AN IN VITRO MURINE MODEL TO STUDY
INTESTINAL MESENTERIC AFFERENT ACTIVITY
IN RESPONSE TO LUMINAL FATTY ACID STIMULI

by

William Andrew Webster

A thesis submitted to the Department of Physiology
In conformity with the requirements for
the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
(June, 2010)

Copyright ©William Andrew Webster, 2010
Abstract

Obesity is pandemic. Pharmacological treatment development depends on modeling the regulation of feeding, particularly by free fatty acids (FFA). Most models have been employed in the rat in vivo, and show FFA-stimulated intestinal satiety signals are dependent on the fat’s acyl chain-length, involve cholecystokinin (CCK) secretion, and are mediated by vagal afferents. I hypothesized that an in vitro mouse model could be employed, with sensitivity to measure afferent responses to nutrient stimuli.

Male C57BL/6N mice were killed, the intestine harvested en bloc, and a jejunal section dissected with neurovascular mesenteric arcade emanating centrally. The tissue was placed in a Krebs-superfused chamber, the lumen cannulated with the outlet open to drain, and Krebs or other mediators were continuously perfused intraluminally. The dissected afferent nerve was placed in a suction electrode for extracellular recording. Afferent responses to distension and the perfusion of mediators (e.g. CCK or FFA) were tested. Preparations from normal mice (no surgery), or from mice following chronic subdiaphragmatic vagotomy or sham operation, were used to assess vagal afferent contributions.

Luminally-perfused CCK (100 nM) increased afferent firing. This response was abolished with the CCK-1 receptor antagonist lorglumide (10 µM). The short-chain fatty acid (SCFA) sodium butyrate (30 mM) potentiated firing. The long-chain fatty acid (LCFA) sodium oleate (1-300 mM) activated concentration-dependent firing (EC\textsubscript{50}=25.35 mM) that was significantly greater at 30 mM than that evoked by butyrate. Lorglumide (30 µM) abolished the oleate (30 mM) response. The L-type Ca\textsuperscript{2+} channel (LTCC) inhibitor nicardipine (3 µM), intraluminally, potentiated the oleate response, while bath application abolished it. Vagotomy
attenuated the oleate response. Vagotomy abolished the intraluminal CCK (100 nM) response, and attenuated the response to bath-superfused CCK.

These findings support FFA chain-length-dependent mesenteric afferent activation and CCK involvement in oleate-induced firing, and suggest LTCC mediation of excitatory and inhibitory oleate response transduction pathways. The murine oleate response was shown to be mostly vagally-mediated, with some spinal contribution, and both vagal and spinal contributions to CCK responses were suggested. These data provide a basis for further investigation in vitro of cellular and molecular mechanisms of afferent satiety signals, and ultimately of obesity pathogenesis.
Acknowledgements

My sincere thanks to Dr. Michael Beyak for his mentorship and support throughout my graduate program. I am honoured to have been your first graduate student, and appreciate how you have enriched my scientific acumen and knowledge of GI physiology through the enthusiasm that you bring to the field.

Thank you to Dr. Sung Jin Park, Dr. Donna Daly, Ms. Ala’a Al-Helaili, and Ms. Iva Kosatka, my colleagues in the Beyak lab. I have greatly appreciated your assistance, congeniality, and kindness, and you have each contributed much to the quality of my experience at Queen’s.

I would like to acknowledge and express my appreciation to the Gastrointestinal Diseases Research Unit and the Department of Physiology of Queen’s University, and to the benefactors of the Franklin Bracken Fellowship (2008-09) for supporting my graduate studies.

Finally, I would like to thank my wife, Colleen Webster, for her ongoing and unwavering support and enthusiasm for my endeavours. You inspire me to keep going, especially when the going is difficult.
Table of Contents

Abstract ................................................................................................................................. ii
Acknowledgements ............................................................................................................. iv
Table of Contents ................................................................................................................. v
List of Figures ........................................................................................................................ vii
List of Abbreviations .......................................................................................................... ix
Chapter 1  Introduction ........................................................................................................ 1
  1.1 Obesity and the Physiology of Food Intake ................................................................. 1
  1.2 Gastrointestinal Epithelial Satiation Signals ............................................................. 2
    1.2.1 Intestinal Satiation Signals .................................................................................. 5
      1.2.1.1 Production of Satiety Mediators by Enteroendocrine Cells ......................... 5
      1.2.1.2 Production of Satiety Mediators by Enterochromaffin Cells ....................... 7
  1.3 CNS Regulation of Food Intake ..................................................................................... 9
  1.4 The Vagus Nerve and Afferent Satiety Signals ......................................................... 11
  1.5 Fatty Acid Binding Receptors ..................................................................................... 12
    1.5.1 G-Protein-Coupled Receptors: Transducers of Nutrient Intake Stimuli to Afferent Satiety Signaling? ................................................................. 12
    1.5.2 Orphan Receptors .............................................................................................. 14
    1.5.3 GPR40 ................................................................................................................. 16
    1.5.4 GPR41 ................................................................................................................. 19
    1.5.5 GPR43 ................................................................................................................. 20
    1.5.6 GPR120 ................................................................................................................. 21
  1.6 Vagal Afferent Responses to Fatty Acids .................................................................... 23
  1.7 Unresolved Issues ....................................................................................................... 25
  1.8 Hypothesis .................................................................................................................... 27
Chapter 2  Methods ............................................................................................................. 28
  2.1 Animal care .................................................................................................................. 28
  2.2 Surgical preparation: vagotomized animals .............................................................. 28
  2.3 Tissue preparation ...................................................................................................... 30
  2.4 Nerve and intraluminal pressure recording .............................................................. 31
  2.5 Experimental protocols .............................................................................................. 32
2.6 Drugs................................................................................................................................. 34
2.7 Data analysis ....................................................................................................................... 35

Chapter 3  Results ...................................................................................................................... 36
3.1 Ramp distension of jejunum preparation produces characteristic afferent response ........ 36
3.2 Intraluminal ATP perfusion potentiates afferent firing .................................................... 36
3.3 Attenuation of intraluminal CCK perfusion-induced afferent firing increase by lorglumide ......................................................................................................................... 39
3.4 The concentration-response relationship of luminally-perfused sodium oleate .......... 43
3.5 Intraluminal sodium butyrate perfusion potentiates afferent firing ................................. 46
3.6 Lorglumide attenuates afferent firing increases in response to intraluminal sodium oleate perfusion .................................................................................................................. 48
3.7 Nicardipine attenuates afferent firing increases in response to intraluminal sodium oleate perfusion .................................................................................................................. 50
3.8 Vagotomy suppresses baseline jejunal afferent firing, and attenuates afferent firing increases in response to perfusion of the saline vehicle, sodium oleate, and CCK ............ 55

Chapter 4  Discussion .................................................................................................................. 63
4.1 Characteristic afferent response to jejunal distension ..................................................... 65
4.2 Lorglumide abolishes CCK-induced afferent responses ................................................. 65
4.3 Luminal sodium oleate perfusion and concentration-dependent afferent responses ...... 66
4.4 Afferent response to intraluminal sodium butyrate ......................................................... 66
4.5 Lorglumide-induced abolition of oleate responses ......................................................... 67
4.6 Nicardipine-induced modulation of the oleate response .................................................. 68
4.7 Vagotomy pre- and post-surgical considerations and confirmation of effectiveness at removing jejunal vagal innervation ................................................................................. 71
4.8 Vagotomy-induced abolition of oleate responses ............................................................. 73
4.9 Vagotomy-induced modulation of CCK responses ............................................................ 74
4.10 Future directions .............................................................................................................. 75
4.11 Broader significance: a novel in vitro murine model to study intestinal afferent responses to nutritive intraluminal stimuli .................................................................................. 76

References..................................................................................................................................... 79
List of Figures

Figure 1. A schematic representation of the short- and long-term regulation of appetite and food intake.................................................................................................................................................. 3
Figure 2. Locations of production of GI peptides implicated in the regulation of feeding.............. 4
Figure 3. Basic layout of secretory enteroendocrine cells and absorptive enterocytes comprising a villus within the small-intestinal wall.................................................................................................................................................. 6
Figure 4. Similarities in nutrient-sensing mechanisms of lingual taste-receptor cells (left) and intestinal enteroendocrine cells (right).................................................................................................................................................. 8
Figure 5. CNS control mechanisms of food intake............................................................................. 10
Figure 6. Classical example of G protein-coupled receptor (GPCR) signaling............................ 15
Figure 7. Subdiaphragmatic vagotomy surgery in anaesthetized mouse....................................... 29
Figure 8. Extracellular mesenteric afferent nerve recording, using the suction electrode technique, from an isolated, lumen-perfused, in vitro murine small-intestinal preparation........... 33
Figure 9. The afferent response to jejunal ramp distension........................................................ 37
Figure 10. Afferent responses to repeat jejunal ramp distensions.............................................. 38
Figure 11. The afferent response to intraluminal ATP perfusion.................................................. 40
Figure 12. The afferent response to intraluminal CCK perfusion, and the lorglumide-mediated attenuation of that response......................................................................................................................... 42
Figure 13. The afferent response to intraluminal sodium oleate perfusion.................................... 44
Figure 14. The sodium oleate concentration-response relationship............................................ 45
Figure 15. The afferent response to intraluminal sodium butyrate perfusion............................. 47
Figure 16. The afferent response to intraluminal sodium oleate perfusion, and the lorglumide-mediated attenuation of that response......................................................................................................................... 49
Figure 17. The afferent response to intraluminal sodium oleate perfusion, and the nicardipine-mediated (via intraluminal perfusion) modulation of that response................................. 52
Figure 18. The afferent response to intraluminal sodium oleate perfusion, and the nicardipine-mediated (via bath superfusion) attenuation of that response.................................................. 53
Figure 19. Evidence that chronic subdiaphragmatic vagotomy was effective in removing vagal innervation of the jejunum......................................................................................................................... 56
Figure 20. Vagotomy-mediated attenuation of the afferent response to intraluminal sodium oleate perfusion......................................................................................................................... 58
Figure 21. Vagotomy-mediated attenuation of the afferent response to intraluminal CCK perfusion...

Figure 22. Vagotomy-mediated attenuation of the afferent response to bath superfusion of CCK...
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>SCFA</td>
<td>short-chain fatty acid</td>
</tr>
<tr>
<td>LCFA</td>
<td>long-chain fatty acid</td>
</tr>
<tr>
<td>LTCC</td>
<td>L-type Ca²⁺ channel</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine; serotonin</td>
</tr>
<tr>
<td>EE</td>
<td>enteroendocrine</td>
</tr>
<tr>
<td>EEC</td>
<td>enteroendocrine cell</td>
</tr>
<tr>
<td>ECC</td>
<td>enterochromaffin cell</td>
</tr>
<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>PYY</td>
<td>peptide YY</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>PP</td>
<td>pancreatic polypeptide</td>
</tr>
<tr>
<td>APO AIV</td>
<td>apolipoprotein A-IV</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>FFAR</td>
<td>free fatty acid receptor</td>
</tr>
<tr>
<td>[Ca²⁺]ᵢ</td>
<td>intracellular Ca²⁺ concentration</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus tractus solitarius</td>
</tr>
<tr>
<td>DVC</td>
<td>dorsal vagal complex</td>
</tr>
<tr>
<td>GDP</td>
<td>guanine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>FFA₁R</td>
<td>free fatty acid receptor 1</td>
</tr>
<tr>
<td>RNAᵢ</td>
<td>ribonucleic acid interference</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco-endoplasmic reticulum Ca²⁺-ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>FFA₂R</td>
<td>free fatty acid receptor 2</td>
</tr>
<tr>
<td>TRPV₁</td>
<td>transient receptor potential vanilloid 1</td>
</tr>
<tr>
<td>sc</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>half-maximal (50%) effective concentration</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half-maximal (50%) inhibitory concentration</td>
</tr>
<tr>
<td>OEA</td>
<td>oleoylethanolamide</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1 Obesity and the Physiology of Food Intake

Obesity and its associated morbidity is on the verge of becoming the most major health concern of our time. Among Canadian adults, estimates indicate that >25% are obese, and >50% are overweight (Canadian Community Health Survey, 2005). Despite our short-term variation of food intake and energy expenditure, our individual maintenance of long-term energy balance is surprisingly precise. Our evolutionary history of coping with famine, however, has “tuned” this homeostatic system toward fat storage and weight gain (Konturek et al., 2005). As well, patterns of overconsumption of high energy foods, particularly those high in fats, and decreased energy expenditure in Western societies over the past few decades have contributed significantly to the current obesity epidemic. The pending health crisis posed by this issue has led to the urgent need for both appropriate lifestyle changes and for the development of novel pharmacological therapies for obesity. Solutions to this problem depend on the elucidation of the control mechanisms of food intake, with specific inquiry about how humans can override normal satiety signals, despite decreasing energy demands, to overeat. Currently, this research is somewhat hindered by the relative dearth of detailed knowledge about the physiological means by which nutrient-related gut satiety signals are received and transduced into afferent nervous traffic to the brain. Understanding the mechanisms of physiological signaling arising from the FFA products of lipid digestion is seen to be central to understanding the physiological causes of the obesity epidemic. More so than the products of protein or carbohydrate digestion, these high energy-density molecules are thought to be important both as macronutrients that are closely associated with obesity causation (Astrup et al., 2008) and as generators of physiological satiety signals that
communicate gut content to the brain (Beglinger & Degen, 2004). It is therefore the intent of this research program to advance knowledge of the mechanisms of FFA-induced gut satiety signal reception and transduction into afferent nervous signals.

1.2 Gastrointestinal Epithelial Satiation Signals

Efficient nutrient digestion and absorption are primary gut functions. Gastrointestinal (GI) satiation signals optimize those processes by modulating gut motility and secretion, and by limiting meal size by inducing a sense of fullness, thereby controlling the rate at which ingested nutrients reach the gut (Strader & Woods, 2005; Cummings & Overduin, 2007; Valassi et al., 2008). Multiple GI sites signal satiety, including the stomach, proximal and distal small intestine, colon, and pancreas. Satiation is evoked by ingested food through the effects of gastric distension and the release of peptide and non-peptide (e.g. 5-HT) products from enteroendocrine (EE) cells (EECs) and enterochromaffin cells (ECCs) of the GI tract, and secretory cells of the pancreas. These short-acting signals are transmitted neurally and hormonally to the brain regions responsible for the control of food intake, which are also modulated by adiposity hormones (e.g. leptin, insulin) to achieve long-term energy balance (Schwartz et al., 2000; Morton et al., 2006) (Fig. 1).

The humoral contributions of the GI epithelium to satiation signaling are both sophisticated and fundamental to health. The enteroendocrine (EE) system of the GI tract (GIT) is the largest endocrine organ in the body, and secretes most of those factors. Specific gut content, such as the FFA products of lipid digestion, influences the secretion of many GI hormones from the specialized EECs, which makes the peptides well-suited to CNS signaling about nutrient status (Chaudhri et al., 2008). EE hormone production is distributed throughout the GIT (Fig. 2), and gastric and intestinal satiation signals function in unison, augmenting each
Figure 1. A schematic representation of the short- and long-term regulation of appetite and food intake

Multiple GI sites signal satiety, including the gut (stomach, proximal and distal small intestine, and colon), liver and pancreas. Satiation is evoked by ingested food through the effects of gastric distension, and also by the release of peptide products (e.g. CCK, GLP-1) from enteroendocrine cells (EECs) of the GI tract. These short-acting signals are conveyed to the brain regions responsible for the control of food intake (primarily the hypothalamus), which are also modulated by adiposity hormones (e.g. leptin, insulin) to achieve long-term energy balance.

(Modified from Konturek et al, 2005)
Figure 2. Locations of production of GI peptides implicated in the regulation of feeding

Shown are the main locations of synthesis for each mediator, although many of these molecules are detectable in smaller quantities at other sites in the GI system. In addition, most of them are also synthesized within the brain, including CCK, GLP1, PYY, and possibly PP.

(Modified from Cummings & Overduin, 2007)
other’s effects (Ritter, 2004). Among the many EE satiety peptides, the most important to intestinal satiation responses are cholecystokinin (CCK), peptide YY (PYY), glucagon-like peptide-1 (GLP-1), and pancreatic polypeptide (PP). Other peptides including ghrelin, leptin, apolipoprotein A-IV (APO AIV), oxyntomodulin, amylin, enterostatin, glucagon, insulin, and neuropeptide Y (NPY) are also involved in the regulation of feeding by the GIT.

1.2.1 Intestinal Satiation Signals

1.2.1.1 Production of Satiety Mediators by Enteroendocrine Cells

In response to nutrient ingesta (e.g. FFAs) sensed within luminal contents by EECs, satiety mediators (e.g. CCK) are secreted from the basolateral membrane of small-intestinal EECs. The peptides diffuse through interstitial fluids to enter circulation, and/or directly activate nearby neuronal afferents (vagal, spinal, submucosal, and myenteric), which innervate the intestinal wall and lie very close to EECs (Berthoud et al., 2004; Cummings & Overduin, 2007) (Fig. 3). The sensing or “tasting” of luminal nutrients by EECs of the small intestine, which initiates the release of satiety mediators and their subsequent signaling cascade, is thought to be mediated by apical nutrient-sensing guanine nucleotide-binding protein- (G-protein-) coupled receptors (GPCRs). Ligand binding domains of these seven-transmembrane proteins are specific to various nutritive molecules within the luminal contents of the intestine. In the case of apical GPCRs that are specifically activated by the binding of FFAs, the discovery of that ligand-receptor pairing resulted in that family of nutrient-sensing receptors being termed “free fatty acid receptors” (FFARs) (Kotarsky et al., 2003). Activation of nutrient-sensing GPCRs by ligand binding and the resultant signaling cascade within the cell cause an increase of intracellular
Figure 3. Basic layout of secretory enteroendocrine cells and absorptive enterocytes comprising a villus within the small-intestinal wall

Enteroendocrine cells sense nutrient content luminally, and respond with the secretion of satiety mediators from their basolateral membrane. The molecules diffuse through interstitial fluids to enter circulation for distant action as hormones, and/or directly activate nearby neuronal afferents (vagal, spinal, and enteric) innervating the intestinal wall.

(Modified from Cummings & Overduin, 2007)
calcium concentration ([Ca$^{2+}$]$_i$) within the EEC, possibly by activation of voltage-gated LTCC. The intracellular calcium surge ultimately results in the basolateral exocytotic release of satiety mediators from the cell. Collectively, the nutrient-sensing and signal-transduction pathways that result in the release of satiation signaling molecules by EECs are similar to the taste-transduction mechanisms of lingual taste-receptor cells, and were previously thought to be expressed solely by taste-bud cells (Cummings & Overduin, 2007) (Fig. 4). This functional linkage between taste and EE cells is further supported by work that suggests that the intestinal satiety mediator CCK may work as a taste transmission regulator, as it has been shown to be expressed by taste-receptor cells (Herness et al., 2002). Moreover, expression of some G-proteins operative in taste-receptor cells has been found in gut cells (Hofer et al., 1996; Wu et al., 2002), and some GPCRs relevant to reception of specific tastes by lingual taste-buds have been found in EECs of the gut (Wu et al., 2002).

Satiety mediators released by the EECs of the intestine cause the perception of fullness, in conjunction with gastric distension, and thus act to promote meal termination (Lal et al., 2004; Cummings & Overduin, 2007). Through the generation of multiple signaling mechanisms, meals of varying content are matched with specific satiety responses (Dockray, 2003).

1.2.1.2 Production of Satiety Mediators by Enterochromaffin Cells

Other specialized secretory cells of the intestinal epithelium include enterochromaffin cells (ECCs), which secrete 5-HT across the basolateral membrane in response to chemical and mechanical stimuli. There are significant data implicating ECCs in the detection of bacterial enterotoxins in the GIT lumen, which triggers 5-HT release and in turn the coordinated physiological response to eliminate the pathogen from the body (Farthing, 2000). This is
Figure 4. Similarities in nutrient-sensing mechanisms of lingual taste-receptor cells (left) and intestinal enteroendocrine cells (right)

In both cell types, activation of apical nutrient-sensing GPCRs (taste receptors or FFARs, respectively) by binding of luminal nutrient ligands initiates a signaling cascade within the cell. The final common pathway in both cell types is an increase of intracellular calcium concentration via LTCC. The intracellular calcium surge causes the basolateral exocytotic release of the peptide messenger products of the cell. These nutrient-sensing and signal transduction pathways were previously thought to be expressed only by taste-receptor cells. (Modified from Cummings & Overduin, 2007)
accomplished by increasing GIT motility, and by inhibiting further consumption of the material by inducing nausea. ECCs are also thought to participate in luminal nutrient signaling, particularly in response to sugars (Raybould, 1999). Like EECs, ECCs seem positioned to strategically “taste” the luminal contents, then generate satiety signals through the generation of action potentials in the periluminal vagal afferent nerve terminals via the release of 5-HT (Gershon, 1999). Studies indicate that 5-HT released from ECCs acts on mucosal afferents directly via activation of 5-HT receptors on the afferent terminals (Grundy, 2006), which are also located near the basolateral surface of both EECs and ECCs (Berthoud et al., 2004).

1.3 CNS Regulation of Food Intake

The hypothalamus, one of the most evolutionarily-ancient central nervous system (CNS) structures, plays a significant regulatory role in feeding. The arcuate nucleus (ARC), in the mediobasal hypothalamus, serves as an integrator of neurological and endocrine signals and a transporter of hormones across the blood-brain barrier. Within the ARC, the regulation of appetite involves two major subpopulations of neurons (Chaudhri et al., 2008), which act to inhibit and increase food intake, respectively (Cone et al., 2001). Both populations project to the paraventricular nucleus (PVN) of the hypothalamus, and other loci of importance in the regulation of feeding (Schwartz et al., 2000). The brainstem, in particular the nucleus tractus solitarius (NTS) division of the caudal brainstem’s dorsal vagal complex (DVC), is also important in satiety signaling (van der Kooy et al., 1984; Ter Horst et al., 1989). The NTS may also act as an integrator of endocrine and neuronal signals, given the extensive reciprocal connectivity of the brainstem and hypothalamus (Chaudhri et al., 2008) (Fig. 5).
The hypothalamus plays a significant regulatory role in feeding. The arcuate nucleus (ARC) serves as an integrator of neurological and endocrine signals. Within the ARC, appetite regulation involves two major subpopulations, which act to modulate food intake. Both populations project to the paraventricular nucleus (PVN) of the hypothalamus. The nucleus tractus solitarius (NTS) region of the brainstem is also important in satiety signaling, and may also act as an integrator of endocrine and neuronal signals, given the extensive reciprocal connectivity of the brainstem and hypothalamus. (Modified from Valassi et al, 2008)
1.4 The Vagus Nerve and Afferent Satiety Signals

Information about the state of the small intestine is conveyed to the CNS by mixed mesenteric afferent nerve bundles, the endings of which innervate the intestine. These mixed nerves are comprised of both spinal and vagal fibers, however the vagus nerve is considered by many to be a major neuroanatomical link between the gut and the brain, often termed the “gut-brain axis”. Vagal afferents convey information about direct mechanical or chemical stimulation of the GIT by ingesta to cell bodies of the nodose ganglia, whose central terminals innervate the brainstem’s DVC, which sends output via the NTS to the hypothalamus and higher cortical centers (Schwartz, 2000; Chaudhri et al., 2008).

Vagal mechanosensitive fibers are stimulated dose-dependently by gastric loads within the physiological range, independently of nutrient content of the load (Schwartz et al., 1991; Mathis et al., 1998). Mechanoreceptor activation due to gastric distension has been shown to suppress feeding (Phillips & Powley, 1996), and is abolished by subdiaphragmatic vagotomy (Phillips & Powley, 1998). Post-prandial satiation, however, is not completely explained by gastric distension. Indeed, the expression of receptors on the vagus nerve with specificity for a number of satiety hormones implicates the vagus nerve as a major site of EE signaling and therefore of the physiological regulation of food intake (Chaudhri et al., 2008). This concept is reinforced by the findings that the actions of CCK (Moran & Kinzig, 2004), PYY (Abbott et al., 2005), GLP-1 (Abbott et al., 2005; Koda et al., 2005), and PP (Asakawa et al., 2003) on food intake/satiety are each abolished by vagotomy.

Preliminary results from in vivo experiments support a role for the vagus nerve in the detection of luminal fatty acids in the intestine. Vagal afferent activity is increased to a greater extent by intestinal infusion of FFAs than by saline infusion (Randich et al., 2000). Also, the pattern of increased vagal afferent firing is characteristic of the fatty acid chain-length, and the
activation responses are abrogated by chronic subdiaphragmatic vagotomy. Mechanistically, long-chain fatty acids (LCFAs) are thought to act via a CCK-mediated mechanism, while short-chain fatty acids (SCFAs) seem to act directly on afferent terminals (Lal et al., 2001). To this point, however, the mechanisms by which FFA ingesta stimuli are transduced into afferent nerve signals, or the specific receptors that are involved, remain to be fully elucidated (Dockray, 2003).

1.5 Fatty Acid Binding Receptors

1.5.1 G-Protein-Coupled Receptors: Transducers of Nutrient Intake Stimuli to Afferent Satiety Signaling?

As discussed (1.2), the mechanisms by which nutrient ingesta stimuli are transduced into afferent nerve satiety signals by the GIT’s secretory EEC and EC cells, or the specific receptors that are involved, remain to be fully elucidated. The ability of those cells to “taste” luminal ingesta and initiate a cascade to signal the brain about the nutrient content is thought to be mediated by GPCRs, and there is a growing body of supporting evidence to that effect (Dockray, 2003; Kotarsky et al., 2003). As such, further understanding of the GPCR family is integral to an advanced understanding of the physiology of food intake and the pathophysiology of obesity.

Sequence homology within GPCRs enables the grouping of the thousands of known receptors of this type into three distinct families. Little sequence identity beyond the characteristic 7TM architecture is evident between families, but proteins within each family share at least 25% sequence identity in the core 7TM region, and a distinctive and highly conserved pattern of residues and motifs. The putative receptors for GI satiety hormones comprise part of the relatively small GPCR Family B. This receptor sub-group includes only approximately 25 proteins, all of which are coupled mainly to the G-protein Gs, which activates the second
messenger effector adenylyl cyclase. In contrast, there is evidence that apical GPCRs on EECs may couple with the G-protein $G_{olf}$, which activates calcium channels, since LTCC-mediated $Ca^{2+}$ influx is thought to precede the exocytotic release of satiety mediators by EECs (Pierce et al., 2002). Thus, G proteins are the functional connections between 7TM receptors and effectors. This activity is enabled in G proteins due to their heterotrimeric structure of $\alpha$, $\beta$, and $\gamma$ subunits. Four subfamilies of G proteins are recognized, based both on their $\alpha$ subunits, which bind the ‘inactive’ guanine nucleotide guanosine diphosphate (GDP), and the second-messenger effector processes with which they are linked. For example, $G_{s}$ proteins contain $G_{\alpha s}$ and couple to adenylyl cyclase stimulation, while $G_{olf}$ proteins contain $G_{\alpha olf}$ and couple to calcium channel activity (Pierce et al., 2002).

GPCR activation by the binding of an agonist, such as a FFA ligand, causes conformational changes from the low-affinity state to an activated form (Farahbakhsh et al., 1995; Ballesteros et al., 2001). An activated receptor transiently couples to the heterotrimeric G protein, forming a high-affinity complex of agonist, activated receptor, and G protein. The activated heterotrimeric complex serves as a guanine nucleotide exchange factor (GEF) to facilitate GDP dissociation from the $\alpha$ subunit, followed by binding of the ‘active’ nucleotide guanosine triphosphate (GTP) (Pierce et al., 2002). This exchange triggers the dissociation of the heterotrimer into an $\alpha$ subunit/GTP complex and a $\beta\gamma$ dimer (Gilman, 1987), although there is some unsettled controversy about whether physical dissociation actually occurs versus a more orderly molecular rearrangement of the components (Klein et al., 2000). Following dissociation, the receptor is available to activate the next G protein.

Downstream signaling is accomplished independently by both the $\alpha$ subunit and the $\beta\gamma$ dimer via the activation or inhibition of one or more effectors, the modulation of which leads to classic physiological responses (Pierce et al., 2002). For example, activation of the effector
adenyl cyclase causes intracellular cyclic AMP (cAMP) to increase, which in turn activates protein kinase A (PKA), which phosphorylates many different substrates (Pierce et al., 2002) (Fig. 6). Finally, the transduced signal is terminated by the hydrolysis of GTP to GDP by the native enzymatic activity of the $G_\alpha$ subunit. This hydrolytic change allows the reassociation of the G protein heterotrimer, renewing its availability for the next activation cycle (De Vries et al., 2000; Ross & Wilkie, 2000).

1.5.2 Orphan Receptors

GPCRs for which the sequence but neither the ligand nor the function are known are termed “orphan receptors”. The “deorphanization” of such receptors remains one of the primary goals of GPCR research (Lefkowitz, 2007). Progress in GPCR deorphanization continues to provide new insights into processes regulated by the receptors (Howard et al., 2001; Lee et al., 2002), including the physiological regulation of food intake, and thus the revelation of novel GPCR drug targets. GPCR research is also advancing by the development of transgenic mice in which specific 7TM receptors have been knocked out (Pierce et al., 2002).

The deorphanization of some GPCRs by the identification of FFA ligands has enabled critical and relatively recent progress in the understanding of obesity and the physiology of satiety. The receptors GPR40, GPR41, GPR43, and GPR120 have been deorphanized, with the finding that their common endogenous ligands are FFAs (Briscoe et al., 2003; Brown et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003; Nilsson et al., 2003; Hirasawa et al., 2005; Stewart et al., 2006). Those findings were significant because of the physiologically-important roles of FFAs that had been previously elucidated, and because of the relative paucity of prior information regarding receptors for FFA ligands.
Figure 6. Classical example of G protein-coupled receptor (GPCR) signaling

In the absence of a ligand, GPCRs (e.g. β2 adrenergic receptor [β2-AR]) are in a low-affinity state. After agonist binding, a transient, high-affinity complex of ligand + activated receptor + G protein is formed. The G protein’s α subunit exchanges GDP for GTP, which causes dissociation of the G-protein complex into its α subunit and βγ dimer. The dissociated G protein constituents activate effectors (e.g. adenylyl cyclase), which causes intracellular cAMP to increase. PKA is thereby activated, and in turn phosphorylates many different substrates, including GPCRs, other kinases, and transcription factors, producing the cell response. (Modified from Pierce et al, 2002)
Lipids, particularly the FFA products of lipid digestion, are recognized for their provision of energy to cells, their structural contributions to cellular components, and their roles among the body’s chemical messengers (Chawla et al., 2001). This functional combination makes FFAs unique as both nutritive substances and potential mediators of diseases such as obesity and type 2 diabetes (Unger & Zhou, 2001; Boden & Shulman, 2002). In the past decade, it has been reported that the messenger role of some FFAs (Nunez, 1997) may be mediated by nuclear peroxisomal proliferator-activated receptors (PPARs) (Berger & Moller, 2002). These mechanisms, however, do not explain all reported biological effects of FFAs. Indeed, some appear to be independent of PPAR mediation (Sauer et al., 2000; Louet et al., 2001) and moreover are characteristic of involvement by cell-surface receptors including GPCRs (Funk, 2001). The identification of GPR40/41/43/120 as putative surface receptors for FFAs therefore represented a major advance in knowledge regarding the physiological signaling pathways of FFAs. The salient aspects of the knowledge regarding the interactions of these specific ligand/receptor pairings are summarized in the following sections of this review.

1.5.3 GPR40

The functional role of GPR40 was reported independently by the Briscoe (Briscoe et al., 2003) and Kotarsky (Kotarsky et al., 2003) groups. Both teams reported that GPR40 (termed Free Fatty Acid Receptor 1 [FFA1R] by Kotarsky et al) is activated by medium- and long-chain FFAs. It was demonstrated in cultured HEK293 cells transfected with GPR40 that challenges with saturated and unsaturated FFAs with chain-lengths greater than 6 carbons (e.g. elaidic acid, palmitic acid), but not SCFAs (e.g. succinic acid, formic acid), dose-dependently elevated \([\text{Ca}^{2+}]_i\). GPR40 expression was found in human, rat, and mouse brain and pancreas, with murine pancreatic expression localized to β-cells, where insulin production occurs. These data suggested
that GPR40 may mediate some physiological effects of FFAs in the brain and pancreatic islets, and may therefore be involved in the regulation of food intake (Briscoe et al., 2003).

Further discoveries regarding the tissue distribution of GPR40, and its specific ligands resulted in the proposal that the receptor acts in the capacity of a “nutrient-sensing receptor”, a transducer linking dietary nutrients and signaling molecules. Broad expression of GPR40 was found in skeletal muscle, heart, liver, and placenta, and the possible chain-length specificity of GPR40 was reaffirmed by the demonstration that a broad spectrum of FFAs with carbon chain-lengths greater than 10 activated GPR40 in reporter cell lines expressing it, while SCFAs did not (Kotarsky et al., 2003). The evidence of Stewart et al was contradictory in that regard, however, as they reported for the first time the activation of GPR40 by SCFAs, specifically those with carbon chain-lengths of 4 or greater, such as butyric acid, in addition to medium- and long-chain FFAs (Stewart et al., 2006). The tissue distribution of this putative nutrient-sensing receptor was further investigated in the rat tongue, with the hypothesis that it would be expressed there as an oral sensor of ingested dietary fat. GPR40 mRNA was not detectable in the sensory papillae of the rat, however, suggesting that dietary nutrient intake signal transduction may be substantially limited to the GI regions of the alimentary tract (Matsumura et al., 2007).

GPR40 has also been implicated as a putative mediator of insulin release, through the determination that activation of native GPR40 receptors in pancreatic β-cell lines mobilizes $[\text{Ca}^{2+}]_i$ (Kotarsky et al., 2003). It was also demonstrated that the activation of GPR40 in pancreatic β cells by LCFAs amplifies glucose-stimulated insulin secretion, an important discovery that elevated the potential for the development of GPR40 agonists and/or antagonists as new anti-diabetic drugs (Itoh et al., 2003). Significant expression of GPR40 mRNA was found in human pancreas and in isolated human pancreatic islets, as well as in tissue extracts from human insulinoma, but not in those from glucagonoma or gastrinoma. Expression in isolated pancreatic
islets was shown to be particularly high, with levels comparable to other genes known to have abundant human pancreatic islet expression. These findings indicate the probable expression of GPR40 in human pancreatic β cells and further implicate GPR40 in human insulin secretion, and therefore potentially in the pathophysiology of obesity (Tomita et al., 2005; Tomita et al., 2006).

GPR40-specific RNA interference (RNAi) was used to document the impairment of the expected increase of insulin secretion and [Ca$^{2+}$], in response to receptor activation by the FFA palmitic acid in a cultured β cell line. The attenuation of FFA-dependent [Ca$^{2+}$], increase was also demonstrated by application of inhibitors of G$\alpha_{q}$, phospholipase C (PLC), and sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA), as well as with LTCC blockers. These data suggest that GPR40 mediates increased [Ca$^{2+}$], and insulin secretory up-regulation via the G$\alpha_{q}$-PLC pathway, which facilitates Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) and leads to increased Ca$^{2+}$ influx via LTCC (Shapiro et al., 2005). The concentration-dependent increase of [Ca$^{2+}$], in rat pancreatic β cells stimulated with the LCFA sodium oleate, and its attenuation in cells transfected with small interfering RNA (siRNA) against GPR40, or in which PLC inhibitors, Ca$^{2+}$-free conditions, or the LTCC blocker nitrendipine had been applied, was also demonstrated. Increased insulin release was also associated with oleate stimulation of isolated islets, and was attenuated significantly by LTCC and PLC inhibition (Fujiwara et al., 2005). GPR40-specific antibodies were subsequently used to localize GPR40 expression to the plasma membrane of pancreatic β cells, which are the manufacturers of insulin, further implicating GPR40 in the insulinogenic pathway and therefore in the regulation of feeding (Salehi et al., 2005).

GPR40 may be an important mediator in the linkage of obesity and type 2 diabetes, since that receptor is a putative regulator of both acute and chronic insulin secretory impairment associated with elevated FFAs. This further links GPR40 signaling to dysregulated glucose homeostasis. Specifically, it was demonstrated that insulin secretion is reduced in response to
FFAs in GPR40-deficient β cells. Conversely, it was shown that, in mice with β cells in which GPR40 is overexpressed, impaired β cell function, hypoinsulinemia, and diabetes are induced (Steneberg et al., 2005). Latour et al reported that GPR40 may modulate approximately 50% of insulin secretion in the full acute response to FFAs in mice. They observed, through in vivo and in vitro experiments with GPR40-/- animals, that insulin secretion following short-term challenges with FFAs was reduced by about one-half, and FFA-potentiated insulin release in isolated islets was significantly reduced. However, their findings did not support GPR40 mediation of insulin secretory impairment resulting from chronic FFA exposure. Indeed, they reported that islets from GPR40 knockout mice, upon prolonged FFA exposure, were as prone to FFA-dependent attenuation of insulin secretion as islets from wild-type (WT) mice (Latour et al., 2007), suggesting that GPR40 is involved in multiple and differential mediatory pathways for feeding regulation. It is noteworthy that most studies to date have focused on FFARs (e.g. GPR40) as putative sensors of circulating, not luminal, FFAs, therefore luminal interactions of FFAs with their putative receptors continues to be a research avenue of great interest.

1.5.4 GPR41

The deorphanization of GPR41 was reported in 2003 by Brown et al. SCFAs had previously been identified as human GPR43 agonists, with activation confirmed by [Ca\(^{2+}\)]\(_i\) mobilization assays. Given the significant sequence homology between GPR41 and GPR43, it was unsurprising that similar ligands were found to activate GPR41, but with different carbon chain-length specificity. Adipose tissue was initially identified as the locus of primary expression of GPR41 (Brown et al., 2003). Le Poul et al also reported the deorphanization of GPR41, with the SCFA propionate being the most potent agonist of the receptor. They found broad GPR41 expression, in lymph node, spleen, bone marrow, and peripheral blood mononuclear cells
(PBMCs; e.g. lymphocytes, monocytes). Given the broad distribution, they were unable to make clear inferences regarding putative physiological functions of GPR41 (Le Poul et al., 2003), however its reported SCFA ligands do suggest that it may play a role as a nutrient-sensing receptor.

Xiong et al demonstrated that the application of physiological concentrations of SCFAs with carbon chain-lengths of 2 to 6 stimulated expression of the anorexigenic, adipose-derived hormone leptin in both mouse adipose tissue and the mouse adipocyte cell line Ob-Luc, via a pathway modulated by GPR41. They showed that the stimulatory effect of propionic acid on leptin expression was almost completely abolished by interference with siRNA against three different coding regions of GPR41 mRNA, and by application of pertussis toxin, a G-protein inhibitor (Xiong et al., 2004). This discovery may indicate an important role for GPR41 in leptin production, and therefore its relevance in pathways in which leptin mediation has been demonstrated, including the modulation of food intake. A recent study has also shown a role for GPR41 in mediating the effects of gut microflora on host adiposity, which further implicates GPR41 in the regulation of feeding and obesity (Samuel et al., 2008).

### 1.5.5 GPR43

As previously discussed (1.5.4), ligands of GPR43 were reported by Brown et al, who identified several SCFA agonists of the receptor. They determined that the highest levels of GPR43 localization were in immune cells including B-lymphocytes, monocytes, and neutrophils, but were unable to elucidate clear physiological roles for the receptors (Brown et al., 2003). It was subsequently demonstrated that the stimulatory effects of GPR43 on neutrophils are similar to those of SCFAs, in terms of chemotaxis and [Ca\textsuperscript{2+}], mobilization responses (Le Poul et al., 2003). This suggests a possible role for GPR43 as a transducer of SCFA-stimulation in other
pathways involving the modulation of LTCC-mediated Ca\(^{2+}\) uptake, such as those implicated in the secretion of satiety mediators in the GIT.

The functional role of GPR43 was also reported by Nilsson et al, who termed it Free Fatty Acid Receptor 2 (FFA\(_2\)R), and also identified SCFA ligands of the receptor. They found predominant human GPR43 expression in leukocytes, and the spleen to a lesser extent (Nilsson et al., 2003). Recent discoveries have indicated putatively-important GPR43 activity in the GIT. Karaki et al localized GPR43 to rat intestinal EECs containing PYY and to intestinal mucosal mast cells containing 5-HT, leading them to hypothesize that those cells use the GPR43 receptor to sense luminal SCFAs (Karaki et al., 2006). Prior studies had demonstrated the release of 5-HT (Fukumoto et al., 2003) and PYY (Cherbut et al., 1998) from the ileum and colon following stimulation with SCFAs, which led to the proposal that GPR43 may be mechanistically involved in these pathways as the transducer between nutrient stimuli and satiety mediator release in the distal GIT (Karaki et al., 2006). More recently, GPR43 mRNA expression was found throughout the rat gut, in all areas of the GIT distal to the esophagus, with highest levels in the colon (Dass et al., 2007). Most recently, the expression of GPR43 in the human colon was documented, in EEL-cells immunoreactive for PYY (Karaki et al., 2008). GPR43 remains an interesting candidate as a putative transducer of FFA stimuli in pathways involved in the control of feeding and obesity.

1.5.6 GPR120

GPR120 activation was detected, using \([\text{Ca}^{2+}]_i\) mobilization assays, upon stimulation with LCFAs with carbon chain-lengths of 14-22. GPR120 mRNA expression was found in both mouse and human tissues, in both cases with highest levels in the lung and distal GIT. GPR120 mRNA expression was also found in the cultured murine EEC line STC-1, but not in other EE
cell lines. It was elegantly demonstrated that GPR120 stimulation with unsaturated LCFAs dose-dependently promoted the secretion of GLP-1 \textit{in vitro} and \textit{in vivo}, causing circulating insulin levels to correspondingly increase (Hirasawa \textit{et al.}, 2005). GLP-1 is the most potent insulinotropic incretin hormone (MacDonald \textit{et al.}, 2002; Drucker, 2003) and is secreted by the gut’s EE L-cells in response to food intake. The putative modulation of the FFA-stimulated cascade of GLP-1 and insulin release by GPR120 suggests that receptor may be a therapeutic target of importance for diabetes (Freeman, 2009).

GPR120 has recently been implicated in the regulation of adipogenesis, which is indeed of importance in the pathophysiology of obesity. GPR120 mRNA are found to be highly expressed in adipose tissues from multiple loci in the mouse, and to be up-regulated in the same tissues of mice fed a high-fat diet. It was demonstrated that GPR120 mRNA levels increased during adipocyte differentiation in 3T3-L1 cells, and this effect was similarly observed in cultured adipocytes, human preadipocytes, and human adipose tissue. Furthermore, it was shown that the down-regulation of GPR120 with siRNA inhibited adipocyte differentiation (Gotoh \textit{et al.}, 2007). These results suggest that GPR120 may have a physiological role of importance in adipose tissue development and, and therefore in the pathophysiology of obesity.

GPR120 has also recently been implicated as the transducer of FFA-induced CCK secretion (Tanaka \textit{et al.}, 2007). CCK is released from EE I-cells (Liddle, 1997) in response to nutrient ingesta, particularly fat, and helps to optimize fat digestion via the regulation and integration of a range of intestinal responses, including gallbladder emptying, inhibition of gastric motility, and stimulation of pancreatic secretion (Hopman \textit{et al.}, 1985; Liddle \textit{et al.}, 1986; Smith & Gibbs, 1994; Higham \textit{et al.}, 1997; Liddle, 1997). It was documented that intra-gastric LCFA administration increased plasma CCK levels in the mouse, a system that was subsequently modeled using cultured murine STC-1 cells. CCK secretion was promoted by LCFA application
to the cells, and was abolished either by LTCC blocking with nicardipine, removal of extracellular Ca\(^{2+}\), or inhibition by transfection of short hairpin RNA specific to GPR120. These results suggest that CCK secretion is induced by LCFA stimulation of GPR120-coupled Ca\(^{2+}\) signaling, and represent another significant knowledge gain with regard to the putative involvement of GPR120, and FFA receptors in general, in the physiology of the regulation of feeding (Tanaka et al., 2007).

1.6 Vagal Afferent Responses to Fatty Acids

The mechanisms by which dietary lipid in the intestinal lumen is transduced into vagal afferent activation, and subsequently to GI reflex control, are incompletely defined, as are the means by which FFAs of different chain-lengths differentially activate the vagus nerve. While the pathways that link the differential effects on GI function to FFA chain-length are not yet fully understood, they are indeed believed to involve the vagus nerve since many are ablated by vagotomy (Yox et al., 1991; Lloyd et al., 1993; Holzer et al., 1994). The body of evidence established to date suggests a sub-family of GPCRs as transducers of FFA ingesta into vagal afferent satiation signals. The specificity of FFA-activated GPCRs for ligands of particular carbon chain-length (i.e. short, medium, long) has been elucidated, as discussed (1.5), and it has been demonstrated that some of those receptor/ligand pairs mediate pathways of importance to the physiology of food intake and to the pathogenesis of obesity and diabetes.

It has long been recognized that the presence of lipids in the intestine inhibits both gastric emptying (Holzer et al., 1994) and gastric acid secretion (Lloyd et al., 1993) and reduces food intake (Walls et al., 1995). It has furthermore been established that the FFA components of a lipid meal provide its effective stimulus (Hunt & Knox, 1968; Guimbaud et al., 1997) and that the carbon chain-length determines the effectiveness of the FFA with respect to the induction of
satiation responses (Melone & Mei, 1991). For example, CCK secretion was shown to be dose-dependently increased in humans by the GIT infusion of FFAs with carbon chain-lengths of 12 or longer, but not 11 or shorter, and the response was blocked in isolated murine EECs by the application of the LTCC antagonist nicardipine (McLaughlin et al., 1998; McLaughlin et al., 1999). The satiety-related delay in gastric emptying is thought to be mediated via a vagovagal reflex. When vagal afferents are stimulated by CCK released in response to FFA ingesta, they signal the brainstem to cause vagal efferent activation, which then slows gastric emptying. This reflex promotes gastric distension and the activation of mechanoreceptors that induce the perception of fullness, which thereby acts to terminate feeding. Gastric mechanoreceptors are themselves believed to be sensitized in humans by CCK release (Lal et al., 2004), and therefore may be subject to modulation that is dependent on FFA chain-length due to the acyl chain-length specificity of CCK release (McLaughlin et al., 1998; McLaughlin et al., 1999). Lal et al Furthermore demonstrated that vagal afferent activation in vivo, in response to infusion of FFAs into the intestinal lumen of rats, is characteristic of the carbon chain-length of the agonist (Lal et al., 2001). Thus, the FFA chain-length specificity of fatty acid-binding GPCRs, CCK release by EECs (and other satiation responses), and vagal afferent activation appear to be similar, and therefore putatively linked in pathways of importance in the physiology of food intake regulation.

The work done by Lal et al with respect to differential vagal afferent activation in response to stimulation with FFAs of different chain-lengths was potentially of great importance to the advancement of our understanding of the physiology of the control of feeding and the pathophysiology of obesity and its co-morbidities. That laboratory used extracellular recording techniques to record jejunal mesenteric afferent nerve discharge, in in vivo preparations of anesthetized rats, in response to luminal perfusion of saline, the LCFA sodium oleate, and the SCFA sodium butyrate. Characteristic chemosensitive afferent nerve responses were evoked by
both FFAs. Since the whole perivascular mesenteric nerve bundles contained both vagal and spinal nerves, chronic subdiaphragmatic vagotomy was performed, which abolished the afferent responses to both FFAs and thus confirmed that the responses were due to the stimulation of vagal fibers. The abolition of the oleate effect by devazepide, a CCK-1 receptor antagonist, was also reported, while the effect of butyrate was not abolished by either the application of devazepide or the LTCC inhibitor nifedipine. Collectively, these results indicate that intestinal mesenteric vagal afferents are differentially activated by long- and short-chain FFAs. Furthermore, these data suggest that the effect of oleate is mediated in a paracrine manner by EE CCK release, probably by activation of CCK-1 receptors on vagal afferent nerve terminals. Conversely, butyrate may act directly on vagal afferent nerve endings, which could make its pathways more difficult to discern due to in vivo factors, such as circulatory effects or the putative involvement of other secreted hormones (Lal et al., 2001).

1.7 Unresolved Issues

In vivo experimentation is often fraught with difficulties due to the complexity of the model. It is often difficult to discern the activity of specific mechanisms in vivo due to the tendency of an organism to recruit multiple compensatory mechanisms to restore homeostasis in response to a given stimulus. In vivo preparations also pose difficulties regarding the maintenance of appropriate anaesthesia and analgesia throughout the experimental protocol without causing death, make systemic drug residence durations difficult to ascertain, and offer very limited numbers of preparations from each animal model. Conversely, while their primary weakness is that they are not fully representative of the whole organism from which they are taken, in vitro preparations often confer the ability to use multiple preparations from the same animal as controls, and allow better concentration-response-type pharmacological experiments
due to improved – although still imperfect - ability to perfuse with and to wash out drugs. Furthermore, and perhaps most importantly, *in vitro* preparations can simplify the elucidation of pathways under investigation, due to the inherently greater simplicity of the model.

To date, the use of *in vitro* preparations for the study of vagal afferent nerve responses to intraluminal stimulation of the intestine with FFAs has not been reported. The *in vitro* methods reported by Rong *et al* in their study of jejunal afferent nerve sensitivity to intraluminal stimulation in WT and TRPV1 receptor knockout mice involved the development of an isolated, lumen-perfused small-intestinal preparation (Rong *et al.*, 2004). This model may be of future benefit if directed toward the study of intestinal vagal afferent stimulation by FFAs, and the signal transduction mechanisms that are involved in that pathway. Also, while the work of Lal *et al* elucidated important new information regarding the FFA chain-length-dependent differential activation of vagal afferents *in vivo* in the rat, there are limitations of that model beyond those inherent to *in vivo* models in general (Lal *et al.*, 2001). The use of transgenic and knockout animals is a powerful, important, and increasingly common technique for the elucidation of genetic function. Currently, the mouse is the most commonly-used platform for the development of such models, while rats are used much less often. Therefore, the development of an isolated, murine, lumen-perfused small-intestinal *in vitro* model of the nutrient-sensing transduction pathways, in which nutrient-induced afferent activation may be recorded, is an attractive experimental goal. Such a model will be of great utility for the mechanistic study of satiation via nutrient-induced intestinal afferent activation, and therefore for our understanding of the regulation of feeding and obesity.
1.8 Hypothesis

I hypothesized that an *in vitro* preparation of the murine small intestine is a viable and experimentally reliable model for characterization of vagal afferents. To test this, I examined the sensitivity of the model to measure significant changes in vagal afferent activation in response to luminally-applied stimuli, in ways that demonstrated the model’s general mechanosensitivity, general chemosensitivity, and specific chemosensitivity to EE satiety mediators and FFAs. I further hypothesized that the *in vitro* model can be used to demonstrate that LTCC-mediated Ca$^{2+}$ influx and subsequent CCK release have important roles in the pathways that transduce luminal FFA stimuli into vagal afferent activation in the small intestine of the mouse.
Chapter 2

Methods

2.1 Animal care

All experimental protocols conformed to the guidelines of the Canadian Council on Animal Care and the regulations of the Queen’s University Animal Care Committee. Experiments were performed with adult male C57BL/6N mice (Charles River, St. Constant, QC, Canada), weighing 20-25 g, which were housed under standardized conditions (access to regular mouse chow and water ad libitum except where indicated otherwise below, 12:12-h light/dark cycle with lights on at 7:30 AM).

2.2 Surgical preparation: vagotomized animals

Subdiaphragmatic vagotomy was performed to eliminate vagal afferent fibers in the mesenteric nerve bundles, to allow for the study of relative vagal and spinal contributions to the afferent signals. Animals were fed on a liquid diet (Ensure High Protein, chocolate flavoured) for one week before surgery and had free access to water. Mice were acclimated to the liquid diet prior to surgery since vagotomy is known to result in impaired gastric emptying. Mice were fasted for 18 h before surgery, and continued to have access to water ad libitum. Due to suspected dehydration following fasting in early surgical animals, subsequent mice were hydrated with lactated Ringer’s solution (1 mL subcutaneously (sc)) 18 h before surgery, again following closure of the surgical incision, and daily thereafter for 3 days. Under general anaesthesia by ketamine-xylazine (125-150 mg/kg [ketamine] + 13-15 mg/kg [xylazine], sc) and under aseptic conditions, a midline laparotomy was performed. The abdominal walls were retracted laterally and fixed to maintain the open abdominal cavity (Fig. 7), the liver was retractedcranially to
Figure 7. Subdiaphragmatic vagotomy surgery in anaesthetized mouse
Views showing abdominal walls retracted following midline laparotomy (top), representative view (15x magnification) of vagus n. and surrounding anatomy (middle), and surgical wound closed with suture following completion of vagotomy (bottom).
expose the subdiaphragmatic esophagus, and the esophagus was retracted anteriorly to facilitate visual identification of the vagal trunks. The dorsal and ventral vagal trunks were freed along the distal 1 cm of the esophagus and sectioned to achieve the subdiaphragmatic vagotomy. In addition, to ensure the completeness of vagotomy, the left gastric artery was isolated and stripped of all connective tissue and nerves, which includes the accessory branch of the vagus n. (Fig. 7). Another group of mice underwent a sham vagotomy operation, which entailed opening of the abdomen and manipulation of the stomach and esophagus. After closure of the incision with surgical suture (Fig. 7), mice were treated with Buprenex (0.05 mg/kg, sc) for analgesia, then again after 24 h. Animals were housed individually after surgery with liquid food and water ad libitum. Mice were allowed to recover for 6-10 days after surgery before afferent recording experiments were performed.

2.3 Tissue preparation

Following the induction of deep anesthesia by an overdose of inhaled isofluorane, mice were killed by decapitation. A mid-line laparotomy was performed, and the small and large intestine were harvested en bloc. Sections of jejunum were removed between 10 and 20 cm proximal to the ileocecal junction. Each piece of jejunum was 30-35 mm in length, and was dissected out such that a non-bifurcated neurovascular mesenteric arcade emanated centrally. The intestinal segments were placed immediately into carbogenated (95% O₂ + 5% CO₂) Krebs-bicarbonate (Krebs) solution (composition, mM: NaCl 119.8; KCl 4.5; NaH₂PO₄ 0.5; MgCl₂ 1.3; NaHCO₃ 24.4; CaCl₂ 2.5; glucose 11.1) at room temperature. One of the jejunal segments was gently flushed with carbogenated Krebs by syringe to expel luminal contents. The jejunum, complete with its mesenteric bundle, was then placed into a purpose-built tissue chamber (20 mL) through which Krebs solution (34 °C) was constantly perfused by gravity feed at approximately
2.2 mL/min and removed by continuous suction. The jejunal lumen was catheterized, and secured with silk suture at the cannulae. Two Aladdin syringe pumps (World Precision Instruments (WPI), USA), were connected in parallel via a T-connector to the inlet cannula to allow intraluminal perfusion of Krebs solution or different test solutions at room temperature. A DTX-Plus pressure transducer (Becton, Dickinson (BD), USA) was also connected to the inlet cannula with a T-connector to monitor intraluminal pressure. The outlet cannula was open for free drainage. The mesenteric bundle was pinned to the Sylgard base of the tissue chamber, and the intact perivascular mesenteric nerve innervating the jejunal segment was dissected to isolate it for suction electrode recording. This in vitro model sustained oxygenated, functional intestinal preparations with viabilities extending beyond the 5-7 h that they were typically used. Since the mesenteric afferent bundles that were recorded contain both spinal and vagal fibres, to assess the relative contribution of vagal afferents, control experiments were conducted with preparations from mice that had undergone chronic subdiaphragmatic vagotomy (or a sham vagotomy operation) via prior survival surgery.

2.4 Nerve and intraluminal pressure recording

Multiunit extracellular nerve recording was conducted using the suction electrode methodology, because of its utility for the recording of afferent signals that are readily discernible due to the high signal:noise ratios that are possible with the technique. The intact and isolated mesenteric nerve was placed in a suction electrode filled with carbogenated Krebs solution. Whole-nerve electrical activity was conveyed by the electrode to a Neurolog headstage (NL100, Digitimer, UK), pre-amplified (NL104, Digitimer), filtered (NL125/126, Digitimer; band pass 125-4000 Hz), and re-filtered by a Hum Bug (50/60 Hz noise eliminator, Quest Scientific, USA). The nerve signal was acquired (19.96 kHz sampling rate) by a computer through an analogue-to-
digital converter (ADC) interface (Micro 1401 Mk. II, 500 kHz, 16-bit ADC, Cambridge Electronic Design (CED), UK) and Spike2 software (CED, version 6.01). The recorded nerve activity was both displayed in real time on the computer monitor and saved to hard disk. Mesenteric afferent nerve firing frequency (imp/s) and amplitude (μV) were recorded over the time course of the experiment and plotted orthogonally by Spike2 software. Sequential rate histograms (1 s bin size) were also concurrently plotted to more readily illustrate and facilitate interpretation of changes in firing frequency throughout the duration of the experiment. Individual action potentials above a given amplitude were counted, with the threshold set at 200% of the baseline noise of the recording. The histograms were subsequently converted to moving average spike frequency (10 s time constant) for downstream analysis. Intraluminal pressure was conveyed by the pressure transducer to a pressure amplifier (NL108, Digitimer), was acquired (99.8 Hz sampling rate) by a computer via an ADC interface and Spike2 software, and both displayed on the monitor and saved to disk. Intraluminal pressure (mmHg) was recorded throughout the experiment and plotted orthogonally by Spike2 software.

2.5 Experimental protocols

The tissue preparation was allowed to stabilize for ½ h-1 h. Throughout the experiment, Krebs solution was constantly perfused intraluminally at 10 mL/h (room temperature) and through the tissue bath at approx. 2.2 mL/min (34 °C), except when FFAs and/or drugs were being applied intraluminally and/or to the bath. Unless the jejunum was being distended, the outlet cannula was kept open to allow free drainage so that any intestinal contractions would dispel luminal content rather than raise intraluminal pressure. This recording configuration was used so as to minimize afferent activation secondary to variation in intraluminal pressure and to
Figure 8. Extracellular mesenteric afferent nerve recording, using the suction electrode technique, from an isolated, lumen-perfused, in vitro murine small-intestinal preparation

The tissue preparation is continuously superfused with carbogenated Krebs-bicarbonate solution that is removed by suction. The jejunal segment is cannulated at both ends, with tandem syringe pumps supplying the inlet side of the lumen with Krebs or other test solutions, and the outlet side open to drain (except during ramp distension protocols). A pressure transducer at the inlet side records the intraluminal pressure of the preparation. The afferent nerve is placed intact in the suction electrode, which is filled with Krebs solution, and which conveys nerve firing signals to the PC via the headstage, preamplifier, filter, noise attenuator, and A/D converter interface.
allow continuous perfusion with fresh bathing solution. To distend the intestine, a three-way tap on the intraluminal outlet cannula was closed while intraluminal perfusion with Krebs continued. In this way, the jejunum was distended to 60 mmHg in approximately 90 s before the three-way tap was opened to return intraluminal pressure to baseline. This was repeated 3 times, at regular intervals of 15 min, to test the reproducibility of the nerve response to ramp distension. To test the effect of intraluminal ATP, CCK, sodium oleate, or sodium butyrate, the perfusate was switched from Krebs solution to ATP (10 µM), CCK (100 nM), various concentrations of sodium oleate (1-300 mM), or sodium butyrate (30 mM) in carbogenated normal saline (0.9% NaCl) for 5 min. To test the effect of bath-applied CCK, the perfusate was switched from Krebs solution to CCK (100 nM) in carbogenated Krebs for 15 min. A minimum interval of 20 min was left between sequential intraluminal or bath applications of test solutions. Antagonists for CCK-1 receptors (lorglumide) or LTCC (nicardipine) were added to the Krebs solution perfusing the tissue chamber at a rate of approximately 2.2 mL/min and/or were perfused intraluminally in carbogenated Krebs at a rate of 10 mL/hr. Lorglumide (10-30 µM) and nicardipine (1-3 µM) were applied to the lumen and/or bath for 10 min prior to intraluminal (CCK, sodium oleate) or bath (CCK) agonist applications, concurrently with the agonist applications (blended with the agonist for intraluminal antagonist applications), during the 20 min interval between sequential agonist applications, and for at least 10 min following the final agonist application.

2.6 Drugs

CCK, (octapeptide; Tocris Bioscience, USA), ATP (disodium salt), sodium oleate, and sodium butyrate (Sigma-Aldrich, Canada) were dissolved in deionized and demineralized water (ddH₂O) and were diluted in carbogenated normal saline (0.9% NaCl) (or carbogenated Krebs solution, for bath applications of CCK) to required concentrations before use. Saline (not Krebs)
was chosen as the vehicle for all intraluminal perfusions because sodium oleate precipitated in Krebs, probably due to interaction with the Ca$^{2+}$ present in Krebs. Lorglumide (Sigma-Aldrich, Canada) was dissolved in carbogenated normal saline and was diluted in carbogenated Krebs solution (or carbogenated normal saline, when co-applied intraluminally with an agonist) to required concentrations before use. Nicardipine (Sigma-Aldrich, Canada) was dissolved in methanol (MtOH) and was diluted in carbogenated Krebs solution (or carbogenated normal saline, when co-applied intraluminally with an agonist) to required concentrations before use. The maximum final bath or intraluminal concentration of MtOH was 0.03%. Vehicle controls were included in all experiments.

2.7 Data analysis

The mean firing frequency (imp/s) was measured with a time constant of 10 s and plotted continuously by Spike2 software, and was subsequently sampled every 10 s for time-course analyses, while the peak mean afferent firing rate was obtained from the continuous data for peak-firing analyses. In the case of both time-course and peak-firing analyses, Δ firing rate (imp/s) was calculated as the change from the baseline firing rate, that is, the difference between the mean firing rate at the time point and the mean firing rate measured over the 180 s period prior to the application of the drug/mediator of interest. In figures showing time-course curves, every third data point was plotted to reduce clutter. Statistical analysis and graphing were performed using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, USA). Where appropriate, data are expressed as means ± SEM (n=number of animals) and compared using paired or unpaired Student’s $t$ tests for parametric data, or 2-way ANOVA with Bonferroni’s post hoc analysis for multiple comparisons. A $P$ value of < 0.05 is considered statistically significant.
Chapter 3

Results

3.1 Ramp distension of jejunum preparation produces characteristic afferent response

Mechanosensory and chemosensory elements both contribute to intestinal afferent nerve signals. Ramp distension experiments were undertaken to validate the mechanosensitivity of the isolated, lumen-perfused, murine small-intestinal model in vitro. The jejunal segment was distended to approximately 60 mmHg by closure of the tap on the intraluminal outlet cannula, while intraluminal perfusion with Krebs solution continued at 10 mL/h (Fig. 9). Distensions were repeated 3 times at regular intervals of 15 min. The resultant biphasic afferent nerve activation that was recorded for each distension was found to be substantially stable, repeatable, and reproducible, which is consistent with previous studies in vivo in the rat (Booth et al., 2001; Booth et al., 2008) and in vitro in the mouse (Rong et al., 2004). It is noted, however, that comparative analyses of the 3 serial time courses of the distension-induced afferent firing changes in the present model indicated that they are significantly different (P<0.0001), indicating slightly attenuated responsiveness, primarily between the first and the subsequent two serial distensions (Fig. 10). The responses were essentially identical at lower pressures, however, and these responses are likely mediated by vagal afferents (Booth et al., 2008). Collectively, the present findings confirmed the mechanosensitivity of this in vitro small-intestinal preparation.

3.2 Intraluminal ATP perfusion potentiates afferent firing

To assess the sensitivity of the small-intestinal preparation to luminal drug application in vitro, electrophysiological studies were conducted while ATP was perfused intraluminally. ATP
Figure 9. The afferent response to jejunal ramp distension

A) Representative trace showing the rise in intrajejunal pressure during ramp distension with Krebs solution at 10 mL/h to a maximal intraluminal pressure of 60 mmHg; B) The sequential rate histogram depicting the increase in whole mesenteric afferent nerve firing rate evoked by ramp distension; C) Raw nerve trace showing the increased afferent activity in response to distension. Below are expanded time bases for nerve recordings at the points indicated, contrasting (L-R) the action potential firing rate at baseline, during the first phase of distension, and during the second phase of distension.
Figure 10. Afferent responses to repeat jejunal ramp distensions

A) Representative trace showing ramp distensions carried out using Krebs solution at a filling rate of 10 mL/h to a maximum intrajejunal pressure of 60 mmHg. Distensions were carried out three times, at 15 minute intervals. Pressure (top), biphasic firing rate (middle), and raw neuronal (bottom) responses are shown (n=6); B) Changes in mesenteric afferent nerve firing rate induced by ramp distension. There was a small reduction in responses with repeated distension, particularly between the first and subsequent two distensions. At lower pressures, however, responses were essentially identical. These responses are likely mediated by vagal afferents (Booth et al., 2008) (n=6; *** P<0.0001; 2-way ANOVA).
is a P2 receptor agonist known to stimulate nerve activity throughout the GIT of the mouse (Giaroni et al., 2002) and guinea pig (Bertrand & Bornstein, 2002), and expression of P2X2/3 receptors has been shown on the endings of most vagal afferents of the gut (Castelucci et al., 2002; Castelucci et al., 2003; Wang & Neuhuber, 2003). The luminal perfusate of the jejunum preparation was switched from Krebs solution (10 mL/h) to 10 µM ATP in saline, at the same perfusion rate for 5 min. The intraluminal application of this agonist evoked a significant increase (P<0.0001) in mean firing rate over the time course of the application (Fig. 11). These data are consistent with prior findings in vivo in the rat (Kirkup et al., 1999) and in vitro in the mouse (Rong et al., 2009), and confirmed the sensitivity of the mucosal terminals in this small-intestinal model to intraluminal drug perfusion in vitro. Furthermore, these findings demonstrated that the preparation is sensitive to the intraluminal application of a much lower concentration (10 µM) of ATP, and elicited approximately the same change in afferent firing, than that reported recently when ATP (1 mM) was applied luminally in a similar in vitro murine jejunal preparation (Rong et al., 2009).

3.3 Attenuation of intraluminal CCK perfusion-induced afferent firing increase by lorglumide

The satiety mediator CCK is released by lipid ingesta. The current investigation focused on modeling the afferent response to intraluminal perfusion of the small intestine with fatty acids in vitro. Thus, it was important to demonstrate the responsiveness of the jejunum preparation to exogenous luminal application of CCK, and furthermore to demonstrate the ability to attenuate that response by applying a CCK receptor antagonist. It has previously been demonstrated in vitro in the rat that 1 mM CCK elicits a significant increase in multi-unit jejunal afferent activity (Eastwood et al., 1998). 100 nM CCK was chosen for the present study, based on preliminary
Figure 11. The afferent response to intraluminal ATP perfusion

A) Representative recording of raw nerve trace (bottom) and sequential rate histogram (top) showing increased afferent activity in response to ATP perfusion; B) Perfusion of ATP significantly increased mesenteric afferent nerve firing (n=3; *** P<0.0001; 2-way ANOVA).
work done in our lab, to determine if the mouse \textit{in vitro} preparation is responsive to lower [CCK].

The intraluminal perfusate was switched from Krebs solution (10 mL/h) to 100 nM CCK in saline, for 5 min at the same perfusion rate. CCK application evoked a pronounced increase in the afferent firing rate (Fig. 12A), a change that was significant (P<0.0001) over the time course of the application and at several points therein (P<0.05; P<0.01) (Fig. 12C). The sensitivity of this mouse preparation to CCK was thus confirmed, and at a much lower concentration than that reported in the rat.

Lorglumide, the CCK-1 receptor antagonist, was chosen for the blocking experiments. 10 min prior to the next luminal CCK application, the intraluminal perfusate was switched from Krebs solution (10 mL/h) to 10 µM lorglumide in Krebs, for 10 min at the same perfusion rate. The bath superfusate was switched from Krebs (2.2 mL/min) to 10 µM lorglumide in Krebs, at the same flow rate, for 10 min prior to the CCK application, for the 5 min duration of the CCK challenge, and for at least 10 min following that application. The intraluminal perfusate was then switched from 10 µM lorglumide in Krebs solution (10 mL/h) to 10 µM lorglumide+100 nM CCK in saline, for 5 min at the same perfusion rate. Following this CCK challenge, the intraluminal perfusate was switched back to 10 µM lorglumide in Krebs (10 mL/h) for at least 10 min. The co-application of lorglumide and CCK abolished the afferent firing rate change previously elicited by CCK alone (Fig. 12B), an antagonistic effect that was significant (P<0.0001) over the time course of the application (Fig. 12D) and at an individual time point therein (P<0.01). These data show the same trend as prior work demonstrating the ability of devazepide (1 mM) to attenuate CCK-induced increases in afferent firing \textit{in vitro} in the rat jejunum (Eastwood \textit{et al.}, 1998), and thereby confirm the effectiveness of lorglumide used for the same purpose in the present model, and at a substantially lower concentration (10 µM).
Figure 12. The afferent response to intraluminal CCK perfusion, and the Lorglumide-mediated attenuation of that response

A) Representative recording of raw nerve trace (bottom) and sequential rate histogram (top) showing increased afferent activity in response to CCK perfusion; B) Representative trace showing attenuation of the CCK response by co-application (in bath and lumen) of the CCK-1 receptor antagonist Lorglumide with CCK; C) Perfusion of CCK significantly increased mesenteric afferent nerve firing (n=11; *** P<0.0001, # P<0.05, ## P<0.01; 2-way ANOVA with Bonferroni post-tests where applicable); D) Co-application of Lorglumide with CCK abolished this response (n=11 CCK perfusions, 6 CCK+Lorglumide perfusions; *** P<0.0001, ## P<0.01; 2-way ANOVA with Bonferroni post-test where applicable).
3.4 The concentration-response relationship of luminally-perfused sodium oleate

Sodium oleate is a representative LCFA (18 C chain backbone) that has previously been shown to evoke characteristic jejunal afferent responses when applied intraluminally in vivo ([oleate]=10 mM) in the rat (Lal et al., 2001). As such, it was selected for use in the present in vitro model, and concentration-response experiments were conducted. The intraluminal perfusate was switched from Krebs solution (10 mL/h) to sodium oleate (1, 3, 10, 30, 100, or 300 mM) in saline, for 5 min at the same perfusion rate. Each complete concentration-response experiment (n=8) encompassed the application of each of the six different oleate concentrations in the same jejunal preparation, with a minimum of 20 min between applications. The six oleate concentrations were applied in random order, except for one set of applications of sequentially-increasing concentrations that was completed for illustrative purposes (Fig. 13). As that recording shows, each intraluminal application of sequentially-increasing oleate concentrations resulted in progressively-greater afferent discharge responses, which confirmed the in vitro responsiveness to this FFA, the concentration-dependent nature of the responses that it evokes, and that good quality recordings of this type can be maintained in this preparation for a number of hours. Graphical analyses of the mean changes in firing evoked over the time-course of the applications (Fig. 14A) and during the peak response phase of each application (Fig. 14B) further illustrate the concentration-response relationship for sodium oleate applied in this model, and therefore the range of receptor activity in response to these stimuli. The peak response data plotted for each applied oleate concentration are different between the time-course (Fig. 14A) and peak firing rate (Fig. 14B) curves due to the manner in which the continuous mean firing frequency (10 s time constant) data were sampled; in the case of time-course analyses, the data were sampled every 10 s, while the peak mean afferent firing rate was obtained as a discrete value from the continuous data. From the peak response data (Fig. 14B), the calculated EC₅₀ of 25.35
Figure 13. The afferent response to intraluminal sodium oleate perfusion

Representative recording of raw nerve trace (bottom) and sequential rate histogram (top) showing progressively-increased mesenteric afferent nerve activity in response to perfusion of progressively-increased concentrations of oleate (1-300 mM; n=8).
Figure 14. The sodium oleate concentration-response relationship

A) Time courses showing the changes in mesenteric afferent nerve firing evoked by intraluminal perfusion of different concentrations of sodium oleate (1-300 mM; n=8); B) Concentration-response curve for sodium oleate in the isolated murine jejunal preparation, described by the increase in peak firing rate evoked by application of each concentration of sodium oleate (n=8; curve is a non-linear fit with variable slope). An EC$_{50}$ of 25.35 mM was indicated for sodium oleate in this preparation by this analysis (Hill equation).
mM (by Hill equation) was thereby indicated for sodium oleate in this *in vitro* jejunal preparation, which informed further studies of the appropriate use of 30 mM sodium oleate for robust and repeatable afferent responses in experiments investigating the effects of perfusion of this LCFA in this model. This oleate concentration is within the physiological range, as determined by prior work that demonstrated CCK secretion in humans in response to the infusion of FFAs in the 25-100 mM range (McLaughlin et al., 1999). It is also only modestly greater than the oleate concentration (10 mM) used by Lal et al to demonstrate vagal afferent responses to FFAs *in vivo* in the rat (Lal et al., 2001), a shift that would rationally be expected to be necessitated by the transition to an *in vitro* system.

3.5 Intraluminal sodium butyrate perfusion potentiates afferent firing

Sodium butyrate is a representative SCFA (4 C-chain backbone) that has previously been shown to evoke characteristic jejunal afferent responses when applied intraluminally *in vivo* ([butyrate]=10 mM) in the rat (Lal et al., 2001). While the present study was most focused on modeling responses to LCFAs in the *in vitro* model, sodium butyrate was applied to the preparation, at the same concentration as the EC$_{50}$ previously determined for sodium oleate (30 mM), to determine if a SCFA would also produce characteristic jejunal afferent activation. The intraluminal perfusate was switched from Krebs solution (10 mL/h) to sodium butyrate (30 mM) in saline, for 5 min at the same perfusion rate. The intraluminal application of this FFA evoked a significant increase (P=0.0005) in mean afferent firing (Fig. 15). The change in mean firing rate in response to butyrate perfusion was significantly smaller than that evoked by application of 30 mM sodium oleate (Fig. 14A) (n=8; P<0.0001; 2-way ANOVA; graphical comparison not shown). These data confirm the sensitivity of the mucosal terminals in this small-intestinal model to intraluminal perfusion of a representative SCFA *in vitro*, and provide evidence of the
Figure 15. The afferent response to intraluminal sodium butyrate perfusion

Perfusion of butyrate significantly increased mesenteric afferent nerve firing (n=23 vehicle control perfusions, 8 butyrate perfusions; *** P=0.0005; 2-way ANOVA).
differential response elicited by a SCFA, compared to that induced by application of a LCFA at the same concentration.

3.6 Lorglumide attenuates afferent firing increases in response to intraluminal sodium oleate perfusion

As discussed (1.6), the abolition of jejunal afferent firing increases induced by intraluminal perfusion of sodium oleate by the application of a CCK-1 receptor antagonist has been demonstrated in vivo in the rat (Lal et al., 2001). Having validated the effectiveness of CCK-1 receptor blockade with lorglumide in the abolition of afferent responses induced by intraluminal CCK perfusion in the present in vitro mouse model (3.3), experiments were conducted to determine if co-application of lorglumide with sodium oleate would attenuate firing in this preparation. First, the intraluminal perfusate was switched from Krebs solution (10 mL/h) to 30 mM oleate in saline, for 5 min at the same perfusion rate. Oleate application caused the afferent firing rate to increase significantly (P<0.0001) (Fig. 16A).

To test the effect of lorglumide (10 µM) on the afferent firing response to sodium oleate, 10 min prior to the next luminal oleate application, the bath superfusate was switched from Krebs (2.2 mL/min) to 10 µM lorglumide in Krebs, at the same flow rate, for the 10 min period before the oleate application, for the 5 min duration of the oleate challenge, and for at least 10 min following that application. At approximately the same time, the intraluminal perfusate was switched from Krebs solution (10 mL/h) to 10 µM lorglumide in Krebs, for 10 min at the same perfusion rate. At the onset of the combined oleate+lorglumide (10 µM) intraluminal challenge, the intraluminal perfusate was then switched to 10 µM lorglumide+30 mM oleate in saline, for 5 min at the same perfusion rate. Following this oleate challenge, the intraluminal perfusate was switched back to 10 µM lorglumide in Krebs (10 mL/h) for at least 10 min. The co-application of
Figure 16. The afferent response to intraluminal sodium oleate perfusion, and the Lorglumide-mediated attenuation of that response

A) Perfusion of oleate significantly increased mesenteric afferent nerve firing (n=9; *** P<0.0001; 2-way ANOVA); B) Co-application (in bath and lumen) of the CCK-1 receptor antagonist Lorglumide (10 µM) with oleate did not significantly change this response (n=9 oleate perfusions, 2 oleate + Lorglumide (10 µM) perfusions); C) Co-application of Lorglumide (30 µM) with oleate abolished this response (n=9 oleate perfusions, 7 oleate + Lorglumide (30 µM) perfusions; *** P<0.0001; 2-way ANOVA).
lorglumide (10 µM) and oleate did not significantly decrease the afferent firing rate change previously elicited by oleate alone (Fig. 16B).

In a separate set of experiments, the protocol described above, to determine if co-application of lorglumide (10 µM) with sodium oleate would modulate firing in this preparation, was repeated with an increased concentration of lorglumide (30 µM) applied to the bath and lumen during the intraluminal oleate challenge. The co-application of lorglumide (30 µM) and oleate significantly decreased (P<0.0001) the afferent firing rate change previously elicited by oleate alone (Fig. 16C). There was no significant difference between the afferent response to intraluminal perfusion of the vehicle and to that of co-applied oleate+lorglumide (30 µM), indicating abolition of the oleate response by lorglumide (n=9 vehicle, 7 oleate + lorglumide (30 µM) perfusions; 2-way ANOVA; graphical comparison not shown).

These data are consistent with prior work demonstrating the ability of CCK-1 receptor blockers to abolish oleate-induced increases in afferent firing in vitro in the rat jejunum, and thereby confirm the effectiveness of lorglumide used for the same purpose in the present in vitro murine model. The concentration-dependent nature of lorglumide used in this application is also suggested, as 30 µM lorglumide more effectively inhibited the jejunal afferent responses than did 10 µM lorglumide.

3.7 Nicardipine attenuates afferent firing increases in response to intraluminal sodium oleate perfusion

It was previously reported, using the cultured EE cell line STC-1, that fatty acid stimuli result in CCK secretion via a mechanism that is dependent on both the acyl chain-length of the fat and an increase in intracellular calcium that is primarily mediated by LTCC, and which was abolished with nicardipine (5 µM) (McLaughlin et al., 1998). Having demonstrated that CCK-1
receptor blockade with lorglumide significantly abolishes the afferent firing response induced by intraluminal sodium oleate perfusion in the present in vitro mouse jejunum preparation (3.6), further experiments were conducted to determine if co-application of the LTCC antagonist nicardipine with sodium oleate would attenuate firing in the preparation. First, the intraluminal perfusate was switched from Krebs solution (10 mL/h) to 30 mM oleate in saline, for 5 min at the same perfusion rate. Oleate perfusion caused the afferent firing rate to increase significantly (P<0.0001) (Fig. 17A; Fig. 18C).

To test the effect of nicardipine (1 µM; luminal) on the afferent firing response to sodium oleate, 10 min prior to the next luminal oleate application, the intraluminal perfusate was switched from Krebs solution (10 mL/h) to 1 µM nicardipine in Krebs, for 10 min at the same perfusion rate. The intraluminal perfusate was then switched to 1 µM nicardipine+30 mM oleate in saline, for 5 min at the same perfusion rate. Following this oleate challenge, the intraluminal perfusate was switched back to 1 µM nicardipine in Krebs, at the same flow rate, for at least 10 min. The intraluminal co-application of nicardipine (1 µM) and oleate significantly decreased (P=0.0023) the afferent firing rate change previously elicited by oleate alone (Fig. 17B).

The protocol described above, for LTCC blockade via luminal nicardipine application, was repeated in a separate set of experiments in which greater [nicardipine] (3 µM) was utilized. Paradoxically, the intraluminal co-application of nicardipine (3 µM) and oleate significantly increased (P<0.0001) the afferent firing rate change previously elicited by oleate alone (Fig. 17C).

LTCC blockade via bath superfusion of nicardipine was then undertaken in a separate set of experiments while sodium oleate was again perfused intraluminally. Following initial intraluminal challenges with the vehicle control and with 30 mM sodium oleate, and 10 min prior to the next luminal oleate application, the bath superfusate was switched from Krebs (2.2
Figure 17. The afferent response to intraluminal sodium oleate perfusion, and the Nicardipine-mediated (via intraluminal perfusion) modulation of that response

A) Perfusion of oleate significantly increased mesenteric afferent nerve firing (n=23; *** P<0.0001, ### P<0.001; 2-way ANOVA with Bonferroni post-test where applicable); B) Intraluminal co-application of the LTCC antagonist Nicardipine (1 µM) with oleate significantly attenuated this response (n=23 oleate perfusions, 11 oleate + Nicardipine (1 µM) perfusions); ** P=0.0023; 2-way ANOVA); C) Intraluminal co-application of Nicardipine (3 µM) with oleate significantly potentiated this response (n=23 oleate perfusions, 4 oleate + Nicardipine (3 µM) perfusions); *** P<0.0001; 2-way ANOVA).
Figure 18. The afferent response to intraluminal sodium oleate perfusion, and the Nicardipine-mediated (via bath superfusion) attenuation of that response

A) Co-application (in bath) of the LTCC antagonist Nicardipine (1 µM) did not significantly change mesenteric afferent nerve firing evoked by sodium oleate (n=23 oleate perfusions, 4 oleate + Nicardipine (1 µM) perfusions); B) Co-application (in bath) of Nicardipine (3 µM) abolished this response (n=23 oleate perfusions, 4 oleate + Nicardipine (3 µM) perfusions); *** P<0.0001; 2-way ANOVA); C) Representative recording of raw nerve trace (bottom) and sequential rate histogram (top) showing increased afferent activity in response to oleate perfusion; D) Representative trace showing abolition of the oleate response by co-application (by bath superfusion) of Nicardipine with oleate.
mL/min) to 1 µM nicardipine in Krebs, at the same flow rate, for the 10 min period before the oleate application, for the 5 min duration of the oleate challenge, and for at least 10 min following that application. The next intraluminal challenge with 30 mM sodium oleate was then applied (10 mL/h; 5 min). Bath superfusion of nicardipine (1 µM) during luminal oleate perfusion did not significantly attenuate the afferent firing rate change previously elicited by oleate alone (Fig. 18A).

The above protocol, for LTCC blockade via bath superfusion of nicardipine, was repeated in a separate set of experiments in which greater [nicardipine] (3 µM) was utilized. The concurrent bath application of nicardipine (3 µM) during intraluminal oleate perfusion abolished (P<0.0001) the afferent firing rate change previously elicited by oleate alone (Fig. 18B; Fig. 18D).

Intraluminal pressure traces that were recorded from the present preparation did not indicate the induction of apparent motor responses, as would be indicated by changes in pressure, by the intraluminal perfusion of sodium oleate. Furthermore, throughout the set of experiments in which nicardipine was co-applied with oleate, the intraluminal pressure trace was specifically monitored for changes indicating modulation of intestinal motor activity due to the use of nicardipine, and in no cases was it found to occur.

These experiments confirmed the effectiveness of nicardipine in attenuating the mesenteric afferent response to luminal oleate perfusion in the in vitro murine jejunal model. It was further demonstrated that the effect of nicardipine in the preparation is both concentration- and delivery mode-dependent, as 3 µM nicardipine more effectively abolished the afferent response to luminal sodium oleate challenges than did 1 µM nicardipine, and nicardipine application by bath superfusion was more effective than it was shown to be by luminal application.
3.8 Vagotomy suppresses baseline jejunal afferent firing, and attenuates afferent firing increases in response to perfusion of the saline vehicle, sodium oleate, and CCK

To discern the contribution of the vagus nerve to the afferent firing recorded \textit{in vitro} from the mixed mesenteric fibers of the jejunal preparation, chronic subdiaphragmatic vagotomy surgeries were performed to ablate the vagus nerve in one group of mice, while sham vagotomy surgeries that involved identical surgical manipulations, except for the vagotomy itself, were completed in the control group of animals. \textit{In vitro} jejunal preparations for extracellular afferent recording were harvested from 6 vagotomized animals, and were compared to those from 9 sham-vagotomized mice.

Evidence that subdiaphragmatic vagotomies were effective at removing vagal innervation of the jejunum was first provided by the significant attenuation (P=0.0318) of baseline afferent nerve firing that was found to occur in preparations from vagotomized mice, compared to the measured rate in preparations from nonvagotomized animals (Fig. 19A). Baseline firing was measured during the 780 s period prior to the first intraluminal administration (of the vehicle control) in preparations from vagotomized and sham-vagotomized animals. In the sham group, the mean rate of afferent discharge measured during that period was $21.02 \pm 3.24$ imp/s, while the mean firing rate in the vagotomized group was $11.44 \pm 3.10$ imp/s, which indicates an attenuation of 45.6% of mean baseline firing. These data are consistent with the findings of Bulmer \textit{et al.}, who reported an attenuation of 46.2% of baseline firing in rats that had undergone chronic subdiaphragmatic vagotomy (the effectiveness of which was confirmed by the absence of an afferent response to stimulation by CCK or a 5-HT$_3$ receptor agonist, and the absence of a jejunal pressure increase following electrical stimulation), compared to those that had undergone sham vagotomies (Bulmer \textit{et al.}, 2005).
Figure 19. Evidence that chronic subdiaphragmatic vagotomy was effective in removing vagal innervation of the jejunum

A) Vagotomy significantly decreased the mean rate of baseline mesenteric afferent nerve firing over the 780 s period prior to the onset of the first intraluminal challenge (n=9 sham vagotomy surgeries, 6 vagotomies; * P=0.0318; unpaired Student’s t-test); B) Vagotomy abolished the afferent response to intraluminal perfusion of the saline vehicle (n=9 shams, 6 vagotomies; P=0.0002; 2-way ANOVA); C) On dissection immediately following death, the majority of vagotomized mice were found to have stomachs tightly distended with contents that appeared to consist primarily of hair and bedding material, ± visually-empty distal guts, while no animals that underwent sham vagotomies exhibited these signs (n=9 sham vagotomies, 14 vagotomies; subjective evaluations).
The response to intraluminal perfusion of the vehicle (carbogenated saline) was compared in nonvagotomized and vagotomized groups (Fig. 19B). The intraluminal perfusate was switched from carbogenated Krebs solution (10 mL/h) to carbogenated saline, for 5 min at the same perfusion rate. Vehicle perfusion caused the afferent firing rate to increase significantly more (P=0.0002) in preparations from sham-vagotomized mice than in those from vagotomized animals, which was as hypothesized due to the vagotomy-mediated proportional decrease in baseline afferent firing previously demonstrated.

Further evidence of successful vagotomies was subjectively provided by morphological comparisons of the stomachs and distal guts in vagotomized and sham-vagotomized animals at the time of killing (Fig. 19C). Of the vagotomized animals, 10 of 14 (71.4%) had visibly-distended stomachs upon death, with gastric contents that appeared to consist primarily of hair and bedding material, and distal guts that did not appear to have any substantial contents. This is consistent with anticipated impaired gastric emptying secondary to vagotomy, and suggests that the passage from the stomach of hair and bedding material consumed by the mice was impeded due to the dysfunction. This, in turn, caused accumulation of the material in the stomach and the resultant distention. In contrast, all animals that underwent sham vagotomies presented normal-looking stomachs at death, and had distal guts that visibly-contained digesta and fecal pellets.

The response to intraluminal perfusion of sodium oleate (30 mM) was compared in nonvagotomized and vagotomized groups by two, serial oleate applications separated by a 20 min washout period. For each application, the intraluminal perfusate was switched from Krebs solution (10 mL/h) to sodium oleate (30 mM) in saline, for 5 min at the same perfusion rate. The first oleate perfusion caused an afferent firing rate change that was significantly greater (P<0.0001) in the nonvagotomized group than in the vagotomized cohort (Fig. 20), where a substantial attenuation of the oleate response is noted. Interestingly, the mesenteric afferent
Figure 20. Vagotomy-mediated attenuation of the afferent response to intraluminal sodium oleate perfusion

A) Representative recording of raw nerve trace (bottom) and sequential rate histogram (top) showing increased mesenteric afferent nerve firing in response to oleate perfusion in a preparation from a sham-vagotomized mouse; B) Representative trace showing attenuation of the oleate response by vagotomy; C) Vagotomy significantly attenuated, but did not abolish the afferent nerve response to oleate perfusion (n=9 sham vagotomy surgeries, 6 vagotomies; *** P<0.0001; 2-way ANOVA).
response to oleate perfusion in preparations from vagotomized mice is still significantly greater than the response to the vehicle (n=6; P<0.0001; 2-way ANOVA; graphical comparison not shown), which indicates partial attenuation, not abolition of the oleate response by vagotomy. Responses to the second, serial oleate applications were very similar to those of the first. A pronounced attenuation of the amplitude of afferent nerve spikes was also noted in recordings from vagotomized mice (Fig. 20B; Fig. 21B; Fig. 22B). Collectively, these results demonstrate the repeatability, through two serial oleate perfusions, of the attenuation of the afferent response to intraluminal perfusion of sodium oleate in jejunal preparations from vagotomized mice. These data further indicate that much of the oleate response is vagally-mediated, but also introduce the possibility of a small spinal component to this response in this model. The finding of significant attenuation by vagotomy of the oleate response in the in vitro murine preparation is consistent with that of Lal et al in rat jejunal preparations (Lal et al., 2001), however the present evidence of putative spinal contributions is novel.

The response to intraluminal perfusion of CCK (100 nM) was compared in nonvagotomized and vagotomized groups (Fig. 21). The intraluminal perfusate was switched from Krebs solution (10 mL/h) to CCK (100 nM) in saline, for 5 min at the same perfusion rate. CCK perfusion caused afferent firing rate increases that were significantly greater (P<0.0001) in sham-vagotomized mice than in vagotomized animals, where abolition of the intraluminal CCK response is noted.

Finally, the response to bath superfusion of CCK (100 nM) was compared in nonvagotomized and vagotomized groups (Fig. 22). The bath superfusate was switched from Krebs (2.2 mL/min) to 100 nM CCK in Krebs, at the same flow rate, for 13 min at the same perfusion rate. CCK superfusion caused afferent firing increases that were significant greater (P<0.0001) in preparations from sham-vagotomized animals than in those from vagotomized
Figure 21. Vagotomy-mediated attenuation of the afferent response to intraluminal CCK perfusion

A) Representative recording of raw nerve trace (bottom) and sequential rate histogram (top) showing increased mesenteric afferent nerve firing in response to CCK perfusion in a preparation from a sham-vagotomized mouse; B) Representative trace showing attenuation of the CCK response by vagotomy; C) Vagotomy abolished the afferent nerve response to CCK perfusion (n=9 sham vagotomy surgeries, 6 vagotomies; *** P<0.0001; 2-way ANOVA).
Figure 22. Vagotomy-mediated attenuation of the afferent response to bath superfusion of CCK

A) Representative recording of raw nerve trace (bottom) and sequential rate histogram (top) showing increased mesenteric afferent nerve firing in response to bath superfusion of CCK in a preparation from a sham-vagotomized mouse; B) Representative trace showing attenuation of the CCK response by vagotomy; C) Vagotomy significantly attenuated, but did not abolish the afferent nerve response to bath superfusion of CCK (n=7 sham vagotomy surgeries, 4 vagotomies; *** P<0.0001; 2-way ANOVA).
animals, where only a partial attenuation, not abolition of the response to bath-applied CCK is noted. This evidence of putative spinal contributions to the jejunal CCK response in the mouse is novel.
Chapter 4

Discussion

Key to our understanding of the regulation of feeding is knowledge of the gut-brain pathways that convey afferent information about nutrient ingesta. The FFA products of lipid digestion, and the means by which their presence in the gut is transduced into afferent traffic to the brain, are of special interest. This is due to the putative roles of lipids as mediators of diseases of feeding dysregulation, such as obesity and type 2 diabetes, owing to their high nutrient density and multiple roles as nutritive, structural, and signaling molecules (Chawla et al., 2001; Unger & Zhou, 2001; Boden & Shulman, 2002). The development and utilization of appropriate models with which to study the relevant pathways is of fundamental importance to the advancement of this science. Vagal afferent responses to FFAs of different chain-lengths have been characterized in vivo in the rat (Lal et al., 2001), and the isolated, lumen-perfused, murine small-intestinal model was established to study afferent nerve sensitivity to non-nutritive stimuli (Rong et al., 2004). I extended the approach initially developed by Rong by developing a novel murine model in which the response to luminal nutrient stimuli was studied, enabling the elucidation of novel mechanistic information about the response of the model to FFAs and to the endogenous satiety mediator CCK, which is secreted in response to lipid ingesta. I validated the model by assessing the general mechanosensitivity with ramp distension experiments, the general chemosensitivity with intraluminal ATP perfusion, since ATP stimulates nerve activity throughout the GIT (Bertrand & Bornstein, 2002; Giaroni et al., 2002) and is an agonist of P2X2/3 receptors known to be expressed on most vagal afferents (Castelucci et al., 2002; Castelucci et al., 2003; Wang & Neuhuber, 2003), and the sensitivity to CCK via intraluminal perfusion. Further, the ablation of the CCK response by lorglumide application was
demonstrated. With respect to the study of FFAs, the present work established the ability to discern concentration-dependent afferent activation \((EC_{50}=25.35 \text{ mM})\) in response to luminal perfusion of the LCFA sodium oleate in the murine model, as well as to detect the relatively less-robust afferent activation in response to perfusion of the SCFA sodium butyrate at 30 mM.

In an effort to elucidate underlying mechanisms for the transduction of LCFA stimuli into afferent signals, and to assess similarities/differences between the mouse model and \textit{in vivo} in the rat, the effect of both the CCK-1 receptor antagonist lorglumide and the LTCC blocker nicardipine on the sodium oleate response was examined. It has previously been demonstrated that LCFAs may act via a CCK-mediated mechanism, by which FFAs stimulate CCK release from EE cells (which, in turn, is reliant on \(\text{Ca}^{2+}\) influx through LTCC), which activates CCK-1 receptor-expressing vagal afferent fibers (Lal \textit{et al.}, 2001). I demonstrated in the murine model the abolition of the afferent response to sodium oleate by both lorglumide and nicardipine, both of which suggest CCK is the putative mediator of the oleate response.

Subdiaphragmatic vagotomy resulted in significant attenuation of the oleate response compared to sham-vagotomy controls. My findings \textit{in vitro} in the murine model are reminiscent of those described \textit{in vivo} for the rat (Lal \textit{et al.}, 2001). Additionally, I documented the modulation, by vagotomy, of the model’s response to CCK administration. The novel and interesting findings of the present study are that the jejunal mesenteric afferent response to intraluminal CCK administration is ablated by vagotomy, while the response to CCK application by bath superfusion is only partially attenuated. These data suggest that the CCK response is both vagally- and spinally-mediated, and that afferent endings of each type are differentially expressed in the intestinal mucosa and in the extramucosal lamina.
4.1 Characteristic afferent response to jejunal distension

Ramp distensions with Krebs solution generated pronounced biphasic responses in the present model, consistent with those characterized by Rong et al in a similar murine model (Rong et al., 2004). The two distinct phases of afferent response signify the activation of two sub-populations of fibers in the multi-unit afferent nerve: low-threshold fibers that fire vigorously at low distension pressures (approximately 2-15 mmHg) and with low-amplitude spikes (suggesting slower conduction velocity), and high-threshold fibers that generate high-amplitude firing (suggesting faster conduction velocity) at higher pressures (approximately 15-60 mmHg). These observations are consistent with previous work in both the rat (Booth et al., 2001) and the mouse (Rong et al., 2004), validating the present in vitro model. Importantly, with respect to the jejunal distensions performed for this study, which were typically conducted at the end of 5-7 hour periods of experimental usage of the preparations, the mesenteric afferent responses were substantially stable, repeatable, and reproducible, particularly at lower distension pressures. This expands the putative future utility of this in vitro murine preparation through the demonstration that extended durations of use, involving multiple intra- and extra-luminal applications of various mediators of interest, are possible with this model. The demonstrated mechanosensitivity of the preparation will also avail it for possible future study of the interaction between nutrient stimuli and mechanosensation in the GIT.

4.2 Loroglumide abolishes CCK-induced afferent responses

It was important to validate the effectiveness of loroglumide for CCK-1 receptor blockade in the present model prior to its use downstream in the experimental plan, when loroglumide was co-applied with sodium oleate. With the intraluminal application of CCK (100 nM), the generation of a robust afferent response that was significantly greater than that resulting from the
intraluminal administration of the vehicle was demonstrated. With co-application of lorglumide (10 µM) in the tissue bath and jejunal lumen during intraluminal CCK application, abolition of the afferent response attributable to CCK, that is, the response beyond that which would be generated by the vehicle alone, was demonstrated. This finding indicated lorglumide as an effective CCK-1 antagonist for further application in the present model.

4.3 Luminal sodium oleate perfusion and concentration-dependent afferent responses

Six serial applications, in random order in most cases, of the LCFA sodium oleate in the 1-300 mM range generated afferent responses in a concentration-dependent manner. These findings validated the present model for the study of jejunal afferent responses to luminally-applied FFAs, again demonstrated the extended duration of use that is possible with single preparations of this in vitro murine preparation, and facilitated the calculation of the EC$_{50}$ for sodium oleate (25.35 mM) in this model. This calculation indicated that 30 mM would be an appropriate concentration for further oleate usage in this study. Subsequent oleate administration of the same concentration resulted in reproducible responses. Intraluminal perfusion of [oleate]>300 mM was toxic to the preparation, as indicated by the subsequent rapid loss of afferent signals, suggesting nerve death.

4.4 Afferent response to intraluminal sodium butyrate

The luminal application of the SCFA sodium butyrate (30 mM) in the jejunal model generated a response that was significantly smaller than that generated by the same concentration of the LCFA sodium oleate. These characteristic afferent response to FFAs of different chain-
length are consistent with the findings of Lal et al in the rat in vivo (Lal et al., 2001), and thereby validate the present in vitro murine model as being sensitive with respect to discerning the acyl chain-length-dependent effects of FFA administration. Prior research has linked SCFA receptor activation with GLP-1 secretion in the intestine (Dumoulin et al., 1998). We have observed that the intestinal mesenteric afferent response to GLP-1 perfusion is not nearly as potent as the response to CCK, which may indicate that there are comparatively few GLP-1 receptors in the jejunum. Thus, it is possible that the jejunal response to SCFA perfusion is mediated by GLP-1 release, which could explain the smaller afferent response seen in the present model upon sodium butyrate perfusion than when LCFAs (sodium oleate) are perfused.

4.5 Lorglumide-induced abolition of oleate responses

In earlier experiments we established that lorglumide (10 µM) abolishes afferent responses to exogenous CCK (100 nM) in the present murine jejunal model (4.2). Lorglumide was not effective at concentrations of 10 µM, applied in the tissue bath and intrajejunally, as an antagonist to the afferent response to intraluminal sodium oleate applied to this preparation. Increasing the lorglumide concentration to 30 µM resulted in abolition of the oleate response. The published IC$_{50}$ for lorglumide at CCK-1 receptors is 63.1 nM (Alexander et al., 2008), therefore it is expected that both concentrations used in the current experiments were in the supramaximal range. The current lorglumide-mediated abolition of the jejunal afferent oleate response in the in vitro murine preparation is consistent with the evidence of Lal et al, who demonstrated abolition of the afferent response to luminal oleate perfusion with the CCK-1 receptor antagonist devazepide, in vivo in the rat (Lal et al., 2001). This suggests that the intestinal oleate signal transduction pathways, and the involvement of CCK as a paracrine mediator, are similar in both animal models. It furthermore indicates that the transition from an
in vivo system to the present in vitro preparation does not interfere with the ability to discern this relationship. These data clearly indicate that CCK is involved in the afferent response to sodium oleate. It is also apparent from prior work, however, that multiple, interacting and/or differential pathways are likely involved in this response, and must be considered while attempting to discern the contribution of the CCK pathway in the present model (Bi & Moran, 2002; Bi et al., 2004; Moran & Bi, 2006; Whited et al., 2006; Bi et al., 2007). Our development and validation of the in vitro murine model provides a new approach to assess the role of CCK through the targeted deletion of the CCK-1 receptor in knockout mice or via the use of receptor-specific siRNA. CCK-1 receptor-deficient animals have been used previously in studies of the regulation of food intake and obesity (Ohta et al., 2000; Bi & Moran, 2002; Moran & Bi, 2006; Bi et al., 2007; Donovan et al., 2007; Kim et al., 2008), and remain promising platforms for the future determination of the role of CCK in those processes.

### 4.6 Nicardipine-induced modulation of the oleate response

LTCC-mediated Ca\(^{2+}\) influx is important in GI physiology, specifically because it is thought to precede secretions of the EE mediators CCK (McLaughlin et al., 1998) and GLP-1 (Reimann et al., 2005), and the EC mediator 5-HT (Racke & Schworer, 1993). Having demonstrated the abolition of the oleate response in the in vitro murine jejunal model with the application of the CCK-1 receptor antagonist lorglumide, further investigation was conducted to determine whether inhibition of Ca\(^{2+}\) influx would likewise attenuate the oleate response. Nicardipine has been reported to be a very potent LTCC antagonist at concentrations above 1 nM (Kenny et al., 1990), and McLaughlin et al demonstrated the abolition of FFA-stimulated CCK secretion in cultured STC-1 cells with 5 µM nicardipine (McLaughlin et al., 1998). Therefore, nicardipine in the 1-3 µM range was chosen for use in the present in vitro intestinal preparation.
It is recognized that nicardipine can cause changes in smooth-muscular contractility (Kunze et al., 1999), however no evidence of this effect, by changes in intraluminal pressure tracings or baseline afferent firing upon nicardipine application, was seen in the present experiments.

Luminal application of 1 µM nicardipine during intraluminal perfusion of sodium oleate partially, but significantly attenuated the afferent response to sodium oleate over the time course of the application. This supports the rationale that a pathway involving Ca\(^{2+}\) influx via LTCC, such as the release of CCK or other satiety mediators, is at least partially-implicated in the jejunal afferent response to luminal stimulation with sodium oleate. Paradoxically, when these experiments were repeated, now using 3 µM nicardipine luminally-applied during the oleate administration, the afferent response robustly and significantly increased. This is interesting, as it is not supported by any other data in the current study. Furthermore, it is inconsistent with prior findings that indicate that EE mediators secreted via LTCC-mediated pathways, especially CCK, tend to increase afferent firing in the gut. As such, future study may be warranted to determine, through attempting the demonstration of repeatability and reproducibility of these results in subsequent experiments, the legitimacy of the current finding. Rational explanations to putatively explain the current, seemingly counterintuitive finding include, but are not limited to: a) the possibility that nicardipine applied intraluminally in the present preparation, at elevated concentrations, modulates LTCC-dependent pathway(s) that regulate the tonic release of mediators that inhibit jejunal afferent firing, hence the elevated firing that was observed when LTCCs were maximally blocked. Such a scenario has been demonstrated previously in satiety pathways, with findings that the somatostatin-sst2 ligand-receptor interaction may inhibit visceral afferent sensitivity (Rong et al., 2007), and that Ghrelin may also inhibit vagal afferent firing (Date et al., 2002); b) the possibility that there could be differences in the roles of LTCC of the apical and basolateral surfaces of jejunal EECs; and, c) the knowledge that antagonist
applications at relatively high concentrations, as was known to be the case in the current study, are seldom absolutely specific for the intended receptor or ion channel. The implication in the present case, then, is that the luminal application of 3 µM nicardipine, concurrent with intraluminal sodium oleate perfusion, may have interacted with receptors or ion channels other than LTCC, with unpredictable effects that could include the potentiation of afferent firing.

Bath superfusion of 1 µM nicardipine during the intrajejunal oleate perfusion did not significantly change the afferent response, however the use of 3 µM nicardipine in the tissue bath abolished afferent firing. This again supports the hypothesis that the afferent response to oleate is partially mediated by a pathway requiring Ca$^{2+}$ influx through LTCC, possibly including CCK secretion, or by the secretion of other mediators. It is acknowledged that, while the data of the current study may implicate both some paracrine action of CCK and Ca$^{2+}$ influx via LTCC in the intestinal afferent response to sodium oleate, any attribution of the LTCC mechanism as causal for the secretion of CCK in the present model must be made with caution. The link between LTCC-mediated Ca$^{2+}$ influx and FFA-stimulated CCK release has heretofore been demonstrated only in cultured STC-1 cells (McLaughlin et al., 1998), and therefore may not apply in the in vitro murine intestinal platform. However, the current work is the first to show involvement of LTCC in the transduction of nutrient-related vagal afferent signaling. Finally, the present study has demonstrated that the bath application of high-concentration nicardipine is more effective at decreasing the afferent oleate response than is intrajejunal application of the antagonist. This suggests that a greater proportion of LTCC are expressed on intestinal loci accessible via bath versus luminal drug perfusion, which is consistent with prior work indicating the probable location of LTCC on the basolateral surface of EE cells (Cummings & Overduin, 2007). It is also possible that some aspect of the intrajejunal milieu, such as a mucous coating, may sterically hinder access of the antagonist to its ion channel target.
4.7 Vagotomy pre- and post-surgical considerations and confirmation of effectiveness at removing jejunal vagal innervation

Techniques previously described for achieving subdiaphragmatic vagotomy in the mouse (Gao et al., 2001; Lal et al., 2001; Gschossmann et al., 2002) were utilized in the current studies, and the experience gained may be useful for improving the methodology in the future. First, the initial protocol of establishing pre-surgical mice on an ad libitum liquid Ensure diet (plus water) for one week, followed by fasting the animals for 18 h prior to surgery, led to mice being dehydrated at the time of surgery, and therefore more likely to struggle with anaesthetic complications and/or post-operative recovery from this invasive operation. Mice would routinely lose ≥10% of body weight while fasting, which can only rationally be explained by dehydration. The unconfirmed hypothesis is that the mice received sufficient nutrition and hydration from the liquid diet, so when fasted after one week, they did not continue drinking, as water then was an uninteresting or unfamiliar choice. These speculations could be tested in the course of future work, to determine the consumption habits of mice given ad libitum access to liquid diets and water. This issue was solved by hydrating subsequent mice (1 mL lactated Ringer’s, sc) at the start of the fasting period, again just prior to surgical anaesthesia, after closure of the surgical incision, and daily thereafter for three days. This seemed to facilitate better tolerance of the surgery by the mice, and a more rapid recovery.

Second, it was observed that the majority of vagotomized mice had stomachs tightly distended with hair and bedding material at the time of killing, and distal guts that seemed to contain little digesta or waste material. Together, these suggest that delayed gastric emptying secondary to vagotomy was occurring, and was impeding the passage of the liquid diet ingesta, which often resulted in weight loss during the post-surgical survival period. In the future, it is recommended that post-surgical vagotomized mice be housed in bedding-free cages to prevent
this problem. If that does not obviate the issue, it is further recommended that pyloroplasty be performed at the time of vagotomy to more readily facilitate passage through the gastric pylorus of all ingested material.

Third, it is strongly recommended that the provision of surgical anaesthesia via ketamine-xylazine to mice should be via sc *versus* ip delivery when possible. When administered ip, care was taken with dosing and gentle ip injection so as not to inject into viscera, however a number of mice died from anaesthesia prior to surgical manipulation. This problem was solved by switching to sc injections of the same anaesthetic dose that would have been administered ip, and no further deaths from that cause occurred. Sc delivery of ketamine-xylazine did not result in latency to the onset of the surgical plane of anaesthesia that was appreciably greater than when ip administration was used.

The effectiveness of the vagotomy technique at removing jejunal vagal afferent innervation was compelling; vagotomy attenuated both baseline firing and the response to the saline vehicle. The determination that the majority of vagotomized mice had clearly distended stomachs, probably caused by impaired gastric emptying secondary to vagotomy, at their time of killing, while no sham-vagotomized animals did, was also interesting. It is acknowledged that the subjective nature of the latter metric, and that those evaluating the distension (or not) of the stomach were not blinded to the surgery that had been performed, diminish the utility of these observations. Thus, possible changes in future work to further strengthen the case for the effectiveness of vagotomy are suggested. First, if a metric such as stomach distention is to be used to assess the success of vagotomy, quantifiable elements, such as stomach diameter or displacement volume, should be compared between vagotomized and sham-vagotomized subjects to bring more objective comparison to such assessment. Furthermore, photographic comparisons of non-distended *versus* distended stomachs would be compelling evidence. Techniques such as
intralaminal injection of retrograde neural tracers (e.g. Fast Blue) in the jejunal wall at the time of surgery could be used to more concretely demonstrate the effectiveness of vagotomy. This technique would result, following the post-surgical survival period, in labeled vagus nerve cell bodies in the nodose ganglion in non-vagotomized animals, while no such labeling would be expected to occur in animals that had undergone subdiaphragmatic vagotomy. These techniques have previously been established (Powley et al., 1987), and could elegantly demonstrate the ablation of the vagus nerve for work such as that of the current study. Finally, CCK-induced satiety has also been used in prior work as a marker for the integrity of vagotomy (Ghia et al., 2007).

The pronounced attenuation of the amplitude of afferent nerve spikes noted in recordings from vagotomized preparations is interesting, and may provide additional confirmation of the effectiveness of the vagotomy techniques utilized in the current study. The spike amplitude modulation by vagotomy may indicate that the vagal fibers innervating the murine jejunum contain a sub-population of A-delta fibers. These small, thinly-myelinated fibers are fast conductors of neural signals, and thus increase the amplitude of the signals, which would result in decreased spike amplitude when they are ablated; however, the relative mix of C and A-delta fibers in jejunal vagal versus spinal afferents is unknown. Another explanation for this phenomenon may be that the ablated (“dead”) vagal fibers have an insulating effect on the remaining fibers of the mixed mesenteric nerve, impeding signal transmission to the measuring electrode and thus decreasing the amplitude of the measured spikes.

4.8 Vagotomy-induced abolition of oleate responses

By vagotomy, the abolition of the portion of the jejunal afferent response to intraluminal sodium oleate perfusion that is greater than that generated by perfusion of the saline vehicle has
been demonstrated in the present model. This finding indicates that the oleate response is vagally- and not spinally-, mediated in the mouse, which is consistent with the results of Lal et al using rat jejunal preparations (Lal et al., 2001). This is important, as it suggests that there are not major intraspecies differences with respect to the gut-brain transmission of LCFA-induced afferent signals, and specifically that there does not appear to be any significant spinal component to this signal in either animal.

**4.9 Vagotony-induced modulation of CCK responses**

In the current study, the portion of the afferent response to intraluminal CCK administration that was greater than the response to the vehicle was abolished by vagotony. This is of interest, because the present work further elaborates prior findings that the intestinal mucosa is innervated by both vagal and spinal endings (Furness et al., 1998), by indicating that intraluminal CCK reception in the mouse jejunum is primarily mediated by mucosal vagal, not spinal, afferent endings.

When CCK was bath-applied, however, the portion of the afferent response to CCK that was greater than the response to saline was significantly attenuated, but not abolished, by vagotony. Prior reports have indicated that the intestinal wall is innervated by both vagal and spinal endings (Furness et al., 1998). The current findings strengthen this concept with the suggestion that the portion of the murine jejunal afferent response to bath-applied CCK that is not abolished following vagotony must then be mediated by spinal or intestinofugal fibers that remain, although the contribution of the latter to the total number of fibers would be small. This discovery, and indeed the possibility that CCK can act on spinal afferents, is novel and therefore an important addition to the science of this field, and suggests important species differences in the mouse model.
The current differential findings regarding the modulation by vagotomy of the CCK-induced murine jejunal mesenteric afferent response to CCK administration, via intrajejunal versus bath superfusion, beyond indicating both vagal and spinal contributions to the CCK response, also therefore suggests a differential distribution of vagal and spinal endings between mucosal and extramucosal loci. These data suggest that mucosal innervation is primarily vagal, hence abolition of the afferent response to intraluminal CCK administration by vagotomy. This is consistent with prior findings in the rat, which showed that afferent responses to luminal CCK and 5-HT administration are vagal (Hillsley & Grundy, 1998; Jiang et al., 2000; Kreis et al., 2002). The present study furthermore demonstrated that extramucosal innervation is both vagal and spinal, thus vagotomy induced only partial attenuation of the afferent response to bath superfusion of CCK.

4.10 Future directions

Further studies will exploit the numerous opportunities that this novel model offers for the study of intestinal sensing of nutrients. While the current study has focused on the study of responses to one representative LCFA, the preparation will be of broad utility for future research about signal transduction in response to FFAs in general, as well as to carbohydrates and amino acids.

Future research using this model will also help to advance our understanding of the roles of specific nutrient-sensing receptors in the transduction of nutrient ingesta stimuli into afferent nerve signals. Investigation of these pathways will be greatly enhanced by the use of transgenic or knockout mice lacking various receptors with putative involvement in nutrient signal transduction. Pathways involved in responses to the FFA products of lipid digestion will continue to be of special interest due to the multi-faceted potential contributions of these
substances in the pathogenesis of obesity. As such, receptors with FFA ligands, particularly in the GPCR family, are anticipated to be potential targets of therapeutic intervention, and should therefore be explored with urgency. Findings to date indicate that GPR40 (Briscoe et al., 2003; Kotarsky et al., 2003), GPR41 (Brown et al., 2003; Le Poul et al., 2003), GPR43 (Brown et al., 2003; Nilsson et al., 2003), GPR120 (Hirasawa et al., 2005; Tanaka et al., 2007) are FFA receptors with probable involvement in satiety mediation and obesity, and it will therefore be of interest to explore their roles in these pathways. Patterns of vagal afferent activation are characteristic of FFA acyl chain-length, an effect that seems to be mediated by FFA receptors with specificity for FFAs of particular chain-lengths. Of these, GPR40 and GPR120 are probably the most important for further study in light of the current findings.

More recently, it has also been reported that oleoylethanolamide (OEA), an endogenous lipid mediator produced by intestinal mucosal cells using dietary oleate as a substrate, may transduce LCFA nutrient ingesta stimuli into a mesenteric afferent satiety response by engaging PPAR-α receptors in the gut and recruiting local afferents (Schwartz et al., 2008). As such, the putative mechanistic involvement of that receptor and the OEA-mediated pathway, using the present, in vitro murine model as a research platform, will be interesting and potentially valuable to explore as research is undertaken to increase understanding about the satiety-related factors that may predispose us to obesity.

4.11 Broader significance: a novel in vitro murine model to study intestinal afferent responses to nutritive intraluminal stimuli

The current study has validated a novel in vitro murine platform which has significant potential for the future study of intestinal afferent responses to luminal nutrient contents. By perfusing FFAs and satiety mediators typically associated with the intestinal response to the
presence of FFAs, it has been demonstrated that the present model is durable for these purposes, and will avail longer (multi-hour) experiments involving multiple applications of various mediators from a single preparation, and multiple preparations from a single animal. In contrast to the *in vivo* models, typically rat, that have been used in this field to date, this *in vitro* model will confer greater experimental flexibility due to the durability of the preparation, greater simplicity of analysis due to the isolated nature of the system, and better opportunities to study genetic function in nutrient signal transduction by the use of transgenic and knockout mice.

The *in vitro* model displays appropriate mechanosensitivity and chemosensitivity, and it is sensitive to both the application of the endogenous intestinal satiety mediator CCK and to CCK-1 receptor blockade. The preparation is responsive to the application of the LCFA sodium oleate, in a concentration-dependent fashion, and a differential response to the SCFA sodium butyrate is discernible. Furthermore, it is reported that CCK-1 receptor antagonism ablates the afferent response to sodium oleate in this murine model, which does support the putative paracrine contribution of CCK in the response. The novel and paradoxical possibility has been discovered that, while some LTCC-mediated process(es) seem to be involved in potentiating the afferent response to sodium oleate, there may be also be an inhibitory role mediated by those ion channels in this response. It was shown that, as in the rat, the murine response to LCFA in the jejunum seems to be mostly vagally-, not spinally-mediated, although new finding of a small spinal contribution were documented. Finally, the novel finding that there may be spinal, as well as vagal, contributions to the afferent response to CCK, is also reported by the present study.

In summary, the current work has contributed a novel, *in vitro* murine model that will be of great utility for the mechanistic study of satiation via nutrient-induced intestinal afferent activation, and of the contributing cellular and molecular pathways, and therefore for our understanding of the regulation of feeding and obesity. Several novel findings regarding putative
modulatory pathways of signal transduction associated with FFA- and CCK-mediated responses in the intestine have been contributed. The importance of both FFAs and CCK in the mediation of satiety has long been recognized, and there is strong evidence that vagal afferents and CCK signaling pathways play important roles in intestinal LCFA-induced satiety (Lo et al., ; Raybould, 1999; Bray, 2000; Lal et al., 2001; Beglinger & Degen, 2004). It is anticipated that the present model and findings will ultimately enhance the ability to identify and exploit novel therapeutic targets for the prevention of obesity and its associated co-morbidities by the modulation of satiety, and thereby to improve health in our populations that are currently suffering the modern scourge of obesity.
References

Data tables from the Canadian Community Health Survey (CCHS) 2005.


