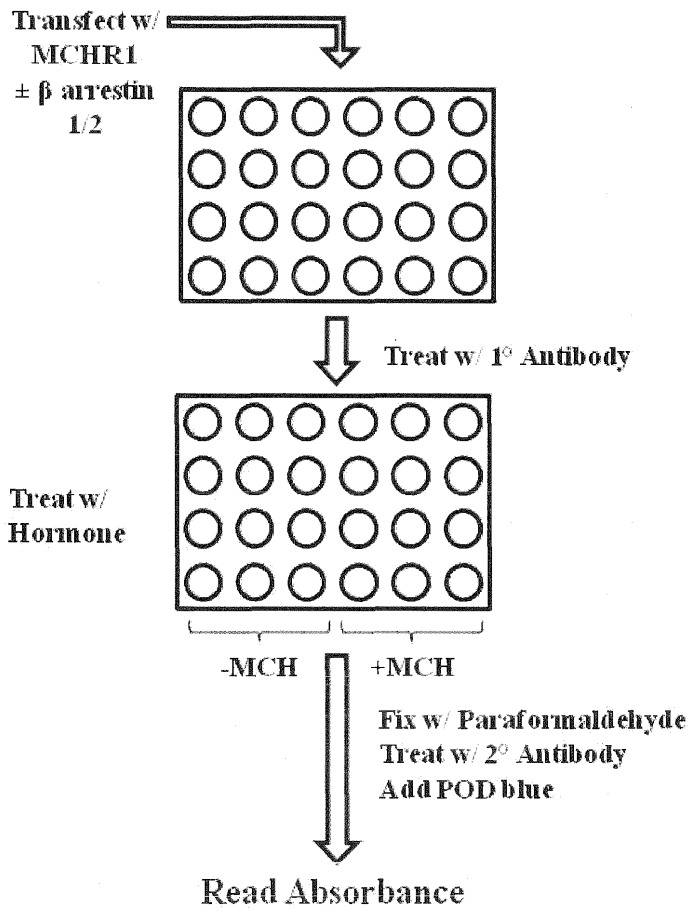

Figure 10: A modified cell based ELISA was developed to measure the loss of MCHR1 from the plasma membrane. A) The optimized ELISA protocol required seeding of BHK570 cells in 24 well plates, subsequent transfection with VSVg MCHR1 and accessory proteins, treated with primary antibody for 1 hour prior to hormone treatment for 30 minutes. The cells were then fixed in paraformaldehyde prior to treatment with HRP conjugated secondary antibody and treatment with POD blue. The absorbance was read at 450nm. Note: experiment was also performed transfecting MCHR1 withGRK2 and DN-GRK2.

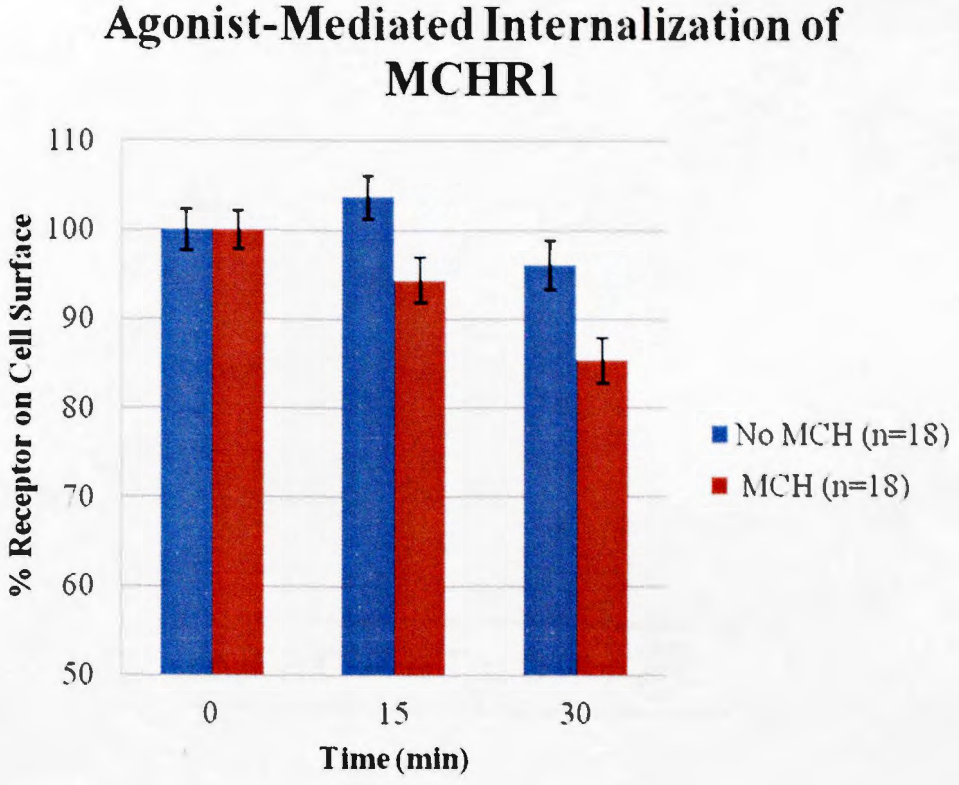

Figure 11: MCHR1 is Sequestered from the Plasma Membrane Under Agonist Exposure. B) The loss of MCHR1 from the plasma membrane was measured using the modified cell based ELISA protocol. MCH treated BHK570 cells exhibited lose of VSVg-MCHR1 from the cell surface over 30 minutes. Cells treated with vehicle showed no internalization.

receptor at the 0 minute time point 100% ± 2.2%, the 15 minute time point 94% ± 2.2%, and the 30 minute time point 85% ± 2.5% (Figure 11).

Role of β–arrestin 1 and 2 in Regulating the Internalization of MCHR1

By successfully designing a Cell-based ELISA protocol to measure agonist-induced VSVg-MCHR1 internalization by measuring the concentration of receptor on the plasma membrane during prolonged agonist treatment, we were now able to address the question of whether MCHR1 was internalized by a clathrin-mediated method. Clathrin-mediated endocytosis has been shown to regulate the desensitization of a multitude of different GPCRs [18] but the affinity of MCHR1 to this type of internalization has yet to be studied in detail. Clathrin-mediated endocytosis follows a relatively conserved pathway where the C-terminal tail of the GPCR is phosphorylated, usually by GRKs and sometimes secondary messengers such as PKC. The β -arrestins then bind to the phosphorylated tail and interact with clathrin heavy chain to facilitate receptor endocytosis [18].

The over expression of β -arrestin has been shown to increase GPCR internalization [59] and it was hypothesized that β -arrestin would act increase the rate of MCHR1 internalization. To measure the effect β -arrestin had on MCHR1 internalization, BHK cells were cotransfected with VSVg-MCHR1 and either β -arrestin 1 or β -arrestin 2 in 24-well plates. The ELISA protocol was performed as described above and in the materials and methods. A total of 7 experiments were performed using BHK570 cells cotransfected with VSVg-tagged MCHR1 and GFP

tagged β -arrestin 1 and a total of 5 experiments were performed using BHK570 cells cotransfected with VSVg tagged MCHR1 and GFP tagged β -arrestin 2. Like the control, each experiment had a triplicate set of wells for each time point ± MCH. The results from the β -arrestin experiments were averaged and normalized prior to plotting.

The β -arrestin 1 experiment indicated the level of MCHR1 on the plasma membrane of BHK570 cells decline with and without the addition of agonist. Without the addition of hormone, the percentage of receptor localized to the plasma membrane equaled 100% ± 3.4%, after 15 minutes the percentage of receptor on the membrane fell to 92% ± 2.6%, and after 30 minutes the percentage receptor fell to 79% ± 2.2%. Treatment with MCH resulted in a significant decrease in the concentration of surface receptor with percentage of receptor on the plasma membrane equaling 100% ± 2.2%, after 15 minute of agonist exposure it equaled 88% ± 2.9%, and after 30 minutes of exposure it equaled 64% ± 2.4%, (Figure 12). The increase in receptor internalization would be expected with the addition of hormone if MCHR1 is internalized through clathrin-mediated endocytosis while the internalization without the addition of MCH seems to indicate hormone-independent basal internalization.

Results from the β -arrestin 2 experiments were similar to that of the β -arrestin 1 experiment in regard to internalization of MCHR1 post treatment with MCH. The level of MCHR1 on the plasma membrane of BHK570 cotransfected with VSVg MCHR1 and GFP β -arrestin 2 declined with the addition of MCH with the

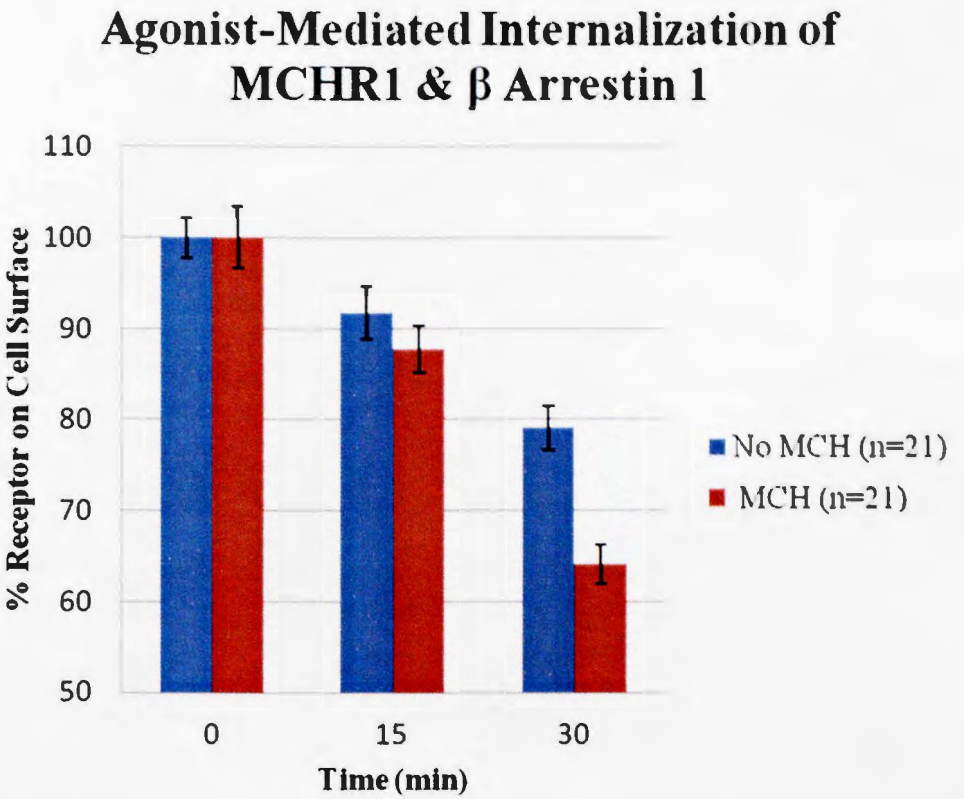

Figure 12: Transient cotransfection of β-arrestin 1 increases the loss of MCHR1 from the cell surface over time. BHK570 cells were transfected with VSVG-MCHR1 and either β arrestin 1 or β arrestin . Cells were treated with MCH or vehicle over a 30 minute time course and internalization was measured utilizing a modified cell-based ELISA. β arrestin 1 increases the loss of MCHR1 from the plasma membrane in both a agonist dependent and agonist independent manner. Ave ± SEM plotted.

percent of receptor on the cells surface 100% ± 6.5% after initial hormone treatment, after 15 minutes agonist exposure, surface receptor fell to 83% ± 4.6%, and following 30 minutes of hormone exposure, surface receptor declined to 62% ± 4.0%. Treatment with placebo resulted in no significant change in the concentration of MCHR1 localized to the plasma membrane with the percentage of receptor at the cells surface being 100% ± 4.0%, 103% ± 5.7%, and 97% ± 4.5% following 0, 15, and 30 minutes agonist exposure respectively (Figure 13).

Receptor internalization was compared between the control, β -arrestin 1, and β -arrestin 2 experiments for the 30 minute time point. Statistical analysis was performed utilizing Student's t-test to determine if β-arrestin 1or β-arrestin 2 significantly influenced receptor internalization. Cotransfection of MCHR1 and β -arrestin 1 resulted in an agonist independent net loss of 17.0% of MCHR1 from the plasma membrane ($p<0.005$) and an agonist-dependent net loss of 21.1%. The cotransfection of β -arrestin 2 resulted in no appreciable agonist-independent net loss and an agonist-dependent net loss of 24.7% ($p<0.01$) (Figure 14). Based, the increase in receptor internalization would be expected with the addition of MCH if MCHR1 is internalized through clathrin-mediated endocytosis.

Role of GRK 2 in Regulating the Internalization of MCHR1

G protein-coupled receptor kinase 2 is a family member of a group of protein kinases that generally function to regulate GPCR signaling through the phosphorylation of serine and threonine residues located on the third intracellular

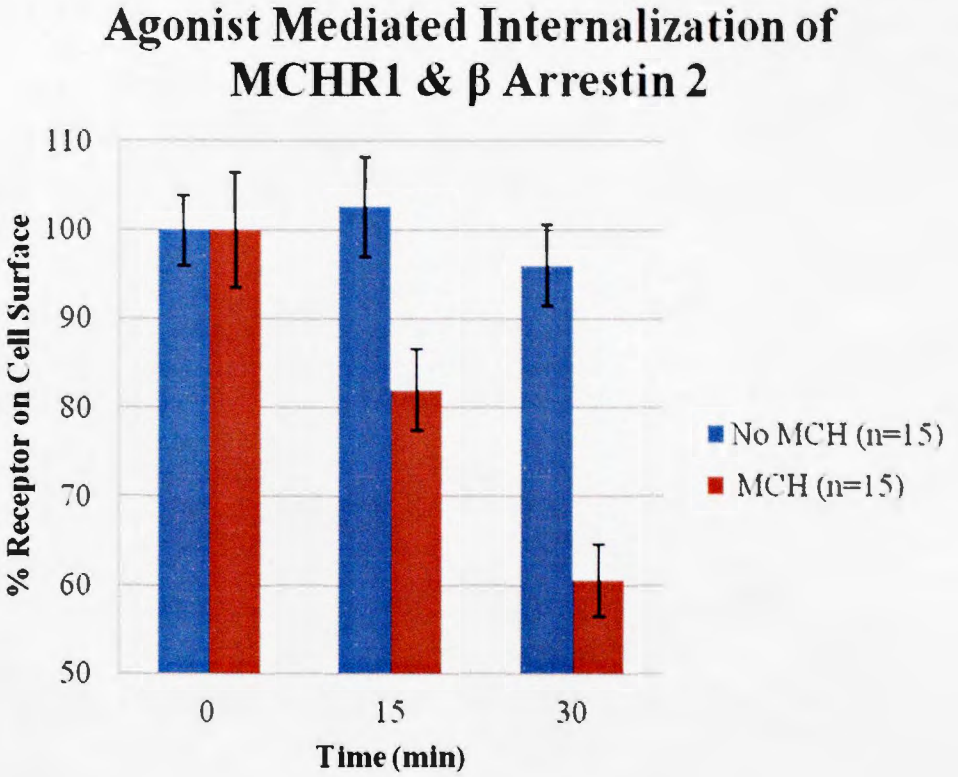

Figure 13: Transient cotransfection of β-arrestin 2 increases the loss of MCHR1 from the cell surface over time. BHK570 cells were transfected with VSVG MCHR1 and either β arrestin 1 or β arrestin . Cells were treated with MCH or vehicle over a 30 minute time course and internalization was measured utilizing a modified cell-based ELISA. β arrestin 2 increases the loss of MCHR1 from the plasma membrane in an agonist dependent manner. Ave ± SEM plotted.

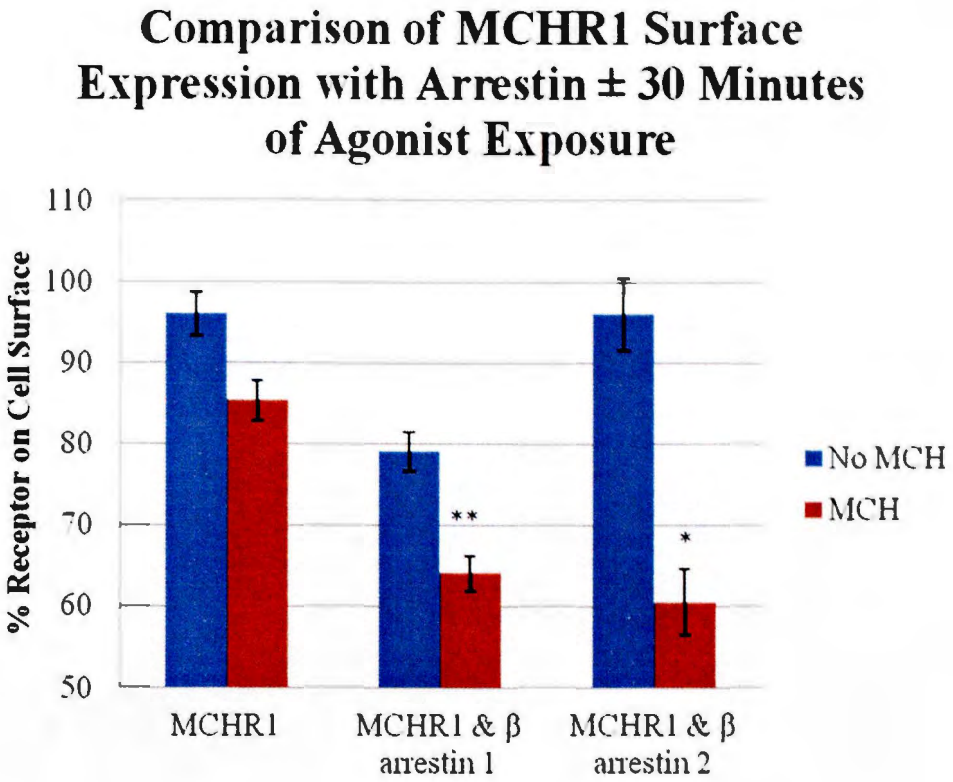

Figure 14: The decrease of MCHR1 mediated by β arrestin 1 and β arrestin 2 from the plasma membrane is statistically significant. Data from the 30 minute time point of the VSVg MCHR1, VSVg MCHR1 β arrestin 1, and VSVg MCHR1 β arrestin 2 ELISA's were compared and statistically analyzed using students T test. Ave ± SEM plotted. ** $p > 0.005$, * $p > 0.01$,

loop and C-terminal tail of GPCR's. The addition of phosphate groups to these areas has been shown to terminate signaling by inhibiting G protein binding and to help facilitate receptor internalization by increasing the affinity of β -arrestin. A mutant form of GRK2, GRK2 K220R, has also been characterized. This dominant negative form of GRK2 is known to knock down the expression of wild type GRK2 when co-expressed. Based on previous studies [60], it was hypothesized that the over expression of GRK2 would increase the rate of MCHR1 internalization while transfection with GRK2 K220R would knock down endogenous GRK 2 expression resulting in MCHR1 internalization being greatly inhibited. ELISAs to measure receptor internalization were performed using the same method as those with β -arrestin.

The GRK2 results indicated the level of MCHR1 on the plasma membrane of BHK570 cells decline both with and without the addition of agonist. Without the addition of hormone, the percentage of receptor localized to the plasma membrane equaled 100% ± 8.8%, after 15 minutes the percentage of receptor on the membrane remained relatively the same at 107% ± 10.3%, and after 30 minutes the percentage receptor fell to 91% ± 9.7%. Treatment with MCH decreased in concentration of surface receptor with percentage of receptor on the plasma membrane equaling 100% ± 11.4 %, after 15 minute of agonist exposure, the level of surface receptor decreased to 91% ± 11.6%, and after 30 minutes of exposure it fell to 79% ± 9.7%, (Figure 15). The agonist-induced internalization results for GRK 2, though less pronounced, were

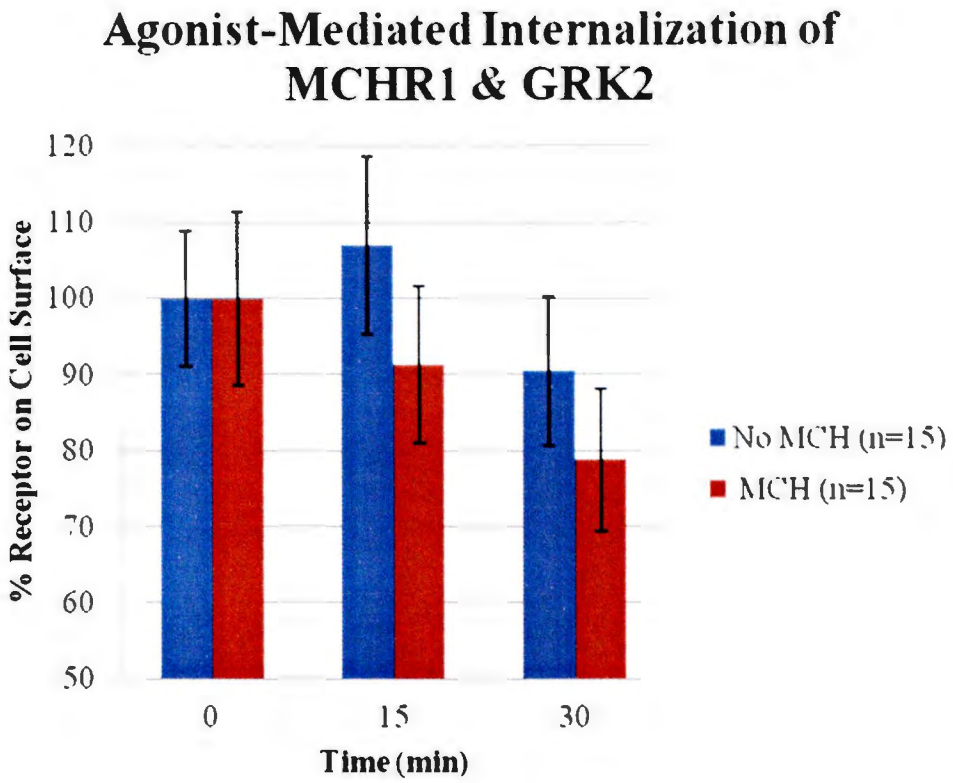

Figure 15: Transient cotransfection GRK2 increases the loss of MCHR1 from the cell surface over time. BHK570 cells were transfected with VSVG MCHR1 and either GRK2 or dominant negative GRK2 . Cells were treated with MCH or vehicle over a 30 minute time course and internalization was measured utilizing a modified cell based ELISA. GRK2 increases the loss of MCHR1 from the plasma membrane in both a agonist dependent and agonist independent manner although at a lower level than β arrestin. Ave ± SEM plotted.

similar to those of cells cotransfected with β -arrestin 2. The level of surface receptor on the plasma membrane of control cells remained stable at approximately 100 percent until the 30 minute time point where it fell to 90.5 percent. The results point toward an intermediary reaction of GRK 2 between both endogenous β -arrestin 1 and β -arrestin 2 with the agonist-dependent internalization being mediated through β -arrestin 1 and β -arrestin 2 while the agonist-independent internalization being solely mediated through β -arrestin 1.

The dominant-negative GRK 2 results indicated the level of MCHR1 on the plasma membrane of BHK570 cells remained relatively constant over time both with and without the addition of agonist. Without agonist addition, the percentage of receptor localized to the plasma membrane equaled 100% ± 3.4%, at 15 minutes, the percentage of receptor on the membrane was 101% ± 2.4%, and after 30 minutes the percentage receptor was to 89% ± 2.4%. The percent surface receptor after the addition of agonist was 100% ± 4.9 %, after 15 minute of agonist exposure, the level of surface receptor was 106% ± 4.5%, and after 30 minutes of exposure it was 97% ± 5.1%, (Figure 16). The agonist-independent internalization values from the dominant negative GRK 2 studies were similar to those of the control remaining relatively constant near 100%. However, agonist treatment showed a decrease in the amount of MCHR1 being internalized post agonist treatment as would have been expected.

Receptor internalization was also compared between the control, GRK2, and dominant-negative GRK2 experiments for the 30 minute time point and tested to determine if the significantly influenced receptor internalization as previously

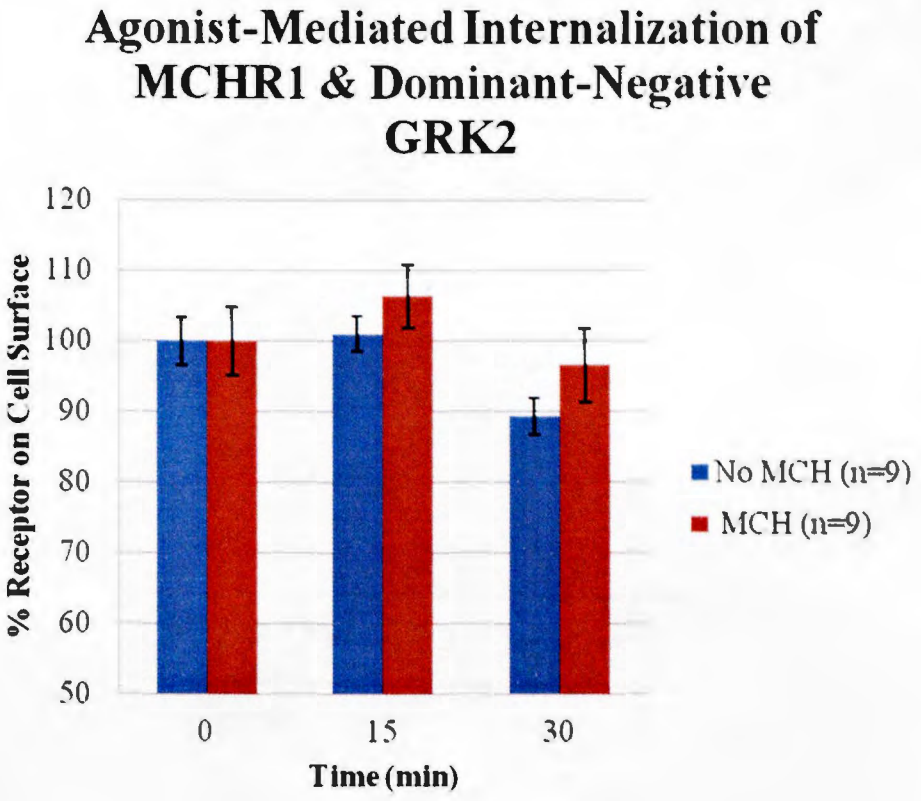

Figure 16: Transient cotransfection GRK2 but not dominant negative GRK2 increases the loss of MCHR1 from the cell surface over time. BHK570 cells were transfected with VSVG MCHR1 and either GRK2 or dominant negative GRK2 . Cells were treated with MCH or vehicle over a 30 minute time course and internalization was measured utilizing a modified cell based ELISA. Dominant negative GRK2 inhibits the loss of MCHR1 from the plasma membrane in response to agonist. Ave ± SEM plotted.

described. Cotransfection of MCHR1 and GRK2 resulted in no appreciable agonist-independent net loss and an agonist-dependent net loss of 6.5% ($p<0.005$). The cotransfection of dominant negative GRK2 again resulted in no appreciable agonist-independent net loss of receptor but revealed an agonist-dependent MCHR1 net gain of 11.3% ($p<0.005$) on the plasma membrane (Figure 17).

Characterizing the interaction between MCHR1, β -arrestin 1, and β -arrestin 2

G protein-coupled receptors can be divided into two classes. Class A receptors preferentially bind β -arrestin 2 over β -arrestin 1 and their interaction is transient in nature with β -arrestin and the receptor dissociating prior to entering the endosome. Class B receptors have equal affinity for both β -arrestin 1 and β -arrestin 2 and form stable interactions resulting in the translocation of both receptor and β -arrestin into the endosome [31]. To characterize the interaction between MCHR1, β -arrestin 1, and β -arrestin 2, BHK570 cells split on coverslips were co-transfected with both VSVg-MCHR1 and GFP β-arrestin. Cells were treated with agonist for 30 min, fixed, permeabilized, and stained with VSVg and AlexaFluor red fluorescent secondary antibody before observation under a fluorescent microscope at 100× magnification.

In cells cotransfected with VSVg-MCHR1 and β -arrestin 1, in the absence of hormone, the MCHR1 fluorescence is evenly distributed across the surface of the plasma membrane while the β -arrestin 1 fluorescence exhibits an even distribution throughout the cytoplasm. Cells fixed after 30 minutes of hormone treatment

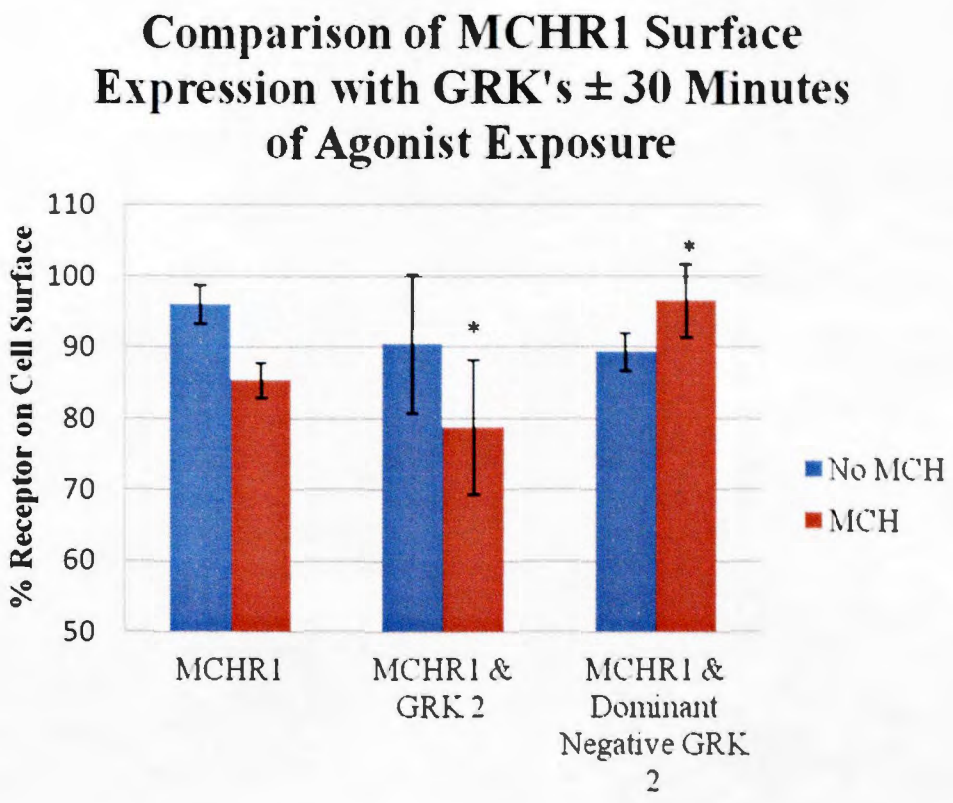

Figure 17: The decrease of MCHR1 on the plasma membrane mediated by GRK2 is statistically significant. Data from the 30 minute time point of the VSVg MCHR1, VSVg MCHR1 GRK2, and VSVg MCHR1 dominant negative GRK2 ELISA's were compared and statistically analyzed using students T test. Ave ± SEM plotted. * $p > 0.005$

exhibited distinct red fluorescent foci on the plasma membrane representing aggregation of MCHR1. Observation of the GFP tagged β -arrestin 1 revealed that it had remained distributed throughout the cytoplasm with evidence of weak interaction through the observation of co localized fluorescence with the MCHR1 (Figure 18). Cells cotransfected with VSVg-MCHR1 and β -arrestin 2 produced similar results with only a weak interaction between MCHR1 and β -arrestin 2 being observed (Figure 19). There was no indication that either β -arrestin 1 or β -arrestin 2 were internalized with MCHR1 suggesting that MCHR1 is a class A receptor since their interactions were transient in nature which is consistent with previous findings [55].

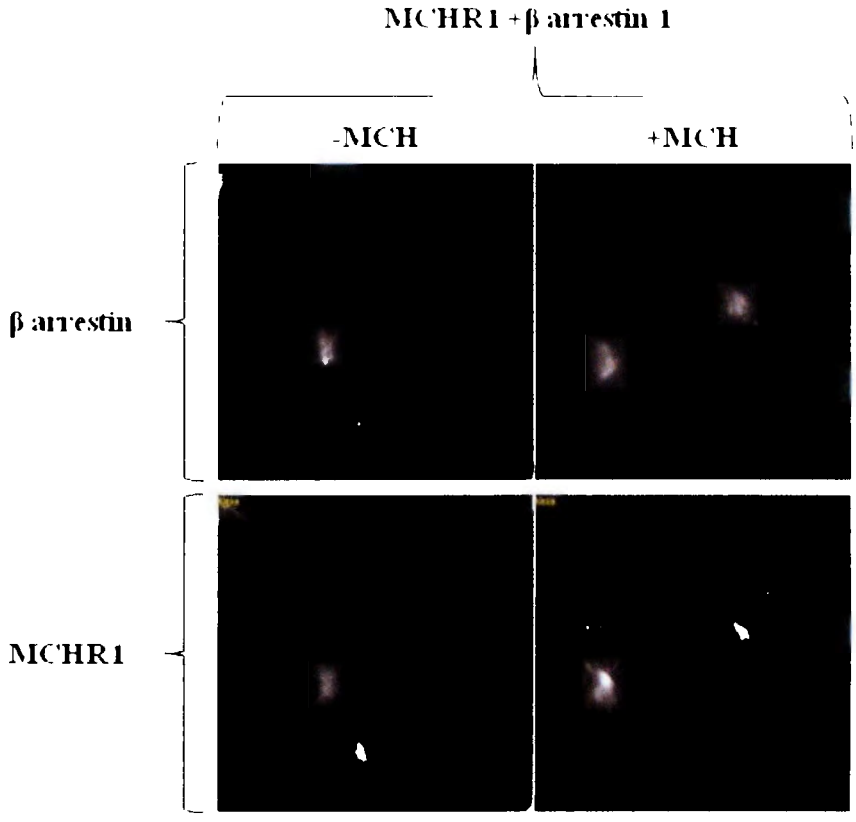

Figure 18: The interaction between VSVg MCHR1 and β arrestin 1 and 2 are transient in nature. BHK 570 cells split on coverslips were transfected with VSVG MCHR1 and GFP β arrestin 1. Cells were treated with hormone or vehicle, fixed and the VSVg MCHR1 was immunostained. Cells treated with vehicle express even GFP β arrestin fluorescence throughout the cytosol while VSVG MCHR1 is present at the plasma membrane. Cells treated with MCH show recruitment of GFP β arrestin to the plasma membrane but no evidence of internalization. Experiment performed by Katrina Haude.

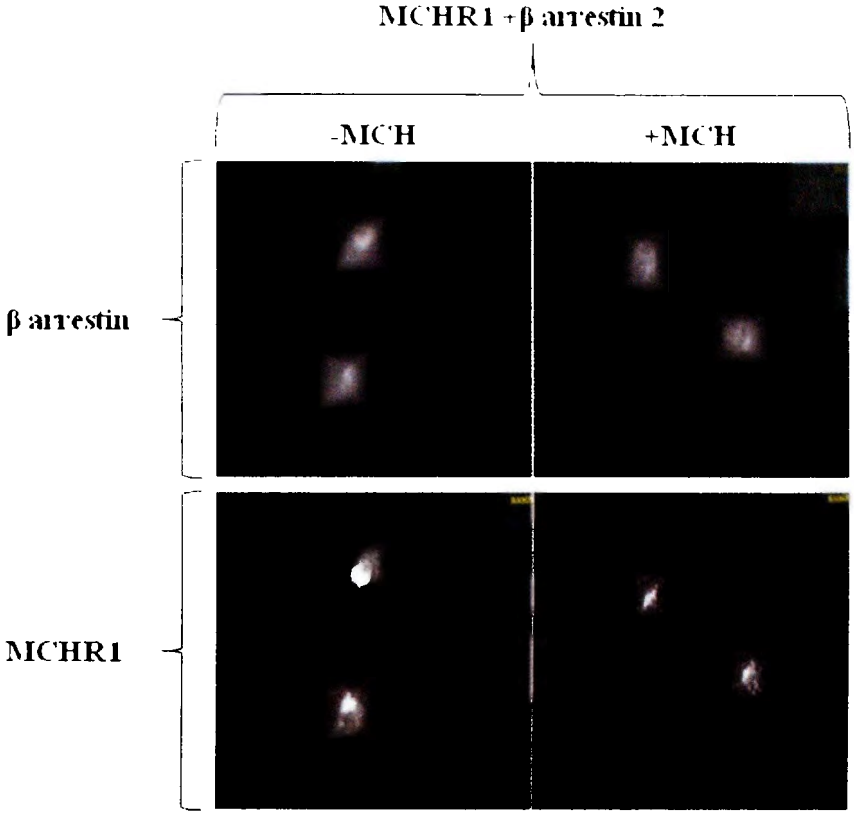

Figure 19: The interaction between VSVg MCHR1 and β arrestin 1 and 2 are transient in nature. BHK 570 cells split on coverslips were transfected with VSVG MCHR1 and GFP β arrestin 2. Cells were treated with hormone or vehicle, fixed and the VSVg MCHR1 was immunostained. Cells treated with vehicle express even GFP β arrestin fluorescence throughout the cytosol while VSVG MCHR1 is present at the plasma membrane. Cells treated with MCH show recruitment of GFP β arrestin to the plasma membrane but no evidence of internalization. Experiment performed by Katrina Haude.

DISCUSSION

Obesity has reached epidemic levels in the United States with greater than 61 million Americans being defined as obese [1]. Obese individuals with a BMI of 45 or greater can expect to lose up to 12 years of their life expectancy [7]. The recent increase in the prevalence of obesity can partially be attributed to the increase of leisure time, decrease in physical exertion, and high fat diets [4]. It's hypothesized that these environmental factors could contribute to the expression of certain genes increasing the likelihood of an obese phenotype [5].

The mammalian brain regulates energy homeostasis through a complex signaling system [9, 10]. One of the hormones of this system, MCH has been shown to help regulate energy homeostasis through MCHR1-mediated signal transduction pathways via both MCH and MCHR1 knockout mouse models [49, 50]. Regulation of the MCH signaling pathway has been implicated as a potential obesity treatment through the modulation of MCHR1 yet little is definitively known about its desensitization [55]. In humans and other upper mammals, a second MCH receptor has been identified. However, little is currently known about MCHR2 primarily since it isn't natively expressed in rodents [53].

In order to further the understanding of both MCHR1 and MCHR2 as potential therapeutic targets to obesity, we decided to create epitope-tagged expression vectors. The creation of these vectors would allow for the transient expression of MCHR1, MCHR2, and the transient co-expression of MCHR1 and MCHR2 in BHK570 cell models to test for co-localization on the plasma membrane,

to characterize the desensitization of both following agonist treatment, and to determine the roles β -arrestin1, β -arrestin 2, and GRK2 had in receptor endocytosis. Several N-terminal epitope-tagged MCHR1 vectors, utilizing FLAG and VSVg tags had been previously constructed with positive results [56,61].

Ideally, mammalian expression vectors should have several features including a high expression level in transfected cells and a highly immunoreactive tag [62]. The pCMV-Myc and pCMV-HA vectors utilized for cloning had a Cytomegalovirus promoter driving transcription, allowing for constitutive protein expression and a high copy number allowing for up to 500 plasmids in each individual transfected cell to further boost protein expression. The c-Myc and Hemagglutinin (HA) tags featured by the plasmids were both highly immunoreactive and well-characterized tags allowing for a high signal to background noise ratio in multiple assays including, ELISAs, fluorescence microscopy, and western blotting, while the small physical size of the tags shouldn't interfere with the function of MCHR1 or MCHR2 [63].

The apparent cytotoxicity of the pCMV-HA MCHR1, pCMV-MYC MCHR1, pCMV-HA MCHR2, and pCMV-MYC MCHR2 plasmids in BHK cells and the subsequent lack of expression could potentially be attributed to several factors including the expression level and copy number of the plasmid as well as the alteration of the proteins structure expressed by the plasmid. The pCMV-Myc and pCMV-HA vectors both had a copy number of 500 meaning that the average transfected cell had approximately 500 copies of the plasmid within its nucleus. The plasmids also utilized a cytomegalovirus promoter that drove transcription in a

constitutive manner. The transcription and translation machinery of the transfected cell is utilized to produce the recombinant protein from the plasmid as well as the standard contingent of proteins normally expressed by the cell. It's possible that the combination of copy number and constitutive expression of the plasmids overtaxed the cellular machinery resulting in insufficient expression of endogenous protein within the cell creating a cytotoxic environment.

As an alternative, MCHR1 and MCHR2 could be cloned into mammalian expression vectors exhibiting inducible promoters. Inducible promoters can be turned on or off by either chemical means, such as antibiotics, steroids, and metals or physical means, such as light exposure or temperature variations. The use of plasmids with inducible promoters allows for the regulation of where and when the cloned cDNA would be expressed. A common inducible promoter utilized in mammalian expression vectors utilizes the antibiotic tetracycline to regulate transcription allowing for not only the regulation of when the cloned gene is transcribed but also how strongly the cloned gene is transcribed since transcription would be dependent on tetracycline concentration. By cloning MCHR1 and MCHR2 into such a vector it would be possible to optimize the expression level of the receptor in a temporal manner as well as modulate their cellular concentration [64].

Alterations in protein confirmation may also induce cell cytotoxicity. The pCMV-Myc and pCMV-HA vectors both utilized N-terminal epitope tags. The N-terminus of G protein-coupled receptors has been shown to play a role in receptor signaling through stabilization of ligand binding. The addition of an immunoreactive

tag to the N-terminal, however small, could potentially interfere with ligand signaling resulting in either the prevention of receptor activation in the presence of hormone or constitutive activation of the receptor in the absence of hormone. Protein structure is based on the sequence of amino acids found in the protein. The secondary structure of a protein is formed starting at the amino or N-terminal of the peptide, continuing to fold as it works its way to the C-terminal [65]. The addition of a small polypeptide tag to the N-terminal of MCHR1 and MCHR2 could have potentially altered its conformation. It's possible that a conformational change to the protein could make it cytotoxic to the cell. A conformational change could interfere with chaperone binding resulting in an excess accumulation of receptor in intracellular compartments such as the ER.

Whether the pCMV-HA MCHR1, pCMV-MYC MCHR1, pCMV-HA MCHR2, and pCMV-MYC MCHR2 induced cell cytotoxicity was a result of protein misfolding due to the N-terminal tags or simply the over expression of the recombinant protein, characterizing the localization and desensitization of MCHR1 and MCHR2 proved not feasible with the pCMV-Myc and pCMV-HA vectors. As an alternative method, we utilized pAcGFP1-N1 and pDsRed-Monomer-Hyg-N1C-terminal tagged vectors. The use of C-terminal tagged vectors would eliminate the potential for N-terminal tag interference with protein folding and our lab as well as others had previously utilized an eYFP-tagged MCHR1 vector that was successfully expressed in both BHK570, CHO-K1, and HEk293 cell lines [66].

The cloning process of the pAcGFP1-N1 and pDsRed-Monomer-Hyg-N1C-terminal tagged vectors involved selecting for the plasmids by plating on agar pates with the appropriate antibiotic at the appropriate concentration. Following incubation with ampicillin at a concentration of 100μg/ml, only colonies of pDsRed-Monomer-Hyg-N1 were visibly present. One possibility why no pAcGFP1-N1plasmid colonies were present was that the transformation of *E. coli* with the pAcGFP1-N1plasmid was faulted in some way, creating E. coli clones that were not resistant. This theory was quickly discounted by performing several further transformations. Upon further examination of the literature, it was clear that the reason no pAcGFP1-N1plasmid colonies were present on amp plates was simply because the pAcGFP1-N1plasmid had a neomycin resistance and not the ampicillin gene originally thought.

While trying to determine the reason for the failure of the pAcGFP1-N1plasmid cloning, the process of constructing the MCHR1 and MCHR2 pDsRed-Monomer-Hyg-N1 constructs continued. The MCHR1 and MCHR2 cDNA engineered without their stop codons were successfully digested with EcoR1 and Xho I and gel purified along with the pAcGFP1-N1plasmid, allowing for their ligation. Following ligation, the recombinant vectors were transformed into E. coli and plated on agar plus ampicillin for selection. Unfortunately, there were no colonies present on the plates. The transformation process was repeated several times using both electrocompetent and calcium competent cells with the same null results indicating that the transformation step wasn't likely the cause of the failure. The RE digest utilized two restriction enzymes; EcoR1 and Xho1. It is possible that the vector or

cDNAs were only cut by one or the other nuclease and not both simultaneously. If this were the case, during the ligation process, only one side of the cDNA would be successfully joined to the vector. The subsequent recombinant vector would be linear in conformation instead of circular and this would have resulted in its targeted degradation when transformed into its host.

Another possibility was that the DNA ligase was inactive or its ligase buffer contents were degraded. In order for DNA to be ligated, a high energy phosphoanhydride bond has to be formed. The DNA ligase utilizes ATP as the energy source for this reaction. Adenosine triphosphate is highly instable and can readily be hydrolyzed into inorganic phosphate and ADP was exposed to temperature. It is possible the ligase buffer had undergone too many freeze and thaw cycles resulting in the breakdown of the ATP it contained. It is also possible that the DNA ligase itself may have gone bad. In order for proteins to function properly, they must maintain the correct 3 dimensional confirmation. The conformation of proteins is dependent on their primary sequence and it is affected by changes in pH, temperature, or the exposure to reducing agents. It is possible that pipette cross contamination could have introduced a reducing agent to the ligase causing its structural breakdown.

ELISA Optimization

The desensitization of G protein-coupled receptors can be performed through the use of an ELISA as well as many other methods including flow cytometry and fluorescence microscopy [56, 57]. The ability to read absorbance of 96 well plates

via a Biotek H1 fluorescent plate reader in lab made the ELISA option the most cost effective and least time consuming option. The cloning of the N and C-terminal tagged expression vectors was performed in the hopes of utilizing them for this purpose. However, their inherent cytotoxicity necessitated the use of VSVg-tagged MCHR1. Previous experiments designed by our lab to measure MCHR1 internalization utilizing a cell based ELISA had proven unsuccessful. Controls treated with vehicle showed that the apparent internalization of the MCHR1 wasn't due to endocytosis but rather the VSVg antibodies rate of dissociation from the VSVg peptide tag being too great to allow meaningful measurement.

In an effort to decrease the K_d of the VSVg antibody-antigen complex, several methodological variations were performed to the assay. When the protocol for the ELISA was initially developed, stably-expressing VSVg-MCHR1 CHO cells were utilized. In an effort to increase MCHR1 expression, transiently-transfected BHK cells were used. The protocol was additionally modified by performing the hormone time course prior to treatment with primary antibody and by modifying the fixation steps by altering the concentrations of paraformaldehyde used to between 2 and 4%. The twenty minute blocking step with RIPA and goat serum was also removed from the original protocol and the 40 minute incubation period with goat anti rabbit HRP secondary was performed in PBS without a blocker instead of RIPA with 5% goat serum. Unfortunately, all of the modifications that were made to the procedure didn't enhance the outcome of the assay with the signal from the receptor remaining at or near background.

Examining the literature allowed for the discovery of a modified cell based ELISA technique optimized by the Hinkle lab at the University of Rochester [58,67]. This ELISA protocol utilized a 2 hour incubation period in buffered media containing primary mouse anti VSVg antibody, treatment with hormone, fixation in 3% paraformaldehyde, and then treatment with secondary antibody. ELISA's performed with this procedure produced signal to background ratios of 5:1 or greater. As to why this procedure worked while the others previously performed didn't is a good question. It is possible the cells maintained a more homeostatic atmosphere while being treated with antibody at room temp in buffered media allowing for better antibody binding than when antibody was in RIPA or PBS at room temp as opposed to 4°C. It is also possible that fixing the cells in 3% paraformaldehyde after primary antibody treatment fixed the antibody to the receptors and therefore the cells through protein cross linking. To verify the protocol was functional, controls were performed utilizing a mock time course treating with vehicle. The results were positive and verified only a ± 5% variation after 30 minutes.

Had the modified ELISA not worked, several other variations to the protocol could have been performed. It would be possible to eliminate the entire secondary antibody and POD blue section of the protocol by utilizing fluorescently tagged IgG primary antibodies. Such tagging kits are readily available from companies such as Invitrogen and covalently link fluorescent dies to the IgG antibodies [67]. Another option would be to produce monoclonal antibodies to the MCHR1 and MCHR2

receptors themselves through the formation of a hybridoma cell line. This would allow the specific targeting of extracellular regions of the receptor.

MCHR1 Internalization

The desensitization of MCHR1 was first described by Lembo et al., who observed internalization of MCHR1 through fluorescent microscopy following agonist treatment [68]. Subsequent studies by the labs of Evans et al. and Saito et al. attempted to characterize MCHR1 internalization in respect to the β -arrestin and clathrin-mediated pathway. Saito measured MCHR1 internalization in stably transfected HEK293T cells via flow cytometry and fluorescence microscopy. Their results showed that the concentration of Flag tagged MCHR1 located on the plasma membrane fell by 44.2% after 30 minutes of agonist treatment [56]. Our ELISA data indicated a 15% decrease of receptor on the plasma membrane after 30 minutes.

There could be several reasons for the discrepancy in the rate of internalization of MCHR1. Saito et al. used HEK293 cells for their experiments [56] while the ELISA utilized BHK570 cells. There could be differential levels in the expression of MCHR1 as well as accessory proteins involved in endocytosis between the cell types. This hypothesis is further supported by the fact that the HEK293 cells line was a stable transfection where as the BHK570 line was a transient transfection. The stable transfection would require random recombination into the cellular genome which could interfere with endogenous protein expression altering the results. The

number of plasmids in a transiently-transfected cell is also generally much greater than a stably-transfected cell further altering the expression levels.

The clathrin-mediated endocytosis pathway has been widely characterized from studies of other GPCRs [69]. The β -arrestins are an important mediator of this pathway, helping to recruit clathrin heavy chain to the receptor [29]. Other GPCR's such as the β_2 adrenergic receptor are known to desensitize through this pathway [70]. Saito further studied this potential method of MCHR1 desensitization by co-transfecting stably expressing HEK293 cells with both dominant negative β -arrestin 1 and dominant negative β-arrestin 2 and measuring receptor internalization [56]. A dominant-negative mutation is a mutant form of a protein, in this case β-arrestin 1 and 2, that prevents its proper function while at the same time inhibits the expression of wild-type protein [71]. According to their findings, the expression of dominant negative β-arrestin 1 had no effect on the rate of MCHR1 internalization. Co-transfection with dominant-negative β-arrestin 2 resulted in a 38% reduction in the number of receptors undergoing endocytosis suggesting only β-arrestin 2 plays a role in MCHR1 endocytosis [56].

Based on the assertion that dominant negative β -arrestins would knock down normal function of β -arrestins thus interfering with internalization and that the over expression of wild type β -arrestin would be expected to enhance receptor internalization, it would be expected that over expression of β -arrestin 2 would increase MCHR1 internalization while β-arrestin 1 would have no effect. The ELISA results for BHK570 cells cotransfected with VSVg MCHR1 and either β -arrestin 1 or

β-arrestin 2, both showed a significant increase in the level of agonist mediated internalization while β-arrestin 1 showed evidence of agonist independent. This directly contradicts the previous findings on β -arrestin 1 [56].

The increase in the rate of MCHR1 desensitization due to β -arrestin 2 over expression is consistent with Saito's finding with dominant negative β -arrestin 2. What is interesting is that HEK293T cells transiently overexpressing wild type β -arrestin 2 showed no significant change in the rate of receptor internalization [57]. This suggests that the transfection may have not been successful or that the β -arrestin 2 cDNA may have been degraded to a point making it nonfunctional. The significant differences in the β -arrestin 1 results point toward the possibility of a cell type specific mechanism of receptor internalization, where the rate and method of MCHR1 internalization is mediated by the expression levels of accessory proteins in each individual cell type.

The protein kinase GRK2 is known to phosphorylate serine threonine residues on the third intracellular loop and C-terminal tail of GPCRs increasing their affinity for β -arrestins in a agonist dependant manner [23]. The affect of GRK2 over expression on the desensitization of MCHR1 had yet to be explicitly measured although Evans et al. utilized GRK2 transfected cells while observing the nature of the interaction of β -arrestins with MCHR1 [56]. The ELISA results indicated that over expression of GRK2 enhanced the agonist induced internalization of MCHR1, though to a lesser extent than cells expressing β -arrestin 1 or 2. These results mirror those from a previous study done with β_2 adrenergic receptor [72]. The β_2 adrenergic

receptor study went further and triple transfected β_2 adrenergic receptor, β_2 adrenergic receptor kinase, and β-arrestins showing a significant synergistic effect. This would suggest that the rate-limiting step in receptor desensitization, at least for β_2 adrenergic receptor, is dependent on the cellular concentration of β-arrestins. It is also possible that the phosphorylation of the C-terminal tail and third intracellular loop is not an explicit requirement for MCHR1 internalization.

There was also evidence of agonist-independent internalization with GRK2 over expression. The results from the β -arrestin 1 ELISA suggested that β -arrestin 1 could mediate agonist-independent endocytosis and the BHK570 cells would natively express β -arrestin 1 at endogenous levels. What is unclear is why GRK2 enhanced this pathway since it functions in an agonist-dependant manner. Agonist-independent regulation of GPCRs by GRK2 has been previously reported but generally involves regulation of the G proteins through their sequestration and not receptor internalization [73].

The transient over expression of dominant-negative GRK2 significantly inhibited MCHR1 internalization in response to agonist treatment. Agonist-induced internalization of MCHR1 while overexpressing dominant negative GRK 2 showed no significant increase in the rate of receptor internalization. These findings were similar to the μ-opioid receptors, which are dependent on phosphorylation by GRKs for the recruitment of β-arrestins and contrast that of the β_2 adrenergic receptor, which can interact with β-arrestins in the absence of GRK2 phosphorylation sites

[74,75]. This suggests that MCHR1 is dependent on GRK2 phosphorylation for the recruitment of β-arrestins.

We also observed the interaction between VSVg-MCHR1 and β -arrestin 1 and 2. Evans et al. observed the interaction between the β -arrestins and MCHR1 through confocal microscopy. Both β -arrestin 1 and β -arrestin 2 were tagged with GFP and transiently co-transfected with GRK2 into HEK293T cells stably expressing MCHR1. Their results showed that treatment with MCH didn't result in any appreciable co localization of β -arrestin 1 GFP with MCHR1 at the cells surface. MCH treatment of cells expressing β -arrestin 2 GFP however resulted in fluorescent foci at the plasma membrane of the cells with some evidence of internalization through the visualization of punctate vesicles within the cell. Prolonged hormone treatment showed no increase in the number of fluorescent vesicles internalized into the cell [56].

The fluorescent microscopy results for β -arrestin 2 mirrored those previously described. However, the fluorescence microscopy of β -arrestin 1 suggested its affinity for MCHR1 was similar to that of β -arrestin 2, contradicting previous findings with colocalized fluorescence following agonist treatment. The findings strongly suggest that MCHR1 may be a hybrid receptor that has characteristics of both Class A and Class B GPCRs. The Class A family of GPCRs is characterized by the dissociation of β -arrestin 1 and 2 from the receptor prior to endocytosis and the rapid recycling of the receptor back to the plasma membrane. Class A receptors affinity toward β -arrestin 2 is also greater than their affinity toward β -arrestin 1 [31].

This preferential affinity wasn't observed for VSVg MCHR1 which is characteristic of Class B receptors.

CONCLUSIONS

The aims of this thesis included creating epitope-tagged expression vectors for both MCHR1 and MCHR2, characterizing their interaction with GRK2, β-arrestin 1, and β-arrestin 2, and to visualize if MCHR1 and MCHR2 are colocalized at the plasma membrane when coexpressed. The N-terminal epitope tagging of both MCHR1 and MCHR2 were plagued with cell cytotoxicity issues. Whether these issues were a result of a conformational shift to the receptors impeding their transport to the plasma membrane and subsequent accumulation in the cytoplasm and cellular organelles or the result of overexpression due to too strong of a promoter, we were unable to satisfactorily express protein in any cells transfected. Both N-terminal and C-terminal epitope tagging of MCHR1 has been performed successfully in the past with the creation of C-terminal eYFP tagged MCHR1 and N-terminal tagged VSVg MCHR1 and Flag tagged MCHR1 [56,66]. It would be interesting to create vectors with inducible promoters to see if the cytotoxicity issues could be minimized while still producing a strong level of expression.

The agonist-induced desensitization of GPCR's has been widely characterized. Desensitization studies of MCHR1 had been previously performed but the results reported were somewhat contradictory [55,56,68]. The results from the ELISAs indicate that MCHR1 doesn't readily internalize when exposed to agonist. The

overexpression of GRK2, β-arrestin 1 and β-arrestin 2 assisted in desensitization but the percentage of MCHR1 sequestered was never above 40% in any case. The interaction between MCHR1 and β-arrestins seemed to be transient suggesting the receptor would be more readily recycled to the membrane rather than degraded. Examining the literature of other GPCRs, such as β_2-adrenergic receptor, gonadotropin-releasing hormone receptor, μ opioid and δ opioid receptors, show that the level of sequestration is dependent on the receptor itself, the ligand binding to it if the receptor has multiple agonists, and the cell type the receptor is expressed in [54,72,76,77].

It is likely that the differences in receptor sequestering in various types of cells is due to differential expression of proteins such as GRK's and β-arrestins. It would be interesting to triple transfect cells with MCHR1, GRK2, and β-arrestin and measure the percentage of receptor sequestered to see if there is a difference. Any such differences suggest that MCH-mediated signaling and its affects are regulated in a cell type specific manner. It is also possible MCHR1 is desensitized in a clathrin-independent manner. Lipid rafts on the plasma membrane have been shown to be enriched in MCHR1 and could potentially be involved in signal mediation [61].

Although further experimentation needs to be performed, such as measuring MCHR1 desensitization over time based on its signal to leptin, it is possible to form a hypothesis on MCHR1 desensitization based on the results from the ELISA's. In this model, MCHR1 is localized in lipid rafts. Under agonist exposure, it is sequestered in a clathrin-dependent manner where it is internalized and rapidly recycled back to the

plasma membrane. It is possible that there are as of yet unknown compartments within the cell holding native receptor that replenish MCHR1 on the plasma membrane under agonist exposure. This would explain the up regulation of MCHR1 on the cell surface when cells expressing dominant negative GRK2 were treated with agonist. Receptor up regulation has been previously reported in other receptors in response to agonist treatment [78].

APPENDIX

1

Primer Name	Sequence 5' - 3'	Tm (°C)
Jay's MCHR1 No Stop Up	GCC CGA ATT CTA TCA GTG GGA GCC AT	63.6
MCHR1 Xho Down	GCC TCG AGT CAG GTG CCT TTG	60.6
Jay's MCHR2 No Stop Up	GCC CGA ATT CTA AAT CCA TTT CAT GC	59.4
MCHR2 Xho Down	GCC TCG AGT CAA AAG TGT GTG ATT TC	58.3
MCHR1 Up Nhe	GTA GCT AGC TAC CAT GTC AGT GGG AG	64
MCHR1 Down Xho	TCT AGA CTC GAG TAA GGT GCC TTT GC	63
MCHR2 Up Nhe	GTA GCT AGC TAC CAT GAA TCC ATT TC	60.2
MCHR2 Down Xho	TCT AGA CTC GAG CAA AAA GTG TGA TT	60.5

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