Melanin-Concentrating Hormone Receptor-1 is Enriched in Lipid Rafts and the Effects of Lipid Raft Integrity on Receptor Signaling

Elizabeth Delorme-Axford

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MELANIN-CONCENTRATING HORMONE RECEPTOR-1 IS ENRICHED IN LIPID RAFTS AND THE EFFECTS OF LIPID RAFT INTEGRITY ON RECEPTOR SIGNALING

A Thesis Submitted to the Department of Biological Sciences of the State University of New York College at Brockport in Partial Fulfillment of the Requirements for the Degree of Master of Science

by Elizabeth B. Delorme
July 2008
# Thesis Defense

**Elizabeth Delorme**  
**THESIS DEFENSE**

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Biographical Sketch

Elizabeth Delorme graduated from Thomas A. Edison High School in Elmira Heights, NY in June 2002. She received her B.S. in biological sciences with a minor in chemistry from The College at Brockport, State University of New York in May 2006. Delorme continued her studies at Brockport in the laboratory of Dr. Laurie Cook, and earned her M.S. in biological sciences in July 2008. She will pursue her Ph.D. at the University of Pittsburgh, School of Medicine through the interdisciplinary biomedical graduate program in August 2008.
Dedication

This thesis is dedicated to my parents — Jeff and Kim Delorme, whose love and support made the completion of this work possible.
Acknowledgements

Dr. Laurie Cook – thank you for giving me the opportunity to learn from you and work in your lab. Your guidance throughout my Masters thesis is much appreciated.

Dr.’s Rey Sia, Stuart Tsubota, and Tracey Householder – thank you for your time and your advice with my comprehensive examination, thesis, defense, and future career plans.

To my love and husband-to-be, Brian Axford – I know that these past few years haven’t been easy for either of us. Thank you for supporting and loving me throughout everything.

To my family: my parents Jeff and Kim Delorme, my sister Katherine Delorme, my brother Matt Delorme, Aunt Michele and Bob, and my grandparents Bill and Sharon Shutt – thank you for your endless love and support. Without you, none of this would have been possible.

Dawn Newman – thank you for the laughter and friendship we’ve shared over the past few years.

To the members of the Cook lab: Kelsi Robinson, Scott Portwood and Bryon Tuthill – thank you for your help with these experiments and for your companionship throughout the completion of the graduate program.

Dr. Adam Rich – thank you for your guidance and for your help with the confocal microscope.

Dr.’s Ferruccio Galbiati (University of Pittsburgh, School of Medicine), Masuko Ushio-Fukai (University of Illinois at Chicago), and Richard Wojcikiewicz (SUNY Upstate Medical Center) – thank you for your conversation and your ideas during my graduate school interviews.
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ABSTRACT

The melanin-concentrating hormone receptor-1 (MCHR-1) is a member of the G protein-coupled receptor (GPCR) superfamily, and functions in the regulation of food consumption and energy metabolism. MCHR-1 is expressed in neural, pancreatic, and fat tissue. Fat cells begin their journey as pre-adipocytes, culminating in the formation of mature adipocytes as differentiation occurs. Of interest to note, fat cells accumulate caveolae markers - caveolin-1 and cholesterol - during this process. Certain GPCRs and their associated downstream signaling molecules co-localize with caveolae membranes.

This thesis seeks to demonstrate that MCHR-1 is enriched in lipid rafts and that a possible consequence of this may be altered signal transduction in obese individuals, such as downregulated ERK1/2-mediated leptin transcription. Increased amounts of adipose tissue, and thus caveolae, may play a central role in the regulation of MCHR-1 signaling.

The first aim of this project addressed the question of MCHR-1 localization to lipid rafts. To test this, caveolae membranes were isolated via sucrose density gradient ultracentrifugation. Subsequent fractionation and western blotting confirmed that MCHR-1 is enriched in lipid raft fractions containing caveolin-1. The second objective assessed ligand dependence on MCHR-1 localization. MCH exposure (1.0-µM) had no obvious effect on receptor localization to lipid raft fractions containing caveolin-1 over an expanded time course. The third aim examined the effect of lipid rafts on MCHR-1 signaling. Pharmacological disruption of caveolae by cholesterol depletion with methyl-β-cyclodextrin (MβCD) dampened MCH-mediated ERK1/2 activation, suggesting that lipid rafts may significantly impact the regulation of MCHR-1 signaling in cells.
INTRODUCTION

Obesity

The Centers for Disease Control (CDC) estimates that at least 34% of American adults were obese in 2006, and that this trend is steadily increasing (1). Obesity is present when an individual has an excess amount of adipose tissue, due to increased caloric intake and reduced energy expenditure. The medical community diagnoses an obese state when an individual has a body mass index (BMI) of 30 or higher. Body mass index calculations are based on a person’s weight to height ratio. The side effects of obesity have been well-documented and include: type II diabetes, sleep apnea, stroke, cancer, heart disease, hypertension, dyslipidemia, and osteoarthritis. Most recently, several theories have been developed to explain the relatively recent phenomenon of prevalent adult and childhood obesity. The CDC suggests that a combination of food consumption behaviors, lack of physical activity, environment, genetics, and underlying medical conditions are responsible (1).

Molecular appetite regulation mechanisms are complex and involve interplay between hormonal and neural signals, moderated by feedback loops (rev 2). The central nervous system (CNS), particularly the hypothalamus, has been implicated as a key source of systemic hunger signals. Specifically, the hypothalamus releases a variety of neuropeptides, such as neuropeptide Y (NPY), agouti-related peptide (AGRP), galanin, melanocortins, glucagon-like peptides, corticotropin releasing factor (CRF), orexin, serotonin, and neurotensin. The peripheral nervous system (PNS) has been shown to interact with the feeding centers of the brain, through gastrointestinal, hepatic, and pancreatic-secreted hormones, including ghrelin, peptide YY, cholecystokinin (CCK),
leptin, amylin, insulin, and bombesin as shown in Figure 1 (Appendix I). Appetite signals are initially released by peripheral tissues, and are received by the hypothalamus. The hypothalamus then secretes additional hormones that prompt an individual to eat. Peripheral responses are generated by gastrointestinal organs, such as the large intestine and stomach, and by metabolic regulators, such as adipose tissue and pancreatic β cells, that loop back to the brain to shut off the hunger signal (3).

The relationship between overeating and obesity appears obvious, but fails to address why obese individuals continually feel the need to eat despite high caloric diets. The “thrifty genotype hypothesis” suggests that excess intake of high fat, high carbohydrate food sources have been evolutionarily selected for (3,4). In the past, humans experienced periods of famine and food deprivation. Those who had the ability to continue eating during times of provisional availability would have been conferred with a selective advantage (4). However, uncontrolled or malfunctioning cellular signaling may also lead to dysregulation of the hunger-satiety pathway. An underlying genetic abnormality, epigenetic changes in gene expression, or alterations of normal cellular functioning may play a role in modulating signaling in the complex neural-endocrine appetite pathway. Any form of molecular defect may offer a possible explanation for the increasing propagation of a global obese population.

**Appetite hormones use G protein-coupled receptors to elicit intracellular responses**

Appetite hormones, such as NPY, orexin, and neurotensin, bind to G protein coupled receptors to induce cellular and systemic responses (2). G protein-coupled receptors (GPCRs) are characterized by their seven hydrophobic, transmembrane spanning domains and association with guanosine nucleotide-binding proteins (G
proteins) as second messengers (5). GPCRs interact with large, heterotrimeric G proteins composed of three individual subunits – \( \alpha \), \( \beta \), and \( \gamma \) (6). The \( \alpha \)- and \( \gamma \)-subunits are tethered to the plasma membrane by short, modified lipid tails. In the inactivated state, ligand is absent, and the \( \alpha \)-subunit is bound to guanosine diphosphate (GDP). When the appropriate ligand – hormone, stimulus, or other peptide – binds to the receptor at the plasma membrane, a conformational change occurs within the receptor. This molecular signal is passed onto the \( \alpha \)-subunit of the G protein, and the \( \alpha \)-subunit loses its affinity for bound GDP. The \( \alpha \)-subunit switches GDP for guanosine triphosphate (GTP), and GTP binds the \( \alpha \)-subunit in the GPCR activated state. The \( \beta \)- and \( \gamma \)-subunits are bound together in an obligatory complex. The \( \alpha \)-subunit dissociates from the \( \beta \)-\( \gamma \) complex, whereby both the \( \alpha \)-subunit and the \( \beta \)-\( \gamma \) complex can contact downstream signaling molecules.

G proteins can elicit a wide array of intracellular signaling cascades. These signal transduction pathways dictate the particular cellular response that will occur. Well documented pathways include the following: \( G_{s/1} \), \( G_0 \), and \( G_q \) proteins may be activated following GTP binding, and each individual G protein acts on a target pathway as shown in Figure 2 (Appendix I). \( G_s \) stimulates adenylyl cyclase, cyclic AMP (cAMP), and protein kinase A (PKA). \( G_i \) works antagonistically to \( G_s \) and inhibits adenylyl cyclase activation and its associated downstream signaling events. \( G_q \) activates phospholipase C (PLC), which leads to the activation of inositol triphosphate (IP\(_3\)), diacylglycerol (DAG), and calcium (Ca\(^{2+}\)). DAG and Ca\(^{2+}\) work synergistically to initiate protein kinase C (PKC) activation. \( G_o \) protein alone may also trigger the PKC pathway. PKC phosphorylates Ras, which signals to Raf and the mitogen-activated protein kinase
(MAPK) pathway. The end result of GPCR signal transduction is often a change in gene regulation, and ultimately, transcription.

If a sustained cellular signal is prolonged, its receptor may undergo desensitization (5) to "shut off" the activated pathway by failing to respond to the presence of ligand (7). The receptor becomes phosphorylated by its respective G protein coupled receptor kinase (GRK) on the intracellular C-terminal tail that would otherwise interact with $G_\alpha$ (7,8). This molecular event creates a binding site for $\beta$-arrestin. Once $\beta$-arrestin is bound, the G protein can no longer associate with the receptor C-terminus tail, which terminates downstream signaling. Desensitization to ligand is one mechanism by which receptors are downregulated from the cell surface (9). Internalization of membrane receptors may also reduce or eliminate a signal transduction response. Areas containing receptors along the plasma membrane may form vesicles and remove receptors from the cell surface through endocytosis events. Thus, less receptor is available for ligand binding, and subsequently, signal transduction is altered. Receptor desensitization and internalization may occur independently, or desensitization may precede internalization (8).

Following receptor internalization, which decreases the number of receptors available at the cell surface, intracellular trafficking then occurs (10). The receptor may be targeted to early endosomes for degradation in the lysosomes or for recycling back to the plasma membrane (8) as shown in Figure 3 (Appendix I). Receptors, such as the $\beta_1$-adrenergic receptor, may be internalized through clathrin-coated pits or through caveolae, depending on the kinase that phosphorylates the C-terminus (11). For the transforming growth factor-$\beta$ receptor (TGF-$\beta$-R), the pathway of internalization
determines the intracellular fate of the receptor (12). If clathrin targets TGF-β-R, then the receptor is recycled for continued signaling. However, the caveolae pathway directs TGF-β-R for degradation.

Certain GPCRs, such as the type I angiotensin receptor, utilize the clathrin pathway (112). Clathrin-coated pits are transported from the endoplasmic reticulum (ER) to the cytosolic surface of the plasma membrane and assemble into a lattice, creating a clathrin-coated vesicle (CCV) with the help of adaptor proteins, such as the AP-2 complex and dynamin at the neck of the vesicle (6,10,13). The pits then ‘pinch off’ from the plasma membrane, with their molecular cargo secured inside the formed vesicle as shown in Figure 4A (Appendix I) (10). Shortly after entering the cell, CCVs lose their clathrin coats, and fuse with early endosomes for sorting (13). Recent studies have suggested that specific amino acid sequences, such as di-leucine and tyrosine-based motifs, may target specific cargo to clathrin-coated pits.

**Lipid rafts and caveolae**

Another possible internalization pathway involves caveolae lipid rafts. Lipid rafts form highly ordered, yet adaptable, platforms within the plasma membrane bilayer (14). Rafts are dynamic plasma membrane structures composed of proteins, sphingolipids, phospholipids, and cholesterol that are assembled in the Golgi complex (14,15). Cholesterol is a key component in the formation of lipid rafts, and is viewed as the “glue” that “keeps the raft assembly together” (16). The removal of cholesterol from the lipid raft structure and the plasma membrane by pharmacological agents causes a dissociation of proteins from the lipid rafts, rendering them non-functional. Proteins that assemble within the lipid raft complex are dependent upon their association with these
detergent-resistant membranes (DRMs) (17). Caveolae are a subset of the lipid raft membrane microdomains characterized by the presence of caveolin.

G protein-coupled receptors and their associated G protein subunits have been isolated in lipid rafts and caveolae (18-20). The muscarinic cholinergic receptor and its \( G_{aq} \) and the adenosine A1 receptor and its \( G_{\beta\gamma} \) subunits have been localized to caveolae (20). The role of caveolae and its effects on GPCR signaling have been hypothesized, although the exact mechanisms through which caveolae modulates GPCR signaling, internalization, and trafficking are still under investigation (21). Caveolae have been implicated as a potential mediator of GPCR signal transduction and as an anchor for second messengers (19).

Caveolae were initially observed in endothelial cells by George Pallade in 1953, with the aid of electron microscopy (EM) as shown in Figure 4B (Appendix I) (22). Pallade termed these 50-100-nm invaginations of the plasma membrane, 'plasmalemmal vesicles,' due to their budding capacity from the plasma membrane for intracellular endocytosis and transport. The integral membrane protein caveolin has been identified as the molecular marker of caveolae lipid rafts (23). The most prevalent isoform of caveolin is caveolin-1 (cav-1), and is found throughout adipose, fibroblast, smooth muscle, and endothelial cells (24,25). Caveolin-2 (cav-2) is also found in the same tissues as cav-1 (rev 26). Caveolin-3 (cav-3) has been exclusively localized to smooth, skeletal, and cardiac muscle (rev 27). However, caveolin-1 will be discussed, unless otherwise mentioned, from this point forward.

As stated previously, lipid raft regions of the plasma membrane are rich with sphingolipids, phospholipids, cholesterol, and membrane proteins. Caveolae lipid rafts
are identified as such due to the presence of caveolin. The gene for caveolin-1 localizes to chromosome 7 (7q31.1), a suspected tumor locus (28). Biochemically, cav-1 directly binds cholesterol in such a way that allows both the amino and the carboxy termini to face the interior of the cytoplasm (29,30). A 33-amino-acid hydrophobic domain anchors cav-1 into the plasma membrane. Caveolin is palmitoylated at cysteine residues 134, 144, and 157 (30,31). Caveolae are abundant with G proteins and their receptors, signaling molecules, glycosylphosphatidylinositol- (GPI) anchored proteins, and other downstream signaling targets (32). Actin, myosin, adenylyl cyclase, PKC, MAPK, and DAG are only a handful of the molecules enriched in caveolae membranes. Co-localization of molecules within caveolae is determined by fractionation methods that isolate caveolae membranes. Distinct fractionation of caveolae is possible because the density of the caveolar fraction is very light compared to the bulk of the plasma membrane (33). It has been hypothesized that caveolae may also behave as a scaffolding domain within the cell membrane, allowing for the necessary interactions between signaling molecules to occur (34).

Caveolae are also involved in non-coated pit-mediated internalization events (35). Sharma et al. proposed that the balance between cav-1, cholesterol, and glycosphingolipids (GSLs) may regulate caveolar endocytosis at the plasma membrane (36). Way and Parton maintain that caveolar budding for endocytosis events is a plausible mechanism as well (37). Clearly, cholesterol plays a distinct function in mediating caveolar endocytosis. Cholesterol depletion from the plasma membrane by pharmacological agents, such as methyl-β-cyclodextrin, filipin, or nystatin (38), interferes with caveolar endocytosis by inhibiting vesicle formation (32). Caveolae are
disassembled following cholesterol depletion (39), hindering internalization and trafficking. It is important to note that the protein dynamin, which also associates with clathrin-coated pits, is necessary for caveolae budding and fission from the plasma membrane during these events (40,41).

It has been suggested that the actin cytoskeleton has a role in regulating caveolar endocytosis. Mundy and colleagues conducted a study that examined the trafficking of GFP-tagged cav-1 in a Chinese hamster ovary (CHO) cell model (34). Without stimulation, GFP-cav-1 was observed to be present at the cell surface within invaginated caveolae and near centrosomal regions of the cell in caveosomes. The administration of the actin depolymerizing drug latrunculin A resulted in the rapid movement of caveolin-associated vesicles towards the interior of the cell, near the centrosomes. Caveolae have been observed in three different contexts: first, at the cell surface tethered with actin; second, near centrosomal caveosomes intracellularly; and third, as components of cavicles, or caveolar vesicles. Cytoskeletal components actin and microtubules influence caveolae, and thereby, may affect GPCR signaling components as well (42). Thus, both cholesterol and actin play critical roles in maintaining caveolae and its functions.

Melanin-concentrating hormone

Melanin concentrating-hormone (MCH) was originally isolated in the teleost fish, where it was demonstrated to have effects on skin pigmentation (rev 43). Teleost fish secrete MCH as an adaptive response to environmental stimuli and stress. The result is an overall lightening of color in the scales of the fish. MCH was also found to be
present in the pituitary glands of salmon (44), wherein it was hypothesized to exert similar effects.

The cyclic MCH fish peptide is 17-amino acids in length (44). A disulfide bond forms the ring structure, creating a dicysteine bridge that links residues 4 and 13 (Cys\textsuperscript{7} and Cys\textsuperscript{16} in mammals) (44-46). Matsunaga et al. deemed the cyclic nature of MCH to be essential to its biological functioning (45). A mammalian MCH homologue exists in humans, rats, mice, rabbits, and dogs, shown in Figure 5 (Appendix I) (43,46). The mammalian MCH peptide is identical in all of these species, as evident by a conserved primary amino acid structure (43). However, there are differences in primary amino acid structure between the fish and the mammalian MCH variants. Mammalian MCH is 19-amino acids in length, with two additional residues and four substituted residues – an N-terminal phenylalanine (Phe\textsuperscript{18}) and aspartate (Asp\textsuperscript{19}) – not observed in the fish homologue (46,47). The mammalian MCH peptide will be discussed from this point forward unless stated otherwise.

MCH is one of several protein products derived from the preprohormone MCH (pMCH) gene (48). Post-translational processing of the pMCH peptide may result in multiple gene products, including neuropeptide E-I (NEI) and neuropeptide G-E (NGE). NEI has been shown to act antagonistically to MCH and agonistically to melanin-stimulating hormone (MSH) in cell culture studies (49). MCH promotes feeding behaviors, while NEI and MSH inhibit feeding (44). The role of NGE remains to be elucidated (49). The human pMCH gene is located at 12q23-24 on the long arm of chromosome 12 as seen in Figure 6 (Appendix I) (43). pMCH is 165-amino acids in length, and generates MCH following proteolytic cleavage at arginine residues 145 and
In humans, MCH mRNA expression is restricted to the lateral hypothalamus and pancreas (50,51).

A role for MCH has been established in the regulation of pathways involved in appetite motivation and energy expenditure (52). MCH peptide synthesis occurs in magnocellular neurons and in the lateral hypothalamus and zona incerta (46). Previous studies associated the location of the MCH peptide within both the central nervous system (CNS) – the hypothalamus – and the peripheral nervous system (PNS) – the pancreas (51,52). Therefore, MCH may act through a systemic mechanism involving hunger responses and energy output. The CNS and the hypothalamus, in particular, have been implicated as centers of feeding, energy homeostasis, and body weight regulation (44,53). The hypothalamus is a key neural area where the CNS communicates with the periphery (53). In addition, the hypothalamus is one of the only areas of the brain where the blood-brain barrier is absent, conferring a unique potential for peripheral influence.

**MCH mouse models**

Early evidence for the role of MCH in feeding behavior was derived from the Maratos-Flier lab’s leptin *ob/ob* knockout (KO) mouse (54). Leptin (*ob*) is the satiety hormone that allows an individual to feel ‘full’ after a meal. *Ob/ob* mice demonstrate elevated levels of MCH mRNA and overexpression of the mature peptide. Deletion of leptin appears to be coupled with a marked overexpression of MCH. *Ob/ob* mice are characterized by obese phenotypes compared to control mice; this is most likely due to MCH-induced hyperphagia (50,54). The authors of the study also administered MCH by central hypothalamic injection into rats, which resulted in excessive feeding behaviors
compared to controls (54). Control mice were observed to have higher MCH mRNA expression patterns under fasting conditions.

Shimada and colleagues engineered a $pMCH^{-/-}$ KO mouse model (55). These mice are characterized by lean phenotypes with 25-30% less body fat than controls. $pMCH^{-/-}$ mice are also shown to be hypophagic with increased metabolic rates, increased resting energy expenditures, and low levels of circulating leptin. Similarly, a transgenic pMCH overexpressing mouse model was created (56). When given a normal diet, pMCH mice were found to be mildly obese and hyperphagic with signs of insulin resistance. However, pMCH mice became markedly obese on a high fat diet with symptoms of hyperinsulinemia and islet cell hyperplasia, compared to controls. This is consistent with the role for MCH as an appetite stimulating hormone. The accumulation of excess body fat in the $pMCH^{-/-}$ mouse also has a negative effect on the body’s response to insulin secretion. Although caloric intake is high, cells of the body fail to absorb circulating glucose from the bloodstream. This suggests the possibility of an interaction between the absence of MCH and a malfunction of the insulin response.

Segal-Lieberman et al. produced a double null leptin-deficient $ob^{-/-} MCH^{-/-}$ mouse as observed in Figure 7 (Appendix I) (50). The double KO shows a dramatic reduction in overall body fat compared to the $ob^{-/-}$ mouse, but with similar hyperphagic behaviors. The $ob^{-/-} MCH^{-/-}$ mouse also demonstrates increased activity, resting energy expenditure, and body temperature. Secondary to this, the double null mouse displays a lean phenotype with decreased adiposity and improved glucose tolerance and insulin sensitivity. This observation is consistent with the role for a functional interaction to exist between MCH and cellular insulin responsiveness.
Studies have also been conducted involving MCH injections into the body and regions of the brain other than the hypothalamus. Ito et al. performed chronic infusions of MCH in mice, which led to the previously noted characteristics of obesity and hyperphagia, along with decreased core body temperature and oxidation of fatty acids in brown fat (57). Zheng and associates injected MCH into the fourth ventricle of the rat brain (58). Decreases in core body temperature and physical activity were observed. Although no effects on feeding behavior were evident, the role for MCH as a mediator of energy conservation is apparent. MCH was also injected into the lateral ventricle of the brain in rats. Dose-dependent effects upregulated feeding behavior to levels two to three times that of controls for up to 6-hours post-injection (43). An investigation carried out by Stricker-Krongrad and colleagues concluded that in the obese Zucker rat model, circulating plasma MCH levels were higher compared to controls (59).

The MCH KO mouse was further characterized during a study that examined the influence of MCH on pancreatic islet cells, conducted by Pissios et al. (51). Earlier observations indicated that MCH overexpressing mice exhibited increases in pancreatic β cell mass. Pancreatic islet β cells are responsible for insulin secretion. Islet hyperplasia was evident in control mice, but not in MCH KO mice when both were fed a high-fat, hypercaloric diet, as represented in Figure 8 (Appendix I) (51). In human and mice cultured islet β cells, MCH was found to enhance insulin secretion in a dose dependent manner, resulting in altered Ca\(^{2+}\) influx, ERK1/2 activation, and Akt phosphorylation. Thus, a role for MCH in modulating peripheral insulin responses has become increasingly clear.
Melanin-concentrating hormone receptors

Two MCH receptor isoforms have been identified thus far – MCH receptor-1 (MCHR-1) and MCH receptor-2 (MCHR-2); both are G protein-coupled receptors. MCHR-1 was originally identified as SLC-1/GPR24, an orphan somatostatin-like G protein-coupled receptor (GPCR) (60-63). The ligand for SLC-1 was previously unknown until Lembo et al. demonstrated that MCH (1.0-nm) activated the orphan receptor (60). As previously described, MCH has orexigenic, or appetite-stimulating, properties, and has been isolated in the hypothalamus and pancreatic islets. MCHR-1 mRNA has been isolated throughout diverse areas of the brain and CNS, including: hypothalamus, thalamus, olfactory tubercle, hippocampus, cerebral cortex, substantia nigra, amygdala, locus ceruleus, and medial nucleus accumbens (53,64,65). Muscle, eye, tongue, and adipose tissues are additional areas of MCHR-1 expression. MCHR-1 has also been identified in mouse and human pancreatic islet β cells (51).

The receptor is highly conserved in rats, mice, and humans (rev 43,66). There is 95% identity between the human and mouse variants and 96% identity between the human and rat variants (rev 43). In humans, the MCHR-1 gene is localized to chromosome 22 (22q13.3), and the MCHR-1 peptide is 353-amino acids in length. MCHR-1 has a classical G protein-coupled receptor structure as shown in Figure 9 (Appendix I), including seven transmembrane helices, a DRY motif at the end of the second intracellular loop, and three potential glycosylation sites at the extracellular N-terminus (rev 43,67). The DRY (Asp\textsuperscript{141}, Arg\textsuperscript{142}, Tyr\textsuperscript{143}) motif is essential for signal transduction, and glycosylation sites are necessary for linking the receptor at the cell
surface (rev 43). The intracellular C-terminal tail of the receptor has been identified as being important, but not required, for internalization (68).

MCHR-1 couples to G\textsubscript{i}, G\textsubscript{o}, and G\textsubscript{q} proteins for intracellular signaling events (62,70,71). Therefore, MCH binding to MCHR-1 leads to cellular changes in the inhibition of cAMP through G\textsubscript{i}, increases in IP\textsubscript{3} and Ca\textsuperscript{2+} through G\textsubscript{q}, and MAPK activation through either G\textsubscript{q} or G\textsubscript{o}. If MAPK is triggered via G\textsubscript{o}, PKC will also be activated; this is not the case if MAPK is phosphorylated by way of G\textsubscript{q}. In addition to cell culture models, MCH-induced MCHR-1 coupling to G\textsubscript{i} and G\textsubscript{q} has also been characterized in the rat brain (70).

Saito et al. investigated the possibility of MCHR-1 internalization via clathrin coated vesicles (68). Acetic acid treatment was performed to inhibit clathrin-mediated endocytosis events in HEK293 cells transfected with a Flag-tagged MCHR-1. Cells were exposed to 300-nm MCH for 40-minutes. MCHR-1 internalization was only partially inhibited by this treatment (66% inhibition), suggesting that non-clathrin-mediated endocytosis was also occurring. However, MCHR-1 internalization was found to be dependent on: protein kinase C-mediated phosphorylation of three C-terminal amino acid residues (Thr\textsuperscript{317}, Ser\textsuperscript{325}, Thr\textsuperscript{342}), β-arrestin-2 binding, and endocytosis by a dynamin dependent pathway.

In addition to MCHR-1, a second GPCR for MCH exists – MCHR-2. MCHR-2 has been found to exhibit 38% homology with MCHR-1, as portrayed in Figure 10 (Appendix I) (72). The highest sequence similarities exist within the amino acid residues of the receptor transmembrane domains of the MCH-binding pocket. Although
38% amino acid sequence correspondence may seem minimal, MCHR-2 is actually more highly homologous to MCHR-1 than any other related GPCR (63). The gene for MCHR-2 has been mapped to the long arm of chromosome 6 (6q16.2-3), and has multiple coding regions (63,72). MCHR-2 has been shown to couple to Gq, resulting in intracellular Ca\(^{2+}\) release (72). Northern blot analysis and \textit{in situ} hybridization studies performed by Sailer and colleagues confirmed the presence of MCHR-2 mRNA in areas of the brain known for body weight regulation – the arcuate nucleus and the ventral medial hypothalamus. MCHR-2 has also been detected in the amygdala, an area of the brain known for storing emotional memories (63). These results indicate a role for MCHR-2 as an additional mediator of the MCH feeding response pathway with potential involvement in the emotional aspect of food consumption.

\textit{MCHR-1 mouse models}

A study conducted by Kokkotou et al. investigated levels of MCHR-1 expression in the leptin-deficient \textit{ob}/\textit{ob} mouse model (73). The absence of leptin was found to negatively regulate MCHR-1 mRNA expression. During leptin deficiency (\textit{ob}/\textit{ob}), MCHR-1 mRNA expression increases seven-fold. Exogenous administration of leptin in \textit{ob}/\textit{ob} mice decreases MCHR-1 mRNA levels. However, MCHR-1 mRNA fails to change within a 48-hour period of fasting for both \textit{ob}/\textit{ob} and control mice. This evidence supports a role for leptin-dependent regulation of MCHR-1 expression in the brain.

The relationship between leptin and MCHR-1 has been further characterized in research conducted by Bradley and colleagues using 3T3-L1 adipocytes (74). MCH exposure to rat adipocytes \textit{in vitro} initiates leptin transcription. Further studies concluded
that 1.0-µM MCH exposure in 3T3-L1 cells lead to the activation of MAPK p42/44, or extracellular-related kinase 1/2 (ERK1/2) (70,75). One transcription product of MCH mediated MAPK p42/44 phosphorylation was later identified to be leptin (75). MCHR-1 expression was also examined between pre-adipocytes and mature, differentiated adipocytes. No significant differences in MCHR-1 mRNA levels were observed in either the immature or differentiated adipocyte state in the absence of MCH administration. In fully differentiated 3T3-L1 cells, MCH exposure was found to downregulate MCHR-1 mRNA expression in a time course-dependent manner. It is possible that MCH-mediated changes in MCHR-1 levels are the result of internalization or another, still unknown, molecular event may be occurring.

A series of MCHR-1 KO mice have been created to better understand the physiological consequences of receptor deletion. Marsh and associates generated the first MCHR-1 KO mouse, which displayed normal body weight with a lean phenotype and reduced overall body fat mass, due to hyperactivity and altered metabolism (76). These mice also demonstrated hyperphagia with a resistance to diet-induced obesity. A second aim of the study examined the effects of chronically administered MCH infusions. Control mice showed hyperphagia with mild obesity; this was absent in MCHR-1 KO mice. These results suggest that MCHR-1 may potentially negatively mediate systemic energy expenditure responses in the body.

Bjursell et al. further characterized the phenotype of the MCHR-1 KO by creating an ob/ob MCHR-1−/− mouse, and by examining the effects of receptor deletion on insulin sensitivity in an obese model (77). Following a high glucose meal, the
The ob−/ob− MCHR-1−/− mouse developed lower blood insulin levels than its ob−/ob− counterpart. The authors attributed this to increased insulin responsiveness in the ob−/ob− MCHR-1−/− model, and hypothesized that MCHR-1 affects insulin sensitivity via an unknown mechanism that is not dependent on body weight. The double null mouse also demonstrated high locomotive activity, leaner body mass, decreased body fat, and altered body temperature compared to the obese ob−/ob− model. The authors also noted that obesity is a contributing factor towards the development of insulin resistance and type II diabetes, and that both conditions can often be reversed due to decreases in body weight. The hypothesis of the study was that mutations in ob can lead to obesity and insulin resistance/type II diabetes. This study provides further evidence for the accumulation of body fat, and its effects on possible cross-talk between MCHR-1 and cellular insulin sensitivity.

**MCHR mutations are linked to obesity**

Mutations may arise within the MCH receptor, creating aberrant cell signaling patterns, any of which can potentially lead to obesity. The following studies have investigated mutation patterns in MCHR-1 and its effects on obese populations. Gibson and colleagues examined MCHR-1 and MCHR-2 for possible relationships between receptor gene mutations and obesity phenotypes (78). Test subjects with a history of hyperphagia and early onset obesity were selected to have their MCHR-1 and MCHR-2 genes sequenced. Two MCHR-1 missense variants were identified – Y181H and R248Q – that were not present in the normal weight control participants. In addition, of the total 541 white test subjects studied, no significant associations were found between MCHR-1
single nucleotide polymorphisms (SNPs) and obesity. However, two non-coding variants of MCHR-2 were discovered.

A study conducted by Bell *et al.* researched the MCHR-1 gene sequence further for evidence of SNPs and a relationship between early-onset extreme obesity (67). The 3.5-kb coding region, including untranslated and intronic regions, plus the 1-kb assumed promoter region of the MCHR-1 gene sequence were analyzed in a study population of 180 morbidly obese adults and 87 morbidly obese children. Thirty-nine SNPs were recognized, seven of which encoded for missense mutations, or amino acid substitutions as shown in Figure 11 (Appendix I). One of the identified SNPs – R248Q, which had been acknowledged by Gibson *et al.*, was verified to be highly associated with early onset obesity. The R248Q mutation exists within the third intracellular loop of MCHR-1. This GPCR region is associated with β-arrestin binding and receptor downregulation (79,80). Perhaps MCHR-1 fails to undergo desensitization and/or internalization in these individuals. The remaining six SNPs were further characterized in a sample population comprised of 557 morbidly obese adults and 552 obese children (67). An SNP found within the supposed promoter region – rs133068 – demonstrates protection against obesity in the children under study. The authors proposed that disruption of the promoter may downregulate MCHR-1 gene expression. Low receptor levels may decrease the orexigenic MCH response in these individuals.

**Caveolae, insulin, and adipocytes**

Of interest to note, adipocytes, or fat cells, accumulate caveolae as differentiation occurs from the pre-adipocyte to the mature adipocyte states (81,82). The caveolae levels of mature adipocytes may be up to ten times that of the pre-adipocyte.
In addition, insulin receptors have been localized to adipocyte caveolae (83). Upon exposure to insulin, the insulin receptor, a tyrosine kinase receptor, phosphorylates cav-1; this results in ERK1/2 activation in 3T3-L1 adipocytes (84,85). The above findings are significant in that adipocytes are also recognized as sources of metabolic regulation (86,87). Hormones termed ‘adipokines’ are key mediators in maintaining the critical balance between energy storage and expenditure.

In addition, insulin is produced by pancreatic islet β cells in response to high blood glucose levels (5). An attractive discovery – Ca^{2+} influx in the β cells precedes the secretion of insulin (88). Insulin activates insulin receptors in adipocytes, which ultimately signal via IP3 for the inhibition of continued food intake (5). A possible mechanism for cross-talk to exist between the insulin and leptin receptors has been hypothesized. Evidence derived from the caveolin-1 KO mouse model demonstrates an alteration of insulin receptor levels (89), which further promotes the possibility for links to exist between MCHR-1, caveolae, and insulin signaling.

**Caveolin-1 mouse models**

Caveolin-1 KO mice were initially discussed in the literature only several years ago, when Drab et al. published an article in Science outlining the consequences of cellular caveolae loss (90). In mice lacking the gene for cav-1, caveolae are also destroyed. This emphasizes that the presence of cav-1 is critical for caveolar maintenance, and that all caveolae are associated with the presence of caveolin proteins. Furthermore, caveolar invaginations of the plasma membrane are a result of caveolin polymerization. Cav-1 KO mice were studied for further physiological abnormalities, particularly in the presence of a high fat diet (91). These mice exhibited hyperphagia
with a resistance to diet-induced obesity and reduced adiposity. Coincidentally, this phenotype is similar to that observed in the MCHR-1 KO model. However, insulin, glucose, and cholesterol levels were within normal limits. Circulating blood plasma quantities of leptin were decreased. There were noted defects in lipid metabolism and adipocyte functioning, as presented by abnormally increased triglyceride and free fatty acid levels. The conclusions were drawn that perhaps cav-1 plays a role in hyperlipidemia and obesity.

In addition, cav-1 KO mice demonstrate consistent levels of insulin receptor mRNA with that of the control (89). However, actual insulin receptor expression at the adipocyte surface is reduced to less than 90% in cav-1-deficient mice. A separate study indicated conflicting results with regards to free cholesterol levels in cav-1 knockouts. Le Lay and associates suggest that cav-1 is responsible for maintaining cellular cholesterol content, due to the absence of detectable cholesterol levels in cav-1 KO mice. Cholesterol expression was rescued when the gene for cav-1 was reintroduced into the defective mice under an adenovirus promoter. Once cav-1 expression was restored, cellular cholesterol levels were restored as well.

However, skeptics remain unconvincing as to the validity of the conclusions drawn by the studies involving cav-1 KO mice. The foremost question is, are the observed phenotypes a result of the loss of cav-1 gene expression, or do the mice physiologically compensate for the loss of cav-1, consequentially causing the apparent phenotypes (92)? One such explanation for this phenomenon is the caveolin signaling hypothesis, which states that the binding of signaling molecules coupled to G protein pathways inhibits transduction. Deletion of caveolin, and subsequent release of these
downstream signaling components due to the loss of caveolins, allows signaling pathways to be activated without restraint. Knockout model gene deletion induces loss of the target gene from all tissues of the body. An siRNA approach has been suggested as a method of studying phenotypic alterations due to gene inactivation in a tissue-specific manner.

**Significance**

At the present time, little is known about the localization, the internalization, and the trafficking of MCHR-1. Previous work has suggested that MCHR-1 partially internalizes by a form of non-clathrin-mediated endocytosis (68). MCHR-1 is expressed in neural, pancreatic, and adipose tissues. Adipocytes accumulate caveolae markers - caveolin-1 and cholesterol - during their maturation process. Certain GPCRs and their associated downstream signaling molecules localize to, or are enriched in, caveolae membranes. The first aim of this thesis was to demonstrate that MCHR-1 is enriched in lipid rafts. A possible consequence of lipid raft enrichment may be altered signal transduction in obese individuals, such as downregulated ERK1/2-mediated leptin transcription. The second aim of this thesis was to examine the effect of ligand exposure on MCHR-1 localization to lipid raft fractions containing caveolin-1. Receptor movement into lipid rafts may indicate the occurrence of MCHR-1 caveolar-mediated endocytosis. The third aim was to disrupt caveolae by pharmacological cholesterol depletion to determine the effect of lipid rafts on MCHR-1 signaling pathways, particularly the ERK1/2 response. This work is significant because the elucidation of receptor signaling pathways and mechanisms proposed to be involved in obesity is necessary for potential pharmacological targeting and drug development.
MATERIALS AND METHODS

Cell culture

Stably expressing VSVg-tagged MCHR-1 CHO-K1 cells were cultured in F12k media (CellGro) containing 5% fetal bovine serum (FBS) and selected with 0.50-g/mL G-418 (A.G. Scientific). Cell lines were cultivated as monolayer cultures at 37°C, 95% air, and 5% CO₂ in a humidified environment. All cells were passaged when 80-90% confluent.

Melanin concentrating hormone (MCH) treatment

MCH (1.0-µM, American Peptide) was added to cells in F12k media for the indicated times.

Cholesterol depletion with methyl-β-cyclodextrin

VSVg-MCHR-1 CHO-K1 cells were serum starved overnight or 1-hour prior to cholesterol depletion, as specified. Methyl-β-cyclodextrin (5.0-mM or 10.0-mM, Sigma) was added to cells in F12k media for 1-hour preceding MCH exposure.

Cholesterol repletion with soluble cholesterol

VSVg-MCHR-1 CHO-K1 cells were serum starved in F12k media 1-hour prior to cholesterol repletion. Soluble cholesterol (1.0-mM, Sigma) was added to cells in F12k media for 1-hour prior to MCH exposure.

Immunocytochemistry

Stable VSVg-MCHR-1 CHO-K1 cells were grown to 50% confluency on coverslips in 35-mm dishes. Cells were exposed to MCH for the designated time points.

1 Performed by Kelsi Robinson, B.S.
All coverslips were washed twice with 1x PBS, fixed with 4% paraformaldehyde in PBS for 10-minutes, and washed three times with 1x PBS. Cells were blocked with 5% goat serum/0.1% Triton X-100 (Sigma) for 20-minutes in a humidified chamber. Blocking solution was removed, and rabbit anti-VSVg antibody (1:1000, Sigma) in blocking solution was added for 2-hours to overnight. Cells were washed three times, 5-minutes each with 1x PBS shaking at room temperature. Anti-rabbit Alexa Fluor 488 antibody (1:500, Invitrogen) in blocking solution was added to coverslips for 45-minutes to 1.5-hours in a dark, humidified chamber. DAPI (1.0-µg/mL, Roche) stain was applied to cells for 20-minutes in a dark, humidified chamber. All coverslips were washed with 1x PBS for 5-minutes, then washed with dH2O for 5-minutes shaking at room temperature. Coverslips were removed from dishes, and dried at room temperature. ProLong Gold (Invitrogen) was applied to coverslips on blank slides and allowed to cure. Clear nail polish completed the coverslip mounting. All slides were allowed to dry overnight at room temperature in a dark environment.

Florescence and confocal microscopy

Fluorescence microscopy was carried out using the Zeiss Axiocam MRm fluorescence microscope with AxioView imaging software. Confocal microscopy was performed with the structured light imaging OptiGrid confocal microscope with Image ProPlus software.

Detergent-free sucrose gradient caveolae isolation

Stable VSVg-MCHR-1 CHO-K1 cells were cultured to confluency on four, 100-x-20-mm dishes. The dishes were placed on ice, washed twice with cold 1x PBS,
and harvested in 4.0-mL, 500-mM sodium carbonate (pH 11.0) with protease inhibitors cocktail (1:1000, Sigma). Cells were allowed to swell on ice for 30-minutes. Total cell lysate was prepared by forty strokes with a chilled, dounce homogenizer. Caveolae membrane microdomains were isolated via detergent-free sucrose density gradient ultracentrifugation as previously described by others (93) with modifications. Cell lysate (2.0-mL) was transferred to a 13-mL ultracentrifuge tube (Beckman). To the tube, 2.0-mL, 90% sucrose in Mes-buffered saline (MBS; 25-mM Mes, pH 6.5, 0.15-M NaCl) was added and vortexed. Carefully and slowly, 4.0-mL, 35% sucrose in MBS with 250-mM sodium carbonate (pH 11.0), followed by 4.0-mL, 5% sucrose in MBS with 250-mM sodium carbonate (pH 11.0) was added dropwise to the top of the gradient. Gradients were ultracentrifuged at 39,000-rpm for 16-18-hours at 4°C with an SW-41 rotor. Thirteen fractions (1.0-mL each) were collected from each gradient on ice, and 100-µL of each fraction was added to 25-µL, 5x Laemelli sample buffer. All remaining samples were frozen at -80°C for storage.

SDS-PAGE

SDS-PAGE gels (10-12% running gel, 4% loading gel) were prepared as described in Appendix III. Samples were boiled for 2-4-minutes, then centrifuged at 16,000-rpm for 4-minutes. EZ Run™ pre-stained Rec protein ladder (5-µL/lane, Fisher) was used as the molecular weight standard. Gels were run at 100-120-volts for 1-2-hours. SDS gels were removed, and incubated with blotting paper (BioRad) and nitrocellulose (BioRad) for 20-minutes in semi-dry transfer buffer. Transfer to nitrocellulose occurred at 15-volts on a Trans-Blot® SD semi-dry transfer cell (BioRad) for 1-hour.
Western blotting

Nitrocellulose blots were blocked for 1-hour at room temperature or overnight at 4°C, shaking in 5% dry milk/TBS-T. Primary antibody in blocking solution with 0.01% sodium azide was added overnight at 4°C shaking. Nitrocellulose membranes were washed three times, 10-minutes each with blocking solution, shaking at room temperature. Secondary conjugated-HRP antibody (1:2000 or 1:5000) in blocking solution was added for 45-minutes to 1.5-hours shaking at room temperature. Nitrocellulose membranes were washed three times, 10-minutes each with blocking solution, shaking at room temperature. Nitrocellulose was subjected to 1.5-mL oxidizing and enhanced luminol solutions of western lighting™ chemiluminescence reagent plus kit (Perkin Elmer) for 1-minute. Blots were exposed to film (Kodak) and developed using developer (Kodak) and fixer/replenisher (Kodak) for varying times.

Bradford assay

The assay was performed using a bovine serum albumin (BSA) standard (0-100-µg) for comparison with experimental protein levels. Bradford reagent (1.0-mL) was added to each BSA control and vortexed. All samples were transferred to a cuvette, blanked, and measured for absorbency in a spectrophotometer OD595 (ThermoSpectronic Genesys 10-UV). Bradford reagent (1.0-mL) was added to 100-µL experimental sample and vortexed. All samples were transferred to a cuvette, blanked, and measured for absorbency in a spectrophotometer OD595 as described.

2 1:2000 rabbit anti-VSV g (Sigma), 1:5000 rabbit anti-caveolin (BD Biosciences), 1:1000 rabbit anti-p42/44 P-MAPK (Cell Signaling), 1:1000 mouse anti-Total p42/44 MAPK (Cell Signaling).

3 Typically exposures were taken at 1-, 3-, and 5-min and/or less than 1-min exposures, if needed.
Densitometry with Adobe Photoshop

Western blot images were scanned into the computer. The images were converted from color to grayscale, and then inverted. Densitometry was measured with control cells reported as the baseline (1.0 relative units).
RESULTS

CHO-K1 cells stably expressing VSVg-tagged MCHR-1: a model system for studying MCHR localization

The data shown in Figure 1 (Appendix II) are representative of two immunocytochemistry experiments. MCHR-1 localization and internalization was investigated in stably expressing VSVg-MCHR-1 CHO-K1 cells, a mammalian cell culture model. Cells were subjected to MCH (1.0-μM) to assess changes in MCHR-1 localization and internalization following ligand exposure. At 0-minutes MCH exposure, MCHR-1 is visualized along the plasma membrane with additional, possibly immature receptor found intracellularly near the nucleus. At 10-minutes MCH exposure, MCHR-1 partially internalizes from the plasma membrane. This is evident by vesicle formation and by an absence of MCHR-1 membrane staining. The observed staining pattern resembles that of Rab5, an early endosomal marker (94). Cellular nuclei are DAPI stained in blue.

MCHR-1 co-localizes with caveolin-1 enriched lipid raft fractions

The data presented in Figure 2 (Appendix II) are demonstrative of four replicated experiments. To determine if MCHR-1 co-localizes with caveolae, caveolae membranes were isolated by sucrose density ultracentrifugation and fractionation by a detergent-free method with modifications (93). Thirteen fractions were collected from the gradient, run on 12% SDS-PAGE gels, and transferred to nitrocellulose. Western blotting using antibodies toward VSVg and cav-1 confirmed the presence of VSVg-MCHR-1 in caveolae fractions 4 and 5. Caveolin-1 localized to fractions 4, 5, 6, and 7, which is consistent with the current literature as caveolae is commonly isolated in

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4 Western blots were assisted by Kelsi Robinson, B.S.
fractions 4 and 5 (93). The wider distribution of caveolin-1 enriched fractions relative to fractions containing VSVg-MCHR-1 may be explained by the high concentrations of cellular caveolae present relative to receptor. This was also observed for the scavenger receptor CD36 using a similar caveolae isolation method (129). A Bradford assay was performed to evaluate the total protein levels of each fraction from the gradient. Caveolar content peaked at fraction 5. The western blot also demonstrated that fraction 5 contained more VSVg-MCHR-1 and cav-1 than fractions 4, 6, or 7. Fraction 13 fell out of range for the standard BSA curve.

MCHR-1 localization to caveolin-1 enriched fractions is independent of 1.0-µM MCH treatment at 10-minutes with equal protein loading

The results shown in Figure 3 (Appendix II) are representative of three experiments. To evaluate the effects of MCH exposure on MCHR-1 localization to caveolae, stable VSVg-MCHR-1 CHO-K1 cells were cultured to confluency. Cells received MCH (1.0-µM) in F12k media for 0- or 10-minutes as designated prior to harvesting. Caveolae membranes were isolated as previously described (93) with modifications, and fractions were removed. A Bradford assay was performed to determine the total protein content of each fraction. Equal amounts of protein (approximately 100-ng) were loaded onto the gel for fractions 4, 5, 6, and 7 from each gradient. SDS-PAGE gels (10%) were run and transferred to nitrocellulose. Western blotting with antibodies toward VSVg and cav-1 established that VSVg-MCHR-1 and cav-1 localize to both fractions 4 and 5 for each gradient at both 0- and 10-minutes MCH exposure. The majority of VSVg-MCHR-1 and cav-1 localized to fraction 4 and smaller amounts to fraction 5 with equal quantities of protein loaded. Trace amounts of VSVg-
MCHR-1 and cav-1 were present in fraction 6. Neither cav-1 nor VSVg-MCHR-1 was present in fraction 7.

**MCHR-1 localization to caveolae lipid raft fractions is independent of MCH exposure across time points**

The data shown in Figures 4 and 5 (Appendix II) are representative of one experiment. To establish the effects of varying lengths of MCH exposure on MCHR-1 localization to caveolae, VSVg-MCHR-1 CHO-K1 cells received MCH (1.0-µM) in F12k media for 0-, 5-, 10-, and 30-minutes as indicated. Caveolae membranes were isolated as previously described (93) with modifications, and fractions removed from each gradient. SDS-PAGE gels (10-12%) were run for all samples, and transferred to nitrocellulose. Western blotting using anti-VSVg antibody towards tagged MCHR-1 was necessary to analyze the distribution of receptor across the time points, as seen in Figure 4A (Appendix II). At 0-minutes MCH exposure, VSVg-MCHR-1 is evident in fraction 4, with less receptor in fraction 5 – in between the 55- and 72-kDa molecular weight (MW) markers. VSVg-MCHR-1 is found in fraction 4 between the 55- and 72-kDa MW bands, and in fraction 5 and 6 with a slight MW upshift at 5-minutes MCH exposure. Following 10-minutes MCH exposure, VSVg-MCHR-1 is present in fraction 4 near the expected MW, and in fraction 5 and 6 with a slight MW upshift. Receptor is observed in fraction 5 with smaller levels of receptor in fraction 4 at 30-minutes MCH exposure, and the MW upshift is absent.

Western blotting using anti-caveolin antibody towards cav-1 was necessary to analyze the distribution of isolated caveolae in each gradient as evident in Figure 4B (Appendix II). At 0- and 5-minutes MCH exposure, caveolae was isolated in fractions 4

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5 Sucrose gradient performed by thesis author and Laurie Cook, Ph.D.
and 5, with lesser amounts present in fractions 6-8, and trace amounts in fractions 9-12. Small amounts of caveolae were present in fraction 4, with the most caveolae in fraction 5, followed by fraction 6 at 10-minutes MCH exposure. The majority of the caveolae is localized to fractions 4 and 5, followed by fraction 6, with trace amounts in fractions 7 and 8 at 30-minutes MCH exposure.

A Bradford assay was performed on all the fractions of each MCH time point sucrose gradient. At 0-minutes MCH exposure, protein levels peaked at 0.095-mg in fraction 4, followed by 0.080-mg in fraction 5, as shown in Figure 5B (Appendix II). Protein levels peaked at 0.125-mg in fraction 5, followed by 0.085-mg in fraction 4 and 0.072-mg in fraction 6 at 5-minutes MCH exposure, as seen in Figure 5C (Appendix II). Figure 5D (Appendix II) shows that at 10-minutes MCH exposure, protein levels peaked at 0.135-mg in fraction 5, followed by 0.085-mg in fraction 6. Protein levels peaked at 0.076-mg in fraction 5, followed by 0.065-mg in fraction 6 at 30-minutes MCH exposure, as evident in Figure 5E (Appendix II). These results were consistent with those observed in the corresponding western blots for each MCH time point.

**Manipulation of membrane cholesterol alters the distribution of MCHR-1 in stably transfected CHO-K1 cells**

The data shown in Figure 6 (Appendix II) are representative of one immunocytochemistry experiment. To further explore the possibility of MCHR-1 enrichment in caveolae, cellular membranes were deprived of cholesterol with MβCD (5.0-mM) or supplemented with exogenous cholesterol (1.0-mM). VSVg-MCHR-1 CHO-K1 cells were also subjected to MCH (1.0-µM) for indicated time points to

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6 Bradford assay and protein calculations completed by Laurie Cook, Ph.D.

7 Experiment performed by Kelsi Robinson, B.S.
determine changes in receptor localization following hormone treatment. Alterations in cellular cholesterol content may modulate MCHR-1 localization to caveolae. The reduction of membrane cholesterol levels lead to the destruction of caveolae lipid rafts, and the accumulation of soluble cholesterol in the plasma membrane replenish caveolae already present. All images were taken for the same exposure time to capture MCHR-1 internalization following MCH treatment for 0-, 30-, and 60-minutes. In control cells, partial receptor internalization is observed at 30-minutes MCH exposure, with possible receptor return to the cell surface at 60-minutes. Cells that have undergone cholesterol depletion with MβCD demonstrate severe morphological deformities and alterations in membrane structure. Cell viability was significantly diminished following cholesterol depletion (data not shown), as evidence by the few cells remaining on coverslips during microscopy. Due to the observed morphological defects in the cell membrane, it was difficult to evaluate evidence of MCHR-1 internalization following MCH treatments. The addition of soluble cholesterol caused MCHR-1 to redistribute itself within the cell. MCHR-1 appeared to localize to areas, such the endoplasmic reticulum (ER), surrounding the nucleus after 30- and 60-minutes of MCH exposure. The staining pattern resembles that of calnexin, an ER molecular marker (95).

**Cholesterol depletion with methyl-β-cyclodextrin dampens MCH-dependent ERK 1/2 activation**

The data displayed in Figure 7 (Appendix II) are representative of three replicated experiments⁸. To investigate the effects of cholesterol depletion and caveolae disruption on MCHR-1 signaling, transiently transfected CHO-K1 cells expressing

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⁸ MβCD and ERK1/2 experiments performed by thesis author and Laurie Cook, Ph.D.
untagged MCHR-1 were grown to confluency in a 12-well dish\(^9\). Cells were serum starved for 1-hour, cholesterol depleted with 10.0-mM MβCD for 1-hour, exposed to 1.0-µM MCH for the designated times, and harvested. Samples were run on 12% SDS-PAGE gels, and transferred to nitrocellulose for western blotting with ERK1/2 antibodies. Total ERK1/2 levels were constant across both cell types. MCH induced a peak phosphorylated ERK1/2 response at 5-minutes and sustained the signal at 10-minutes exposure in control cells. In MβCD treated cells, activated ERK1/2 appeared at 5-minutes and was prolonged up to 30-minutes MCH exposure as shown in Figure 7A (Appendix II).

Densitometry was performed using Adobe Photoshop software\(^{10}\) as shown in Figure 7B (Appendix II). Baseline levels (1.0 relative units) of control cells were reported. Control cells demonstrated peak ERK1/2 activation at 5-minutes MCH exposure with 52 relative units; the signal was sustained at 10-minutes MCH exposure with 42 relative units. Levels of phosphorylated ERK1/2 declined at 30-minutes with 2.0 relative units. In MβCD treated cells, ERK1/2 activation appeared at 5-minutes with 7.5 relative units and peaked at 10-minutes with 10 relative units. After 30-minutes of MCH exposure, P-ERK levels remained at 5.0 relative units. The MCH-dependent ERK1/2 response was dampened in cholesterol depleted/caveolae disrupted cells compared to controls.

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\(^9\) Transfection carried out by Laurie Cook, Ph.D.
\(^{10}\) Densitometry for this blot was performed by Laurie Cook, Ph.D.
DISCUSSION

**CHO-K1 cells stably expressing VSVg-tagged MCHR-1: a model system for studying MCHR localization**

Partial internalization of VSVg-MCHR-1 was evident following 10-minutes MCH exposure in this preliminary immunocytochemistry experiment. The procedure was only performed twice so the results should not be considered definite. However, this early evidence has suggested that 10-minutes of exposure to 1.0-µM MCH is sufficient to cause receptor internalization from the plasma membrane in the stable VSVg-MCHR-1 CHO-K1 cell model. This is consistent with the literature for MCHR-1 internalization. Saito et al. demonstrated that 5-minutes of 300-nm MCH exposure led to 21.9 +/- 2.6% loss of surface receptor, and that 30-minutes of hormone exposure led to 44.2 +/- 2.5% loss of surface receptor (68).

**MCHR-1 co-localizes with caveolin-1 enriched lipid raft fractions**

Fractionation methods using insolubility detergents such as Triton X-100 or detergent-free methods such as sucrose gradients are widely used to isolate caveolae and lipid rafts from the rest of the plasma membrane and cellular contents (32,93). These techniques are successful because the density of caveolar membranes is very light compared to the bulk of the plasma membrane (33). In addition, caveolae membrane components – glycosphingolipids, GPI-anchored proteins, and caveolin – resist solubilization by detergents, giving rise to the term “detergent- resistant” membrane microdomains (rev 98). The advantage of sucrose gradient preparation methods include: a more delicate membrane preparation and a purer caveolar fraction (93,99).
Co-localization and/or enrichment of various signaling molecules or membrane receptors within caveolae fractions, following isolation procedures, are an accepted method of confirming the presence of these molecules within caveolae. Song et al. established that the Ras protein co-localizes with caveolae membranes, along with c-Src, \(G_{12\alpha}\), and \(G_{13}\), by a detergent free, sodium carbonate-based sucrose density gradient method (93). The protocol used for identifying the co-localization of MCHR-1 with lipid raft fractions containing caveolin-1 has been adapted from Song et al.

VSVg-tagged MCHR-1 was found to co-localize with caveolin-1 enriched fractions – 4 and 5 – subsequent to sucrose density gradient ultracentrifugation as shown in Figure 2 (Appendix II). The majority of VSVg-MCHR-1 and caveolae were localized to fraction 5, as evident by western blot and Bradford assay. A protein peak at fraction 5 confirmed that caveolae was separated from the remainder of the cellular contents. Song and colleagues observed caveolae localization to fraction 5 in the previously mentioned investigation (93). Fractionation methods that isolate caveolae and co-immunoprecipitation with caveolin are the two approaches discussed in the literature for establishing the presence of caveolae enrichment and/or co-localization (20). Thus, MCHR-1 was found to be enriched in characteristic caveolin-1 containing fractions as demonstrated by caveolar fractionation and western blotting. The same results were apparent in each of the four replicative experiments. This provides strong evidence for MCH receptor enrichment to lipid raft regions of the plasma membrane.
MCHR-1 localization to caveolin-1 enriched fractions is independent of 1.0-μM MCH treatment at 10-minutes with equal protein loading

Exposure to 1.0-μM MCH appeared to have no effect on receptor localization to caveolin-1 fractions within 10-minutes, with equal amounts of fractionated protein loaded onto SDS-PAGE gels as seen in Figure 3 (Appendix II). At 0-minutes and at 10-minutes MCH exposure, both MCHR-1 and cav-1 remain localized to fractions 4 and 5. A hypothesized explanation for this is that the receptor has not yet undergone internalization from the plasma membrane and remains within lipid rafts at 10-minutes. To test this, a longer time course of hormone treatment was performed to investigate whether or not the MCH receptor moved out of lipid raft fractions when exposed to MCH.

MCHR-1 localization to caveolae lipid raft fractions is independent of MCH exposure across time points

An expanded time course, including 0-, 5-, 10-, and 30-minutes of MCH exposure was carried out as observed in Figures 4 and 5 (Appendix II). This experiment was only performed once, so the results must be interpreted with the knowledge that repetition is necessary to be certain of the outcome. Poor caveolae distribution during the 0- and 5-minute MCH exposures was most likely due to poor technique in removing the fractions off the gradient, rather than an error of the gradient itself. The co-localization of VSVg-MCHR-1 and cav-1 is apparent throughout each point of the MCH time course. However, the majority of MCHR-1 appears to be present in fraction 4 at 0- and 5-minutes MCH exposure, with a transition to fraction 6 at 10-minutes, and with a transfer back to fraction 5 at 30-minutes as seen in Figure 4A (Appendix II). These results may suggest a role for lipid rafts in the internalization of MCHR-1.
Receptor enrichment to caveolae membranes may or may not be accompanied by caveolar endocytosis \((rev \ 8)\). First, in general, receptor localization to caveolae may be coupled with caveolar-mediated endocytosis. Second, opposite to this scenario, receptor transition out of caveolae fractions may be evidence of clathrin-dependent receptor-mediated endocytosis events \((rev \ 8)\). Receptors that reside in lipid rafts may move into clathrin-coated pits for internalization upon ligand exposure. Third, receptors may transition into caveolae lipid rafts for endocytosis following agonist exposure. Fourth, ligand administration may prompt other receptors to enter caveolae for signaling events, but then later internalize via clathrin-coated vesicles. MCHR-1 does not appear to be entirely caveolae-dependent since the inhibition of clathrin endocytosis partially abolished receptor internalization \((68)\). MCHR-1 has been shown to reside in lipid raft fractions containing caveolin-1 as seen in Figure 2 (Appendix II). Thus, MCHR-1 movement into caveolae is not dependent on MCH exposure. The most plausible explanation is that MCHR-1 is enriched in caveolae, and internalization may occur by either clathrin-coated pits or lipid rafts. This is the case for the \(\beta_1\)-adrenergic and the transforming growth factor \(\beta\) \((TGF \ \beta)\) receptors. Dependency on either endocytotic pathway may be determined by specific kinase phosphorylation events \((11)\). For the \(\beta_1\) adrenergic receptor, protein kinase A directs the receptor to caveolae internalization, and GRK-mediated phosphorylation targets the receptor to clathrin-coated vesicles.

The small upshift in MCHR-1 molecular weight may be due to phosphorylation of the C-terminal tail of the receptor prior to internalization as seen in Figure 4A (Appendix II). G protein-coupled receptors such as the thyrotropin releasing hormone receptor \((TRHR)\) are phosphorylated on their C-tails before internalization.
occurs (100,101). A small upshift in molecular weight of the TRH receptor has also been observed once it has been phosphorylated (101). The addition of phosphatases reverted the molecular weight to that of the mature TRH receptor. The MCHR-1 upshift was initially observed at 5-minutes MCH exposure. Confirmation of MCHR-1 phosphorylation could be performed using a method similar to that of Zhu et al. with the TRH receptor (101). Alternative explanations for the change in molecular weight of MCHR-1 following ligand treatment include dimerization or ubiquitination. GPCR dimerization has recently been cited as a common event that enables molecular cross-talk to occur between receptor families (102). However, dimerization is probably unlikely as it would result in a doubling of the molecular weight rather than only a small upshift as was observed. Ubiquitination is also doubtful as this molecular event would produce a much larger increase in molecular weight than noted for MCHR-1 (102,103).

Co-localization of the MCH receptor to lipid raft fractions containing cav-1 provides strong evidence supporting the presence of MCHR-1 within caveolae. Although MCHR-1 does not appear to move out of caveolae lipid rafts as longer hormone exposure occurs, this does not rule out caveolae as a possible mechanism of MCH receptor internalization. The next step would be to assess whether or not MCHR-1 and cav-1 form a protein complex with each other; this can be achieved through co-immunoprecipitation studies of isolated caveolae membrane fractions containing receptor. If MCHR-1 and cav-1 do form a protein complex with each other, then movement from caveolae membranes would not be as readily apparent in fractionation and western blot studies such as those performed. It is also important to note that although MCHR-1 appears to localize to caveolae in CHO-K1 cells, the receptor may
actually be enriched in caveolae in certain cell types, yet found in other locations of the plasma membrane in other cell types.

**Manipulation of membrane cholesterol alters the distribution of MCHR-1 in stably transfected CHO-K1 cells**

Cholesterol depletion and repletion techniques are standard procedures used to disrupt or restore cellular caveolae, respectively. If MCHR-1 is enriched in caveolae lipid rafts, and cholesterol levels are manipulated, internalization, trafficking, and receptor signaling will subsequently be affected. Immunocytochemistry was utilized as a means to evaluate cholesterol depletion and repletion on MCHR-1 internalization. This preliminary experiment was only performed once, so the results must be considered with this in mind. No conclusions can be made regarding the effect of cholesterol depletion, and subsequent destruction of caveolae, on MCHR-1 internalization. Imaging of VSVg-MCHR-1 CHO-K1 cells, following MβCD treatment, shows a severely deformed cellular structure. There were very few cells to image post-MβCD. Immunocytochemistry studies with cholesterol depletion were not a good method of confirming MCHR-1 localization to caveolae lipid rafts. An alternative may be to perform immunocytochemistry with fluorescence-tagged antibodies to establish co-localization between MCHR-1 and caveolin-1. Fluorescence imaging, a widely established approach, has been used to assess the co-localization of molecules in vitro. Often, green and red fluorescence-conjugated secondary antibodies are used towards the two proteins of interest. Co-localization may be observed if the two fluorescent antibodies overlap, producing yellow fluorescence.
The addition of soluble cholesterol appears to redistribute MCHR-1 compared to control cells. At 30- and 60-minutes post-MCH exposure, MCHR-1 is observed to internalize to areas surrounding the nucleus. This differs from control cells since this form of receptor relocation is not evident. These areas are hypothesized to be endoplasmic reticulum (ER). Further studies involving immunocytochemistry aimed at determining co-localization of MCHR-1 and molecular markers of the ER in cholesterol repleted cells would be necessary to verify this finding. Organelle markers that could be utilized include calnexin, an ER marker involved in protein folding (95).

**Cholesterol depletion with methyl-β-cyclodextrin dampens MCH-dependent ERK 1/2 activation**

Cholesterol depletion with methyl-β-cyclodextrin (MβCD) disassembles caveolae lipid rafts. If MCHR-1 were enriched in caveolae, lipid raft disruption would subsequently be expected to alter receptor signaling. MCHR-1 activates ERK1/2 via G0 with protein kinase C, or Gq with calcium release (62). The phosphorylated ERK1/2 response in cholesterol depleted cells was dampened and peaked later than the control cell response. These results suggest that caveolar destruction downregulates MCHR-1 mediated ERK1/2 activation. Similar results were observed over the course of four repeated experiments, both with stable and transiently transfected MCHR-1 expressing CHO-K1 cells; and thus, can be considered valid.

Previous studies that did not involve MCH or MCHR-1 demonstrated hyperactivation of ERK1/2 (p42/44 MAPK) as a by-product of membrane cholesterol depletion and caveolar loss (28,97). Cholesterol depletion with pharmacological agents is often utilized as a technique for disrupting caveolae lipid rafts during experimental
Hyperactivation of the p42/44 MAPK cascade was observed in at least two separate investigations. Furuchi et al. noted phosphorylated ERK overexpression following cholesterol depletion with cyclodextrin in rat-1 cells (97). The study indicated that membrane cholesterol levels may also order the molecular components of signal transduction cascades. It was hypothesized that interference with membrane cholesterol may lead to cellular disorganization and dysregulated signaling patterns. Galbiati and colleagues documented hyperactivation of p42/44 MAPK following siRNA knockout of cav-1 in NIH 3T3 cells (28). This work theorized that cav-1 may negatively regulate the p42/44 MAP kinase (ERK1/2) pathway.

If MCHR-1 were enriched in lipid rafts, cholesterol depletion would be expected to disrupt the lipid raft scaffolding domain and alter signaling by reducing the availability of downstream targets. Thus, the signaling response of MCHR-1 would be predicted to decrease following cholesterol depletion. The data presented in Figure 7 (Appendix II) indicate a role for MCHR-1 enrichment to lipid rafts. Downregulated phosphorylated ERK1/2 levels suggest that such events are likely occurring due to MCHR-1 enrichment in raft regions. Hyperactivation of the p42/44 MAPK cascade is absent, indicating that the results obtained are due to MCH-dependent ERK1/2 responses rather than an anomalous consequence of lipid raft disassembly.

Interestingly, ERK1/2 activation was previously shown to induce leptin transcription in 3T3-L1 cells (74). Leptin acts as an appetite suppressor, while MCH stimulates feeding and energy conservation. A role for lipid raft regulation of MCHR-1 signaling is plausible, as demonstrated by the functional ERK1/2 signaling data presented in this thesis. If MCHR-1 is enriched within the caveolae of adipose cells, normal
ERK1/2 signaling would occur upon MCH exposure, resulting in leptin transcription. Systemically, leptin feedback would turn off the activated MCH signals for the body to consume food. As an individual gains excess fat and cholesterol, and thus weight, their pre-adipocytes differentiate to become mature adipose (fat) cells. Caveolae accumulates in the adipocyte during this process. The normal signaling pathways and functioning of the MCH receptor enriched in adipocyte caveolae may become dysregulated at this point due to an unknown mechanism. In an overweight individual, this alteration of signaling pathways could lead to an absence of satiety due to low leptin levels, loss of the MCH-leptin feedback loop, and hyperactive MCH secretion by the hypothalamus. This person would continue to feel hungry, despite high caloric intake, due to the disruption in leptin secretion. Increased hunger inevitably leads to food consumption, an accrual of body mass, and an obese body type.

**Further support for MCHR-1 enrichment to lipid rafts**

Further evidence for the enrichment of MCHR-1 to lipid rafts arises from the primary amino acid sequence of the receptor itself. Somatostatin receptors are known to localize to lipid rafts (69), and MCHR-1 was originally identified as orphan GPCR SLC-1, or somatostatin-like receptor-1. In addition, preliminary co-immunoprecipitation studies currently underway in the lab indicate that a protein complex interaction between MCHR-1 and cav-1 is occurring (data not shown). If conclusive, this evidence would further support MCHR-1 enrichment to caveolae lipid rafts.

The finding that MCHR-1 is enriched in lipid rafts provides another attractive question – what about other tissues that MCHR-1 has been discovered in? Although this thesis seeks to demonstrate that MCHR-1 localizes to the lipid rafts of CHO-K1 cells,
MCHR-1 has also been isolated in adipose and pancreatic islet β cells and within the brain and central nervous system. Although caveolae are present in adipocytes and pancreatic islet β cells (107), caveolae is undetectable in neurons (67). The possibility exists that MCHR-1 is present within the lipid rafts of certain cell types, such as pre- and mature adipocytes, but not in all MCH receptor-associated cells. This is characteristic of receptors that are enriched in, but do not solely localize to, caveolae lipid rafts.

Proposed peripheral model

MCH secretion from the hypothalamus begins as a central nervous system response that induces peripheral feeding behaviors. The mechanism that triggers MCH release has not yet been elucidated. MCH release also appears to result in energy conservation. MCH receptors have been isolated in pancreatic islet β, adipose, CNS, eye, tongue, and muscle cells (51,64,65). The discovery of MCHR-1 in pancreatic islet β cells and the presence of the insulin receptor in the caveolae of adipocytes suggest an interesting possibility for cross-talk to exist between these two receptors. Previous work has suggested that Ca\(^{+2}\) influx precedes insulin secretion in pancreatic islet β cells (88). Similarly, MCH administration results in a dose-dependent insulin secretion in β cells preceded by Ca\(^{+2}\) release (51). Insulin causes ERK1/2 activation, an MCHR-1 G\(_{q}\)/G\(_{q}\) coupled response, in adipocytes (84).

It is possible that low systemic circulating insulin levels prompt MCH release in a fasting state. Due to increased levels of MCH, an individual consumes food. Circulating MCH binds to the MCH receptors on the pancreatic β cells, causing a G\(_{q}\) coupled pathway to become activated, which then causes Ca\(^{+2}\) and insulin release.
Insulin enters the bloodstream to prepare the cells of the body to take up newly released glucose from the meal. Insulin binds to insulin receptors, while MCH binds to MCHR-1, both localized to adipocyte caveolae. The insulin receptor, a tyrosine kinase receptor, dimerizes and is able to mediate cross-talk with the activated MCH receptor. The body is now in a fed state, and activated MCHR-1 couples to $G_o$ or $G_q$; this results in leptin transcription. Leptin release from adipocytes results in an overall 'full feeling' once leptin binds to its hypothalamic receptor (96), and the individual stops eating. Systemic MCH levels then decrease to prevent continued food intake.

The overweight or obese individual varies from this model in several respects. Obesity may result if a person has molecular defects that result in one of the following: hyperactive MCH release, failure of MCHR-1 to elicit the leptin response, or disruption of the leptin-MCH feedback mechanism. Too much MCH or too little leptin release may cause an individual to continue to consume food, leading to weight gain without feeling calorically satisfied. Increased weight is the result of pre-adipocyte accumulation of triacylglycerides, causing increases in fat cell size, differentiation, and ultimately cell division (74,96). Unfortunately, once an increase in the number of fat cells has been achieved, even weight loss does not decrease the number of cells present in an individual.

As stated previously, increased levels of caveolae within an adipocyte may lead to dysregulated MCHR-1 signaling and/or its proposed cross-talk with the insulin receptor by an unknown mechanism. Presumably, higher amounts of ERK1/2 activation would lead to corresponding increases in leptin transcription in vivo. Systemically, this individual would probably feel full and stop eating. However, in an obese individual, ERK1/2 activation and leptin transcription may be decreased, and MCH and insulin
levels would remain high. High insulin may lead to insulin receptor desensitization, and the individual would continue eating, propagating and encouraging an obese phenotype. This proposed peripheral model offers an explanation as to why obese individuals are at risk for type II insulin-resistant diabetes. Weight loss often results in a reversion of the condition, restoring cellular responsiveness to insulin.

Future directions

There are several potentially promising directions that this project could take. First, the characterization of a protein complex interaction between MCHR-1 and cav-1 by co-immunoprecipitation is critical. Co-immunoprecipitation with cav-1 is one of the accepted methods for establishing enrichment within caveolae (20,32). This experiment could be performed on isolated caveolae fractions containing the MCH receptor. If such a protein interaction were to be discovered, it would provide substantial evidence for the enrichment of MCHR-1 to caveolae lipid rafts. Second, the use of an endogenous cell line, such as the 3T3-L1 pre-adipocyte or differentiated adipocyte, is critical for assessing the functional role of MCHR-1 in adipocyte caveolae. Third, the use of siRNA to delete caveolin-1 solely rather than pharmacological cholesterol depletion, which may have possible side-effects unrelated to receptor signaling, is advised as well. This method will better assess the consequences of caveolar loss on MCHR-1 signaling and functioning.

Fourth, the use of MCHR-1 inhibitors in combination with signaling assays for cAMP or Ca\(^{2+}\) may be used to further determine the consequences of signal alteration due to caveolae disruption in an endogenous system. Fifth, evaluation of leptin transcription levels at various MCH exposure time points with and without caveolae lipid raft disassembly would provide further supporting evidence for caveolar regulation of
MCHR-1. Sixth, the use of electron microscopy with immunogold labeling towards MCHR-1 in 3T3-L1 cells to localize the receptor to caveolae would be highly appealing, and may provide more detailed evidence than immunocytochemical studies alone.

Finally, further analysis of the possible cross-talk between MCHR-1 and the insulin receptor could be pursued to establish molecular and physiological connections between both of these important metabolic mediators.
CONCLUSION

Investigation into MCHR-1 and lipid raft regulation of receptor functioning is important for several reasons. First, the MCHR-1 pathway provides a promising target for pharmacological drug development towards fighting the obesity epidemic. However, knowledge of this receptor and its signal transduction mechanism must first be elucidated before drug targeting is possible. Second, the medical and the social impacts of uncovering a possible obesity therapeutic are extremely positive, and at this point, necessary. The ever-growing global obese population is in desperate need of an answer and a hope. Although everyday lifestyle changes such as improved eating habits and physical activity allow for incremental weight loss, even these apparently obvious adjustments are not enough for some morbidly obese individuals. Molecular and cellular disruption of key homeostatic energy and metabolic pathways is also contributory. The only answer is to continue the scientific pursuit of appetite hormones, such as MCH and associated receptors to provide evidence of, and solutions to, abnormal molecular functioning as an underlying cause of obesity.
Figure 1. **Central nervous regulation of peripheral hormone responses** *(previously published, 3).* Appetite signals are initially released by peripheral tissues, and are received by the hypothalamus. The hypothalamus then reacts by secreting additional hormones that prompt an individual to eat. Peripheral responses are generated by gastrointestinal organs, such as the large intestine and the stomach, and by metabolic regulators, such as adipose tissue and pancreatic islet $\beta$ cells, that loop back to the brain to shut off the hunger signal.

*Diagram:*
- **orexigenic effects**
  - ↑ appetite
  - ↑ energy expenditure
  - ↑ thermogenesis
- **anorexigenic effects**
  - ↓ appetite
  - ↓ energy expenditure
  - ↑ thermogenesis

**MCH, orexins A and B, TRH, CRH**

**downstream neurons**

**orexigenic signals**

**anorexigenic signals**

**POMC**

**(n-MSH)/CART neurons**

**arcuate nucleus**

**NPY/AgRP neurons**

**GHRHR**

**LEPR**

**insR**

**PYy**

large intestine

**ghrelin**

stomach

**leptin**

adipose tissue

**insulin**

$\beta$-pancreatic cells

49
Figure 2. G protein-coupled receptor signaling pathways involving G$i$, G$0$, and G$q$. Coupling of GPCRs to G$i$ stimulates the adenylyl cyclase pathway, while G$i$ inhibits adenylyl cyclase and its downstream signaling components cyclic AMP (cAMP) and protein kinase A (PKA). The G$0$ pathway activates protein kinase C (PKC) and the Ras/Raf MAPK cascade. G$q$ leads to phospholipase C (PLC), inositol triphosphate (IP$_3$) and diacylglycerol (DAG) activation, followed by calcium (Ca$^{2+}$) release.
Figure 3. G protein-coupled receptors are targeted for degradation or recycling following internalization (previously published, 10). After sorting in early endosomes, receptors may be targeted for degradation or recycled back to the plasma membrane for further signaling.
Figure 4. Clathrin-coated vesicles and caveolae differ in characteristic morphology, as shown by electron microscopy. A) Clathrin-coated pits (previously published, 6) assemble into a lattice, creating clathrin-coated vesicles (CCVs) with the help of adaptor proteins, such as the AP-2 complex and dynamin (6,10,13). These pits 'pinch off' from the plasma membrane, with their molecular cargo secured inside the formed vesicle (10). B) Caveolae are distinguished by forming invaginations along the plasma membrane (previously published, 32). Lipid rafts are lipid- and cholesterol-rich regions of the plasma membrane. An abundance of signaling molecules and membrane proteins sequester within these areas.
Figure 5. The mammalian MCH peptide is 19-amino acids in length with two additional and four substituted residues (adapted, 46). The primary amino acid sequence of the MCH cyclic neuropeptide varies between the mammalian and the fish homologues. The dicysteine bridge forms the essential ring structure between residues Cys\textsuperscript{7} and Cys\textsuperscript{16} in the mammalian MCH peptide (Cys\textsuperscript{4} and Cys\textsuperscript{13} in fish). The mammalian MCH variant (shown) has an additional N-terminal aspartate (Asp\textsuperscript{1}) phenylalanine (Phe\textsuperscript{2}) and with substitutions at methionine (Met\textsuperscript{4}), leucine (Leu\textsuperscript{9}), leucine (Leu\textsuperscript{9}), and glutamine (Gln\textsuperscript{18}). The fish MCH peptide homologue is 17-amino acids in length, differing from the mammalian MCH with variable amino acids at threonine (Thr\textsuperscript{4}), methionine (Met\textsuperscript{5}), valine (Val\textsuperscript{5}), and glutamate (Glu\textsuperscript{18}) (not shown).
Figure 6. Post-translational processing of the MCH preprohormone (pMCH) gene in humans results in multiple protein products (previously published, 43). MCH is only one of three peptides that may be encoded by the second and third exons of the pMCH gene. Neuropeptide E-I (NEI) and neuropeptide G-E (NGE) are also potential gene products. NEI has been shown to stimulate systemic effects that are antagonistic to MCH, such as inhibiting feeding behaviors. pMCH is found on chromosome 12 (12q23-24).
Figure 7. The $ob^+/ob^+$ MCH$^{+/}$ mouse demonstrates an overall reduction in body fat and adiposity compared to the leptin deficient $ob^+/ob^+$ mouse (previously published, 50). The $ob^+/ob^+$ MCH$^{+/}$ mouse (right) exhibits hyperphagic behavior, similar to that of the obese $ob^+/ob^+$ mouse (left). Increases in resting metabolism, body temperature, and physical activity characterize the $ob^+/ob^+$ MCH$^{+/}$ mouse. Improvements in glucose tolerance and insulin sensitivity were also noted.
Figure 8. MCH regulates hypothalamic feeding responses and insulin secretion from pancreatic islet β cells (previously published, 51). Mice with unregulated pMCH expression show increases in both feeding behaviors and pancreatic β cell mass with insulin resistance and hyperglycemia. Mice lacking the functional pMCH gene demonstrate decreases in feeding and insulin secretion with hypoglycemia.
Figure 9. MCHR-1 is a G protein-coupled receptor with seven transmembrane spanning domains (adapted, 67). The extracellular N-terminus is designated by Met at 'start', and the intracellular C-terminus tail extends into the cytoplasm of the cell. Each of the 353-amino acids of MCHR-1 is shown according to single letter designations. MCHR-1 couples to G_i, G_o, and G_q proteins to activate a variety of signaling molecules.
Figure 10. MCHR-1 and MCHR-2 share 38% sequence homology (previously published, 72). The primary amino acid sequences for MCHR-1 and MCHR-2—identical residues are highlighted in red, and predicted transmembrane domains are underlined in blue.
Figure 11. MCHR-1 single nucleotide polymorphisms (SNPs) are linked to obesity (previously published, 67). The MCHR-1 gene contains a 1-kb promoter with a 3.5-kb coding region. Seven of the 39 MCHR-1 SNPs were identified as encoding for single amino acid substitutions. R248Q has been associated with early onset extreme obesity. The remaining six SNPs (shown below) were characterized in an obese population of 557 adults and 552 children. Dominant linkages were found between the SNPs and obesity. SNP rs133068 has been shown to provide protection against childhood obesity.
APPENDIX II: Table of Results
Figure 1. CHO-K1 cells stably expressing VSVg-tagged MCHR-1: a model system for studying MCHR localization. VSVg-MCHR-1 CHO-K1 cells were grown to partial confluency on coverslips, and exposed to 1.0-μM MCH (American peptide) for the indicated times. Immunocytochemistry was performed using rabbit anti-VSVg (1:1000, Sigma) and anti-rabbit Alexa Fluor 488 (1:500, Invitrogen) antibodies. DAPI (1-µg/mL, Roche) was applied, and coverslips were washed and mounted with ProLong Gold (Invitrogen) on blank slides. Images were taken with the Zeiss Axiocam MRm fluorescence microscope, using AxioView imaging software. At 0-minutes MCH exposure, MCHR-1 is observed along the plasma membrane (arrows). Following 10-minutes MCH exposure, MCHR-1 internalization is evident due to the absence of membrane staining and possible vesicle formation (arrows).
Figure 2. VSVg-MCHR-1 co-localizes with caveolin-1 enriched fractions, as demonstrated by fractionation and western blotting. VSVg-MCHR-1 CHO-K1 cells were cultured to confluency, and caveolae membranes were isolated using a detergent free sucrose gradient ultracentrifugation method as previously described (93) with modifications. Fractions were collected and separated by 12% SDS-PAGE. A) Western blotting was performed using rabbit anti-VSVg (1:2000, Sigma) and rabbit anti-caveolin (1:5000, BD Biosciences) antibodies. VSVg-MCHR-1 and cav-1 co-localize in fractions 4 and 5. B) A Bradford assay assessed total protein of each fraction. Cellular protein content peaks at fraction 5, following caveolae isolation.

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1 Western blots were assisted by Kelsi Robinson, B.S.
Figure 3. MCHR-1 localization to caveolae enriched fractions is independent of 1.0-µM MCH treatment at 10-minutes with equal protein loading. VSVg-MCHR-1 CHO-K1 cells were cultured to confluency, and exposed to 1.0-µM MCH in F12k media for 0- or 10-minutes as indicated. Caveolae membrane microdomains were isolated using a detergent-free sucrose gradient ultracentrifugation method as previously described (93) with modifications. Fractions were collected from each gradient, and a Bradford assay was performed. Equal protein (approximately 100-ng) from fractions 4, 5, 6, and 7 for each treatment was loaded onto 10% SDS-PAGE gels. Western blotting was performed using rabbit anti-VSVg (1:2000, Sigma) and rabbit anti-caveolin (1:5000, BD Biosciences) antibodies. The majority of cellular VSVg-MCHR-1 and cav-1 continue to localize to fraction 4 with MCH exposure.
Figure 4. MCHR-1 localization to caveolae lipid raft fractions is independent of 1.0-μM MCH exposure across time points, as shown by sucrose-density gradient isolation and western blotting. VSVg-MCHR-1 CHO-K1 cells were cultured to confluency, and exposed to 1.0-μM MCH in F12k  media for the time points indicated. Caveolae membrane microdomains were isolated using a detergent-free sucrose gradient ultracentrifugation method as previously described (93) with modifications. Fractions were collected from each gradient. A) Samples were run on 10% SDS-PAGE gels, and western blotting was performed using rabbit anti-VSVg antibody (1:2000, Sigma) towards VSVg-MCHR-1. At 0-minutes MCH exposure, VSVg-MCHR-1 localizes to fractions 4 (arrow) and 5. The majority of MCH receptor localizes to fraction 4, with a molecular weight upshift of MCHR-1 in fraction 5 following 5- and 10-minutes MCH exposure (arrows). At 30-minutes MCH exposure, receptor returns to fraction 5, and the molecular weight upshift is absent (arrow). B) Samples were run on 12% SDS-PAGE, and western blotting was performed using rabbit anti-caveolin (1:5000, BD Biosciences) antibody towards cav-1. Cav-1 co-localization with MCHR-1 is evident in fractions 4 and 5 at 0-minutes, in fractions 4, 5, and 6 at 5-minutes, in fractions 5 and 6 at 10-minutes, and in fraction 5 at 30-minutes MCH exposure.

- Sucrose gradient performed by author and Laurie Cook, Ph.D. (PI).
Figure 5. MCHR-1 localization to caveolae is independent of 1.0-µM MCH exposure across time points, as shown by Bradford assay. A Bradford assay was performed on the isolated fractions. A) Combined sucrose gradient profile for 0-, 5-, 10-, and 30-minutes MCH exposure. B) Sucrose gradient profile at 0-minutes MCH exposure. Caveolae peaks at fraction 4. C) Sucrose gradient profile for 5-minutes MCH exposure. Caveolae peaks at fraction 5. D) Sucrose gradient profile for 10-minutes MCH exposure. Caveolae peaks at fraction 5. E) Sucrose gradient profile for 30-minutes MCH exposure. Caveolae peaks at fraction 5.

Bradford assay completed by Dr. Laurie Cook.
Figure 6. Manipulation of membrane cholesterol alters the distribution of MCHR-1 in stably transfected CHO-K1 cells\(^4\). VSVg-MCHR-1 CHO-K1 cells were grown to partial confluency on coverslips. Cells were serum starved 1-hour prior, treated with 5.0-mM methyl-β-cyclodextrin (MβCD; Sigma) or 1.0-mM soluble cholesterol (Sigma) for 1-hour, and then exposed to 1.0-µM MCH for the indicated times during incubation. Immunoctytochemistry was performed using mouse anti-VSVg (1:1000, Sigma) and donkey anti-mouse Cy3 (1:500, Jackson Labs) antibodies. DAPI (1-µg/mL, Roche) was applied, and coverslips were washed and mounted with ProLong Gold (Invitrogen) on blank slides. Images were taken with the structured light imaging OptiGrid confocal microscope using ImagePro Plus software. A) Cholesterol depletion with MβCD alters the morphology of VSVg-MCHR-1 CHO-K1 cells significantly. Cell membranes are observed to be severely deformed. B) Loading with soluble cholesterol causes MCHR-1 redistribution to cellular areas surrounding the nucleus, possibly endoplasmic reticulum (ER), following MCH exposure.

\(^4\) Immunocytochemistry and imaging performed by Kelsi Robinson, B.S.
Figure 7. Cholesterol depletion with methyl-β-cyclodextrin dampens MCH-dependent ERK 1/2 activation. CHO-K1 cells were grown to confluency in a 12-well dish, and transiently transfected with untagged MCHR-1 plasmid (UMR cDNA Resource Center). Cells were serum starved in F12k media for 1-hour prior to harvesting, then received 10.0-mM methyl-β-cyclodextrin or F12k alone for 1-hour as indicated. MCH (1.0-µM) was added for the designated time points. Cells were washed with 1x PBS on ice, harvested in 50-µL, 2x Laemelli buffer, and run on 12% SDS-PAGE. A) Western blotting was performed using mouse anti-P-MAPK p42/44 (1:1000, Cell Signaling) antibody toward activated ERK1/2 and rabbit anti-MAPK p42/44 (1:1000, Cell Signaling) antibody toward total ERK1/2. ERK1/2 is activated following 5-minutes, and sustained through 10-minutes MCH exposure in control cells. From the western blot, the ERK1/2 response appears at 5-minutes, and is prolonged up to 30-minutes MCH exposure in the MβCD-treated cells. B) Densitometry was performed using Adobe Photoshop software. Total ERK1/2 levels from control cells are reported as the baseline (1.0 relative units). In control cells, ERK1/2 phosphorylation peaks at 5-minutes and declines at 10-minutes MCH exposure. MCH-dependent ERK1/2 activation is dampened at 5-minutes and peaks at 10-minutes MCH exposure following cholesterol depletion with MβCD, as shown.

\[ \text{ERK1/2 activation with MβCD experiments performed by author and Laurie Cook, Ph.D. Transient transfection and densitometry (for this blot) were performed by Dr. Cook.} \]
APPENDIX III: Standard Recipes
G-418

1) 0.5-mg/mL G-418 (A.G. Scientific) in sterile dH₂O
2) Filter sterilize.

10x Phosphate-buffered saline (PBS)

To approximately 800-mL dH₂O, add:

80-g NaCl
2-g KCl
14.4-g Na₂HPO₄
2.4-g KH₂PO₄

pH to 7.4 with 10-N NaOH, then bring to 1-L with the necessary volume of dH₂O.

PBS-T

1x PBS with 0.1% Tween-20

Methyl-β-cyclodextrin (MβCD) stock solution

1) 0.50-g/mL (Sigma) in sterile dH₂O
2) Filter sterilize.

Soluble cholesterol stock solution

1) 0.39-g/mL Soluble cholesterol (Sigma) in sterile dH₂O to achieve 100-mM stock
2) No need to filter sterilize.

5x Bradford reagent

1) Dissolve 100-mg Coomassie blue (90%) in 50-mL ethanol
2) Add 100-mL, 85% Phosphoric acid
3) Add 50-mL dH₂O
4) Gravity filter.
**Mes-buffered saline (MBS)**

25-mM Mes (Acros Organics)
0.15-M NaCl
pH to 6.5

**90% Sucrose gradient**

45-g Sucrose

Bring to 50-mL with the necessary volume of MBS.

**35% Sucrose gradient**

35-g Sucrose

Bring to 100-mL with the necessary volume of MBS with 250-mM \( \text{Na}_2\text{CO}_3 \), pH 11.

**5% Sucrose gradient**

5-g Sucrose

Bring to 100-mL with the necessary volume of MBS with 250-mM \( \text{Na}_2\text{CO}_3 \), pH 11.

**2x Sample buffer by Hoeffer Scientific (prepare under fume hood)**

2.5-mL, 0.5-M Tris HCl, pH 6.8
4.0-mL, 10% SDS
2.0-mL Glycerol
1.0-mL Concentrated 2-mercaptoethanol
0.4-mg Bromophenol blue

Bring to 10-mL with the necessary volume of dH\(_2\)O.
**5x Laemelli sample buffer** (prepare under fume hood)

- 3.75-mL, 1.0-M Tris HCl, pH 6.8
- 1.5-g SDS
- 0.075-g Bromophenol blue
- 1.16-g Dithiothreitol (Cleland’s reagent)

Bring to 7.5-mL with the necessary volume of dH$_2$O. Add 7.5-mL glycerol.

**10x SDS running buffer**

For 1-L in dH$_2$O:

- 30.3-g Tris base
- 144-g Glycine
- 10-g SDS

**Semi-dry transfer buffer**

To 250-mL of dH$_2$O, add:

- 5.8-g Tris base
- 2.9-g Glycine
- 0.37-g SDS
- 200-mL Methanol

Bring to 1-L with the necessary volume of dH$_2$O.

**10x Tris-buffered saline (TBS)**

- 87.66-g NaCl
- 12.11-g Tris base
- 4-mL HCl

pH to 8.0, then bring to 1-L with the necessary volume of dH$_2$O.

**TBS-T**

- 1x TBS with 0.1% Tween-20
**SDS-PAGE gels (10-12%)**

A) 10-12 % Running gel, B) 4% Stacking gel.

<table>
<thead>
<tr>
<th>Percentage</th>
<th>10%</th>
<th>12%</th>
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<tbody>
<tr>
<td></td>
<td>7.5-mL</td>
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<tr>
<td>40% Bis-acryl</td>
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<tr>
<td>4x Tris-Cl/SDS, pH 8.8</td>
<td>1.875-mL</td>
<td>2.5-mL</td>
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<tr>
<td>dH₂O</td>
<td>3.75-mL</td>
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<tr>
<td>10% APS</td>
<td>25.0-µL</td>
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<tr>
<td>TEMED</td>
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<table>
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<td>40% Bis-acryl</td>
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<td>dH₂O</td>
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<tr>
<td>TEMED</td>
<td>2.5-µL</td>
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