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Modulation of Fat Taste by Diet and Hormones

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MODULATION OF FAT TASTE BY DIET AND HORMONES

By

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ABSTRACT

The prevalence of obesity worldwide continues to rise despite efforts to reverse the trend. While many factors contribute to the onset and maintenance of obesity, caloric intake and dietary composition have been shown be primary contributors. The oral cavity is one of the first systems to encounter food and determine its hedonic value. As the gateway to ingestion, the taste system plays a unique role in the initial decisions surrounding the control of food intake. Nutrients like carbohydrates, protein, minerals, and fat all have dedicated systems to allow their recognition at this outermost site of the enteric nervous system. Recent research has shown this system to have a high degree of plasticity, where it may tune itself to the nutritional needs of an organism. The work in this dissertation examined how circulating hormones and dietary changes alter fatty acid detection in the oral cavity thereby altering fat intake. Firstly, we examined the role high dietary fat intake has on fatty acid taste responses. We concluded that high dietary fat intake significantly increases inward currents elicited by linoleic acid (LA) in taste cells, these changes are dependent on the type of dietary fatty acids consumed, and only occur in a subset of fatty acid responding taste cells that are not thought to be the classical receptor cells of the taste bud. Additionally, to better understand physiological factors modulating fat taste sensing, we examined the effects of the orexigenic hormone ghrelin in the taste system. Through a conditioned taste aversion assay, systemic *Ghrl*$^{-/-}$ male mice exhibit diminished fat taste sensitivity compared to wild type (WT) mice with corresponding decreased calcium responses to fatty acids in taste cells. Lastly, ghrelin receptor (GHSR) agonists increased calcium responses to taste cells in WT mice. These data suggest that ghrelin plays a modulatory role in fat taste sensitivity. To further examine these effects using *Ghsr*$^{-/-}$ mice we observed *Ghsr*$^{-/-}$ females

consume significantly less high fat diet than their WT counterparts. *Ghsr*$^{-/-}$ females also showed a significant reduction in fatty acid detection via a conditioned taste aversion assay with no threshold changes observed in males. Collectively, these studies demonstrate that the taste system is plastic and is modulated by diet, circulating hormone levels, and sex to selectively alter food intake.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

5-HT	5-hydroytyrptamine (serotonin)
AgRP	Agouti-related peptide
ATP	Adenosine-5’-triphosphate
BAPTA	1,2-Bis(o-aminophenoxy)ethane-N,N,N’N’-tetraacetic acid
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
CALHM1	Calcium homeostasis modulator 1
CD36	Cluster of differentiation 36
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CTA	Conditioned taste aversion
DAG	Diacylglycerol
DIO	Diet induced obese
DRK	Delayed rectifier potassium
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ENaC	Epithelial sodium channel

ER	Endoplasmic reticulum
EtOH	Ethanol
FA	Fatty acids
GAD67	Glutamic acid decarboxylase 67
GDP	Guanylyl diphosphate
GFP	Green fluorescent protein
Ghrl$^{-/-}$	Ghrelin Knock-out
Ghsr$^{-/-}$	Growth hormone secretagogue knock-out mice
GHSR	Growth hormone secretagogue receptor
GHRP-6	Growth hormone-releasing peptide 6
GLAST	Glutamate-aspartate transporter
GPCR	G protein-coupled receptor
GLP-1	Glucagon-like peptide-1
GTP	Guanosine -5'-triphosphate
HFD	60% High fat diet (1:1 UFA to SFA)
HSFD	60% High saturated fat diet (1:10 UFA to SFA)
HUFD	60% High unsaturated fat diet (3.3:1 UFA to SFA)

FFA	Free fatty acids
IP3	Inositol trisphosphate
IP3R3	Receptor for inositol trisphosphate
KCl	Potassium Chloride
KOH	Potassium hydroxide
LA	Linoleic Acid
LCFA	Long chain fatty acids
LiCl	Lithium Chloride
Mg^{2+}	Magnesium ions
MgCl	Magnesium Chloride
MSG	Monosodium glutamate
N_2	Nitrogen
Na^+	Sodium ions
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NCAM	Neural cell adhesion molecule
NPY	Neuropeptide Y

O_2	Oxygen
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLCβ2	Phospholipase C beta 2
POMC	Proopiomelanocortin
PUFA	Polyunsaturated fatty acid
ROMK	Renal outer medullary potassium
SFA	Saturated fatty acid
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment receptor
SSO	Sulfosuccinimidyl oleate
TPPO	Triphenylphosphine oxide
TRC	Taste receptor cells
TRPM5	Transient receptor potential type melastatin subtype 5
UFA	Unsaturated fatty acid
US	Unconditioned stimulus
WT	Wild-type

CHAPTER 1: INTRODUCTION

In recent decades, obesity has become a major health concern both globally and more specifically within the United States. The U.S. currently leads in obesity incidence with approximately 42% of individuals being categorized as obese with prevalence rising 11% in the last 15 years alone [1]. Additionally, obesity increases incidence of other maladies such as cardiovascular disease, diabetes, stroke, and cancer all of which are among the leading causes of death within the U.S [2, 3]. Obesity is a complex disease with both biological and environmental factors implicated in its onset. A positive energy balance (higher caloric intake than energy expenditure) and an environment that promotes this imbalance are thought to be primary contributors [4]. These dietary changes commonly referred to as the westernized diet is characterized by readily accessible calorically dense foods high in saturated fats, simple sugars, and salt [5]. These dietary alterations paired with environmental changes that leave many living a much more sedentary lifestyle have created a metabolic health crisis. Further research aimed at understanding the drivers of nutrient intake are needed to determine potential therapeutic interventions to improve metabolic status.

The Taste System

The oral cavity plays a vital role in determining caloric intake. Nutrients in the oral cavity are assessed for hedonic value and nutritional content. Evolutionarily, the taste system provided two critical roles in survival, first the detection of toxins or spoiled food through aversive taste

qualities, and secondly nutrient detection through appetitive stimuli. While olfactory cues, temperature, and texture all play a role in overall chemosensory perception this dissertation will focus primarily on taste through taste cell activation.

The major taste organ, the tongue, contains three types of taste sensing papillae: fungiform, circumvallate, and foliate. Large numbers of fungiform papillae populate the anterior region of the tongue. Between one and four taste buds populate the most superficial region of these papillae, with majority of papillae containing just one taste bud. The foliate and circumvallate papillae are situated in the more posterior regions of the tongue with the foliate on the two lateral posterior regions and the circumvallate in the most posterior medial region. Similar in structure, these two papillae contain deep crevices on either side of the papillae which house hundreds of taste buds [6]. In contrast to mice which only have one circumvallate papilla, humans can have up to a dozen of these papillae [7].

Taste information from the tongue is carried primarily through two cranial nerves the chorda tympani branch of the facial nerve which innervations to anterior 2/3 of the tongue, and glossopharyngeal which innervates the posterior region of the tongue. Taste information is transduced onto these nerves which synapse with second order neurons in the nucleus of the solitary tract which carry the information to the gustatory cortex in the insula.

Taste Cell Types

Taste buds embedded in the epithelial layer of the tongue are comprised of 50-100 taste cells tightly bundled together to form the taste bud. These polarized taste cells arise from

epithelial precursors though they contain many neuron-like characteristics such as voltage-gated channels, neurotransmitter release, and form synapses [9]. Nutrients and saliva within the oral cavity access the taste bud via taste pores on the surface of the tongue. Tastants bind to receptors on apical regions of taste cells near the taste pore. Taste buds contain different taste cell types that communicate with one another and synapse with afferent nerve fibers located on the basolateral portion of the taste bud to inform of nutrients within the oral cavity. Taste cells are currently divided into primarily three categories: Type I, II, and III [10]. These subtypes are essential for proper functioning of the taste bud.

Type I: Supporting Cells

Type I cells are glial-like support cells. They have a half-life of eight days and are the most prevalent of the three taste cell types with approximately 50% of the taste cells within a bud being Type I [11, 12]. These cells extend appendages to neighboring cells within the taste bud to control uptake of signaling molecules and to induce/inhibit the activation of a cell. Taste buds are compact structures with cells in close proximity, Type I cells aid in regulation of the microenvironment of the taste bud through the uptake of neurotransmitters and ions. Glutamate, secreted from nerve fibers near the basolateral regions of taste buds is taken up by Type I cells through the glutamate-aspartate transporter (GLAST) [13, 14]. Additionally, Type I cells hydrolyze ATP secreted primarily from Type II cells through NTPDase [12, 13]. During cellular excitation potassium leaves the cell to allow repolarization, Type I cells aid in regulation of extracellular potassium concentrations through the uptake of potassium via renal outer medullary potassium (ROMK) channels [15]. The supporting role of Type I cells maintain the extracellular

space of the taste bud allowing appropriate taste cell activation of Type II and III cells. Research suggests that Type I cells may respond to salt taste [16].

Type II: Receptor Cells

Type II cells (receptor cells) are the most studied of the cell types due to their role in detecting several tastants. Similar to Type I taste cells they have a half-life of approximately eight days [11]. They are less prevalent then Type I cells, making up approximately 30% of the cells in a taste bud. Type II cells are recognized as the main taste receptor cells as they have been shown to respond to bitter, sweet, umami, and fat tastants. They contain G-protein coupled receptors (GPCR's) and downstream elements necessary for signal transduction [17, 18]. Additionally, Type II taste cells contain voltage-gated sodium and potassium channels necessary for cellular depolarization leading to action potential formation. ATP released from Type II taste cell activation is key for proper transduction of taste information through receptors on both Type III taste cells and afferent nerve fibers [19]. Currently Type II taste cell responses are thought to be restricted to GPCR-mediated tastants and therefore do not respond to salt and sour compounds.

Type III: Presynaptic Cells

Type III cells are the presynaptic cells, they have a much higher longevity than Type I or Type II cells, with a half-life of 22 days and are the least prevalent of the three cell types discussed with 2-20% of taste cells being Type III [11, 12]. These cells form synapses with afferent nerve fibers and contain SNARE proteins (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) involved in vesicular release of neurotransmitters into the synaptic cleft [10, 20]. Additionally, Type III cells express purinergic receptor P2Y which

activates upon binding of ATP released from Type II cells. Binding of ATP activates Type III cells leading to the release of 5-HT [10]. Type III cells have been shown to respond to sweet, bitter, and umami via paracrine activation dependent upon Type II cells [21]. Lastly Type III cells are thought to respond to sour tastants though the mechanism remains unclear at this time [10, 12].

The distinct roles these three cell types play within the taste bud are critical to creating the proper extracellular environment for taste responses. While it is unclear whether Type I cells detect any tastants they play a vital role in maintaining the extracellular environment for Type II and Type III cells to respond to tastants. In addition, activation of Type II cells to release ATP is necessary to stimulate both Type III and afferent nerve fibers for the detection of bitter, sweet, and umami tastants. Lastly activation of Type III cells leads to release of serotonin a secondary mechanisms of nerve fiber activation. While much remains to be understood in taste system transduction it is clear the differing roles of these cell types are necessary for proper cell maintenance and nutrient detection.

The Five Basic Tastants

Evolutionarily, the ability to detect different molecules has been important for animal survival and fulfills one of two main roles. Aversive tastants detect the presence of poisons, toxins, or indicators of food spoilage and are routinely avoided. On the other hand, the appetitive tastes reflect the body's ability to detect essential nutrients to provide adequate energy and maintain normal physiological processes [22]. It has become clear in recent years that the body is

able to detect nutritional deficiencies, altering chemosensitivity and ultimately regulating the types and amounts of specific foods that are consumed. To date, there are five basic tastants that fulfill these two disparate roles; bitter and sour represent aversive aspects of taste while, sweet, salty, and umami are generally considered appetitive tastes. Additionally, fat as a sixth basic tastant has gained traction and will be discussed as well.

Bitter, Sweet, and Umami

Bitter, sweet, and umami tastants have similar transduction pathways despite the divergent role of bitter from the other tastants. Bitter substances are aversive to warn of potential toxins, while sweet and umami are appetitive stimuli and signal carbohydrates and amino acids, respectively. All three bind to G protein-coupled receptor's (GPCR) located on the plasma membrane of Type II taste cells. Once bound a signaling cascade is activated where the βγ subunit dissociates from the GPCR. This subunit activates PLCβ2 which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). IP_3 then binds to its receptor IP_3R_3 on the endoplasmic reticulum (ER). IP_3R_3 is a ligand-gated calcium channel, its activation leads to the release of calcium from intracellular stores. The rise in intracellular calcium then activates a calcium-dependent sodium channel on the plasma membrane. The influx of sodium leads to cellular depolarization and release of ATP [23]. Previously PanX1 was thought to be required for ATP release [17] however, recent data suggests a potential role of calcium homeostasis modulator 1 (CALHM1) in ATP release of Type II taste cells [24]. While the channel has yet to be conclusively identified ATP is necessary for proper taste transduction [19] and activates purinergic receptors on both Type III taste cells and afferent nerve fibers.

Salt and Sour

While both salt and sour taste use independent pathways, they are both thought to directly activate ion channels. Salty tastants are appetitive at low concentrations but aversive at higher concentrations. For years researchers have known about an amiloride-sensitive pathway selective for sodium detection [25] and have long thought ENaC may be responsible. Knock-out of epithelial sodium channel (ENaC) in mice shows an abolishment of appetitive taste qualities of sodium while the aversion to high concentrations remains intact [26]. These data suggest ENaC may be responsible for lower concentrations of sodium taste transduction. However, much is still unknown about salt taste including the cell types it activates. Additionally an amiloride-insensitive pathway for salt taste exists yet remains to be elucidated. Similar to salt taste much is still unknown about sour taste transduction. Research suggests sour taste occurs in Type III taste cells [21] where sour tastants acidify the cellular environment to induce activation through a proton sensitive ion channel [10]. Little is known about the mechanism of sour taste, and research in this area is ongoing.

Fat as the Sixth Tastant

Fatty acids play an essential role in an array of physiological pathways that are critical to maintaining homeostasis in an organism, indicating a need to detect and tightly regulate fatty acid intake. Approximately 95-99% of dietary fat is consumed in the form of triglycerides (three fatty acids attached to a glycerol backbone). Even though concentrations of FFAs in food are enough to stimulate taste receptor cell's (TRCs), lingual lipase in the saliva cleaves fatty acids from the glycerol backbone providing additional FFAs. Previously, it was thought that the only

salient cues from fatty acids were through its textural properties (oiliness or slipperiness). However, in the late 1990's, Gilbertson and colleagues found that fatty acids activate isolated rat taste cells through delayed rectifier potassium channels (DRKs), and that these effects were dependent upon fatty acid type. Polyunsaturated fatty acids (PUFAs) blocked DRK channels while monounsaturated and saturated fatty acids had no significant effect [27]. In addition to these finding, Takeda and colleagues found that mice prefer corn oil to xanthan gum. To prevent indirect cues such as olfaction and texture, mice were anosmic through administration of $ZnSO_4$, and xanthan gum was used to mimic the textural cues of fat [28]. Since these initial studies, researchers have been working to elucidate the fatty acid taste transduction pathway.

The proposed fatty acid taste transduction pathway is shown in Figure 1. Research suggests taste cell activation elicited by fatty acids is through primarily GPR120 and fatty acid translocase (CD36), a commonly known transporter of fatty acids throughout the body [29]. CD36 KO mice show a reduced preference for fatty acids, however KO of ATP signaling mechanisms (shown to be critical for proper taste transduction) shows even greater reduction in lipid preference [30]. These data suggest that mice still detect fatty acids in the absence of CD36 indicating the presence of additional fatty acid receptors. CD36 may play a role in facilitating binding of fatty acids cleaved by lingual lipase, to GPR120 in the oral cavity.

Cluster of differentiation 36 (CD36)

Cluster of differentiation 36 (CD36) is broadly expressed throughout a variety of cell types including macrophages, hepatocytes, and adipocytes. While it binds to multiple ligands it has a high binding affinity for polyunsaturated fatty acids (PUFAs) and plays an important role in fatty acid metabolism including detection, absorption, and utilization [31]. CD36 is selectively

expressed in the taste system with no expression in the surrounding epithelium. Its expression is highly reserved to the apical regions of the taste bud near the taste pore [32]. *Cd36* expression is dynamic in rodents with decreases during dark phases when food intake is increased and rises during light phases during low food intake. These levels also peak during fasting and immediately lower following feeding for a slow rise back to pre-prandial concentrations. Lastly mice lacking CD36 show a reduced preference for linoleic acid (LA) compared to wild-type (WT) counterparts [33]. These data implicate CD36 in fat taste transduction and its fluctuation in expression based on metabolic status suggest it may be involved in adapting fatty acid taste responses based on physiological conditions. While inhibition of CD36 in isolated taste cells show a significant reduction in calcium responses to LA it does not abolish the calcium signal [29]. CD36 may be important in fat taste transduction but it is clear that it is not the only receptor whereby taste cells respond to fatty acids.

G Protein-Coupled Receptors (GPCR)

GPCRs contain seven transmembrane folds with the amino terminus in the extracellular environment and the carboxyl terminus in the intracellular environment. In general, G-proteins are activated when a ligand binds to the receptor domain on the extracellular surface of the cell. Binding of the ligand allows phosphorylation of guanylyl diphosphate (GDP) bound to the α subunit of the GPCR. This phosphorylation causes mobilization of the α subunit, which dissociates from β and ϒ subunits, allowing activation of downstream signaling pathways. Different fatty acids bind to various GPCRs. This project will focus mainly on one of the essential fatty acids, that the human body requires but cannot produce, LA. Both GPR40 and GPR120 have been shown to bind to long chain fatty acids [34].

GPR40

GPR40 binds both medium and long-chain fatty acids with no affinity for short chain fatty acids. While there is a low-level widespread expression of GPR40 throughout the body, the highest concentrations are in the pancreas and brain [35]. Due to its high expression in the pancreas and more specifically in β-cells it is involved in glucose regulation and insulin secretion [36]. *Gpr40* expression in the taste system has yet to be clearly established. Studies have shown expression of GPR40 receptors in the circumvallate, foliate, and to a lesser extent in the fungiform papillae in rodents while others have shown no expression [34, 37]. Additionally, GPR40 protein expression does not appear to be present in human fungiform or circumvallate taste papillae [38]. During a two-bottle preference test *Gpr40* knock out (KO) mice showed a reduced preference for linoleic and oleic acid when compared to their wild type (WT) counterparts. Furthermore, glossopharyngeal nerve recording in these KO mice during fatty acid stimulation showed decreased responses [34]. Additional studies are needed to fully understand if GPR40 plays a functional role in fatty acid taste transduction within the taste system.

GPR120

GPR120 binds to long chain unsaturated fatty acids throughout the body. It is highly expressed in the large intestine, lungs, mature adipocytes, and macrophages [39, 40]. It is involved in the regulation of adipogenesis, appetite, and food preference. GPR120 is expressed in the taste system, more specifically in the fungiform and circumvallate papillae with little to no expression in the surrounding epithelium. Immunostaining revealed a higher expression of GPR120 in the apical end of papillae, similar to that of CD36 [37]. In another study, researchers stained the fungiform and circumvallate papillae with α-gustducin, 1-phosphatidylinositol-4, 5-

bisphosphate phosphodiesterase beta (PLCβ2), neural cell adhesion molecule (NCAM), and GPR 120. α-gustducin and PLCβ2 are indicative of Type II taste cells, while NCAM is indicative of Type III. GPR120 co-localized with α-gustducin and PLCβ2, but rarely with NCAM suggesting it is largely reserved to Type II taste cells [41]. GPR120 knockout mice exhibit a reduced preference for LA compared to WT counterparts and nerve recording in these mice revealed diminished responses to fatty acids in both the glossopharyngeal and chorda tympani nerves [34]. In a 2011 study, researchers observed changes in GPR120 expression throughout a 24-hour period. When comparing animals in a fasting state to those on a 30% fat diet no significant fluctuations in *Gpr120* were observed [33].

Delayed rectifier potassium (DRK) Channels:

Delayed rectifier potassium (DRK) channels are an important site of regulation for fatty acids in the taste system. DRK channels are voltage-gated potassium channels that are activated by changes in membrane potential. After cellular activation potassium slowly returns the membrane potential back to resting levels by leaving the cell through DRK channels. Fatty acids, in particular long chain polyunsaturated fatty acids, have been shown to block DRK channel function thereby prolonging depolarization of the cell [27]. Research showed that obesity-resistant (OR) rats had a higher blockage of DRK channels than obesity-prone (OP) rats. OR rats also show reduced preferences for fatty acids compared to OP rats suggesting a possible inverse correlation between fat preference and fat taste sensitivity [42]. Liu, et al., (2005) extensively characterized the DRK channels present in the rat taste system. The three major subtypes of DRK channels present in the taste system are KCNA, KCNB, and KCNC. KCNA5 (Kv1.5) is broadly expressed in rat taste cells and Kv2.2, Kv3.2, Kv3.1, and Kv1.3 also show high

expression levels. Researchers also showed expression of Kv1.5 was not limited to the apical portion of taste cells (Liu, 2005). Smithers and colleagues examined the binding site of K^+ channels for fatty acids. Using a fluorescent tag, they were able to observe the interaction of fatty acids with the central cavity of the K^+ channel (KcsA). The binding affinity of fatty acids to the K^+ channel increased in direct correlation with the fatty acid chain length up to 20 carbons (Smithers, et al., 2012). These studies provide evidence of the interaction of fatty acids with K^+ channels and their presence in the taste system. Additionally, altered expression of potassium channels is correlated with fat taste preference levels, suggesting DRKs may play a role in cellular plasticity and overall responses to LA.

Plasticity of fatty acid sensing by diet

The recognition that fatty acids activate taste cells, in coordination with the increasing health concerns of rising obesity incidence has led to significant interest in fat taste plasticity. Fat is the most energy dense macronutrient and increased fat consumption is thought to be a contributing factor in the rising obesity incidence in the last half century. Research has shown either a negative correlation between BMI and fat taste sensitivity in humans or no correlation at all [43-47]. Additionally, studies suggest that recent fat intake alters fat taste sensitivity [47]. A study in lean and obesity participants found that after four weeks of a low-fat diet both groups had higher fat taste sensitivity, in contrast when both groups were placed on a high fat diet only lean participants exhibited decreased fat taste sensitivity [48]. These data suggest that increased fat consumption decreases fat taste sensitivity thereby driving increased consumption of dietary fats.

Additional studies in rodent models have sought to better elucidate the role of diet and metabolic status on fat taste sensitivity. Several studies have examined difference between subgroups of rats with obesity-prone (Osborne-Mendel; OP) and obesity-resistant (S5B/Pl; OR) phenotypes. On a standard chow diet OP rats are approximately 50% heavier than OR rats and twice as heavy on a 20 week 60% high fat diet [49]. When given a three-choice preference test with protein, fat, and carbohydrates OP rats prefer fat while OR rats prefer carbohydrates [50]. Additionally, function studies of these rats revealed that delayed rectifier potassium channels (DRK's) were differentially blocked among the two strains. Though binding affinity of the channels were the same, OP rats showed substantially less block of DRK channels by LA suggesting the composition of channels making up the total DRK current was altered in the OP rats with a lower concentration of fatty acid sensitive DRK channels expressed in OP rats. As mentioned above (*DRK channels section*) DRK channels repolarize the cell following excitation. Fatty acid block of these channels leads to prolonged depolarization [42, 51]. Based on human studies as fat taste sensitivity increases preference for fat decreases. These data would suggest then that OP rats are less sensitive to fat than their resistant counterparts. Interestingly using a conditioned taste aversion assay OP rats showed a greater sensitivity to LA than OR rats [49].

When placed on a 5-week 60% high fat diet feeding study OP rats show no change in fat sensitivity while OR rats significantly increase their sensitivity to fatty acids [52]. Additionally, data shows mice on a high fat diet exhibit a reduced preference for fatty acids and this preference can then be returned to normal levels following caloric restriction. Further, *Cd36* expression in diet-induced obese (DIO) rodent models do not show post-prandial decreases in *Cd36* following mealtime [53]. These data taken together suggest the taste system loses plasticity in both human

and rodent models of obesity and these changes may be via CD36 or other mechanistic changes. Lastly single nucleotide polymorphisms in *Cd36* alter fat taste sensitivity and may contribute to the large distribution of fat taste sensitivity seen in individuals [54, 55].

Plasticity of fatty acid sensing by hormones

In addition to modulation of fat sensing within the taste system, it is also susceptible to modulation through outside forces such as circulating hunger/satiety hormones that contribute to the regulation of metabolic homeostasis. In addition, many of these hormones become dysregulated in an obesogenic state raising the question if diet-induced changes within the taste system are a result of altered hormone secretion or changes in the fatty acid taste transduction pathway.

Significant focus has been on understanding the action of hunger and satiety hormones on central neuronal circuitry to drive or inhibit further caloric intake. However, many of these hormones have been shown to be present in the taste system and play a direct role in modulating responses to various tastants. Studies have shown suppression of sweet taste by leptin and divergent actions of glucagon-like peptide-1 (GLP-1) on sweet and umami tastants [56, 57].

Ghrelin is an orexigenic hormone secreted primarily by X/A cells of the stomach. It activates Agouti-related peptide (AgRP) and neuropeptide Y (NPY) neurons and inhibits proopiomelanocortin (POMC) neurons within the hypothalamus to stimulate orexigenic effects [58]. Ghrelin levels fluctuate rapidly throughout the day with elevated levels prior to mealtime and lower levels post consumption [59]. It is synthesized in the taste system and its receptor

growth hormone secretagogue receptor (GHSR) is present in the taste system [60]. The rapid rise and fall of ghrelin plasma levels associated with mealtime paired with its orexigenic effects and presence in the taste system suggest a potential action on taste cells to further promote caloric intake. Previous data showed expression of both ghrelin and GHSR are broadly expressed in taste cells [60, 61]. However, there is still an incomplete picture on the role of ghrelin and GHSR in modulating taste responses. GHSR knockout (*Ghsr*$^{-/-}$) mice show reduced sensitivity to salt and sour tastants but no change in sweet or bitter tastants [60]. Ghrelin knockout (*Ghrl*$^{-/-}$) mice showed no change in responsiveness to sweet, sour, or bitter but showed a delayed aversion to high concentrations of salt and a significantly reduced response to fatty acids [61]. These data suggest that ghrelin may be playing a role in taste modulation. The high caloric density of fat paired with reduced preferences for fatty acids in *Ghsr*$^{-/-}$ mice merit further investigation of the role of ghrelin in fat signaling in the peripheral taste system.

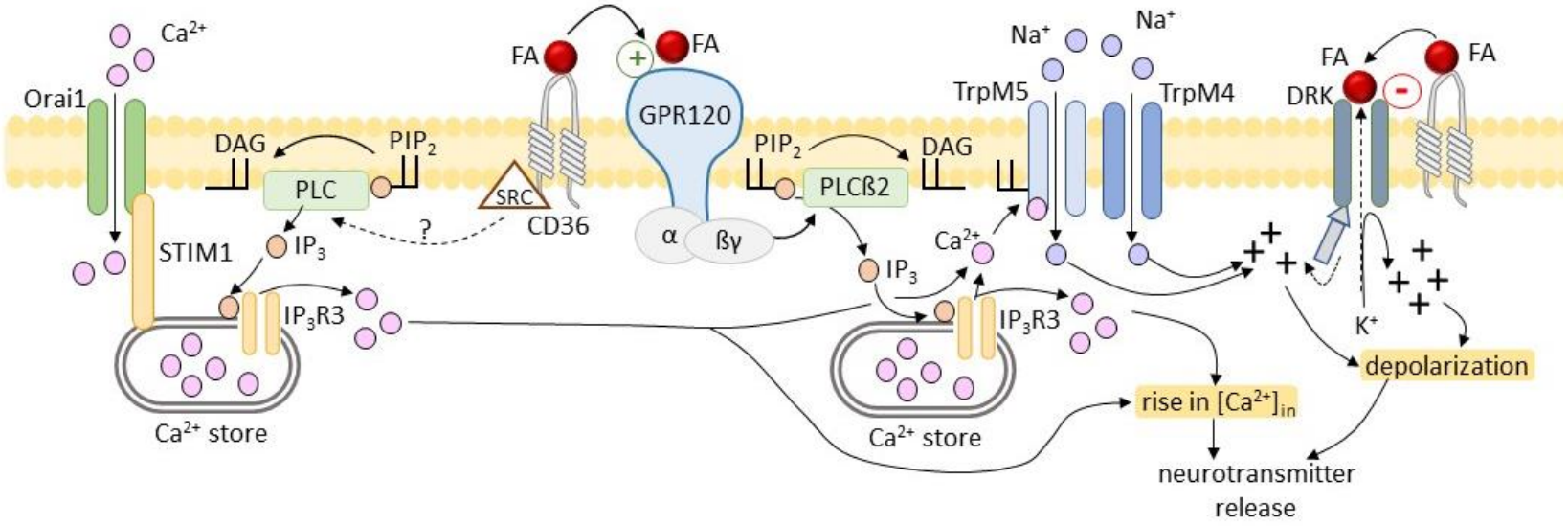

Figure 1: Proposed fatty acid taste transduction pathway.

Similar to other G-protein mediated tastants fatty acids utilize a G-protein signaling cascade involving PLCβ2 and TrpM5. Fatty acids are also able to block DRK channels thought to increase cellular depolarization.

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CHAPTER 2: DIETARY MODULATION OF PERIPHERAL TASTE SIGNALING

Abstract:

As obesity rates have continued to rise over the past 50 years, recent data suggests the adaptation of a "Western diet", a diet high in fats among other things, may lead to metabolic imbalance. The initial recognition of nutrients occurs in the oral cavity where sapid molecules are detected by the peripheral taste system which integrates this information prior to sending it to various central nuclei on its way to the gustatory cortex. While questions remain concerning how metabolic status and diet selection affect these chemosensory signals, emerging evidence suggests the taste system is altered by food selection, nutritional status, and disease. The goal of this study was to elucidate the effects of high fat diet feeding on the detection of free fatty acids (the prototypical stimuli for the taste of fat) in the taste system. Using high fat diets with differing fatty acid saturation levels we observed limited changes in the polyunsaturated fatty acid taste transduction pathway. Following 8 weeks of high fat diet feeding, our data showed increased food intake and weight gain across all high fat diet groups compared to control diet mice. Additionally, high fat diet mice had significant increases in adipose stores while showing no changes in liver weights. These metabolic disturbances were accompanied by increased expression of *Cd36* and decreased expression of delayed rectifier potassium channels in high fat diets. Furthermore, high fat diets showed increased inward currents elicited by linoleic acid (LA) that appeared to depend on fatty acid saturation levels. Further examination revealed these currents did not occur in PLCβ2 expressing cells and did not appear to be TRPM5-dependent. Pharmacological inhibition of CD36 also yielded increased remaining current and no reduction

in depolarizations compared to control counterparts. Overall, our data suggest high dietary fat intake and its metabolic sequalae may elicit limited functional changes to taste cells within the canonical peripheral fat taste transduction pathway. Diet induced changes, however, may occur via pathways and targets not directly examined in the present study.

Introduction:

Obesity has become increasingly prevalent within the past 50 years, and is linked to several comorbidities including diabetes, cancer, and heart disease (World Health Organization). While many factors contribute to the onset of obesity, a positive energy balance is believed to be the primary cause. Much of this energy imbalance is attributed to changes in dietary habits including increased consumption of calorically dense foods, high in simple sugars and fats. Despite extensive examination of nutrient intake and metabolic regulation, obesity remains a major health concern. Further research to elucidate mechanisms underlying food intake and metabolism are essential to finding successful therapeutic interventions.

The taste system is the earliest detector of nutrients in the body. It is where the body receives initial information about nutrient composition, allowing the determination of ingestion (appetitive stimuli) or avoidance (aversive stimuli). Understanding recognition of macronutrients within the oral cavity and how dietary changes modulate these pathways is critical to our understanding of how nutrient sensing drives consumption.

Previously, fatty acid taste perception was thought to occur largely through textural and olfactory cues. However, studies in recent decades have established rodents maintain preferences

for fatty acids despite the removal of textural and olfactory cues [1]. Additionally, isolated taste cells are activated by fatty acids to release calcium and elicit cellular depolarization [2]. Free fatty acids are present in many foods at effective concentrations and may be generated from triglycerides in the oral cavity by lingual lipase activity within the saliva. Long chain polyunsaturated free fatty acids have been shown to bind to multiple targets within the taste system including CD36, GPR120 and delayed rectifier potassium channels [2, 3]. Through CD36 and GPR120, fatty acids elicit a signaling cascade similar to that of other G-protein mediated tastants, involving release of calcium from intracellular stores and activation of TRPM5 channels, ultimately resulting in cellular depolarization [2]. The rise in calcium and cellular depolarization have been shown to induce ATP release in Type II taste cells [4]. These data have led to the current working model of the fat taste transduction system (see Figure 1).

As fat taste has become more established in the field, questions regarding its role in obesity has been an area of particular interest. While several studies have sought to better understand modulation of the taste system by diet and metabolic status, research has yet to clearly establish where these changes are occurring. Human studies correlating weight status and fat taste sensitivity remain unclear as some studies show an inverse relationship between weight status and fat taste sensitivity while others show no such correlations [5-9]. Dietary fat intake alters fat taste sensitivity, fat taste thresholds can be increased following a 4-week low fat diet in both obese and lean participants, but a high fat diet only decreased sensitivity in the lean participants [5-7, 10]. Additionally, many human studies find correlations between fat taste sensitivity and single nucleotide polymorphisms to fat taste receptors, particularly CD36 (see review [11]). Studies in rodents have shown that obesity prone rats have lower fat sensitivity

compared to their obesity resistant counterparts. Delayed rectifier potassium channels are a key site for fatty acid modulation of the fat taste transduction pathway. Obesity resistant rats also have an increased ratio of fatty acid sensitive DRK to insensitive DRK channels and show a greater block in these channels thereby prolonging cellular activation by LA. These data suggest the taste system adapts based on dietary experience, but how these changes occur remains largely unknown.

The relationship between weight status, food intake, and fat taste sensitivity is complex and multidimensional. As such most research to date examine fat taste threshold changes in behavioral models of mice or human studies with limited abilities to look at the direct impact of diet-induced obesity (DIO) and high dietary fat consumption on taste cell signaling. While behavioral changes are important to understanding the overall effects, observing changes within the taste system allow for a more targeted approach of determining where these changes may occur whether in the gustatory system or in upstream effectors.

This study examines both functional cellular changes and their molecular underpinnings in taste cell changes isolated from mice on 8 weeks of control diet or one of three 60% high fat diets with differing ratios of unsaturated to saturated fatty acids (see table 1). We observed weight gain and increase food intake that resulted in increased adipose deposits in mice on all three high fat diets. While mice on high fat diets had similar weight gain, food intake among the three diets increased as unsaturated fatty acid levels in diet increased. Further examination of adipose deposits revealed mice on a high unsaturated fat diet (HUFD) and a standard high fat diet (HFD) had significant increases in visceral fat while high saturated fat diet (HSFD) fed mice did not. Additionally, we show increased gene expression of *Cd36* in HSFD and HUFD fed mice

and decreased expression of delayed rectifier potassium channels Kv2.2 and Kv3.2 across all high fat diets. Taste cells isolated from mice on a HUFD and HFD elicited greater inward current densities in response to LA. Unexpectedly, we found that these increased current densities were not observed in PLCβ2–expressing cells. Additionally, inhibition of TRPM5 in HUFD taste cells did not present a significant current reduction as observed in control mice. Lastly, CD36 inhibition in control taste cells elicited a significant reduction in depolarizations while having little to no effect on HUFD isolated taste cells. Taken together these data suggest fatty acid taste cell signaling is altered in high fat diets via cell types other than Type II taste cells.

Materials and Methods:

Animals

7–9-week-old Male C57BL/6J were obtained from Jackson laboratories and creation of the PLCβ2-EGFP mouse line has been described previously [12] and was generously provided by Dr. Nirupa Chaudhari (University of Miami). All mice strains were housed according to IACUC protocols and procedures at the University of Central Florida.

Feeding Studies

Mice were group housed and allowed to acclimate for 1 week prior to the start of feeding studies. Automatic watering systems were removed, and mice were given water bottles for water intake measurements. Mice were placed on an 8-week diet of one of 3 60% high fat diets or control diet. Water, food intake, and weights were measured weekly.

Diets

Diets were obtained from research diets and stored at proper temperatures (-20°C for all high fat diets and -4°C for the control diet). All three high fat diets consisted of 60% fat, 20% protein, and 20% carbohydrates with an energy density of 5.24 kcal/gram. The high unsaturated diet (HUFD, D06062303) contains 3.3:1 unsaturated fatty acids to saturated fatty acids with the majority of the fat coming from lard and safflower oil. The high fat diet (HFD, D12492) contains a 1:1 ratio of unsaturated to saturated fatty acids with most fat coming from lard and soybean oil. The third high fat diet, a high saturated fat diet (HSFD, D06062302) contains 1:10 unsaturated to saturated fatty acids with its primary fat sources being coconut oil and lard. The control diet (D07020902) is a 10% fat diet containing equal parts saturated to unsaturated fatty acids with 3.85 kcal/g energy density. For full diet composition refer to Table 1.

Body Composition

Measurements were taken prior to the start of the feeding study and immediately following the 8-weeks of diet. Body composition measurements were determined using the Bruker minispec series. Following calibrations mice are placed in the bottom of the NMR tube, a smaller tube was then placed inside to minimize movement of the animal. Measurement readings were taken for each individual mouse and weights were also recorded. Cage differences were calculated between subtracting average initial measurements from average final measurements for each cage.

Adipose stores

Following 8-weeks of diet mice were euthanized using CO_2 followed by secondary cardiac puncture. Animals were then dissected, and fat pads were isolated. Subcutaneous adipose tissue included fat stores outside of the central cavity between the connective tissue and the skin. Visceral fat included the mesenteric, perirenal and retroperitoneal fat stores. Gonadal fat pads consisted of only the fat surrounding the gonads in the lower abdominal region. Liver weights were also collected.

Solutions

Saline solution (Tyrode's): 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 10 mM Na pyruvate; adjusted to a pH of 7.40 with NaOH; and maintained an osmolarity of 305-315 mOsm. Stock solutions of LA (Sigma-L1012) 25 mg/mL were made in 100% ethanol and stored under nitrogen (N_2) at -20°C. All LA solutions were made fresh each day and the stock were not used longer than 3 months. Fura-2AM was dissolved in DMSO to a concentration of 1 mM and stored at -20°C prior to use. For imaging experiments stock of Fura-2AM was then dissolved to a concentration of 4 μM in 0.05% pluronic acid (dissolved in Tyrodes). Intracellular low chloride solution contained 140 mM K-Gluconate, 1 mM CaCl2, 2 mM $MgCl_2$, 10 mM HEPES, 11 mM EGTA and stock solutions were made and frozen at -20°C. Stock solution was thawed and 1.2 mM ATP and 0.45 mM GTP were added the day of use, pH was adjusted to 7.20 using KOH and maintained an osmolarity of 290-310 mosM. Sulfosuccinimidyl oleate (SSO) was reconstituted in DMSO to a stock concentration of 25 mg/mL and stored at -20°C under N_2 prior to use. SSO was diluted the day of for each experiment to a concentration of 100 μM in Tyrode's. Triphenylphosphine oxide (TPPO) was

diluted in DMSO to a concentration of 25 mg/ml and stored at -20°C for no longer than 5 days prior to use. Stock solutions were diluted the day of experiment to a TPPO concentration of 100 μM in Tyrode's.

Taste Cell Isolation

For a more thorough description of taste cell isolation see previously published work [3]. Briefly, following euthanasia, the tongue was excised and placed in a Tyrode's solution. 0.25 to 0.3 mL of an enzyme cocktail containing dispase II (2.0mg/mL), collagenase (0.5 mg/mL), and trypsin inhibitor (1 mg/mL) in Tyrodes was injected between the muscle and epithelial layers throughout the tongue. The injected tongue was bubbled in O_2 for approximately 40 minutes. Next, the lingual epithelium was peeled from the underlying muscle layer with forceps, pinned out in a Sylgard™-lined petri dish. The epithelium was then incubated in Ca^{2+} Mg^{2+} free Tyrodes for 5 minutes, washed with standard Tyrodes, and then incubated from an additional 2 minutes in the enzyme cocktail described above at room temperature. Taste cells/taste buds were removed by gentle suction using a glass fire polished pipette under a dissection microscope. Taste cells were then gently expelled onto coverslips containing Corning® Cell-Tak™ Cell and Tissue Adhesive (Corning, PN 354240) for live cell assays.

RNA isolation

Following the feeding studies, RNA was collected from all groups on the three days following the end of the feeding study. RNA was pooled from 4-5 mice for each collection. Mice were sacrificed via CO_2 followed by cervical dislocation. Following euthanasia, the tongue was removed and injected with the enzyme cocktail described above. Tongues were bubbled in O_2 while on ice for approximately 30 minutes. Following incubation, the epithelium was peeled

away from the muscle, and tissue sections were taken from the fungiform and circumvallate papillae. Once sections were cut from the epithelium, they were immediately placed in RNAzol and vortexed vigorously. Following collection, the RNAzol protocol, followed by the Zymo clean and concentrator kits (-25) were followed. These included an in-column DNase treatment. Upon completion of RNA isolation samples were aliquoted and frozen at -80°C. RNA integrity was verified using an Agilent Bioanalyzer chip and analysis kit (ExperionTM RNA HighSens Analysis Kit).

Gene Expression

RNA samples were converted to cDNA using reverse transcriptase. Following which they were used for Taqman gene expression assays. *Gapdh* was used as the internal control, as a Taqman with primer limiting properties. The following TaqMan were obtained from Fisher scientific: *Gpr120* (Mm00725193_m1), *Gpr84* (Mm02620530_s1), *Trpm5* (Mm01129032_m1), *Cd36* (Mm00432403_m1), *Kcnb2* (Mm03057813_m1), *Kcnc1* (Mm00657708_m1), and *Kcnc2* (Mm01234233_m1). Each sample was run in triplicate and averaged. The average of triplicates was then used for the different tissue samples from mice in the same treatment. Relative gene expression was calculated, and samples were averaged together from the same treatment. All samples were run on the Quant Studio 5. Data was analyzed using the ΔΔCT method, where the fold change between the gene of interest (GOI) and the calibrator (CAL) as shown in the equations below (adapted from [13].

$$\Delta C_T^1 = C_T^{GOI} - C_T^{GAPDH}$$

$$\Delta C_T^2 = C_T^{Cal} - C_T^{GAPDH}$$

$$\Delta\Delta C_T = C_T^2 - C_T^1$$

$$Relative\ Expression = \frac{1}{(2^{-\Delta\Delta CT})}$$

Calcium Imaging

Isolated taste cells adhered to Corning® Cell-Tak™ coated coverslips for approximately 20 minutes. Following adherence, they were then incubated in a Pluronic acid/Fura-2AM mixture for 45 minutes to 1 hour prior to imaging. Cells ready for imaging were placed in a perfusion chamber (Warner Instruments, RC-25F). During imaging, the cells were perfused continuously at a rate of 4 mL/minute. Imaging was performed using an Olympus CKX53 microscope with a Basler acA720 camera and Incyt Im2 software was used to capture calcium change data. Cells were excited at 340 and 380 nm and recorded at 510 nm and the 340/380 ratio was converted to $[Ca^{2+}]i$ based on the calcium calibration buffer kit (Invitrogen). LA concentrations were introduced in a random order following by a 0.1% BSA solution and Tyrodes to remove fatty acids from the bath and until the calcium signals returned to baseline. The criteria used for calcium responses were determined by amplitudes greater than twenty standard deviations above baseline for each cell and the reversibility of response. Area under the curve and amplitude were calculated for each cell.

Patch Clamp Recording

Following 8-weeks of HUFD or CD mice were euthanized, and taste cells were isolated as stated above for electrophysiology assays. Taste cells adhered to the Corning® Cell-Tak™ coated coverslips at room temperature for twenty minutes prior to use. Low chloride intracellular

solution was kept on ice prior to use to avoid hydrolysis of ATP and GTP. Gentle suction was applied to cells sealed onto the pipette at ≥ 1 gΩ to break the membrane into whole cell patch recording. Patched taste cells were only used with a leak less than 200 pA throughout testing. Depolarizations from taste cells with a resting membrane potential of -35 or lower were used for analysis. The step protocol consists of a pre and post holding potential of -100mV with 15 steps starting at -100 mV and increasing 10mV/step until 40 mV. Total inward current was measured by holding the cell at -100 mV and using a Picospritzer III (Parker Hannifin Corp.) to apply focal application of LA onto the cells for five seconds. Depolarizations consisted of holding the current at 0 pA and using the Picospritzer III to apply focal application of LA (5 seconds) onto the cells. Delayed rectifier potassium channel (DRK) block was determined by running steps prior to bath perfusion of 30 µM LA and again following 8 to 10 minutes of LA perfusion. The 40-mV step was then baselined and compared between time 0 and 8 minutes. Percent current remaining is the average current at 8 minutes divided by the average current at 0 minutes multiplied by 100. Isolated taste cells were incubated in an irreversible CD36 blocker (SSO) or concentration matched DMSO for controls 20 minutes prior to patching studies.

Table 1 Dietary composition from Research Diets

Diet Composition								
Diet Name	**Control Diet**		**HFD**		**HSFD**		**HUFD**	
Diet Number	**D07020902**		**D12492**		**D06062302**		**D06062303**	
Ratio of UFA: SFA	**1:1 UFA to SFA**		**1:1 UFA to SFA**		**1:10 UFA to SFA**		**3.3:1 UFA to SFA**	
Macronutrient Composition	% gm	% kcal	% gm	% kcal	% gm	% kcal	% gm	% kcal
Protein	19.2	20	26.2	20	26.2	20	26.2	20
Carbohydrate	67.3	70	26.3	20	26.3	20	26.3	20
Fat	4.3	10	34.9	60	34.9	60	34.9	60
Total		100		100		100		100
kcal/gm	**3.85**		**5.24**		**5.24**		**5.24**	
Ingredients	gm	kcal	gm	kcal	gm	kcal	gm	kcal
Casein, 80 Mesh	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	3	12
Corn Starch	500	2000	0	0	0	0	0	0
Maltodextrin 10	100	400	125	500	125	500	125	500
Sucrose	100	400	68.8	275	68.8	275	68.8	275
Cellulose BW200	50	0	50	0	50	0	50	0
Soybean Oil	10	90	25	225	10	90	10	90
Lard	5	45	245	2205	25	225	130	1170
Coconut Oil (hydrogenated)	0	0	0	0	235	2115	0	0
Safflower Oil	0	0	0	0	0	0	130	1170
Cocoa Butter	30	270	0	0	0	0	0	0
Mineral Mix S 10026	10	0	10	0	10	0	10	0
Dicalcium Phosphate	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H20	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0
FD&C Yellow Dye #5	0	0	0	0	0.025	0	0	0
FD&C Red Dye #40	0	0	0	0	0	0	0.025	0
FD&C Blue Dye #1	0	0	0.05	0	0.025	0	0.025	0
Total	**1055**	**4057**	**773.85**	**4057**	**773.85**	**4057**	**773.85**	**4057**

Results:

8 Weeks of high fat diet consumption leads to increased adipose stores and caloric intake.

To establish a baseline in metabolic changes occurring in mice on 8 weeks of 60% high fat diets or control diet; body weights, food, and water intake were measured weekly. Cumulative water and caloric intake were measured for mice on high fat diets (n=4 cages, 16 mice) or control diet (n=4 cages, 10 mice). Mice on all three high fat diets consumed significantly more calories than those on a control diet. Additionally, among the three high fat diets caloric intake increased as unsaturated fatty acid concentrations increased (Fig. 2A). Corresponding to the increased food intake all three high fat diet groups of mice gained significantly more weight than control diet mice with no significant differences in weight between the three high fat diet groups (Fig. 2B). Lastly mice on a high saturated fat diet (HSFD) consumed significantly less water than the control group, but no significant differences were found between high fat diet groups (Fig. 2C).

To better characterize weight gain localization and type in the three high fat diets, NMR was used to determine body composition. Fat, lean, and fluid mass change was determined by subtracting initial body composition measurements from final measurements (8 weeks). A 2-way ANOVA with Bonferroni's multiple comparison's test across all diet groups for statistical significance. As expected, mice on all three high fat diets had significant increases in fat mass from control diet but were not significantly different from each other (p-value<0.0001 for all high fat diets compared to control). Lean mass and free body fluid were not significantly different among the four diet groups (Fig. 3A), demonstrating that the observed weight gain came primarily from increased fat accumulations and not changes in muscle mass or fluid levels.

Lastly to characterize where the increased fat mass was located within the body, visceral, subcutaneous, and gonadal fat pat measurements were taken along with liver weights (Fig. 3B). A two-way ANOVA was used to assess statistical significance across all diet groups with Bonferroni's multiple comparisons test. HFD and HUFD fed mice had significant increases in visceral fat while HSFD showed no significant change compared to control diet mice (HFD p-value: 0.0466; HUFD p-value: 0.0436). All three groups of high fat diet fed mice had increased subcutaneous (p-value: <0.0001 for all three high fat diets compared to controls) and gonadal fat stores (HFD p-value: 0.0005; HUFD p-value: 0.0055; and HSFD p-value: 0.0008). No significant differences were observed among the different high fat diet groups for any of the adipose store regions, and no significant differences were found in liver weights among the four diet groups.

High fat diets lead to changes in expression of fat taste components in the fungiform papillae.

To determine the effects of high fat diets on components of the polyunsaturated fatty acid taste transduction pathway, RNA was pooled from 4-5 mice following an 8-week feeding study (Fig. 4A). Both CD36 and GPR120 are thought to be the primary receptors responsible for taste cell activation via LA. As such, *Cd36* expression increased in the fungiform papillae of mice fed a HUFD (p-value: 0.0093) and a HSFD (p-value: 0.0116). A 2-way ANOVA with Bonferroni's test for multiple comparisons was used to determine significance for all gene expression data. No significant expression changes were observed for *Gpr120*. Lastly, *Trpm5* a key downstream calcium activated sodium channel in the fatty acid taste expression remained constant regardless of diet in both papillae. Expression of *Trpm5* in the circumvallate of mice in control diet was used as the calibrator, the highest expressing gene in all the groups. Previous data has shown that long chain polyunsaturated fatty acids including LA acted as an open channel blocker of delayed

rectifier potassium channels [14]. We observed gene expression of three DRK channels shown to be highly expressed in rat taste cells: *Kv2.2, Kv3.1* and *Kv3.2* [15] Both *Kv2.2* (HFD p-value: 0.0173, HUFD p-value: 0.0007, HSFD p-value: 0.0004) and *Kv3.2* (HFD p-value: 0.0055, HUFD p-value: 0.0003, HSFD p-value: 0.0016) showed a downregulation in the fungiform papillae for all groups of high fat diet fed mice, while *Kv3.1* was unaltered in the either papillae of mice regardless of diet. No significant changes were observed for *Kv2.2* or *Kv3.2* in the circumvallate papillae (Fig. 4B).

Calcium responses of taste cells from control diet and HUFD fed mice were similar in response to LA.

To better understand diet effects on taste cells, calcium responses were measured from of taste cells isolated following 8 weeks of high fat or control diet feeding. Representative calcium traces show the dose dependent response of taste cells to LA (Fig. 5A). Calcium responses did not appear to change across any of the high fat diets and control diet except the HSFD which had significant increase in calcium response at high concentrations of LA (Fig. 5B). Overall, these data trend toward similar or slight increases in taste cells that were not statistically significant, suggesting that calcium response are not significantly altered after 8 weeks of high fat diet feeding.

LA elicits greater inward current densities in taste cells isolated from high fat diet fed mice than control mice.

As differences were not observed in calcium responses from taste cells of mice on differing diets, we next wanted to determine if the fatty acid-induced inward currents and membrane potential changes elicited by these taste cells showed any differences. Using whole-cell patch clamp recording, cells were held at -100 mV for current measurements in voltage clamp mode or 0 pA

in current clamp mode for measuring changes in membrane potential. Isolated taste buds for patch clamp experiments are shown (Fig. 6A). Cells were exposed to focal application of 30 µM LA for 5 seconds and current/depolarization measurements were recorded. Cells from mice on a HUFD (p-value: 0.0406) and HFD (p-value: 0.0244) had greater inward currents than cells from control diet mice (Fig. 6B and 6E). However, no significant differences in inward current were observed between HSFD and control diet taste cells. Additionally, although not significant, cell membrane potentials increased in cells from both the HFD and HUFD compared to that of control diet (Fig. 6C and 6F). Lastly cell capacitance decreased in mice on a HSFD (p-value: 0.0239) compared to control diet, but no differences were observed in HFD and HUFD fed mice (Fig. 6D). A one-way ANOVA with Bonferroni's test for multiple comparisons was used for statistical analysis.

Delayed rectifier potassium (DRK) channel block by fatty acids is not significantly altered following high fat diet feeding.

Previous data suggests fatty acid sensitive/insensitive delayed rectifier potassium channels differ between obesity prone and resistant rats [13]. As such, to determine if these differences were observed in mice on high fat and control diets, potassium currents were measured prior to exposure to LA and following a 8-10 minute bath perfusion of 30 µM LA to calculate percentage voltage-activated potassium current remaining. Taste cells from mice on control diet and the three high fat diets did not show significant differences in the percentage of potassium channel current remaining following LA block (Fig. 7A). Additionally, no significant differences were observed between current-voltage relationships of cells on HUFD and control

diet mice (Fig. 7B). Representative voltage step protocol from -100 to 40 mV at 10 mV increments in Tyrodes (Fig. 7C) and following 8 minutes of LA exposure (Fig. 10D).

LA responses are not altered in PLCβ2-GFP taste cells regardless of diet.

To better understand where the increased inward currents in HUFD and HFD isolated taste cells were coming from, we analyzed taste cell responses from transgenic PLCβ2-GFP mice. PLCβ2 has previously been characterized as an indicator of Type II taste cells, the primary cells responding to G-protein mediated tastants. Using PLCβ2-GFP mice on either a HUFD or control diet, we were able to isolate the fatty acid-induced calcium responses, inward current alterations, membrane potential changes, and DRK channel block specifically in Type II taste cells. PLCβ2-GFP cells from HUFD mice exhibited similar calcium responses elicited by LA regardless of diet (Fig. 8A and D). Additionally, currents and depolarization amplitudes were unaltered in HUFD isolated taste cells (Fig. 8B and 8E). Lastly, no significant differences were observed in DRK channel currents remaining following LA block (Fig. 8C). Taken together these results indicate that the increased currents observed did not occur in Type II taste cells. A patched GFP cell with focal LA application is shown in Fig. 8F.

HUFD isolated taste cells are less dependent upon TRPM5 for fatty acid responses.

Based on data in Figure 8 increased inward currents of taste cells from HUFD fed mice do not appear to come from PLCβ2 taste cells as expected. Since PLCβ2 is thought to be necessary for the majority of LA-induced currents [2] the increased currents in HUFD and HFD fed mice did not appear to come from the prototypical Type II taste cells. Previous data suggests TRPM5 as a major contributor to inward currents elicited by LA in Type II taste cells, and mice genetically lacking *Trpm5* show a significant decrease in LA-induced inward currents [2]. To

determine the contribution of TRPM5 to the observed inward current changes observed in mice on HUFD, taste cells were treated with focal application of LA or LA with 100 µM TPPO. Inward currents were significantly reduced in taste cells from control diet fed mice (p-value: 0.0238) (Fig. 9A and 9C). However, while the current was also reduced in HUFD isolated taste cells it did not reach significance. As shown, greater current remains in the HUFD isolated taste cells than control diet when TRPM5 is inhibited. Additionally, taste cells from both diet groups show reductions in depolarization with TRPM5 inhibition yet neither reach statistical significance (Fig. 9B & 9D).

CD36 is less important for taste cell activation in HUFD fed mice.

Lastly, *Cd36* was the only gene tested in the fat taste pathway that had significant increases in expression. To determine the role of CD36 in fatty acid induced taste cell responses, we inhibited CD36 in taste cells prior to experiments by incubating cells in an irreversible CD36 antagonist (SSO) for 20 minutes prior to patching. Current measurements were significantly reduced in both control (p-value: <0.0001) and HUFD (p-value: <0.0001) isolated taste cells (Fig. 10A and 10C). Depolarization amplitudes were also significantly reduced in control diet (p-value: 0.0012) isolated taste cells and showed a similar trend in HUFD taste cells though it did not reach statistical significance (Fig. 10B & 10D). These data suggest CD36 is still important for fatty acid responses following a HUFD but may not be as necessary as in low fat or normal diets.

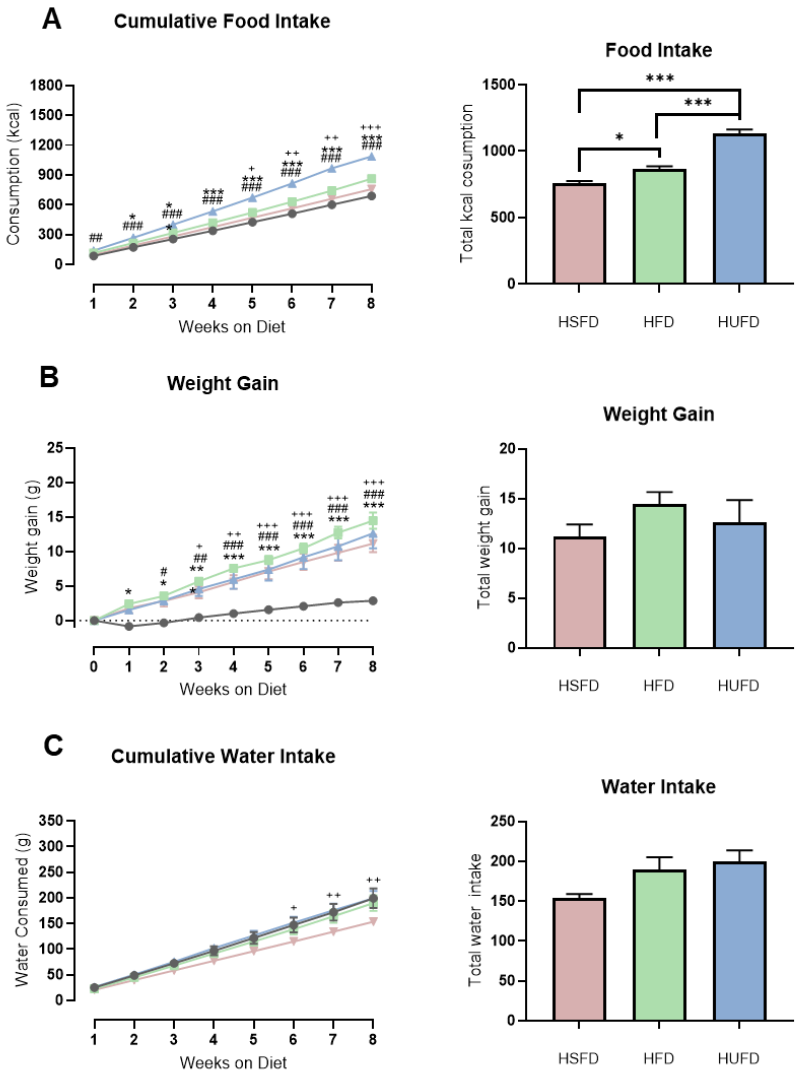

Figure 2: C57BL/6 Males on high fat diets consume more calories leading to increased weight gain.

Feeding study outcomes from mice given control or high fat diets. **A)** Mice on all three high fat diets consumed more kilocalories than control mice. Food intake increased as unsaturated fatty acid concentrations increased among high fat diets. **B)** Mice on high fat diets gained more weight than control diet, but no significant differences were found in weight gain among the three high fat diet groups. **C)** Mice on a HSFD consumed less water than control diet mice. Water intake did not vary significantly between high fat diet fed mice. Sample sizes: Control (n=10 mice), HFD (n=16 mice), HUFD (n=16 mice), HSFD (n=16 mice). A 2-way ANOVA with Bonferroni's multiple comparisons test was used to determine significance. HFD (*), HUFD (#), HSFD (+), *p<0.05, **p<0.01, ***p<0.001.

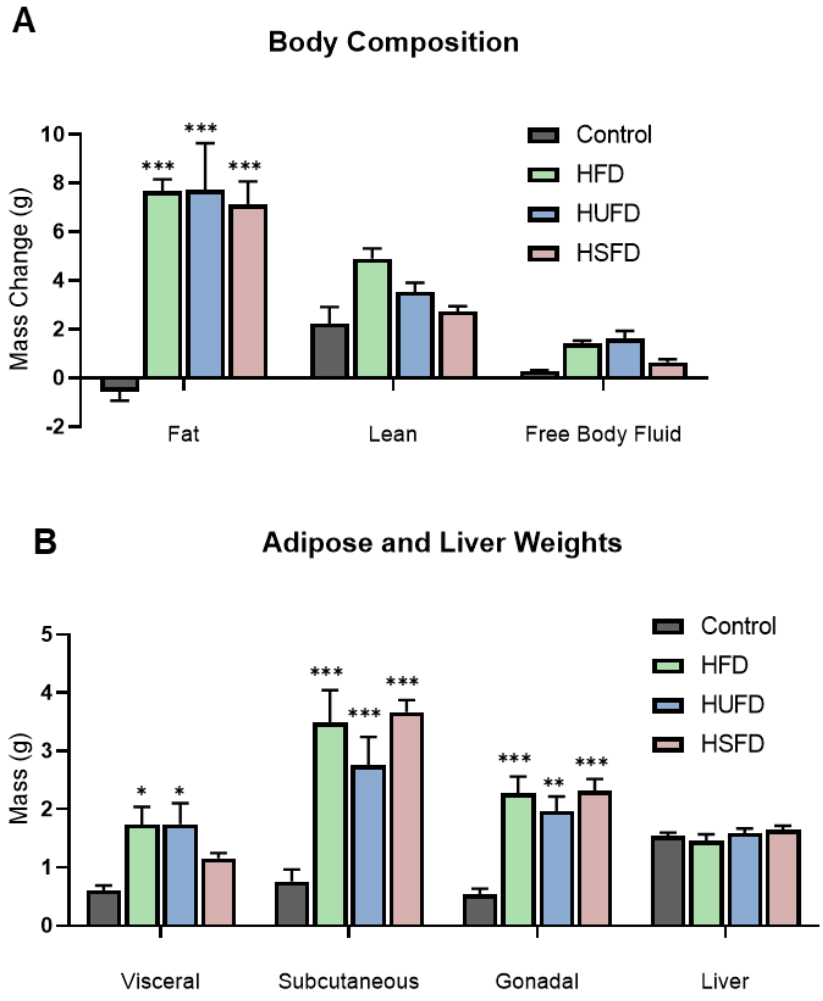

Figure 3: High fat diets led to increased adipose stores and fat mass in male mice.

A) NMR data shows increased fat mass among all three high fat diet groups compared to control diet mice. No significant differences were found in lean mass or free body fluid among the various diet groups. Sample sizes: Control (n=10 mice), HFD (n=16 mice), HUFD (n=16 mice), HSFD (n=16 mice). B) Fat pads and liver weights of mice upon completion of the 8-week feeding study. Mice on high fat diet (1:1) (HFD) and high unsaturated fat diet (HUFD) had increased visceral adipose tissue. All three high fat diets had increased subcutaneous and gonadal fat pads. No differences were found in liver weights across the four diet groups. Sample sizes: Control (n=4 mice), HFD (n=5 mice), HUFD (n=5 mice), HSFD (n=4 mice). A 2-way ANOVA with Bonferroni's multiple comparisons test was used to determine statistical significance. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

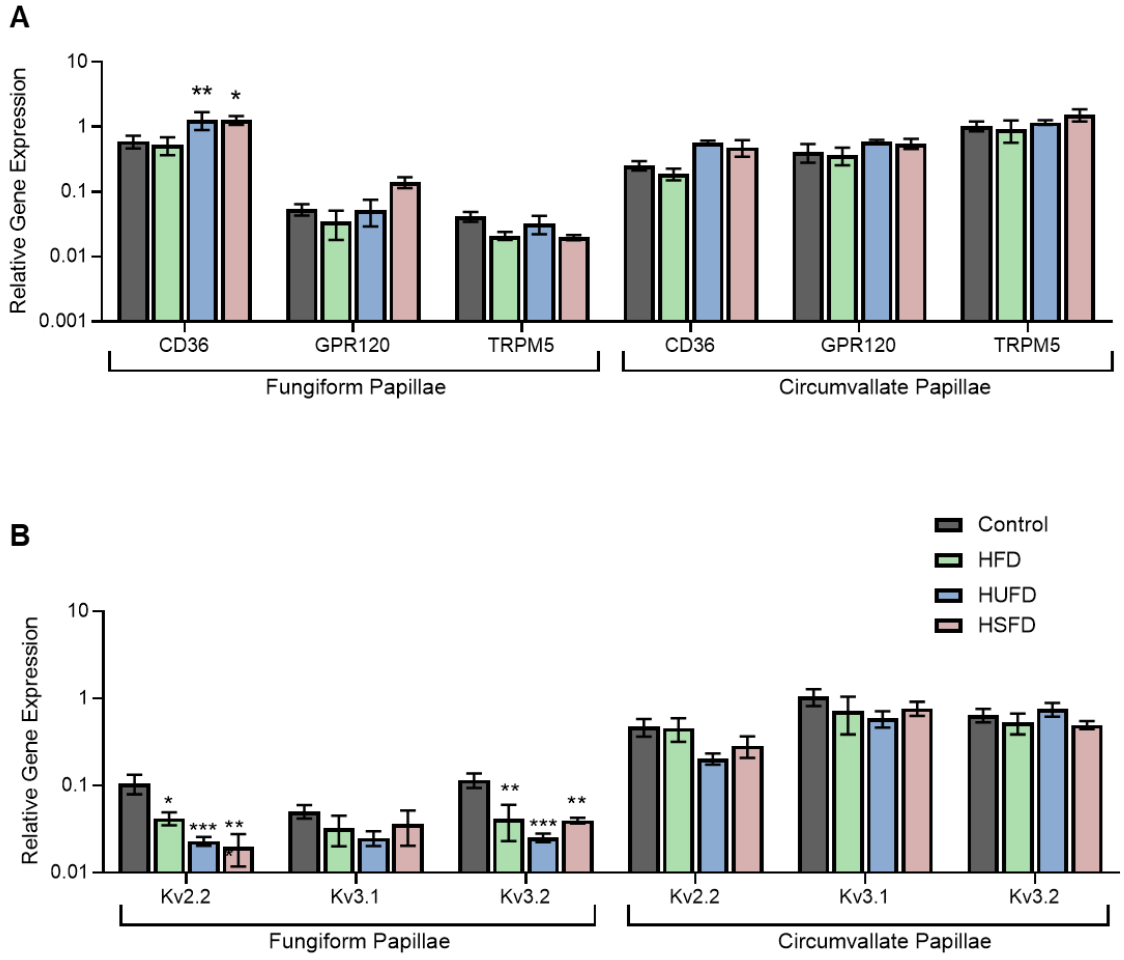

Figure 4: 8-week consumption of high fat diets leads to modest changes in gene expression of the fungiform papillae.

A) Relative expression of key components of fat taste transduction. *Cd36* was significantly upregulated in the fungiform papillae of HUFD and HSFD fed mice. Control diet circumvallate *Trpm5* expression was used as the calibrator. B) Relative expression of delayed rectifier potassium channels (DRK's). *Kv2.2* (*Kcnb2*) and *Kv3.2* (*Kcnc2*) are significantly downregulated in all high fat diets in the fungiform papillae. Expression of *Kv3.1* (*Kcnc1*) from mice on a control diet was used as the calibrator. A two-way ANOVA with Bonferroni's correction for multiple comparisons was used to determine statistical significance between control and HFD's. *p<0.05, **p<0.01, ***p<0.001.

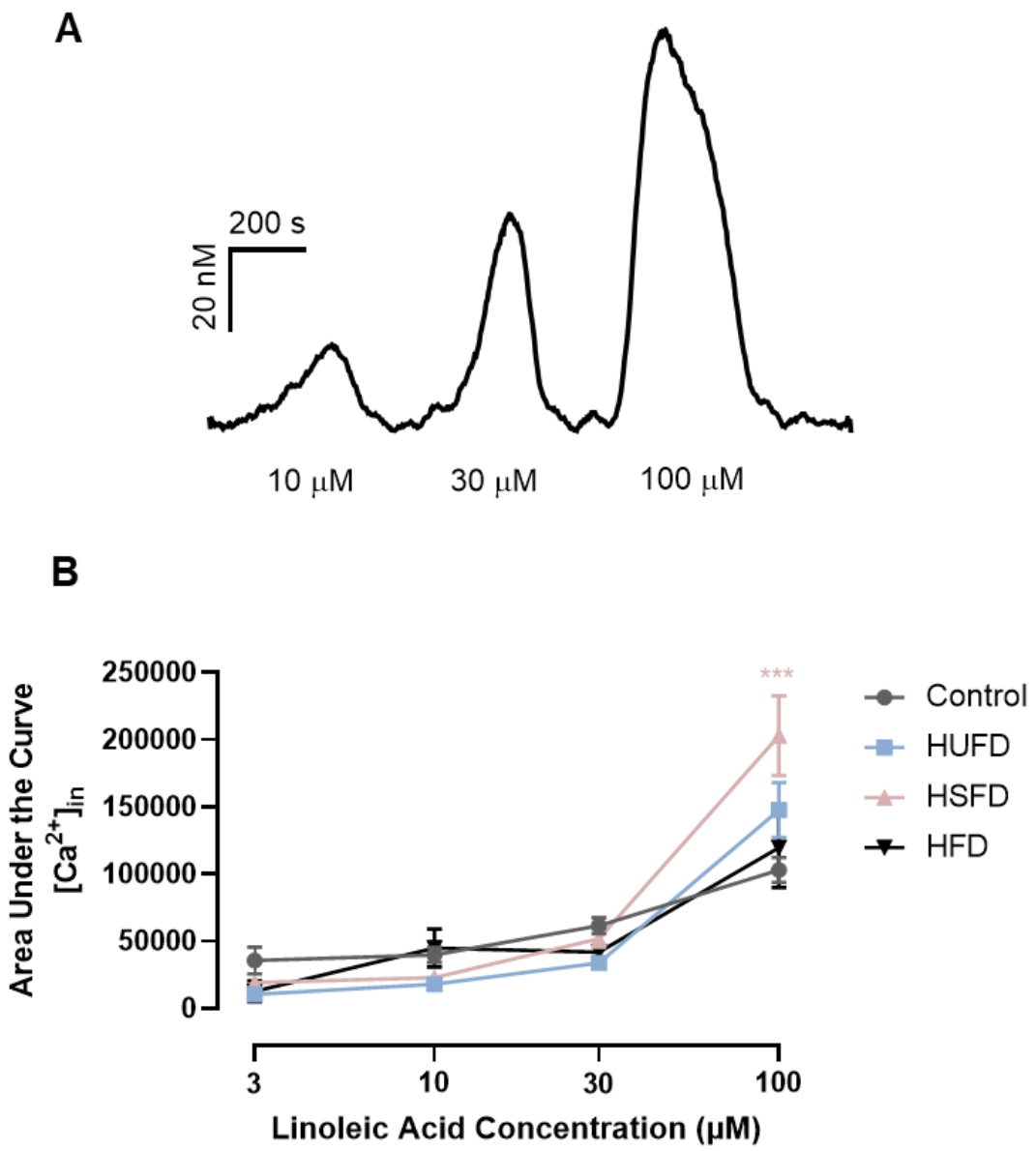

Figure 5: High fat diet does not significantly alter calcium responses to LA in taste cells.

Ratiometric calcium responses of taste cells isolated after 8 weeks of control or high fat diet feeding. **A)** Representative calcium trace showing responses to increasing fatty acid concentrations. **B)** Taste cells exhibit similar calcium responses regardless of diet at lower concentrations. HSFD taste cells had increased calcium responses to LA at 100 μM than control diet taste cells. A 2-way ANOVA with Bonferroni's multiple comparisons test was used to determine statistical significance. *p<0.05, **p<0.01, ***p<0.001.

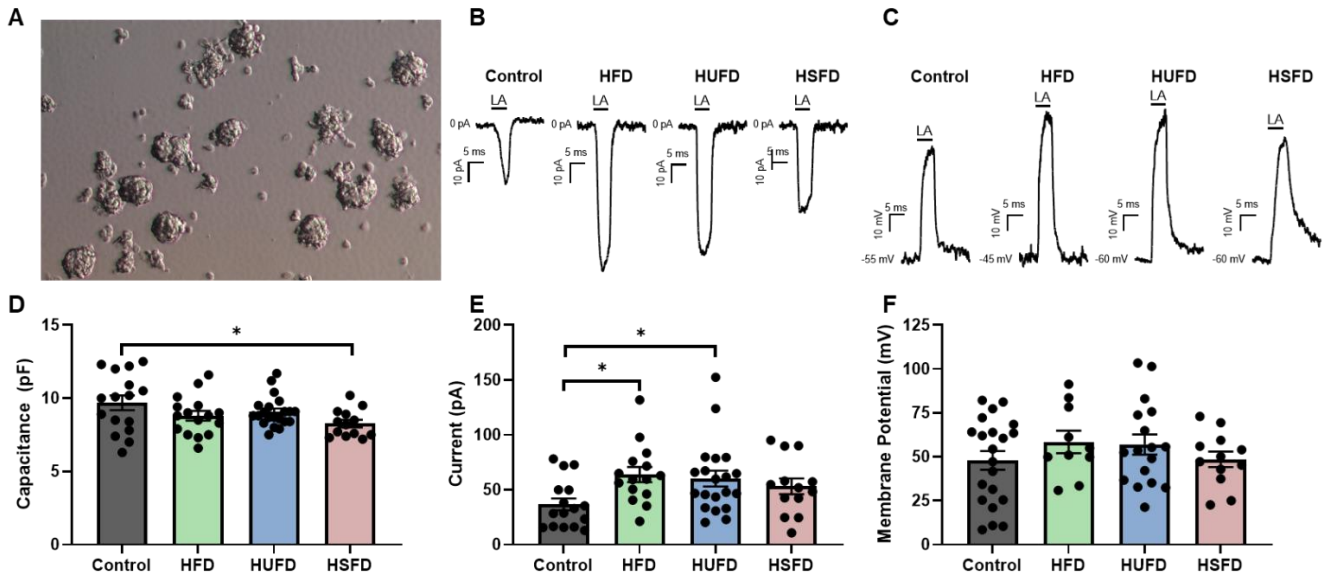

Figure 6: High fat diets significantly increase inward currents elicited by LA.

Inward currents and changes in membrane potential in taste cells stimulated with 30 µM LA from mice on 8 weeks of high fat or control diets. **A)** Representative current traces from high fat and control diets. **B)** Representative depolarizations from taste cells isolated from mice on the various diets. **C)** Isolated taste buds for patching clamp experiments. **D)** Inward current amplitudes elicited from LA in mice on control or high fat diets. Cells from mice on HFD and HUFD stimulated with LA had significantly increased current density compared to control diet. No significant differences were found in taste cells from HSFD and control diet mice. Sample sizes: Control (n= 16 cells), HFD (n=15 cells), HUFD (n=20 cells), HSFD (n=13 cells). **E)** No significant differences were found between depolarization amplitudes of taste cells regardless of diet. Sample sizes: Control (n= 21 cells), HFD (n=10 cells), HUFD (n=17 cells), HSFD (n=12 cells). **F)** HSFD isolated taste cells had lower capacitance than control diet counterparts, both other high fat diets did not exhibit significant changes. Sample sizes: Control (n=16 cells), HFD (n=16 cells), HUFD (n= 21 cells), and HSFD (n=13 cells). Statistical significance was determined using a One-way ANOVA with Bonferroni's multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001.

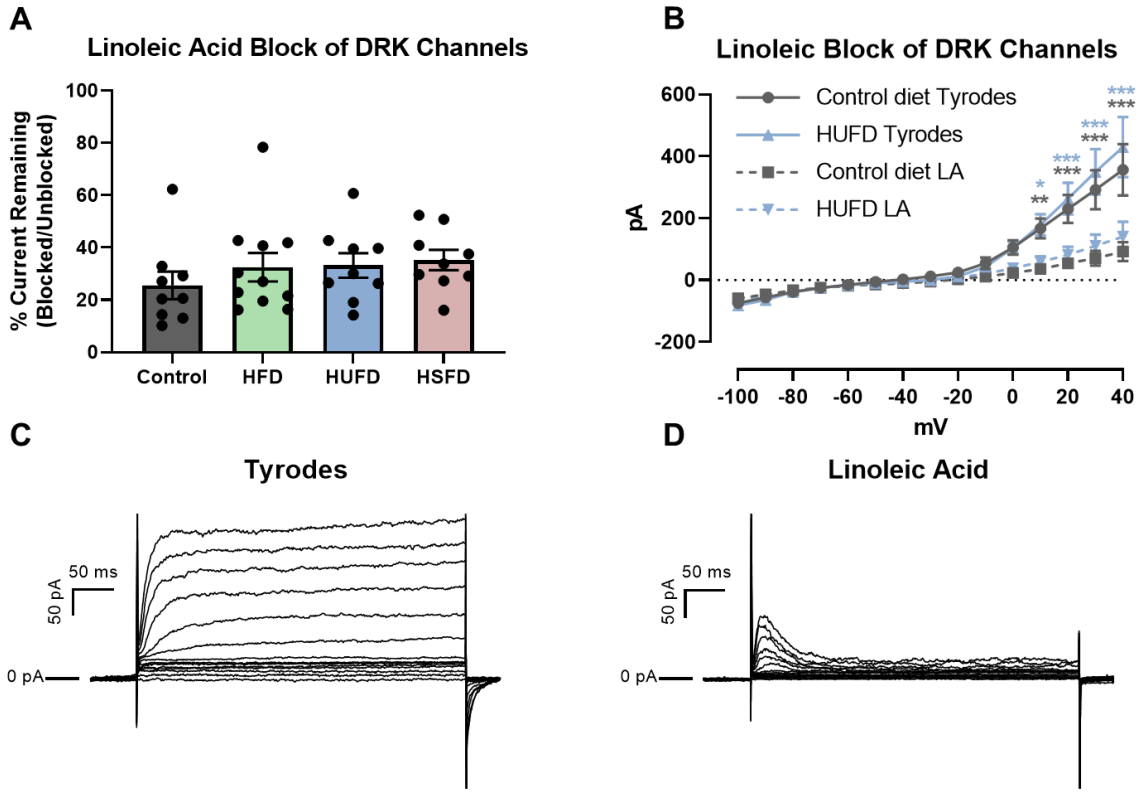

Figure 7: High fat diets did not significantly alter DRK channels.

Taste cells isolated from control and high fat diet fed mice following an 8-week feeding study. **A)** LA block of DRK channels were similar in taste cells regardless of diet type. Cells were perfused with 30 µM LA for 8-10 minutes to achieve maximum block of DRK channels. Sample sizes: Control (n=13 cells), HFD (n=11 cells), HUFD (n=9 cells), and HSFD (n=9 cells). **B)** Representative I-V curves in a taste cell isolated from control and HUFD fed mice. Both show a similar current voltage relationship in both *T*yrodes and similar reduction in current during DRK block. Statistical analysis shows a significant block of outward currents at 10mV in both control and HUFD taste cells by LA. **C)** Representative voltage step during Tyrodes perfusion. **D)** Representative voltage step during 30 µM LA block. Statistical significance was determined using a *one*-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001

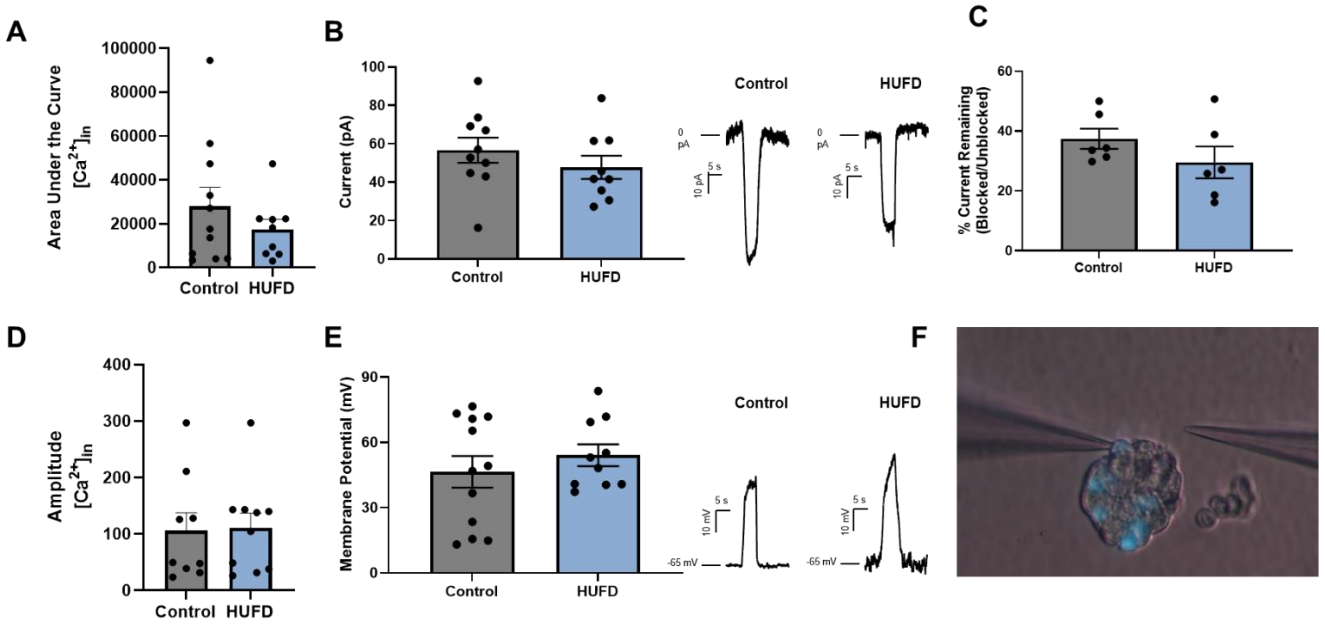

Figure 8: PLCβ2 expressing taste cells show no change in fat responses by diet.

GFP positive taste cells were isolated from PLCβ2-GFP expressing mice on 8 weeks of HUFD or control diet. A & D) PLCβ2 cells isolated from HUFD fed mice show no significant changes in area under the curve (AUC) or amplitude (AMP) calcium responses elicited by LA. Sample sizes: control (n=9 cells) and HUFD (n=9 cells). B) PLCβ2 cells show no significant change in inward currents elicited by LA compared to control diet. Representative inward current traces elicited by 30 µM LA. Sample sizes: control (n= 10 cells) and HUFD (n=8 cells). C) No significant differences were found in DRK percent block by LA between HUFD and control diet. Sample sizes: control (n=6) and HUFD (n=6). E) Depolarization amplitude did not change between taste cells isolated from control diet and HUFD. Representative current clamp traces for cells isolated from control and HUFD fed mice. F) Pictures depicted patched PLCβ2 cells with drug pipette positioned for focal application of 30 µM LA. Outliers were identified using ROUT method with a Q=1%. An unpaired student's t-test was used to determine statistical analysis. *p<0.05, **p<0.01, ***p<0.001..

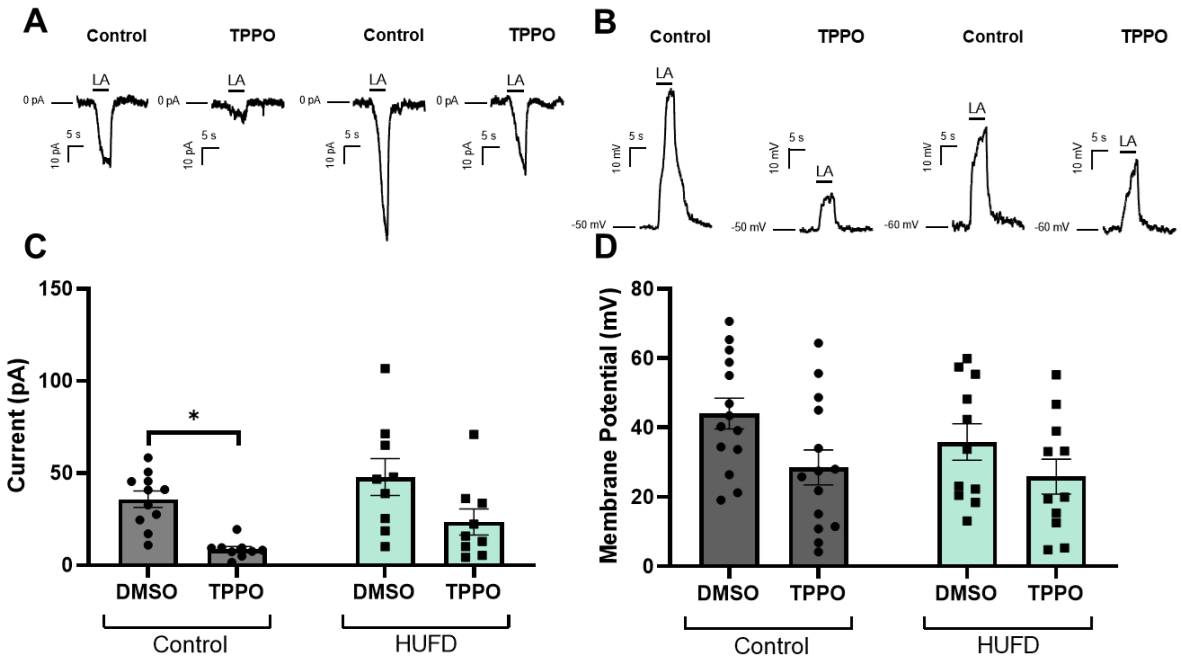

Figure 9: LA induced currents are less dependent on TRPM5 in HUFD isolated taste cells.

The effect of TRPM5 antagonist TPPO on fatty acid responses. A-B) Representative current and depolarization traces from control and HUFD taste cells. **C)** Inward currents were significantly reduced in control taste cells following inhibition of TRPM5. While current reductions were observed in HUFD taste cells they did not reach significance. Sample sizes: Control (DMSO n=11, TPPO n=9) and HUFD (DMSO n=9, TPPO n=9). **D)** Depolarizations were not significantly reduced in control or HUFD taste cells during TRPM5 inhibition. Sample sizes: Control (DMSO n= 14 cells, TPPO n= 14 cells) and HUFD (DMSO n= 11 cells, TPPO n= 11 cells). A two-way ANOVA was used to determine statistical significance. *p<0.05, **p<0.01, ***p<0.001.

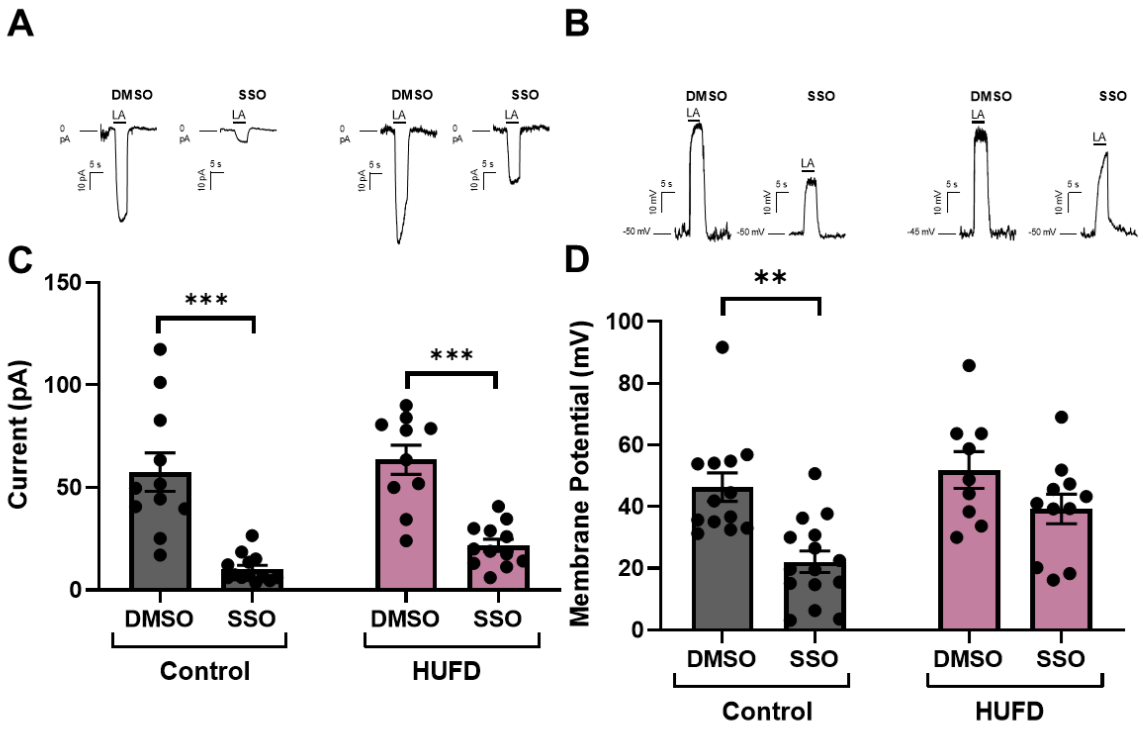

Figure 10: LA induced depolarizations are not dependent on CD36 in HUFD isolated taste cells.

Taste cells were incubated for 20 minutes in a CD36 antagonist (SSO) or DMSO of similar concentration prior to experiments. **A-B)** Representative current and depolarization traces from control and HUFD taste cells. **C)** Inward currents were significantly reduced in control and HUFD taste cells following inhibition of CD36. Greater current still remained in HUFD taste cells following CD36 inhibition. Sample sizes: Control (DMSO n= 11 cells, SSO n= 13 cells) and HUFD (DMSO n= 10 cells, SSO n= 13 cells). **D)** Depolarizations were significantly reduced in control diet but remained unaffected in HUFD taste cells. Sample sizes: Control (DMSO n=13 cells, SSO n= 15 cells) and HUFD (DMSO n= 9 cells, SSO n= 11 cells). A two-way ANOVA was used to determine statistical significance. *p<0.05, **p<0.01, ***p<0.001.

Discussion:

Fatty acids are the most calorically dense macronutrient and contribute significantly to dietary caloric intake. Several studies in both humans and rodents suggest potential plasticity in fatty acid taste signaling through weight status or dietary intake of fatty acids though its mechanism remains unclear. Some studies show BMI in humans is negatively correlated with fat taste sensitivity while others show no apparent associations [5, 16-18]. Studies on human and rodent models suggest correlations between fat taste detection levels and dietary fat intake [5, 14, 19]. Rodent studies also show obesity prone rats prefer fat to carbohydrates while obesity resistant rats prefer carbohydrates [14]. Further behavioral assays show obesity resistant rats on a high fat diet increase sensitivity to fatty acids while no change occurs in obesity prone rats [20]. These data mirrored human studies showing that dietary fat intake can alter fat taste thresholds in participants with a healthy BMI, but data are conflicting on if thresholds are altered due to diet in obese participants [10, 21, 22]. Taken together, the research thus far suggests that fat intake may be a contributor to changes in fat taste while the role of weight status on fat taste sensitivity largely remains unclear. Additionally, genetic predispositions may play a role in fat taste sensitivity and metabolic status. In humans increased BMI is correlated with genetic variants of *Cd36*, suggesting a genetic component to fat taste threshold levels (see review [11]). These data together begin to give a picture of the complex relationship of fat taste sensitivity, dietary fat consumption, and metabolic status.

In this study, we sought to better understand the role of dietary fat, particularly PUFAs, and weight gain on taste cells responses to LA. By observing cellular changes within the taste system, we can better extrapolate the cellular and molecular underpinnings of these diet induced

changes. To better understand the importance of dietary PUFA intake in fatty acid induced taste responses we performed all studies on mice following either 8 weeks of 60% high unsaturated fat diet (HUFD), high saturated fat diet (HSFD), high fat diet (HFD), or control diet (10% fat). We recorded increased caloric intake and adiposity in all high fat diet fed mice that resulted in significant visceral, subcutaneous, and gonadal fat pad weights. Additionally, HUFD upregulated gene expression of *Cd36*, with no gene expression changes occurring in *Gpr120* or *Trpm5* across diets. DRK channels *Kv2.2* and *Kv3.2* had significant downregulation in the fungiform across all high fat diet groups compared to the controls. Functionally taste cells had similar calcium responses to LA regardless of diet, with the exception of the HSFD at high concentrations of LA which had significantly higher calcium responses than control taste cells. In the patch clamp experiments, HUFD and HFD led to increased currents elicited by 30 μM LA but no significant change in depolarization size. Upon further investigation, we found this increased current did not appear to occur in Type II taste cells. Lastly, we showed that while CD36 and TRPM5 contribute to the inward currents elicited by LA in HUFD fed mice, pharmacological inhibition of both resulted in smaller current reductions in HUFD cells compared to controls. Our data suggest high fat diets increase taste cells responses a result that was repeated in both HUFD and HFD. Additionally, we found the observed increased current density responses in subtype of cells that are not PLCβ2 positive in HUFD fed mice, suggesting a broader signaling mechanism than previously observed [2] .

Several publications using obesity prone (OP) and resistant (OR) rats suggest a relationship between macronutrient preference, taste sensitivity, and metabolic status. Previous data in rat models showed that when given a three-diet choice (fat, carbohydrate, or protein), obesity prone (OP) rats prefer fat while obesity resistant (OR) rats prefer carbohydrates [19].

Furthermore, there are inherent differences in the expression of DRK channels within these two subgroups of rats. Delayed rectifier potassium (DRK) channels are responsible for repolarizing the cell following depolarization. Blocking of DRK channels is thought to cause prolonged cellular depolarization following stimulation. DRK channel currents are blocked by long chain polyunsaturated fatty acids with little to no effect seen in monounsaturated or saturated fatty acids in isolated rat taste cells [3]. When stimulated with LA, OP rats display a significantly smaller block of DRK channels compared to OR rats. They are also thought to contain a lower ratio of fatty acid sensitive to insensitive DRK channels compared to their OR counterparts [15]. Additional findings by Pitman and colleagues., found when placed on a HFD, OR rats displayed a decreased threshold for fatty acids where little to no effect was observed in OP rats, suggesting that in OR rats the taste system was plastic and fat taste thresholds could be altered [20]. These data provide supporting evidence that the taste system is plastic, and modulation occurs through diet, metabolic status, or both; with potential DRK channel involvement. Based on these findings we originally hypothesized that taste cells from mice on high fat diets would exhibit a diminished DRK channel block compared to taste cells from control diet mice. However, functional experiments showed no significant differences in LA block of DRK channels between taste cells isolated from high fat and control diets (Fig. 6A). Additionally, no changes were observed in DRK channel block of PLCβ2 positive (Type II) taste cells. These data indicate that overall DRK channel block by LA following 8 weeks of high fat diet feeding of male mice was not changed by dietary differences. Differences observed in OP and OR rats may be due to inherent genetic differences that result in lower DRK channel expression driving changes in fat taste thresholds rather than fat consumption driving DRK expression. Alternatively, more directed studies in mice observing specific fatty acid sensitive DRK channels are needed to

determine if changes occur in subsets of DRK channels. Specifically, in rats Kv1.5 was broadly expressed and thought to be a major contributor to the DRK currents. Future experiments should include pharmacological block of Kv1.5 to determine if diet induced changes occur in these particular DRK channels. Furthermore, it is possible that changes to DRKs may take longer to induce. Potentially a longer duration of elevated FFA levels in the plasma may contribute to changes in DRK channel expression within the taste system and if given longer (e.g.,12+ weeks) on diet these changes could be observed.

To better understand the effect that diet and weight status may have on the rest of the fat taste transduction pathway, we performed functional calcium imaging and measured inward currents and changes in membrane potential elicited by LA. Calcium dose response curves largely remained unchanged based on diet. In contrast significant differences in inward currents elicited by 30 μM LA were observed among the diets. All high fat diets showed increased currents though only the HFD and HUFD inward currents were statistically significant. Corresponding trends were observed in both HFD and HUFD for depolarization amplitude (though not statistically significant). These data suggest modulation of taste cells by diet. The taste system is generally thought to contain 3 main types of taste cells: Type I cells are glial-like and function as the support cells of the taste bud, Type II taste cells respond to GPCR dependent tastants (bitter, sweet, umami, fat), and Type III cells are the pre-synaptic cells (see reviews [23, 24]). To better ascertain if the observed larger inward currents in high fat diet taste cells were in Type II taste cells, we utilized a transgenic mouse model with a PLCβ2-GFP tag (a common marker for Type II taste cells) [25]. We measured calcium responses, current density, depolarization, and DRK channel block elicited by 30 μM LA in PLCβ2 positive cells only in HUFD and control diet taste cells. Additionally, based on the increased overall current densities

observed above, we were interested to see if the increased current from HFD and HUFD mirror again in Type II specific cells. Surprisingly, we found no significant differences in calcium response, current, depolarization, or DRK channel block in HUFD and control diet Type II cells. These results indicate that diet induced changes to fat taste do not occur in Type II taste cells but must occur in one of the other two remaining types. Previously researchers thought that Type III cells largely do not respond to GPCR-mediated tastants. Recently published data suggests a subset of Type III cells respond to bitter, sweet, and umami tastants through a separate PLC isoform, PLCβ3 [26]. While the pathway involving GPCR-mediated tastants in these Type III cells is not yet well understood, fatty acids may utilize a similar pathway. Dando et al., also found that following 8 weeks of high fat diet mice expressed fewer taste buds in the circumvallate papillae than their WT counterparts but observed no changes in expression levels of Type I, II, or III markers [1]. These data along with our increased inward currents suggest that it is the cells responsiveness to fatty acids that is changing in the taste system rather than the prevalence of these taste cells. Regardless, further research focused on the cellular changes we observed perhaps focused on Type III cells are needed to better understand the role of diet on taste signaling.

To further examine how components of the elucidated fat taste pathway effect the observed increased inward currents (in all cell types), we measured gene expression of three key components of the fat taste pathway: CD36, GPR120, and TRPM5. *Cd36* gene expression significantly increased in the circumvallate papillae of HUFD and HSFD fed mice. CD36 protein expression has been show in both human and mouse taste papillae [28]. This target was of particular interest as several studies suggest an association between *Cd36* mutations and fatty acid detection levels in humans. The *Cd36* gene contains several polymorphisms with

correlations between certain SNP's and decreased fat taste thresholds (see review [11]). In human taste cells, CD36 is co-expressed with GPR120 (the other primary fatty acid taste receptor) and PLCβ2 [29, 30]. Research in rodent models also suggest the importance of CD36, as *Cd36* knockout leads to decreased preference for fat in mice [31-33]. Its expression is also restricted to the apical portion of the taste bud, near the pore where tastants bind to receptors to stimulate responses [31]. *Cd36* mRNA levels in the circumvallate papillae decrease during the dark period when food intake increases, while *Gpr120* mRNA show slight increases. Additionally, *Cd36* increases significantly during a fasted state and immediately following feeding with a gradual rise to fasting level concentrations in the hours post ingestion. Once the cell type which contains enhanced fat responses is determine additional studies into the involvement of CD36 are needed to determine if the increased currents are via a CD36 mediated pathway.

In contrast *Gpr120* expression levels remained fairly constant regardless of diet state [32]. Suggesting that while GPR120 may be important for basal fatty acid detection, CD36 may play a more dynamic role in modulating fat taste based on metabolic status or feeding state. This divergence in roles is further shown in fatty acid stimulation of taste cells. CD36 appears to play a more important role in taste cell calcium response to fatty acids at low levels while GPR120's involvement occurred primarily at higher fatty acid concentrations [30]. To better understand the role of CD36 in the observed enhanced inward currents, we used a common irreversible CD36 blocker (SSO). Following a 20-minute incubation of SSO, we observed a decreased current in cells isolated from control and HUFD fed mice (Fig. 13A and C). HUFD isolated taste cells did not show a significant difference in depolarization size as was shown in control diet taste cells (Fig. 13B and D). Interestingly, in addition to no effect in total cellular depolarization, HUFD

taste cells had a larger inward current remaining following CD36 inhibition compared to taste cells from control mice. Based on these data, CD36 contributes less to the cellular response of taste cells to LA following high fat diet feeding.

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CHAPTER 3: THE EFFECTS OF GHRELIN ON GUSTATORY FAT DETECTION

Abstract

The importance of ghrelin in energy intake has long been established. In recent years, the presence of ghrelin and its receptor (GHSR) have been shown to influence taste activity [1, 2]. While studies have suggested a role for ghrelin in macronutrient detection and regulation, little is known about its role in the taste system. Our study provides further insight into the role of ghrelin in modulating fatty acid detection within the oral cavity. Here we show that taste cells isolated from ghrelin knock-out (*Ghrl*$^{-/-}$) mice had decreased linoleic acid (LA)-induced calcium responses. Additionally, our data demonstrate that ghrelin plays an acute role in the taste system. Fatty acid calcium responses in the presence of a GHSR agonist (GHRP-6) are elevated when compared to fatty acids alone. Lastly, behavioral assays showed that a lack of ghrelin decreased sensitivity to fatty acids in mice undergoing a conditioned taste aversion. This work suggests ghrelin plays a role in the modulation of fatty acid sensing in the peripheral taste system.

Introduction

Since the early 1970's, adult obesity prevalence has almost tripled, and childhood obesity rates have more than quadrupled [3]. One major contributor to the onset of obesity is a state of positive energy balance caused by increased caloric intake and decreased physical activity. Vital to understanding how the body regulates energy balance and caloric intake is determining how nutrients are sensed and the endocrine factors involved. Many hormones secreted by the gut are involved in energy homeostasis and can become dysregulated in an obesogenic state [4].

Research in recent years has demonstrated hormones such as leptin, glucagon-like peptide-1 (GLP-1), and ghrelin modulate the taste system [1, 2, 5-8].

The orexigenic hormone ghrelin has been shown to play a key role in mechanisms of metabolism including food intake, weight gain, insulin release, gastric acid secretion, and gut motility [9-15]. Peripheral ghrelin crosses the blood-brain-barrier and acts on the arcuate nucleus of the hypothalamus. Here it activates orexigenic AgRP/NPY neurons while inhibiting anorexigenic POMC neurons to increase food intake [14-17]. In addition to ghrelin's role in the CNS, studies suggest a peripheral action of ghrelin within the oral cavity [1, 2]. While most of the ghrelin is produced in the stomach, small amounts are produced elsewhere in the periphery including the salivary glands and taste cells of the oral cavity [2, 18, 19]. Additionally, salivary ghrelin levels fluctuate with food intake similar to changes observed in plasma ghrelin concentrations [20]. The rapid rise and fall of circulating ghrelin, correlated with food intake, suggests a potential role for ghrelin in taste signaling.

Moreover, the ghrelin receptor (GHSR) is expressed in Type II taste cells which have been shown to respond to bitter, sweet, umami, and fat tastants [21-23]. Additional studies have since investigated the role of ghrelin's involvement in the detection and signaling of several different tastants, yet its role in taste largely remains unclear [1, 2, 24]. To our knowledge only one article has looked at the effect of ghrelin on fat taste detection. Cai et al., showed that ghrelin KO mice had a significant decrease in responsiveness to fatty acids compared to their WT counterparts [1].

The aim of the present study was to assess the mechanistic role of ghrelin in the taste system and more specifically on fatty acid-induced cellular activation. A global knockout of ghrelin attenuated taste cell responses and the use of a GHSR agonist showed increase cellular

responses. The use of GHSR agonist alone induced little to no taste responses while the combination of GHSR agonist and fatty acids enhanced taste cell responses. This was further supported by the finding that mice lacking ghrelin exhibit lower taste responsiveness than their wild-type counterparts. When taken together these data demonstrate the modulatory role of ghrelin in fat taste.

Materials and Methods

Animals

Ghrelin knockout (*Ghrl*$^{-/-}$) mice were obtained from Dr. Yuxiang Sun's lab at Baylor College of Medicine. *Ghrl*$^{-/-}$ mice were bred at Utah State University following proper Institutional Animal Care and Use Committees (IACUC) protocols and procedures. *Ghrl*$^{-/-}$ mice were verified using endpoint polymerase chain reaction showing a lack of ghrelin expression. Wild-type C57BL/6J mice were obtained from Jackson Laboratories and housed according to proper IACUC protocols and procedures at Utah State University and the University of Central Florida.

Solutions

Standard saline solution (Tyrode's) contained (in mM) 140 NaCl, 5 KCl, 1 CaCl2, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 305-315 mOsm. LA (L1012) was purchased from Sigma (St. Louis, MO) and diluted in 100% ethanol to a concentration of 25 mg/mL and stored under nitrogen at -20°C until the day of experiment. GHRP-6 (HOR-298) was purchased from ProSpec (Rehovot, Israel) and was made in ddH2O to 1 mg/mL and stored at -20°C until use. Enzyme cocktail components consisted of collagenase A, dispase II, and trypsin inhibitor and were purchased from Sigma (St. Louis, MO).

Taste Cell Isolation

Immediately following euthanasia, the tongue was removed and placed in a Tyrode's solution. Taste cell isolation has been described previously (Gilbertson et al., 1997). In brief, 0.2 mL of an enzyme cocktail containing dispase II (2.0 mg/mL), collagenase (0.5 mg/mL), and trypsin inhibitor (1 mg/mL) in Tyrode's was injected between the muscle and epithelium throughout the tongue. Next, the lingual epithelium was removed from the underlying muscle layer with forceps, pinned out in a Sylgard™-lined petri dish and taste buds were removed by gentle suction with a fire polished pipette (100-150 µm bore) under a low magnification dissection microscope. Taste cells were then gently collected and placed on coverslips containing Corning® Cell-Tak™ Cell and Tissue Adhesive (Corning, PN 354240) for calcium imaging.

Calcium Imaging

Once cells adhered (approximately 20 minutes), coverslips were incubated in 0.05% pluronic acid/Fura-2AM (4 µM) (Invitrogen) for approximately 45 minutes. Next, cells were placed in a perfusion chamber (Warner Instruments, RC-25F), and perfused continuously at a flow rate of approximately 4 mL/minute. Imaging was performed using an Olympus CKX53 microscope with a Basler acA720 camera and Incyt Im2 software was used to capture calcium change data. Cells were excited at 340 and 380 nm and recorded at 510 nm and the 340/380 ratio was converted to intracellular calcium concentration ($[Ca^{2+}]i$) based on the calcium calibration buffer kit (Invitrogen). LA concentrations were introduced in a random order. The minimum criterion for a calcium response was determined by a reversible response with an amplitude greater than twenty standard deviations above the prestimulus baseline variance for each cell. Area under the curve responses were calculated for each cell and raw responses were recorded. Relative responses were determined by calculating the area under the curve for each LA

concentration relative to the control concentration (30 µM LA). For the acute stimulation experiments, taste cells were stimulated using LA and GHRP-6 (ghrelin agonist) and calcium responses were recorded. GHRP-6 was used at a concentration of 100 nM diluted in Tyrode's. For the short-term incubation with ghrelin agonist, cells were incubated for 20 minutes with 100 nM GHRP-6 in Tyrode's or Tyrode's alone prior to imaging cells using various concentrations of LA.

Conditioned Taste Aversion

Mice were water deprived for approximately 23.5 hours a day throughout testing. Animals were trained on the MS-160 Davis Rig to lick water presentations continuously between shutter openings until they reliably performed in the chamber for a minimum of 30 presentations. Following training, animals underwent conditioning days in which mice were separated into a control group which received intraperitoneal (i.p.) injection of 150 mM NaCl and a treatment group which received 150 mM LiCl to induce gastrointestinal malaise and paired with oral application of 100 µM LA. All mice in the LiCl group showed signs of gastric distress following injection. Aversion was observed in all mice of the LiCl group to 100 µM LA by conditioning day 3. Following the final day of conditioning, lick responses in mice were determined for varying concentrations of LA (0.1, 0.3, 1, 3, 10, 30, and 100 µM) and control solutions (100 mM sucrose, 100 µM capric acid, 100 mM NaCl, 3 mM denatonium benzoate (DB), and water) (refer to Fig. 11). After each conditioning and testing day, animals were given 30-minute access to water. Efforts were made to minimize other sensory (olfactory) cues by running fans perpendicular to the shutter opening during experiments in a quiet room.

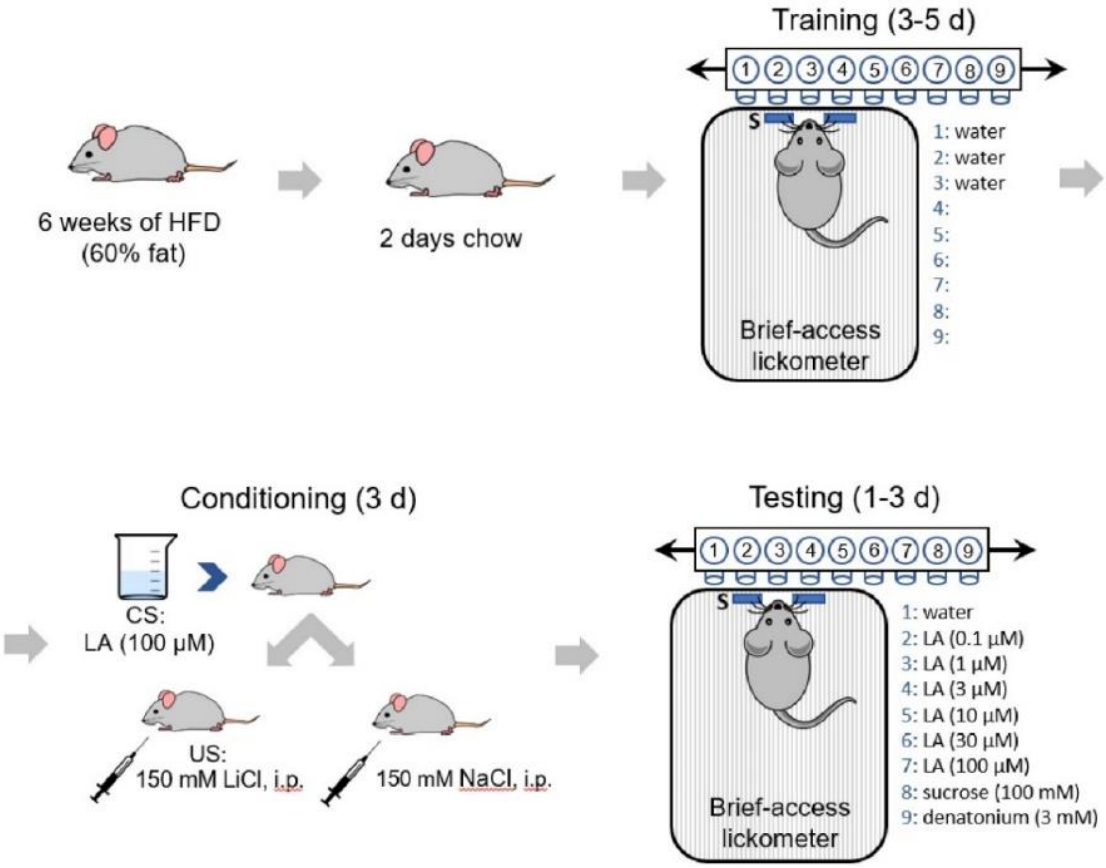

Figure 11: Conditioned taste aversion paradigm.

Following 6 weeks of high-fat diet (HFD) (60%), the mice underwent the following conditioning paradigm. Throughout the study mice were water deprived for 23.5 hours/day. The mice were trained to lick from a Davis rig for 3–5 days. Following which conditioning occurred for 3 days with a conditioned stimulus of 100 µM LA and i.p. injections of 150 mM NaCl or LiCl. Mice in the LiCl treatment group were observed post-injection for signs of gastric distress. During testing days, mice were given access to LA at concentrations of 0.1, 1, 3, 10, 30, and 100 µM; 100 mM sucrose; 3 mM denatonium benzoate; and water in a randomized sequence. Mice had access to test solutions for 5 s followed by a rinse solution (water) for 2 s before presentation of the next test solution [2].

Results

Ghrl$^{-/-}$ mice exhibit a reduced aversion to LA in a conditioned taste aversion assay.

Previous data suggests alterations in intralipid preferences between ghrelin knockout (*Ghrl$^{-/-}$*) and WT mice [1]. To investigate the effects of ghrelin on fat taste responsiveness, we performed a conditioned taste aversion (CTA) assay on both C57-BL6J (WT) and *Ghrl$^{-/-}$* mice with LA. Over days 2 and 3 of testing *Ghrl$^{-/-}$* mice showed a significantly reduced aversion to LA compared to WT mice. While *Ghrl$^{-/-}$* mice showed an aversion at 100 µM LA, WT mice showed aversions to concentrations as low as 10-30 µM LA (Fig. 12A and 12B). A 2-way ANOVA with multiple comparisons was used to determine statistical significance. Additionally, we found the LiCl-induced aversion did not generalize to other tastants in either *Ghrl$^{-/-}$* or WT mice (Fig. 12C; Student's unpaired t-test).

Mice lacking ghrelin show diminished calcium responses to LA.

To better understand differences in behavioral responses of *Ghrl$^{-/-}$* and WT mice, we attempted to determine if there was a change to the taste cell responses that could lead to these behavioral changes. Using calcium imaging, we calculated the area under the curve (AUC) and amplitude for taste cells responses in WT and *Ghrl$^{-/-}$* cells ($n \geq 50$ cells across all groups and concentrations). Representative calcium traces are shown for *Ghrl$^{-/-}$* and WT mice (Fig. 13A). WT mice had significantly reduced calcium responses in the fungiform papillae to both 10 µM LA (p-value: 0.002) and 30 µM LA (p-value <0.001) (Fig. 13B). Similar reductions in calcium responses to LA were observed in the circumvallate papillae where *Ghrl$^{-/-}$* mice had significantly reduced calcium responses to LA (p-value: <0.001 for all concentrations) (Fig. 13C). These changes were observed in both AUC and amplitude measurements of calcium responses. A two-way ANOVA with Bonferroni's test for multiple comparisons was used to determine statistical

significance. These data show that taste cells isolated from mice lacking ghrelin have significantly lower calcium responses to LA and these effects are independent of concentration or papillae type.

Acute co-stimulation of wild-type taste cells with GHSR agonist and LA significantly increased calcium responses.

Our data in *Ghrl*$^{-/-}$ and WT mice show the long-term effects of ghrelin on cellular and behavioral mechanisms. However, ghrelin levels fluctuate throughout the day with circulating levels which rise prior to mealtime and dramatically drop following food intake. Thus, we sought to determine if ghrelin plays a more immediate role within the taste system and more specifically in fatty acid detection. A representative trace shows calcium responses to: LA, LA+GHRP-6, and GHRP-6 alone (Fig. 14A). As shown, GHRP-6 alone elicited little to no calcium response. The simultaneous addition of GHRP-6 and 30 μM LA to taste cells significantly increased calcium responses compared to 30 μM LA alone in both the fungiform (AUC p: 0.0001, amplitude p: 0.0001) and circumvallate papillae (AUC p: 0.003, amplitude p: 0.001) (Fig.14 B). An unpaired student's t-test was used to determine significance (p: **<0.01, ***<0.001).

Wild-type taste cells pre-incubated in GHSR agonist (GHRP-6) and stimulated with LA alone showed no change in calcium response.

In order to investigate the temporal effects of ghrelin on the taste system we performed an additional experiment to determine if ghrelin must be present at the time of stimulation or if recent exposure is adequate to enhance LA responses. Cells were pre-incubated in either a GHRP-6/Tyrode's mixture or Tyrode's alone (control). Following incubation, cells were rinsed with Tyrode's and stimulated with 30 μM LA. Representative calcium traces of taste cells incubated in Tyrode's (control) or GHRP-6/Tyrode's are shown (Fig. 15A). As shown in Figure 15 no significant differences were found between calcium responses of cells pre-incubated in

GHRP-6/Tyrode's mixture or Tyrode's alone across the fungiform or the circumvallate papillae (Fig. 15B). A Student's t-test was used to determine statistical significance.

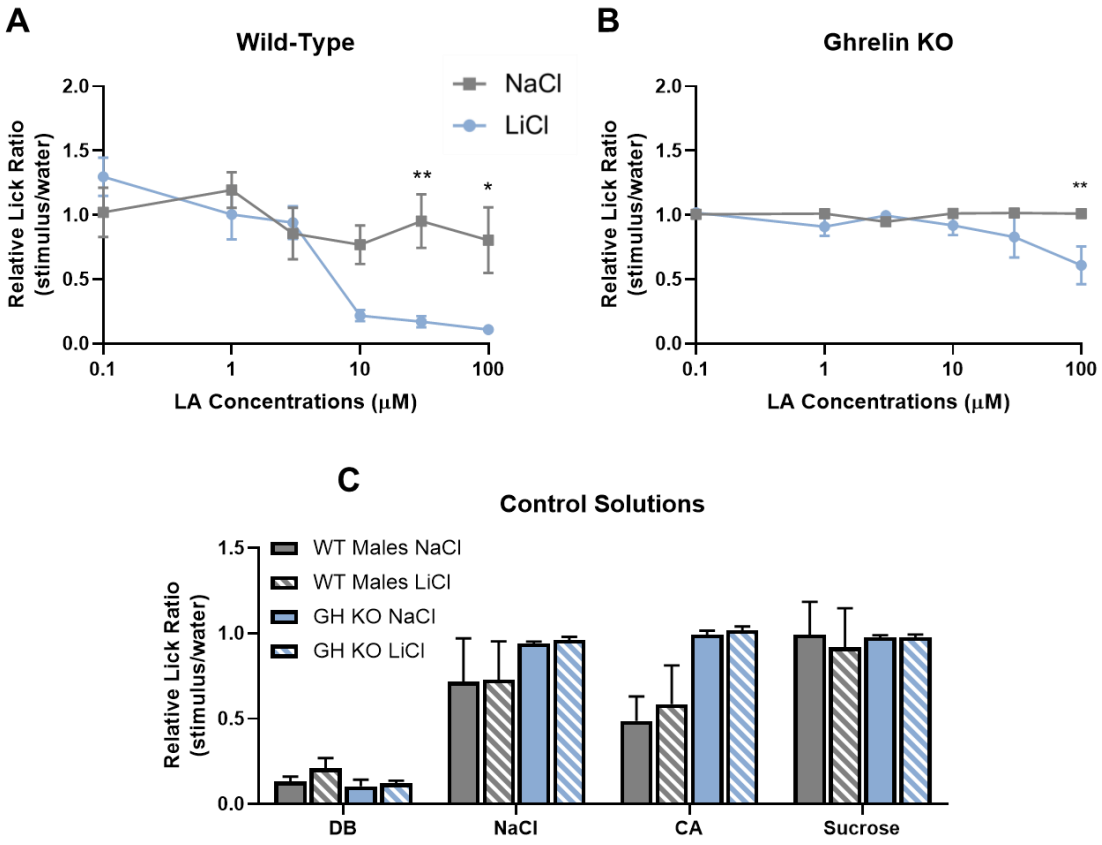

Figure 12: *Ghrl*$^{-/-}$ mice show diminished aversion to LA compared to Wild-type male mice.

A) Wild-type males treated with LiCl mice show an aversion to LA at 30 µM. Sample sizes: LiCl (n=7) and NaCl (n=6). **B)** *Ghrl*$^{-/-}$ males treated with LiCl mice did not show an aversion to LA until 100 µM. Sample sizes: LiCl (n=6) and NaCl (n=5). **C)** LA aversion did not cross generalize to any of the other tastants in either wild-type or *Ghrl*$^{-/-}$ mice. A 2-way ANOVA with multiple comparisons was used for statistical analysis. *$p<0.05$ and **$p<0.01$.

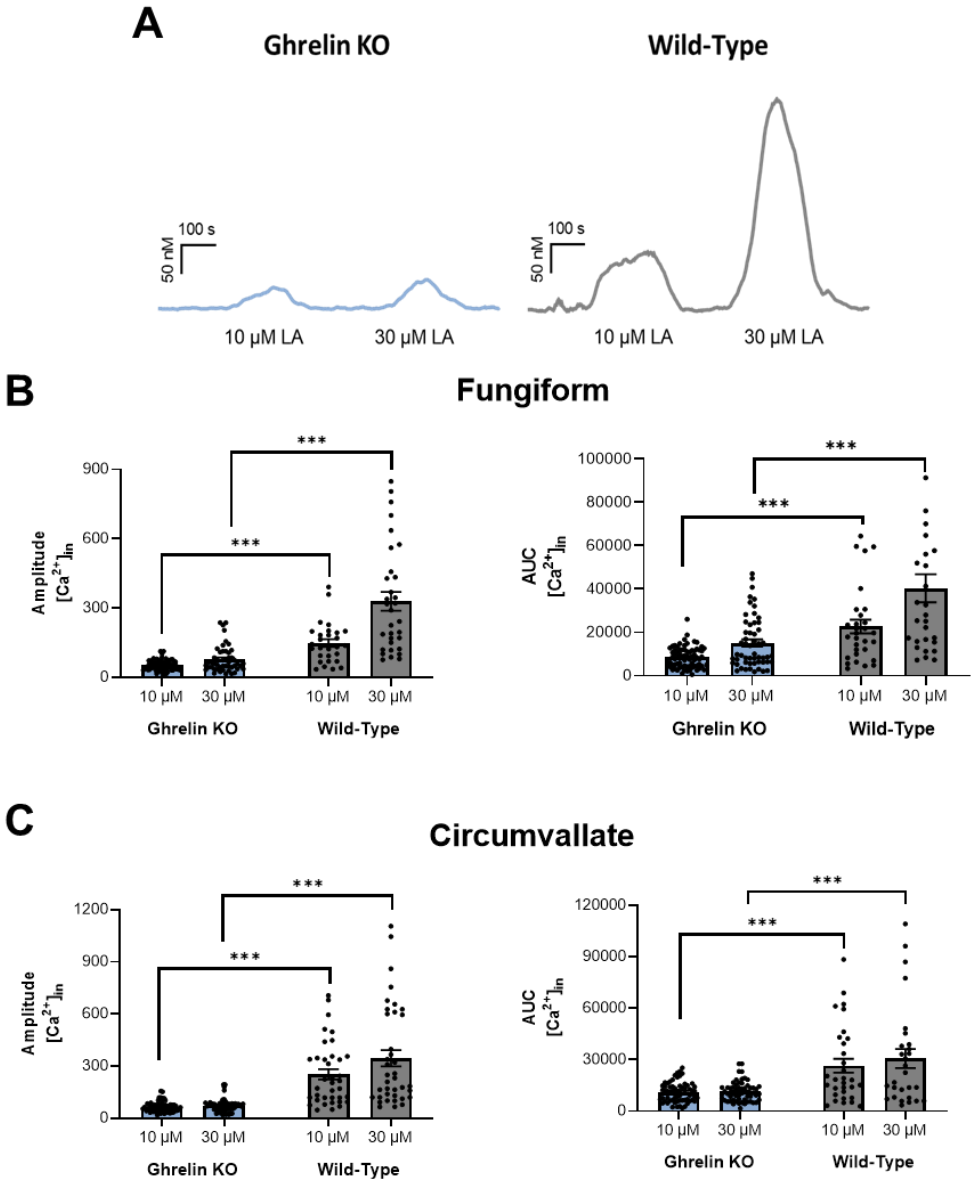

Figure 13: *Ghrl*$^{-/-}$ taste cells have lower responses to LA.

Ratiometric calcium responses to LA in *Ghrl*$^{-/-}$ and wild-type mice. **A)** Representative calcium traces from a fungiform taste cells of *Ghrl*$^{-/-}$ and wild-type mice. **B)** Peak responses and area under the curve (AUC) were measured in taste cells of *Ghrl*$^{-/-}$ and wild-type mice. Taste cells from *Ghrl*$^{-/-}$ mice have lower calcium responses to 10 and 30 µM LA. **C)** Calcium responses in taste cells from the circumvallate of *Ghrl*$^{-/-}$ mice had lower responses to both concentrations of LA similar to the fungiform papillae. A 2-way ANOVA with Bonferroni's multiple comparisons was used for statistical analysis.***p<0.001.

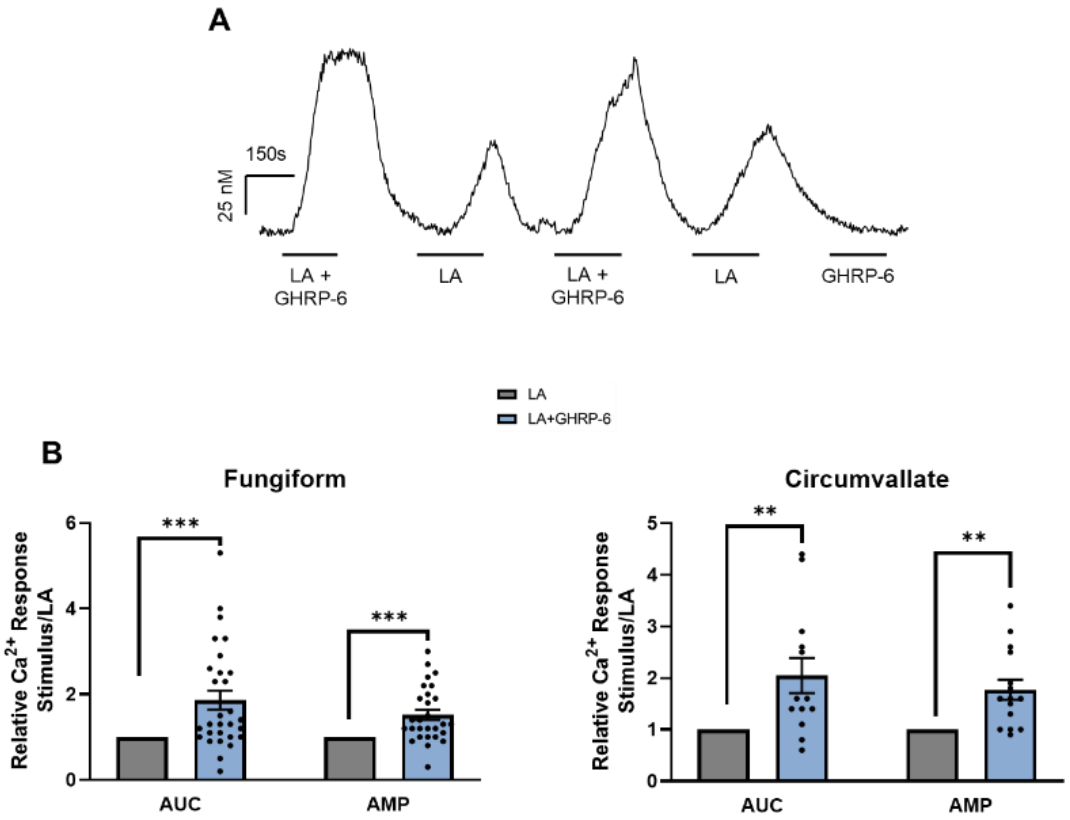

Figure 14: Acute stimulation of taste cells with GHSR agonist increases calcium responses to LA.

Ratiometric calcium responses to 30 µM LA alone or LA/100 nM GHRP-6 mixture in taste cells isolated from WT mice. AUC and peak (AMP) calcium responses were measured and set relative to 30 µM LA average response. **A)** Calcium trace showing fungiform taste cell responses to LA/GHRP-6 mixture, LA alone, and GHRP-6 alone. As shown in the figure GHRP-6 alone exhibited low to no calcium response in isolated taste cells. **B)** AUC and peak responses showed significant increases in calcium response when exposed to

LA/GHRP-6 mixture in fungiform taste cells. Sample sizes: 28-29 cells. Similar to the fungiform, circumvallate taste cells show increased calcium responses to the LA/GHRP-6 mixture compared to LA alone. Sample sizes: 13-15 cells. An unpaired student's t-test was used to determine statistical significance. *p<0.05, **p<0.01, ***p<0.001.

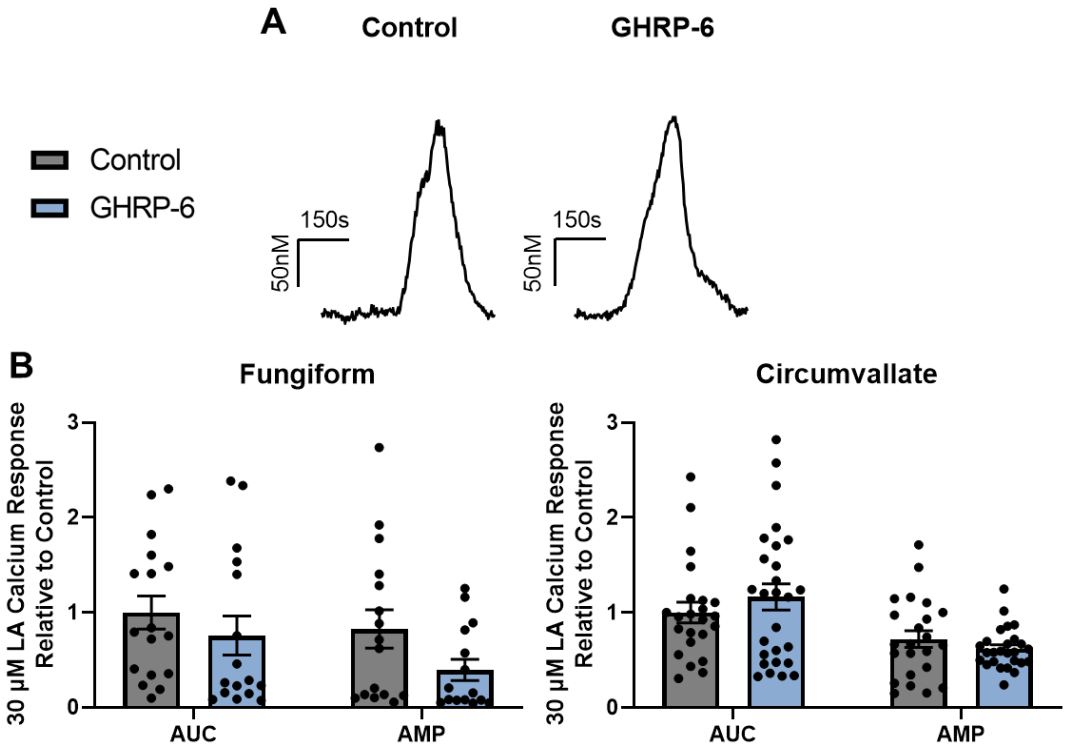

Figure 15: Wild-type taste cells incubated with GHRP-6 showed no significant changes to LA-induced calcium responses.

A) Calcium traces of taste cells responding to 30 µM LA incubated for 20 minutes in Tyrode's (control) or GHRP-6. **B)** AUC and amplitude (AMP) measurements for taste cells incubated in GHRP-6 or Tyrode's (control) in the fungiform and circumvallate papillae. No significant differences in calcium responses were observed in either the papillae regardless of treatment.

Discussion

Readily accessible calorically dense foods are thought to be a primary contributor to the rising incidence of obesity [25]. This food availability has led to an increased importance in understanding the mechanisms driving personal dietary choices. As such, several hormones secreted primarily from the digestive tract and adipose tissues have been implicated in regulating energy homeostasis [26]. Much of the research thus far has focused on the role of these hormones in the CNS and particularly within the hypothalamus. Less studied is the role of these hormones in regulating nutrient sensing in the taste system to drive or inhibit intake. Studies have shown that mice with impaired leptin receptors exhibit elevated neural responses and indicate suprathreshold preferences for sweet [7]. Furthermore, in WT mice leptin suppresses nerve responses to sweet tastants in a dose dependent manner via activation of outward potassium currents [5]. Glucagon-like peptide-1 (GLP-1) and its receptor are expressed in taste cells [6]. Examination of GLP-1 KO mice revealed reduced taste sensitivity and nerve responses to sweet tastants. In contrast, GLP-1 KO mice exhibited enhanced umami taste sensitivity [6, 8]. These data suggest hormone modulation is tastant specific.

Although ghrelin's role in energy homeostasis has been studied extensively, there has been limited investigation into its role in the taste system. Injections of exogenous ghrelin in rodent models have been shown to increase food intake and decrease locomotor activity [27]. Additionally, central administration of ghrelin altered macronutrient preferences driving fat intake over carbohydrates [28]. Previous data demonstrated that ghrelin, its receptor (GHSR),

and enzymes necessary for activation are present in the taste system [2]. GHSR is mainly co-localized with PLCβ2 expressing cells, the primary taste cells responsible for responding to fatty acids [29]. Understanding the role of ghrelin in fat taste may provide new insights into the mechanisms regulating the taste system and peripheral factors contributing to food intake.

This work utilizes global *Ghrl*$^{-/-}$ and WT mouse models to determine the effects of ghrelin on fat taste. Conditioned taste aversion assays and functional calcium imaging were used to establish the behavioral and cellular effects of ghrelin signaling in the oral cavity. Our data show *Ghrl*$^{-/-}$ mice exhibited reduced responsiveness to LA when compared to WT mice in a conditioned taste aversion assay. These data were further supported by significantly reduced responses to LA in *Ghrl*$^{-/-}$ compared to WT taste cells. We next utilized a GHSR agonist (GHRP-6) to better understand the acute role of ghrelin in fat taste signaling. To determine the temporal effects of ghrelin on taste cells, we performed two experiments. First, we incubated cells in GHRP-6 prior to imaging with LA. We found that pre-incubating WT taste cells in GHRP-6 led to no alteration in calcium responses to LA. Second, we co-stimulated taste cells with GHRP-6 and LA which led to significant increases in calcium responses. These results indicate that ghrelin acutely enhances taste cell responses to fatty acids, and that a lack of ghrelin leads to diminished fatty acid taste sensitivity.

A previous study has shown that *Ghsr*$^{-/-}$ mice have lowered aversions to high concentrations of salt and sour tastants during a brief access test [2]. Furthermore, *Ghrl*$^{-/-}$ mice also showed a reduced aversion to high concentrations of salt, though no changes in sour tastants were observed [1]. Both *Ghsr*$^{-/-}$ and *Ghrl*$^{-/-}$ mice showed no alterations in sweet and umami tastes

[1, 2]. Additionally, *Ghrl*$^{-/-}$ mice showed a reduced intralipid responsiveness in a brief access test [1]. These data suggest ghrelin's role in the taste system is tastant specific. Our findings that *Ghrl*$^{-/-}$ mice have a reduced detection of LA compared to WT counterparts builds upon previous results and confirms a role of ghrelin in fatty acid taste signaling.

To further investigate the cellular underpinnings of these behavioral changes we examined calcium responses in taste cells isolated from *Ghrl*$^{-/-}$ and WT mice. Our data showed significant reductions in calcium responses to LA in *Ghrl*$^{-/-}$ compared to WT mice. These changes may be due to a significant downregulation of the two primary fatty acid receptors *Cd36* and *Gpr120* as observed by Cai, et al., (2013) in *Ghrl*$^{-/-}$ mice. Interestingly, they found no such changes in expression of *Trpm5*, a calcium-dependent sodium channel involved in the transduction of fat, bitter, sweet, and umami tastes [22, 30, 31]. The lack of change in *Trpm5*, paired with co-localization of GHSR primarily with Type II taste cells, suggests ghrelin's role in these cells may be specific to fatty acids as there appear to be no apparent changes in umami or sweet taste pathways.

Studies in WT mice have explored differences in male and female fat taste signaling with females exhibiting significantly greater sensitivity to LA. Additionally, estradiol modulated taste cell responses to fatty acids across the estrous cycle. These changes were largely dependent upon G Protein-Coupled Estrogen Receptor 1 (GPER1) [32]. Research has also shown interactions between estrogen signaling and ghrelin. Weight gain in ovariectomized mice is largely mitigated in *Ghsr*$^{-/-}$ ovariectomized mice. Further examination revealed that both endogenous and exogenous administration of estrogen inhibits elevated food intake induced by ghrelin [33].

Moreover, *Ghsr*$^{-/-}$ females were shown to have diminished fat taste responsiveness compared to WT mice, but these effects were largely unseen in *Ghsr*$^{-/-}$ male mice [29]. This suggests that ghrelin/GHSR signaling in fat taste is sex dependent. Future studies in females are needed to examine the modulatory role of ghrelin and potential interactions with estradiol signaling in fat taste.

Another potential avenue of future research examines the role of ghrelin in obesity. Obesity results in several physiological changes including dysregulation of many of the hormones responsible for energy homeostasis, such as ghrelin. Obese individuals exhibit lower fasting ghrelin levels when compared with lean individuals [34]. Our data, in conjunction with others, demonstrate the modulation of the taste system as an ancillary role for ghrelin in altering food intake. Based on our data we would assume that lower circulating levels of ghrelin found in obesity would result in decreased sensitivity to fatty acids. In this circumstance it is reasonable to theorize that individuals may consume higher concentrations of fat to compensate for the diminished detection of fatty acids. Future studies examining the role of ghrelin in fat taste during an obesogenic state are needed to determine how it contributes to the maintenance of obesity. A greater understanding of this relationship could provide potential therapeutic targets to aid in reducing caloric intake while maintaining taste quality.

In conclusion, our studies show that the presence of ghrelin in the oral cavity enhances fatty acid responses. Behavioral assays showed that mice lacking ghrelin have reduced responsiveness to fatty acids. In conjunction with these findings, cellular assays showed *Ghrl*$^{-/-}$ taste cells had diminished calcium responses to fatty acids when compared to those from WT

mice. In keeping with these findings, our data show acute stimulation of taste cells with GHSR agonist and fatty acids enhances taste cell responses to fatty acids. Finally, this work provides a basis for the role of ghrelin in modulating fatty acid sensing in the peripheral taste system.

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CHAPTER 4: GHRELIN RECEPTOR GHSR IN FAT TASTE DETECTION

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Abstract

Ghrelin is a major appetite-stimulating neuropeptide found in circulation. While its role in increasing food intake is well known, its role in affecting taste perception, if any, remains unclear. In this study, we investigated the role of the growth hormone secretagogue receptor's (GHSR; a ghrelin receptor) activity in the peripheral taste system using feeding studies and conditioned taste aversion assays by comparing wild-type and *Ghsr* knockout models (*Ghsr*$^{-/-}$). Using GFP transgenic mice, we demonstrated GHSR expression in the taste system in relation to PLCβ2 (Type II taste cell marker) - and GAD67 (Type III taste cell marker) - expressing cells using immunohistochemistry. We observed high levels of co-localization between PLCβ2 and GHSR within the taste system, while GHSR rarely co-localized in GAD67-expressing cells. Additionally, following 6 weeks of 60% high-fat diet, female *Ghsr*$^{-/-}$ mice exhibited reduced responsiveness to linoleic acid (LA) compared to their wild-type (WT) counterparts, while no such differences were observed in male *Ghsr*$^{-/-}$ and WT mice. Overall, our results are consistent with the interpretation that ghrelin in the taste system is involved in the complex sensing and recognition of fat compounds. Ghrelin-GHSR signaling may play a critical role in the recognition of fatty acids in female mice, and this differential regulation may contribute to their distinct ingestive behaviors.

Introduction

Ghrelin is a peptide hormone primarily produced by the endocrine cells in the stomach, with its most established function associated with the stimulation of food intake [1]. Circulating ghrelin levels rise between meals, which peak during a fasting state and fall within one hour after a meal [2]. Instead of directly reflecting the physiological fasting level, ghrelin is generally considered to be a meal anticipation signal, a food-entrainable circadian clock in both humans and mice [3,4]. Despite this fact, ghrelin's actual role in both metabolic and feeding behaviors remains unclear. Interestingly, both fasted human and rodent models display elevated taste thresholds compared to their fed counterparts [5,6]. These studies are coincident with elevated ghrelin levels, suggesting a role for hormones such as ghrelin in impacting taste detection. However, this physiological connection between ghrelin and taste sensitivity, if any, is largely unexplored.

The current understanding of ghrelin's orexigenic and metabolic effects is focused on its actions in the hypothalamus of the brain, which has been eloquently reviewed [7]. Interestingly, experimenters using an alternate Ghsr knockout model observed that the ghrelin receptor (growth hormone secretagogue receptor (GHSR))-knockout (*Ghsr*$^{-/-}$) mice were resistant to high-fat diet (HFD)-induced obesity, with a reduction in food intake [8]. From these findings, one might predict that at least part of the diet-induced obesity (DIO) resistance observed in these *Ghsr*$^{-/-}$ mice was due to a lower HFD intake [8]. In contrast, the *Ghsr*$^{-/-}$ mice by Sun et al. [9,10], which are used in the present study, showed no significant changes in food intake after being on a 35% high-fat diet for 10 weeks. To determine whether a 60% HFD elicits caloric intake or body

weight differences in *Ghsr*$^{-/-}$ male and female mice, we performed a 6-week feeding study. Further, we investigated whether *Ghsr*$^{-/-}$ mice have an altered responsiveness to the chemical cues contained in dietary fat.

Ghrelin signaling elements have already been found in taste buds, the primary taste-sensing organelle in the peripheral sensory system. First, ghrelin can be produced by the salivary glands, with subsequent excretion of the hormone into saliva [11]. Second, both ghrelin and GHSR have been found in Type I, II, III, and IV taste cells [12,13]. Ghrelin signaling has been shown to alter sensitivities to certain tastants in the brief-access lickometer test. *Ghrl*$^{-/-}$ mice have reductions in both NaCl aversion and intralipid preference [12], and *Ghsr*$^{-/-}$ mice have reduced sensitivities to NaCl and citric acid [13]. While NaCl (salt) and citric acid (sour) sensitivities also contribute to the overall gustatory experience, the reduction in intralipid responsiveness in ghrelin KO mice suggests that the ghrelin/GHSR axis plays a role in the initial events surrounding the taste of fat.

Palatable foods rich in lipids are known to be attractive to humans and rodents. Lipids can be easily hydrolyzed to free fatty acids (FFAs) by lingual lipase provided by von Ebner's gland in the oral cavity [14,15]. Additionally, there is a sufficient concentration of free fatty acids present in fat-containing food where they act as gustatory cues for dietary fat [16–18]. Over the past 20 years, molecular mechanisms of FFA recognition in the taste system have slowly emerged, with delayed rectifier potassium channels (DRKs), fatty-acid sensitive G protein-coupled receptors (i.e., GPR40 and GPR120), and the fatty acid transporter CD36 as the top candidates for sensors of FFAs in the oral cavity [18–22]. The somatosensory system also contributes to the sensory detection of FFAs. Several FFAs of varying chain lengths have been

reported to be able to induce calcium responses in trigeminal neurons [23]. Therefore, the idea that fat sensing occurs during the initial events in peripheral chemosensory pathways, playing a significant role in the overall flavor experience in foods, is gaining increasing popularity. However, whether fat taste sensing can be modulated by other factors, especially those related to the modulation of food intake, remains unclear. Given that ghrelin KO mice previously showed a decrease in lipid taste responsiveness [12] and considering the observed reduction of HFD intake in *Ghsr*$^{-/-}$ mice, we hypothesize that loss of ghrelin receptors in mice leads to a reduction in the peripheral signals carrying fat taste information emanating from the oral cavity. To test this, we examined whether GHSR plays a role in taste-mediated fat detection by comparing taste responsiveness to LA, the prototypical fatty acid stimulus and one that is abundant in food) in *Ghsr*$^{-/-}$ animals and their WT counterparts using conditioned taste aversion (CTA) assays.

Materials and Methods

Animals and High-Fat-Diet Feeding

Eight-week *Ghsr*$^{-/-}$ and littermate wild-type (WT) control mice were obtained from the laboratory of Dr. Yuxiang Sun, where the mice were backcrossed with a C57BL/6J background over 10 generations [10]. All mice were bred at the Laboratory Animal Research Center (LARC), and all procedures were approved by the Institutional Animal Care and Use Committees (IACUC) of Utah State University and the University of Central Florida. Our goal was to assess the effects of loss of GHSR in mice that have been maintained on a high-fat diet. Therefore, *Ghsr*$^{-/-}$ and WT mice were fed a high-fat diet (HFD; 60% calories from fat, Research

Diets D12492) for 6 weeks, with ad libitum access to food and water. Body weights were recorded at the beginning of the feeding study and then weekly until the end of the study. MRI data were collected prior to the start of HFD feeding and immediately following completion of the feeding study. All mice were switched to a chow diet (Teklad rodent diet #8604) for a minimum of 2 days to facilitate the formation of a conditioned taste aversion to LA. A total of 37 WT mice (21 females, 16 males) and 29 *Ghsr*$^{-/-}$ mice (12 females, 18 males) were used.

Immunohistochemistry

To determine the expression pattern of GHSR in cell types within the taste bud, adult PLCβ2-GFP and GAD67-GFP transgenic mice on a C57Bl/6 background were used. The PLCβ2-GFP mice were a generous gift from Dr. Nirupa Chaudhari (University of Miami School of Medicine), and the GAD67-GFP mice were purchased from the Jackson Laboratory (Bar Harbor ME). The PLCβ2-GFP and GAD67-GFP transgenic mice were deeply anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). The tongues were excised and immersed in the same fixative for 1 h at room temperature first and cryo-protected in 30% sucrose in phosphate-buffered saline (PBS, pH 7.4) overnight. After cryoprotection, tissue sections containing circumvallate and fungiform papillae were embedded in OCT, frozen and sectioned at 20 µm using a cryostat, and mounted on Superfrost Microscope Slides (Fisher Scientific). After 3× 10 min rinses with PBS, the sections were blocked with 10% normal goat serum and 2% bovine serum albumin in PBST (0.05% Tween® 20) for 1 h and incubated with 1:500 rabbit GHSR (extracellular) (Alomone, Jerusalem, Israel) overnight in a blocking solution without Tween® 20. Following another 3× 10 min rinsing with PBS, the sections were incubated with 1:500 goat-anti rabbit Alexa Fluor 594 (Invitrogen) for 2

h in the same diluent as the primary antibody. To validate the specificity of our antibody, *Ghsr*$^{-/-}$ mice served as controls for the immunofluorescence assays and treated in a similar fashion as the experimental sections. Subsequently, all the sections were rinsed 3× for 10 min each in PBS, counterstained with 1:2000 Hoechst 33342 (Invitrogen, A10027) in PBS for 10 min for nuclei staining and mounted with Fluoromount G (Southern Biotech). We used a laser scanning confocal microscope (Zeiss, LSM710) equipped with 405, 488, 561, and 633 laser lines for images acquisition. Images were processed by ImageJ, and PLCβ2- and GAD67-positive taste cells were counted using the Cell Counter plug-in in ImageJ (V1.51s).

Conditioned Taste Aversion (CTA) Assay

The scheme of our CTA assay is shown in Figure 1. Four groups of mice (*Ghsr*$^{-/-}$ females and males, WT females and males) were used in the study. Each group was further divided into two subgroups to receive either LiCl (experimental manipulation, CTA) or NaCl (control) injections with the following sample sizes that successfully completed training: *Ghsr*$^{-/-}$ female LiCl (n = 7), NaCl (n = 4); *Ghsr*$^{-/-}$ male LiCl (n = 10), NaCl (n = 8); WT female LiCl (n = 9), NaCl (n = 6); and WT male LiCl (n = 8), NaCl (n = 7). The details of using CTA assays to assess the taste sensitivity were described previously [24]. Briefly, the whole paradigm consisted of three stages: training, conditioning, and testing. Mice had ad libitum access to water until 24 h prior to the first training day, when mice were started on a 23.5 h water restriction schedule for the whole duration of the experiment. On water-restricted days, 2 h after the start of training/conditioning/testing, animals were given 30 min access to water to facilitate rehydration. Training days were designed to familiarize mice to the lickometer chamber and testing procedures using water as the stimulus for the training trials (MS-160 Davis Rig gustatory

behavioral apparatus, DiLog Instruments, Tallahassee, FL). Training was followed by three conditioning days, where animals were trained to avoid the conditioned stimulus (100 μM LA). Briefly, on each conditioning day, mice were first given 5 min access to 100 μM LA. Once the mice stopped licking, they were given the same solution orally with syringes. Immediately after the intraoral application, either 150 mM LiCl or 150 mM NaCl (control) was administered through intraperitoneal injections (20 mL/kg body weight). All mice receiving LiCl injections showed behavioral signs of gastric malaise within 10 min of the injection. There were three testing sessions (days 0, 1, and 2) performed. Day 1 data were reported, when mice were behaving more consistent after day 0, where significant neophobia was evident across all stimulus classes, but the associated aversion had not weakened. On the testing days, 9 bottles (8 stimuli and 1 water) were used on a Davis rig. To reduce olfactory cues, a fan was placed near the chamber to provide constant airflow and to serve as white noise. The effectiveness of the fan was evident as mice rarely accessed the spout without initiating licking behavior. The test session included 2 blocks of 9 trials (8 stimuli plus 1 water) with stimulus durations of 5 s, a water rinse of 2 s, and wait times for the first lick of 150 s. The stimulus order within each block was randomly assigned. Total numbers of licks per stimulus were summarized across the two trials per test session and normalized using a lick ratio (licks per test stimulus/licks to water) in order to account for individual variances in the water-restricted motivation across the mice. Zero lick trials, while rare, were not included in subsequent analyses. Thus, all mice included in the data analysis sampled each stimulus at least once during each daily test session.

Stimuli

All taste stimuli were prepared from reagent grade chemicals and presented at room temperature. In addition to water, there were 8 test stimuli in the study, which consisted of 0.1, 1, 3, 10, 30, and 100 μM LA; 100 mM sucrose; and 3 mM denatonium benzoate. All LA solutions were made fresh daily on conditioning/testing days. Sucrose and denatonium benzoate were made fresh on day 0 of testing.

Statistics

The normalized lick rates of female and male WT or *Ghsr*$^{-/-}$ mice were examined using two-way ANOVA treating the unconditioned stimulus (LiCl or NaCl) and days (day 1 or day 2) as between-subject variables. Test solutions (6 concentrations of LA) were treated as within-subject variables. The simple effects within test solutions were corrected with Bonferroni's multiple-comparison test. Results are presented as the mean ± SEM. For body weights and MRI analysis, the two-way ANOVA method with Bonferroni's multiple-comparison test was used for correcting multiple comparisons. Unpaired t-tests were used in food intake analysis. The alpha value was set as 0.05. All the analyses were done using GraphPad Prism 7.

Results

GHSR Is Expressed Predominantly in Type II Taste Cells

Although it was previously reported that the GHSR antibody co-labels with markers from all taste cell types [12,25], here, we examined cell-type specific extracellular GHSR expression, again using PLCβ2-GFP and GAD67-GFP mice, which faithfully label Type II and Type III

cells. As shown in Figure 16A–C, GHSR was expressed in some but not all PLCβ2-positive Type II cells from circumvallate papilla. In contrast, it was almost completely absent in GAD67-positive Type III cells from circumvallate papilla (Figure 16D–F, Table 2). Immunohistochemistry from fungiform papillae showed a similar pattern (Figure 17). After counting GHSR and PLCβ2 or GHSR and GAD67 co-expression cells, we found that in circumvallate papilla, 71.1% of GHSR cells were Type II and 2.9% were Type III, while in fungiform papilla, 100% of GHSR cells that we counted were Type II and 4.2% were Type III (Table 2). This indicates that GHSR is expressed mainly in Type II and possibly in Type I or other supportive basal cells but rarely in Type III cells. We compared the GHSR expression pattern in both sexes of mice; no obvious differences were observed.

Ghsr$^{-/-}$ Males and Females Express Divergent Metabolic Phenotypes

Ghsr$^{-/-}$ and WT males and females were placed on 6 weeks of 60% high-fat diet (HFD) feeding. Female mice showed no significant differences in weight gain ($F(1, 217) = 0.5382$, $p > 0.05$) (Figure 18A). In contrast, however, *Ghsr$^{-/-}$* males gained less weight on the HFD than WT males ($F(1, 224) = 11.15$, $p < 0.01$) (Figure 18C). While they did not gain weight, *Ghsr$^{-/-}$* females consumed less HFD than their WT counterparts (WT 82.4 ± 0.9 g vs. Ghsr-/- 78.6 ± 1.5 g, $p < 0.05$) (Figure 18B). No significant changes in food consumption were observed between WT and *Ghsr$^{-/-}$* males (WT 93.8 ± 1.7 g vs. *Ghsr$^{-/-}$* 89.8 ± 1.3 g, $p > 0.05$) (Figure 18D). Studies have seen a similar metabolic phenotype for these *Ghsr$^{-/-}$* males where they show reduced body weight but similar HFD consumption [9] These metabolic trends were further observed in the MRI data where no significant changes were found between WT and *Ghsr$^{-/-}$* females in fat, lean,

or water mass ($F(1, 93) = 0.2414$, $p > 0.05$), (Figure 19A). *Ghsr*$^{-/-}$ males, however, showed a significant decrease in fat mass but not in water or lean mass ($F(1, 96) = 13.14$, $p < 0.001$) (WT 4.8 ± 0.4 vs. *Ghsr*$^{-/-}$ 3.0 ± 0.4 $p < 0.01$; Figure 19B).

Female Ghsr$^{-/-}$ Mice Show Reduced Avoidance to LA in CTA Assays

Since *Ghsr*$^{-/-}$ mice are known to have altered feeding behavior and metabolic status, we hypothesized that the taste detection of fat contributes, at least in part, to this phenomenon by altering fatty acid responsiveness at the peripheral level. Therefore, we performed brief-access behavioral assays after forming a CTA to LA (conditioned stimulus, 100 μM LA) to investigate the alteration of taste responsiveness to LA in both sexes of *Ghsr*$^{-/-}$ and WT mice.

Using the CTA assay with 100 μM LA as the conditioned stimulus, the WT female mice developed an aversion to LA at concentrations as low as 10 μM ($F(1, 78) = 51.71$, $p < 0.0001$) (Figure 20A). In contrast, *Ghsr*$^{-/-}$ female mice did not develop a significant aversion to LA ($F(1, 54) = 3.085$, $p > 0.05$) (Figure 20C), though there was evidence of an aversive pro-file at higher concentrations. These findings suggested that the LA taste responsiveness in female *Ghsr*$^{-/-}$ mice was reduced compared to the WT controls. Due to our IHC findings showing high levels of co-localization between GHSR and PLCβ2 (Type II cells), we used two other G-protein-mediated tastants requiring PLCβ2, bitter and sweet, to test the over-generalization of LA aversion to other tastants. The preference for the sweet stimulus sucrose and the rejection of the bitter stimulus denatonium benzoate showed no differences between the *Ghsr*$^{-/-}$ and WT animals (WT females ($F(1, 28) = 3.097$, $p > 0.05$); *Ghsr*$^{-/-}$ females ($F(1, 18) = 0.7361$, $p > 0.05$)) (Figure 20E).

Interestingly, the male *Ghsr*$^{-/-}$ mice did not display the reduced aversion to LA, as shown in the female *Ghsr*$^{-/-}$ mice, which corresponds with similar high-fat diet intake among the *Ghsr*$^{-/-}$ and WT males. As shown in Figure 20B, WT mice developed a normal taste aversion to LA, starting from 10 µM, and male *Ghsr*$^{-/-}$ mice presented a similar trend in LA aversion. As shown in Figure 20D, male *Ghsr*$^{-/-}$ mice showed evidence of aversion to LA, beginning at concentrations of 10 µM (WT males ($F(1, 78) = 38.12$, $p < 0.0001$); *Ghsr*$^{-/-}$ males ($F(1, 96) = 55.72$, $p < 0.0001$). These data suggest that reduction in LA taste responsiveness in *Ghsr*$^{-/-}$ mice is restricted to female mice, as in the case of females, loss of GHSR did not affect behavioral responses to either sucrose or denatonium in the CTA assay (WT males ($F(1, 26) = 0.5446$, $p > 0.05$; *Ghsr*$^{-/-}$ males ($F(1, 32) = 3.247$, $p > 0.05$) (Figure 20F).

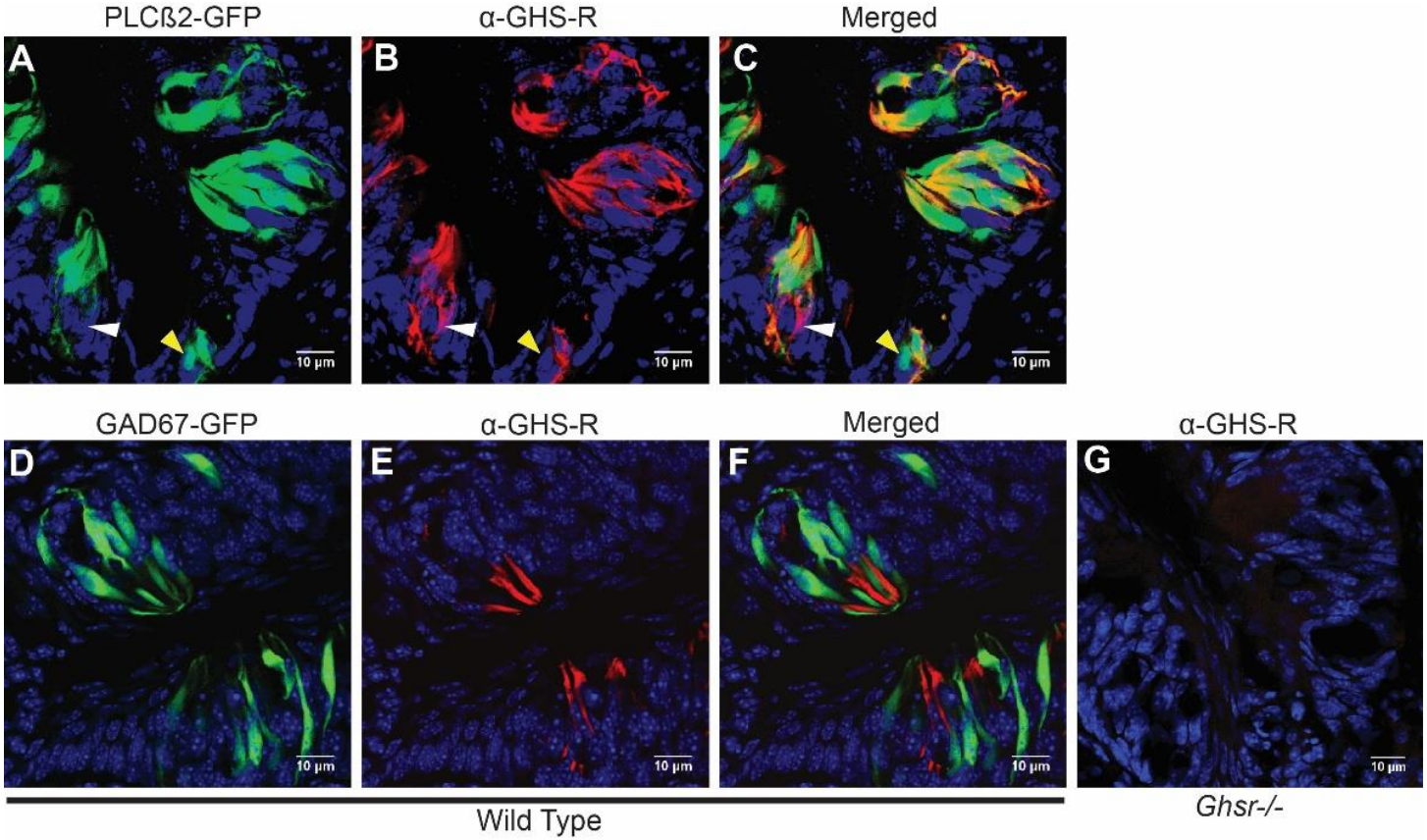

Figure 16: Growth hormone secretagogue receptor (GHSR) is expressed in Type II but rarely in Type III taste cells of circumvallate papillae.

(A–C) PLCβ2-GFP, green; anti-GHSR, red; and merged images, respectively. In **(C)**, the yellow arrow points to a representative taste cell that expresses PLCβ2 but not GHSR, and the white arrow highlights a PLCβ2-negative, GHSR-positive taste cell. **(D–F)** GAD67-GFP, green; anti-GHSR, red; and merged images, respectively. **(G)** Anti-GHSR antibody incubated on a representative section of circumvallate papillae from a GHSR-deficient mouse (negative control). Nuclear staining is shown in blue in all figures.

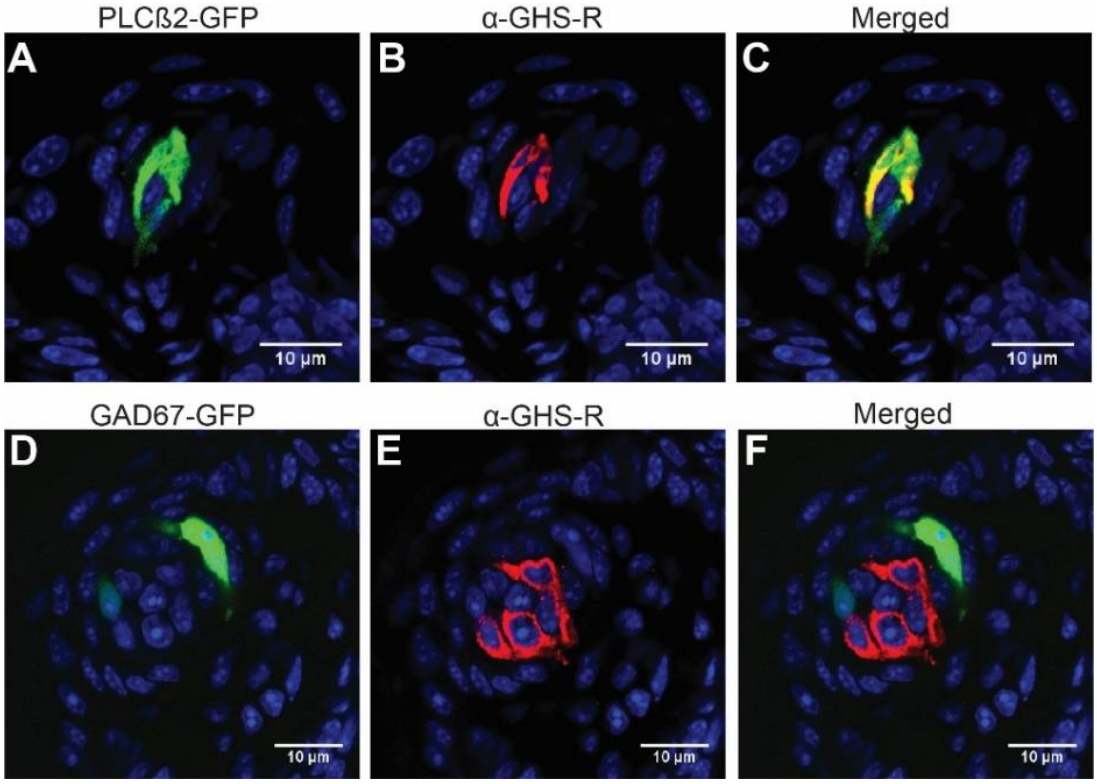

Figure 17: GHSR is expressed in Type II but rarely in Type III taste cells of the fungiform papillae.

(A–C) PLCβ2-GFP, green; anti-GHSR, red; and merged images, respectively. **(D–F)** GAD67-GFP, green; anti-GHSR, red; and merged images, respectively.

Table 2: Relative proportion of Type II (PLCß2-positive) and Type III (GAD67-positive) taste cells expressing GHSR.

	PLCß2-GFP, n	**GHSR(+), n**	**Co-expressing, n (%)**
Circumvallate	101	97	69 (71.1)
Fungiform	12	8	8 (100)
	GAD67-GFP, n	**GHSR(+), n**	**Co-expressing, n (%)**
Circumvallate	114	103	3 (2.9)
Fungiform	9	24	1 (4.2)

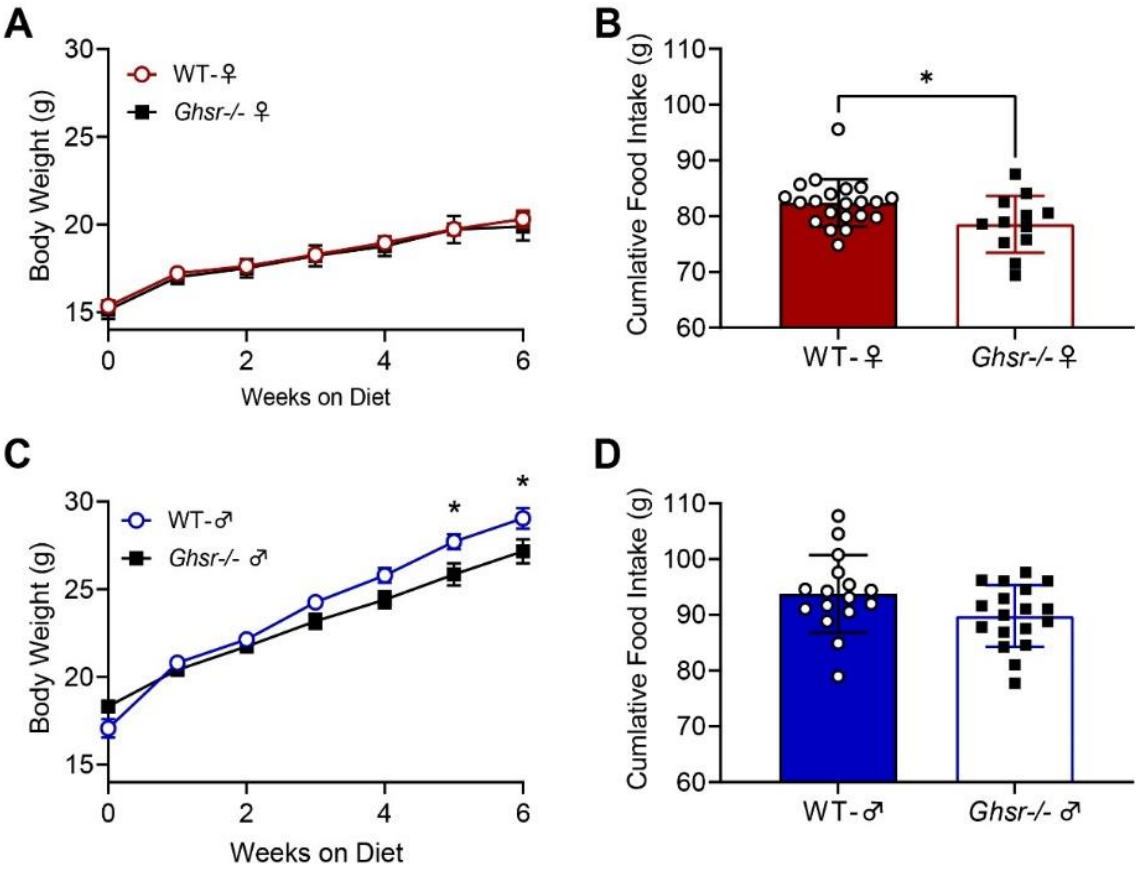

Figure 18: Body weight and food intake in wild-type (WT) and GHSR-deficient mice on a high-fat diet.

While not showing a significant difference in body weight **(A)**, *Ghsr*$^{-/-}$ females consumed less compared to WT females **(B)**. Alternatively, male *Ghsr*$^{-/-}$ mice **(C, D)** showed a decrease in body weight by week 5 of the HFD and no significant differences in food intake compared to their WT counterparts. * $p < 0.05$.

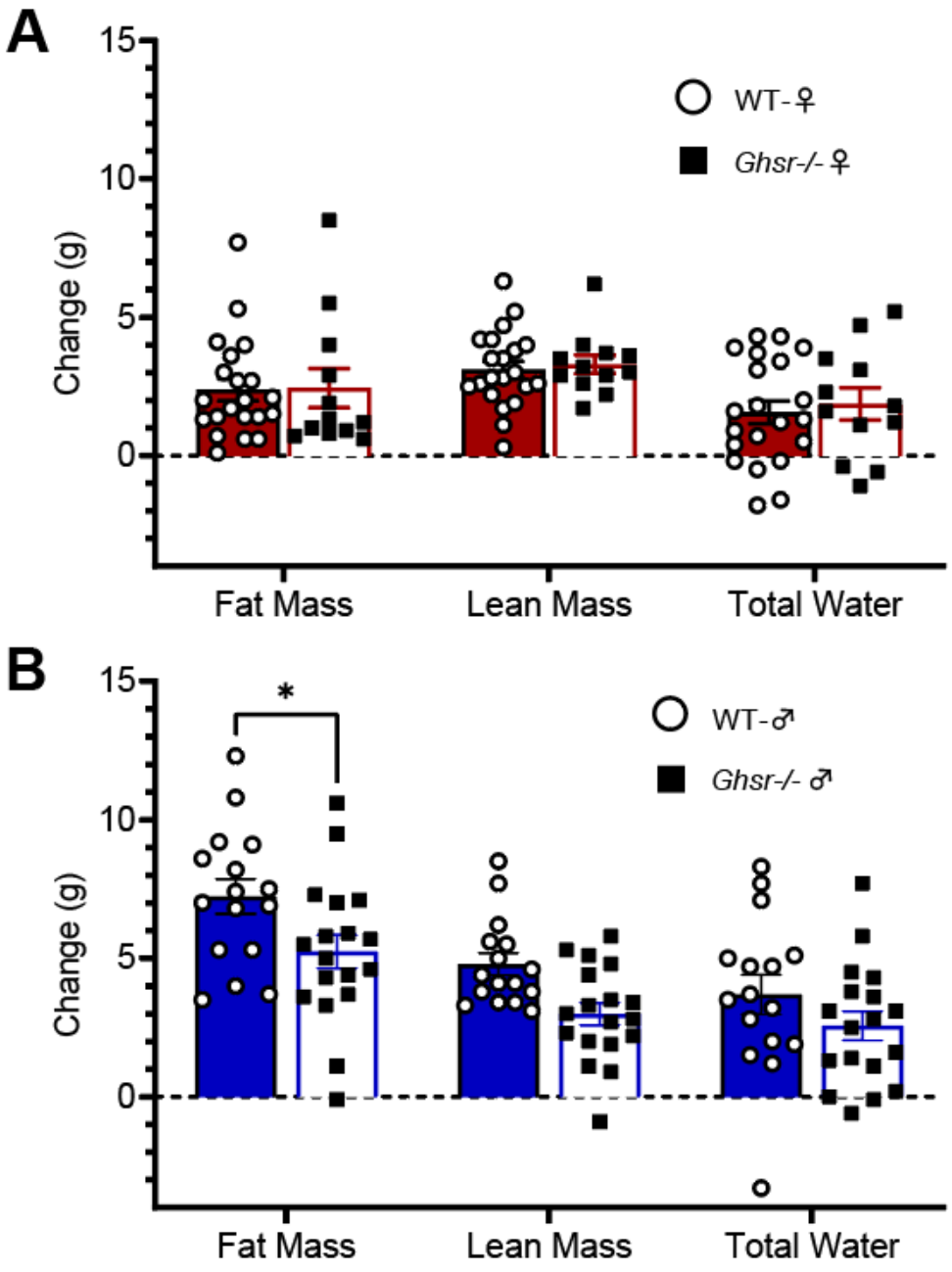

Figure 19: Body composition changes in WT and *Ghsr*$^{-/-}$ mice on a high-fat diet.

Changes in body compositions calculated from MRI data collected before the HFD and after 6 weeks of the HFD. **(A)** No significant changes in body composition were found in WT and *Ghsr*$^{-/-}$ females on 6 weeks of the HFD. **(B)** WT males gained more fat mass on 6 weeks of HFD compared to *Ghsr*$^{-/-}$ males.* $p < 0.05$.

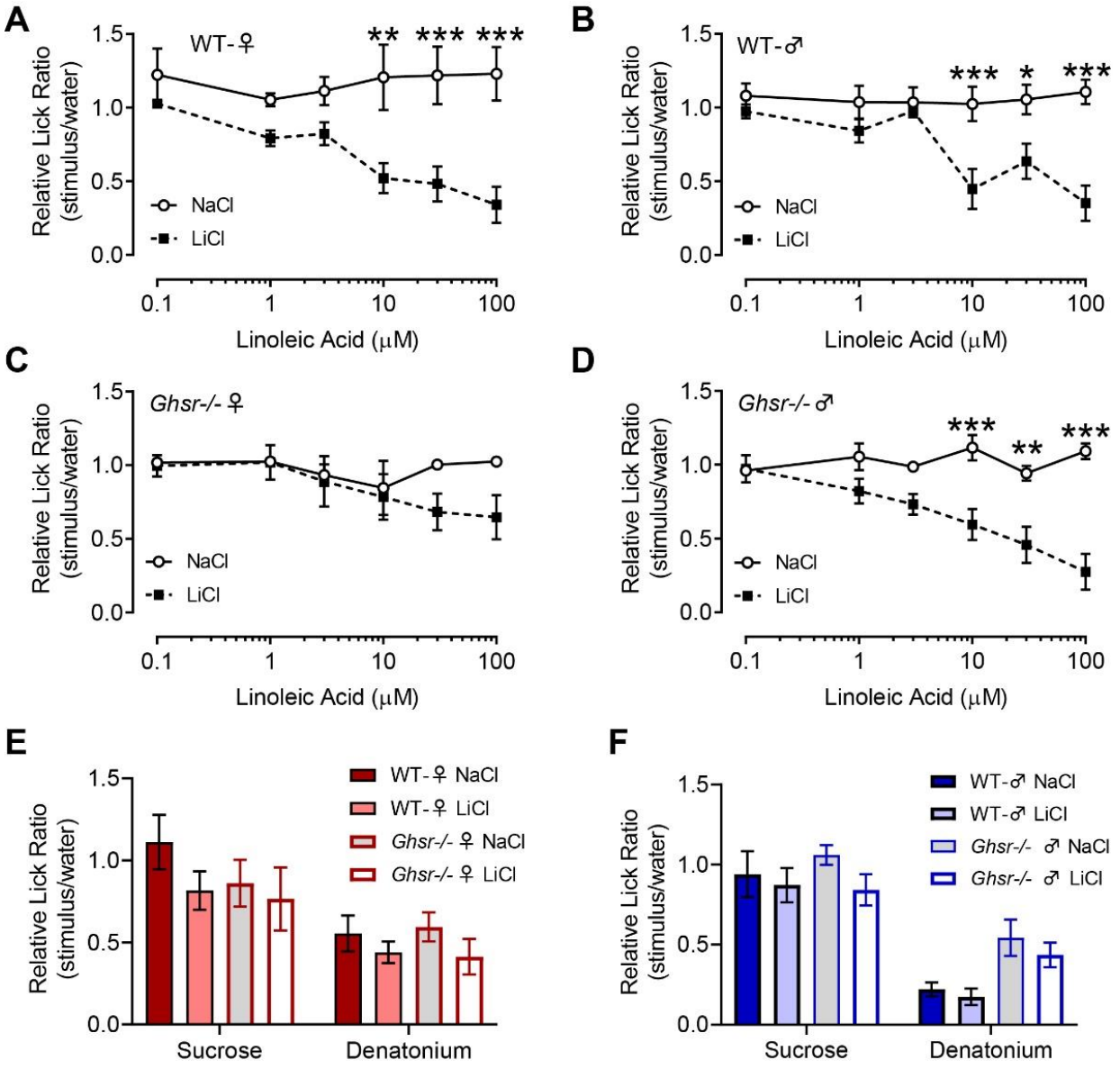

Figure 20: LA responsiveness assessed in a conditioned taste aversion assay showed changes in *Ghsr*$^{-/-}$ female mice.

(A) WT female mice (n = 15) revealed a significant aversion to LA at 10 μM, similar to that seen in WT males (n = 15) **(B)**. **(C)** *Ghsr*$^{-/-}$ females (n = 11) showed no significant differences in the LiCl compared to the NaCl group across all concentrations of LA. **(D)** male mice lacking GHSR (n = 18) showed aversion at 10 μM LA, similar to WT mice. WT and *Ghsr*$^{-/-}$ females **(E)** and males **(F)** exhibited similar lick ratios to the control solutions, sucrose (100 mM), and denatonium benzoate (3 mM). * $p < 0.05$, ** $p \leq 0.01$, and *** $p < 0.0001$.

Discussion

It is well known that numerous hormones regulate eating behaviors through higher level processing in the brain. However, many of these same hormones, like ghrelin, are present in the circulatory system and have secondary targets throughout the peripheral systems involved in metabolism and food intake. A recent study has shown that neuronal specific deletion of GHSR alone is able to prevent HFD-induced obesity in male mice [26]. Additionally, ghrelin has been shown to interfere with eating behavior at many levels. Bitter taste receptors and α-gustducin stimulate ghrelin secretion in the stomach, promoting consumption and then later delaying stomach emptying [27]. Centrally administered ghrelin (intracerebroventricular or intraventral tegmental area) acutely (3–6 h) increases chow and lard intake but not sucrose intake [28]. On the other hand, peripheral ghrelin injections (intraperitoneal) increase saccharin ingestion for 4 h post-injection [29]. While research has focused mainly on the role of ghrelin in macronutrient and caloric intake, less research has been done to understand whether the contributing role of ghrelin in orexigenic behaviors is due to manipulation of nutrient detection in the taste system. To better understand its role in taste (more specifically fat taste detection) and to limit off target effects of ghrelin, we used a global *Ghsr*$^{-/-}$ mouse model to focus specifically on the ghrelin–GHSR pathway.

In this report, we examined the effects of the ghrelin receptor, GHSR, on the taste system. We showed that GHSR is expressed in PLCβ2-positive Type II taste cells but rarely in GAD67-positive Type III taste cells, indicating possible interactions with sweet, bitter, umami, and fatty acid taste sensing. In addition, previous data by Sun et al. demonstrated that on a 35%

HFD, *Ghsr*$^{-/-}$ males had caloric intake and body weight similar to WT counterparts [9]. To better understand metabolic changes in *Ghsr*$^{-/-}$ males and to further understand whether there are sex-dependent differences in these *Ghsr*$^{-/-}$ mice, we performed feeding studies and body composition measurements on both male and female *Ghsr*$^{-/-}$ and WT mice. Behaviorally, we observed differing roles of ghrelin among the sexes in *Ghsr*$^{-/-}$ mice. Following 6 weeks of a 60% HFD, *Ghsr*$^{-/-}$ males had significantly less fat mass compared to their WT counterparts, with no change in HFD intake. Additionally, *Ghsr*$^{-/-}$ female mice consumed less food than their WT counterparts, with no significant differences in weight gain or fat mass. Sex-dependent differences were also present in conditioned taste aversion assays, where *Ghsr*$^{-/-}$ females showed reduced aversion to LA but *Ghsr*$^{-/-}$ males showed no significant changes compared to WT mice.

Previous data published by Shin et al. reported the expression of ghrelin and GHSR in all taste cell types using double-labeling of the GHSR antibody and other taste cell-type-specific antibodies [13]. Our data support their finding that GHSR co-localizes in PLCβ2 (Type II)-expressing cells. Additionally, our data show GHSR expressed in a subset of cells that did not express PLCβ2 (about 30% of GHSR-expressing cells) and had little co-localization with GAD67 (Type III), supporting their findings of GHSR in non-PLCβ2 expressing cells such as Type I and basal taste cells. Contrary to their findings, we observed little expression of GHSR in Type III cells. These differences, however, could be due to the use of different Type III markers (NCAM vs. GAD67) or a different methodology, as our study used a genetically expressed GFP under the control of a Type III-specific gene (GAD67), and the previous study used dual-labeling of a Type III marker and GHSR. Our data provide new insight into the potential role of GHSR in taste signaling. Relatively high levels of co-expression of GHSR and PLCβ2 suggest a more

targeted role of ghrelin/GHSR in the taste system, as Type II cells respond to G-protein-mediated tastes: bitter, sweet, umami, and fat.

CD36 and GPR120 are thought to be the primary receptors for the long-chain polyunsaturated fat taste pathway. The majority of ghrelin-expressing cells of the stomach express GPR120, and both GPR120 and long-chain unsaturated fatty acids have been shown to inhibit ghrelin secretion [30–32]. In addition, ghrelin-deficient mice exhibit decreased Gpr120 expression in isolated taste buds [12]. These data together suggest a necessary crosstalk between ghrelin and fatty acid pathways to maintain metabolic balances. While it has been shown that fatty acid activation of GPR120 inhibits secretion of ghrelin, it may be that ghrelin also plays a role in sensing pathways for fatty acids in the oral cavity to help further drive metabolic needs. Future studies are needed to determine how or whether ghrelin/GHSR pathways interact with GPR120 to regulate both ghrelin secretion and fat taste sensitivity.

Cai et al. reported that ghrelin (*Ghrl*$^{-/-}$) and ghrelin O-acyltransferase-knockout (*Goat*$^{-/-}$) male mice had reduced intralipid (a fat emulsion) sensitivity but did not appear to have altered preference for intralipid compared to their WT counterparts [12]. Additionally, they showed that ghrelin-deficient mice had reduced expression of fatty acid receptors (CD36 and GPR120) thought to play a crucial role in fat taste transduction, while they found no significant expression changes in the components of bitter, sweet, and umami taste pathways. Following this and other studies suggesting that *Ghsr*$^{-/-}$ mice are resistant to high-fat diet-induced obesity [8], we focused on the role of GHSR in lipid sensing using *Ghsr*$^{-/-}$ mice. Lipids can be easily hydrolyzed to FFAs by lingual lipase, and FFAs exist in food at concentrations that can be detected by taste cells. For rodents, fatty acid solutions by themselves are less preferred [24]. To better separate the

sensitivity differences between *Ghsr-/-* and WT mice, we used CTA assays to assess the taste responsiveness to LA. Our results demonstrated that ghrelin-GHSR signaling is involved in the lipid/fatty acid taste thresholds in mice, but future studies are still needed to explore additional tastants. While our CTA assay did not show changes in the LA thresholds of male mice, we did observe changes in the apparent LA thresholds of female mice. This is interesting in light of our data showing that loss of GHSR in males leads to a reduction in body fat (cf. Figure 5B) but does not do so in females (Figure 5A). This suggests that there are significant sex differences in fatty acid taste and its metabolic regulation, a finding that has recently received additional empirical support [33]. Our results in *Ghsr*$^{-/-}$ mice, coupled with those of Cai et al. in ghrelin KO mice [12], may provide further insight into the role ghrelin plays in the taste system and whether it is through the GHSR signaling pathway or through alternative mechanisms. Therefore, while it is clear from our data that ghrelin receptors are pre-sent in the peripheral taste system, whether the effects of loss of GHSR in the present study are attributable to a direct action on the gustatory system or whether its regulatory effect is restricted to the descending central pathways remains unknown. Additional functional and mechanistic studies are needed to clarify the extent to which peripheral ghrelin directly targets the taste system and, more specifically, the pathways devoted to fat taste.

An important finding in this study is that *Ghsr*$^{-/-}$ females demonstrated increased taste thresholds to LA, as assessed by CTA assays after the 6-week high-fat diet feeding, while male mice showed no evidence of such an effect. While limited publications discuss the role of ghrelin in the taste system, research has shown sex-dependent effects of ghrelin on feeding behavior. Clegg et al. [34] reported increased food intake during peripheral injection of ghrelin in male

rats, while no effects were seen in intact female rats. Additionally, females demonstrated reduced sensitivity to the orexigenic effects of centrally administered ghrelin. Furthermore, these sex-dependent effects of ghrelin were found to be estradiol mediated. Ovariectomized females displayed increased feeding in response to ghrelin; however, when given estradiol supplementation, the effects of ghrelin were again lost [34]. Previous studies also indicate that differences in taste preference exist between the two sexes. In a lickometer behavioral study using rats, ovariectomized female rats supplemented with estrogen responded to a lower LA + sucrose concentration than male counterparts [25]. LA can also increase the preference for lower MSG concentrations (40 mM) in male rats and higher MSG concentrations (100 mM) in female rats [35]. In one crowdsourcing human study, women and girls rated high concentrations of LA as more intense than men and boys [36]. Recently, it was shown that there are sex differences in fat taste detection and that estradiol acts as the key regulator in altering fatty acid taste responsiveness [33]. Females responded to lower concentrations of fatty acids than males, while loss of ovarian hormones reversed this effect by decreasing taste responsiveness to fat. Furthermore, fatty acid taste responsiveness varied significantly within the estrous cycle in females, where high levels of taste responsiveness coincided with high secretion of estradiol [33]. Of note, our CTA experiments illustrated similar fat taste thresholds between WT males and females, whereas significant differences were observed in previous studies [33,37]. A question of physiological interest is whether taste responsiveness is altered during the estrous cycle; therefore, it is possible that both the high-fat diet in our experiments and estrous cycle variation complicate apparent fat taste thresholds and contribute to these differences. Although the interplay between the effects of estradiol and ghrelin signaling in the taste system are

uncertain, our data suggest that ghrelin may play a significant role in fatty acid detection in females and the interaction of both endocrine hormones may contribute to the observed sex differences. While the beginning of these effects may be seen in the slight decrease in the caloric intake of *Ghsr*$^{-/-}$ females, longer-term food intake studies in females need to be performed to better understand whether these effects lead to significant behavioral changes. Additionally, these changes in fat taste responsiveness may play a more significant role in preference when mice are presented different tastants simultaneously but was not as apparent as only one choice (high-fat diet) was present.

Previous research shows that individuals with high fat sensitivity tend to consume less fat and gain less weight [38]. This suggests a negative correlation between fat taste threshold levels and food intake. However, we did not observe a similar pattern in *Ghsr*$^{-/-}$ females, as they showed decreased responsiveness to LA in the behavioral assay yet consumed slightly fewer calories than WT females. It is possible that much of the overall caloric reduction seen in these mice may be due to the central role of ghrelin/GHSR. Central administration of ghrelin has been shown to increase caloric intake by acting on neuro-peptide Y and agouti-related peptide [39]. This central role of ghrelin is well established and a potent driver of caloric intake. Central KO of GHSR may be obfuscating the behavioral impact of ghrelin/GHSR signaling that is present within the taste system. Further research is needed to better delineate the peripheral role of ghrelin/GHSR in the taste system with central ghrelin/GHSR signaling intact to better understand the importance of ghrelin signaling in the taste system.

In this report, we investigated ghrelin receptor expression patterns in taste cells and explored the change in LA taste thresholds and metabolic phenotypes in the presence and

absence of growth hormone secretagogue receptor (GHSR). Our results suggest that ghrelin-GHSR signaling may have a direct action on the peripheral taste system, independent of descending central pathways. Additionally, ghrelin-GHSR effects on the taste system appear to be sex specific, which may have important implications in differential weight regulation in men and women. Moreover, GHSR and estrogen receptor (ERα) are highly co-expressed in a number of hypothalamic regions, indicating a dual role of GHSR and ERα in mediating metabolic signals [40]. ERα is also expressed in taste cells [33], and it is possible that estradiol signaling through ERα is convergent with GHSR signaling in the taste system. These data help further elucidate the peripheral role of ghrelin in the taste system, likely linked to sex-dependent fatty acid taste pathways. Future studies exploring the mechanism by which ghrelin alters fat signaling in the taste system and its differential effects among the sexes will provide valuable insights into and understanding of the fundamentals of how endocrine factors affect taste perception and drive caloric intake.

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CHAPTER 5: CONCLUSIONS

Obesity is a complex disease with many contributing factors and associated diseases. The onset of obesity leads to an increased risk of cardiovascular disease, diabetes, and several types of cancer. While a positive energy balance is commonly thought to be the primary cause, other factors such as a genetic predispositions and hormonal imbalances, can be major contributors. The overarching goal of this dissertation was to understand how oral fatty acid sensitivity contributes to the onset and maintenance of obesity by altering fat intake. Understanding how fat taste is modulated by dietary fat intake and orexigenic hormones such as ghrelin provides insight into how hormonal and metabolic status may regulate fat intake through the peripheral taste system.

Dietary Fat Intake and Fat Taste

Obesity prevalence is increasing world-wide, with the U.S. currently leading in obesity prevalence (World Health Organization). Dietary changes are thought to be a major driver for this. These changes, commonly referred to as a "Western diet" include high intake of calorically dense foods such as fats and simple sugars. Previous research shows a negative correlation between fat intake and fat taste sensitivity [3-5] and research on weight status and fat taste thresholds demonstrate either no correlation or a negative correlation between BMI and fat taste [3, 4, 6-8]. The first studies in this dissertation (Chapter Two) examined the effects of increased dietary fat intake on fat taste thresholds. We sought to understand the role high fat diets play in changing fat taste. As such, we hypothesized that altering dietary fat composition would decrease

peripheral fat detection and thus increase fat intake and disrupt metabolic regulation. As expected, our results showed metabolic disruption with similar weight gain among all three high fat diets. Additionally, food intake was increased in all high fat diets compared to control diet. Among the high fat diets food intake increased as unsaturated fatty acids concentrations increased. We also observed significant increases in subcutaneous and gonadal adipose stores across all high fat diets. Mice on a high saturated fat diet (HSFD) did not have significant increases in visceral adipose stores, while both of the other two high fat diets showed a significant increase. Based on the literature and previous rodent data [9-11], we further hypothesized that taste cell responses to fatty acids would decrease in high fat diet fed mice and these changes would be further exacerbated as dietary unsaturated fatty acids increased. We also hypothesized that we would see a downregulation in fatty acid receptors and components of the fat taste pathway that correlated with the diminished cell responses. Contrary to expectations we observed increased expression of *Cd36* in high fat diet (HFD) and high unsaturated fat diet (HUFD) isolated taste cells. Additionally, we observed significant increases in fatty acid-induced inward currents among HFD and HUFD taste cells. While these increased inward currents did not result in statistically significant increases in membrane potential, both HFD and HUFD depolarization sizes trended larger than control diet taste cells. Interestingly these changes did not occur in Type II taste cells (previously thought to be the primary fatty acid responding cells). Further pharmacological manipulation revealed these inward currents did not appear dependent upon TRPM5 and were at least in part independent of CD36. Lastly, previous data in rats showing correlations between delayed rectifier potassium channels (DRK's) and fat taste preference where DRK channel block resulted in prolonged depolarization and thereby

enhancing cellular responses to fatty acids [12]. Based on these data we further hypothesized that mice fed a high fat diet would show a substantial decrease in the DRK currents blocked by fatty acids. However, in mice, we found little to no change in fatty acid block of DRK channels in high fat diet fed mice in all cell types and again in specifically Type II cells. These data suggest that taste cell responses in mice following high fat diet feeding increase in response to fatty acids. These changes appear to be dependent on the fatty acid type as observed cellular responses in HSFD mice were not as strong and did not result in statistical significance. Furthermore, these changes occur in a subset of taste cells not expressing PLCβ2, a Type II taste cell marker. Based off these findings additional research is needed determine which cell types are responsible for modulating fat taste in response to diet.

Fat taste modulation via Type III taste cells

Within the taste field current consensus is that Type II taste cells respond to G-protein mediated tastants and release of ATP from these cells activate Type III cells to transmit signals to nerve fibers [13, 14]. However, our data suggest that increased dietary fat intake leads to increased taste cell responses to fatty acids and these changes are largely unseen in Type II taste cells. Our data show unaltered calcium responses, inward currents, membrane potentials, and DRK channel block elicited by LA in Type II taste cells. Therefore, the observed increased currents observed in high fat diet taste cells suggest these changes are occurring in a cell type other than Type II taste cells. Recent data suggests that Type I, Type II, and Type III taste cell role may not be as distinct as once thought. “Broadly responding” Type III taste cells have been shown to respond to tastants previously reserved to Type II taste cells namely bitter, sweet, and umami [16]. All three of these tastants have previously been shown to use a similar G-protein

mediated pathway in Type II taste cells to that of fat. Additionally, preliminary data in our lab suggests Type III taste cells also respond to fatty acids. It is then possible that the observed inward currents observed may be in these broadly responding Type III taste cells. These cells have been shown to use the PLCβ isoform PLCβ3 rather than PLCβ2 which is used in Type II taste cells, potentially providing another avenue by which Type III taste cells are able to respond to tastants [16].

Based on findings in this dissertation we would expect that future experiments in Type III cells would reveal increased fatty acid taste cell responses induced by a high fat diet. Initial studies should focus on elucidating the fat taste pathway in Type III cells as it appears to be via a different mechanism than Type II cells. These results may also help to better our understanding of the role of CD36 and GPR120 in fat taste. Second, based on our finding's electrophysiological studies in Type III taste cells are needed to confirm if the observed inward currents are in Type III taste cells specifically and the molecular underpinnings of these changes.

Ghrelin and Fat Taste

It has been previously shown that circulating orexigenic and anorexigenic hormones play a key role in regulating food intake and metabolic status. Ghrelin has been shown to act on hypothalamic neurons to drive caloric intake, with high levels prior to mealtime and rapid postprandial decreases. These central actions of ghrelin paired with the rapid fluctuations in ghrelin associated with increased intake beg the question of ghrelin's peripheral effects to also drive ingestive behavior within the taste system. Therefore, we investigated the role of ghrelin

and its receptor, growth hormone secretagogue receptor (GHSR), in modulating fat taste sensitivity in rodents and the cellular basis of these changes.

Previous data has shown mice lacking ghrelin have decreased lipid responsiveness [9]. Based on these data along with the role of ghrelin in driving food intake we hypothesized that ghrelin's action in the taste system would be to enhance fat taste. Therefore, ghrelin knock-out (*Ghrl*$^{-/-}$) mice would be expected to exhibit diminished sensitivity to fatty acids. Using a conditioned taste aversion assay we found that *Ghrl*$^{-/-}$ mice had a significant decrease in fat taste sensitivity compared to WT mice. Consistent with this reduced fat taste sensitivity in *Ghrl*$^{-/-}$ mice, we also discovered LA elicited significantly lower calcium responses in their taste cells. Further examination in WT mice revealed that calcium responses to LA increased significantly when simultaneously perfused with a GHSR agonist (GHRP-6). These data show that *Ghrl*$^{-/-}$ mice have impaired sensitivity to fat taste and these changes are seen in taste cell responses. Additionally, we found that the enhancement of taste cell responses requires ghrelin be present at the time of cellular activation. Next, we characterized expression of *Ghsr* within the taste system and found that it is relatively highly co-localized to Type II taste cells (the taste cells primarily involved in fat taste). Providing further evidence that ghrelin/GHSR signaling is modulating fat taste responses. Following these data, we utilized a *Ghsr*$^{-/-}$ model to determine if these changes in fat taste sensitivity were specific to the ghrelin receptor. Previous data in these specific *Ghsr*$^{-/-}$ had found delayed changes to weight gain in males on a high fat diet but had yet to determine *Ghsr*$^{-/-}$ effects in females [11]. To determine the metabolic disturbance (if any) in these *Ghsr*$^{-/-}$ mice we performed a 6-week 60% high fat diet feeding study. Interestingly we found that *Ghsr*$^{-/-}$ females had reduced caloric intake but no significant changes in weight gain, while *Ghsr*$^{-/-}$ male

exhibited a reduced weight gain but no change in caloric intake. Surprisingly male *Ghsr*$^{-/-}$ mice showed similar fat taste sensitivity to WT males while female *Ghsr*$^{-/-}$ mice showed a reduced sensitivity with only a slightly aversive profile at high concentrations of LA. These reduced sensitivities in *Ghsr*$^{-/-}$ females may explain, in part, the reduced high fat diet intake of the *Ghsr*$^{-/-}$ females. While our data demonstrate ghrelin/GHSR signaling is involved in regulating fat taste how these changes occur remain largely unexplored. Further research is needed to understand the role ghrelin plays in the different sexes and how these effects are modulating fat taste.

Sex differences in ghrelin signaling

The differences in fat taste thresholds in males between *Ghrl*$^{-/-}$ and *Ghsr*$^{-/-}$ mice suggest ghrelin may be play dual roles in the taste system. One via its known receptor GHSR which appears to be sex dependent and another independent of GHSR. Studies by Dahir, et al., demonstrate sex differences in fat taste between males and females, and within the female estrous cycle driven by circulating estradiol levels [10]. Further studies investigating the sex dependent effects of ghrelin/GHSR signaling would clarify how fat taste is regulated in female mice and how these circulating hormones drive sensitivity and thereby intake. Initial studies focused on how/if fat taste is altered in *Ghrl*$^{-/-}$ female mice via conditioned taste aversion assays and functional cell-based assays will provide a better understanding of ghrelin's sex-dependent role in the taste system.

Ghrelin and obesity

Secondly, differences in fat taste thresholds of male *Ghrl*$^{-/-}$ and *Ghrl*$^{-/-}$ mice suggest a role for ghrelin independent of GHSR. Based on our changes in fatty acid taste responses suggest these actions may be through direct actions on Type II fatty acid cells. Further studies are needed

to elucidate the role of ghrelin on the fat taste pathway. Limited studies show a binding site for hexarelin (a synthetic ghrelin analog) on CD36 [17, 18]. Though not much is known about the role of ghrelin in fat taste interactions ghrelin may be mediating fat taste through regulation via CD36. Initial studies inhibiting CD36 while stimulating cells would quickly determine ghrelin's actions in fat taste are via CD36 or other potential targets.

Lastly several studies in humans show negative correlations between BMI and fat taste thresholds [5, 19, 20]. Yet we observed increased taste cell responses in mice given high fat diets. Our studies in ghrelin may provide insight as we demonstrated that a lack of ghrelin decreases fat taste sensitivity and obese individuals have been shown to have reduced circulating ghrelin levels [21]. Therefore, it is plausible that the observed threshold changes shown in obesity are a result of changes in ghrelin levels as opposed to direct changes to the fatty acid taste pathway. An initial study looking at how ghrelin supplementation alters fat taste thresholds in diet induced obese mice may provide preliminary insight into if these observed changes are ghrelin mediated.

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APPENDIX A: IACUC PERMISSIONS

8/6/2018

Timothy Gilbertson

Internal Medicine
6900 Lake Nona Blvd
Orlando, FL 32827

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Timothy Gilbertson:

This letter is to inform you that your following animal protocol was approved by the IACUC. The IACUC Use Approval Form is attached for your records.

Animal Project #: 18-22

Title: Multidisciplinary studies in nutrient chemoreception and food intake

First Approval Date: 8/6/2018

Please be advised that IACUC approvals are limited to one year maximum and must be renewed annually. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur.

If the protocol is over three years old, it must be rewritten and submitted for IACUC review and approval. A renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval.

This letter does not serve as Environmental Health and Safety (EHS) approval. EHS approval must be handled separately. EHS approval must be obtained prior to initiating animal use or procedures that need EHS approval. The contact phone number is 407-823-6300

Should you have any questions, please do not hesitate to call the office of Animal Welfare at (407) 882-1164.

Please accept our best wishes for the success of your endeavors.

Best Regards,

Cristina Caamaño
Associate Director
Office of Animal Welfare
Copies: Appropriate Facility Manager (when applicable)

THE UNIVERSITY OF CENTRAL FLORIDA
INSTITUTIONAL ANIMAL CARE and USE COMMITTEE (IACUC)
Approval to Use Animals

Dear Timothy Gilbertson,

Your application for IACUC Approval has been reviewed and approved by the UCF IACUC Reviewers.

Approval Date:	8/6/2018
Title:	Multidisciplinary studies in nutrient chemoreception and food intake
Department:	
Animal Project #:	18-22
Expiration:	8/6/2019

You may purchase and use animals according to the provisions outlined in the above referenced animal project.

Deborah A Altomare

Deborah Altomare, Ph.D.
IACUC Chair

APPROVAL OF IACUC PROTOCOL SUBMISSION

June 8, 2020

Timothy Gilbertson
407-266-7245
Timothy.Gilbertson@ucf.edu

Dear Timothy Gilbertson:

The IACUC reviewed the following submission:

Type of Review:	New Protocol Application
Title of Protocol:	Taste and Food Intake
Investigator:	Timothy Gilbertson
IACUC ID:	PROTO202000079

- The protocol was approved on 6/8/2020.
- Your next annual review is due before 6/8/2021.
- Your next triennial review is due 6/8/2023.

Please be advised that IACUC approvals are limited to one year maximum and must be renewed annually. Should there be any technical or administrative changes to the approved protocol, they must be submitted as an Amendment to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur.

If the protocol is over three years old, it must be re-submitted as a Triennial Review for IACUC review and approval. Annual Reviews must be submitted for approval at least three months prior to the Annual Review end date.

You may purchase and use animals according to the provisions outlined in the above referenced animal project.

This letter does not serve as Environmental Health and Safety (EHS) or Institutional Biosafety Committee (IBC) approval. EHS and IBC approval must be handled separately. You must contact EHS prior to initiating animal work to confirm if EHS or IBC approval is required for your project.

Should you have any questions, please do not hesitate to call the office of Animal Welfare at (407) 266-2235.

Please accept our best wishes for the success of your endeavors.

Sincerely,
UCF - Office of Animal Welfare

APPROVAL OF IACUC ANNUAL REVIEW

April 30, 2021

Timothy Gilbertson
407-266-7245
Timothy.Gilbertson@ucf.edu

Dear Timothy Gilbertson:

The IACUC reviewed the following submission:

Type of Review:	Annual Review
Title of Protocol:	Annual Review for PROTO202000079
Investigator:	Timothy Gilbertson
IACUC Protocol ID:	PROTO202000079
IACUC Annual Review ID:	AR202100058

- The annual review was approved on 4/30/2021.
- Your next annual review is due before 6/8/2022.
- Your next triennial review is due 6/8/2023.

Please be advised that IACUC approvals are limited to one year maximum and must be renewed annually. Should there be any technical or administrative changes to the approved protocol, they must be submitted as an Amendment to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur.

If the protocol is over three years old, it must be re-submitted as a Triennial Review for IACUC review and approval. Annual Reviews must be submitted for approval at least three months prior to the Annual Review end date.

You may purchase and use animals according to the provisions outlined in the above referenced animal project.

This letter does not serve as Environmental Health and Safety (EHS) or Institutional Biosafety Committee (IBC) approval. EHS and IBC approval must be handled separately. You must contact EHS prior to initiating animal work to confirm if EHS or IBC approval is required for your project.

Should you have any questions, please do not hesitate to call the office of Animal Welfare at (407) 266-2235.

Please accept our best wishes for the success of your endeavors.

Sincerely,
UCF - Office of Animal Welfare