$NA_V1.8+$ VISCERAL AFFERENT NEURONS: ROLES IN METABOLISM AND INFLAMMATION

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To

My parents, Padmashri and Udit

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by

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The word mentor is used widely in many different fields, but it's particularly applicable to ours. As the story goes, the term originates from the name of an older counselor to Telemachus. Athena, the goddess of wisdom, assumed Mentor's form to urge Telemachus to go out into the world and seek the truth about his father's fate. In short, then, the term encapsulates wisdom in the form of a trusted advisor urging one to seek the truth. I've been fortunate to have multiple people in my life who embody mentorship in this truest sense.

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The increasing prevalence of obesity and its related comorbidities are major health issues facing western societies. Obesity involves a long-term, chronic perturbation of energy balance, in which food intake is not properly matched to calories expended. It is also closely linked with low-grade inflammation characterized by increased levels of circulating inflammatory cytokines and acute-phase reactants, concomitant with the activation of a network of inflammatory signaling pathways. As vagal afferents are a neuronal population that can rapidly respond to both food-related stimuli as well as inflammatory agents and cytokines, I hypothesized that intact vagal afferent function may be

required for proper regulation of energy balance and particularly diet-induced inflammation.

The sensory component of the vagus nerve has long been considered the main neural relay by which nutritional signals from the gut reach brain sites maintaining homeostasis. To study the function of this neuronal population, we generated a genetic mouse model with specific ablation of sensory vagal neurons by driving expression of diphtheria toxin (DTA) in a Cre-dependent manner through crossing the DTA mouse, which expresses DTA upon Cre activity, with the well-characterized $Na_v1.8$-Cre mouse, which expresses Cre recombinase under the control of $Na_v1.8$, a voltage gated sodium channel present only in peripheral sensory neurons, including 80% of vagal sensory afferent neurons

Metabolic phenotyping of these ablated mice in comparison to control littermates did not reveal differences in body weight or food intake on chow diet . However, input from vagal afferents does appear to be important for linking ingested nutrients to acute changes in energy expenditure. This work also suggests that vagal afferents may be involved in checking inflammation to certain stimuli, possibly dietary high fat. As activity of vagal afferents has been shown to be decreased in obesity, dysfunction in this group of neurons could contribute to the diet induced inflammation associated with obesity.

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PRIOR PUBLICATIONS

Gautron, L., I. Sakata, **S. Udit**, J. M. Zigman, J. N. Wood and J. K. Elmquist (2011). "Genetic tracing of Nav1.8-expressing vagal afferents in the mouse." J Comp Neurol 519(15): 3085-3101.

Cordaux, R., **S. Udit**, M. A. Batzer and C. Feschotte (2006). "Birth of a chimeric primate gene by capture of the transposase gene from a mobile element." Proc Natl Acad Sci U S A 103(21): 8101-8106.

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5-HT - Serotonin

α7 nAChR - Alpha7 nicotinic acetylcholine receptor

AGTR - Angiotensin receptor

CART - Cocaine and amphetamine regulated transcript

CB - Cannabinoid

CCK - Cholecystokinin

CGRP - Calcitonin-gene related peptide

DRG - Dorsal Root Ganglion

DTA - Diphtheria toxin subunit A

DTR - Diphtheria toxin receptor

FGA - Fibrinogen

G-CSF - Granulocyte – colony stimulating factor

GM-CSF - Granulocyte macrophage – colony stimulating factor

GHSR - Growth hormone secretagogue receptor

GI - Gastrointestinal

GLP-1 - Glucagon-like peptide 1

HFD - High fat diet

HPA - Hypothalamic-pituitary-adrenal

iDTR - Inducible diphtheria toxin receptor

IL-1α - Interleukin 1 alpha

IL-1β - Interleukin 1 beta

IL-6 - Interleukin 6

IL-10 - Interleukin 10

LPS - Lipopolysaccharide

MBP - Myelin Basic Protein

NG - Nodose Ganglion

NR - Nuclear receptor

NTS - Nucleus tractus solitarius

PPARα - Peroxisome proliferator-activated receptor alpha

PPARγ - Peroxisome proliferator-activated receptor gamma

PYY3-36 - Peptide YY (3-36)

SAA-1 - Serum amyloid alpha 1

TLR4 - Toll-like receptor 4

TNF-α - Tumor necrosis factor alpha

TRP - Transient Receptor Potential Channel

TTX - Tetrodotoxin

TZD - Thiazolidinedione

VGSC - Voltage gated sodium channel

VMH: Ventral medial hypothalamus

VNS: Vagal nerve stimulation

Chapter 1: Vagal Sensory Neurons and Roles in Sensing Energy Status and Mediators of Inflammation

1.1 Overview

The vagus nerve, the tenth cranial nerve, innervates the viscera, covering the neck, chest, and abdomen. Along with parasympathetic motor (efferent) innervation of various organs in the body, the vagus nerve conveys sensory information about the state of the body's organs to the central nervous system. Eighty to ninety percent of the nerve fibers in the vagus nerve are sensory afferents. The cell bodies of these pseudounipolar sensory neurons reside in the nodose ganglion (NG), and signals are transduced from the nerve ending in the viscera to the cell body in the nodose ganglion and then terminate at the nucleus tractus solitarius (NTS) in the brainstem.

The location of the vagus nerve, bridging the hormonal, nutritional, and immunological milieu of the viscera and the central nervous system, makes it an ideal candidate for involvement in the central homeostatic mechanisms which maintain energy balance and limit inflammation in response to changing nutritional status. While much of parasympathetic efferent vagal function has been characterized, however, the role of the sensory vagus remains less understood.

1.2 Anatomy of the Vagus Nerve

The vagus nerve has a long history of being studied by humans. Though his writings do not survive, Marinus of Alexandria was reported to have arranged the cranial nerves in numbered pairs in the first century A.D. The following century, Galen described seven paired cranial nerves, the sixth pair of which included the vagus along with the glossopharyngeal (9^{th}) and spinal accessory (11^{th}) nerves. Much of the anatomy of the vagus was already understood, with Galen correctly describing the recurrent laryngeal nerve, vagal innervation of the liver, and the gastric plexus. Despite this early anatomical understanding, when Thomas Willis described the cranial nerves in the 17^{th} century, the vagus, the glossopharyngeal nerve, and the cranial portions of the spinal accessory were still considered one nerve, and it was 1778 before the vagus was finally recognized as an individual pair by Soemmerring. While the vagus was considered but a portion of a larger nerve, its distinctive course had already earned it the name of second or wandering part (*par vagum*). In the nineteenth century, the nerve was referred to by the name pneumogastric or vagus, with vagus becoming the accepted term in the twentieth century. (Skandalakis, Gray et al. 1986)

As its name suggests, the course of the vagus is circuitous, and thus a thorough description is far beyond the scope of this dissertation. Briefly, afferent and efferent rootlets converge to form the left and right vagus nerves as they exit the skull through the jugular foramen. The jugular ganglion and the nodose ganglion, which contains the neuronal cell bodies of the sensory neurons, are

located immediately as the nerves leave the cranium. In the neck, the cervical vagus travels with the common carotid artery and internal jugular vein inside the carotid sheath. The first branches given off are the auricular and meningeal branches at the level of the jugular foramen. The pharyngeal branch and superior laryngeal nerve branch off at the level of the nodose ganglion, and a cervical cardiac branch is also given off in the neck. The recurrent laryngeal nerve splits off the vagus nerve at the level of the subclavian artery on the right side, and the aortic arch on the left side. The recurrent laryngeal nerve actually ascends with the trachea and esophagus upon leaving the main vagus. In the upper thorax, both vagal nerves give off branches to the main bronchi, lungs, and hearts; these branches form the cardiac and pulmonary plexuses. In the lower thorax, both vagal nerves become intimately linked with the esophagus and form the esophageal plexus in its adventitia. After passing through the esophageal hiatus in the diaphragm, the left and right vagus form the anterior and posterior vagal trunks, respectively. Crossover of fibers has been described between the two sides at this level, and it's estimated that as many as 20% of afferent axons cross over in the rat. (Berthoud and Neuhuber 2000)

In the abdomen, the anterior trunk branches into the common hepatic, ventral gastric and ventral celiac branches, while the dorsal trunk forms the dorsal gastric and dorsal celiac branches. The common hepatic branch runs with the common hepatic artery and then divides into branches following either the right

gastric artery to the gastric antrum and pyloric sphincter or the ventral and dorsal duodenopancreatic arteries to the proximal duodenum and head of the pancreas, so note that the majority of the common hepatic branch innervation is of the stomach and duodenum, not the liver. The ventral gastric branch innervates the ventral side of the stomach and also supplies the proximal duodenum through transpyloric fibers. The dorsal gastric branch runs along the left gastric artery and innervates the dorsal side of the stomach and also contributes to the innervation of the proximal duodenum. The ventral celiac branch joins with the dorsal celiac branch to descend with the celiac artery. Both branches contribute to the innervation of the small and large intestines. Vagal innervation continues along the gut until the splenic flexure, about 2/3 of the way across the transverse colon. (Berthoud and Neuhuber 2000)

The patterns of sensory innervation suggested by gross understanding of nerve anatomy have been confirmed by various methods of neuronal tracing. Here I'll focus solely on the organs and tissues relevant to the studies outlined in this dissertation, namely the organs of the gastrointestinal tract and lymphoid tissues. Multiple methods have shown vagal afferent axons terminate near intrahepatic triads, extrahepatic bile ducts, portal vein, and the hepatic arterial plexus in the liver hilus (Berthoud, Kressel et al. 1992) (Gautron, Sakata et al. 2011). However, liver parenchyma does not appear to be a primary target of vagal innervation in rats and mice (Metz and Forssmann 1980) (Gautron, Sakata et al. 2011). In the

stomach, distinctive vagal endings have been identified in both external muscle layers (Phillips, Baronowsky et al. 1997) (Phillips and Powley 2000), the myenteric plexus (Berthoud, Patterson et al. 1997), and the mucosal lamina propria (Berthoud and Neuhuber 2000). Similar innervation at all three levels exists in the small and large intestines as well (Berthoud, Kressel et al. 1995) (Berthoud, Patterson et al. 1997) (Gautron, Sakata et al. 2011). Mucosal terminals in the gut form networks of multiple branching axons close to the basal lamina, and thus are ideally positioned to detect both ingested nutrients and any secreted substances from mucosal cells (Berthoud and Neuhuber 2000) (Gautron, Sakata et al. 2011). In the pancreas, afferent terminals have been reported in close proximity to the islets of Langerhans (Neuhuber 1989) (Gautron, Sakata et al. 2011).

Despite studies by multiple and varied approaches, no vagal innervation of the spleen has been demonstrated (Nance and Burns 1989) (Cano, Sved et al. 2001). Likewise, most tracing studies in the literature suggests that the thymus receives only a spinal innervation (Nance and Sanders 2007).

As its extensive anatomical coverage would indicate, the vagus nerve supplies motor parasympathetic innervation to most of the organs of the viscera. Thus, the motor function of the vagus nerve is implicated in many vital physiological processes. Again, a thorough review of vagal parasympathetic function is beyond the scope of this work. As an overly simplistic summation,

vagal parasympathetic function is required for much of “rest-and-digest” functions of the body at rest; these include slowing heart rate, dilation of bronchioles, and digestion. Loss of bilateral vagal function is thus incompatible with life. The overriding importance of these motor functions has thus limited our ability to study the roles of the sensory afferents of the vagus. However, these sensory afferents comprise the majority of the fibers in the vagus nerve. For example, in the rat subdiaphragmatic trunks, there are approximately 11,000 total fibers; of these, about 8,000 are afferent fibers, while only 3,000 are efferent fibers (Prechtl and Powley 1990).

1.3 The Role of the Sensory Vagus in Metabolism

The sensory vagus nerve is known to transduce many signals which could potentially contribute to metabolic regulation. Vagal afferents have been shown to respond to several gut peptides, including cholecystokinin (CCK) (Blackshaw and Grundy 1990), serotonin (5-HT) (Hillsley and Grundy 1998; Hillsley and Grundy 1998), somatostatin (Nakabayashi, Niijima et al. 1994), and glucagon-like peptide 1 (GLP-1) (Nishizawa, Nakabayashi et al. 1996). However, sensing by vagal afferents has been demonstrated to have varying degrees of importance in the satiating or anorectic effects of these hormones. CCK, a hormone secreted by mucosal enteroendocrine cells of the duodenum and jejunum in response to food within the intestinal lumen, sends satiety signals to the brain. Vagotomy abolishes the anorectic effect of CCK (Smith, Jerome et al. 1981). However, other studies

suggest that CCK does not act directly on vagal afferents but through potentiating vagal responses to distending loads (Schwartz and Moran 1996).

GLP-1 and Peptide YY(3-36) (PYY3-36) are likewise secreted from enteroendocrine L cells in response to luminal nutrients. Administration of PYY3-36 stimulates vagal afferent nerves via a Y2 receptor expressed at nerve terminals, and abdominal vagotomy inhibits the anorectic effect of PYY3-36 (Koda, Date et al. 2005). Similarly, the anorectic effects of peripheral GLP-1 administration are abolished by vagotomy (Abbott, Monteiro et al. 2005).

Ghrelin is a peptide produced in the stomach which acts on growth hormone secretagogue receptor (GHSR) to induce feeding through activation of orexigenic NPY-and GHRH-producing neurons (Chen, Trumbauer et al. 2004). The ghrelin receptor is also expressed in vagal afferent terminals, and ghrelin suppresses vagal afferent firing. Blockade of gastric vagal afferents can abolish all ghrelin effects (Date, Murakami et al. 2002).

Taken together, these findings indicate vagal afferents are involved in conveying signals regarding satiety as well as starvation from the gut to the brain. However, there is also competing evidence for each of these gut hormones that suggests the humoral route is as or more important in the modulation of food intake. Reidelberger et al. reported that CCK receptor antagonists that penetrated the blood brain barrier could partially rescue feeding in vagotomized animals, suggesting that endogenous CCK acts directly on the brain as well as through

vagal afferents (Reidelberger, Hernandez et al. 2004). Similarly, direct action of PYY3-36 on the hypothalamus can inhibit feeding (Batterham, Cowley et al. 2002), and peripheral GLP-1 has been demonstrated to activate area postrema neurons (Yamamoto, Kishi et al. 2003). One study found that while levels of ghrelin affected vagal afferent activity, ghrelin could still stimulate feeding in rats with subdiaphragmatic vagotomies or subdiaphragmatic deaffrentation (Arnold, Mura et al. 2006). Thus, the role and relative importance of vagal afferents remains to be delineated.

There is also evidence that vagal afferents can sense dietary nutrients directly. For example, raising portal vein glucose levels leads to a decrease in vagal afferent discharges reaching NTS neurons, which then leads to activation of sympathetic efferents throughout the body. All reflex sympathetic activation can be blocked by vagotomy, suggesting that signals triggered by high levels of portal glucose are transmitted through vagal afferents (Adachi, Shimizu et al. 1984; Niijima 1984).

There is also some evidence suggesting that vagal afferents can sense dietary lipids, either directly or through lipid-sensing nuclear receptors. Administration of the fatty acid oleoylethanolamide produces satiety and reduces body weight gain through a peroxisome proliferator-activated receptor alpha (PPARα) dependent mechanism. Subdiaphragmatic vagotomy or vagal deafferentation can abolish the reduction in food intake by oleoylethanolamide

administration (Fu, Gaetani et al. 2003). Gut vagal deafferentation can also abolish the suppression of hepatic glucose production induced by the presence of upper intestinal lipids (Wang, Caspi et al. 2008). Other suggestive evidence that vagal afferents can mediate responses to lipids come from work with peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ is activated by endogenous arachidonic acid metabolites such as 15-deoxy-delta 12, 14 prostaglandin J2 (Forman, Tontonoz et al. 1995) and is also the target for binding of the thiazolidinediones (TZD), a class of compounds used to treat type 2 diabetes that act as insulin sensitizers (Lehmann, Moore et al. 1995). In mouse models, adenovirus mediated hepatic PPARγ overexpression leads to severe hepatic steatosis but decreased peripheral adiposity. Peripheral insulin sensitivity and glucose tolerance are markedly improved, while metabolic rates were increased, recapitulating the effect of thiazolidinedione treatment. Selective hepatic branch vagotomy could reverse the distal beneficial effects of PPARγ overexpression, including reduction in peripheral adiposity and increase in energy expenditure. Hepatic vagotomy also abolished the PPARγ-mediated increase in resting oxygen consumption and expression levels of PPARγ target genes in adipose tissue after TZD treatment (Uno, Katagiri et al. 2006).

Along with chemoreception, vagal afferents can convey mechanosensory information about contraction and distension of the gastrointestinal tract. Afferent fibers in the gastrointestinal tract have been shown to be dose-dependently

responsive to increased gastric volume (Mathis, Moran et al. 1998), with increases in gastric volume able to reduce meal size. This mechanosensory information can act synergistically with other vagal signals. For example, combinations of CCK and gastric loads produce greater neuronal activity in the NTS than either stimulus alone (Schwartz, McHugh et al. 1993).

Taken together, these findings in multiple individual pathways suggest a possible role for the sensory vagus as a site of integration and control of feeding behavior and glucose homeostasis. However, the relative importance of vagal signaling versus other indicators of nutritional status, especially hormonal signals, remains to be defined. One of the earliest studies to try to address this was in 1886, when Gaskell conducted a study in which he sectioned the cervical portion of one or both vagus nerves in 11 young crocodiles. He found that although the animals ate and seemed to digest food normally, they died of starvation, with complete disappearance of fat bodies and signs of energy depletion in most tissues (Gaskell 1886).

The first systematic studies of vagal role in global metabolism came with investigations into the role of the autonomic nervous system on the effects of hypothalamic lesions. Lesions of the ventral medial hypothalamus (VMH) were known to cause hyperphagic obesity, and various studies had suggested that vagal parasympathetic activity was increased in animals with VMH lesions (Ridley and Brooks 1965; Berthoud and Jeanrenaud 1979). Subdiaphragmatic vagotomy was

then shown to abolish the obesity seen with VMH lesions (Powley 1974; Inoue and Bray 1977; Eng, Gold et al. 1978). However, the true role of the vagus in mediating the obesity seen in VMH lesions remained confusing, as vagotomy prior to VMH lesioning was shown to be unable to prevent development of obesity (King, Carpenter et al. 1978). Furthermore, other groups reported that vagotomy failed to reverse obesity in VMH-lesioned rats (Wampler and Snowdon 1979) or exacerbated obesity when conducted prior to lesioning (Chikamori, Masuda et al. 1977). In particular, these results prove difficult to interpret because vagotomy is known to disturb normal digestive functions, for example by reducing gastric motility and induce esophageal distention, which could contribute to reducing food intake and preventing obesity. Moreover, there was considerable variation between vagotomy procedures and verification of completion in these early studies (Grijalva and Lindholm 1982). Truncal vagotomy in severely obese humans also been reported to lead to weight loss (Kral 1978). However, results from human studies remain difficult to interpret due to wide variances in type and extent of vagal lesioning and limited numbers.

1.4 The Sensory Vagus and Inflammation

Along with information about the body's nutritional status, vagal afferents can also convey information about inflammatory markers. It's been demonstrated that vagal electrical activity increases upon injection of Interleukin 1 beta (IL-1β) into the hepatoportal vein (Niijima 1996), and IL-1β can also induce c-fos

expression, a marker of neuronal activation, in vagal afferent neurons (Goehler, Gaykema et al. 1998). Receptors for IL-1β have been demonstrated on vagal paraganglia (Goehler, Relton et al. 1997). The most striking evidence that the vagus is important in sensing IL-1β comes from studies that looked at sickness behavior in response to IL-1β in mice with subdiaphragmatic vagotomy. Vagotomy was demonstrated to mitigate the thermogenic and corticosterone response to IL-1β (Fleshner, Goehler et al. 1998) without changing circulating levels of the cytokine (Gaykema, Goehler et al. 2000). However, other studies found no effect of subdiaphragmatic vagotomy on IL-1β-induced anorexia (Schwartz, Plata-Salaman et al. 1997; Porter, Hrupka et al. 1998). One interpretation of these conflicting findings is that vagal importance in sensing IL-1β may be dependent on cytokine dose. Indeed, a further study reported that intraperitoneal IL-1β acted to induce fever via the vagal route at low doses, but fever induction could not be blocked by vagotomy at higher doses (Hansen, O'Connor et al. 2001). Similar findings have been reported with other cytokines, with fever and corticosterone responses to tumor necrosis factor (TNF-α) also attenuated by subdiaphragmatic vagotomy (Fleshner, Goehler et al. 1998).

There is also considerable evidence that the vagus nerve is involved in sensing foreign molecules directly. One study demonstrated expression of activation marker c-fos in vagal sensory ganglia upon administration of bacterial endotoxin or lipopolysaccharide (LPS) (Gaykema, Goehler et al. 1998). Multiple

studies reported that subdiaphragmatic vagotomy attenuated fever in response to intraperitoneal LPS in guinea pigs (Sehic and Blatteis 1996; Goldbach, Roth et al. 1997). Similarly, vagal deaffrentation in rats was demonstrated to result in lack of a pyrogenic response to LPS (Szekely, Balasko et al. 1997). Further work demonstrated that this was a specific response and not secondary to vagotomy-associated malnutrition (Romanovsky, Kulchitsky et al. 1997). Other studies also demonstrated that pyrogenic responses to other stimuli, such as cold, were preserved in vagotomoized rats (Romanovsky, Kulchitsky et al. 1997). There is also evidence that, as with IL-1β, these responses too could be dose-dependent effects. In rats, vagotomy ablated fever in response to low doses of LPS but not at high doses (Romanovsky, Simons et al. 1997).

This role of the sensory vagus in detecting inflammatory signals is particularly important in light of recent work showing the motor vagus can act to modulate inflammation. In the mid-1990s, dissection of the mechanism of action of CNI-1493 first indicated the possibility of the vagus nerve being able to suppress inflammation. Studies had shown that administration of minute doses of intracereberal CNI-1493 could suppress peripheral TNF-α expression, suggesting that it was activating a population of neurons that then suppressed TNF-α production. Further work showed that vagotomy could abolish the anti-inflammatory effect of CNI-1493, indicating that the vagus nerve was transmitting the anti-inflammatory signal (Borovikova, Ivanova et al. 2000).

This finding was confirmed by the demonstration that direct electrical stimulation of the vagus nerve could suppress TNF-α production in the spleen (Borovikova, Ivanova et al. 2000). Pharmacologically mimicking the activation of the vagus nerve via cholinergic agonists could also suppress cytokine production in isolated splenic macrophages. This effect was lost in mice with genetic knockout of the α7 nicotinic acetylcholine receptor (α7 nAChR), establishing that vagal acetylcholine acted on macrophages via the α7 nAChR (Wang, Yu et al. 2003).

The actual anatomy of this pathway remained ambiguous for a long time. Suppression of cytokine production by vagal stimulation was shown to be lost in splenectomized animals, suggesting the spleen to be the major target tissue of this pathway (Huston, Ochani et al. 2006). Initial reports of the vagal anti-inflammatory pathway contended that the vagus directly released acetylcholine at the spleen. However, this contradicted decades of anatomical tracing that reported no direct innervation of the spleen by the vagus nerve.

Attempting to activate the cholinergic anti-inflammatory pathway in mice with surgical ablation of the splenic nerve or with pharmacological depletion of catecholamines by reserpine demonstrated that the catecholaminergic splenic nerve was required for suppression of inflammation (Rosas-Ballina, Ochani et al. 2008). This suggested that the cholinergic anti-inflammatory pathway required a multisynaptic neural pathway involving vagal preganglionic neurons and

sympathetic postganglionic neurons projecting to the spleen. However, this finding was confusing in light of prior work showing that it was acetylcholine acting through the α7 nAChR to suppress cytokine production on macrophages, as the splenic nerve is purely catecholaminergic. One possible explanation of this conundrum came with the identification of a population of splenic T cells that could produce acetylcholine. Rosas-Ballina et al. demonstrated that these T cells were required for inhibition of cytokine production by the vagal anti-inflammatory pathway (Rosas-Ballina, Olofsson et al. 2011). Taken together, these multiple findings suggest the vagus nerve can operate an anti-inflammatory reflex and act to limit inflammation in physiological and pathological settings.

1.5 Current Techniques of Vagal Manipulation

Progress in the understanding the role of vagal afferents has been limited by the current techniques for vagal manipulation. As the vagus is mixed nerve, surgical techniques along with being technically challenging also result in full or partial loss of efferent function, with severe effects on gut motility as a result.

Subdiaphragmatic vagotomy is performed by cutting the two roots of the vagus running on either side of the esophagus below the diaphragm. Additionally, the connectors between organs such as the hepato-duodenal and falciform ligaments are also severed because it’s believed they carry vagal fibers. Ensuring complete transection of the posterior vagal trunk may require ligating and cutting the left gastric artery. This can result in antral ulceration of the stomach; however,

animals generally recover prior to experimentation due to generation of collateral circulation. A second method for full subdiaphragmatic vagotomy involves the independent identification and transection of all abdominal vagal branches. Selective vagotomies are obtained by identifying and sectioning one branch or another. (Louis-Sylvestre 1983)

One caveat of this method of vagal manipulation is that vagal branches may partially regenerate post-transection. A study on vagotomized rats reported that visual verification upon the conclusion of experiments 12 months after surgery showed no signs of regeneration (Kraly, Smith et al. 1978). However, in dogs, normal nerve fibers were reported in the stomach wall 12 – 16 weeks after vagotomy (Loup, Ghavami et al. 1981). Similarly, studies on the rat pancreas after vagotomy reported differences between vagotomized and normal pancreas to be negligible as early as 5 weeks after surgery (Wesolowski and Snop 1975).

The other major caveat of this approach in studying afferent function is that it necessarily comprises vagal efferent function as well. Following vagotomy, rats rapidly lose weight, as much as 10-30% of preoperative weight at 20 days after surgery, and even after several months of recovery, the body weight of vagotomized rats remains significantly below that of sham-operated controls. Generally, daily food intake remains reduced in the long term after vagotomy, with some groups reporting a specific decrease in carbohydrate ingestion as opposed to fat and protein. Post vagotomy, rats also eat smaller, more frequent

meals accompanied by a decrease in water consumption. Much of these effects can be attributed to disruption of vagal motor output as disruption of the gastric branches of the vagus results in gastric distension and retention of solid foods in the stomach. Vagotomy has also been shown to reduce total exocrine secretion from the pancreas, as well as reducing basal insulin levels and abolishing the cephalic phase of insulin secretion. With these myriad, significant effects attributed to loss of vagal motor output, it is difficult to assess vagal afferent contribution in vagotomy models.

One way to circumvent some of the confounding effects of vagotomy is through vagal deafferentation. Commonly, however, vagal deafferentation consists of unilateral afferent rhizotomy at the level of the skull where vagal afferents rootlets are separate from efferent rootlets and a transection of the vagal branch of interest on the opposite side (Walls, Wang et al. 1995). This method results in bilateral vagal deafferentation but only unilateral efferent transection. However, even unilateral loss of vagal motor output can be expected to significantly impact metabolism.

A pharmacological technique, the administration of capsaicin to destroy TRPV1 expressing neurons, not only targets small-diameter sensory neurons but also destroys some neurons in the central nervous system (Ritter and Dinh 1988) without destroying all vagal sensory neurons. Additionally, by 60-days post-capsaicin treatment, neuronal nuclei in the nodose of rats are not different from

controls (Czaja, Burns et al. 2008), limiting its applicability towards studying long-term regulation by the sensory vagus.

Vagus nerve stimulation (VNS) has recently emerged as an efficacious approach for the treatment of certain forms of depression and epilepsy. However, due to the fact that vagal stimulators are too large for implantation in small laboratory animals, basic research on its mechanisms of action remains very limited.

1.6 Rationale and Approach for Studies

Obesity and its related comorbidities, such as diabetes, are now epidemic in the United States. If left unchecked, the growing rate of these diseases will cause a tremendous increase in morbidity and mortality, as well as ensuing health care costs. Although the need could not be greater, few effective pharmacological therapies for obesity exist currently.

Obesity involves a long-term, chronic perturbation of energy balance, in which food intake is not properly matched to calories expended. It is also closely linked with low-grade inflammation characterized by increased levels of circulating inflammatory cytokines and acute-phase reactants, concomitant with the activation of a network of inflammatory signaling pathways (Hotamisligil 2006; Lumeng and Saltiel 2011). This obesity-dependent inflammation uniquely differs from other inflammatory paradigms by its association with a chronic activation of the innate immune system and its multi-organ involvement (Olefsky

and Glass 2010; Lumeng and Saltiel 2011), and multitudes of experimental evidence causally links this inflammatory state to the development of the comorbidities that emerge from obesity, including insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, dementia, respiratory diseases and cancers (Hotamisligil 2006; Lumeng and Saltiel 2011).

As vagal afferents are a neuronal population that can rapidly respond to both food-related stimuli (Berthoud and Neuhuber 2000; Moran, Ladenheim et al. 2001) as well as inflammatory agents and cytokines (Goehler, Gaykema et al. 2000; Julius and Basbaum 2001), I hypothesized that intact vagal afferent function may be required for proper regulation of energy balance and particularly diet-induced inflammation.

To test this hypothesis, I utilized a genetic mouse model of primary afferent neuron ablation by crossing two mouse lines, floxed-Stop-DTA and $Na_v1.8$-Cre mice. Floxed-Stop-DTA mice express Diphtheria toxin subunit A (DTA) after the Cre-mediated, cell-type specific removal of a transcriptional blocker. The well-characterized $Na_v1.8$ knock-in Cre mouse expresses Cre-recombinase under the control of $Na_v1.8$ (Stirling, Forlani et al. 2005), a tetrodotoxin-resistant, voltage-gated sodium channel previously implicated in nociception (Dib-Hajj, Cummins et al. 2010). $Na_v1.8$ is present in 75-82% of vagal afferent neurons in the nodose ganglion (NG) (Stirling, Forlani et al. 2005; Gautron, Sakata et al. 2011), preferentially in visceral chemoreceptors/multimodal

receptors. Hence, $Na_v1.8$-Cre mice are ideal for targeting large subsets vagal afferent neurons that are thought to be concerned with nutrients and/or inflammatory stimuli sensing. A major limitation of this approach is that $Na_v1.8$- is also expressed in many dorsal root ganglion (DRG) neurons (~70%), principally Aδ and C fiber neurons (Djouhri, Fang et al. 2003; Stirling, Forlani et al. 2005; Fukuoka, Kobayashi et al. 2008). While these spinal afferents are mainly thought to be involved in nociception, a possible role in metabolism cannot be discounted, and all phenotypes I present in this dissertation have to be considered as possibly resulting from both vagal and spinal sources. There is also expression in the trigeminal ganglion and the vestibular/geniculate ganglionic mass (Gautron, Sakata et al. 2011).

I was able to successfully generate the ablation model mice and confirm efficient ablation of $Na_v1.8$ neurons. The work detailed in this dissertation will focus on the metabolic phenotyping of these animals.

Abbott, C. R., M. Monteiro, et al. (2005). "The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway." Brain Res **1044**(1): 127-131.

Adachi, A., N. Shimizu, et al. (1984). "Convergence of hepatoportal glucose-sensitive afferent signals to glucose-sensitive units within the nucleus of the solitary tract." Neurosci Lett **46**(2): 215-218.

Arnold, M., A. Mura, et al. (2006). "Gut vagal afferents are not necessary for the eating-stimulatory effect of intraperitoneally injected ghrelin in the rat." J Neurosci **26**(43): 11052-11060.

Batterham, R. L., M. A. Cowley, et al. (2002). "Gut hormone PYY(3-36) physiologically inhibits food intake." Nature **418**(6898): 650-654.

Berthoud, H. R. and B. Jeanrenaud (1979). "Acute hyperinsulinemia and its reversal by vagotomy after lesions of the ventromedial hypothalamus in anesthetized rats." Endocrinology **105**(1): 146-151.

Berthoud, H. R., M. Kressel, et al. (1992). "An anterograde tracing study of the vagal innervation of rat liver, portal vein and biliary system." Anat Embryol (Berl) **186**(5): 431-442.

Berthoud, H. R., M. Kressel, et al. (1995). "Vagal sensors in the rat duodenal mucosa: distribution and structure as revealed by in vivo DiI-tracing." Anat Embryol (Berl) **191**(3): 203-212.

Berthoud, H. R. and W. L. Neuhuber (2000). "Functional and chemical anatomy of the afferent vagal system." Auton Neurosci **85**(1-3): 1-17.

Berthoud, H. R., L. M. Patterson, et al. (1997). "Distribution and structure of vagal afferent intraganglionic laminar endings (IGLEs) in the rat gastrointestinal tract." Anat Embryol (Berl) **195**(2): 183-191.

Blackshaw, L. A. and D. Grundy (1990). "Effects of cholecystokinin (CCK-8) on two classes of gastroduodenal vagal afferent fibre." J Auton Nerv Syst **31**(3): 191-201.

Borovikova, L. V., S. Ivanova, et al. (2000). "Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation." Auton Neurosci **85**(1-3): 141-147.

Borovikova, L. V., S. Ivanova, et al. (2000). "Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin." Nature **405**(6785): 458-462.

Chen, H. Y., M. E. Trumbauer, et al. (2004). "Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein." Endocrinology **145**(6): 2607-2612.

Chikamori, K., K. Masuda, et al. (1977). "Effect of vagotomy on hyperinsulinemia in obese rats with hypothalamic lesions." Endocrinol Jpn **24**(3): 251-258.

Czaja, K., G. A. Burns, et al. (2008). "Capsaicin-induced neuronal death and proliferation of the primary sensory neurons located in the nodose ganglia of adult rats." Neuroscience **154**(2): 621-630.

Date, Y., N. Murakami, et al. (2002). "The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats." Gastroenterology **123**(4): 1120-1128.

Dib-Hajj, S. D., T. R. Cummins, et al. (2010). "Sodium channels in normal and pathological pain." Annu Rev Neurosci **33**: 325-347.

Djouhri, L., X. Fang, et al. (2003). "The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons." J Physiol **550**(Pt 3): 739-752.

Eng, R., R. M. Gold, et al. (1978). "Hypothalamic hypoactivity prevented but not reversed by subdiaphragmatic vagotomy." Physiol Behav **20**(5): 637-641.

Fleshner, M., L. E. Goehler, et al. (1998). "Thermogenic and corticosterone responses to intravenous cytokines (IL-1beta and TNF-alpha) are attenuated by subdiaphragmatic vagotomy." J Neuroimmunol **86**(2): 134-141.

Forman, B. M., P. Tontonoz, et al. (1995). "15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma." Cell **83**(5): 803-812.

Fu, J., S. Gaetani, et al. (2003). "Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha." Nature **425**(6953): 90-93.

Fukuoka, T., K. Kobayashi, et al. (2008). "Comparative study of the distribution of the alpha-subunits of voltage-gated sodium channels in normal and axotomized rat dorsal root ganglion neurons." J Comp Neurol **510**(2): 188-206.

Gaskell, W. H. (1886). "On the Structure, Distribution and Function of the Nerves which innervate the Visceral and Vascular Systems." J Physiol **7**(1): 1-80 89.

Gautron, L., I. Sakata, et al. (2011). "Genetic tracing of Nav1.8-expressing vagal afferents in the mouse." J Comp Neurol **519**(15): 3085-3101.

Gaykema, R. P., L. E. Goehler, et al. (2000). "Subdiaphragmatic vagotomy blocks interleukin-1beta-induced fever but does not reduce IL-1beta levels in the circulation." Auton Neurosci **85**(1-3): 72-77.

Gaykema, R. P., L. E. Goehler, et al. (1998). "Bacterial endotoxin induces fos immunoreactivity in primary afferent neurons of the vagus nerve." Neuroimmunomodulation **5**(5): 234-240.

Goehler, L. E., R. P. Gaykema, et al. (1998). "Interleukin-1 induces c-Fos immunoreactivity in primary afferent neurons of the vagus nerve." Brain Res **804**(2): 306-310.

Goehler, L. E., R. P. Gaykema, et al. (2000). "Vagal immune-to-brain communication: a visceral chemosensory pathway." Auton Neurosci **85**(1-3): 49-59.

Goehler, L. E., J. K. Relton, et al. (1997). "Vagal paraganglia bind biotinylated interleukin-1 receptor antagonist: a possible mechanism for immune-to-brain communication." Brain Res Bull **43**(3): 357-364.

Goldbach, J. M., J. Roth, et al. (1997). "Fever suppression by subdiaphragmatic vagotomy in guinea pigs depends on the route of pyrogen administration." Am J Physiol **272**(2 Pt 2): R675-681.

Grijalva, C. V. and E. Lindholm (1982). "The role of the autonomic nervous system in hypothalamic feeding syndromes." Appetite **3**(2): 111-124.

Hansen, M. K., K. A. O'Connor, et al. (2001). "The contribution of the vagus nerve in interleukin-1beta-induced fever is dependent on dose." Am J Physiol Regul Integr Comp Physiol **280**(4): R929-934.

Hillsley, K. and D. Grundy (1998). "Sensitivity to 5-hydroxytryptamine in different afferent subpopulations within mesenteric nerves supplying the rat jejunum." J Physiol **509 (Pt 3)**: 717-727.

Hillsley, K. and D. Grundy (1998). "Serotonin and cholecystokinin activate different populations of rat mesenteric vagal afferents." Neurosci Lett **255**(2): 63-66.

Hotamisligil, G. S. (2006). "Inflammation and metabolic disorders." Nature **444**(7121): 860-867.

Huston, J. M., M. Ochani, et al. (2006). "Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis." J Exp Med **203**(7): 1623-1628.

Inoue, S. and G. A. Bray (1977). "The effects of subdiaphragmatic vagotomy in rats with ventromedial hypothalamic obesity." Endocrinology **100**(1): 108-114.

Julius, D. and A. I. Basbaum (2001). "Molecular mechanisms of nociception." Nature **413**(6852): 203-210.

King, B. M., R. G. Carpenter, et al. (1978). "Hyperphagia and obesity following ventromedial hypothalamic lesions in rats with subdiaphragmatic vagotomy." Physiol Behav **20**(5): 643-651.

Koda, S., Y. Date, et al. (2005). "The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats." Endocrinology **146**(5): 2369-2375.

Kral, J. G. (1978). "Vagotomy for treatment of severe obesity." Lancet **1**(8059): 307-308.

Kraly, F. S., G. P. Smith, et al. (1978). "Abdominal vagotomy disrupts food-related drinking in the rat." J Comp Physiol Psychol **92**(2): 196-203.

Lehmann, J. M., L. B. Moore, et al. (1995). "An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma)." J Biol Chem **270**(22): 12953-12956.

Louis-Sylvestre, J. (1983). "Validation of tests of completeness of vagotomy in rats." J Auton Nerv Syst **9**(1): 301-314.

Loup, P. W., B. Ghavami, et al. (1981). "Can vagal regeneration account for gastric motility after vagotomy?" Scand J Gastroenterol Suppl **67**: 13-17.

Lumeng, C. N. and A. R. Saltiel (2011). "Inflammatory links between obesity and metabolic disease." J Clin Invest **121**(6): 2111-2117.

Magni, F., F. Bruschi, et al. (1987). "The afferent innervation of the thymus gland in the rat." Brain Res **424**(2): 379-385.

Mathis, C., T. H. Moran, et al. (1998). "Load-sensitive rat gastric vagal afferents encode volume but not gastric nutrients." Am J Physiol **274**(2 Pt 2): R280-286.

Metz, W. and W. G. Forssmann (1980). "Innervation of the liver in guinea pig and rat." Anat Embryol (Berl) **160**(3): 239-252.

Moran, T. H., E. E. Ladenheim, et al. (2001). "Within-meal gut feedback signaling." Int J Obes Relat Metab Disord **25 Suppl 5**: S39-41.

Nakabayashi, H., A. Niijima, et al. (1994). "A unique receptor-mediated mechanism in vagal chemoreception of somatostatin in the hepatoportal area." J Auton Nerv Syst **50**(1): 45-50.

Nance, D. M. and J. Burns (1989). "Innervation of the spleen in the rat: evidence for absence of afferent innervation." Brain Behav Immun **3**(4): 281-290.

Neuhuber, W. L. (1989). "Vagal afferent fibers almost exclusively innervate islets in the rat pancreas as demonstrated by anterograde tracing." J Auton Nerv Syst **29**(1): 13-18.

Niijima, A. (1984). "Reflex control of the autonomic nervous system activity from the glucose sensors in the liver in normal and midpontine-transected animals." J Auton Nerv Syst **10**(3-4): 279-285.

Niijima, A. (1996). "The afferent discharges from sensors for interleukin 1 beta in the hepatoportal system in the anesthetized rat." J Auton Nerv Syst **61**(3): 287-291.

Nishizawa, M., H. Nakabayashi, et al. (1996). "The hepatic vagal nerve is receptive to incretin hormone glucagon-like peptide-1, but not to glucose-dependent insulinotropic polypeptide, in the portal vein." J Auton Nerv Syst **61**(2): 149-154.

Olefsky, J. M. and C. K. Glass (2010). "Macrophages, inflammation, and insulin resistance." Annu Rev Physiol **72**: 219-246.

Phillips, R. J., E. A. Baronowsky, et al. (1997). "Afferent innervation of gastrointestinal tract smooth muscle by the hepatic branch of the vagus." J Comp Neurol **384**(2): 248-270.

Phillips, R. J. and T. L. Powley (2000). "Tension and stretch receptors in gastrointestinal smooth muscle: re-evaluating vagal mechanoreceptor electrophysiology." Brain Res Brain Res Rev **34**(1-2): 1-26.

Porter, M. H., B. J. Hrupka, et al. (1998). "Vagal and splanchnic afferents are not necessary for the anorexia produced by peripheral IL-1beta, LPS, and MDP." Am J Physiol **275**(2 Pt 2): R384-389.

Powley, T. L., & Opsahl, C. A. (1974). "Ventromedial hypothalamic obesity abolished by subdiaphragmatic vagotomy." American Journal of Physiology **174**(226): 25-33.

Prechtl, J. C. and T. L. Powley (1990). "The fiber composition of the abdominal vagus of the rat." Anat Embryol (Berl) **181**(2): 101-115.

Reidelberger, R. D., J. Hernandez, et al. (2004). "Abdominal vagal mediation of the satiety effects of CCK in rats." Am J Physiol Regul Integr Comp Physiol **286**(6): R1005-1012.

Ridley, P. T. and F. P. Brooks (1965). "Alterations in Gastric Secretion Following Hypothalamic Lesions Producing Hyperphagia." Am J Physiol **209**: 319-323.

Ritter, S. and T. T. Dinh (1988). "Capsaicin-induced neuronal degeneration: silver impregnation of cell bodies, axons, and terminals in the central nervous system of the adult rat." J Comp Neurol **271**(1): 79-90.

Romanovsky, A. A., V. A. Kulchitsky, et al. (1997). "Cold defense mechanisms in vagotomized rats." Am J Physiol **273**(2 Pt 2): R784-789.

Romanovsky, A. A., V. A. Kulchitsky, et al. (1997). "Febrile responsiveness of vagotomized rats is suppressed even in the absence of malnutrition." Am J Physiol **273**(2 Pt 2): R777-783.

Romanovsky, A. A., C. T. Simons, et al. (1997). "The vagus nerve in the thermoregulatory response to systemic inflammation." Am J Physiol **273**(1 Pt 2): R407-413.

Rosas-Ballina, M., M. Ochani, et al. (2008). "Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia." Proc Natl Acad Sci U S A **105**(31): 11008-11013.

Rosas-Ballina, M., P. S. Olofsson, et al. (2011). "Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit." Science **334**(6052): 98-101.

Schwartz, G. J., P. R. McHugh, et al. (1993). "Gastric loads and cholecystokinin synergistically stimulate rat gastric vagal afferents." Am J Physiol **265**(4 Pt 2): R872-876.

Schwartz, G. J. and T. H. Moran (1996). "Sub-diaphragmatic vagal afferent integration of meal-related gastrointestinal signals." Neurosci Biobehav Rev **20**(1): 47-56.

Schwartz, G. J., C. R. Plata-Salaman, et al. (1997). "Subdiaphragmatic vagal deafferentation fails to block feeding-suppressive effects of LPS and IL-1 beta in rats." Am J Physiol **273**(3 Pt 2): R1193-1198.

Sehic, E. and C. M. Blatteis (1996). "Blockade of lipopolysaccharide-induced fever by subdiaphragmatic vagotomy in guinea pigs." Brain Res **726**(1-2): 160-166.

Skandalakis, L. J., S. W. Gray, et al. (1986). "The history and surgical anatomy of the vagus nerve." Surg Gynecol Obstet **162**(1): 75-85.

Smith, G. P., C. Jerome, et al. (1981). "Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat." Science **213**(4511): 1036-1037.

Stirling, L. C., G. Forlani, et al. (2005). "Nociceptor-specific gene deletion using heterozygous NaV1.8-Cre recombinase mice." Pain **113**(1-2): 27-36.

Szekely, M., M. Balasko, et al. (1997). "Peripheral neural inputs. Their role in fever development." Ann N Y Acad Sci **813**: 427-434.

Uno, K., H. Katagiri, et al. (2006). "Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity." Science **312**(5780): 1656-1659.

Walls, E. K., F. B. Wang, et al. (1995). "Selective vagal rhizotomies: a new dorsal surgical approach used for intestinal deafferentations." Am J Physiol **269**(5 Pt 2): R1279-1288.

Wampler, R. S. and C. T. Snowdon (1979). "Development of VMH obesity in vagotomized rats." Physiol Behav **22**(1): 85-93.

Wang, H., M. Yu, et al. (2003). "Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation." Nature **421**(6921): 384-388.

Wang, P. Y., L. Caspi, et al. (2008). "Upper intestinal lipids trigger a gut-brain-liver axis to regulate glucose production." Nature **452**(7190): 1012-1016.

Wesolowski, H. and S. Snop (1975). "Histochemical studies on the albino rat pancreas in different periods following vagotomy and simultaneous pyloromyotomy." Folia Histochem Cytochem (Krakow) **13**(1-2): 85-92.

Yamamoto, H., T. Kishi, et al. (2003). "Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites." J Neurosci **23**(7): 2939-2946.

Chapter 2: Biochemical Characterization of Vagal $Na_v1.8$ Neuronal Population

2.1 Introduction

$Na_v1.8$ is present in 75-82% of vagal afferent neurons in the nodose ganglion and in around 70% of all DRG neurons (Stirling, Forlani et al. 2005; Gautron, Sakata et al. 2011). $Na_v1.8$ fibers in the DRG principally consist of Aδ and C fiber neurons (Djouhri, Fang et al. 2003; Stirling, Forlani et al. 2005; Fukuoka, Kobayashi et al. 2008), which correspond to DRG nociceptors and a small number of low-threshold mechanoreceptors. The electrophysiological properties of DRG $Na_v1.8$-expressing neurons have been extensively studied as well (Djouhri, Fang et al. 2003) (Rush, Dib-Hajj et al. 2006) and their functional roles have been well characterized. $Na_v1.8$-expressing spinal sensory neurons mediate sensing of cold, inflammatory, and mechanical pain. However, they are not implicated in neuropathic pain or heat sensing (Abrahamsen, Zhao et al. 2008).

In contrast, we know little about the identity and specific roles of the vagal $Na_v1.8$ neuronal population. Work done in the Elmquist lab by Laurent Gautron used $Na_v1.8$-Cre mice to induce tdTomato fluorescent protein in $Na_v1.8$-expressing fibers. These $Na_v1.8$-Cre-tdTomato mice allowed for the labeling of $Na_v1.8$-expressing vagal afferents fibers and endings in tissues. By examining the morphological features of afferent endings in target tissues, we were able to

identify the receptor subtypes represented by $Na_v1.8$ neurons. The GI tract is innervated by two types of specialized mechanoreceptors: intraganglionic laminar endings (IGLEs), which are thought to be tension receptors, and intramuscular arrays (IMAs), which are thought to serve as stretch receptors. IGLEs could be seen in the myenteric plexus of the duodenum and stomach in $Na_v1.8$-Cre-tdTomato mice, but at a lower density than seen with labeling of total vagal innervation. However, no IMAs were formed by $Na_v1.8$ neuronal endings. In contrast, mucosal endings, which are putative chemoreceptors, were labeled throughout the stomach and entire gut. Similar results were seen outside the GI tract, as aortic bodies that contain chemoreceptors were labeled by tdTomato but mechanoreceptors in the aortic arterial wall and heart were not labeled. (Gautron, Sakata et al. 2011)

These results suggest that the $Na_v1.8$ is preferentially expressed in chemoreceptors in the nodose ganglion. To confirm and extend this work, I generated mice with specific ablation of $Na_v1.8$ neurons and systemically compared the expression of various genes in control nodose ganglia to that in nodose ganglia with ablation of $Na_v1.8$ neurons.

2.2 Validation of Genetic Model of $Na_v1.8$ Neuronal Ablation

I utilized a genetic mouse model of primary afferent neuron ablation by crossing two mouse lines, floxed-Stop-DTA and $Na_v1.8$-Cre mice. Floxed-Stop-DTA mice express Diphtheria toxin subunit A (DTA) after the Cre-mediated, cell-

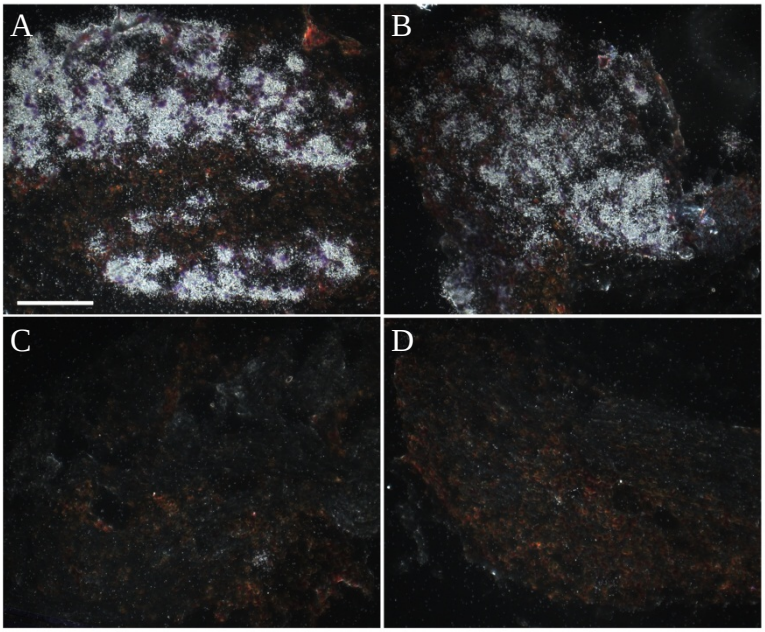

Figure 2.2.1: Ablation of $Na_v1.8$-expressing neurons using diphtheria toxin (A-D). In situ hybridization of NG (A,B) and DRG (C,D) with *$Na_v1.8$* antisense probe in control littermates (A,C) and ablated mice (B,D). Scale Bar indicates 100 µm.

type specific removal of a transcriptional blocker. Mice carrying one copy of $Na_v1.8$-Cre and one floxed-Stop-DTA allele were generated, thus resulting in the irreversible ablation of all $Na_v1.8$-expressing neurons from late embryogenesis, and systematically compared to $Na_v1.8$-Cre littermates with intact neurons. I'll refer to the mice with $Na_v1.8$ neuronal ablation as ablated mice, while control $Na_v1.8$-Cre littermates will be control.

The ablation of $Na_v1.8$-expressing neurons was validated using a number of histological, biochemical and physiological experiments. In situ hybridization for *$Na_v1.8$* mRNA in NG and DRG showed that no *$Na_v1.8$*-expressing neurons

were retained in ablated mice (Figure 2.2.1; conducted by Charlotte Lee). The loss of *Nav1.8* mRNA expression was confirmed by quantitative RT-PCR (Figure 2.2.2).

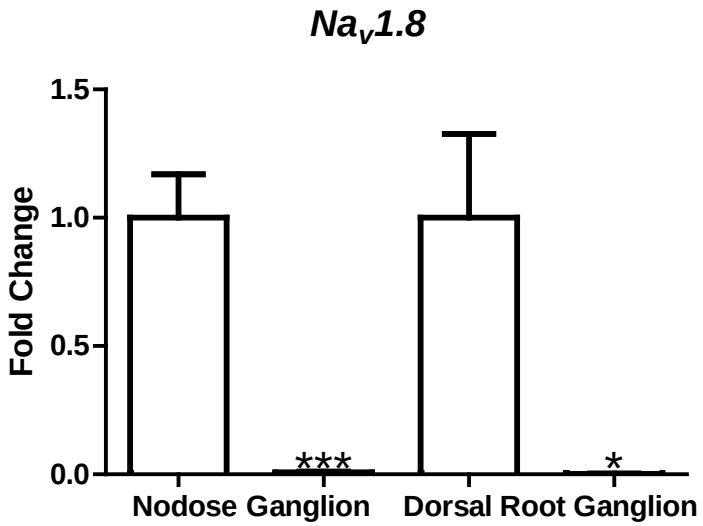

Figure 2.2.2: Loss of *Na$_v$1.8* mRNA expression in ablated animals. Expression of *Na$_v$1.8* mRNA in NG and DRG of control littermates (white bars) and ablated mice (black bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 5–6 mice) Asterisks denote significant differences when compared with littermate controls (*P <0.05, ***P < 0.001 Student's t test, unpaired). Error bars indicate SEM.

Phox2b is a transcription factor found in all vagal neurons. Thus, by quantifying *Phox2b* mRNA expression in the nodose ganglion, I have a surrogate measure of neuronal loss. As expected, in the ablation model mice, *Phox2b* expression is reduced by 80% (Figure 2.2.3a). The expression of calcitonin-gene related peptide (*Cgrp*), a neuropeptide found in subsets of vagal and spinal Nav1.8-expressing neurons (Abrahamsen, Zhao et al. 2008; Gautron, Sakata et al.

2011), was significantly reduced in both NG and DRG (Figure 2.2.3b), and cutaneous CGRP immunoreactivity was lost in ablated mice (Figure 2.2.4). Similar changes were seen in Cocaine and amphetamine regulated transcript (*Cart*), another neuropeptide found in subsets of NG sensory neurons (Figure 2.2.3c). In contrast, levels of myelin basic protein (MBP), which is not found in neurons, were unchanged in NG and DRG (Figure 2.2.3d). In addition, innervation by motor autonomic and enteric neurons remained intact in ablated mice (Figure 2.2.4; immunohistochemistry by Laurent Gautron).

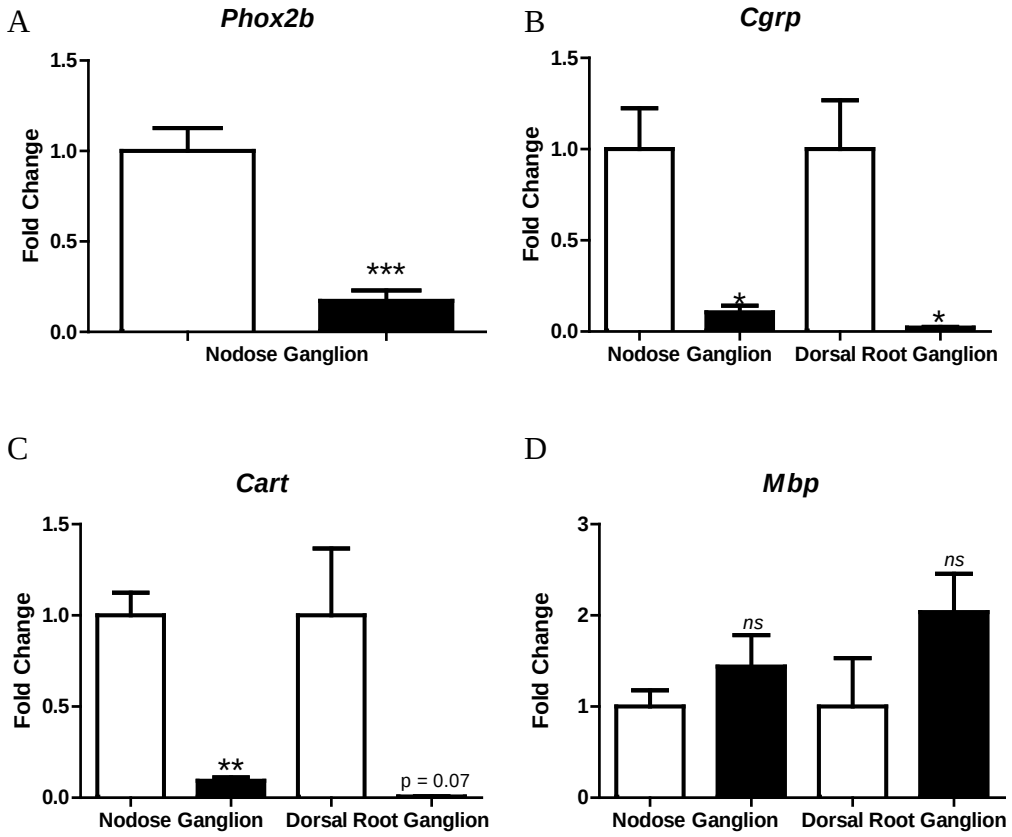

Figure 2.2.3: Validation of $Na_v1.8$ Neuronal Ablation by Quantification of Neuronal and Non-neuronal Markers in NG and DRG. Expression of Phox2b (A), Cgrp (B), Cart (C) and Mbp (D) in NG and DRG of control littermates (white bars) and ablated mice (black bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 3–8 mice). Asterisks denote significant differences when compared with littermate controls (*P <0.05, **P<0.01, ***P<0.001 Student's t test, unpaired). Error bars indicate SEM.

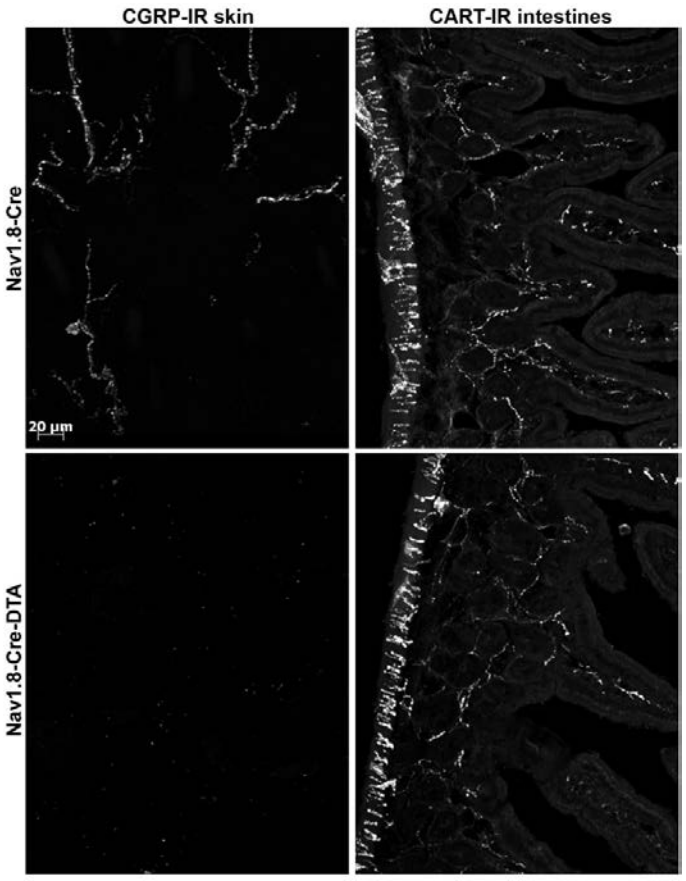

Figure 2.2.4: Sensory neurons are lost in ablated animals, but motor autonomic and enteric neurons are retained. Immunohistochemistry of tissue from control (top row) and ablated mice (bottom row) show loss of sensory afferents but retention of motor autonomic and enteric neurons. On left, CGRP staining shows sensory fibers in the skin in control animals but not in ablated mice. On right, CART-positive terminals in the gut are retained in both control and ablation model mice.

2.3 Characterization of $Na_v1.8$ Vagal Neurons

Next, I attempted to characterize biochemically the neuronal population that was lost in the ablation model by systemically comparing the expression profiles of left nodose ganglia in control $Na_v1.8$-Cre expressing only mice and their ablated littermates via qPCR.

$Na_v1.9$ is, like $Na_v1.8$, a tetrodotoxin (TTX)-resistant voltage gated Na^+ channel (VGSC). In the DRG, it's known to be selectively expressed in nociceptors as $Na_v1.8$ (Wood, Boorman et al. 2004). I see a similar pattern in the nodose ganglia, with an almost complete loss of *$Na_v1.9$* mRNA in our ablated mice (Figure 2.3.1a). The other VGSC I looked at are TTX sensitive. *$Na_v1.1$* and *$Na_v1.6$* (Figure 2.3.1b and c) show about a 50% reduction in expression level in the ablated model, suggesting that they're preferentially expressed in the non-$Na_v1.8$ vagal afferent population, while *$Na_v1.7$* (Figure 2.3.1d) mRNA loss matches that of *Phox2b*, suggesting no preferential expression in either population.

Several members of the Transient Receptor Potential Channel (TRP) family are known to be expressed in sensory neurons, including vagal afferents. Six members of the TRP family are thermosensitive ion channels, with each exhibiting unique thresholds for thermal activation: TRPV4 (>25ºC), TRPV3 (>31ºC), TRPV1 (>43ºC), TRPV2 (>52ºC), TRPM8 (<28ºC) and TRPA1 (<17ºC) (Dhaka, Viswanath et al. 2006). TRPV1 is of particular interest because it is also

the capsaicin receptor (Caterina, Schumacher et al. 1997). *Trpv1* (Figure 2.3.2a) mRNA expression is completely lost in the ablated mice. In contrast, *Trpm8* (Figure 2.3.2b) expression is not significantly changed upon $Na_v1.8$+ neuronal ablation. This is consistent with data in the DRG which shows that neurons that express *Trpv1* do not generally express *Trpm8*, although there has been a report of a small population that expresses both receptors (Okazawa, Inoue et al. 2004). Likewise, *Trpv4* (Figure 2.3.2c) expression is also not significantly changed in ablated mice, while *Trpv2* shows about 80% reduction in expression (Figure 2.3.2d). As TRPV4 is also purported to have a mechanosensory role (Moran, McAlexander et al. 2011), this data is consistent with morphological data suggesting that $Na_v1.8$+ vagal neurons are chemosensory neurons.

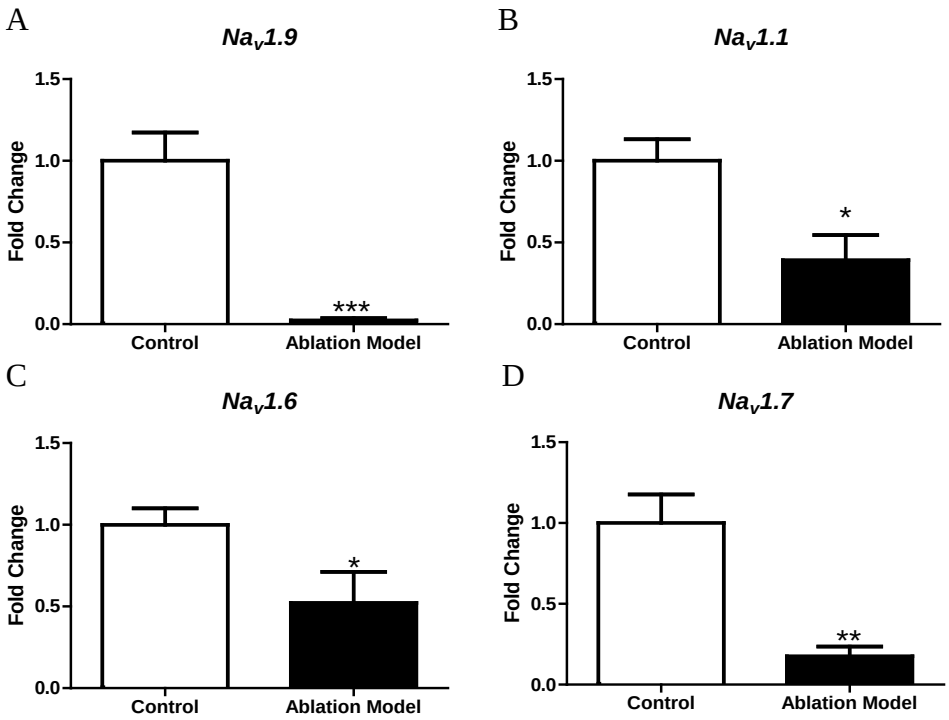

Figure 2.3.1: Expression of *Na$_v$1.9* (A), *Na$_v$1.1* (B), *Na$_v$1.6* (C) and *Na$_v$1.7* (D) in NG of control littermates (white bars) and ablated mice (black bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 5–6 mice). Asterisks denote significant differences when compared with littermate controls (*P<0.05, **P<0.01, ***P<0.001 Student's t test, unpaired). Error bars indicate SEM.

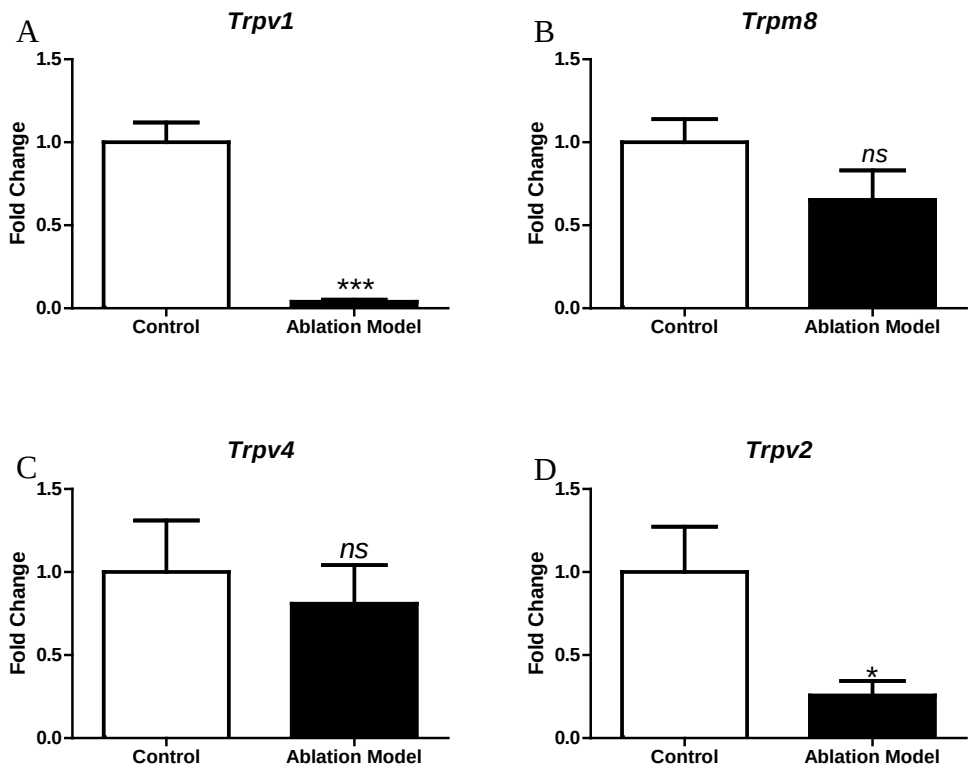

Figure 2.3.2: Expression of *Trpv1* (A), *Trpm8* (B), *Trpv4* (C), and *Trpv2* (D) in NG of control littermates (white bars) and ablated mice (black bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 5–6 mice). Asterisks denote significant differences when compared with littermate controls (ns = not significant, *P<0.05, ***P<0.001, Student's t test, unpaired). Error bars indicate SEM.

Not much is known about the function of TRPC members of the TRP family in sensory neurons. In this study, I found levels of *Trpc1* and *Trpc3* mRNA were significantly decreased in ablated nodose ganglia, while levels of *Trpc6* mRNA were not significantly changed but showed a trend towards reduction (Figure 2.3.3).

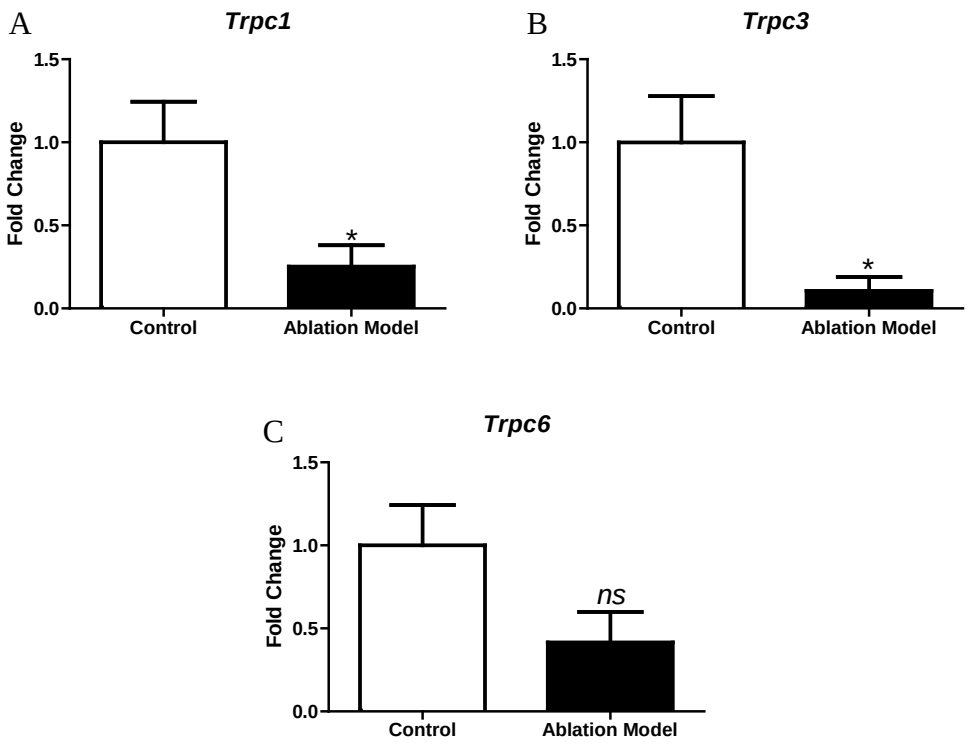

Figure 2.3.3: Expression of *Trpc1* (A), *Trpc3* (B), and *Trpc6* (C) in NG of control littermates (white bars) and ablated mice (black bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 5–6 mice). Asterisks denote significant differences when compared with littermate controls (ns = not significant, *P<0.05, Student's t test, unpaired). Error bars indicate SEM.

The two-pore-domain potassium channels are substrates for resting K^+ currents in neurons. All 7 members of this family are expressed in the DRG (Talley, Solorzano et al. 2001). Of the five channels I profiled here, only *Kcnk2* and *Kcnk4* showed significant reductions in ablated nodose, suggesting that the others, Kcnk3, 5, and 6, are found largely in non-$Na_v1.8$ vagal afferents (Figure 2.3.4).

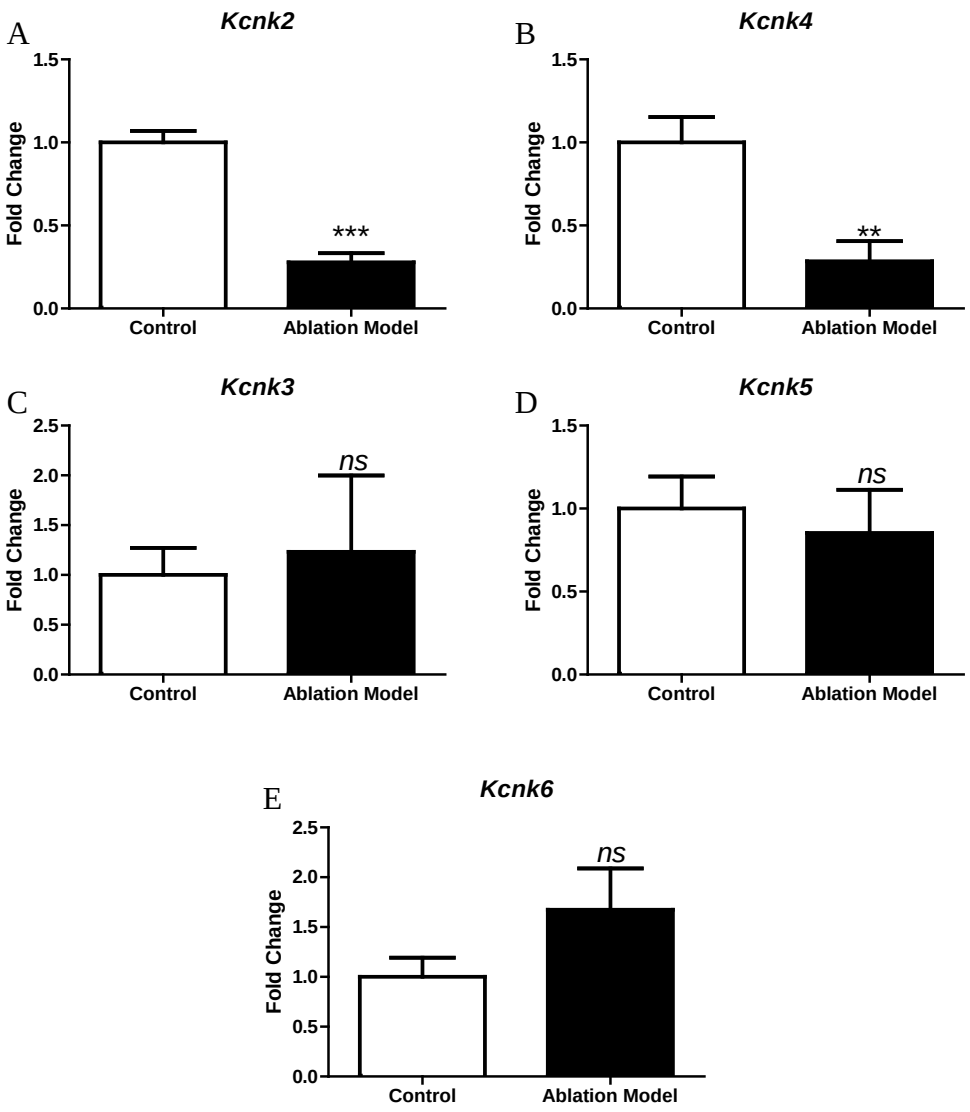

Figure 2.3.4: Expression of *Kcnk2* (A), *Kcnk4* (B), *Kcnk3* (C), *Kcnk5* (D), and *Kcnk6* (E) in NG of control littermates (white bars) and ablated mice (black bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 5–6 mice). Asterisks denote significant differences when compared with littermate controls (ns = not significant, **P<0.01, ***P<0.001 Student's t test, unpaired). Error bars indicate SEM.

Morphological characterization of the sensory terminals of $Na_v1.8$ vagal neurons suggested that this group of neurons comprised the chemosensory and multimodal vagal neurons but not the mechanosensory ones. If so, we would expect to see significantly attenuated expression of vagal chemoreceptors in ablated nodose. This is indeed the case.

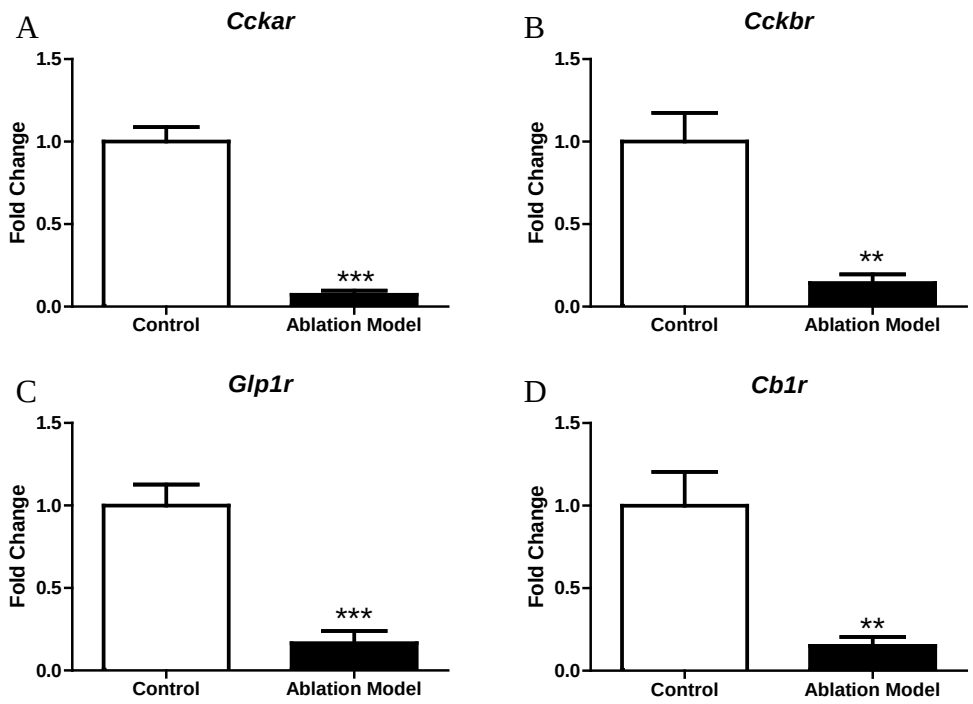

Figure 2.3.5: Expression of *Cckar* (A), *Cckbr* (B), *Glp1r* (C), and *Cb1r* (D) in NG of control littermates (white bars) and ablated mice (black bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 5–6 mice). Asterisks denote significant differences when compared with littermate controls (**P<0.01, ***P<0.001, Student's t test, unpaired). Error bars indicate SEM.

The gut peptide cholecystokinin has been shown to decrease feeding through actions on vagal afferents (Smith, Jerome et al. 1981); mRNA expression of both A and B isoforms of CCK receptor (*Cckar* and *Cckbr*) in the NG is significantly reduced in ablated animals (Figure 2.3.5a and b). GLP-1 is another gut peptide that has been shown to activate vagal afferents (Nishizawa, Nakabayashi et al. 1996), and expression of *Glp-1r* is likewise reduced in ablated NG (Figure 2.3.5c). Neurons in the vagal nodose ganglion also express cannabinoid (CB_1) receptors, and *Cb1r* mRNA levels have been shown to be differentially regulated in the nodose in response to fasting (Burdyga, Lal et al. 2004). We do see decreased expression of *Cb1r* mRNA in ablated animals as well (Figure 2.3.5d). The serotonin receptor HTR3a is a ligand-gated ion channel receptor, thought to be expressed in chemosensory vagal neurons. As expected, we see almost total loss of expression in ablated mice (Figure 2.3.6a).

Another important channel involved in pain processing is the P2X3 ion channel. The P2X3 channel belongs to a ligand gated ion channel family that is activated by extracellular ATP. It is thought that ATP released from damaged or inflamed cells activates the P2X3 receptor, initiating nociceptive signals. The P2X3 receptor is highly expressed in sensory neurons in the DRG, both as a homomer and as a heteromer with the P2X2 receptor (Surprenant and North 2009). Expression of both *P2x3* and *P2x2* mRNA are significantly reduced in ablated nodose (Figure 2.3.6b and c). In contrast, the angiotensin receptor

(AGTR1a) is thought to be largely expressed on mechanosensory vagal neurons (Li and Zheng 2011). Consistent with this idea, *Agtr1a* expression is not significantly changed in ablated neurons, although there is a trend towards reduced expression (Figure 2.3.6d).

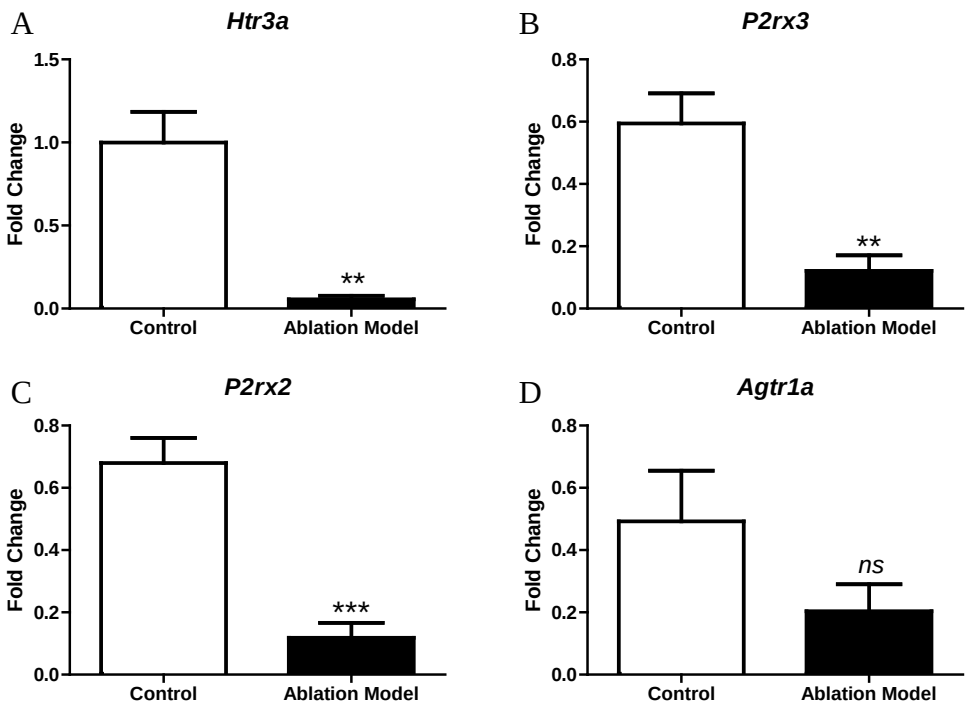

Figure 2.3.6: Expression of *Htr3a* (A), *P2rx3* (B), *P2rx2* (C), and *Agtr1a* (D) in NG of control littermates (white bars) and ablated mice (black bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 5–6 mice). Asterisks denote significant differences when compared with littermate controls (ns = not significant, **P<0.01, ***P<0.001, Student's t test, unpaired). Error bars indicate SEM.

2.4 Discussion

Previously published work from the Elmquist lab suggested that $Na_v1.8$ vagal afferent neurons represented the chemoreceptor or multimodal receptor population of vagal afferents. This dissertation has expanded upon that to characterize the expression profile of various other channels in this neuronal group, as well as to demonstrate that $Na_v1.8$ neurons do express various chemoreceptors known to act on vagal afferents. Moreover, upon loss of $Na_v1.8$ expressing neurons in the ablated mice, there is almost complete loss of certain chemoreceptors. The results of this expression profiling in the nodose ganglion, along with values from the dorsal root ganglion for comparison, are presented in Table 2.4.1.

While these results were not surprising, they were crucial to know before moving on to the next step in my dissertation work. As my ablation model does not target a random 80% or so of vagal afferent neurons, it was important to clearly define the population that is affected. The obvious next question becomes to delineate the functional role of this neuronal group. As I demonstrated that I could successfully and specifically ablate $Na_v1.8$ neurons, I was set to move on to functional characterization of the ablated animals.

	NG			DRG
	Fold Change	SEM	p-value	Fold Change
Nav1.8	0.01	< 0.01	0.0005	0.00
Phox2b	0.17	0.06	0.0004	0.00
TRPV1	0.04	0.01	< 0.0001	0.06
TRPV2	0.26	0.09	0.0412	0.12
TRPV4	0.81	0.23	0.6474	0.25
TRPM8	0.65	0.18	0.1533	0.23
TRPA1	0.04	0.02	0.0001	0.01
Nav1.1	0.39	0.15	0.0146	0.66
Nav1.6	0.52	0.19	0.0443	0.32
Nav1.7	0.18	0.06	0.0028	0.19
Nav1.9	0.02	0.01	0.0006	0.00
TRPC1	0.25	0.13	0.0316	0.44
TRPC3	0.11	0.08	0.0355	0.02
TRPC6	0.42	0.18	0.0979	0.00
Kcnk3	1.23	0.77	0.7657	0.32
Kcnk5	0.85	0.26	0.6549	1.77
Kcnk6	1.67	0.42	0.1527	3.18
Kcnk4	0.28	0.12	0.0062	0.29
Kcnk2	0.28	0.06	<0.0001	0.73
HTR3A	0.06	0.02	0.0013	0.08
CCKAR	0.07	0.03	<0.0001	0.33
CCKBR	0.14	0.05	0.0019	0.00
GLP1R	0.17	0.07	0.0004	0.00
CB1R	0.15	0.05	0.0052	0.23
AGTR1A	0.41	0.09	0.1743	0.75
P2RX3	0.20	0.05	0.0027	0.03
P2RX2	0.17	0.05	0.0003	0.19

Table 2.4.1 Summary of mRNA expression levels of genes in NG and DRG of ablated animals normalized to expression levels of controls. Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 5–6 mice). Values were normalized to average value of control animals in the same tissue. Genes with fold change values in green indicate those that are preferentially expressed in $Na_v1.8$ neurons, while those in red indicate those that are preferentially expressed in non- $Na_v1.8$ neurons. SEM and p-values are as indicated (Student's t test, unpaired).

Abrahamsen, B., J. Zhao, et al. (2008). "The cell and molecular basis of mechanical, cold, and inflammatory pain." Science **321**(5889): 702-705.

Burdyga, G., S. Lal, et al. (2004). "Expression of cannabinoid CB1 receptors by vagal afferent neurons is inhibited by cholecystokinin." J Neurosci **24**(11): 2708-2715.

Caterina, M. J., M. A. Schumacher, et al. (1997). "The capsaicin receptor: a heat-activated ion channel in the pain pathway." Nature **389**(6653): 816-824.

Dhaka, A., V. Viswanath, et al. (2006). "Trp ion channels and temperature sensation." Annu Rev Neurosci **29**: 135-161.

Djouhri, L., X. Fang, et al. (2003). "The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons." J Physiol **550**(Pt 3): 739-752.

Fukuoka, T., K. Kobayashi, et al. (2008). "Comparative study of the distribution of the alpha-subunits of voltage-gated sodium channels in normal and axotomized rat dorsal root ganglion neurons." J Comp Neurol **510**(2): 188-206.

Gautron, L., I. Sakata, et al. (2011). "Genetic tracing of Nav1.8-expressing vagal afferents in the mouse." J Comp Neurol **519**(15): 3085-3101.

Li, Y. L. and H. Zheng (2011). "Angiotensin II-NADPH oxidase-derived superoxide mediates diabetes-attenuated cell excitability of aortic baroreceptor neurons." Am J Physiol Cell Physiol **301**(6): C1368-1377.

Moran, M. M., M. A. McAlexander, et al. (2011). "Transient receptor potential channels as therapeutic targets." Nat Rev Drug Discov **10**(8): 601-620.

Nishizawa, M., H. Nakabayashi, et al. (1996). "The hepatic vagal nerve is receptive to incretin hormone glucagon-like peptide-1, but not to glucose-dependent insulinotropic polypeptide, in the portal vein." J Auton Nerv Syst **61**(2): 149-154.

Okazawa, M., W. Inoue, et al. (2004). "Noxious heat receptors present in cold-sensory cells in rats." Neurosci Lett **359**(1-2): 33-36.

Rush, A. M., S. D. Dib-Hajj, et al. (2006). "A single sodium channel mutation produces hyper- or hypoexcitability in different types of neurons." Proc Natl Acad Sci U S A **103**(21): 8245-8250.

Smith, G. P., C. Jerome, et al. (1981). "Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat." Science **213**(4511): 1036-1037.

Stirling, L. C., G. Forlani, et al. (2005). "Nociceptor-specific gene deletion using heterozygous NaV1.8-Cre recombinase mice." Pain **113**(1-2): 27-36.

Surprenant, A. and R. A. North (2009). "Signaling at purinergic P2X receptors." Annu Rev Physiol **71**: 333-359.

Talley, E. M., G. Solorzano, et al. (2001). "Cns distribution of members of the two-pore-domain (KCNK) potassium channel family." J Neurosci **21**(19): 7491-7505.

Wood, J. N., J. P. Boorman, et al. (2004). "Voltage-gated sodium channels and pain pathways." J Neurobiol **61**(1): 55-71.

Chapter 3: Role of $Na_v1.8$ Expressing Afferents in Long Term Energy Balance

3.1 Introduction

The visceral afferents innervate the gastrointestinal tract, providing information about the chemical, hormonal, and mechanical milieu of the gut to the metabolic regulatory regions of the brain. Considering the relatively slow rates at which postabsorptive and humoral signals can be mobilized, it is generally accepted that visceral afferents supply much of the short-term, rapid feedback that controls feeding. In particular, the mechanical and chemical signals conveyed by the afferents in the vagus nerve are known to be crucial in meal termination and satiation.

However, how this short-term feedback associated with visceral signaling contributes to long term control of energy balance is controversial. Suggestive albeit often contradictory evidence comes from several animal models with impaired visceral afferent function. Sprague–Dawley rats that underwent selective vagal de-afferentation with capsaicin gained less weight and had less abdominal fat on high fat diet (HFD) (Stearns, Balakrishnan et al. 2012), while mice which show a loss of vagal afferent innervation of the small intestines as a result of NT-4 gene knockout are unable to reduce food intake in response to calorie loads and show increased weight gain (Fox, Phillips et al. 2001). The OLETF rat lacks the receptor for CCK, an endogenous gut peptide that signals satiety largely through action on the vagal afferents (Reidelberger, Hernandez et al. 2004). These rats

show an increase in meal size without a reduction in meal number, leading to the development of obesity (Covasa and Ritter 2001; Bi and Moran 2002). However, the CCK-A receptor knockout mouse shows increase in meal size without accompanying long term positive energy balance and obesity (Bi, Scott et al. 2004). These discrepant findings are not unexpected considering the physiologic variables necessarily introduced by targeting different subsets of visceral afferent activity.

While the results of these studies vary, they do demonstrate that perturbation of these traditionally short-term loops may translate into long term disturbances in energy balance. However, other work suggests that vagal afferents may be required for short term but not long term regulation of body weight. Work done in humans with epilepsy showed that electrical stimulation of vagal afferents does not appear to decrease food intake and body weight (Koren and Holmes 2006). Vagal deafferentations have been shown to produce increases in meal size in rats (Schwartz, Salorio et al. 1999) (Kelly, Morales et al. 2000) , but this does not appear to result in excessive weight gain due to compensatory changes in meal number (Powley, Chi et al. 2005). However, this work cannot be considered to definitively answer the question as it was noted in the long term study that vagal afferents regenerate post-vagotomy, such that reinnervation of the stomach was already noted at 4 weeks, and meal patterning was studied during this period of regeneration of vagal fibers (Powley, Chi et al. 2005).

One final piece of evidence that could suggest that visceral afferents play a role in global metabolic regulation comes from the long term metabolic changes induced by bariatric treatments for obesity. Surgical remodeling of the gastrointestinal (GI) tract must undoubtedly alter patterns of visceral afferent stimulation, and patients who have undergone bariatric surgery regularly report symptoms consistent with altered visceral afferent activity, such as discomfort associated with large or frequent meals, excessive feelings of distension, and nausea.

To directly assess the role of visceral afferents in global energy homeostasis, we have generated a mouse model with specific ablation of visceral afferents without affecting visceral efferents. By utilizing this unique genetic model, we can assess the contributions of visceral afferents in the long term regulation of food intake, energy expenditure, and glucose/insulin homeostasis.

3.2 Perturbations in Energy Expenditure Regulation in Ablated Animals

To assess the requirement of visceral afferent signaling in the appropriate regulation of body weight, I analyzed weight gain in ad libitum fed ablated model mice versus control littermates. Ablated mice showed no differences in body weight from control littermates on chow diet (Figure 3.2.1). Body composition was also not altered (Figure 3.2.2), and body length was also unchanged between the two groups (data not shown). As the body weight data would suggest, there was no difference in food intake (Figure 3.2.3).

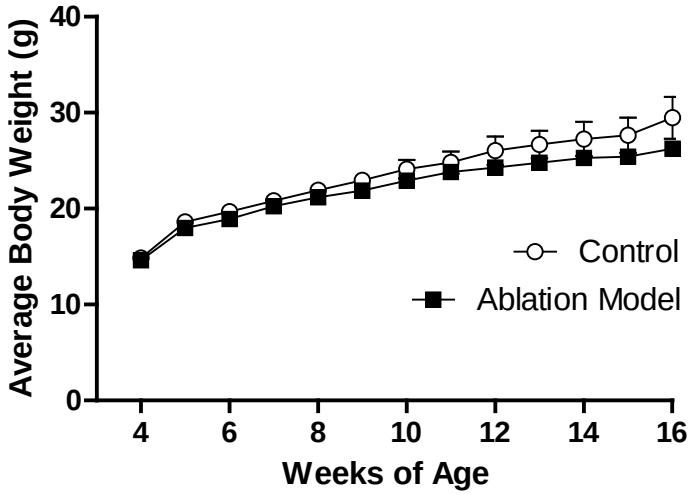

Figure 3.2.1: Ablated mice show normal body weight on chow diet. Body weight was measured weekly in singly housed male control (open circles) and ablated mice (black boxes) fed chow diet starting at weaning (n = 6/genotype). Error bars indicate SEM.

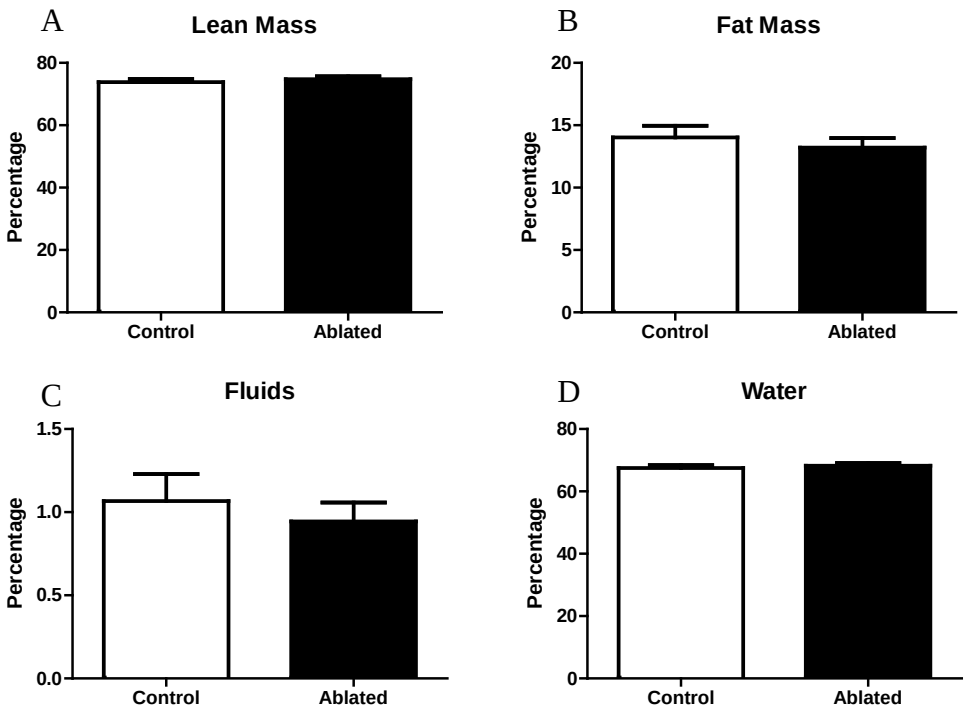

Figure 3.2.2: Body composition of ablated mice (black bars) and control littermates (white bars) on chow (n = 8/ genotype) by NMR. A. Body percentage composed of lean mass. B. Body percentage composed of fat mass. C. Body percentage composed of fluids. D. Body percentage composed of water. Error bars indicate SEM.

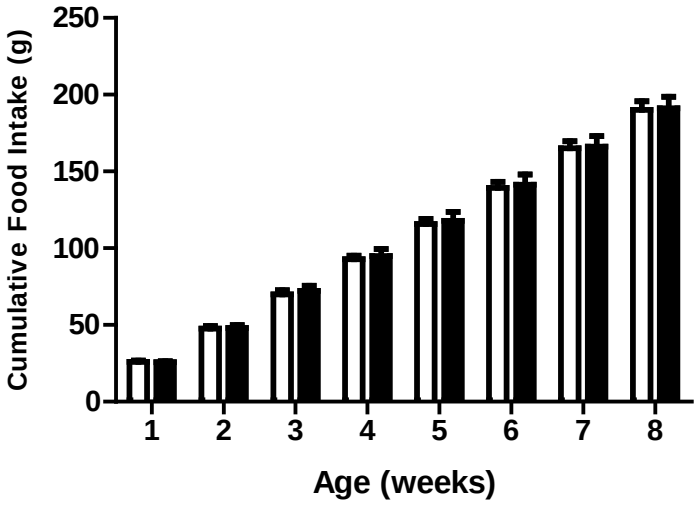

Figure 3.2.3: Ablated Mice show no difference in food intake. Weekly food intake was recorded in singly housed ablated mice (black bars) and control littermates (white bars) (n = 6/genotype). Error bars indicate SEM.

Ablated mice showed a trend towards reduced energy expenditure (O_2 consumption, CO_2 production and heat production) compared to their body weight-matched control littermates (Figure 3.2.4) but it was not significant. However, a similar magnitude of depression was seen in four separate cohorts of animals (data not shown). Components of total energy expenditure include energy required for physical activities, basal metabolism, and thermogenesis (Castaneda, Jurgens et al. 2005; Butler and Kozak 2010). Neither ambulatory movements nor rearing activities were significantly changed in ablated mice (Figure 3.2.5). Taken together, all these findings suggest little, if any, perturbation in long term food intake and energy balance in ablated animals.

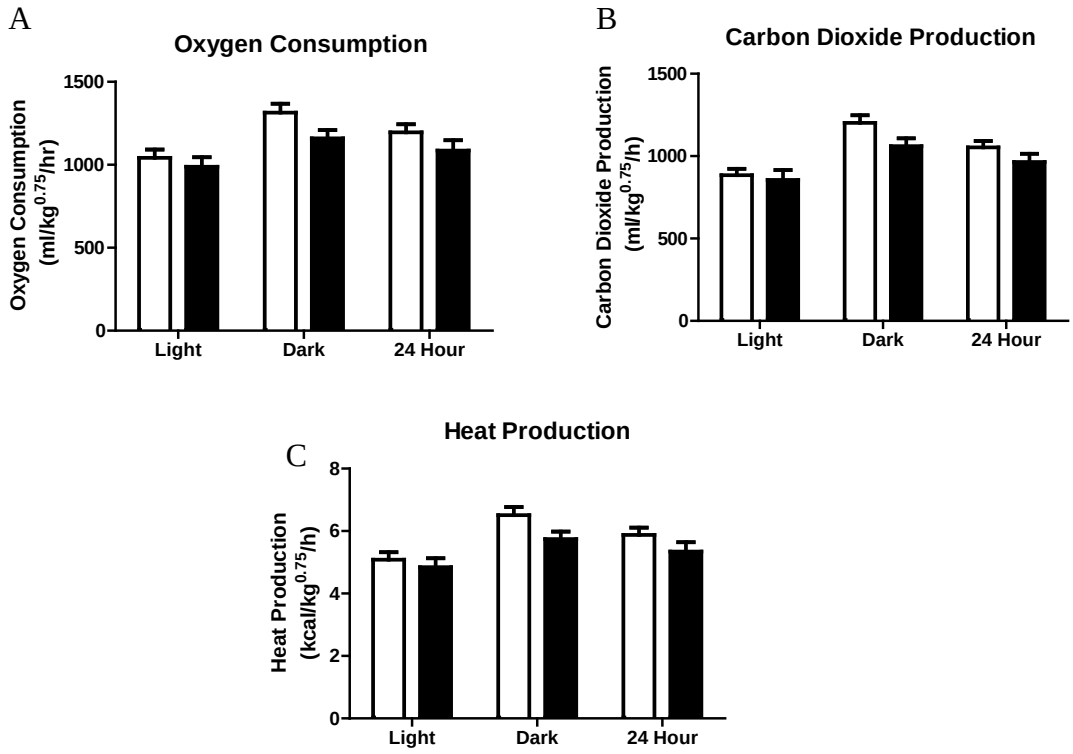

Figure 3.2.4: Ablated mice show trend towards depressed energy expenditure. Oxygen consumption (A), carbon dioxide production (B), and heat production (C) were measured in control littermates (left) and ablated mice (right) in the light phase, dark phase, and over the entire 24 hour period (n = 6-9/ genotype).

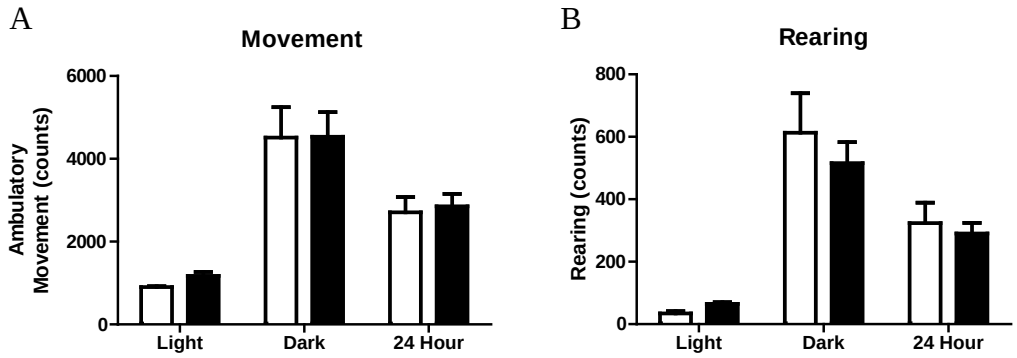

Figure 3.2.5: Ablated mice show no differences in voluntary movement. Activity of control littermates (white bars) and ablated mice (black bars) were measured in metabolic cages over a 5 day period (n = 4-5/genotype). A. Ambulatory movement in the light phase, dark phase, and over a 24 hour period. B. Rearing in the light phase, dark phase, and over a 24 hour period. Error bars indicate SEM.

However, as vagal mechanisms are mostly involved in rapid, short-term regulation, I hypothesized that although the animals might be able to maintain energy balance on the long term, short term responses might be impaired. Thus, I next examined whether intact visceral afferent signaling is required for appropriate thermogenic responses to excess nutrition. When acutely exposed to HFD feeding, body weight-matched control mice increase their O_2 consumption, CO_2 production and heat production. However, the increase in energy expenditure in ablated mice was significantly less than that in controls (Figure 3.2.6). The impaired thermogenic responses to HFD feeding in ablated mice were not due to reductions in food intake, as food intake after being switched to HFD was comparable between control littermates and ablated mice.

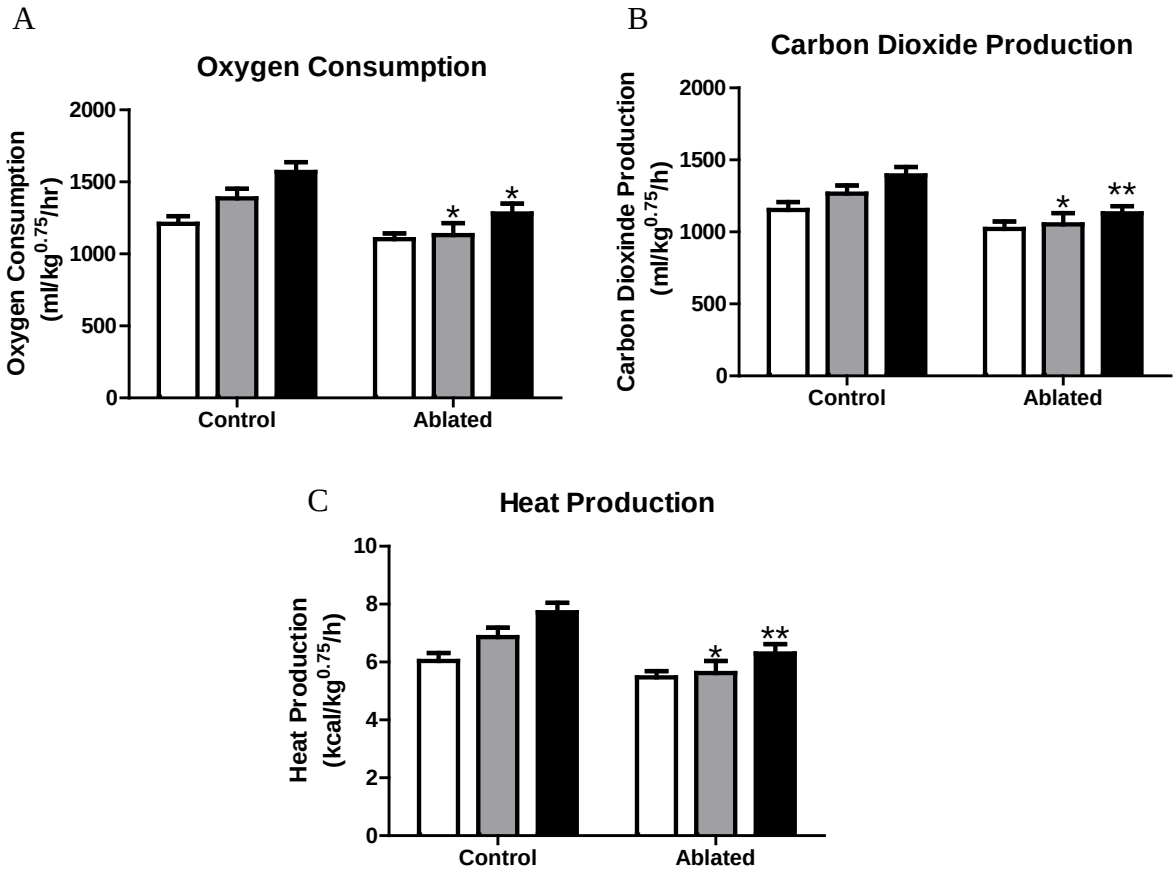

Figure 3.2.6: Ablated mice do not show diet induced thermogenesis upon acute high fat diet exposure. Oxygen consumption (A), carbon dioxide production (B), and heat production (C) were measured in control littermates (left) and ablated mice (right) in the dark phase before (white bars), immediately after (gray bars), and one day later (black bars) switching to high fat diet (n = 6-7/ genotype). There was significant effect of genotype by two-way repeated measures ANOVA. Asterisks indicate significant differences (*P < 0.05, *P < 0.01, Bonferroni multiple comparisons) from control mice at same time point.

3.3 Meal Patterning Perturbations in Ablated Animals

Visceral afferents are known to be particularly crucial for satiation and meal termination, so I hypothesized that the changes in food intake were a result of perturbed meal patterning. Meal size, meal duration, and meal frequency were studied in ablated mice and control littermates on chow diet. Ablated mice tended to eat shorter and more frequent meals (Figure 3.3.1). Meal size also trended towards being decreased. However, differences in meal characteristics between the two groups are small, and repetition of this experiment in a second cohort showed similar but not statistically significant changes.

Several gut peptides are thought to signal satiety through actions on visceral afferents. As $Na_v1.8$ is preferentially expressed in visceral chemoreceptors/multimodal receptors, I hypothesized that the observed effects stemmed from functional loss of these chemoreceptors. To test this, food intake was measured in animals after the intraperitoneal administration of CCK-8. Control mice showed a reduction in food intake as expected, but ablated mice failed to reduce food intake upon CCK administration, suggesting functional loss of CCK signaling (Figure 3.3.2).

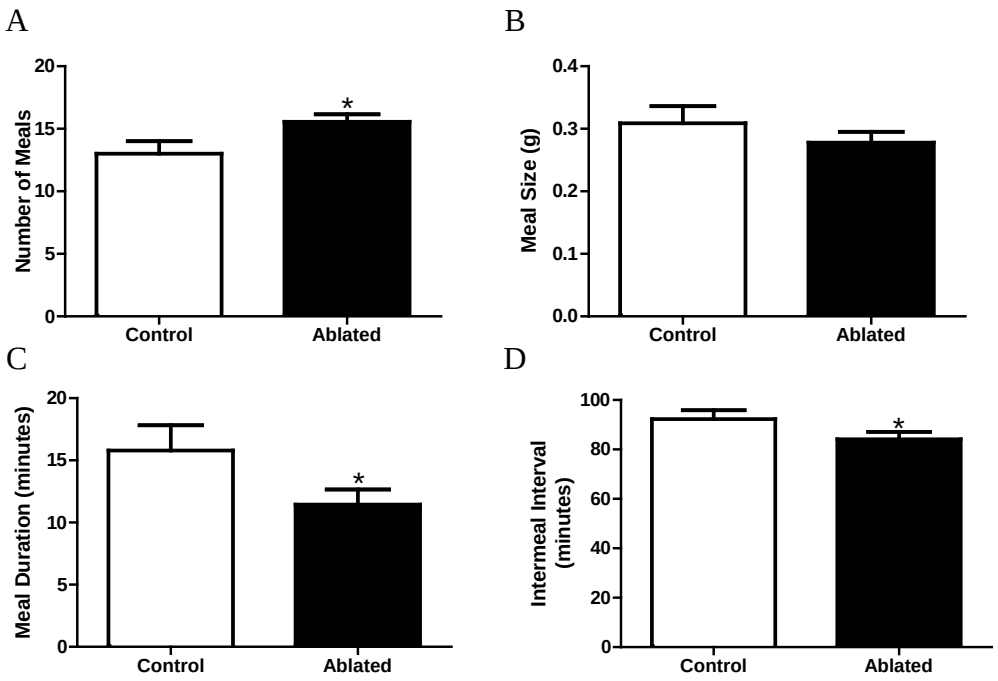

Figure 3.3.1: Ablated mice (black bars) show differences in meal patterning from control littermates (white bars). A. Number of meals in a 24 hour period. (n = 9/genotype) B. Average size of meal. (n = 7-9/ genotype) C. Length of meal. (n = 12-14/genotype) D. Average interval between meals. (n = 8-9/genotype) Asterisks indicate significant differences between groups. (*P < 0.05, Student's t test, unpaired). Error bars indicate SEM.

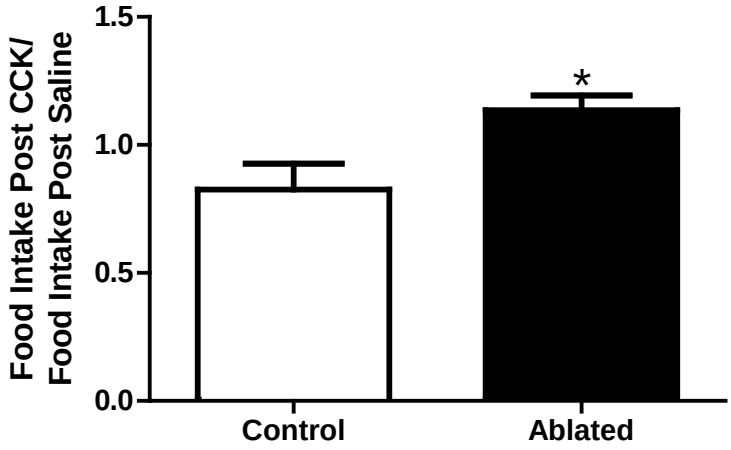

Figure 3.3.2 Food intake in control littermates (white bars) and ablated mice (black bars) at 90 minutes post-CCK injection. Values are expressed relative to food intake in vehicle-treated animals (n = 6–7/ genotype). Asterisks denote significant differences when compared with littermate controls (*P <0.05, Student's *t* test, unpaired). Error bars indicate SEM.

3.4 Discussion

The genetic mouse model of afferent ablation allowed us to examine the role of the visceral afferents in modulating food intake without the potential confounding variables that arise from pharmacologic and/or surgical manipulations, which necessarily target motor efferents or other neuronal populations as well. When control mice were observed after an intraperitoneal injection of CCK-8, a decrease in food intake was evident. In contrast, ablated mice show no change in food consumption after administration of exogenous CCK-8. These observations support the conclusion that CCK-induced inhibition of food intake is mediated through the visceral afferents. They also validate that

our genetic model of visceral afferent ablation successfully targets visceral chemoreceptor population.

To examine the physiologic role of visceral afferents over an extended period of time, weight as a function of age was assessed in wild-type vs. ablated mice that were fed ad libitum. Ablated mice had body weights comparable to the corresponding wild-type littermates. The similarity in weights between ablated and wild-type animals persisted through the rapid growth phase and extended into adulthood. Ablated mice were indistinguishable from littermate controls in terms of body length and composition. Taken together, these data indicate that visceral afferents are not essential for the maintenance of normal body weight or growth.

Despite no changes in body weight, I did observe slight differences in meal patterning. The observation that loss of $Na_v1.8$+ visceral afferents results in more frequent but shorter meals is in contrast to studies showing visceral afferent function is critical in meal termination and thus would predict that loss of these neurons would result in longer meals. However, it is likely that since $Na_v1.8$ is expressed preferentially in chemoreceptors so our model leaves mechanoreceptors intact, responses to gut stretch and distension might be sufficient to terminate meals appropriately. In fact, it is possible that in the absence of visceral chemosensation, which mediates anorectic signals as well as orexigenic signals, satiety signals from mechanoreceptors are unopposed or more sensitive.

The small but not significant depression in energy expenditure, observed with the loss of $Na_v1.8$-expressing visceral afferents in the ablated mouse model, was unexpected. However as body weight was unchanged with comparable food intakes, this effect, if real, is miniscule. More significant is the loss of diet induced thermogenesis in the ablated mice, which suggests that sensing of the increased calorie load by sensory afferents is required to initiate diet-induced thermogenesis.

Despite the subtle and complex metabolic effects seen, these findings, do not establish that visceral afferents do not contribute to global regulation of energy balance and food intake. As all $Na_v1.8$ expressing neurons are irreversibly ablated in late embryogenesis in our model, it is possible that the body is able to develop compensatory pathways. Also, as the body is able to compensate for the perturbations seen here and maintain normal body weight, clearly multiple redundant pathways exist to regulate these crucial functions.

Bi, S. and T. H. Moran (2002). "Actions of CCK in the controls of food intake and body weight: lessons from the CCK-A receptor deficient OLETF rat." Neuropeptides **36**(2-3): 171-181.

Bi, S., K. A. Scott, et al. (2004). "Differential roles for cholecystokinin a receptors in energy balance in rats and mice." Endocrinology **145**(8): 3873-3880.

Butler, A. A. and L. P. Kozak (2010). "A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes." Diabetes **59**(2): 323-329.

Castaneda, T. R., H. Jurgens, et al. (2005). "Obesity and the neuroendocrine control of energy homeostasis: the role of spontaneous locomotor activity." J Nutr **135**(5): 1314-1319.

Clerc, P., M. G. Coll Constans, et al. (2007). "Involvement of cholecystokinin 2 receptor in food intake regulation: hyperphagia and increased fat deposition in cholecystokinin 2 receptor-deficient mice." Endocrinology **148**(3): 1039-1049.

Covasa, M. and R. C. Ritter (2001). "Attenuated satiation response to intestinal nutrients in rats that do not express CCK-A receptors." Peptides **22**(8): 1339-1348.

Fox, E. A., R. J. Phillips, et al. (2001). "Neurotrophin-4 deficient mice have a loss of vagal intraganglionic mechanoreceptors from the small intestine and a disruption of short-term satiety." J Neurosci **21**(21): 8602-8615.

Reidelberger, R. D., J. Hernandez, et al. (2004). "Abdominal vagal mediation of the satiety effects of CCK in rats." Am J Physiol Regul Integr Comp Physiol **286**(6): R1005-1012.

Stearns, A. T., A. Balakrishnan, et al. (2012). "Relative contributions of afferent vagal fibers to resistance to diet-induced obesity." Dig Dis Sci **57**(5): 1281-1290.

Chapter 4: Loss of $Na_v1.8$ Neurons Leads to Uncontrolled Inflammation Upon High Fat Feeding

4.1 Introduction

Obesity is closely linked with low-grade inflammation characterized by increased levels of circulating inflammatory cytokines and acute-phase reactants, concomitant with the activation of a network of inflammatory signaling pathways (Hotamisligil 2006; Lumeng and Saltiel 2011). While it is well appreciated that nutrient excess from carbohydrate-rich and fatty foods is directly responsible for lipotoxicity and inflammation in obesity (Unger and Scherer 2010), much of how diet-induced inflammation is regulated at the organismal level remains to be understood. As discussed in the introduction to this dissertation, visceral afferent neurons can mediate responses to both nutritional and inflammatory stimuli, I hypothesized that they may be involved in the coordinating the inflammatory response to nutrition and thus wanted to test their function in a paradigm of diet-induced inflammation.

To determine whether these neurons are important in diet-induced inflammation, I continued to utilize my mouse model with selective ablation of $Na_v1.8$-expressing afferent neurons. Strikingly, the ablated mice developed a severe, ultimately lethal response to high-fat feeding characterized by splenomegaly, cachexia and acute phase response. This phenotype appeared to be mediated by excessive inflammatory cytokine induction upon high-fat feeding

which was rescued by pharmacological activation of the cholinergic anti-inflammatory pathway. This suggests visceral afferent neurons could be involved in checking high-fat diet-induced inflammation.

4.2 Severe Weight Loss Upon High Fat Feeding in Ablated Animals

To study the role of homeostatic afferents in diet-induced inflammation, ablated and control mice were started on standard chow (4% fat) and high-fat diet (42% fat). On standard chow, ablated mice showed no difference in body weight from control littermates (Figure 3.2.1). However, on high-fat diet, ablated mice showed weight loss after a short period of initial weight gain (Figure 4.2.1). This loss of body weight presented with overt signs of illness, such as toxic appearance, reduced activity, and matted fur, and eventually required euthanasia of the animals when more than 20-30% of body weight was lost (compared to age-matched controls). Although the onset of weight loss varied between individual mice, 75% of ablation model mice showed signs of illness along with weight loss by 12 weeks of high-fat feeding (Figure 4.2.2). Weight loss was not due to decreased food intake, as high-fat diet consumption was equivalent in ablated mice and control littermates prior and after development of observable illness (Figure 4.2.3). Likewise, weight loss was not due to nutrient malabsorption as measurement of fecal lipid content demonstrated unchanged levels of fat absorption between the two groups (Figure 4.2.4; conducted with Ryan Jones). In fact, a primary deficit in lipid absorption was ruled out as the kinetics of

triglyceride appearance in the bloodstream after lipid gavage was similar between the two groups (Fig. 4.2.5; lipid levels in plasma measured by Joseph Rutkowski).

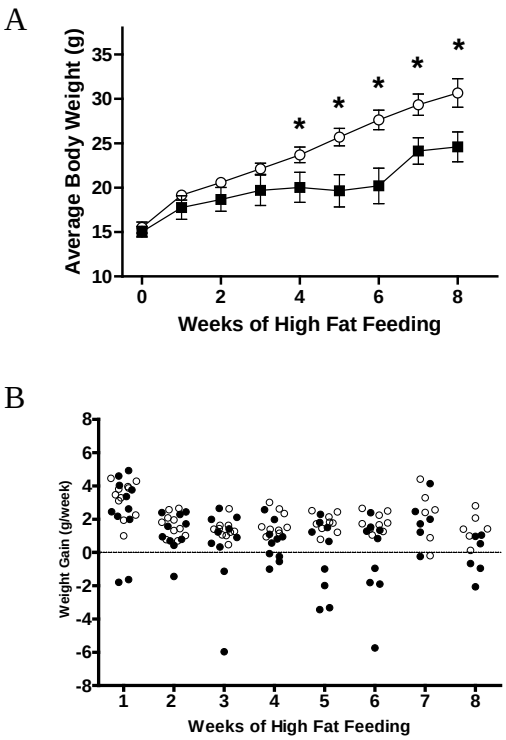

Figure 4.2.1: Ablated mice show severe weight loss upon high-fat feeding. (A) Control littermates (white circles) and ablated mice (black boxes) were started on high-fat at 5 weeks of age and body weight and food intake were recorded weekly (n = 6/genotype). (B) Weight gain per week was recorded after starting high fat feeding in control littermates (white circles) and ablated model mice (black circles) (n = 12/genotype). Asterisks indicate significant differences (*P<0.05, Student's t test, unpaired). Error bars indicate SEM.

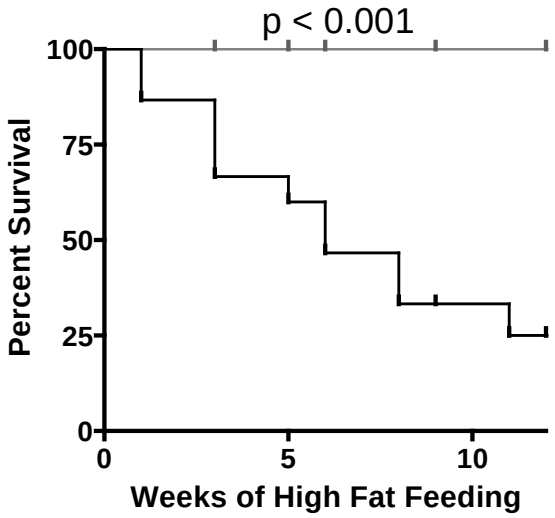

Figure 4.2.2: Illness-free survival was recorded after starting high fat feeding in control mice (gray line) and ablated mice (black line) (n = 18/genotype). P value as indicated (Log-rank test).

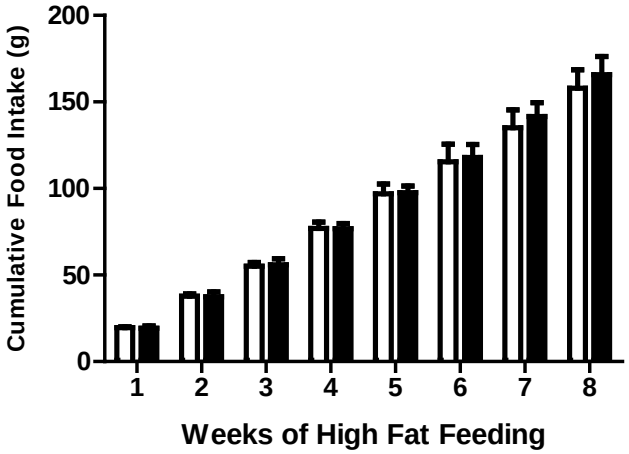

Figure 4.2.3: Ablated mice do not show anorexia upon high-fat feeding. Control littermates (white bars) and ablated mice (black bars) were started on high-fat at 5 weeks of age and food intake were recorded weekly (n = 6/genotype). Error bars indicate SEM.

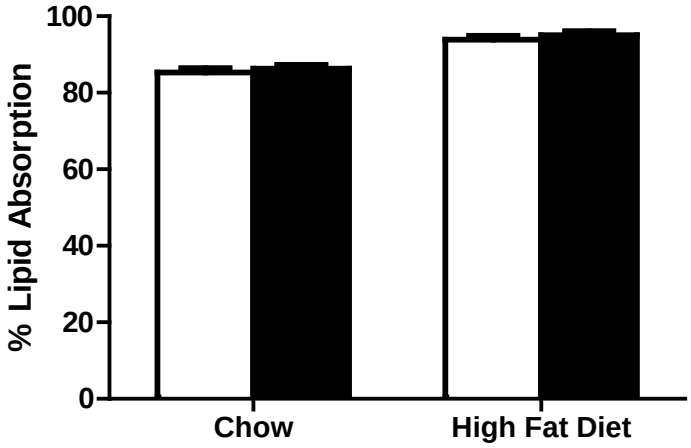

Figure 4.2.4: Fecal lipid absorption on chow and high-fat diet of ablated mice (black bars) and control littermates (white bars) (n = 6–9/ genotype). Error bars indicate SEM.

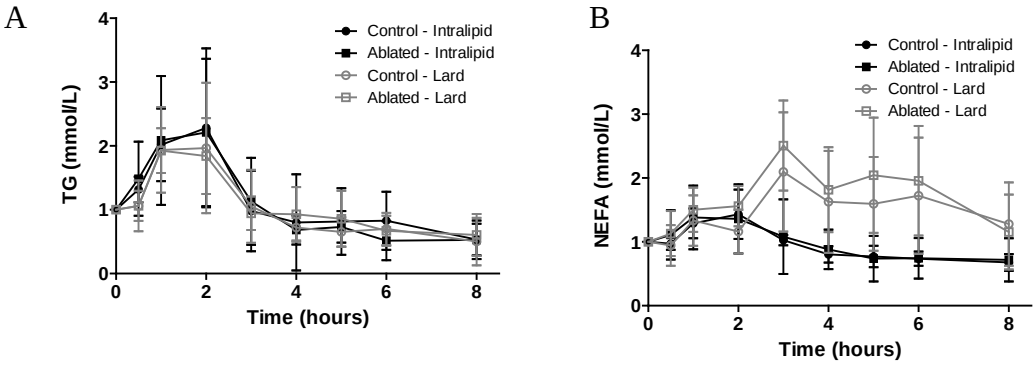

Figure 4.2.5: Ablation model mice show normal kinetics of triglyceride appearance in blood stream after gavage of lipids. (A) Plasma levels of triglyceride or (B) non-esterified fatty acids after gavage of Intralipid (black symbols) or lard oil (open symbols) in control animals (circles) versus ablation model animals (boxes) (n = 6/genotype and experimental condition). Error bars indicate SEM.

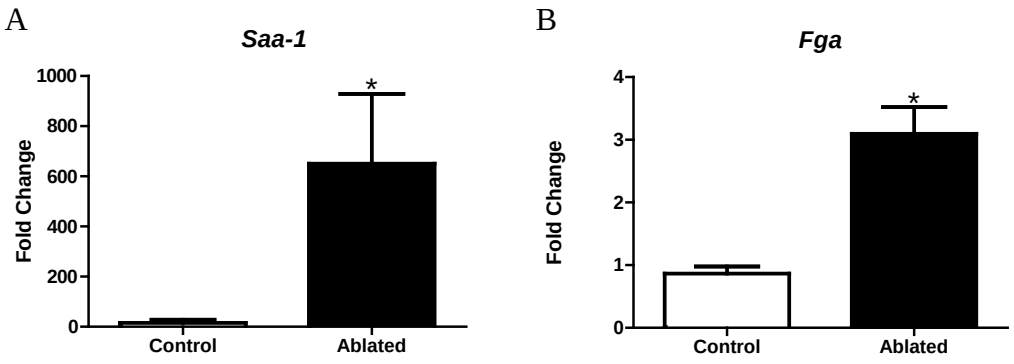

Figure 4.2.6: Expression of acute phase reactants *Saa-1* (A) and *Fga* (B) in livers of terminally ill ablated mice (black bars) and their control littermates (white bars). Transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to 18S levels (n = 4–6/ genotype). Asterisks denote significant differences when compared with littermate controls (*P < 0.05, Student's t test, unpaired). Error bars indicate SEM.

Necroscopy of $Na_v1.8$ ablated mice requiring euthanasia consistently revealed splenomegaly with enlarged red pulp and leucopenia in the white pulp, proteinacious build-up in blood vessels, depletion of glycogen in liver, and shrinking of adipose cells in different fat pads (Figure 4.2.7). However, no apparent foci of infection (e.g. bacterial translocation) or other cause of decline (e.g. portal hypertension) could be identified by pathologist upon examination. This pathology represents a unique response to high-fat feeding, as no similar changes were observed in ablation model animals upon chow feeding, even at advanced ages (data not shown). Furthermore, terminally-ill ablated mice showed massive induction of hepatic mRNA for Serum amyloid α-1 (*Saa-1*) and

fibrinogen (*Fga*), thus implying acute phase response (Fig. 4.2.6). The phenotype observed in $Na_v1.8$ neurons ablated mice is surprisingly reminiscent of, but not identical to, the cachectic phenotype seen in transgenic mice with disrupted lipid metabolism (Lichtenstein, Mattijssen et al. 2010). However, gene expression in major lipid metabolism pathways (i.e. Angplt4, FXR) were not changed in ablated mice prior to high-fat exposure (data not shown), thus indicating that a primary deficit in lipid metabolism is not responsible for the phenotype of ablated mice.

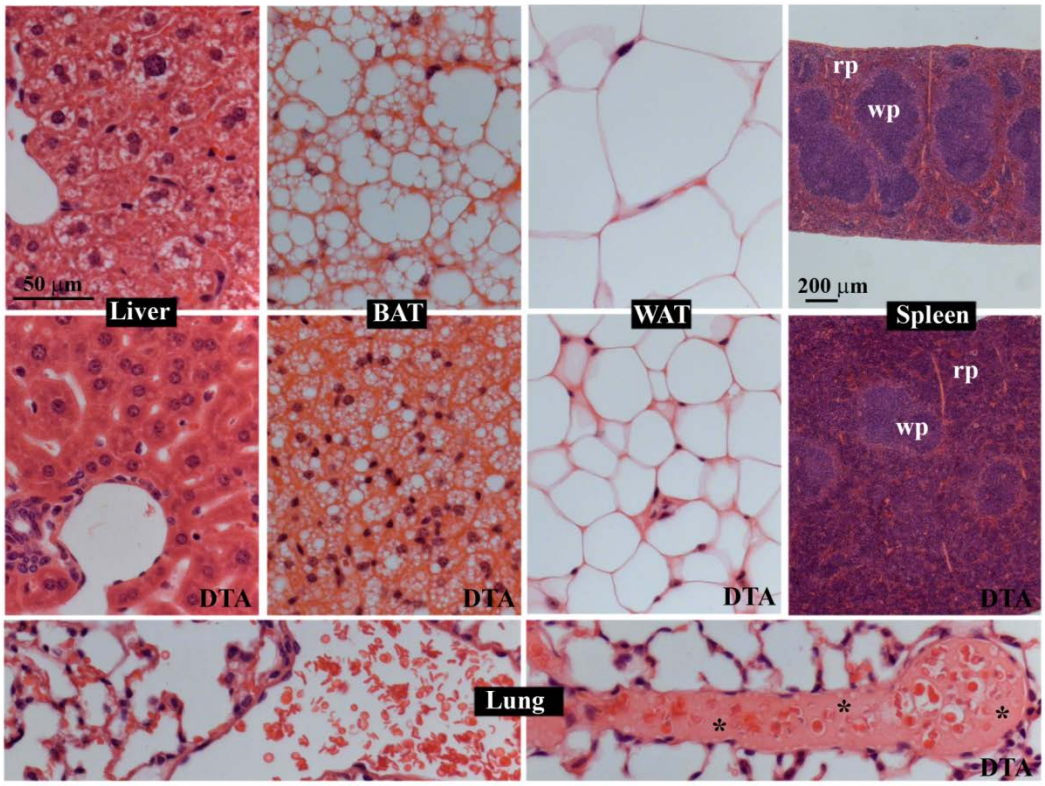

Figure 4.2.7: H&E stained sections of liver, brown adipose tissue, white adipose tissue, lung, and of spleen of control littermates and ablated mice (labeled DTA). White asterisks indicate proteinacious deposits (pink) in blood vessels. Abbreviations: rp, red pulp; wp, white pulp.

4.3 Increased Inflammation on High Fat Diet in Ablated Animals

To gain insight into the cause of this diet-induced cachectic phenotype, mice were studied before onset of overt weight loss and sickness. Although cachexia is multifactorial, a sustained and unwarranted stimulation of inflammatory pathways plays a key role in its initiation (Das, Eder et al. 2011). Strikingly, plasma levels of interkeukin-6 (IL-6) (Figure 4.3.1a) and white blood cells (Figure 4.3.3) were significantly increased in ablated mice as early as one week of high-fat feeding. Similar elevation was also observed in other inflammatory cytokines and chemokines (data not shown), and the increase in spleen size was already significant at two weeks of high-fat feeding (Figure 4.3.2). As expected with rises in inflammatory cytokines, body temperature was also increased in ablated mice (Figure 4.3.1b). Importantly, ablated mice showed no difference in any of the aforementioned parameters prior to high fat feeding (Figure 4.3.1, Figure 4.3.4). Thus, ablation model mice exhibit systemic inflammation in response to high-fat diet, prior to development of clinical signs.

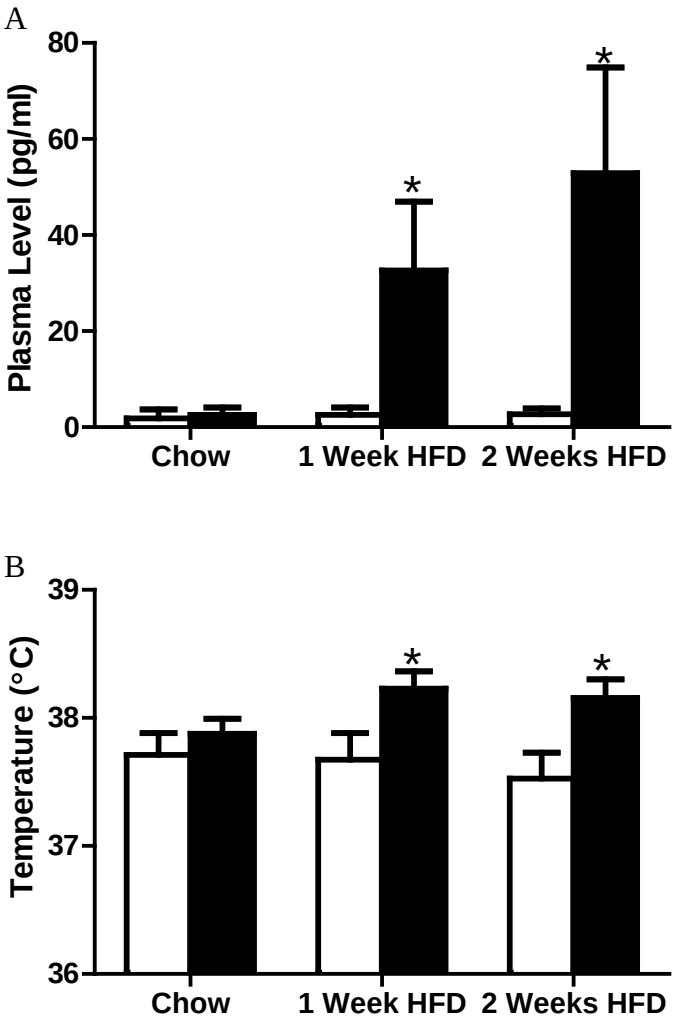

Figure 4.3.1: Loss of $Na_v1.8$ neurons exacerbates diet-induced inflammation in control (white bars) ablated mice red bars). (A) Plasma levels of IL-6 were measured by Luminex Multiplex assay on chow diet, after 1 week and after 2 weeks on high fat diet (n = 5-6/genotype). (B) Body temperature was measured via subcutaneously implanted thermal transponder on chow diet, after 1 week and 2 weeks on high fat diet (n = 7-8/genotype). Asterisks denote significant differences when compared with littermate controls (*$P < 0.05$, Student's t test, unpaired). Error bars indicate SEM.

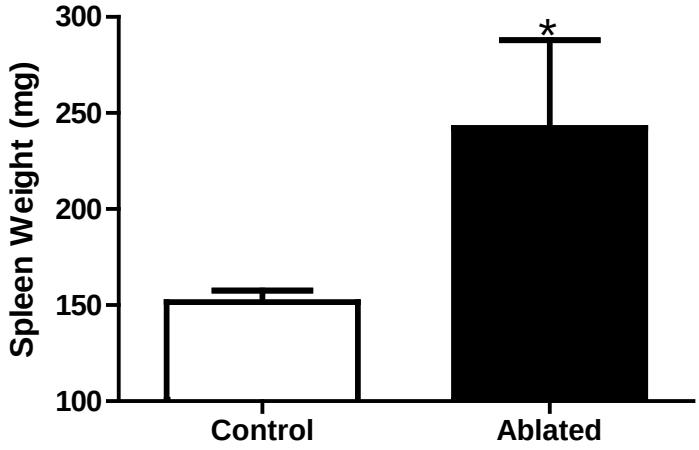

Figure 4.3.2: Spleen weights of mice after 2 weeks on high-fat (n = 5/genotype). Asterisks denote significant differences when compared with littermate controls (*P < 0.05, Student's t test, unpaired). Error bars indicate SEM.

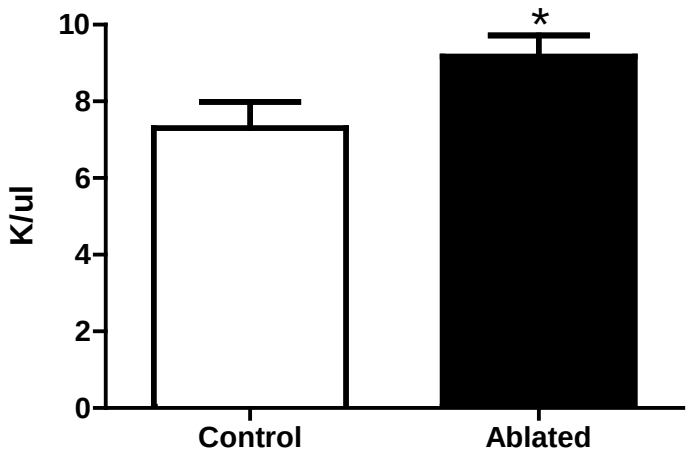

Figure 4.3.3: Circulating white blood cells counts after one week of high-fat feeding in control (white bars) and ablated (black bars) mice (n = 5/ genotype). Asterisks denote significant differences when compared with littermate controls (*$P < 0.05$, Student's *t* test, unpaired). Error bars indicate SEM.

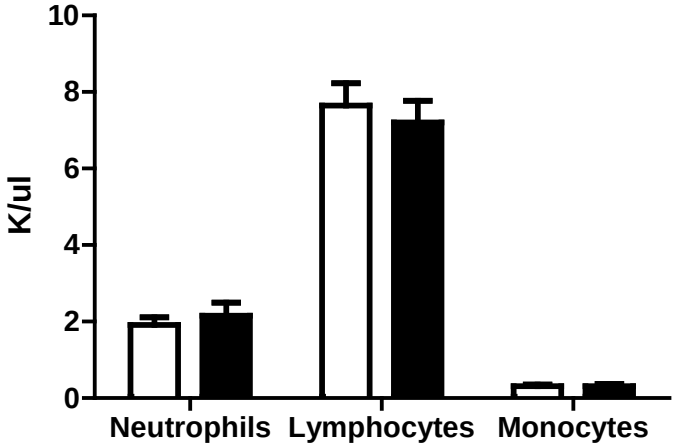

Figure 4.3.4: Blood cells counts of control littermates (white bars) and ablated mice (black bars) on chow diet were measured (n = 5/genotype). Counts of neutrophils, lymphocytes, and monocytes in peripheral blood. Error bars indicate SEM.

4.4 Pharmacological Activation of the Cholinergic Anti-Inflammatory Pathway Can Blunt Inflammation in Ablated Animals

As we see that disruption of $Na_v1.8$ neurons *in vivo* significantly increases high-fat feeding-induced cytokine release, we hypothesized that the activity of these neurons normally activates a compensatory response to inhibit inflammation upon nutrient excess. Stimulation of afferent neurons may inhibit inflammation through activation of the hypothalamic-pituitary-adrenal (HPA) axis or the sympathetic nervous system. To assess activation of the sympathetic nervous system, norepinephrine and epinephrine levels were measured in plasma after animals were started on high-fat feeding and showed no differences between control and ablated mice (Figure 4.4.1). Likewise, plasma levels of corticosterone and ACTH were unchanged between the two groups (Figure 4.4.2). It was recently discovered that the vagus nerve can also modulate inflammatory responses through the so-called “vagal cholinergic anti-inflammatory” reflex (Andersson and Tracey 2011). When inflammatory molecules are detected by peripheral afferents, enhanced activity of vagal efferents leads to acetylcholine release in the organs of the reticuloendothelial system (Andersson and Tracey 2011). Acetylcholine then interacts with α7-nicotinic receptors on tissue cells to inhibit cytokine release and suppress inflammation (Andersson and Tracey 2011). The implication of this pathway is suggested by a recent work demonstrating that

α7-nicotinic receptors knock-out mice are more susceptible to diet induced-inflammation (Wang, Yang et al. 2011).

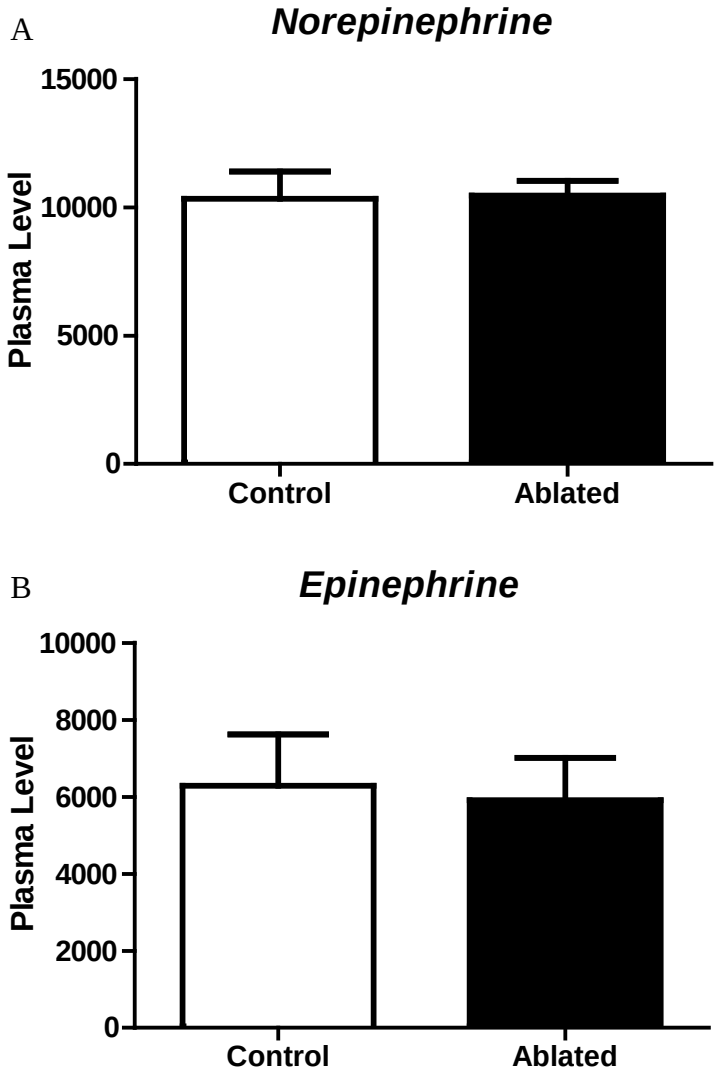

Figure 4.4.1: Plasma levels of norepinephrine (A) and epinephrine (B) in control littermates (white bars) and ablated mice (black bars) 36 hours after starting high-fat diet (n = 5/genotype).

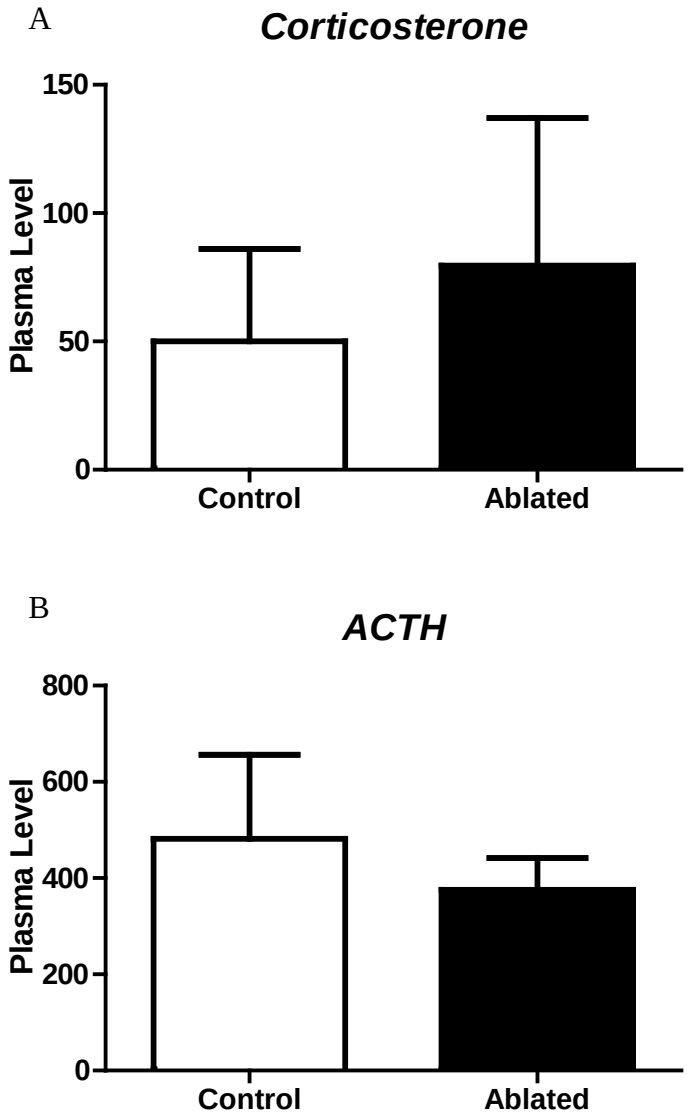

Figure 4.4.2: Plasma levels of corticosterone (A) and ACTH (B) are not significantly different in control littermates (white bars) and ablated model mice (black bars) 36 hours after starting high-fat diet (n = 5/genotype). Error bars indicate SEM.

To test whether this pathway contributes to the exaggerated inflammation in ablated mice upon high-fat feeding, we administered choline, a selective agonist of α7-nicotinic receptors with known anti-inflammatory activity (Parrish, Rosas-Ballina et al. 2008), upon starting high-fat feeding for a period of 2 weeks. Preliminary results show that choline administration was able to normalize levels of inflammatory plasma cytokines IL-6, IL-1α, IL-1β, and G-CSF seen in ablated mice upon high fat feeding (Figure 4.4.3). In contrast, levels of anti-inflammatory cytokine IL-10 were not significantly changed (data not shown). This rescue of cytokine levels was matched by rescue of macroscopic abnormalities, as splenomegaly was also prevented by choline treatment (Figure 4.4.4). These results need to be validated in additional studies but represent an exciting preliminary finding.

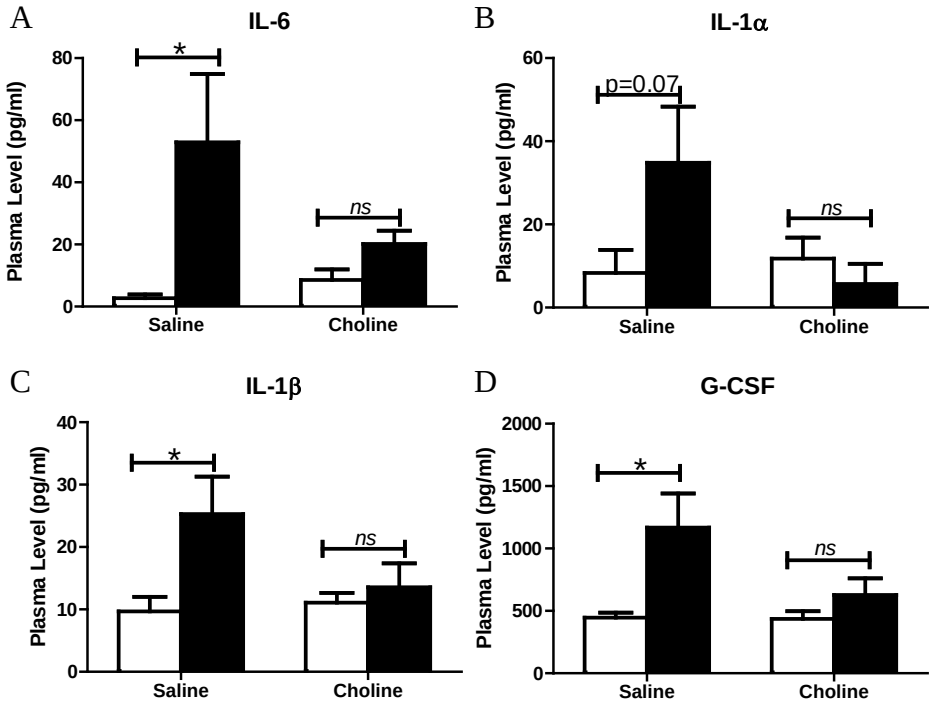

Figure 4.4.3: Choline treatment suppresses diet-induced inflammation in ablated mice. Control littermates (white bars) and ablated mice (red bars) were started on high-fat diet on Day 0 and then received daily vehicle only or 50 mg/kg/day choline i.p. injections for 2 weeks. (A) Plasma levels of IL-6, IL-1α, IL-1β, and G-CSF measured via Luminex Multiplex Assay at Day 14. Asterisks denote significant differences when compared with littermate controls (*P < 0.05, Two-Way ANOVA followed by Bonferroni Multiple Comparisons). Error bars indicate SEM .

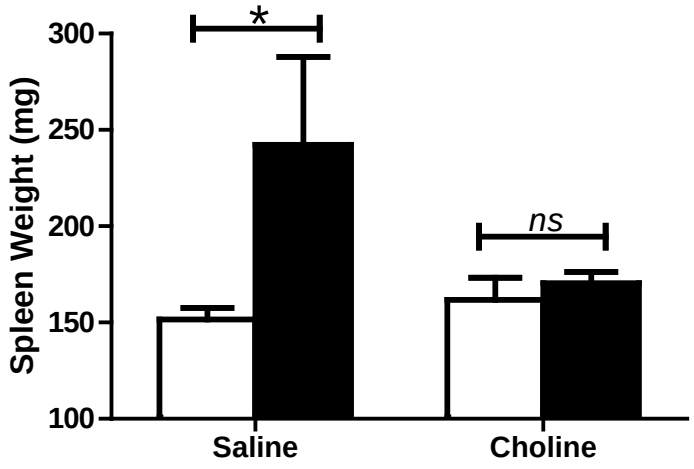

Figure 4.4.4: Choline treatment suppresses diet-induced inflammation in ablated mice. Control littermates (white bars) and ablated mice (black bars) were started on high-fat diet on Day 0 and then received daily vehicle only or 50 mg/kg/day choline i.p. injections for 2 weeks. Spleen weights of mice at Day 14. Asterisks denote significant differences when compared with littermate controls (*P < 0.05, Two-Way ANOVA followed by Bonferroni Multiple Comparisons). Error bars indicate SEM .

4.5 Discussion

Mice with ablation of $Na_v1.8$-expressing neurons show a severe phenotype in response to high fat feeding. This includes weight loss and sickness behavior accompanied by an acute phase response. These observable signs are preceded by massive increase in levels of circulating cytokines. Preliminary results indicate these effects can be blunted by administration of choline, an agonist of the α-7 nicotinic receptor.

This work thus suggests that $Na_v1.8$ neurons act to regulate responses to high fat diet, including limiting induction of inflammatory cytokines. With loss of

these neurons, the inflammatory cascade proceeds unchecked, ultimately leading to depletion of body energy stores and weight loss. Furthermore, blunting of this inflammation through pharmacological targeting of the cholinergic anti-inflammatory pathways suggests that this pathway is normally involved in limiting inflammation through activation downstream of $Na_v1.8$-expressing neurons. Our current understanding of these pathways then naturally suggests a circuit whereby $Na_v1.8$ neurons directly or indirectly sense inflammatory triggers in ingested materials and then act to activate, possibly through other mediators, the cholinergic anti-inflammatory pathway to limit the extent of inflammation initiated. An important caveat in interpreting this data is that high fat diet differs significantly from chow diet in a number of ways besides fat content. Thus, the inflammatory affect may be a result of a trigger other than high fat. Indeed, incomplete penetrance of an otherwise severe phenotype upon high fat feeding would support this. Moreover, a similar phenotype can be seen in ablated animals when subject to other inflammatory triggers, such as infection, in the absence of high fat feeding (data not shown.)

Andersson, U. and K. J. Tracey (2011). "Reflex Principles of Immunological Homeostasis." Annu Rev Immunol.

Bernal-Mizrachi, C., L. Xiaozhong, et al. (2007). "An afferent vagal nerve pathway links hepatic PPARalpha activation to glucocorticoid-induced insulin resistance and hypertension." Cell Metab **5**(2): 91-102.

Bookout, A. L., Y. Jeong, et al. (2006). "Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network." Cell **126**(4): 789-799.

Chawla, A., J. J. Repa, et al. (2001). "Nuclear receptors and lipid physiology: opening the X-files." Science **294**(5548): 1866-1870.

Das, S. K., S. Eder, et al. (2011). "Adipose triglyceride lipase contributes to cancer-associated cachexia." Science **333**(6039): 233-238.

Fu, J., S. Gaetani, et al. (2003). "Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha." Nature **425**(6953): 90-93.

Gofflot, F., N. Chartoire, et al. (2007). "Systematic gene expression mapping clusters nuclear receptors according to their function in the brain." Cell **131**(2): 405-418.

Hotamisligil, G. S. (2006). "Inflammation and metabolic disorders." Nature **444**(7121): 860-867.

Lichtenstein, L., F. Mattijssen, et al. (2010). "Angptl4 protects against severe proinflammatory effects of saturated fat by inhibiting fatty acid uptake into mesenteric lymph node macrophages." Cell Metab **12**(6): 580-592.

Lumeng, C. N. and A. R. Saltiel (2011). "Inflammatory links between obesity and metabolic disease." J Clin Invest **121**(6): 2111-2117.

Luquet, S., F. A. Perez, et al. (2005). "NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates." Science **310**(5748): 683-685.

Nguyen, M. T., S. Favelyukis, et al. (2007). "A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways." J Biol Chem **282**(48): 35279-35292.

Olefsky, J. M. and C. K. Glass (2010). "Macrophages, inflammation, and insulin resistance." Annu Rev Physiol **72**: 219-246.

Parrish, W. R., M. Rosas-Ballina, et al. (2008). "Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling." Mol Med **14**(9-10): 567-574.

Poltorak, A., X. He, et al. (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science **282**(5396): 2085-2088.

Pradel, E., Y. Zhang, et al. (2007). "Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by Caenorhabditis elegans." Proc Natl Acad Sci U S A **104**(7): 2295-2300.

Shi, H., M. V. Kokoeva, et al. (2006). "TLR4 links innate immunity and fatty acid-induced insulin resistance." J Clin Invest **116**(11): 3015-3025.

Stirling, L. C., G. Forlani, et al. (2005). "Nociceptor-specific gene deletion using heterozygous NaV1.8-Cre recombinase mice." Pain **113**(1-2): 27-36.

Sun, J., V. Singh, et al. (2011). "Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes." Science **332**(6030): 729-732.

Unger, R. H. and P. E. Scherer (2010). "Gluttony, sloth and the metabolic syndrome: a roadmap to lipotoxicity." Trends Endocrinol Metab **21**(6): 345-352.

Uno, K., H. Katagiri, et al. (2006). "Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity." Science **312**(5780): 1656-1659.

Wang, X., Z. Yang, et al. (2011). "Activation of the cholinergic antiinflammatory pathway ameliorates obesity-induced inflammation and insulin resistance." Endocrinology **152**(3): 836-846.

Chapter 5: Conclusions and Perspectives

5.1 Summary

The work summarized herein with our mouse model of $Na_v1.8$-expressing homeostatic afferent ablation has revealed several key findings about the role of these neurons in metabolism. Although the primary contribution of vagal afferents has been thought to be mediating satiation in response to ingested nutrients, we do not see major defects in control of food intake in our ablation model despite demonstrated loss of the majority of visceral chemoreceptors. We do observe slight changes in meal patterning. However, there is adequate compensation for these changes, as animals are able to maintain normal body weight on chow. Input from vagal afferents does appear to be important to linking ingested nutrients to acute changes in energy expenditure, as animals with ablation of $Na_v1.8$ neurons do not show induction of appropriate thermogenic response to an acute increase in calorie intake. Thus, it is possible that signaling through visceral afferents is required in short term coupling of nutritional intake to energy expenditure, but other pathways can compensate over a longer time period.

Most critically, $Na_v1.8$ expressing visceral afferents may be involved in checking inflammation in response to dietary lipids, as mice lacking these neurons showed massive cytokine induction, acute phase response, splenomegaly, and cachexia upon high fat feeding, eventually leading to severe weight loss requiring euthanasia. Preliminary findings suggest this inflammatory response can be

rescued by treatment with choline, which acts at the α7-nicotinic receptor and the final step in the vagal efferent-mediated cholinergic anti-inflammatory pathway. These results indicate that sensing of high fat feeding by $Na_v1.8$ neurons is required for activating a response that then checks the resultant inflammation and that a large component of this compensatory response is activation of the cholinergic anti-inflammatory pathway.

Some important considerations in interpreting these results include that while we demonstrate that the vagal anti-inflammatory reflex is involved, we do not exclude that other anti-inflammatory mechanisms are not involved in suppressing inflammation upon visceral afferent signaling. Though we demonstrated that plasma levels of catecholamines and glucocorticoids were unchanged, it is still possible that the activity of these systems are changed; for example, sympathetic signaling may only be decreased in certain tissues and thus not reflected in plasma epinephrine and norepinephrine levels or sensitivity to glucocorticoids is decreased while overall levels are not. Indeed considering the immense level of inflammation seen upon $Na_v1.8$ neuronal loss in a HFD paradigm, I would speculate that multiple anti-inflammatory pathways are normally involved and thus multiple arms of defense are lost in our ablated animals.

A second consideration is that although we typically attribute metabolically relevant nerve activity to vagal afferents, we cannot exclude

contributions from signaling through spinal sensory nerves as the tissues of the viscera show dual innervation from vagal and spinal sources (Gautron, Sakata et al. 2011). Also, as data shown earlier in this dissertation demonstrates, spinal visceral afferents express many of the same chemoreceptors as vagal afferents and and $Na_v1.8$ expressing spinal afferents are ablated in our model as well. One possibility is that vagal and spinal afferent functions overlap, and our severe phenotype results from the loss of both, functionally redundant neuronal populations.

Taken together, these results suggest a model, summarized in Figure 5.1, in which certain attributes of high fat diet, possibly fats, are sensed by $Na_v1.8$ expressing visceral afferents; their signaling then acts to activate compensatory mechanisms to suppress inflammation and maintain energy balance. In the absence of these neurons, the efferent pathway, likely the cholinergic anti-inflammatory pathway, is not activated, and inflammation is unchecked.

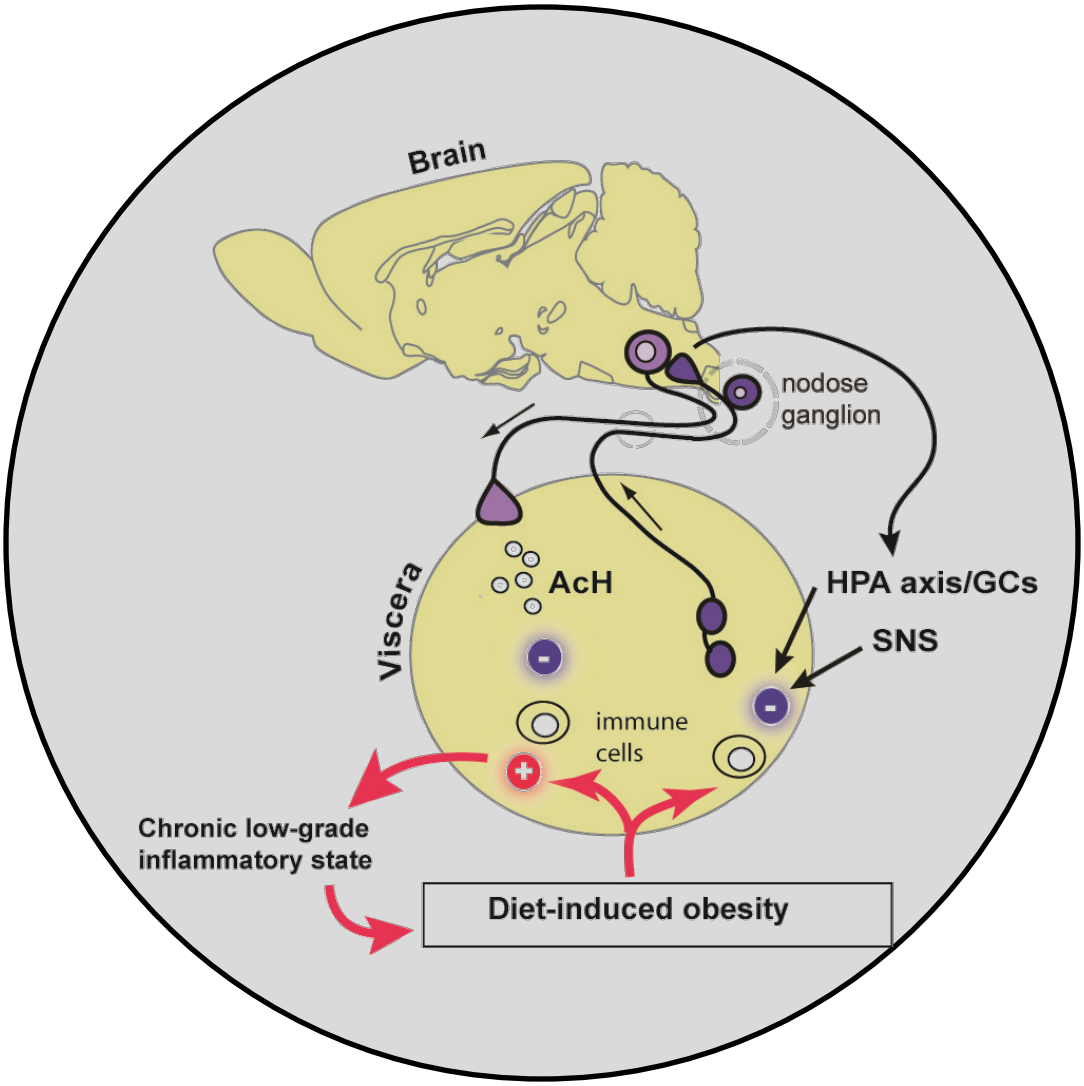

Figure 5.1.1: Proposed model of action of $Na_v1.8$ Neurons.

5.2 Perspectives

This work is most interesting, of course, in the context of obesity. Obesity is a state associated with chronic inflammation. Most critically, this inflammation has been established as a causative factor in the various comorbidities linked to obesity, including diabetes. While the inflammatory response caused by obesity resembles the classic inflammatory response in many ways, including induction of acute phase proteins, increase of levels of circulating cytokines, and activation and recruitment of leukocytes to tissues, obesity-associated inflammation also differs from classic paradigms in several ways. This obesity-associated inflammation is chronic and low grade in nature, with superimposed acute episodes of diet-induced inflammation (high-fat meals). Obesity associated inflammation also primarily involves low-grade activation of the innate immune system. In addition, obesity-related inflammation is unique in its multi-organ involvement.

As detailed earlier in this thesis, the phenotype of animals with ablation of $Na_v1.8$ neurons on high-fat diet resembles an exaggerated version of obesity-associated inflammation. Several key features are present, such as induction of acute phase response and cytokines, increase in circulating leukocytes, preferential activation of the innate immune system, and induction of a pro-thrombotic state. This would suggest that these neurons play an important role in normally suppressing the inflammatory response to nutritional triggers, thus

resulting in an exaggerated response in their absence. As activity of vagal afferents has been shown to be decreased in obesity (Daly, Park et al. 2011), dysfunction in this group of neurons could contribute to the diet induced inflammation associated with obesity.

Furthermore, it has been proposed that functional changes in gut-brain communication could be implicated in the beneficial actions (and perhaps side effects) of bariatric surgery (Berthoud, Shin et al. 2011). If true, the current results may help explain the aversion for fatty foods that commonly occur after Roux-en-Y gastric bypass (Novais, Junior et al. 2011). More broadly, $Na_v1.8$-expressing neurons appear to be an important component of the regulation of immunity. In fact, the control of immune functions by sensory neurons plays a primordial role in survival, and evolutionary ancient animals such as worms possess specialized sensory neurons able to respond to inflammatory stimuli and bacterial endotoxins, and regulate innate immunity (Pradel, Zhang et al. 2007; Sun, Singh et al. 2011). Lastly, this work also suggests that this neuronal population might play a role in gut immune tolerance. Immune response in the gut achieves a delicate balance, allowing precise targeting of immune response to harmful pathogens while tolerating benign foreign antigens. It is tempting to extrapolate for this work to suggest that action of $Na_v1.8$ might be important in maintaining tolerance to the wide array of antigens that act as nutrients.

Lastly, breaking the links between obesity and its associated comorbidities will require therapies that can target diet-induced inflammation. Modulating the activity of autonomic nerves by pharmacological or device-assisted (i.e. stimulator) means holds promise as a therapeutic approach for uncontrolled inflammatory states (Andersson and Tracey 2011), and this study specifically suggests that targeting homeostatic afferents could alleviate obesity-associated inflammation.

5.3 Open Questions

What are the relative contributions of vagal versus spinal $Na_v1.8$+ populations?

One major limitation of these studies is that they are unable to distinguish between the role of spinal $Na_v1.8$ and vagal $Na_v1.8$ populations. $Na_v1.8$-expressing neurons comprise about 70% of DRG neurons (Djouhri, Fang et al. 2003; Stirling, Forlani et al. 2005; Fukuoka, Kobayashi et al. 2008) and about 75-82% of vagal afferent neurons (Stirling, Forlani et al. 2005; Gautron, Sakata et al. 2011), so significant proportions of both neuronal groups are targeted. While spinal sensory neurons have been traditionally thought of as nociceptors in contrast to the metabolically relevant function of vagal afferents, much of the tissues of the viscera show dual innervation from both populations.

Is there developmental compensation for the role of these neurons? Alternatively, are some of the defects we observe developmental in origin, as a result of lack of feedback from $Na_v1.8$ neurons on other systems?

In our ablation mouse model, $Na_v1.8$-expressing cells are ablated irreversibly in late embryogenesis, as Cre expression begins around E15 – 18(Stirling, Forlani et al. 2005). As work with other metabolically important neuronal populations has demonstrated, compensatory mechanisms can develop after the ablation of certain neurons in neonates but does not occur in adults. For example, ablation of NPY/AgRP neurons has minimal effects on feeding in neonates, but rapidly causes starvation in adults(Luquet, Perez et al. 2005). Thus, before concluding that $Na_v1.8$ neurons do not play a major role in regulation of food intake, it is important to test the effect of post-developmental ablation in adults.

An alternate but related consideration is that proper neural development often requires appropriate input from sensory systems. As a result of lack of $Na_v1.8$ neuronal input in development, there may be defects in various related autonomic efferent pathways, and some of our phenotype may be attributed to these developmental defects as opposed to the continuing role of $Na_v1.8$ neurons in adulthood. Thus, again, it is important to test the role of $Na_v1.8$ neurons solely after development is complete.

To conduct this study, we would need a method to allow for ablation of this specific neuronal population in adulthood. I previously attempted this through use of the iDTR (inducible Diphtheria Toxin Receptor) mouse crossed with our $Na_v1.8$-Cre mice. In the iDTR mouse, DTR (Diphtheria toxin receptor) is

expressed upon Cre-mediated excising of a stop codon. Because mouse cells are naturally resistant to diphtheria toxin, injection of diphtheria toxin then allows for cell ablation in a Cre-dependent manner at the time of your choosing. However, although I could verify expression of DTR in neurons of the NG and DRG, I was unable to ablate $Na_v1.8$ neurons in this mouse model, even at the highest doses of toxin reported in the literature.

Thus, a method I would like to suggest is through the use of an inducible $Na_v1.8$ERCre. Through use of a tamoxifen inducible Cre system, Cre expression can be induced upon tamoxifen injection. Preliminary results obtained by Chen Liu in the Elmquist Lab suggest that this approach is working and that the administration of tamoxifen effectively induced Cre activity in Nav1.8 neurons in adulthood. When crossed with the DTA mouse, this will allow for ablation of the $Na_v1.8$-expressing cell population after development. Comparing the results of the studies outlined here in this model versus the old model would delineate the relative contributions of developmental defects versus the adult role of this neuronal population, as well as reveal any compensatory mechanisms that were developed.

What is the trigger for the massive inflammation seen in our ablated mice?

In our mice lacking $Na_v1.8$-expressing neurons, we see uncontrolled inflammation on high fat diet but not on normal chow. While the increased fat in the HFD is the likely culprit, the nutritional content of HFD is too disparate from

regular chow to allow for conclusive determination of the trigger. Further studies with finer modifications in diet are necessary to pinpoint a particular dietary component that is responsible for the massive inflammation seen in the ablated mice.

The major pro-inflammatory ingredients of western style diets are thought to be saturated fats and cholesterol, and the HFD used in these studies was 42% fat, mostly saturated, and 0.2% cholesterol. I would like to do a study in which the animals are switched to an otherwise identical HFD, but in which the fat source is from unsaturated fats. Comparison of the phenotype on these two diets could answer whether saturated fats are the culprit. Similar studies could be conducted for other nutritional components.

Another method of addressing this issue would be through adding particular components to the chow diet, as opposed to subtracting components from the HFD. For example, animals could be maintained on chow diet but also given gavages of lard oil (i.e. saturated fat) daily and inflammatory parameters would be measured after two weeks of treatment. This methodology would allow for attribution of our observed effects to one particular dietary component instead of a nonspecific change.

It's also possible that the difference between chow and high fat diet inducing the changes seen is not an intrinisic component of the diets, but an extrinsic change that results from a different diet. For example, a high fat diet may

induce changes in gut flora or the barrier function of the intestine that could result in inflammatory changes. Such a possibility is supported by the fact that the phenotype is not observed in all animals and the severity is variable. Also, we observed a similar phenotype in ablated animals in the absence of high fat feeding when other inflammatory stimuli was present. Thus, if studies of the nutritional contents are not revealing, we will have to consider these other factors.

What is the putative lipid sensor in $Na_v1.8$ neurons?

One of the most intriguing implications of this study is that Nav1.8 neurons are capable of sensing fats and thus can activate mechanisms to suppress inflammation. However, this raises the question of what serves as the lipid sensor in these neurons. CCK release could contribute to the sensing mechanisms of high-fat diet. However, CCK knockout mice do not show intolerance to HFD. Thus, there must another mechanism which remains to be determined. Several candidates are nuclear receptors. Nuclear receptors (NRs) are ligand-activated transcription factors that act as molecular sensors of lipophilic hormones and dietary-derived lipids (Chawla, Repa et al. 2001) and alter target gene expression in response to changing cellular lipid levels. Nuclear receptors have been demonstrated to be crucial in maintaining normal energy balance, as gene knockouts of several nuclear receptors, such as the peroxisome-proliferator activated receptors (PPARs), have been shown to affect glucose, lipid, and energy homeostasis (Chawla, Repa et al. 2001). Most importantly, data from several

recent studies suggest that the peripheral nervous system may contribute to the effects of NRs on metabolism. In one study, adenovirus-mediated overexpression of PPARg in the liver could enhance whole-body insulin sensitivity and decrease adiposity in rats, but this effect was lost in animals with transection of the hepatic branch of the vagus nerve (Uno, Katagiri et al. 2006). Similarly, dexamethasone-induced insulin resistance mediated by PPARa could be abrogated by hepatic vagotomy and by capsaicin-induced deafferentation (Bernal-Mizrachi, Xiaozhong et al. 2007). Finally, the anorectic effects of upper intestinal administration of oleoylethanolamide, a putative ligand for PPARa, are abrogated in vagotomized rats (Fu, Gaetani et al. 2003). Previous work for our lab has demonstrated that homeostatic afferent neurons of the NG and DRG express NRs such as PPARα and γ which are typically low or absent in the CNS, but abundant in metabolic tissues (Bookout, Jeong et al. 2006; Gofflot, Chartoire et al. 2007).

Another candidate for mediating lipid sensing in $Na_v1.8$ neurons is Toll-like receptor 4 (TLR4), a receptor for lipopolysacharides (LPS) from Gram-negative bacteria (Poltorak, He et al. 1998) that is also believed to be involved in saturated fatty acid-mediated induction of inflammation. Intriguingly, TLR4 is abundantly expressed in neurons of the NG and DRG, but not CNS other neurons. In mice with an inactivating mutation of TLR4, saturated fatty acid mediated induction of inflammatory signaling is lost in adipocytes, and the mice do not show decreased insulin sensitivity on high fat diet despite obesity (Shi, Kokoeva

et al. 2006). Despite this, saturated fatty acids are not believed to be a direct ligand of TLR4 (Glass and Olefsky 2012); thus any mechanism involving TLR4 will involve many other players as well.

Conclusive testing of these candidates will require knockout of these genes specifically in sensory afferents. Fortunately, we have a specific way to target our cellular population of interest with the $Na_v1.8$-Cre, and mice with floxed alleles of PPARα, PPARγ, and TLR4 exist.

Andersson, U. and K. J. Tracey (2011). "Reflex Principles of Immunological Homeostasis." Annu Rev Immunol.

Bernal-Mizrachi, C., L. Xiaozhong, et al. (2007). "An afferent vagal nerve pathway links hepatic PPARalpha activation to glucocorticoid-induced insulin resistance and hypertension." Cell Metab **5**(2): 91-102.

Berthoud, H. R., A. C. Shin, et al. (2011). "Obesity surgery and gut-brain communication." Physiol Behav **105**(1): 106-119.

Bookout, A. L., Y. Jeong, et al. (2006). "Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network." Cell **126**(4): 789-799.

Chawla, A., J. J. Repa, et al. (2001). "Nuclear receptors and lipid physiology: opening the X-files." Science **294**(5548): 1866-1870.

Daly, D. M., S. J. Park, et al. (2011). "Impaired intestinal afferent nerve satiety signalling and vagal afferent excitability in diet induced obesity in the mouse." J Physiol **589**(Pt 11): 2857-2870.

Djouhri, L., X. Fang, et al. (2003). "The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons." J Physiol **550**(Pt 3): 739-752.

Fu, J., S. Gaetani, et al. (2003). "Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha." Nature **425**(6953): 90-93.

Fukuoka, T., K. Kobayashi, et al. (2008). "Comparative study of the distribution of the alpha-subunits of voltage-gated sodium channels in normal and axotomized rat dorsal root ganglion neurons." J Comp Neurol **510**(2): 188-206.

Gautron, L., I. Sakata, et al. (2011). "Genetic tracing of Nav1.8-expressing vagal afferents in the mouse." J Comp Neurol **519**(15): 3085-3101.

Gofflot, F., N. Chartoire, et al. (2007). "Systematic gene expression mapping clusters nuclear receptors according to their function in the brain." Cell **131**(2): 405-418.

Luquet, S., F. A. Perez, et al. (2005). "NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates." Science **310**(5748): 683-685.

Nguyen, M. T., S. Favelyukis, et al. (2007). "A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways." J Biol Chem **282**(48): 35279-35292.

Novais, P. F., I. R. Junior, et al. (2011). "Food aversions in women during the 2 years after Roux-en-Y gastric bypass." Obes Surg **21**(12): 1921-1927.

Poltorak, A., X. He, et al. (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science **282**(5396): 2085-2088.

Pradel, E., Y. Zhang, et al. (2007). "Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by Caenorhabditis elegans." Proc Natl Acad Sci U S A **104**(7): 2295-2300.

Shi, H., M. V. Kokoeva, et al. (2006). "TLR4 links innate immunity and fatty acid-induced insulin resistance." J Clin Invest **116**(11): 3015-3025.

Stirling, L. C., G. Forlani, et al. (2005). "Nociceptor-specific gene deletion using heterozygous NaV1.8-Cre recombinase mice." Pain **113**(1-2): 27-36.

Sun, J., V. Singh, et al. (2011). "Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes." Science **332**(6030): 729-732.

Uno, K., H. Katagiri, et al. (2006). "Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity." Science **312**(5780): 1656-1659.

Chapter 6: Materials and Methods

6.1 Mice and Breeding Strategy

$Na_v1.8$ knock-in Cre mice on a pure C57Bl/6J background were obtained from Dr. John Wood (University College London) (Stirling, Forlani et al. 2005). Importantly, $Na_v1.8$-Cre mice show no Cre activity outside of the peripheral nervous system during development or adulthood (Stirling, Forlani et al. 2005; Gautron, Sakata et al. 2011). Furthermore, we also verified by biochemical and histological means that high-fat exposure did not induce ectopic Cre activity in lymphoid tissues (not shown). Heterozygous $Na_v1.8$-Cre mice were crossed with homozygous ROSA26-eGFP-DTA mice (stock # 006331; The Jackson Laboratory, USA) to generate a 50/50 percent mixture of littermate controls and ablated mice. Mice were housed in a light-controlled (12 hours on/12 hours off; lights on at 6 A.M.) and temperature-controlled environment (21.5–22.5°C). The animals and procedures used were approved by University of Texas Southwestern Medical Center at Dallas Institutional Animal Care and Use Committee.

Genotyping analysis

The genomic DNA was isolated from the tail. The DTA wild-type fragment and floxed fragment were detected by PCR with the following primers:

a) 5’- AAAGTCGCTCTGAGTTGTTAT-3’ (common forward)

b) 5’-GGAGCGGGAGAAATGGATATG-3’ (reverse - WT)

c) 5’- GCGAAGAGTTTGTCCTCAACC-3 (reverse - DTA)

The $Na_v1.8$-Cre fragment or wild-type band was detected by PCR with the following primers:

d) 5'- TGT AGA TGG ACT GCA GAG GAT GGA -3' (common forward)

e) 5' - TTA CCC GGT GTG TGC TGT AGA AAG (reverse - WT)

f) 5 - AAA TGT TGC TGG ATA GTT TTT ACT GCC -3' (reverse - Cre)

6.2 Diet and Study Design

Feeding Studies on Chow and High Fat Diet

Male mice (cohorts were started at 4 – 5 weeks old or at 7 – 8 weeks old) were singly housed and fed chow or high-fat diet, providing 12% or 42% energy percent as triglycerides (2016 or TD.88137, Harlan Teklad), respectively. The high-fat diet was irradiated and vacuum-sealed into one time use packets to minimize pathogen contamination of diet. Food intake and body weight were measured weekly for up to 12 weeks. Mice showing signs of illness were euthanized by chloral hydrate overdose when greater than 20% body weight was lost, after which tissues were collected and directly frozen in liquid nitrogen for RT-PCR or drop-fixed in 10% formalin for histology. Terminally ill animals, along with littermate controls, were transferred to the Molecular Pathology Core at University of Texas Southwestern Medical Center for formal autopsy by a licensed animal pathologist. Body composition was determined using quantitative magnetic resonance (QMR) (Bruker's Minispec MQ10, Houston, TX).

Cytokine Profiling on High Fat Diet

Group housed male mice were maintained on chow or high-fat diet for 7 days and 14 days. After the end of the requisite time period, mice were anesthetized with an i.p. injection of chloral hydrate (350 mg/kg). Blood was collected by cardiac puncture into EDTA tubes. Mice were killed by decapitation, after which tissues were collected and directly frozen in liquid nitrogen or prepared for histology as previously described.

Choline Treatment Studies

Group housed male mice (9 – 12 weeks old) were started on high-fat diet at lights out on Day 0. At lights on the following day (Day 1), mice received an i.p. injection of 67.02 mg/kg choline chloride (Sigma Aldrich) or an equivalent volume of saline. The dose and route of administration were chosen based on previous literature (Parrish, Rosas-Ballina et al. 2008). Injections were repeated at lights on daily for 14 days. On Day 14, mice were anesthetized with an i.p. injection of chloral hydrate (350 mg/kg). Blood was collected by cardiac puncture into EDTA tubes. Mice were killed by decapitation, after which tissues were collected and directly frozen in liquid nitrogen or prepared for histology as previously described.

Temperature Measurement

Group housed adult males were subcutaneously implanted into the back interscapular region with IPTT-300 transponders (BioMedic Data Systems Inc.). After allowing a week for recovery, mice were started on high-fat diet and temperature was recorded through scanning with the corresponding BMDS Smart Probe. All temperatures were recorded at 12 A.M. for a period of two weeks.

CCK Refeeding Studies

Singly housed mice were fasted overnight. 3 hours after lights on, mice were injected with 10 ug/kg CCK-8 (American Peptide Company) or an equivalent volume of saline, and a pre-weighed chow pellet was placed in their cages. Food intake was measured at 90 minutes post-injection. After a week, each animal received the opposite treatment (CCK-8 or saline) from the previous time. Food intake post-CCK administration was normalized to food intake post-saline administration.

6.3 Metabolic Analyses

Metabolic Cage Analysis

Meal pattern, physical activity, and energy expenditure were monitored using a combined indirect calorimetry system (TSE Systems GmbH, Bad Homburg, Germany) in young chow-fed or HFD-fed mice. Mice were housed individually at room temperature (22°C) under an alternating 12 hr light/12 hr dark cycle. After adaptation for 5 days, physical activity was determined using a multidimensional infrared light beam system. Ambulatory movement was defined

as breaks of any two different light beams at cage bottom level. Simultaneously, O_2 consumption, CO_2 production, and heat production were measured and normalized by metabolic body size (body weight$^{0.75}$) to determine the energy expenditure. In addition, meal patterns were determined continuously by integration of weighing sensors associated with the food containers. Meals were defined as food intake events with a minimum duration of 60 seconds and a break of 300 seconds between food intake events.

For analysis of acute responses to HFD, animals were acclimated for five days followed by 2 days of measurement in metabolic cages. On day three, diet was switched to 42% energy percent as triglycerides (TD.88137, Harlan Teklad) 3 hours before lights off, and measurement was resumed for three more days.

Fecal Lipid Absorption

All mice were individually housed for performing stool collections. Stool from each animal was then collected for 7 days. Fecal neutral sterol excretion was measured as previously described (Schwarz, Russell et al. 1998).

Triglyceride Tolerance Test

Four hours prior to the triglyceride tolerance test, mice were singly housed and had food removed. Prior to gavage, blood was collected via tail bleed into EDTA tubes. At time 0, mice were gavaged with 10 µl/g lard oil or 20% Intralipid (Baxter). Blood was then collected at 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, and 6 hours and kept on ice. The samples were then centrifuged at

3000 rcf for 5 minutes, and levels of plasma triglycerides and non-esterfied fatty acids were measured as per manufacturer's instructions using the Infinity Triglycerides kit (Fisher) and the HR Series NEFA-HR(2) kit (Wako Diagnostics) respectively.

6.4 Real-time PCR Analysis of mRNA

Total RNA was isolated from NG and DRG with the Ambion® RiboPure Kit (Invitrogen) and from all other issues with RNA Stat-60 (Tel Test, Inc.) in accordance with the manufacturer's instructions. The DNA in the samples was removed with RNase-Free DNase (Invitrogen). For NG and DRG samples, complementary cDNA was generated from 8 ng of RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and cDNA was then pre-amplified using the TaqMan® PreAmp Master Mix (Applied Biosystems) and the primers listed below. For all other tissues, complementary cDNA was generated from 2 μg of RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR was then performed with SYBR® Green PCR Master Mix (Applied Biosystems) for the genes listed below. The following primer pairs were used:

SAA-1

5'AGATCTGCCCAGGAGACACC3'

5'AGTGTAGGCTCGCCACATGTC3'

Trpv2

5'AAGCTTCTGCAGGAGAAATGG3'

5'CCAAGTAACAGGCGAAGTTGA3'

Trpv4

5'AGCCGCACATCGTCAACTA3'

5'CGCCTCATGTCAGCTTTCTT3'

For the genes listed below, real-time quantitative PCR was then performed with pre-developed Taqman assays were used [5′-FAM; Applied Biosystems] according to published protocols (Bookout, Jeong et al. 2006):

Agtr1a: Mm00616371_m1

Cart (Pomc): Mm00435874_m1

Cb1r (Cnr1): Mm01212171_s1

Cckar: Mm00438060_m1

Cckbr: Mm00432329_m1

Cgrp: Mm00801463_g1

Fga: Mm00802584_m1

Glp1r: Mm00445292_m1

Htr3a: Mm00442974_m1

Kcnk2: Mm01323942_m1

Kcnk3: Mm00807036_m1

Kcnk4: Mm00434626_m1

Kcnk5: Mm00498900_m1

Kcnk6: Mm01176312_g1

Mbp: Mm01266402_m1

$Na_v1.1$ (Scn1a): Mm00450580_m1

$Na_v1.6$ (Scn8a): Mm00488110_m1

$Na_v1.7$ (Scn9a): Mm00450762_s1

$Na_v1.8$ (Scn10a): Mm00501467_m1

$Na_v1.9$ (Scn11a): Mm00449377_m1

P2rx2: Mm00462952_m1

P2rx3: Mm00523699_m1

Phox2b: Mm00435872_m1

Trpc1: Mm00441975_m1

Trpc3: Mm00444690_m1

Trpc6: Mm01176083_m1

Trpm8: Mm01299593_m1

Trpv1: Mm01246302_m1

Levels were normalized to levels of ribosomal RNA 18S. All assays were performed using a performed using an Applied Biosystems Prism 7900HT sequence detection system.

6.5 Hormone/Cytokine Measurement and Blood Counts

Cytokine Measurement

Cytokines were measured using the Luminex® Multiplex System in the University of Texas Southwestern Mouse Metabolic Phenotyping Core.

Hormone Measurements

For measurements of plasma catecholamines, glucocorticoids, and corticosterone, animals were started on high fat diet and blood was collected by decapitation 36 hours later 2 hours after lights on. The samples were then spun at 3000 rcf for 20 minutes at 4º C, and plasma was stored at -80º C until measurement assays were conducted. Epinephrine and norepinephrine were measured in the Vanderbilt University Mouse Metabolic Phenotyping Center Hormone Assay Core (http://hormone.mc.vanderbilt.edu/). Plasma levels of corticosterone were measured using the Corticosterone 3H RIA Kit (MP Biomedicals), and plasma levels of ACTH were measured using the ACTH Double Antibody - ^{125}I RIA Kit (MP Biomedicals) as per manufacturer's instructions.

Complete Blood Counts

Whole blood was collected via submandibular bleed, and CBC panels were conducted by ARC Diagnostic Lab at University of Texas Southwestern Medical Center.

6.6 In Situ Hybridization, Histology, and Immunohistochemistry

In Situ Hybridization

Mice were anesthetized using an i.p. injection of chloral hydrate (350 mg/kg) and then perfused transcardially with DEPC-treated 0.9% saline followed by 10% neutral buffered formalin. NG and DRG were dissected, equilibrated with 20% sucrose solution, and cut at 16 μm into five series. In situ hybridization for $Na_v1.8$ mRNA was then performed as follows. The $Na_v1.8$ probe was designed from Genbank sequence NM_009134 to span nucleotide positions 5825-6229. cDNA generated with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) from total mouse brain RNA (Stratagene). The primers used to clone the probe were: 1) Nav1F TGACAGGGCCAACATTAACA and 2) Nav2R ACCACCAGAAATGTCCTTGC, and fragments were amplified with Taq DNA polymerase (Millipore) The PCR product was cloned with the TOPO TA Cloning® Kit for Sequencing (Invitrogen). Antisense and sense 35S labeled probes were generated with MAXIscript® In Vitro Transcription Kits (Ambion).

Sections were fixed in 4% formaldehyde in DEPC-treated PBS, pH 7.0 (20 minutes at 4°C), dehydrated in ethanol, cleared in xylene, and rehydrated in decreasing concentrations of ethanol. Sections were then incubated in sodium citrate buffer (95–100°C, pH 6.0) and microwaved for 10 minutes (70% power), dehydrated in graded ethanol and air-dried. The 35S-labeled cRNA probes were diluted to 106 cpm/ml in a hybridization solution containing 50% formamide, 10 mM Tris-HCl, pH 8.0, 5 mg tRNA (Invitrogen), 10 mM dithiothreitol (DTT), 10% dextran sulfate, 0.3 M NaCl, 1 mM EDTA, pH 8.0, and 1x Denhardt's

solution. Hybridization solution and a coverslip were applied to each slide, and sections were placed at 57°C for 12–16 hours. Afterwards, sections were washed with 2x SSC buffer and incubated in 0.002% RNase A (Roche Molecular Biochemicals) with 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA for 30 minutes, followed by a 30 minutes incubation in the same buffer without the RNase. The sections were subsequently incubated in 2x SSC, 0.25% DTT at 50°C for 1 hour, in 0.2x SSC, 0.25% DTT at 55°C for 1 hour, in 0.2x SSC, 0.25% DTT at 60°C for 1 hour. Slides were air-dried, placed in X-ray film cassettes with BMR-2 film (Kodak) for 3 days, and then dipped in NTB2 photographic emulsion (Kodak) at 4°C for 2 weeks. Finally, slides were developed with D-19 developer (Kodak), dehydrated in graded ethanols, cleared in xylenes, and coverslipped with Permaslip (Alban Scientific).

Histology

Mice were anesthetized using an i.p. injection of chloral hydrate (350 mg/kg) and then perfused transcardially with DEPC-treated 0.9% saline followed by 10% neutral buffered formalin. Tissues were then collected and stored in 50% ethanol. Hematoxylin and eosin staining was then performed in paraffin-embedded samples using standard protocols by UTSouthwestern Histology Core.

Immunohistochemistry

Mice were anesthetized using an i.p. injection of chloral hydrate (350 mg/kg) and then perfused transcardially with DEPC-treated 0.9% saline followed

by 10% neutral buffered formalin. Tissues were then collected and incubated in a 20% sucrose solution overnight at 4º. Samples were then embedded and frozen on dry ice in OCT solution (source).Immunohistochemistry was performed using antisera against CGRP (Bachem, T4032) or CART (Phoenix Pharmaceutical, H-003-62), followed by secondary AlexaFluor 594-conjugated anti-rabbit (Invitrogen; Cat# A21207).

6.7 **Statistical Analysis**

All values are presented as means ± s.e.m. Data were analyzed with two-way ANOVA followed by Bonferroni multiple comparisons, repeated measures ANOVA and Student's t test as indicated. Differences were considered significant at $P < 0.05$.

Abbott, C. R., M. Monteiro, et al. (2005). "The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway." Brain Res **1044**(1): 127-131.

Abrahamsen, B., J. Zhao, et al. (2008). "The cell and molecular basis of mechanical, cold, and inflammatory pain." Science **321**(5889): 702-705.

Adachi, A., N. Shimizu, et al. (1984). "Convergence of hepatoportal glucose-sensitive afferent signals to glucose-sensitive units within the nucleus of the solitary tract." Neurosci Lett **46**(2): 215-218.

Andersson, U. and K. J. Tracey (2011). "Reflex Principles of Immunological Homeostasis." Annu Rev Immunol.

Arnold, M., A. Mura, et al. (2006). "Gut vagal afferents are not necessary for the eating-stimulatory effect of intraperitoneally injected ghrelin in the rat." J Neurosci **26**(43): 11052-11060.

Batterham, R. L., M. A. Cowley, et al. (2002). "Gut hormone PYY(3-36) physiologically inhibits food intake." Nature **418**(6898): 650-654.

Bernal-Mizrachi, C., L. Xiaozhong, et al. (2007). "An afferent vagal nerve pathway links hepatic PPARalpha activation to glucocorticoid-induced insulin resistance and hypertension." Cell Metab **5**(2): 91-102.

Berthoud, H. R. and B. Jeanrenaud (1979). "Acute hyperinsulinemia and its reversal by vagotomy after lesions of the ventromedial hypothalamus in anesthetized rats." Endocrinology **105**(1): 146-151.

Berthoud, H. R., M. Kressel, et al. (1992). "An anterograde tracing study of the vagal innervation of rat liver, portal vein and biliary system." Anat Embryol (Berl) **186**(5): 431-442.

Berthoud, H. R., M. Kressel, et al. (1995). "Vagal sensors in the rat duodenal mucosa: distribution and structure as revealed by in vivo DiI-tracing." Anat Embryol (Berl) **191**(3): 203-212.

Berthoud, H. R. and W. L. Neuhuber (2000). "Functional and chemical anatomy of the afferent vagal system." Auton Neurosci **85**(1-3): 1-17.

Berthoud, H. R., L. M. Patterson, et al. (1997). "Distribution and structure of vagal afferent intraganglionic laminar endings (IGLEs) in the rat gastrointestinal tract." Anat Embryol (Berl) **195**(2): 183-191.

Berthoud, H. R., A. C. Shin, et al. (2011). "Obesity surgery and gut-brain communication." Physiol Behav **105**(1): 106-119.

Bi, S. and T. H. Moran (2002). "Actions of CCK in the controls of food intake and body weight: lessons from the CCK-A receptor deficient OLETF rat." Neuropeptides **36**(2-3): 171-181.

Bi, S., K. A. Scott, et al. (2004). "Differential roles for cholecystokinin a receptors in energy balance in rats and mice." Endocrinology **145**(8): 3873-3880.

Blackshaw, L. A. and D. Grundy (1990). "Effects of cholecystokinin (CCK-8) on two classes of gastroduodenal vagal afferent fibre." J Auton Nerv Syst **31**(3): 191-201.

Bookout, A. L., Y. Jeong, et al. (2006). "Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network." Cell **126**(4): 789-799.

Borovikova, L. V., S. Ivanova, et al. (2000). "Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation." Auton Neurosci **85**(1-3): 141-147.

Borovikova, L. V., S. Ivanova, et al. (2000). "Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin." Nature **405**(6785): 458-462.

Burdyga, G., S. Lal, et al. (2004). "Expression of cannabinoid CB1 receptors by vagal afferent neurons is inhibited by cholecystokinin." J Neurosci **24**(11): 2708-2715.

Butler, A. A. and L. P. Kozak (2010). "A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes." Diabetes **59**(2): 323-329.

Cano, G., A. F. Sved, et al. (2001). "Characterization of the central nervous system innervation of the rat spleen using viral transneuronal tracing." J Comp Neurol **439**(1): 1-18.

Castaneda, T. R., H. Jurgens, et al. (2005). "Obesity and the neuroendocrine control of energy homeostasis: the role of spontaneous locomotor activity." J Nutr **135**(5): 1314-1319.

Caterina, M. J., M. A. Schumacher, et al. (1997). "The capsaicin receptor: a heat-activated ion channel in the pain pathway." Nature **389**(6653): 816-824.

Chawla, A., J. J. Repa, et al. (2001). "Nuclear receptors and lipid physiology: opening the X-files." Science **294**(5548): 1866-1870.

Chen, H. Y., M. E. Trumbauer, et al. (2004). "Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein." Endocrinology **145**(6): 2607-2612.

Chikamori, K., K. Masuda, et al. (1977). "Effect of vagotomy on hyperinsulinemia in obese rats with hypothalamic lesions." Endocrinol Jpn **24**(3): 251-258.

Covasa, M. and R. C. Ritter (2001). "Attenuated satiation response to intestinal nutrients in rats that do not express CCK-A receptors." Peptides **22**(8): 1339-1348.

Czaja, K., G. A. Burns, et al. (2008). "Capsaicin-induced neuronal death and proliferation of the primary sensory neurons located in the nodose ganglia of adult rats." Neuroscience **154**(2): 621-630.

Daly, D. M., S. J. Park, et al. (2011). "Impaired intestinal afferent nerve satiety signalling and vagal afferent excitability in diet induced obesity in the mouse." J Physiol **589**(Pt 11): 2857-2870.

Das, S. K., S. Eder, et al. (2011). "Adipose triglyceride lipase contributes to cancer-associated cachexia." Science **333**(6039): 233-238.

Date, Y., N. Murakami, et al. (2002). "The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats." Gastroenterology **123**(4): 1120-1128.

Dhaka, A., V. Viswanath, et al. (2006). "Trp ion channels and temperature sensation." Annu Rev Neurosci **29**: 135-161.

Dib-Hajj, S. D., T. R. Cummins, et al. (2010). "Sodium channels in normal and pathological pain." Annu Rev Neurosci **33**: 325-347.

Djouhri, L., X. Fang, et al. (2003). "The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons." J Physiol **550**(Pt 3): 739-752.

Eng, R., R. M. Gold, et al. (1978). "Hypothalamic hypoactivity prevented but not reversed by subdiaphragmatic vagotomy." Physiol Behav **20**(5): 637-641.

Fleshner, M., L. E. Goehler, et al. (1998). "Thermogenic and corticosterone responses to intravenous cytokines (IL-1beta and TNF-alpha) are attenuated by subdiaphragmatic vagotomy." J Neuroimmunol **86**(2): 134-141.

Forman, B. M., P. Tontonoz, et al. (1995). "15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma." Cell **83**(5): 803-812.

Fox, E. A., R. J. Phillips, et al. (2001). "Neurotrophin-4 deficient mice have a loss of vagal intraganglionic mechanoreceptors from the small intestine and a disruption of short-term satiety." J Neurosci **21**(21): 8602-8615.

Fu, J., S. Gaetani, et al. (2003). "Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha." Nature **425**(6953): 90-93.

Fukuoka, T., K. Kobayashi, et al. (2008). "Comparative study of the distribution of the alpha-subunits of voltage-gated sodium channels in normal and axotomized rat dorsal root ganglion neurons." J Comp Neurol **510**(2): 188-206.

Gaskell, W. H. (1886). "On the Structure, Distribution and Function of the Nerves which innervate the Visceral and Vascular Systems." J Physiol **7**(1): 1-80 89.

Gautron, L., I. Sakata, et al. (2011). "Genetic tracing of Nav1.8-expressing vagal afferents in the mouse." J Comp Neurol **519**(15): 3085-3101.

Gaykema, R. P., L. E. Goehler, et al. (2000). "Subdiaphragmatic vagotomy blocks interleukin-1beta-induced fever but does not reduce IL-1beta levels in the circulation." Auton Neurosci **85**(1-3): 72-77.

Gaykema, R. P., L. E. Goehler, et al. (1998). "Bacterial endotoxin induces fos immunoreactivity in primary afferent neurons of the vagus nerve." Neuroimmunomodulation **5**(5): 234-240.

Glass, C. K. and J. M. Olefsky (2012). "Inflammation and lipid signaling in the etiology of insulin resistance." Cell Metab **15**(5): 635-645.

Goehler, L. E., R. P. Gaykema, et al. (1998). "Interleukin-1 induces c-Fos immunoreactivity in primary afferent neurons of the vagus nerve." Brain Res **804**(2): 306-310.

Goehler, L. E., R. P. Gaykema, et al. (2000). "Vagal immune-to-brain communication: a visceral chemosensory pathway." Auton Neurosci **85**(1-3): 49-59.

Goehler, L. E., J. K. Relton, et al. (1997). "Vagal paraganglia bind biotinylated interleukin-1 receptor antagonist: a possible mechanism for immune-to-brain communication." Brain Res Bull **43**(3): 357-364.

Gofflot, F., N. Chartoire, et al. (2007). "Systematic gene expression mapping clusters nuclear receptors according to their function in the brain." Cell **131**(2): 405-418.

Goldbach, J. M., J. Roth, et al. (1997). "Fever suppression by subdiaphragmatic vagotomy in guinea pigs depends on the route of pyrogen administration." Am J Physiol **272**(2 Pt 2): R675-681.

Grijalva, C. V. and E. Lindholm (1982). "The role of the autonomic nervous system in hypothalamic feeding syndromes." Appetite **3**(2): 111-124.

Hansen, M. K., K. A. O'Connor, et al. (2001). "The contribution of the vagus nerve in interleukin-1beta-induced fever is dependent on dose." Am J Physiol Regul Integr Comp Physiol **280**(4): R929-934.

Hillsley, K. and D. Grundy (1998). "Sensitivity to 5-hydroxytryptamine in different afferent subpopulations within mesenteric nerves supplying the rat jejunum." J Physiol **509 (Pt 3)**: 717-727.

Hillsley, K. and D. Grundy (1998). "Serotonin and cholecystokinin activate different populations of rat mesenteric vagal afferents." Neurosci Lett **255**(2): 63-66.

Hotamisligil, G. S. (2006). "Inflammation and metabolic disorders." Nature **444**(7121): 860-867.

Huston, J. M., M. Ochani, et al. (2006). "Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis." J Exp Med **203**(7): 1623-1628.

Inoue, S. and G. A. Bray (1977). "The effects of subdiaphragmatic vagotomy in rats with ventromedial hypothalamic obesity." Endocrinology **100**(1): 108-114.
Julius, D. and A. I. Basbaum (2001). "Molecular mechanisms of nociception." Nature **413**(6852): 203-210.
Kelly, L., S. Morales, et al. (2000). "Capsaicin-treated rats permanently overingest low- but not high-concentration sucrose solutions." Am J Physiol Regul Integr Comp Physiol **279**(5): R1805-1812.
King, B. M., R. G. Carpenter, et al. (1978). "Hyperphagia and obesity following ventromedial hypothalamic lesions in rats with subdiaphragmatic vagotomy." Physiol Behav **20**(5): 643-651.
Koda, S., Y. Date, et al. (2005). "The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats." Endocrinology **146**(5): 2369-2375.
Koren, M. S. and M. D. Holmes (2006). "Vagus nerve stimulation does not lead to significant changes in body weight in patients with epilepsy." Epilepsy Behav **8**(1): 246-249.
Kral, J. G. (1978). "Vagotomy for treatment of severe obesity." Lancet **1**(8059): 307-308.
Kraly, F. S., G. P. Smith, et al. (1978). "Abdominal vagotomy disrupts food-related drinking in the rat." J Comp Physiol Psychol **92**(2): 196-203.
Lehmann, J. M., L. B. Moore, et al. (1995). "An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma)." J Biol Chem **270**(22): 12953-12956.
Li, Y. L. and H. Zheng (2011). "Angiotensin II-NADPH oxidase-derived superoxide mediates diabetes-attenuated cell excitability of aortic baroreceptor neurons." Am J Physiol Cell Physiol **301**(6): C1368-1377.
Lichtenstein, L., F. Mattijssen, et al. (2010). "Angptl4 protects against severe proinflammatory effects of saturated fat by inhibiting fatty acid uptake into mesenteric lymph node macrophages." Cell Metab **12**(6): 580-592.
Louis-Sylvestre, J. (1983). "Validation of tests of completeness of vagotomy in rats." J Auton Nerv Syst **9**(1): 301-314.
Loup, P. W., B. Ghavami, et al. (1981). "Can vagal regeneration account for gastric motility after vagotomy?" Scand J Gastroenterol Suppl **67**: 13-17.
Lumeng, C. N. and A. R. Saltiel (2011). "Inflammatory links between obesity and metabolic disease." J Clin Invest **121**(6): 2111-2117.
Luquet, S., F. A. Perez, et al. (2005). "NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates." Science **310**(5748): 683-685.
Mathis, C., T. H. Moran, et al. (1998). "Load-sensitive rat gastric vagal afferents encode volume but not gastric nutrients." Am J Physiol **274**(2 Pt 2): R280-286.

Metz, W. and W. G. Forssmann (1980). "Innervation of the liver in guinea pig and rat." Anat Embryol (Berl) **160**(3): 239-252.
Moran, M. M., M. A. McAlexander, et al. (2011). "Transient receptor potential channels as therapeutic targets." Nat Rev Drug Discov **10**(8): 601-620.
Moran, T. H., E. E. Ladenheim, et al. (2001). "Within-meal gut feedback signaling." Int J Obes Relat Metab Disord **25 Suppl 5**: S39-41.
Nakabayashi, H., A. Niijima, et al. (1994). "A unique receptor-mediated mechanism in vagal chemoreception of somatostatin in the hepatoportal area." J Auton Nerv Syst **50**(1): 45-50.
Nance, D. M. and J. Burns (1989). "Innervation of the spleen in the rat: evidence for absence of afferent innervation." Brain Behav Immun **3**(4): 281-290.
Nance, D. M. and V. M. Sanders (2007). "Autonomic innervation and regulation of the immune system (1987-2007)." Brain Behav Immun **21**(6): 736-745.
Neuhuber, W. L. (1989). "Vagal afferent fibers almost exclusively innervate islets in the rat pancreas as demonstrated by anterograde tracing." J Auton Nerv Syst **29**(1): 13-18.
Niijima, A. (1984). "Reflex control of the autonomic nervous system activity from the glucose sensors in the liver in normal and midpontine-transected animals." J Auton Nerv Syst **10**(3-4): 279-285.
Niijima, A. (1996). "The afferent discharges from sensors for interleukin 1 beta in the hepatoportal system in the anesthetized rat." J Auton Nerv Syst **61**(3): 287-291.
Nishizawa, M., H. Nakabayashi, et al. (1996). "The hepatic vagal nerve is receptive to incretin hormone glucagon-like peptide-1, but not to glucose-dependent insulinotropic polypeptide, in the portal vein." J Auton Nerv Syst **61**(2): 149-154.
Novais, P. F., I. R. Junior, et al. (2011). "Food aversions in women during the 2 years after Roux-en-Y gastric bypass." Obes Surg **21**(12): 1921-1927.
Okazawa, M., W. Inoue, et al. (2004). "Noxious heat receptors present in cold-sensory cells in rats." Neurosci Lett **359**(1-2): 33-36.
Olefsky, J. M. and C. K. Glass (2010). "Macrophages, inflammation, and insulin resistance." Annu Rev Physiol **72**: 219-246.
Parrish, W. R., M. Rosas-Ballina, et al. (2008). "Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling." Mol Med **14**(9-10): 567-574.
Phillips, R. J., E. A. Baronowsky, et al. (1997). "Afferent innervation of gastrointestinal tract smooth muscle by the hepatic branch of the vagus." J Comp Neurol **384**(2): 248-270.
Phillips, R. J. and T. L. Powley (2000). "Tension and stretch receptors in gastrointestinal smooth muscle: re-evaluating vagal mechanoreceptor electrophysiology." Brain Res Brain Res Rev **34**(1-2): 1-26.

Poltorak, A., X. He, et al. (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science **282**(5396): 2085-2088.

Porter, M. H., B. J. Hrupka, et al. (1998). "Vagal and splanchnic afferents are not necessary for the anorexia produced by peripheral IL-1beta, LPS, and MDP." Am J Physiol **275**(2 Pt 2): R384-389.

Powley, T. L., & Opsahl, C. A. (1974). "Ventromedial hypothalamic obesity abolished by subdiaphragmatic vagotomy." American Journal of Physiology **174**(226): 25-33.

Powley, T. L., M. M. Chi, et al. (2005). "Gastrointestinal tract innervation of the mouse: afferent regeneration and meal patterning after vagotomy." Am J Physiol Regul Integr Comp Physiol **289**(2): R563-R574.

Pradel, E., Y. Zhang, et al. (2007). "Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by Caenorhabditis elegans." Proc Natl Acad Sci U S A **104**(7): 2295-2300.

Prechtl, J. C. and T. L. Powley (1990). "The fiber composition of the abdominal vagus of the rat." Anat Embryol (Berl) **181**(2): 101-115.

Reidelberger, R. D., J. Hernandez, et al. (2004). "Abdominal vagal mediation of the satiety effects of CCK in rats." Am J Physiol Regul Integr Comp Physiol **286**(6): R1005-1012.

Ridley, P. T. and F. P. Brooks (1965). "Alterations in Gastric Secretion Following Hypothalamic Lesions Producing Hyperphagia." Am J Physiol **209**: 319-323.

Ritter, S. and T. T. Dinh (1988). "Capsaicin-induced neuronal degeneration: silver impregnation of cell bodies, axons, and terminals in the central nervous system of the adult rat." J Comp Neurol **271**(1): 79-90.

Romanovsky, A. A., V. A. Kulchitsky, et al. (1997). "Cold defense mechanisms in vagotomized rats." Am J Physiol **273**(2 Pt 2): R784-789.

Romanovsky, A. A., V. A. Kulchitsky, et al. (1997). "Febrile responsiveness of vagotomized rats is suppressed even in the absence of malnutrition." Am J Physiol **273**(2 Pt 2): R777-783.

Romanovsky, A. A., C. T. Simons, et al. (1997). "The vagus nerve in the thermoregulatory response to systemic inflammation." Am J Physiol **273**(1 Pt 2): R407-413.

Rosas-Ballina, M., M. Ochani, et al. (2008). "Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia." Proc Natl Acad Sci U S A **105**(31): 11008-11013.

Rosas-Ballina, M., P. S. Olofsson, et al. (2011). "Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit." Science **334**(6052): 98-101.

Rush, A. M., S. D. Dib-Hajj, et al. (2006). "A single sodium channel mutation produces hyper- or hypoexcitability in different types of neurons." Proc Natl Acad Sci U S A **103**(21): 8245-8250.

Schwartz, G. J., P. R. McHugh, et al. (1993). "Gastric loads and cholecystokinin synergistically stimulate rat gastric vagal afferents." Am J Physiol **265**(4 Pt 2): R872-876.

Schwartz, G. J. and T. H. Moran (1996). "Sub-diaphragmatic vagal afferent integration of meal-related gastrointestinal signals." Neurosci Biobehav Rev **20**(1): 47-56.

Schwartz, G. J., C. R. Plata-Salaman, et al. (1997). "Subdiaphragmatic vagal deafferentation fails to block feeding-suppressive effects of LPS and IL-1 beta in rats." Am J Physiol **273**(3 Pt 2): R1193-1198.

Schwartz, G. J., C. F. Salorio, et al. (1999). "Gut vagal afferent lesions increase meal size but do not block gastric preload-induced feeding suppression." Am J Physiol **276**(6 Pt 2): R1623-1629.

Schwarz, M., D. W. Russell, et al. (1998). "Marked reduction in bile acid synthesis in cholesterol 7alpha-hydroxylase-deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolemia." J Lipid Res **39**(9): 1833-1843.

Sehic, E. and C. M. Blatteis (1996). "Blockade of lipopolysaccharide-induced fever by subdiaphragmatic vagotomy in guinea pigs." Brain Res **726**(1-2): 160-166.

Shi, H., M. V. Kokoeva, et al. (2006). "TLR4 links innate immunity and fatty acid-induced insulin resistance." J Clin Invest **116**(11): 3015-3025.

Skandalakis, L. J., S. W. Gray, et al. (1986). "The history and surgical anatomy of the vagus nerve." Surg Gynecol Obstet **162**(1): 75-85.

Smith, G. P., C. Jerome, et al. (1981). "Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat." Science **213**(4511): 1036-1037.

Stearns, A. T., A. Balakrishnan, et al. (2012). "Relative contributions of afferent vagal fibers to resistance to diet-induced obesity." Dig Dis Sci **57**(5): 1281-1290.

Stirling, L. C., G. Forlani, et al. (2005). "Nociceptor-specific gene deletion using heterozygous NaV1.8-Cre recombinase mice." Pain **113**(1-2): 27-36.

Sun, J., V. Singh, et al. (2011). "Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes." Science **332**(6030): 729-732.

Surprenant, A. and R. A. North (2009). "Signaling at purinergic P2X receptors." Annu Rev Physiol **71**: 333-359.

Szekely, M., M. Balasko, et al. (1997). "Peripheral neural inputs. Their role in fever development." Ann N Y Acad Sci **813**: 427-434.

Talley, E. M., G. Solorzano, et al. (2001). "Cns distribution of members of the two-pore-domain (KCNK) potassium channel family." J Neurosci **21**(19): 7491-7505.
Unger, R. H. and P. E. Scherer (2010). "Gluttony, sloth and the metabolic syndrome: a roadmap to lipotoxicity." Trends Endocrinol Metab **21**(6): 345-352.
Uno, K., H. Katagiri, et al. (2006). "Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity." Science **312**(5780): 1656-1659.
Walls, E. K., F. B. Wang, et al. (1995). "Selective vagal rhizotomies: a new dorsal surgical approach used for intestinal deafferentations." Am J Physiol **269**(5 Pt 2): R1279-1288.
Wampler, R. S. and C. T. Snowdon (1979). "Development of VMH obesity in vagotomized rats." Physiol Behav **22**(1): 85-93.
Wang, H., M. Yu, et al. (2003). "Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation." Nature **421**(6921): 384-388.
Wang, P. Y., L. Caspi, et al. (2008). "Upper intestinal lipids trigger a gut-brain-liver axis to regulate glucose production." Nature **452**(7190): 1012-1016.
Wang, X., Z. Yang, et al. (2011). "Activation of the cholinergic antiinflammatory pathway ameliorates obesity-induced inflammation and insulin resistance." Endocrinology **152**(3): 836-846.
Wesolowski, H. and S. Snop (1975). "Histochemical studies on the albino rat pancreas in different periods following vagotomy and simultaneous pyloromyotomy." Folia Histochem Cytochem (Krakow) **13**(1-2): 85-92.
Wood, J. N., J. P. Boorman, et al. (2004). "Voltage-gated sodium channels and pain pathways." J Neurobiol **61**(1): 55-71.
Yamamoto, H., T. Kishi, et al. (2003). "Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites." J Neurosci **23**(7): 2939-2946.