NOVEL ROLES FOR TRPA1 AND TRPV1 IN NUTRIENT SENSING AND OBESITY

A Dissertation
submitted to the Faculty of the
Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the
degree of
Doctor of Philosophy
in Pharmacology

By

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Washington, DC
June 1, 2011
In the first part of this thesis I explored a role for the capsaicin receptor TRPV1 in the regulation of feeding and body mass. On a diet containing 4.5% fat, wild-type and TRPV1-null mice gained equivalent body mass. On a diet containing 11% fat, however, TRPV1-null mice gained significantly less mass and adiposity; at 44 weeks the mean body weights of wild-type and TRPV1-null mice were 51 and 34 g respectively. Both groups of mice consumed equivalent energy and absorbed similar amounts of lipids. TRPV1-null mice, however, exhibited a significantly greater thermogenic capacity. In contrast to earlier reports, I found that TRPV1-null animals have higher blood glucose levels. Further, I demonstrate that 3T3-L1 preadipocytes expressed functional calcitonin gene-related peptide (CGRP) receptors suggesting a potential neurogenic mechanism by which TRPV1-expressing sensory neurons may regulate adiposity. Taken together, these data support a role for TRPV1 expressing sensory nerves in regulating energy and fat metabolism.

In the second part of this thesis I investigated the role of TRPA1, a nociceptive ion channel, as a fatty acid receptor and modulator of gastric function. Polyunsaturated fatty acids are known agonists for a variety of receptors including members of the transient receptor potential ion channel family. Long chain polyunsaturated fatty acids such as DHA and EPA, predominately found in oily fish, are recognized by their aversive, pungent quality. These properties make DHA a prime candidate to activate TRPA1 within the intestinal mucosa. Indeed,
I found that DHA directly activates TRPA1 without covalently binding to the channel. Further, activation of TRPA1 on intestinal-derived cell lines induces secretion of CCK. Taken together these studies suggest DHA can modulate digestive mechanisms through TRPA1.
This thesis is dedicated to my parents, Robert and Lila Motter.
I am and always will be grateful for your love and support.
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COMMONLY USED ABBREVIATIONS
5-HT  Serotonin
AITC  Allyl isothiocyanate
BMI  Body mass index
CAP  Capsaicin
CaR  Calcium sensing receptor
CCK  Cholecystokinin
CGRP  Calcitonin gene-related peptide
CRAC  Calcium release activated current
CRLR  Calcitonin receptor-like receptor
DAG  Diacylglycerol
DHA  Docosahexaenoic acid
DHA-CoA  Docosahexaenoic acid-coenzyme A
DRG  Dorsal root ganglia
EPA  Eicosapentaenoic acid
ER  Endoplasmic reticulum
GDGF  Glial cell derived growth factor
GI  Gastrointestinal
GLP-1  Glucagon-like peptide 1
GPCR  G-protein coupled receptor
IGF  Insulin-like growth factor
IP$_3$  Inositol trisphosphate
I-V  Current-Voltage
LA  Lauric acid
NAC  n-Acetylcysteine
NG  Nodose ganglia
NGF  Nerve growth factor
OAG  1-oleoyl-2-acetyl-sn-glycerol
PIP$_2$  Phosphatidylinositol 4,5-bisphosphate
PLC  Phospholipase C
PPAR  Peroxisome proliferator-activated receptor
PUFA  Polyunsaturated fatty acid
RT  Room Temperature
RTX  Resiniferatoxin
SOCE  Store-operated calcium entry
SP  Substance P
STIM  Stromal interaction molecule
T1R  Type 1 (taste) receptor
T2R  Type 2 (taste) receptor
<table>
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CHAPTER I

INTRODUCTION
**SIGNIFICANCE OF OBESITY**

Obesity is the result of an imbalance between energy intake and energy expenditure. Clinically obesity is characterized as a body mass index (BMI) >30. An individual is morbidly obese when the BMI is >35. Over two-thirds (68.0%) of Americans are currently considered overweight or obese (Flegal et al., 2010). This disease is no longer limited to westernized countries as globally nearly 1.5 billion adults are obese (2006). The incidence of obesity is also rising among children. Currently, 17% of children in the United States alone are afflicted and in socioeconomic depressed areas these rates are much higher (Ogden et al., 2010). Excess body mass poses a serious health risk as it increases the risk for developing diabetes, hypertension, heart disease, stroke, osteoarthritis, hyperlipidemia gallbladder disease, sleep apnea, and some cancers (breast, endometrial, and colon).

**TRPV1**

**From Structure to Function**

Transient Receptor Potential Vanilloid 1 (TRPV1), also known as the capsaicin receptor, is a 95-kDa, 838-amino-acid protein with 6 transmembrane (TM) domains. Amino acids between transmembrane domain 5 (TM5) and transmembrane domain 6 (TM6) create a pore-forming loop. The channel has a long amino terminus with 3 ankyrin-repeat domains and a shorter carboxyl terminus with a TRP domain (highly conserved region comprised of 23-25 amino acids forming an α-helix) proximal to TM6. Homotetramers are the most predominant form of TRPV1 (Kedei et al., 2001) but it can form heterotetramers with other vanilloid family members.
TRPV1 has been identified in both neuronal and non-neuronal tissue. In the peripheral nervous system the channel is predominately found on dorsal root ganglia (DRG), trigeminal ganglia (TG), and nodose ganglia (NG). Specifically, small- and medium-diameter peptidergic (neurons involved in developing pain and inflammation) and nonpeptidergic neurons (those that mediate chronic pain) express TRPV1. Expression of TRPV1 on peripheral nerves is regulated by nerve growth factor (NGF), glial cell derived neurotrophic factor (GDNF) and insulin-like growth factor (IGF) (Ji et al., 2002; Amaya et al., 2004; Lilja et al., 2007). Within the central nervous system TRPV1 is expressed in the caudal hypothalamus (Cavanaugh et al., 2011). Numerous studies identify TRPV1 in non-neuronal tissue (Lazzeri et al., 2004; Zhang et al., 2007). However, expression may be artificial, resulting from neurons innervating the tissue.

TRPV1 is a nonselective cation channel with near equal selectivity for the monovalent ions sodium (Na$^+$), potassium (K$^+$), lithium (Li$^+$), cesium (Cs$^+$), rubidium (Rb$^+$) (Caterina et al., 1997) and moderate selectivity for divalent ions calcium (Ca$^{2+}$), magnesium (Mg$^{2+}$). It is highly permeable to protons (H$^+$), and has a large 10 Å pore (Jara-Osegua et al., 2008) that can also pass polyvalent cations such as spermine (Ahern et al., 2006), organic cationic dyes (Meyers et al., 2003) and aminoglycoside antibiotics (Myrdal and Steyger, 2005).

The conductance of single channels activated by capsaicin at positive potentials is ~90-100 pS (Pingle et al., 2007). At negative potentials (-60mV) the conductance is much lower, approximately 50 pS (Caterina et al., 1997). TRPV1 displays outward rectification caused by the voltage effect on channel conductance and open probability (Nilius et al., 2005). Inflammatory mediators like prostaglandins sensitize TRPV1 to capsaicin via a PKA-dependent phosphorylation mechanism (Tominaga and Tominaga, 2005). A second mechanism of
sensitization involving PKC-dependent phosphorylation occurs downstream of Gq-coupled receptors (Tominaga and Tominaga, 2005). The channel can undergo both rapid desensitization during a single application of an agonist and slow desensitization after repeated agonist application. Desensitization is a Ca$^{2+}$ dependent mechanism as removal of extracellular Ca$^{2+}$ or addition of Ca$^{2+}$ chelators abolishes this process.

TRPV1 has numerous modes of activation. The most notable agonist of TRPV1 is the vanilloid, capsaicin (the pungent component of chili peppers). Activation of TRPV1 increases intracellular Ca$^{2+}$. Using Ca$^{2+}$ imaging, the EC$_{50}$ for capsaicin activation of TRPV1 ranges from 40 nM (Szallasi and Blumberg, 1999) to 270 nM (Acs et al., 1996). The EC$_{50}$ value of capsaicin using electrophysiological recordings in transfected HEK293 cells is approximately 500 nM (Gunthorpe et al., 2000). A second vanilloid, resiniferatoxin (RTX) is an ultrapotent TRPV1 agonist with an EC$_{50}$ of 1 nM for stimulating Ca$^{2+}$ transients in sensory neurons and an EC$_{50}$ of 40 nM using whole-cell patch clamp recordings (Caterina et al., 1997). Calcium-induced calcium release caused by the influx of Ca$^{2+}$ through TRPV1 or the low saturability of the fluorescent calcium dye may account for the differences in EC$_{50}$ values for these two methods.

Capsaicin and RTX are highly lipophilic and structurally similar to endogenous fatty acids. Indeed endogenous fatty acids such as the endocannabinoid anandamide (Zygmunt et al., 1999), N-arachidonoyl dopamine (NADA) (Movahed et al., 2005), oleoylethanolamide (OEA) (Ahern, 2003) as well as the omega-3 polyunsaturated fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Matta et al., 2006) are TRPV1 agonists. Many of the vanilloids and lipids activate TRPV1 by interacting with intracellular residues.
TRPV1 is directly gated by heat at resting membrane potentials (-60 mV) with a threshold >42°C (Caterina et al., 1997). Further, heat at subthreshold temperatures synergizes with TRPV1 agonists to enhance currents (Babes et al., 2002). Though heat sensitivity is maintained in excised patches (suggesting heat activation is membrane-delimited) the mechanism underlying heat activation remains unknown. Nilius et al. proposed that temperature may regulate the channel by altering its intrinsic voltage-sensitivity (Nilius et al., 2005).

The intrinsic voltage-dependent properties of TRPV1 contribute to outward rectification. The channel is deactivated at negative potentials and is activated in a time-dependent manner at positive potentials. Voltage-dependent activation and deactivation is dependent on temperature and agonist concentration. Without the presence of an agonist, large nonphysiological membrane depolarizations are required to activate TRPV1 ($V_{1/2}$ at 21°C is +150 mV) (Nilius et al., 2005; Ahern et al., 2006). As with the heat sensor, the voltage sensor remains unknown.

Furthermore, TRPV1 is regulated by extracellular protons and cations. At a pH between 6 and 7 TRPV1 is sensitized to agonists such as capsaicin and heat. However, at a pH below 6, the concentration of protons is high enough to directly activate the channel.

Monovalent ($\text{Na}^+$, 190 mM) and divalent cations ($\text{Ca}^{2+}$ and $\text{Mg}^{2+}$, 1-10 mM) sensitize TRPV1 to other agonists while high concentrations of divalent cations (>10 mM) directly gate the channel (Ahern et al., 2006). Further, low concentrations (micromolar range) of polyvalent cations such as gadolinium ($\text{Gd}^{3+}$) and spermine also sensitize and activate TRPV1. The mechanism by which protons and cations regulate TRPV1 is most likely due to electrostatic charge.
Capsazepine, a competitive TRPV1 antagonist, is structurally similar to capsaicin. It blocks capsaicin-induced responses by competing with the capsaicin-binding site. Interestingly, capsazepine also inhibits proton, heat (Tominaga et al., 1998) and voltage (Matta and Ahern, 2007) activation of TRPV1. This suggests capsazepine may also block TRPV1 in a more general manner as an inverse agonist. The IC$_{50}$ for capsazepine is 0.2-5 µM (Szallasi et al., 1993). Further, the iodinated form of RTX acts as an antagonist of TRPV1 with an IC$_{50}$ of 4 nM.

Ruthenium red, an inorganic cationic dye, also acts as a TRPV1 antagonist by blocking the pore (Dray et al., 1990). However, ruthenium red block is not selective for TRPV1, and inhibits other members of the TRP family. Numerous pharmaceutical companies have and continue to develop several selective potent TRPV1 antagonists (AMG 9810, BCTC, A425619, SB-366791 and IBUT).

TRPV1 is a known transducer of noxious chemical and thermal stimuli in nociceptive sensory neurons. After an injury, sensitization of TRPV1 directly contributes to allodynia (pain induced by non-noxious stimuli) and hyperalgesia (increased sensitivity to a painful stimulus). Many inflammatory mediators such as ATP, bradykinin, prostaglandins and lipoxygenase products can act via second messenger signaling pathways to sensitize TRPV1. Tissue acidification that occurs during ischemia and inflammation also potentiates TRPV1. The combination of these factors lowers the threshold for heat activation of TRPV1 rendering TRPV1 active at normal body temperature (37°C).

TRPV1 contributes to normal body temperature regulation. Systemic or hypothalamic injections of capsaicin in animals produce a hypothermic response (Jancso-Gabor et al., 1970b). In contrast, injections of TRPV1 blockers produce a transient hyperthermia (Gavva et al., 2007;
Steiner et al., 2007). TRPV1-null mice show no gross deficits in body temperature but do exhibit an attenuated fever response (Iida et al., 2005). Under extreme fasting conditions mice slowly lose the ability to maintain core body temperature. Interestingly, when TRPV1-null mice were food deprived for 72 hours they displayed normal circadian body temperature fluctuations (Garami et al., 2010).

**TRPV1 and Body Weight**

There is accumulating evidence indicating a role for TRPV1 in body weight regulation. Longstanding anecdotal evidence indicates that consuming chili peppers in the diet promotes leanness. In 1986, Kawada et al. showed in rats that capsaicin (0.021% supplemented in a diet containing 30% lard), significantly reduced peri-renal fat and serum triglycerides (Kawada et al., 1986). Subsequently, several studies have demonstrated that administration of capsaicin or chemically-related vanilloid compounds can reduce weight-gain and adiposity in animals consuming moderate to high-fat diets (Kawada et al., 1986; Kobayashi et al., 2001; Ohnuki et al., 2001; Zhang et al., 2007). Similar anti-obese effects of vanilloids are seen in human studies. Kawabata et al. showed that a two-week intake of CH-19 sweet (a non-pungent red pepper), significantly reduced body weight, BMI, and fat mass in men (Kawabata et al., 2006). Snitker et al. showed that CH-19 sweet significantly reduced abdominal adiposity in a 12-week, calorie-restricted diet (Snitker et al., 2009).

The precise pharmacological actions of vanilloids affecting body weight are unclear, since these compounds initially activate TRPV1 and thereafter produce a long-lasting desensitization of TRPV1 and TRPV1-expressing sensory neurons. Some studies support an
agonistic action of vanilloids. For example, repeated daily administration of vanilloids has been reported to increase metabolism or reduce adipogenesis through the stimulation of TRPV1 (Zhang et al., 2007). In contrast, the results of several other studies suggest that vanilloids affect body weight through TRPV1-desensitization. One method of permanently desensitizing or eliminating TRPV1 is by destroying TRPV1 (capsaicin-sensitive) nerve terminals by administering a high dose of capsaicin to neonate animals. Chemical destruction (in neonates) of capsaicin-sensitive sensory neurons provides protection from diet-induced obesity. Chemically-denervated rats have significantly less adiposity up to one year after treatment (Cui and Himms-Hagen, 1992; Melnyk and Himms-Hagen, 1995). These conflicting phenotypes may result from capsaicin’s lack of specificity at high concentrations. Also chemical lesioning destroys a majority of capsaicin-sensitive afferents and therefore does not selectively target TRPV1.

**TRPV1 and Feeding**

There is evidence for exogenous vanilloids affecting the perception of fullness and satiety. Indeed, capsaicin-sensitive afferents innervate the oral cavity and viscera; trigeminal and vagal sensory afferents (and their cell bodies) highly express TRPV1 (Liu et al., 2000; Zhong et al., 2008). Thus, TRPV1 could participate in transmitting signals arising from the gastrointestinal tract caused by mechanical or chemical stimuli, ultimately modulating satiety. Activation of vagal afferent neurons by gastric distension is reduced in TRPV1-null animals suggesting that TRPV1 participates in mechanotransduction in the stomach (Bielefeldt and Davis, 2008). In humans, consumption of red pepper at breakfast significantly reduces subsequent energy intake at lunch (Yoshioka et al., 1999). Further, oral ingestion of capsaicin is more effective in reducing
food consumption than gastrointestinal application (via a capsule), suggesting an important role for taste/oral sensory perception (Westerterp-Plantenga et al., 2005). This raises the question whether dietary capsaicin modulates food intake through nociception rather than satiety. Indeed in mice, intraperitoneal administration of the putative satiety factor oleoyethanolamide (OEA), which is also a TRPV1 ligand (Liu et al., 2000; Ahern, 2003), can produce malaise and a short-term anorexia in a TRPV1-dependent manner (Wang et al., 2005).

In humans, several studies show that capsaicin or chili pepper can slow gastric emptying (which would promote satiation) while enhancing overall gut transit time (Horowitz et al., 1992; Gonzalez et al., 1998). On the other hand, other studies have found either no change (Rodriguez-Stanley et al., 2000) or an increase (Debreceni et al., 1999) in gastric emptying rate. This discrepancy might be explained by the vanilloid dosage and timing and the bimodal activation/desensitization effects of TRPV1 agonists. It is noteworthy that long-term capsaicin treatment is not effective in humans probably due to the aversive qualities of capsaicin and lack of compliance (Lejeune et al., 2003).

**TRPV1 and Energy Expenditure**

Several studies have demonstrated that dietary capsaicin does not impair intestinal fat absorption in rats (Kawada et al., 1986) and humans (Belza et al., 2007). Thus, this observation indicates that TRPV1-signaling must regulate body weight via altered energy expenditure. Administration of vanilloids triggers a decrease in body temperature. This could be due to changes in heat loss and/or heat production. Indeed capsaicin produces a profound heat loss, mediated by skin vasodilation. Subcutaneous capsaicin reduces core body temperature by up to
7°C (Jancso-Gabor et al., 1970a) while RTX can cause a 10°C drop in body temperature (Figure 5). Accompanying this heat loss is an increase in metabolic rate, primarily due to capsaicin-induced stimulation of catecholamine secretion (Watanabe et al., 1987). In humans, dietary capsaicin induces a decreased respiratory quotient, consistent with a shift from carbohydrate to fat oxidation (Belza et al., 2007). In addition, several studies in rodents show up-regulation of uncoupling proteins after treatment with capsaicin and its analogs (Cui and Himms-Hagen, 1992; Masuda et al., 2003). Thus, the thermogenic effects of chronic daily vanilloid administration may influence long-term body weight and adiposity.

The thermoregulatory effects of TRPV1 deserve special attention in considering potential roles for TRPV1 in energy expenditure. As mentioned, hypothermia is a hallmark of administration of a strong TRPV1 agonist while antagonists produce a transient hyperthermia. These thermoregulatory effects could result from TRPV1-signaling at distinct locations. TRPV1 is expressed in thermoregulatory neurons in the hypothalamus, and central injections of capsaicin produce marked hypothermic effects (Jancso-Gabor et al., 1970b). In addition, TRPV1 is strongly expressed in peripheral afferent nerves which send projections to the hypothalamus. Thus, both central and peripheral mechanisms could contribute to TRPV1-mediated thermoregulation. Recently, several studies point to a prominent role for peripheral warmth-sensing visceral afferents; selective desensitization of visceral TRPV1-expressing afferents block the hyperthermic response of TRPV1 antagonists (Steiner et al., 2007). Thus, these afferents are likely to be tonically active, and as core body temperature rises, serve to provide a negative feedback input to the hypothalamus, activating cooling effector mechanisms (e.g. skin vasodilatation). The hyperthermia produced by TRPV1 antagonists in humans is short-lived (48
hours) (Gavva et al., 2008), indicating that other thermoregulatory mechanisms can compensate. Indeed, TRPV1-null animals do not exhibit overt alterations in temperature regulation (Iida et al., 2005). However, these animals do display subtle thermoregulatory changes including, slightly greater fluctuations in circadian core body temperature (Szelenyi et al., 2004) and an attenuated fever response (Iida et al., 2005). Recently two studies have demonstrated a link between TRPV1, feeding and body temperature. TRPV1-null mice (Kanizsai et al., 2009) and capsaicin-desensitized rats (Garami et al., 2010) were better able to maintain 24-hr body temperature during a 3-day fast, compared with control animals. Both mice and rats exhibited a daytime hypothermia, but the hypothermia was significantly greater in wild-type mice and untreated rats compared to TRPV1-null mice and capsaicin-desensitized rats. Taken together, these studies suggest that TRPV1 contributes to physiologic temperature regulation (operating as a negative feedback mechanisms to dissipate heat). Also, these data suggest that stress/obesity may lead to an augmentation of TRPV1 signaling, thereby depressing body temperature. It is tempting to speculate that a chronic up-regulation of TRPV1 signaling would reduce energy expenditure and lead to increased adiposity. In contrast, inhibition or disruption of TRPV1 signaling would cause the reverse: an increase in energy expenditure and loss of adiposity.

Aside from thermoregulation, a recent study demonstrated that activation of TRPV1 blocks adipogenesis (Zhang et al., 2007). This is controversial as TRPV1 expression on adipocytes has not been confirmed and because capsaicin may produce TRPV1-independent effects in this tissue (Hsu and Yen, 2007; Kang et al., 2007).
**TRPV1 and Insulin Regulation**

Along with weight loss, capsaicin was shown to trigger insulin secretion (Akiba et al., 2004). The mechanism is unclear since both the sensory afferent nerves innervating the pancreas (Wick et al., 2006) and the pancreatic beta cells themselves (Akiba et al., 2004) express TRPV1 channels. TRPV1-signaling produces complex effects on glucose regulation; chemical destruction of TRPV1-expressing neurons in mice (Karlsson et al., 1994), normal rats (Guillot et al., 1996; van de Wall et al., 2005), streptozotocin diabetic rats (Guillot et al., 1996), and Zucker diabetic fatty rats (Gram et al., 2005a; Gram et al., 2007) renders these animals better at removing excess glucose from their blood. In other words chemical destruction of TRPV1-expressing neurons with capsaicin improves glucose tolerance. This improved glucose tolerance is also seen in mice treated with resiniferatoxin (RTX), an ultra potent TRPV1 agonist which produces long-term desensitization of TRPV1 containing nerves (Gram et al., 2005b; Moesgaard et al., 2005). However, in some studies RTX-desensitized animals exhibit increased insulin secretion compared to control animals (Karlsson et al., 1994; Gram et al., 2005b; Gram et al., 2007) while others show no change (Guillot et al., 1996; Gram et al., 2005a) or a decrease (Moesgaard et al., 2005; van de Wall et al., 2005). Hence, it remains unclear if the improved glucose tolerance results from enhanced insulin secretion or better insulin sensitivity. TRPV1-null mice reportedly exhibit greater glucose tolerance and increased insulin sensitivity compared to wild-type counterparts (Razavi et al., 2006). Although, this finding is contradictory to my data (Figure 7).
TRPV1 and Nutrient Sensing

Glucose is the body’s main source of energy for all cell types. Within the central nervous system the hypothalamus (Mayer, 1953), nucleus tractus solitarii (NTS), and dorsal motor nucleus of the vagus (DMV) (Ferreira et al., 2001) contain glucose-sensing neurons. The hypothalamus expresses low levels of TRPV1 in distinct nuclei while the NTS contains TRPV1 expressing vagal terminals. However, it remains unknown if glucose-sensing neurons express TRPV1. As blood glucose increases, 14-19% of hypothalamic glucose-sensing neurons are excited while a separate population (3-14%) is inhibited (Song et al., 2001; Dunn-Meynell et al., 2002). The mechanism underlying glucose excitation of neurons involves the transport of glucose across the membrane via one of the four glucose transporters (GLUT 1-4). Once glucose enters the cell it is converted to ATP. ATP then binds to $K_{ATP}$ channels, inhibiting its activity. This leads to membrane depolarization and $Ca^{2+}$ entry through voltage-dependent $Ca^{2+}$ channels resulting in increased neuronal activity and transmitter or peptide release (Levin et al., 2004). An alternative mechanism employs the electrogenic sodium-glucose cotransporter (SGLT). SGLT couples the uptake of one glucose molecule with the influx of one or two Na$^+$ ions. The net increase in intracellular Na$^+$ can cause depolarization with the same end result as the glucose metabolism mechanism (Gonzalez et al., 2009). Much less is understood about the mechanisms involving inhibition of glucose-sensing neurons. Ultimately, central glucose-sensing neurons maintain energy homeostasis by controlling neuroendocrine function, nutrient metabolism and food intake (Levin et al., 2004).

In addition to the hypothalamus, peripheral vagal neurons are glucose sensitive (Grabauskas et al.). Glucose sensitivity in vagal neurons may underlie the inhibitory effects of
hyperglycemia on food intake and gastric motility (Aylett, 1962; Tordoff and Friedman, 1986; Barnett and Owyang, 1988; Tordoff et al., 1989; Zhou et al., 2008). In support of this hypothesis, glucose infusion into the portal vein (which is densely innervated by vagal afferents) has a greater effect on food consumption compared to glucose infusion into the jugular vein (which is poorly innervated) (Tordoff and Friedman, 1986; Tordoff et al., 1989; Delprete and Scharrer, 1990). Further, hepatic branch vagotomy or capsaicin-denervation attenuates the effects of hyperglycemia (Delprete and Scharrer, 1990; Zhou et al., 2008). Taken together this suggests TRPV1 expressing neurons may be involved in peripheral glucose-sensing.

There is no direct evidence that TRPV1 senses glucose. However, TRPV1 ion channels contain a Walker B type sequence within the ankyrin repeat domains of the N-terminus (Kwak et al., 2000; Lishko et al., 2007) and a Walker A motif within the C-terminal domain (Kwak et al., 2000; Grycova et al., 2007) which can bind ATP (Kwak et al., 2000). Walker A and B motifs are nucleotide binding sites that contain glycine rich sequences which form a loop that is preceded by a beta sheet and followed by an alpha helix. Intracellular application of ATP enhances the response to capsaicin which is lost after single amino acid mutations in either the Walker A or Walker B domains (Kwak et al., 2000). Although ATP can sensitize and prevent desensitization of TRPV1 (Lishko et al., 2007), ATP alone has no effect on the channel (Kwak et al., 2000). Further experiments are needed to determine if increased glucose concentrations can elevate intracellular ATP and modulate TRPV1 activity within a neuron.
MECHANISMS OF NUTRIENT SENSING

Many of the proteins involved in nutrient sensing in the oral cavity are also in the cells lining the small intestines, suggesting the gut also “tastes” food (Wu et al., 2002; Dyer et al., 2005; Hirasawa et al., 2005; Edfalk et al., 2008; Cartoni et al., 2010). The enteroendocrine cell line STC-1 is used as a model to extensively study mechanisms involved in nutrient sensing and subsequent peptide release in the GI tract. These cells express both type 1 and type 2 taste receptors (for a diagram see Figure 1). The type 1 family of taste receptors are G-protein coupled receptors (GPCRs) that detect sweet compounds while type 2 taste receptors are activated by bitter tastants. In response to dietary sugar (Margolskee et al., 2007) or bitter stimulants (Chen et al., 2006) STC-1 cells robustly secrete glucagon-like peptide-1 (GLP-1). For years, long chain fatty acids (specifically saturated fatty acids) have been known to induce CCK (McLaughlin et al., 1998). Only recently orphan GPCRs (GPR40: Itoh et al., 2003; Fujiwara et al., 2005; Stewart et al., 2006; GPR120: Hirasawa et al., 2005; Tanaka et al., 2008; Oh et al.) were identified as fatty acid receptors. The GI tract also senses protein through the calcium sensing receptor (CaR). The CaR detects aromatic amino acids such as phenylalanine and induces CCK secretion (Mangel et al., 1995; Hira et al., 2008).

Peptide secretion is a calcium-dependent event. The current dogma assumes the primary mechanism for Ca$^{2+}$ entry is through voltage-gated Ca$^{2+}$ channels (Mangel et al., 1993; Mangel et al., 1994; Mangel et al., 1995; Mangel et al., 1996a; Mangel et al., 1996b; Scott et al., 1996; Glassmeier et al., 1998; McLaughlin et al., 1998). In short, once nutrient sensing GPCR second messenger signaling pathways are activated it leads to Ca$^{2+}$ release from intracellular stores and K$^+$ channel blockade. Inhibition of K$^+$ channels will cause membrane depolarization and the
opening of voltage-gated Ca\textsuperscript{2+} channels. The influx of Ca\textsuperscript{2+} results in peptide secretion. In Chapter III, I reveal an additional mechanism by which fatty acids directly gate Ca\textsuperscript{2+} permeable TRP channels to elicit CCK and 5-HT secretion.
Figure 1. STC-1 cell diagram
Type 1 taste receptor (T1R); type 2 taste receptor (T2R); calcium sensing receptor (CaR); G-protein coupled receptor 40 (GPR40); G-protein coupled receptor 120 (GPR12); endoplasmic reticulum (ER); cholecystokinin (CCK)
TRPA1
From Structure to Function

TRPA1, also a member of the transient receptor potential family, is known as the mustard oil or wasabi receptor. It is a 127.4 kD protein containing ~1119 AA which varies slightly among species. Similar to other TRP family members, TRPA1 has 6 transmembrane domains with a pore-forming loop between TM5 and TM6. The intracellular N-terminus containing 18 ankyrin repeats accounts for nearly half of the protein (Garcia-Anoveros and Nagata, 2007). The many ankyrin domains have made it a favorable candidate as a mechanosensitive ion channel. However, there is no evidence the channel alone is mechanosensitive but it may contribute to a mechanosensitive complex.

TRPA1 is found in both neuronal and non-neuronal tissue. In the peripheral nervous system TRPA1 is expressed on the small-diameter neurons originating from the dorsal root ganglia (DRG), trigeminal ganglia, and nodose ganglia. Expression is restricted to nociceptive C-fibers with no expression in A-fibers. The channel colocalizes with TRPV1, calcitonin gene related peptide (CGRP), substance P, and peripherin (Bautista et al., 2005; Kobayashi et al., 2005; Nagata et al., 2005). While TRPA1 is not detected in the CNS (Nagata et al., 2005) it has been identified in the vestibular and auditory sensory epithelia of the inner ear. Although TRPA1 was hypothesized to be a mechanotransduction channel for hearing (Kwan et al., 2009), subsequent studies demonstrated that TRPA1-null mice have no gross hearing deficit (Corey et al., 2004; Bautista et al., 2005; Nagata et al., 2005). More recently, reports have identified TRPA1 in the mucosa of the small intestines (Stokes et al., 2006; Purhonen et al., 2008). Many of the biophysical properties of TRPA1 are similar to those of TRPV1. TRPA1 is permeable to both monovalent and divalent cations and it has a single channel conductance of
100 pS (Nagata et al., 2005). It is an outwardly rectifying channel, passing greater current at positive potentials. Calcium also has significant effects on TRPA1 gating. In the presence of extracellular Ca\(^{2+}\), activation of TRPA1 by the agonist allyl isothiocyanate (AITC) produces an inward current. Once the maximum current is reached the channel is inactivated. After potent agonist application the channel remains inactive for many seconds (up to minutes). In a Ca\(^{2+}\) free environment inactivation does not occur.

Numerous pungent compounds found in plants activate TRPA1. Many of which are isothiocyanates found in edibles such as wasabi, mustard, horseradish, and Brussels sprouts (Bandell et al., 2004). Other agonists include caffeine (Nagatomo and Kubo, 2008), nicotine (Talavera et al., 2009), allicin (garlic) (Bautista et al., 2005), acrolein (tear gas) (Bautista et al., 2006), some general anesthetics (Matta et al., 2008), cinnamaldehyde (cinnamon), gingerol (ginger), and eugenol (clove) (Bandell et al., 2004). Application of these compounds is irritating and produces neurogenic inflammation and hyperalgesia.

A key similarity among noxious TRPA1 agonists is their electrophilic properties which enable them to react with cysteine residues. Indeed, TRPA1 has approximately 31 cysteine amino acids in the cytoplasmic N-terminus that are conserved among human, rat and mouse. By mutating these cysteines, Hinman et al. and Macpherson et al. discovered these amino acids are required for channel activation by electrophilic compounds (Hinman et al., 2006; Macpherson et al., 2007). In short, noxious electrophilic compounds act by covalently modifying specific cysteine amino acid residues on TRPA1.

Not all TRPA1 agonists activate the ion channel in this manner. For example, the cooling compound, menthol is known to activate or block TRPA1 in a concentration dependent manner.
Interestingly, this phenomenon only applies to mouse TRPA1, as human TRPA1 is only activated by menthol (Xiao et al., 2008). Further, fly and mosquito TRPA1 orthologs are menthol insensitive. Using a series of human-mouse-fly chimeras Xiao et al. determined that specific amino acid residues in the TM5 domain were critical for menthol sensitivity (Xiao et al., 2008).

Similar to TRPV1, TRPA1 is activated by the endogenous proinflammatory agent, bradykinin, as well as extreme temperatures. Bradykinin indirectly activates TRPA1 via the phospholipase C (PLC) second messenger signaling pathway which leads to phosphorylation of the channel (Bandell et al., 2004; Jordt et al., 2004). TRPA1 is also thermosensitive. Mammalian TRPA1 is activated by noxious cold; TRPA1 currents develop at temperatures as low as 17°C (Story et al., 2003; Bandell et al., 2004). In contrast, Drosophila (Hamada et al., 2008) and snake (Gracheva et al., 2010) TRPA1 are heat gated channels. Further, TRPA1 in the rattlesnake pit organ mediates sensing of infrared radiation (Gracheva et al., 2010).

**TRPA1 and Gastric Function**

As a result of its channel properties and location, TRPA1 is predominatly recognized for its role in pain signaling. However, more recent data suggests that it has a role in gastric function. Indeed, TRPA1 is located in three distinct gastric locations: intrinsic enteric neurons (Boesmans et al., 2011), primary afferent neurons (DRG and vagal) (Cattaruzza et al., 2009; Kondo et al., 2009), and the endocrine cells of the mucosa (Purhonen et al., 2008; Nozawa et al., 2009). Gastric projecting neurons that express TRPA1 are involved in vasodilatation, distention-induced pain, and gastric inflammation (Holzer, 2011). However, the discovery of mucosal
TRPA1 expression suggests TRPA1 may have alternative mechanisms for regulating gastric function.

In addition to expression in native tissue, TRPA1 was also found in the enteroendocrine cell line, STC-1 (Purhonen et al., 2008). This cell line is a well-established model for studying intestinal hormone release (Mangel et al., 1993; Kieffer et al., 1995; Purhonen et al., 2008). Indeed, AITC stimulation of these cells subsequently leads to a Ca\(^{2+}\) influx and CCK secretion (Purhonen et al., 2008). Ultimately, CCK will effect digestion and satiety.

Furthermore, TRPA1 was also identified in the enterochromaffin-like cell lines RIN14B and QGP-1. Both cell lines secrete serotonin in response to ATIC activation of TRPA1 (Doihara et al., 2009; Nozawa et al., 2009). Serotonin is synthesized from tryptophan in enterochromaffin cells and serotonergic neurons. In the gastrointestinal tract, serotonin primarily affects motility and peptide secretion (Gershon and Liu, 2007). Interestingly, TRPA1 activation \textit{in vivo} induces ileum contractions and delays gastric emptying (Nozawa et al., 2009).

**TRP Channels and Fatty Acids**

**Biological Significance of Fatty Acids**

Fatty acids are hydrocarbon chains with a methyl group at one end and a carboxyl group at the other. Fatty acid chain length can vary from 2 to more than 30 carbons. Notably, long chain fatty acids, those greater than 10 carbons in length, have signaling effects in the gut (McLaughlin et al., 1999). Fatty acids are considered saturated if there are no double bonds in the hydrocarbon chain; monounsaturated if there is one double bond and polyunsaturated if there are two or more double bonds. Saturated fatty acids such as lauric acid (LA) and stearic acid are
commonly found in foods such as butter, cheese, and animal fat. High dietary consumption of saturated fat is linked to obesity, hyperlipidemia, cardiovascular disease and some cancers. Conversely, polyunsaturated fatty acids (PUFAs) specifically, omega-3 polyunsaturated fatty acids (n-3 PUFAs) have beneficial health affects (Calder and Yaqoob, 2009).

Omega-3 polyunsaturated fatty acids contain a double bond on the third carbon from the methyl group and consist of linolenic acid (LNA 18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA 22:6) (see Figure 13 for structures). High levels of n-3 PUFAs are primarily found in plants, fish oil, and breast milk. However, they can be synthesized in vivo through bioconversion of LNA, the shorter chain precursor. When ingested, fatty acids are in the form of triglycerides (three fatty acids and a glycerol) and must be broken down in order to be absorbed in the intestine. Digestion of triglycerides begins with lingual lipase in the oral cavity and continues with pancreatic lipase in the small intestines. Both lingual and pancreatic lipases are specific for the primary (sn-1 and sn-3) ester bonds of triglycerides and are more active on short-chain rather than long-chain fatty acids. Ultimately, triglyceride cleavage forms either a monoglyceride and two fatty acids or a diglyceride and one fatty acid (Carey et al., 1983).

In the intestines long chain fatty acids induce secretion of cholecystokinin (CCK) (McLaughlin et al., 1999; Matzinger et al., 2000; Bradford et al., 2008) and glucagon-like peptide-1 (GLP-1) (Adachi et al., 2006; Bradford et al., 2008). Cholecystokinin is secreted by enteroendocrine cells (I cells) located in the mucosa of the duodenum, jejunum, and proximal ileum (Chandra and Liddle, 2007) as well as from autonomic nerves (Liddle, 1997). Release of CCK has multiple effects on the gastrointestinal systems, including gallbladder contractions, pancreatic enzyme and gastric acid secretion, gut motility and gastric emptying by acting on
peripheral CCK₁ receptors (Grider and Jin, 1994; Schwartz et al., 1997). Further, studies have demonstrated that CCK is a satiety factor which in the short-term can reduce meal size (Gibbs et al., 1973; Della-Fera and Baile, 1980; Houpt, 1983). GLP-1 is secreted by L cells in the ileum and colon. GLP-1 stimulates insulin secretion (D'Alessio et al., 1994), reduces gastrointestinal secretions, inhibits motility, and attenuates gastric emptying (Nauck et al., 1997; Giralt and Vergara, 1999). Similar to CCK, GLP-1 also reduces short-term food intake (Hwa et al., 1998).

Fatty acids, particularly n-3 PUFAs have effects beyond the digestive tract. Dietary intake of fish oil reduces the incidence of cardiovascular disease (Kris-Etherton et al., 2003), improves lipid profiles (Harris, 1996) and decreases inflammation (Calder, 2006). There are high concentrations of DHA within the brain and retina (Fleischer and Anderson, 1983) suggesting that DHA plays a role in neuronal function and vision. Indeed, deficits of DHA lead to impaired vision, memory loss and reduced function of sensory systems (Salem et al., 2001) while DHA supplementation is beneficial in psychiatric disorders including, Alzheimer’s disease (Barberger-Gateau et al., 2002), anxiety (Mamalakis et al., 1998), attention deficit hyperactivity disorder and autism (Richardson and Puri, 2002), bipolar disorder (Stoll et al., 1999), depression (Logan, 2003), and schizophrenia (Assies et al., 2001).

**Fatty Acid Modulation of TRP channels**

TRP channels are modulated by lipid signaling. Stimulation of a class of G-protein coupled receptors (Gq) leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C (PLC) and the formation of inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Both DAG and IP₃ are part of second messenger signaling pathways that regulate
multiple cellular functions. DAG which consists of two fatty acids is a potential precursor for PUFAs. Interestingly, DAG is known to activate *Drosophila* TRP and TRPL (Raghu, 2006) as well as vertebrate TRPC2, 3, 6 and 7 channels (Estacion et al., 2001). Further, Chyb *et al.* demonstrated that PUFAs, specifically linolenic, directly gate *Drosophila* TRP and TRPL (Chyb *et al.*, 1999). Recent studies indicate that vertebrate TRP channels (TRPV1, Matta *et al.*, 2006; TRPV3, Hu *et al.*, 2006; and TRPM8, Andersson *et al.*, 2007) are modulated by PUFAs. The mechanism by which PUFAs activate TRP channels or other proteins is largely unknown. Potentially, there may be a common PUFA binding motif on TRP channels. Further, TRP ion channels may mediate some of the cellular signaling functions of PUFAs. This possibility is explored in Chapter IV.
CHAPTER II

MATERIALS AND METHODS
**CELL CULTURE**

HEK293 and mouse 3T3-L1 preadipocytes were cultured in low glucose Dulbecco’s Modified Eagle’s Medium with 10% bovine calf serum. STC-1 cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium supplemented with 15% horse serum and 2.5% bovine calf serum. The RIN14B cell line was cultured in high glucose RPMI with 10% bovine serum. All cell lines were supplemented with 0.1% L-glutamine and 1% penicillin/streptomycin.

Dorsal root ganglia (DRG) were cultured from adult mice (mixed B6/129P background wild-type and TRPA1-null) in Neurobasal supplemented with 2% B-27 medium (Invitrogen), 0.1% L-glutamine and 1% penicillin/streptomycin.

**CELL TRANSFECTION**

HEK 293F cells were transfected using Lipofectamine 2000 (Invitrogen). Rat TRPV1 and TRPA1 cDNA were gifts from David Julius of the University of California, San Francisco. Drosophila TRPA1 cDNA was a gift from Paul Garrity of Brandeis University. Zebrafish A and B cDNA were gifts from Alex Schier at Harvard University. Mouse TRPA1 cDNA was a gift from Jaime García-Añoveros of Northwestern University. Human TRPA1 cDNA was a gift of Sven Jordt at Yale University. Chimeric TRPA1 cDNAs were gifts of Ardem Patapoutian at The Scripps Research Institute.

**Ca²⁺ IMAGING**

Cells were loaded with 1 µM Fluo4-AM (Invitrogen) for 20 min and washed for another 10-20 min before recording. The dye was excited at 488 ± 15 nm. Emitted fluorescence was
filtered with a 535 ± 25 nm bandpass filter, captured by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI) and read into a computer. Analysis was performed offline using Simple PCI software (Compix Inc.). Drugs were applied through a gravity flow system via a micropipette (~5-10 µm diameter) positioned at a distance of ~100 µm from the cell of interest.

**ELECTROPHYSIOLOGY**

Whole cell patch-clamp recordings were performed using an EPC8 amplifier (HEKA Elcetronics). For whole-cell recordings the bath solution contained 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.2 mM CaCl₂, 10 mM HEPES, 5.6 mM glucose, pH 7.3. The pipette solution contained 140 mM NaCl or CsCl, 10 mM HEPES, 5 mM EGTA, pH 7.3 Solutions were applied via a gravity-fed system. Separate outlets were used to apply fatty acids, capsaicin and AITC solutions to prevent contamination. Current-voltage measurements consisted of a 150-ms ramp from -150 mV to +150 mV for transfected HEK293 and STC-1 cells. RIN14B cells contained a 1 second step to +70 mV to inactivate sodium channels before a 150-ms ramp from -50 mV to +150 mV. The baseline currents under control conditions were subtracted (unless otherwise stated).

**CCK AND SEROTONIN SECRETION**

STC-1 and RIN14B cells were plated on 35 x 10 mm plates and cultured for 2-3 days until they reached confluency. Cells were washed twice with 1 ml HBBS and incubated for 20 min at room temperature with HBBS containing stimulants (625 µl/well). Supernatants were collected and spun to remove cell debris. STC-1 cells were analyzed for CCK using an EIA kit.
(Phoenix Pharmaceutical) and RIN14B cells were analyzed for serotonin using an EIA kit (Rocky Mountain Diagnostics, Inc.). Stimulated cells were lysed and analyzed for total protein using a Bradford assay. Peptide secretion was normalized to total protein for each plate.

**RT-PCR**

Total cellular RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. First strand cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen) with the supplied oligo (dT)20 primer. Amplification was performed using Platinum Blue PCR Super Mix (Invitrogen). The following primers were used for CRLR, 5’-GGCGCAACGGATACATTGC-3’ and 5’-ACAAAGCAGCACAACATCGGACC-3’; β-actin, 5’-GCTGGTGTCAAGGCTG-3’ and 5’-CAGGTCCAGACGCGAGGGCATTG-3’; TRPC1, 5’-TCTGGGAAATGTTCTCCTTTG-3’ and 5’-GCTGGTGTGGCAGCAACGGCT-3’; TRPC2, 5’-GATCCGGTTATGTCTCCTTGC-3’ and 5’-GAGCGAGCAACAGGCTG-3’; TRPC3, 5’-CACGCTTCTCGCTGACATC-3’ and 5’-TTCAGAATGGGCTCCTACCC-3’; TRPC5, 5’-ATCTACTGCTAGTACTGGG-3’ and 5’-ATATCATAGATCGCATG-3’; TRPC6, 5’-AAAGATATATCTTCAAATTCATGGTC-3’ and 5’-CACGCTCGCAGTAAATCTTCATGGTC-3’; GAPDH, 5’-GAAGGTCGGTGTCAGAAGCT-3’ and 5’-GAAGACACCAGTAGACTCC-3’.

Real-time amplifications were performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and SYBR Green PCR Master Mix (Applied
Biosystems). RNA was extracted from white and brown adipose tissues and skeletal muscle (quadriiceps containing both fast-glycolytic fibres-\textit{vastus} and oxidative fibres-\textit{rectus femoris}). Oligonucleotide primers for mouse UCP1, UCP2, and UCP3 were the same as those previously published (Masuda et al., 2003). \(\beta\)-actin was amplified in parallel reactions as an endogenous control. The amplification profile included denaturation at 95\(^\circ\)C for 30 sec, annealing at 55\(^\circ\)C for 30 sec, and extension at 72\(^\circ\)C for 30 sec for a total of 40 cycles. Dissociation curve analysis was performed at the end of each real-time PCR run to ensure that primer dimers were not present. Additionally, the real-time PCR products were verified on agarose gel. The comparative threshold cycle (\(C_T\)) method was use to determine the relative quantification of target RNA. The target threshold cycle number was normalized to an endogenous reference (GAPDH), and relative amounts of UCP message were normalized to \(\beta\)-actin.

**Body Weight and Feeding Studies**

All experimental procedures involving animals were approved by the Georgetown University Animal Care and Use Committee and conform to NIH guidelines.

C57BL6 wild-type and TRPV1-null mice were house either individually or in a group. Body mass was monitored from 3 to 44 weeks of age while consuming either a 4.5\% fat diet (Purina diet 5001; 23.0\% protein, 4.5\% fat, 5.3\% crude fiber, 49\% carbohydrate, 3.04 kcal/g metabolizable energy, 12.1\% of calories provided by fat) or an 11\% fat diet (Purina diet 5015; 17.0\% protein, 11.0\% fat, 3.0\% crude fiber, 53.5\% carbohydrate, 3.73 kcal/g metabolizable energy, 25.8\% of calories provided by fat). For paired feeding studies male WT and TRPV1-null mice were housed individually. TRPV1-null mice received food \textit{ad libitum} while WT mice were
restricted to the amount of food consumed by their TRPV1-null counterpart. Both mice received water *ad libitum*.

**BODY TEMPERATURE**

Body temperatures were measured in 9.5 week old mice with a rectal thermometer inserted 1.8 cm before and 1 hour after exposure to 0-2°C. Body temperatures were also measured in the same manner before and after subcutaneous injections of RTX or capsaicin.

**GELATIN CONSUMPTION STUDIES**

Mice were housed in groups and had access to food and water *ad libitum*. Male and female (C57/B6 wild-type, TRPV1-null, B6/129P wild-type and TRPA1-null; 12-16 weeks) were individually placed in an empty cage and allowed to acclimate for 30 min before testing. For studies involving glucose sensing, mice were trained to eat gelatin containing lipid or saccharin prior to the testing. Gelatin for these studies contained 2.5% (w/v) gelatin (Knox/Kraft Foods) plus the indicated stimulant.

For taste preference tests, mice were trained to eat gelatin from two dishes within 60 min. Only mice that eat equally from both dishes were included. On the testing day mice were presented with two dishes of gelatin, one with the stimulant and one without. The gelatin contained 10% (w/v) Polycose (Ross Nutrition/Abbot), 2.5% (w/v) gelatin (Knox/Kraft Foods), 2.5% (w/v) sucrose, and 0.04% (v/v) orange flavoring (McCormick & Co., Inc.). Flavored gelatin was used to minimize effects of fatty acid texture and smell.
LIPOLYSIS IN VITRO AND GLYCEROL ANALYSIS

Lipolysis was assessed in adipose explants using methods previously described (Ort et al., 2005). Approximately 100 mg of mouse gonadal adipose tissues were minced and placed into a microtube containing 500 µl Hanks buffer. Aliquots were removed at 0 and 5 hrs. Glycerol release and plasma glycerol were measured using a glycerol assay kit (Cayman Chemical Co., Ann Arbor, MI).

FAT ABSORPTION

Fat absorption was measured using methods previously described (Jandacek et al., 2004). Wild-type and TRPV1-null mice were fed a diet containing (wt %) 16 fat, 45 non-fat dry milk, and 39 sucrose (30:15:55 fat:protein:carbohydrate energy %). The fat component was a mixture of 95% safflower oil and 5% sucrose octabehenate, a non-absorbable lipophilic marker. Fecal samples from the third day were analyzed by gas chromatography (GC) for fatty acids.

CHEMICALS

Capsaicin (CAP) and HC030031 were obtained from Tocris Bioscience (Ellisville, MO, USA). Allyl isothiocyanate (AITC), ionomycin, thapsigargin, stearic and lauric acid were purchased from Sigma-Aldrich. OAG, Oleic, linoleic, α-linolenic, stearidonic, n-3 and n-6 arachidonic acids, DHA, and EPA with 0.1% BHT were obtained from Cayman Chemical (Ann Arbor, MI, USA). Stock solutions of drugs were prepared in ethanol or DMSO and diluted to physiological solution prior to the experiments; final ethanol and DMSO concentrations were always <0.1%, which had no effect on TRPA1 or TRPV1 activity. CGRP was obtained from
Phoenix Pharmaceuticals, Inc (Burlingame, CA, USA) and prepared as a stock solution in water. Fatty acids are rapidly oxidized in aqueous solutions therefore, the antioxidant, N-acetyl L-cysteine (15 mM), was added and fresh solutions were made every 20 min where indicated. In some experiments BSA (0.01%) was added to improve fatty acid solubility.
CHAPTER III

TRPV1 AND OBESITY
INTRODUCTION

The capsaicin receptor, TRPV1, is an ion channel expressed predominantly in sensory nerves. TRPV1 detects a variety of noxious physical and chemical stimuli including capsaicin, the pungent component of chilli-peppers (Pingle et al., 2007; Szallasi et al., 2007). Numerous studies support a fundamental role for TRPV1 in pain signaling; disruption of the TRPV1 gene (Caterina et al., 2000; Davis et al., 2000) and TRPV1 antagonists (Chizh et al., 2007) markedly attenuate thermal hyperalgesia. Interestingly, there is emerging evidence for the participation of TRPV1 and capsaicin signaling in other physiologic functions, including the regulation of feeding and body weight (Suri and Szallasi, 2008). Dietary administration of capsaicin and other chemically-related “vanilloid” compounds can reduce food intake and increase energy expenditure in animals and humans (Yoshioka et al., 1999; Masuda et al., 2003; Westerterp-Plantenga et al., 2005; Belza et al., 2007). Vanilloids exert both short and long term effects. Acutely, capsaicin can reduce food intake, an effect that may be related to altered satiety (Yoshioka et al., 1999), or alternatively, via visceral malaise and anorexia (Wang et al., 2005). In addition, capsaicin can stimulate secretion of catecholamines producing a transient increase in metabolism (Watanabe et al., 1987). Chronic administration of vanilloids reduces weight gain, adiposity and triglycerides in animals consuming high-fat diets (Kawada et al., 1986; Masuda et al., 2003). Interestingly, chemical destruction of capsaicin-sensitive neurons in neonates also affords protection from diet-induced obesity (Cui and Himms-Hagen, 1992; Melnyk and Himms-Hagen, 1995). The precise role of TRPV1 in these effects is unclear. Vanilloids exert complex pharmacological effects at TRPV1, producing an initial activation followed by a long-lasting desensitization of the channel (Pingle et al., 2007; Szallasi et al., 2007). In addition, capsaicin
may signal independently of TRPV1 (Hsu and Yen, 2007; Kang et al., 2007). Further, capsaicin
treatment in neonates ablates entire sensory nerves and therefore does not selectively target
TRPV1. To better understand the role of TRPV1 we explored the effects of a high-fat diet in
wild type and TRPV1-null animals. **I hypothesize that TRPV1 modulates body weight by
regulating food intake and/or energy expenditure.**
TRPV1-NULL MICE HAVE REDUCED BODY MASS AND ADIPOSITY ON A HIGHER FAT DIET

To investigate a role for TRPV1 in body weight regulation I studied changes in body mass of wild-type (WT) and TRPV1-null mice consuming either a low-fat (4.5%) or higher fat (11%) diet. When placed on a low-fat diet, both groups exhibited a similar weight gain from 3-25 weeks of age (Figure 2A). In contrast, when placed on a higher fat diet, wild-type mice gained greater body mass with significant differences beginning at ~17 weeks of age (Figure 2B). At 44 weeks, the mean body mass of WT and TRPV1-null male mice was ~51g and ~34g respectively (Figures 2D and E). A similar difference was noted in female mice; with a mean body mass of 47g and 31g respectively (Figure 2E) at 40 weeks. We also observed differential weight gain when adult mice raised on a low-fat diet were switched to a higher fat diet (Figure 2C). WT mice gained significantly greater mass compared with TRPV1-null mice within a 6-week period. When placed on a very high-fat diet (45% fat) for 3.5 months WT and TRPV1-null mice gain equivalent mass (WT, 48.00±5.01g n=3; TRPV1-null, 43.61±3.55 n=4) suggesting a ceiling for the underlying mechanism.
Figure 2. TRPV1-null mice gain less body mass than wild-type mice on a high-fat diet

(A&B) Mean body mass of wild-type (WT) or TRPV1-null male mice consuming either a low-fat (4.5%) or high-fat (11%) diet from 3 to 25 weeks of age (n=5 for both). (C) Male mice (n=5) switched from a 4.5% fat to an 11% fat diet at 22 weeks of age. (D) Representative photograph of male WT and TRPV1-null mice on an 11% fat diet at 44 weeks of age. (E) Mean body mass of male (age 34 wks: WT n=11, TRPV1-null n=7 and age 44 wks: WT n=4, TRPV1-null n=5) and female (age 40 wks: n=4 for both) mice consuming an 11% fat diet. T-test * P<0.05, **P<0.01, ***P<0.001
Figures 3A&B show that this increase in body mass was associated with greater adiposity. WT animals raised on an 11% fat diet (28 weeks) had significantly greater abdominal and subcutaneous fat than their TRPV1-null counterparts (Figure 3A and B). In addition, adipose tissue from WT mice had markedly larger adipocytes (Figure 3C). Furthermore, both the fat content of the liver and the size of lipid droplets were greater in WT compared with TRPV1-null animals (Figure 3C and D).
Figure 3. TRPV1-null mice have reduced adiposity
(A) Representative photographs of visceral fat in female WT and TRPV1-null mice. (B) Mass of adipose tissue from the abdominal cavity and subcutaneous fat in WT and TRPV1-null mice on an 11% fat diet (n=3, *P<0.05 T-test). (C) Representative cross-sections of gonadal adipose tissue (Hemotoxilyn and Eosin staining, scale bar indicates 200 µm) and liver (Oil Red-O staining, scale bar indicates 10 µm) from WT and TRPV1-null mice on a high-fat diet. (D) Mean area of hepatocyte lipid droplets (n=300 from 3 WT and 3 TRPV1-null mice; ***P<0.001 T-test).
WILD-TYPE AND TRPV1-NULL MICE CONSUME EQUIVALENT ENERGY BUT POSSESS DIFFERENT THERMOGENIC CAPACITIES

To test whether food intake accounted for differences in body mass and adiposity I monitored cumulative energy intake. Figure 4A shows that WT and TRPV1-null animals consumed equivalent energy on low-fat and high-fat diets. To confirm this result I performed paired-feeding experiments in which WT mice consuming high-fat chow were restricted to the food intake of TRPV1-null counterparts. Figure 4B shows that WT mice still gained significant body mass on this regimen. Thus, these data rule out differences in energy intake between WT and TRPV1-null animals. Further, to test for differences in intestinal fat absorption I performed fecal fat analysis. Figure 4C shows that both WT and TRPV1-null animals absorbed ~98% of dietary fats.

Next, I tested for differences in energy expenditure. Previous studies have found that obese mice have a reduced thermogenic capacity compared with lean animals and this is reflected by an impaired ability to maintain body temperature in a cold environment (Trayhurn and James, 1978). I therefore measured core body temperatures before and after a one-hour cold exposure (0-2°C) in mice fed either a low-fat or high-fat diet. Figure 4D shows that cold exposure produced a 2.0°C drop in rectal temperature in WT mice fed a low-fat diet (n=8, P<0.01). A significantly greater decrease of 3.4°C, was seen in WT animals consuming a high-fat diet (n=, P<0.01). In addition, WT mice consuming a high-fat diet had a lower resting body temperature compared with animals consuming a low-fat diet (P<0.01). In contrast, cold exposure produced no significant change in body temperature in TRPV1-null mice consuming either diet (Figure 4D, n=3-7). Aside from an attenuated fever response, TRPV1-null mice
display normal thermoregulation (Iida et al., 2005). These data suggest that TRPV1-null animals have a greater capacity for thermogenesis than WT animals, and a higher resting metabolic rate when consuming a high-fat diet.
Figure 4. Wild-type and TRPV1-null mice have the same food intake and intestinal fat absorption but different energy expenditure

(A) Cumulative energy intake of male mice on a 4.5% fat or 11% fat diet for 8 days (n=4). (B) Mean gain in body mass for WT and TRPV1-null mice after 7 weeks of paired feeding (n=3; *P<0.05, **P<0.01 T-test). (C) Total fat absorption by WT and TRPV1-null mice (n=3). (D) Mean body temperatures of WT (left) and TRPV1-null (right) mice at room temperature (RT) and after one hour at 0-2°C while consuming either the low-fat (4.5% fat) or high-fat (11% fat) diet (WT: 4.5% fat n=7, 11% fat n=6, **P<0.01 ANOVA; TRPV1-null: 4.5% fat n=7, 11% fat n=3, NS by ANOVA).
TRPV1 contributes to normal body temperature regulation. TRPV1 agonists such as capsaicin, produce a hypothermic response (Jancso-Gabor et al., 1970a, b) while TRPV1 antagonists produce a transient hyperthermia (Gavva et al., 2007; Steiner et al., 2007). Thus far, thermoregulatory affects have only been studied in lean animals. I sought to understand if TRPV1 affects body temperature differently in lean and fat mice. Both lean and obese wild-type mice received subcutaneous injections of RTX (0.01 µmol/kg) or capsaicin (1 mg/kg). Consistent with previous studies RTX and capsaicin produced a 12°C and 4.5°C drop in body temperature respectively (Figure 5A and B). While there were no differences in body temperature for lean and obese mice for either agonist there was a later onset and longer time to recovery for the obese animals. The difference in drug kinetics is most likely due to a larger volume of distribution in the obese mice. Capsaicin and RTX are highly lipophilic compounds and were sequestered in the larger fat stores.

Altered thermogenesis may reflect the activity of uncoupling proteins, particularly in brown fat. Indeed, mice fed a capsaicin-rich diet exhibit increased mRNA levels for several uncoupling proteins (Masuda et al., 2003). However, I observed no differences in mRNA expression of uncoupling proteins (UCP1-3) in brown and white adipose tissue, and skeletal muscle of WT and TRPV1-null mice fed a high-fat diet (data not shown). Further, both the plasma glycerol levels (WT 25.6±1.6 mg/dL; TRPV1-null 25.2±3.2 mg/dL, n=7-9) and in vitro lipolytic capacity (WT, 0.36±0.07 mg glycerol g⁻¹ h⁻¹; TRPV1-null 0.37±0.09 mg glycerol g⁻¹ h⁻¹, n=4) were identical in both sets of animals.
Figure 5. RTX and capsaicin induced decrease in body temperature of lean and obese wild-type mice

(A&B) Rectal temperature changes in lean and obese wild-type (WT) mice after a subcutaneous bolus of RTX (0.01 µmol/kg, A) or capsaicin (1 mg/kg, B). RTX: WT n=3, Obese WT n=2, TRPV1-null n=1 Capsaicin: WT n=1, Obese WT n=1
ADIPOCYTES EXPRESS FUNCTIONAL CGRP RECEPTORS

TRPV1 is predominantly expressed on sensory nerve terminals. In turn, these nerves release the neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP). Therefore, I explored whether TRPV1 could exert an action on preadipocyte function through neurogenic mechanisms. Figure 6A shows that CGRP (50 nM) elicited Ca\(^{2+}\) transients in a subset of 3T3-L1 preadipocytes (30%, 27 of 89 cells), consistent with activation of an inositol trisphosphate (IP\(_3\)) signaling pathway. RT-PCR confirmed expression of the CGRP receptor, calcitonin receptor-like receptor (CRLR) (Figure 6B). In contrast, SP (50 nM) failed to evoke Ca\(^{2+}\) transients (data not shown, n=50). Interestingly, a recent study has described expression of TRPV1 in 3T3-L1 preadipocytes (Zhang et al., 2007). However, I found that the TRPV1 ligand, capsaicin (10 µM), failed to evoke Ca\(^{2+}\) responses (n=64) whereas all cells responded robustly to application of 2 mM extracellular ATP (Figure 6C and D).
Figure 6. Mouse 3T3-L1 preadipocytes express functional CGRP receptors
(A) CGRP (50 nM) produced Ca\(^{2+}\) transients in 3T3-L1 preadipocytes. (B) Adipocytes express mRNA for the CGRP receptor, CRLR. (C&D) Capsaicin (10 µM) failed to produce Ca\(^{2+}\) transients in adipocytes, though cells responded to ATP (2 mM).
ELEVATED BLOOD GLUCOSE LEVELS IN TRPV1-NULL MICE

Diabetes is associated with obesity. Therefore differential diabetic susceptibility between WT and TRPV1-null mice could contribute to body weight differences. Indeed, others suggest that TRPV1 sensory neurons are involved in the pathogenesis of diabetes (Razavi et al., 2006). Razavi and colleagues reported that TRPV1-null mice showed a significantly improved glucose response with accelerated glucose clearance during glucose tolerance and insulin sensitivity tests (Razavi et al., 2006). Therefore, I tested wild-type and TRPV1-null mice for difference in blood glucose on a high-fat diet.

First, random blood glucose measurements were taken from 3 to 5 month old wild-type and TRPV1-null mice while still consuming low-fat chow. Surprisingly, TRPV1-null mice had a higher random blood glucose (168±4 mg/dL) compared to WT mice (148±4 mg/dL; Figure 7A). Next, I monitored blood glucose levels of 12 week old WT and TRPV1-null mice placed on a high-fat diet for 8 weeks. TRPV1-null mice had a higher random blood glucose level (Figure 7C) at the onset though there was no difference in body mass (Figure 7B). Further, blood glucose levels of TRPV1-null mice remained elevated for 8 weeks even though they gained less body weight compared to wild-type animals.
Figure 7. TRPV1-null mice have higher blood glucose levels compared to wild-type mice (A) Random blood glucose of WT and TRPV1-null (3-5 months) on a low fat (4.5%) diet. WT n=30, TRPV1 n=25 (B&C) Body mass (B) and random blood glucose (C) of wild-type (WT) and TRPV1-null mice placed on a high-fat (11%) diet over 8 weeks. WT n=11, TRPV1-null n=7; T-test *p<0.05, **p<0.01, ***p<0.001
ALTERED SHORT-TERM SUGAR CONSUMPTION IN TRPV1-NULL MICE

While conducting feeding studies I observed TRPV1-null mice consistently consume more glucose enriched gelatin than their wild-type counterparts. This observation is interesting because I also found that TRPV1-null mice have higher blood glucose levels (Figure 7A). One explanation for these findings is that TRPV1 expressing vagal afferents are involved in peripheral glucose sensing leading to changes in blood glucose levels and/or glucose intake. To explore this hypothesis, wild-type and TRPV1-null mice were fed a sugar enriched gelatin for 1 hour each day as a supplement to their normal diet for >8 weeks. TRPV1-null mice consumed more gelatin containing either 10% Polycose (a glucose polymer) or 10% sucrose (Figure 8A and B). This increase was observed at 30 minutes (Figure 8A), 60 minutes (Figure 8B) and up to 2 hours (Figure 8C). Eventually the rate of gelatin intake by TRPV1-null mice decreased. Within 4 hours both animals consumed an equivalent amount (Figure 8C). Interestingly, there was no significant difference in gelatin consumption during the first minute of feeding (Figure 8D). This suggests a post-ingestive mechanism rather than a taste specific effect.

To further examine this short-term dysregulation of glucose intake in TRPV1-null animals, I tested naïve mice. Wild-type and TRPV1-null were trained to eat lipid infused gelatin, hence were naïve to sugar taste. Under these conditions both mice consume equal amounts of 10% Polycose gelatin on their first exposure. Taken together these data suggest TRPV1-null mice do not have an enhanced taste preference for Polycose or sucrose. It seems evident TRPV1-null mice learn to eat more sugar infused gelatin.
Lastly, to confirm that this is a TRPV1 specific phenotype, we tested mice lacking the TRPA1 gene. Compared to wild-type counterparts, TRPA1-null mice consumed equivalent amounts of gelatin containing 10% Polycose within 30 minutes (Figure 8F).
Figure 8. TRPV1-null mice consume more sugar within 2 hours compared to wild-type mice
(A&B) Amount of gelatin containing either 10% Polycose or 10% sucrose consumed by wild-type (WT) or TRPV1-null mice within 30 minutes (A) or 60 minutes (B). Control gelatin contains no sugar. WT n=6, TRPV1 n=6 (C) Amount of 10% Polycose gelatin consumed over 8 hours. WT n=6, TRPV1-null n=6 (D) Amount of 10% Polycose or sucrose gelatin consumed in the first minute of eating. WT n=6, TRPV1-null n=6 (E) Amount of 10% Polycose gelatin consumed by naive WT and TRPV1-null mice on their first exposure. WT n=8, TRPV1 n=7 (F) Consumption of gelatin by wild-type and TRPA1-null mice in 30 minutes. WT n=10, TRPA1-null n=11; T-Test *p<0.05, **p<0.01, ***p<0.001


**DISCUSSION**

These results reveal a novel role for TRPV1 and sensory nerves in the regulation of obesity. I show that TRPV1-deficient mice are resistant to diet-induced increases in body mass and adiposity. The phenotype of these knock-out animals closely matches that of animals following chemical destruction of sensory afferent neurons (Cui and Himms-Hagen, 1992; Melnyk and Himms-Hagen, 1995). Our data suggest that TRPV1 is the key target of these sensory nerve lesions. Interestingly, animals that chronically consume dietary vanilloids are also protected from diet-induced obesity (Kawada et al., 1986; Masuda et al., 2003). Vanilloids produce an initial activation followed by long-term desensitization of TRPV1, suggesting that their pharmacological effect may be mediated through inhibition of TRPV1. On the other hand, the possibility that vanilloids signal independently of TRPV1 cannot be excluded (Hsu and Yen, 2007; Kang et al., 2007). Thus, three separate methods of manipulating TRPV1: selective disruption of the TRPV1 gene, destruction of TRPV1-expressing sensory neurons and pharmacological activation/desensitization of TRPV1 produce equivalent effects on body mass and adiposity. Taken together, these data suggest that TRPV1 signaling promotes fat accumulation, and inhibition of this signaling is therefore protective. However, this protective role has a ceiling as TRPV1-null mice can become obese on a 45% fat diet.

Furthermore, it is interesting that I only observed differences in body mass in mice older than ~15 weeks. Similarly, altered weight and adiposity in capsaicin-desensitized rats is only seen after 14 weeks of age (Cui and Himms-Hagen, 1992). These data support a role for TRPV1 in age-onset obesity.
While exploring potential mechanisms, I found that both WT and TRPV1-null animals exhibited equivalent energy intake. Further, intestinal absorption of fat was identical in both groups and this agrees with earlier findings that lipid absorption is unaffected in animals (Kawada et al., 1986) or humans (Belza et al., 2007) treated chronically with capsaicin, which elicits a functional loss of TRPV1. These data suggest that altered energy utilization likely accounts for the differential weight gain. Consistent with this hypothesis, I found that WT mice have a reduced thermogenic capacity compared to TRPV1-null counterparts and this effect was more marked on a higher-fat diet. Resting body temperature was also lower in WT animals consuming a high-fat diet compared with a low-fat diet. This is consistent with a diet-induced reduction in metabolic rate. Obese strains of mice are known to have decreased thermogenesis and a lower resting body temperature (Trayhurn and James, 1978). Interestingly, TRPV1-null animals exhibit normal locomotor activity (Marsch et al., 2007) suggesting that this factor per se does not account for altered energy utilization. In direct contrast, Garami et al. recently reported that TRPV1-null mice are hyperactive. Further studies are needed to confirm if locomotor activity in TRPV1-null mice is dysregulated (Garami et al., 2011).

The precise molecular mechanism by which TRPV1 influences energy and lipid handling is unclear. One potential pathway is through thermoregulation. Both TRPV1 agonists and antagonists have profound effects on normal body temperature (Jancso-Gabor et al., 1970a, b; Gavva et al., 2007; Steiner et al., 2007) yet TRPV1-null mice under normal conditions reportedly show no temperature differences (Szelenyi et al., 2004; Iida et al., 2005). I also found no differences in core body temperature of normal TRPV1-null mice until they were exposed to near freezing temperatures. Under these conditions lean and obese wild-type mice were unable to
maintain core body temperature. Conversely, TRPV1-null mice had a greater capacity to thermoregulate in the cold environment. These findings were also confirmed by Garami and colleagues (Garami et al., 2011). Additionally, TRPV1-null mice (Kanizsai et al., 2009) and capsaicin-desensitized rats (Garami et al., 2010) are able to maintain normal day-time and night-time body temperatures during a prolonged fasting period. Taken together these data suggest TRPV1-null mice have a greater capacity to maintain normal body temperature under strenuous (extreme cold, fasting, obesity) conditions. Hence, small increases in body temperature may contribute to their reduced weight gain on a high-fat diet.

A second potential mechanism may involve diabetes. Indeed, C57Bl/6 mice are prone to age-onset diabetes and obesity and TRPV1-null animals on this background are reported to have increased glucose tolerance and insulin sensitivity (Razavi et al., 2006). Improved glucose and insulin regulation may contribute to enhanced energy utilization and the overall leanness of TRPV1-null animals. However, I found that TRPV1-null mice consistently have elevated blood glucose levels arguing against a diabetic-resistant phenotype. Furthermore, TRPV1-null mice consumed ~60% more glucose-containing gelatin than their wild-type counterparts. No differences in consumption were observed in naïve mice or during the first minute of eating ruling out a greater glucose taste preference in TRPV1-null mice. Rather, it appears mice learn to eat more glucose-rich gelatin over several days of feeding. These findings suggest TRPV1 is involved in glucose sensing and acts to negatively regulate glucose intake. However, further studies are needed to confirm if TRPV1 is directly gated by increased concentrations of glucose.

Lastly, I explored a mechanism whereby CGRP may regulate adipocyte function. Activation of TRPV1 in sensory nerve terminals triggers the secretion of CGRP and SP, two
neuropeptides known to modulate pancreatic islet function (Pettersson et al., 1986; Razavi et al., 2006). Notably, elevated levels of CGRP contribute to insulin resistance (Kreutter et al., 1993). My data points to a potential neurogenic pathway by which TRPV1 may regulate adipocyte function. I found that 3T3-L1 preadipocytes exhibit functional responses to CGRP and express the CGRP receptor, CRLR. Interestingly, levels of CGRP are elevated in obese humans and animals (Zelissen et al., 1991; Kreutter et al., 1993). In support of this Walker et al. recently demonstrated that αCGRP-null mice display a similar phenotype to TRPV1-null mice when chronically challenged with a high-fat diet (Walker et al., 2010). Mice lacking the αCGRP gene had reduced body weight gain and a reduction in adipose tissues which corresponded to an increase in core body temperature, enhanced respiratory quotient and elevated energy expenditure suggesting greater β-oxidation. Thus, it will be important to explore whether CGRP can promote obesity through direct modulation of adipocytes.

In contrast to my findings, Zhang et al. proposed that TRPV1 could directly regulate adipocyte function. Preadipocytes are reported to express TRPV1 (Zhang et al., 2007) and treatment with capsaicin blocks the differentiation of these cells into mature adipocytes (Hsu and Yen, 2007; Zhang et al., 2007). However, whether capsaicin modulates adipocyte function via TRPV1 is uncertain. Capsaicin can inhibit NF-kappa B and modulate adipocyte function independently of TRPV1 (Hsu and Yen, 2007; Kang et al., 2007). Moreover, I could not confirm functional TRPV1 signaling in mouse 3T3-L1 preadipocytes; these cells failed to produce elevations in [Ca^{2+}] in response to capsaicin.

In summary, our data reveal a role for TRPV1 in promoting fat accumulation and weight gain. Pharmacologic inhibitors of TRPV1 are currently under development for pain treatment.
Our data suggest that TRPV1 antagonists or agonists that promote TRPV1 desensitization, may have additional utility in the treatment of obesity.
CHAPTER IV

TRPA1 AND NUTRIENT SENSING
INTRODUCTION

Ingestion of fat is detected both in the mouth and gastrointestinal (GI) tract by the same nutrient sensing proteins (Wu et al., 2002; Dyer et al., 2005; Hirasawa et al., 2005; Edfalk et al., 2008; Cartoni et al., 2010). Most nutrient sensors are G-protein coupled receptors (GPCRs). Recently, GPR40 and GPR120 were identified as fatty acid receptors that through second messenger signaling pathways lead to peptide (CCK and GLP-1) secretion (Itoh et al., 2003; Fujiwara et al., 2005; Hirasawa et al., 2005; Stewart et al., 2006; Tanaka et al., 2008; Oh et al., 2010). In turn, gastric peptides exert numerous effects; they regulate gastric emptying, gastric motility, induce gallbladder and pancreatic secretions and promote satiety. Recently, Purhonen and colleagues identified TRPA1 in the GI tract and proposed that TRPA1 agonists regulate digestive functions through CCK (Purhonen et al., 2008). Additionally, TRPA1 modulates gastric motility through the release of serotonin (Nozawa et al., 2009).

GPCRs are not the only known fatty acid receptors. Indeed, fatty acids activate many TRP ion channels (Chyb et al., 1999; Estacion et al., 2001; Hu et al., 2006; Matta et al., 2006; Raghu, 2006; Andersson et al., 2007). Specifically, longer chain (≥20 carbons) polyunsaturated fatty acids involved in the PLC pathway (arachidonic acid, AA and diacylglycerol, DAG) are recognized TRP agonists. These long chain PUFAs, particularly the omega-3 PUFAs, impart an aversive, almost noxious taste (Verhagen et al., 2003). Both TRPV1 and TRPA1 are nociceptive ion channels located on either intestinal mucosal cells (Purhonen et al., 2008) or nerve terminal innervating the GI tract (Cattaruzza et al., 2009; Kondo et al., 2009). Hence, I hypothesize:

1. PUFAs activate TRPA1 and/or TRPV1 (nociceptive ion channels) on sensory neurons.
2. PUFAs acting on TRPA1 and/or TRPV1 can modulate gastrointestinal function.
DHA PRODUCES Ca\textsuperscript{2+} TRANSIENTS IN TRPA1 EXPRESSING DRG NEURONS

Numerous TRP channels have been identified as fatty acids receptors (Chyb et al., 1999; Estacion et al., 2001; Hu et al., 2006; Matta et al., 2006; Raghu, 2006; Andersson et al., 2007). Consuming polyunsaturated fatty acids (PUFAs), particularly those found in oily fish, can produce an aversive taste (Verhagen et al., 2003). Based on these two observations, I began a quest to identify other potential TRPs that may sense fatty acids. To test if PUFAs can directly activate nociceptors I performed Ca\textsuperscript{2+} imaging experiments in cultured sensory neurons. Figures 9A and B show that in a population of AITC sensitive DRG neurons, DHA (n-3, 22:6) also increased intracellular Ca\textsuperscript{2+} (17.5%, 25 of 143 cells; Figure 9G). Conversely, the saturated fatty acid lauric acid (12:0) failed to substantially activate sensory neurons (3.4%, 2 of 58 cells, Figures 9D, E and G). Notably, all DHA-sensitive neurons responded to the TRPA1 agonist, AITC (Figure 9B, C and G). To confirm a critical role for TRPA1 in sensing DHA, I tested sensory neurons obtained from TRPA1-null mice. As demonstrated in Figure 9F, neurons lacking TRPA1 were mostly unresponsive to DHA (1.7%, 1 of 60 cells; Figures 9F and G). However, 27% (16 of 60 cells; Figure 9G) of cultured DRG neurons were responsive to capsaicin (CAP). Capsaicin was used to determine potential TRPA1 positive neurons as Bautista and colleagues demonstrated that all ATIC sensitive neurons are also capsaicin sensitive (or TRPV1 containing) (Bautista et al., 2006).
Figure 9. DHA activates TRPA1 expressing neurons

(A) Image of DHA-responsive neurons (top panel) and a nonresponsive neuron (bottom panel) Scale bar is 40 µm. (B-E) Representative Ca$^{2+}$ transients evoked by DHA (100 µM), AITC (1 mM), lauric acid (LA, 100 µM), or KCl (140 mM) in DRG neurons from wild-type mice. (F) Representative Ca$^{2+}$ transients evoked by DHA (100 µM) or Capsaicin (CAP, 100 nM) in DRG neurons from TRPA1-null mice. (G) The percentage of WT or TRPA1-null neurons responsive to DHA (WT, n=25 of 143; TRPA1-null, n=1 of 60), LA (WT, n=2 of 58), AITC (WT, n=39 of 201) or CAP (TRPA1-null, n=16 of 60).
TRPA1 CONTRIBUTES TO THE AVERSIVE QUALITY OF DHA

Next, I explored whether TRPA1 contributes to fatty-acid aversion by performing taste preference tests in mice. First, I trained mice to eat sweetened, flavored gelatin out of two dishes. Mice were individually placed in the testing environment and allowed to acclimate for 30 minutes before they received two dishes containing gelatin. Animals were given 1 hour to freely consume the gelatin. The training period lasted for approximately 1 week. Mice that did not eat equally out of both dishes were excluded. On the test day animals were presented with gelatin supplemented with or without DHA (0.5 or 5 mM). Both WT and TRPA1-null mice displayed nearly equal preference for normal gelatin and gelatin containing 0.5 mM DHA (Figure 10A). This suggests a concentration of 0.5 mM DHA was below the limit of detection. However, WT mice had a negative preference to gelatin containing 5 mM DHA while TRPA1-null mice showed no preference (Figure 10A). DHA is an oil and therefore could effect the gelatin texture and contribute to the taste preference. To control for textural affects mice were given a choice of two lipids; DHA a polyunsaturated fatty acid or lauric acid a saturated fatty acid that does not activate TRPA1. During this test, WT type mice preferred 5 mM lauric acid over 5 mM DHA (Figure 10B) ruling out non-specific texture effects. Furthermore, when WT mice were given no choice, that is they received one dish of gelatin containing either 5 mM DHA or lauric acid, they consumed DHA and lauric acid supplemented gelatin as if it were fatty acid free. This argues against a strict avoidance of DHA. Taken together, these data suggest TRPA1 is essential for sensory nerve detection of PUFAs and their ensuing behavioral responses.
Figure 10. TRPA1 senses DHA in vivo
(A) Percentage of gelatin containing DHA (0.5 or 5 mM) consumed over 1 hour by WT (n=10) or TRPA1-null mice (n=7) compared to gelatin without DHA. T-test * p<0.05 (B) Percentage of gelatin containing DHA (5 mM) consumed compared to gelatin with LA (5 mM). (n=3) (C) Percentage of gelatin containing DHA (5 mM) or LA (5 mM) consumed compared to lipid free gelatin consumed the previous day by wild-type mice only. (n=2)
DHA DIRECTLY ACTIVATES TRPA1

To test if DHA directly actives TRPA1, I performed voltage-clamp experiments in HEK293 cells transfected with TRPA1. Consistent with AITC activation of TRPA1, DHA (100 µM) evokes an outwardly rectifying current that reverses at 0 mV (Figure 11A). PUFAs readily oxidize in aqueous solutions forming electrophiles. Electrophilic compounds can activate TRPA1 by covalently modifying cysteine residues (Hinman et al., 2006; Macpherson et al., 2007). Indeed, numerous TRPA1 agonists including AITC activate the ion channel in this manner. Therefore, the DHA response observed in Figure 11A potentially involves a DHA metabolite and not DHA itself. To explore this possibility I co-applied the antioxidant N-acetyl-L-cysteine (NAC) with AITC or DHA. Figure 11B shows that 15 mM NAC completely abolishes an AITC (1mM) evoked response. Hence, NAC effectively sequesters electrophilic compounds. Surprisingly, both DHA and EPA (n-3, 20:5, 100 µM) produce robust currents in the presence of either NAC or ascorbic acid (15 mM, Figures 11C and D). This indicates PUFAs are directly stimulating TRPA1.

DHA activates TRPA1 in a dose-dependent manner at -60 mV with an EC$_{50}$ of 41±5 µM (Figure 11E). The maximal efficacy of DHA is approximately 50% of a saturating dose of AITC (1mM). At depolarizing potentials (+100 mV) DHA activation is equal to AITC (Figure 11A). Hence, DHA synergizes with voltage activation producing the enhanced response. I also performed a dose-response analysis of DHA in a TRPA1-expressing cell line developed from intestinal enteroendocrine cells. However, solutions containing DHA were extensively sonicated and remained at room temperature for more than an hour promoting lipid oxidation. Under these conditions there was no difference in the EC$_{50}$ (45±17 µM) but the maximal response increased
to ~100% at negative potentials (-50 mV, Figure 12). This result suggests that oxidized DHA metabolites impart greater TRPA1 activation than DHA alone.
Figure 11. DHA activates rat TRPA1 in transfected HEK293 cells
(A) I-V relationship for DHA (100 µM) and AITC (1 mM). (B&C) Representative current traces demonstrating n-acetyl-L-cysteine (NAC, 15 mM) prevents the activation of 1 mM ATIC (B) but not 100 µM DHA (C) in HEK293 cells expressing rat TRPA1. (D) Percent EPA (100 µM) current at -60 mV and +100 mV in control buffer (n=4) or with NAC (15 mM, n= 4), or Ascorbic Acid (15 mM, n=4). All responses normalized to 1 mM AITC. (E) DHA activates TRPA1 in a dose dependent manner with an EC$_{50}$ = 41±5 µM, Hill Coefficient = 3.8±1.3 and E$_{max}$ = 49±6% (n=3-5).
Figure 12. Oxidized DHA acts as a full agonist at TRPA1
(A) Dose-response for DHA in STC-1 cells. EC$_{50}$ = 45±17 µM; Hill coefficient=1.6±0.8 µM; $E_{\text{max}}$=106±11% (n=3-12)
Specific structural requirements for PUFA activation of TRPA1

PUFAs vary in carbon-chain length, degree of saturation and position of the first double bond. I used multiple fatty acids to investigate the structural requirements for PUFA activation of TRPA1. Figure 13B shows that activation of TRPA1 increases with both carbon-chain length and polyunsaturation. At least three double bonds are required for activation while maximal activation is achieved with at least four double bonds and 20 carbons. Surprisingly, TRPA1 discriminates between n-3 and n-6 fatty acids. n-3 arachidonic acid (20:4) strongly activated TRPA1 while the response of an equal concentration of n-6 arachidonic acid was ~5 to 10 fold less.
Figure 13. PUFA activation of TRPA1 is dependent on chain length, degree of saturation and location of first double bond
(A) Fatty acid molecular structures. (B) Fatty acid (100 μM) responses normalized to 1 mM AITC at -60 or +100 mV in HEK293 cells transfected with rat TRPA1. (n=4-8)
DHA SENSING IS NOT PHYLOGENETICALLY CONSERVED

The nociceptive function of TRPA1 is highly conserved across many species. Humans to insects employ TRPA1 as a sensor of harmful compounds. Indeed drosophila, zebrafish (A and B), mouse, rat, and human TRPA1 are stimulated by AITC (Figure 14). I investigated whether fatty acid sensing is also conserved. Surprisingly, this property is not. Figure 15 shows that DHA (100 µM) robustly activates mouse, rat and human TRPA1. Conversely, DHA does not activate drosophila (Figure 14A and 15) or either zebrafish TRPA1 ortholog (zebrafish A and B, Figures 14B, C and 15). Thus, fatty acid sensing appears to be selective for mammalian TRPA1 channels.
Figure 14. Species specific $I-V$ relationships for DHA and AITC activation of TRPA1
(A-D) $I-V$ relationship for DHA (100 µM) and AITC (1 mM) responses in HEK293 cells
transfected with drosophila (A), zebrafish A (B), zebrafish B (C), mouse (D), rat (E), or human
(F) TRPA1.
Figure 15. DHA activates mammalian TRPA1
(A) Normalized DHA (100 µM) responses at -60 and +100 mV in HEK293 cells transfected with either drosophila, zebrafish A, zebrafish B, mouse, rat or human TRPA1. (n=3-6)
DHA INDUCES GASTRIC PEPTIDE SECRETIONS THROUGH TRPA1

In addition to sensory nerves, TRPA1 is expressed within the small intestine (Stokes et al., 2006). Recently, TRPA1 was identified in the well-established enteroendocrine cell line, STC-1. Purhonen and colleagues demonstrated that AITC evokes CCK secretion (Purhonen et al., 2008). With an EC$_{50}$ of 40 µM, DHA activation of TRPA1 is biologically relevant in the gut after consumption of a meal high in PUFAs (Kris-Etherton et al., 2003). Using the STC-1 cell line Figure 16A shows that DHA activates TRPA1 currents that are blocked by the selective TRPA1 antagonist HC030031. Strikingly, DHA produced enhanced CCK secretion over the saturated fatty acid, lauric acid (Figure 16B). This suggests that n-3 PUFAs may have a greater contribution to peptide secretion by activating TRPA1.

Further, in situ hybridization revealed that TRPA1 co-localizes with serotonin in the enterochromaffin cells lining the gut mucosa. I used the RIN14B cell line, an enterochromaffin cell model that expresses TRPA1, to study the effects of DHA on TRPA1 (Nozawa et al., 2009). Figure 16C shows DHA evoked outwardly rectifying currents that are blocked by HC030031. Nozawa et al. demonstrated that AITC elicited ileal smooth muscle contractions which were mediated by 5-HT (Nozawa et al., 2009). Potentially, DHA activation of TRPA1 would evoke a similar response.
Figure 16. DHA activation of TRPA1 evokes CCK secretion from STC-1
(A) I-V relationship demonstrating a DHA (100 µM) response and block by the TRPA1 antagonist, HC030031 (50 µM) in STC-1 cells. (B) CCK secretion from STC-1 cells. 100 µM DHA, 50 µM HC030031, 500 µM LA, 100 µM AITC, 140 mM KCl (n=4-12) T-test ** p<0.01
(C) I-V relationship demonstrating a DHA (100 µM) response and block by the TRPA1 antagonist, HC030031 (50 µM) in RIN14B cells.
DHA INTERACTS WITH AN INTRACELLULAR DOMAIN

Finally, I searched for a fatty acid binding site on TRPA1. First, to test the location (intracellular or extracellular) of DHA activation, I used DHA conjugated to coenzyme A (DHA-CoA). The coenzyme A form of DHA not only increases its solubility but also prevents it from crossing the membrane. Figure 17A shows that extracellular application of DHA-CoA (100 µM) does not activate the channel. In contrast, intracellular application produces a response equal to DHA alone. This clearly demonstrates that DHA must interact with an intracellular domain or within the inner leaflet of TRPA1.

Previously, Xiao and colleagues used chimeric TRPA1 channels to determine the menthol activation site (Xiao et al., 2008). Since DHA displays species specific effects on TRPA1 (Figures 14, 15, 17B and C), I employed a similar approach. The fifth transmembrane domain (TM5) of TRPA1 contains amino acid residues required for non-electrophile agonist gating. Replacing TM5 of mouse TRPA1 (mA1) with the drosophila sequence does not alter DHA sensitivity (Figure 17D). Hence TM5 is not required for DHA-induced activation. Next, when the N-terminus of drosophila TRPA1 (dA1) was replaced with the mouse N-terminus, the channel did not gain DHA sensitivity (Figure 17E). This suggests the N-terminus alone is not necessary for activation. The reverse chimera (mouse TRPA1 with the drosophila N-terminus) is needed to adequately rule out involvement of the N-terminus. However, this and other chimeras reportedly do not form functional channels (Xiao et al., 2008).
Figure 17. PUFAs do not interact with the N-terminus nor transmembrane domain 5 of TRPA1

(A) Average responses to control buffer, DHA (20 µM, 100 µM), DHA-CoA (100 µM), and intracellular applications of DHA-CoA (20 µM), and DHA (20 µM) in HEK293 cells expressing rat TRPA. Background not subtracted. (+100 mV; n=3-4) (B-E top) Diagram of chimera TRPA1 channels. (B-E bottom) I-V relationships for DHA (100 µM) and AITC (1 mM) responses in HEK293 cells expressing chimera TRPA1 channels.
**FATTY ACID-INDUCED Ca\(^{2+}\) MOBILIZATION IN STC-1 CELLS**

To study the mechanisms by which fatty acids increase [Ca\(^{2+}\)] in STC-1 cells, I compared fatty acid-evoked responses under Ca\(^{2+}\) and Ca\(^{2+}\) free conditions. Figure 18 shows both DHA (50 µM, Figures 18A and B) and lauric acid (500 µM, LA, Figures 18C and D) induce Ca\(^{2+}\) transients in a normal media (Figures 18A and C) and in a Ca\(^{2+}\)-free environment (Figures 18B and D). Noticeably, DHA evokes a long lasting response in Ca\(^{2+}\) containing medium (Figure 18A) which is not seen in Ca\(^{2+}\)-free conditions (Figure 18B). An antioxidant was not added to the lipid containing solutions for these experiments. It is possible that the long lasting transient is caused by covalent modification of TRPA1 by DHA metabolites. Further, these data confirm that fatty acids increase Ca\(^{2+}\) through two distinct mechanisms – Ca\(^{2+}\) entry and Ca\(^{2+}\) release from internal stores.
Figure 18. Both polyunsaturated and saturated fatty acids increase intracellular Ca$^{2+}$ in STC-1 cells

(A-F) Averaged Ca$^{2+}$ transients in STC-1 cells evoked by DHA (50 µM), lauric acid (LA, 500 µM), AITC (100 µM) and KCl (140 mM) under normal Ca$^{2+}$ (1.2 mM) conditions (A, C, E and F) or Ca$^{2+}$ free conditions (B&D). (n=25-40 cells per experiment)
Others speculate that lauric acid induces CCK secretion by sustaining high intracellular Ca\(^{2+}\) levels (McLaughlin et al., 1998). Indeed, a prolonged application (30 minutes) of lauric acid (500 µM) does maintain higher than background levels of Ca\(^{2+}\) (Figure 19A). However, the sustained level of Ca\(^{2+}\) produced by DHA (50 µM, Figure 19B) is much greater. This difference may underlie the enhanced CCK secretion elicited by DHA compared to lauric acid induced release (Figure 16B).
Figure 19. Ca\textsuperscript{2+} response to prolonged fatty acid application in STC-1 cells
(A-D) Averaged Ca\textsuperscript{2+} transients in STC-1 cells for DHA (50 µM), lauric acid (LA, 500µM), and KCl (140 mM). n=10-22 cell per experiment
IDENTIFICATION OF STORE-OPERATED Ca\(^{2+}\) ENTRY AND OTHER TRPs IN STC-1 CELLS

The activation of GPCRs on STC-1 cells produces DAG and IP\(_3\). In turn, IP\(_3\) releases Ca\(^{2+}\) from internal stores. Depletion of endoplasmic reticulum (ER) Ca\(^{2+}\) triggers store-operated calcium entry (SOCE) through specific Ca\(^{2+}\) channels in the plasma membrane. STC-1 cells display basal fluctuating Ca\(^{2+}\) transients (Figures 18A, 18C and 20B) which significantly contribute to normal intracellular Ca\(^{2+}\) levels (Glassmeier et al., 1998). Figure 20A shows that removal of extracellular Ca\(^{2+}\) decreases intracellular Ca\(^{2+}\) levels (reduction in fluorescence) and the spontaneous transients cease. After the reintroduction of Ca\(^{2+}\) media the intracellular [Ca\(^{2+}\)] returns to baseline (Figure 20A). However, thapsigargin, a sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) inhibitor that depletes ER Ca\(^{2+}\) stores, also produces a large increase in intracellular Ca\(^{2+}\) when cells are reintroduced to 1.2 mM Ca\(^{2+}\) (Figure 20B). This confirms that STC-1 cells have store-operated calcium entry (SOCE).

Store depletion is sensed by stromal interactional molecule (STIM) proteins within the ER. STIM proteins then organize Orai subunits into plasma membrane channels that are highly permeable to Ca\(^{2+}\). The current produced is known as the calcium release-activated calcium (CRAC) current. Recently, TRPC channels were implicated in producing CRAC currents. Indeed STIM not only activates Orai but also TRPC1, C2, C3, C4, and C5 (Huang et al., 2006; Yuan et al., 2007; Liao et al., 2008; Ma et al., 2008; Yuan et al., 2009). Therefore, I sought to indentify TRPCs in STC-1 cells. I identified TRPC1, C2 and C3 expression by RT-PCR (Figure 20C). Both TRPC2 and C3 are activated by the diacylglycerol (DAG) analog, 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Lucas et al., 2003; Fauconnier et al., 2007). Indeed OAG (500 µM) evoked Ca\(^{2+}\) transients in STC-1 cells. However, further studies that directly measure CRAC currents are...
needed to verify the contribution of TRPCs to CRAC currents in STC-1 cells since OAG is a potential TRPA1 agonist (Bandell et al., 2004).

Taken together, these data suggest there is more than one mode of Ca$^{2+}$ entry in STC-1 cells. While Ca$^{2+}$ entry through voltage gated Ca$^{2+}$ channels leads to robust peptide secretion it would be beneficial to determine the contribution of SOCE and TRPC channels.
Figure 20. Additional modes of Ca\(^{2+}\) entry in STC-1 cells

(A&B) Average Ca\(^{2+}\) responses demonstrating store-operated entry. Thapsigargin (10 µM), Ionomycin (5 µM), 1.2 mM Ca\(^{2+}\) n=15-25 cells (A) PCRs of TRPC channels expressed in STC-1 cells. (B) Averaged Ca\(^{2+}\) transients evoked by OAG (500 µM). (n=25)
DISCUSSION

In this study I demonstrate that the TRPA1 ion channel is a polyunsaturated fatty acid receptor. This is not the first TRP channel to be identified as such. Indeed numerous invertebrate and vertebrate TRPs are activated by PUFAs (Chyb et al., 1999; Estacion et al., 2001; Hu et al., 2006; Matta et al., 2006; Raghu, 2006; Andersson et al., 2007). Additionally, Bandell and colleagues reported that TRPA1 can be activated by lipids (arachidonic acid, AA, and OAG) in the phospholipase C pathway (Bandell et al., 2004). These studies demonstrate activation using tens of micromolar amounts of lipid which would not be physiologically relevant except at fatty acid micro domains within the peripheral nervous system. With an EC$_{50}$ of approximately 40 $\mu$M for DHA, activation of TRPA1 on peripheral sensory nerves is highly unlikely under normal conditions. However, these concentrations are highly relevant in the GI tract. For example, a 3oz. serving of salmon alone can provide nearly 2 grams of DHA and EPA (2005). Further, DHA is also a major dietary constituent of suckling animals.

Fatty acids have a multitude of effects on taste. Saturated fats like those in butter and cream enhance the palatability of food while longer chain PUFAs found in fish can produce an aversive “fishy” taste (Verhagen et al., 2003). Further PUFAs can alter the perception of bitter and umami tastants (Kooriyama T, 2000). While Cartoni and Laugerette with their respective colleagues indicate fatty acid receptors GPR40 and GPR120 (Cartoni et al., 2010) and CD36 (Laugerette et al., 2005) mediate a preference for oleic and linoleic acids, the mechanism underlying DHA and EPA taste remains largely unknown. Similar to human studies, I found that WT mice have a negative preference for DHA (Verhagen et al., 2003). Yet mice lacking the TRPA1 gene seem to lack the ability to detect DHA when it is added to a gelatin treat. These
findings suggest TRPA1 is required for producing an aversive taste to n-3 PUFAs (at least at the concentration range tested).

Many of the proteins that comprise the nutrient sensing mechanisms in the mouth are duplicated throughout the intestines where they contribute to secretion of GLP-1, CCK and serotonin (Wu et al., 2002; Dyer et al., 2005; Hirasawa et al., 2005; Edfalk et al., 2008; Cartoni et al., 2010). Using the well established enteroendocrine cell line STC-1, I demonstrated that DHA activates TRPA1 producing a large influx of Ca$^{2+}$. Further, DHA evoked a ~30 fold increase in CCK secretion over basal release where as lauric acid produced only a ~5.5 fold difference. While others have reported similar effects of lauric acid on CCK release (McLaughlin et al., 1998) this is the first measurement of DHA-induced CCK secretion. It appears a majority of secretion is due to TRPA1 activation as the TRPA1 inhibitor, HC030031, reduced release by ~70%. The affects of CCK (satiety, pancreatic contraction, bile secretion, gastric emptying) are well known, hence one might suggest that DHA would produce a greater release of CCK in humans. Despite my cellular evidence, the affect of n-3 PUFAs on CCK secretion in vivo remains unclear (Edelbroek et al., 1992; Wojdemann et al., 1999; Jonkers et al., 2000). However, there are numerous confounds with the way these studies were conducted. For example, Edelbroek and colleagues demonstrate that gastric contents form layers during digestion and oily lipids can be the last to exit the stomach (Edelbroek et al., 1992). This would influence the time required to take adequate blood CCK measurements. Jonkers and others point out that in studies where oil is infused directly into the intestines, pancreatic lipases are not efficient at liberating these larger free fatty acids (Jonkers et al., 2000). Hence free fatty acid concentrations may be
reduced. Better designed studies are required to determine whether saturated or unsaturated fatty acids have a greater effect on gastric peptide secretion.

Additionally, Nozawa et al. demonstrated that AITC can induce 5-HT release from enterochromaffin cells which results in enhanced gastric motility (Nozawa et al., 2009). Using the enterochromaffin cell line, RIN14B which expresses TRPA1 and releases 5-HT, I demonstrated that DHA can activate TRPA1. This suggests DHA would also induce 5-HT secretion and regulate gastric motility. However, further experiments are necessary to confirm this hypothesis.

Long chain PUFAs, particularly DHA and EPA, are readily oxidized which is enhanced in an aqueous environment (Frankel et al., 2002). Surprisingly, very few lipid studies take this into consideration. I found that the oxidative state of DHA greatly influences TRPA1 activation. When DHA becomes oxidized it can activate TRPA1 similar to other electrophilic compounds (Hinman et al., 2006; Macpherson et al., 2007). DHA has 50% efficacy in the presence of antioxidants where as this efficacy is 100% in conditions that promote fatty acid oxidation.

This thesis is not the first report demonstrating differences for oxidized and nonoxidized PUFAs. In 2000 Diep et al. demonstrated that DHA is an agonist of peroxisome proliferator-activated receptors (PPAR, (Diep et al., 2000). Five years later, Yamamoto et al. found that oxidized forms of DHA were significantly better at inducing PPARγ activation and also docked in the thiazolidinedione binding site (Yamamoto et al., 2005). This finding is remarkable as it is often difficult to elucidate a lipid binding site on proteins because of the loose interactions (lack of covalent or hydrogen bonding) between proteins and fatty acids. In our attempt to identify a PUFA binding site on TRPA1 I have ruled out extracellular domains as well as TM5. Further,
the N-terminus alone is not sufficient to cause activation. More experiments are required to definitively confirm a PUFA binding site.

Two other interesting findings emerged from these studies. First, omega-3 but not omega-6 fatty acids activate TRPA1. Omega-3 fatty acids can not be synthesized *de novo* and thus must be obtained directly from the diet or through bioconversion of α-linolenic acid. Infants lack the enzymes involved in bioconversion hence the only source of DHA and EPA are from the mother. The role of TRPA1 as an n-3 PUFA sensor in infants has yet to be determined. Separately, this suggests there are strict structural requirements for lipid activation of TRPA1.

Secondly, DHA has a species specific effect on TRPA1 – only mammalian TRPA1 is activated by DHA. While many TRPA1 properties are highly conserved many non-conserved properties have recently been revealed. Kang and colleagues established TRPA1 as a highly conserved, ancient (~500 million years) noxious chemical sensor based on the ion channel’s ability to detect electrophiles (Kang et al., 2010). Conversely, other thioaminal-containing, electrophilic compounds block human TRPA1 while activating rat TRPA1 (Chen et al., 2008). In addition, caffeine activates rodent (mouse) TRPA1 but inhibits human TRPA1 (Nagatomo and Kubo, 2008). Further, the non-electrophilic compound, menthol, exerts bimodal effects on mouse TRPA1; activation at low concentrations and block at high concentrations (Macpherson et al., 2006; Karashima et al., 2007). The significance of selective n-3 PUFA detection in mammals remains unclear.

Lastly, I explored alternative modes of Ca\(^{2+}\) entry in intestinal enteroendocrine cells. As previously mentioned, the most commonly recognized mode of entry is through voltage-gated Ca\(^{2+}\) channels. However, I have demonstrated Ca\(^{2+}\) influx through both TRPA1 and TRPC ion
channels as well as store-operated calcium entry (SOCE). Although SOCE is crucial for proper immune cell function (Varnai et al., 2009), further studies are need to elucidate its role in GI function.
CHAPTER V:

GENERAL SUMMARY
The first part of this thesis demonstrates a role for TRPV1 in obesity. Mice lacking the TRPV1 ion channel are resistant to diet induced obesity. It appears disruption of TRPV1 expression promotes an increase in energy expenditure and a lean phenotype. Potential altered energy expenditure may result from thermoregulatory effects or CGRP signaling. With ever increasing global obesity rates there is a great need to combat this disease as well as its related health effects. While numerous pharmaceutical companies are fervently developing selective TRPV1 antagonist for the treatment of pain these drugs may also have a utility in weight loss.

TRPV1 is located both in the thermoregulatory center of the hypothalamus and on thermosensitive peripheral neurons. Currently, it is difficult to determine the precise role TRPV1 has in each location. In the future, conditional knock-out mice – mice lacking TRPV1 in either peripheral or central neurons – would be a useful model for studying the contribution of each location in thermoregulation.

Further, I found that TRPV1-null mice exhibit a greater capacity to thermoregulate in a near freezing environment. Kanizsai et al. also demonstrated that TRPV1-null mice maintain normal body temperature during a prolonged fast (Kanizsai et al., 2009). These data may suggest that TRPV1 is involved in regulating body temperature during hibernation. During hibernation periods, ambient temperatures are near or below zero and animals fast for months. A reduction in body temperature resulting in a decrease in energy expenditure during this time would be highly beneficial to survival.

I also discovered differential short-term feeding behaviors in WT and TRPV1-null mice. TRPV1-null mice consume greater amounts of glucose enriched gelatin. In addition, TRPV1-null mice have elevated blood glucose levels. Based on these findings I propose TRPV1 may be a
glucose sensor on vagal afferents. While TRPV1 activity is modulated by intracellular ATP, further studies are needed to determine if hyperglycemic conditions alter intracellular ATP levels and subsequently modulate TRPV1 activity.

The second part of this thesis explored the role of TRPA1 as a fatty acid receptor. TRPA1 is directly activated by omega-3 polyunsaturated fatty acids. Expressed in the GI tract, TRPA1 is prime for modulating gastric function. Indeed, I found DHA activation of TRPA1 induces CCK secretion in STC-1 cells. DHA also activates TPRA1 in enterochromaffin cells. Potentially, n-3 PUFAs can modulate gastric motility through the release of 5-HT.

DHA is essential for proper neuronal and eye development and function. Deficits in n-3 PUFAs are associated with a host of pathologies. Adults possess the ability to synthesize longer chain fatty acids de novo from shorter, less saturated fatty acids. However, infants lack the required enzymes for bioconversion hence their sole source is from breast milk. In the future it would be interesting to investigate if TRPA1 expression or function is developmentally regulated. Potentially, DHA activation of TRPA1 in neonates has significant effects on gastric function resulting in enhanced motility, peptide secretion or even absorption of n-3 PUFAs.

Overall this thesis describes novel roles for TRPA1 and TRPV1 in nutrient sensing and obesity. Both ion channels are predominantly recognized for their ability to detect noxious stimuli and mediate pain. However, it is increasingly evident that TRPA1 and TRPV1 can modulate other homeostatic functions. As these ion channels are explored as potential pharmacological targets it is necessary to be aware of and continue to study their other functions.
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