Activation of the central nervous system
by circulating Glucagon-Like Peptide-1

A dissertation submitted to the
Graduate School of the University of Cincinnati
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy (Ph.D.)

In the Graduate Program in Pathobiology and Molecular Medicine
of the College of Medicine

2009

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ABSTRACT

The aim of this dissertation research was to investigate how Glucagon-Like Peptide-1 activates the central nervous system to illicit changes in blood glucose. Understanding how the body regulates blood glucose levels is important for not only understanding the disease diabetes, but also in developing treatments for the disease. Though numerous studies have looked the actions and mechanisms of GLP-1 action, there still is not a concise and agreed upon mechanism of action. Our hypothesis is that it acts in a neural manner by activating the central nervous system to stimulate efferent pathways to regulate glucose homeostasis.

Two likely places for this signaling to take place are the portal vein and the small intestine. By using retrograde tracing and immunohistochemistry techniques, we were able to identify sensory afferent nerves that were capable of accessing circulation that expressed the GLP-1r. After a visiting student demonstrated that when GLP-1 was infused into the portal vein no resulting increase in insulin was observed, we decided to investigate the GLP-1/GLP-1r signaling system in the small intestine. We were able to identify a population of enterocytes that express the GLP-1r in the duodenum, jejunum and ileum. We attempted to identify which enterocytes were expressing the GLP-1r, but were only able to rule out expression on L-cells, D-cells, and enterochromaffin cells. Additionally, we were able to identify GLP-1r on synaptophysin positive cells, in both the enterocytes and myenteric plexi. Further work is required to identify this unique population of enterocytes, and whether or not GLP-1r are present on vagal nerve elements in the small intestine.

In conclusion, we identified that sensory neurons in the portal vein can access
circulation, that there is a population of enterocytes in the small intestine that express the GLP-1r and that the GLP-1r is can also be found on some neural elements in the small intestine. These findings are important for the understanding of GLP-1’s multiple effects and sites of action to elicit changes in glucose homeostasis, particularly concerning pharmacological manipulation of the GLP-1 system for treatment of diabetes. Further work will continue to expand on our knowledge of this important global glucose homeostasis hormone.
ACKNOWLEDGEMENTS

First I have to thank my mentor, Dr. David D’Alessio for accepting me into his laboratory and taking me on as his first graduate student. Dave was always optimistic about projects and results and never failed to leave me excited and motivated after a meeting. I appreciated the way he tackled problems and thought about things in a bigger picture. I would like to thank him for his unyielding support over the last 4 years.

Secondly, I would like to thank Dr. James Herman for taking on the role as my co-mentor when my research took a turn “to the dark side” into neuroscience. It has been a real pleasure to know both him and his wife Susan. I thank them both for their guidance, suggestions, feedback, and some good times on the microscope.

I would not have been as successful if it wasn’t for my committee: Dr. David D’Alessio, Dr. David Askew, Dr. James Herman, Dr. Randy Seeley, and Dr. Steve Woods. All of you made my committee meetings a great interaction and discussion without it being too formal or intimidating (after the qualifier, of course, that was very formal and very intimidating). Thank you all for being a part of my committee.

I would like to thank the members of the Woods/Seeley lab for making me feel welcome and a part of the lab. I appreciate the friendship, conversation, research discussions, and feedback from the team room and from lab meetings. P.S. Thanks very much Randy for giving me a desk in your team room and allowing me to stay there even when your lab grew so large some people had to move to another building 😊

I would like to thank the members of the Herman lab, particularly Kenneth Jones, for all the help they have given me over the years and for the friends that I have made there. It has been a pleasure to work with you.

I also would like to thank the Pathobiology and Molecular Medicine Program for allowing me to transfer here on short notice. I never regretted leaving Wayne State University for UC and once I was here, the faculty, Regina, and Heather made it feel like home. I am also very grateful to the graduate committee for giving me a second chance to prove myself and remain in the program. Thanks for having faith in me that I would be successful.

Finally, and last but certainly not least, I want to thank my family. Mom & Gary, Dad & Laurie, Grandma & Grandpa E, Grandma & Grandpa O, Ryan, Dennis & Nijole, and Paul have always been supportive during my 10 year “professional student career” and have always been there when needed for anything. I love you all.
DEDICATION

It is with great love and appreciation that I dedicate this Ph.D. dissertation to my husband, Paul. Paul has been an inspiration to me from the day I met him, overcoming considerable odds and working hard to create success here in the US. He has stayed true to our wedding vows - encouraging me in times of doubt, comforting me in times of sorrow, and has been my refuge of strength in times of uncertainty. I can never express how much Paul means to me and I don’t think I would have made it through the last five years without him. Ačiū mano meilė; this is for you
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<td>Glucagon-Like Peptide-1</td>
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<td>NTS</td>
<td>Nucleus of the Solitary Tract</td>
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<td>PC(2 and 3)</td>
<td>Prohormone Convertase</td>
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<td>DPP-IV</td>
<td>Dipeptidyl Peptidase IV</td>
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<td>GIP</td>
<td>Glucose-dependent insulinotropic Polypeptide</td>
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<td>CCK</td>
<td>Cholecystokinin</td>
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<td>PVN</td>
<td>Paraventricular Nucleus</td>
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<td>DMH</td>
<td>Dorsal Motor Nucleus of the Hypothalamus</td>
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<td>VMH</td>
<td>Ventral Medial Hypothalamus</td>
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<tr>
<td>5HT</td>
<td>5-hydroxytryptamine, also known as serotonin</td>
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<td>ENS</td>
<td>Enteric Nervous System</td>
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<td>CTB</td>
<td>Cholera Toxin subunit Beta</td>
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<td>SON</td>
<td>Supraoptic Nucleus</td>
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<tr>
<td>BCP</td>
<td>1-bromo-3-chloropropane</td>
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<tr>
<td>Q-PCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>LCM</td>
<td>Laser Capture Microdissection</td>
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<tr>
<td>WG-HRP</td>
<td>Wheat Germ Agglutinin-Horseradish Peroxidase</td>
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<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
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CHAPTER 1

Overall introduction
1.1. Introduction

Humans have three priorities when it comes to fuel utilization: 1) maintain a steady supply of glucose and oxygen to the brain, 2) maintain protein reserves and 3) replenish liver and muscle glycogen reserves following a meal. The major organs that are involved in maintaining fuel homeostasis are the liver and kidney (producers of glucose), the brain (a key regulator of metabolism with a high dependence on glucose as its energy source), and the muscle and adipose tissue (major sites of storage). Plasma concentrations of glucose are maintained in a narrow region, mainly because low levels of glucose impair brain function and high levels of glucose exceed the renal glucose reabsorption threshold which leads to wasted energy. The three mechanisms the body uses to maintain normal blood glucose levels following a carbohydrate-rich meal are: 1) suppression of hepatic glucose production, 2) stimulation of hepatic glucose uptake and 3) stimulation of glucose uptake by peripheral tissues (predominantly muscle). Insulin is the primary signal that directs these processes. (Schwartz et al., 2003).

Under fed and fasting conditions, insulin is the key hormone that regulates the distribution of fuel between tissues. When insulin signaling is disrupted, glucose homeostasis is severely affected resulting in high levels of blood glucose and the disease diabetes. Both of the major types of diabetes, Type 1 and Type 2, involve abnormal insulin secretion. In Type 1 diabetes, the insulin secreting β-cells of the pancreas are destroyed in an autoimmune attack and patients require insulin injections to control their blood glucose levels. In Type 2 diabetes, subnormal insulin secretion is
coupled with insulin resistance, resulting in defective responsiveness of the major tissues in glucose homeostasis to the actions of insulin and glucose intolerance ensues. Important defects demonstrated in patients with Type 2 diabetes, besides impaired insulin secretion, are increased rates of hepatic glucose release and insufficient peripheral tissue glucose use. All of these factors lead to higher blood glucose levels. This causes not only inefficient energy usage, but also detrimental effects on vascular tissues causing the classic diabetic complications of retinal, kidney, neural and heart disease. (Schwartz et al., 2003).

Insulin is not the only endocrine player in the glucose homeostasis game. When a meal is ingested, the gastrointestinal tract senses that nutrients are coming in and produces peptides that signal to the pancreas and the brain to initiate and amplify insulin secretion. One of those peptides is Glucagon-Like Peptide-1 (GLP-1), a hormone secreted from the intestine that is necessary for normal glucose tolerance.

1.2. Overview of GLP-1

Glucagon-like Peptide 1 (GLP-1) is a product of the proglucagon gene, which consists of six exons and five introns (Bell et al., 1983). Proglucagon is expressed in the L-cells of the intestine, the alpha cells of the pancreas, and a subset of neuronal cells in the nucleus of the solitary tract (NTS) in the brainstem. The proglucagon gene product is preproglucagon, a prohormone that has tissue specific post-translational processing to generate tissue specific products by different prohormone convertases
Figure 1.1 – Differential splicing and gene products of the proglucagon gene.

For example, in the pancreatic α-cells, PC2 cleaves proglucagon into glucagon (Rouille et al., 1994 and 1995). By contrast, intestinal L-cells and hindbrain neurons use PC3 to produce GLP-1 from proglucagon (Rouille et al., 1995 and 1997). Initially,
GLP-1 is formed as a 36-amino acid protein, termed GLP-1[1-36]. Then it is cleaved by an intracellular enzyme that takes off the first 6 amino acids resulting in the active form of GLP-1 termed GLP-1[7-36] which is then secreted from the L-cell. GLP-1 is released by the L-cells following a meal rich in carbohydrates and lipids (Orskov et al., 1994; Herrmann et al., 1995; Kieffer, 1999). In the circulation, GLP-1[7-36] is rapidly metabolized by the protease dipeptidyl peptidase IV (DPP-IV), and once it has undergone cleavage by DPP-IV, the protein is no longer active. DPP-IV is present both in the endothelial tissue of capillaries and in a circulating form and works rapidly to convert GLP-1[7-36] to GLP-1[9-36] (Kieffer et al., 1999) with a half life of 60-90 seconds. In fact, it has been estimated that half of the GLP-1 released is inactivated immediately following release from the intestine (Hansen et al., 1999). GLP-1 works by binding to a single highly specific receptor that is distributed on various tissues throughout the body, most notably on the beta cells of the pancreas, but also the islet α-cells, the heart, the stomach, and the kidney.

GLP-1 contributes to what is known as the incretin effect, and is thus classified as an incretin. Incretins are “factors secreted by the intestine in response to an oral load of glucose or lipid that increase glucose-stimulated insulin secretion” (Burcelin, 2005). The incretin effect helps to match the load of calories coming in from the gut with the amount of insulin secretion that is needed to store these nutrients. Importantly, the incretin effect is abolished in people who have Type 2 diabetes. Even more interesting is that incretin analogues and drugs that block the metabolism of GLP-1 by blocking DPP-IV can be used as a therapy for the disease. Currently, metabolism-resistant GLP-1 receptor agonists (Ilitz et al., 2006; Kieffer et al., 1999; Drucker, 2006)
GLP-1 is not only responsible for the incretin effect, but it is also involved in numerous other processes. GLP-1 has been found to inhibit gastric emptying (Flint et al., 2001; Vilsbøll et al., 2004), induce satiation and suppress food intake in both rats and humans (Flint et al., 1998; Naslund et al., 1999; Rodrique de Fonseca et al., 2000; Edwards et al., 2001; Flint et al., 2001; Larsen et al., 2001; Vilsbøll et al., 2004), reduce glucagon secretion and hepatic glucose production (Drucker, 2006), and even stimulate cardiac output (Drucker, 2006). All of these actions of GLP-1 tend to reduce the rise in blood glucose suggesting that is it more than just an incretin, but a general glucose homeostasis hormone.

1.3. GLP-1 Mechanisms of Action

Classically, it has been believed that GLP-1 acts through a typical endocrine pathway (Figure 1.2): GLP-1 is secreted from the L cells of the intestine, goes into circulation, binds directly to the GLP-1 receptor, a seven transmembrane G-protein coupled receptor, on the beta cells of the pancreas. When GLP-1 binds to its receptor, cyclic AMP is increased, activating protein kinase A (PKA) releasing intracellular calcium stores (Holz et al., 1995) and promoting the exocytosis of insulin granules (Ammala et al., 1993; Gromada et al., 1998).
Figure 1.2 – Classical endocrine mechanism of GLP-1 action.
However, there are some inconsistencies with the classical mechanism. First, the circulating levels of GLP-1 are relatively low and postprandially, the levels of GLP-1 increase only about two-fold over basal, compared to postprandial levels of the other major incretin, Glucose-dependent Insulinotropic Polypeptide (GIP), which increase six-fold (Visboll et al., 2003). Secondly, the rapid cleavage of GLP-1 in the circulation raises some doubt as to whether sufficient amounts of hormone even reach the target tissues. It has been estimated that 90% of GLP-1 secreted by the intestinal L-cells is metabolized by DPP-IV by the time it leaves the hepatic portal circulation to the general circulation (Hansen et al., 1999). Additionally, the emerging data about neural effects of GLP-1 (Knauf et al., 2005; Vahl et al., 2007) raise the possibility that alternative mechanisms are responsible for GLP-1 action.

In light of the remarkable circumstances required for endocrine actions of GLP-1, a new mechanism based on a neural-endocrine pathway has been proposed (Figure 1.3). In 1996 the group of Nakabayashi et al executed a study where they showed that intraportal GLP-1 increases the firing rate in the afferent branches of the hepatic vagus nerve (Nakabayashi et al., 1996). In 2000 Balkan et al demonstrated that intraportal GLP-1 augments insulin release in response to intraportal glucose and that this response could be inhibited by a ganglionic blockade (Balkan et al., 2000). Both of these studies, and others, suggest that vagal nerves are involved in the sensing of glucose in the portal bed. This led to the development of the following hypothesized mechanism of postprandial GLP-1 action: GLP-1 secreted from the intestine acts primarily on GLP-1r expressed by neurons in the wall of the portal vein. Upon binding to its receptor, a signal is sent up the axonal vagal trunks to visceral afferent neurons.
with cell bodies in the nodose ganglion. These neurons project to synapses in the hindbrain, in the Nucleus of the Solitary Tract (NTS), where these visceral afferent signals can initiate appropriate responses, such as beta cell stimulation and insulin release.
There is precedent for the portal GLP-1 pathway we propose in the action of other gut hormones. For example, the gastric hormone ghrelin has been proposed to mediate its actions similarly (Date et al., 2002). Additionally, another system, the renin-
angiotensin-aldosterone system (RAAS), works in a similar manner. Renin is a proteolytic enzyme released into circulation whose release is stimulated by sympathetic nerve activation acting via $\beta_1$-adrenoceptors. Renin stimulates the formation of angiotensin in the blood which in turn stimulates the release of aldosterone from the adrenal cortex. This system plays an important role in regulating blood volume, cardiac output and arterial pressure by integrating systemic and central signaling, similar to the role we are proposing for GLP-1 and metabolism.

Further support for our hypothesis comes from looking at analogous systems of gut hormones acting via peripheral nerves to initiate their function. An analogous system to the model we propose is that of cholecystokinin (CCK). CCK is the major mediator of pancreatic exocrine secretion, but the mechanism by which it does this under physiological conditions was unclear. Li and Owyang demonstrated that at physiological conditions, CCK stimulates pancreatic enzyme secretion through an afferent vagal pathway that is sensitive to capsaicin (Li et al., 1993). Once secreted from I-cells in the small intestine, CCK binds to CCK-receptors on vagal afferent nerves and communicates to the dorsal vagal complexes (DVC) for effector functions on the pancreas via the vagal efferents (Konturek et al., 2003).

This is very similar to what we are proposing for the action of GLP-1 in the portal vein, and also suggests a second alternative site of action of GLP-1, the small intestine. If CCK binds to receptors on vagal afferent nerves to mediate its effects, and it has been shown that the GLP-1r is also present on vagal afferent nerves, perhaps GLP-1 can bind those receptors in the small intestine to mediate its insulinotropic effects.
Figure 1.4 – A second alternative site of action for the neuroendocrine model of GLP-1; GLP-1 secreted from intestinal L-cells binds to GLP-1 receptors on vagal afferent nerves in the gut and signals via visceral afferents to the brain effector functions to the pancreas via visceral efferents.
1.4. GLP-1 and the Brain

Signaling throughout the body is accomplished with the central and peripheral nervous system. The brain is the heart of the central nervous system and cell bodies located there send out projections called axons throughout the body. There are several brain regions that have particular importance in metabolism. The hypothalamus is responsible for the regulation of food intake, water intake, body temperature, heartbeat, and control of secretory activity in the pituitary gland. Recent studies also suggest the nerves in the hypothalamus can regulate hepatic glucose production (Obici et al., 2002). A second key area is the hindbrain which is the first relay center for information coming in from visceral organs. (Van de Graff, 2002). The hindbrain has important roles in the regulation of food intake and the function of the abdominal organs.

The brain receives information via afferent neurons and stimulates effects via efferent nerves. The transport of information to and from the brain often involves long axon projections that travel a great distance from the cell nucleus. This requires cells in the brain to transport important proteins like signaling molecules and receptors to the sites of action. This occurs via two different mechanisms, axoplasmic flow and axonal transport. In axoplasmic flow, rhythmic waves of contractions push cytoplasmic contents from the origin of the axon to the nerve fiber endings. Axonal transport requires ATP and the motor proteins kinesin or dynein to carry cell products along microtubules present on the nerve fibers. Axonal transport is much faster than axoplasmic flow. Axonal transport can either be from the cell body to the nerve terminal, called anterograde transport, or from the nerve terminal to the cell body, called
retrograde transport. (Jung et al., 2004; Oztas, 2003). Retrograde transport explains how small molecules can be taken from the site of the nerve innervations to the cell body.

GLP-1 is a product of a discrete set of neurons in the nucleus of the solitary tract (NTS), a key region of the hindbrain for receiving visceral signals. GLP-1 containing neurons project widely to many brain areas, including the paraventricular nucleus (PVN), periventricular hypothalamus, dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and the arcuate nucleus (Drucker, 1990, Larsen et al., 1997; Sarkar et al., 2003). These GLP-1 neurons are involved in a number of functions such as food intake, stress and illness. (Larson et al., 2004, Seeley et al., 2000). The GLP-1r is also expressed in the above key regions of the brain (Shughrue et al., 1996; Merchenthaler et al., 1999; Tang-Christensen et al., 2001) suggesting that GLP-1 is also acting as a neurotransmitter, as well as expressed on vagal afferent nerve terminals in the periphery (Vahl et al., 2007).

1.5. Vagal Innervation of the Viscera

The vagal afferent nerves transmit sensory information from the periphery to the brain. Vagal nerves have their cell bodies in the nodose and jugular ganglia (Berthoud et al., 2004) and send projections to the heart, lungs, stomach, pancreas, and gastrointestinal tract (Teff, 2008). From the ganglia, they project to the nucleus of the solitary tract (NTS) to relay peripheral sensory information.
The neurons of the nodose ganglia produce various receptors that are actively transported down the long axons to nerve synapses present in the peripheral organs. Many of these receptors are important in metabolism, such as the GLP-1 receptor, the CCK-1a receptor, the ghrelin receptor, and 5HT3 receptors (Kakei et al., 2002; Broberger et al., 1999; Burdyga et al., 2006; Morales et al., 2002). Even though the vagal afferent nerve endings cannot sense luminal nutrients directly, they are present in close position to the basal membranes of enteroendocrine cells (Berthoud et al., 1996) to transmit messages from the enteroendocrine cells to the brain.

1.6. The Enteric Nervous System

The enteric nervous system (ENS) is specific to the gastrointestinal tract, extending from the esophagus to the anus and includes innervations of the associated organs, such as the pancreas, liver, gall bladder, and salivary glands. The ENS functions to regulate gastrointestinal motility, absorption, gastric emptying, secretions, blood flow, and immune responses (Powley et al., 2002). The ENS consists of various ganglia, fibers, and effector cells to carry out its regulatory processes. Of the approximate 100 million neurons (in the human ENS), there are four types of functional neurons: sensory neurons, interneurons, muscle motor neurons, and secretomotor neurons, found in an approximate ratio of 2:1:1:1(Weidmann et al., 2007; Hansen, 2003a and b). The sensory neurons are both extrinsic and intrinsic. The extrinsic sensory neurons are the vagal and spinal afferents with their cell bodies located outside of the wall of the gut. The intrinsic sensory neurons have their cell bodies located within
the wall of the gut. The extrinsic and intrinsic neurons work together and communicate with each other and the brain to survey the gut (Hansen, 2003a and b).

The ENS has three primary ganglionated plexi: the myenteric plexus, the submucosal plexus and the mucous plexus. The myenteric plexus is found between the longitudinal and circular muscle layers, the submucosal plexus is found between the muscularis mucosa and the circular muscle layer, and the mucous plexus is found in the mucous layer (Hansen, 2003a and b) (Figure 1.5). The myenteric plexus also contains the intraganglionic laminar vagal afferent endings (IGLEs) which respond to muscle tension and are found in great numbers throughout the GI tract (Berthoud, 2008).
Another important component of the ENS are enteroendocrine cells (EEC). EEC’s are positioned in the gut in such a way as to sense the luminal contents and release their contents accordingly to activate or inhibit other EEC’s or to activate the appropriate afferent nerve endings located in the lamina propria. EEC’s are known to be innervated, but how they are controlled neurally is still unknown. (Sjolund et al., 1983; Furnes, 2000). Some types of EEC’s include D-cells that secrete somatostatin, I-cells that secrete CCK, K-cells that secrete GIP, L-cells that secrete GLP-1, G-cells that secrete gastrin, and Enterochromaffin cells which contain, among others, 5-HT, a major [22]
1.7. Summary

It is well documented that neurons in the Central Nervous System (CNS) contribute to the regulation of key metabolic processes, such as satiety, insulin and glucagon secretion, and hepatic glucose metabolism (Visboll et al., 2004; Prigeon et al., 2003; Knauf et al., 2005; Demuro et al., 2006). But it is not known how the CNS receives information about circulating nutrient levels, such as glucose, nor how it interacts with the endocrine system. The integration of neural sensors into a working physiologic model remains unclear and this is a critical issue for understanding brain regulation of metabolism. It is essential that an understanding of how the body senses and regulates circulating nutrient and hormone levels is attained to be able to diagnose and treat metabolic disease earlier, and prevent illnesses associated with such diseases.

One of the main players in the regulation of metabolism is GLP-1. Our lab focuses its research on GLP-1, not only because of its interesting characteristics, but also because of its recent discovery of improving glucose tolerance in people with diabetes. GLP-1 not only works at the level of the pancreas, but at the level of the brain to regulate glucose homeostasis. It is still unknown how the central nervous system integrates incoming signals from the periphery and communicates with other major organ systems based on the input signals, but studying the GLP-1 system could shed light on this undiscovered territory and lead to a better understanding of how the brain communicates with target organs to maintain metabolic homeostasis. Though [23]
numerous studies have looked at the actions and mechanisms of GLP-1 action, there
still is not a concise and agreed upon mechanism of action. Our hypothesis is that it
acts in a neural manner by activating the central nervous system to stimulate efferent
pathways to regulate glucose homeostasis.
CHAPTER 2

Sensory nerves in the portal vein expressing the Glucagon-Like Peptide-1 receptor can access circulation

2.1. INTRODUCTION

Glucagon-like Peptide 1 (GLP-1) is a product of the proglucagon gene, which consists of six exons and five introns (Bell et al., 1983). Proglucagon is expressed in the L-cells of the intestine, the alpha cells of the pancreas, and a subset of neuronal cells in the nucleus of the solitary tract (NTS) in the brainstem. The proglucagon gene product is preproglucagon, a prohormone that has tissue specific post-translational processing to generate tissue specific products by different prohormone convertases (PC). For example, in the pancreatic α-cells, PC2 cleaves proglucagon into glucagon (Rouille et al., 1994 and 1995). By contrast, intestinal L-cells and hindbrain neurons use PC3 to produce GLP-1 from proglucagon (Rouille et al., 1995 and 1997). Initially, GLP-1 is formed as a 36 amino acid protein, termed GLP-1[1-36]. Then it is cleaved by an intracellular enzyme that takes off the first 6 amino acids resulting in the active form of GLP-1 termed GLP-1[7-36] which is then secreted from the L-cell. GLP-1 is released by the L-cells following a meal rich in carbohydrates and lipids (Orskov et al., 1994; Herrmann et al., 1995; Kieffer, 1999). In the circulation, GLP-1[7-36] is rapidly metabolized by the protease dipeptidyl peptidase IV (DPP-IV), and once it has undergone cleavage by DPP-IV, the protein is no longer active. DPP-IV is present both in the endothelial tissue of capillaries and in a circulating form and works rapidly to convert GLP-1[7-36] to GLP-1[9-36] (Kieffer et al., 1999) with a half life of 60-90 seconds. In fact, it has been estimated that half of the GLP-1 released is inactivated immediately following release from the intestine (Hansen et al., 1999). GLP-1 works by binding to a single highly specific receptor that is distributed on various tissues throughout the body, most notably on the beta cells of the pancreas, but also the islet α-
Classically, it has been believed that GLP-1 acts through a typical endocrine pathway: GLP-1 is secreted from the L cells of the intestine, goes into circulation, binds directly to the GLP-1 receptor, a seven transmembrane G-protein coupled receptor, on the beta cells of the pancreas. When GLP-1 binds to its receptor, cyclic AMP is increased, activating protein kinase A (PKA) releasing intracellular calcium stores (Holz et al., 1995) and promoting the exocytosis of insulin granules (Ammala et al., 1993; Gromada et al., 1998).

However, there are some inconsistencies with the classical mechanism. First, the circulating levels of GLP-1 are relatively low and postprandially, the levels of GLP-1 increase only about two-fold over basal, compared to postprandial levels of the other major incretin, GIP which increase six-fold. Secondly, the rapid cleavage of GLP-1 in the circulation raises some doubt as to whether sufficient amounts of hormone even reach the target tissues. It has been estimated that 90% of GLP-1 secreted by the intestinal L-cells is metabolized by DPP-IV by the time it leaves the hepatic portal circulation to the general circulation (Hansen et al., 1999). Additionally, the emerging data about neural effects of GLP-1 (Knauf et al., 2005; Vahl et al., 2007) raise the possibility that alternative mechanisms are responsible for GLP-1 action.

In light of the remarkable circumstances required for endocrine actions of GLP-1, a new mechanism based on a neural-endocrine pathway has been proposed. In 1996 it was demonstrated that intraportal GLP-1 increases the firing rate in the afferent branches of the hepatic vagus nerve (Nakabayashi et al., 1996). A few years later,
another group found that intraportal GLP-1 augments insulin release in response to intraportal glucose and that this response could be inhibited by a ganglionic blockade (Balkan et al., 2000). Both of these studies, and others, suggest that the vagal nerves are involved in sensing of glucose in the portal bed. This led to the development of the following hypothesized mechanism of postprandial GLP-1 action: GLP-1 secreted from the intestine acts primarily on GLP-1r expressed by neurons in the wall of the portal vein. Upon binding to its receptor, a signal is sent up the axonal vagal trunks to visceral afferent neurons with cell bodies in the nodose ganglion. These neurons project to synapses in the hindbrain, in the Nucleus of the Solitary Tract (NTS), where these visceral afferent signals can initiate appropriate responses, such as beta cell stimulation and insulin release.

The portal vein is proposed to be a site of sensors that detect changes in the concentration of circulating glucose and hormones in order to initiate neural responses. However, the mechanisms by which neurons sense circulating substrates and hormone levels is not clear. We hypothesize that circulating GLP-1 can bind to nerve receptors in the portal vein, and initiate signals that are carried in vagal afferent nerves (Figure 2.1).
Figure 2.1: Hypothesized mechanism of postprandial GLP-1 action – GLP-1 is secreted from the intestine, binds to receptors in the portal vein, resulting in the regulation of neurally mediated insulin secretion.
2.2. MATERIALS AND METHODS

Animals

All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Male Long Evans rats from Harlan, 250-350g, were housed in individual cages at 23°C on a 12 hour light/dark cycle.

Retrograde tracers

Dextran Amines conjugated to Texas Red (Invitrogen, California); 10mg/mL in sterile water.

Application of Retrograde Tracer

Rats were anesthetized with isofluorane and an abdominal laparotomy performed to expose the visceral organs. A 0.020”x0.37” Silicone catheter 28cm in length was inserted into the hepatic portal vein and secured with suture. The 10mg of tracer was dissolved into 1mL of sterile water, loaded into a syringe and delivered via an infusion pump at a constant rate for one hour. Upon completion of infusion, the catheter was closed, the animal was sutured, stapled and allowed to recover for a one or seven day period. 0.3mL of Buprenex was administered subcutaneously for pain management to all animals.

A second group of animals received the portal vein catheter and additionally received a subdiaphragmatic hepatic vagotomy, severing the vagal afferent nerve connection from the portal vein to the nodose ganglion. For this procedure, the afferent nerve trunk was isolated below the diaphragm and two 4.0 silk sutures tied off two
centimeters apart. The nerve was severed between the sutures using electro-cautery. Then the infusion of retrograde tracer was performed as described above.

A third group of animals received a catheter inserted into the jugular vein (silicone tubing, 0.025"x0.47', 20 cm in length), using the same procedure as the portal vein catheter insertion. The infusion also proceeded as described above.

Tissue preparation for immunohistochemistry

Rats were euthanized by pentobarbital injection and perfused intracardially with 300 mL of normal saline followed by 300 mL of 4% buffered paraformaldehyde. Tissues were postfixed in 4% paraformaldehyde for 4 h and then placed in 30% sucrose in PBS at 4°C for at least 24 h. The portal vein was removed en bloc with the liver hilus and the mesentery attached and placed together in tissue boats, leaving the surrounding anatomy of the hepatoporal region intact, including liver and pancreatic tissue. Tissue Tek freezing medium was added and the tissue blocs were frozen and subsequently cut at 20 µm on a cryostat and then stored directly on slides at -20°C. The right and left nodose ganglia were dissected from the neck by removing the neck muscles and the occipital bone and following the vagal nerve running along the carotid artery to the nodose ganglion, a yellow-ish swelling of the nerve. They nodose ganglia were processed, and sectioned in the same manner.

Immunohistochemistry

To view the retrograde tracer, the frozen slides were dried at room temperature
and coverslipped with Gelvatol. Then the slides were viewed under the Cy3 filter on a standard fluorescence microscope (Zeiss) to detect fluorescence from the Texas Red molecule attached to the dextran amines.

To assess for the GLP-1r, standard immunohistochemical procedure was followed. Briefly: the frozen slides were allowed to dry at room temperature for one hour; washed in 50mM KPBS for 30 min; washed in 0.3% H2O2 for 15 min; washed in 50mM KPBS for 30 min; incubated in a humidity chamber with blocking serum for 30 min at RT; incubated in a humidity chamber with anti-GLP-1r Primary Antibody 1:500 (LS-A1206, MBL, Massachusetts) overnight at 4°C; washed in 50mM KPBS for 30 min; incubated in a humidity chamber with Goat Anti-Rabbit Alexa 488 1:200 (Invitrogen, California) for 60 min at RT; washed in 50mM KPBS for 30min; washed in Millipore H2O for 5 min; dried at RT (in dark); coverslipped with Gelvatol; viewed with FITC filter on a fluorescence microscope (Zeiss Imager.Z1 with Apotome).
2.3. RESULTS

To determine whether sensory nerves in the portal vein have access to small molecules in the circulation, similar in size to GLP-1 and other peptide hormones, the retrograde tracer dextran amine conjugated to the fluorescent molecule texas red was infused into the portal vein. The principle measure of nerve contact with the tracer was examination for its presence in the cell bodies of the sensory nerves located in the nodose ganglion. To control for region specific contact of the tracer an identical amount of the dextran amine was infused into the jugular vein of control animals.

When the retrograde tracer was infused into the portal vein, labeled neurons were detected in the nodose ganglion as soon as one day post infusion (Figure 2.2). In contrast, animals that had the tracer delivered into the jugular vein had no labeling of the nodose ganglion (Figure 2.2).
Figure 2.2: Representative photomicrograph or retrograde tracer labeling of nodose ganglion after infusion of tracer; left panel – nodose ganglion 1 day post portal infusion; right panel – nodose ganglion 7 days post jugular infusion; n= 4 per group; 20x.

There was little to no tracer observed in the nodose ganglion seven days post infusion in rats that had administration of the tracer into the portal vein after the hepatic vagotomy (Figure 2.3).
Figure 2.3: Tracer labeling in the nodose ganglion following portal infusion of tracer under denervated conditions (via hepatic vagotomy); n=4, 2 represented; 20x

To confirm that sensory neurons express the GLP-1r, we assessed the nodose ganglion for presence of the GLP-1r. Immunohistochemistry revealed that the GLP-1r is expressed on nodose ganglion neurons (Figure 2.4).
To see if the sensory nerves that access circulation (the nerves picking up the retrograde tracer) also express the GLP-1r, the nodose ganglion was assessed for the presence of retrograde tracer and the GLP-1r. A population of double labeled neurons is identified (Figures 2.5).
Figure 2.5: Double labeling of nodose neurons for the GLP-1r and portally infused retrograde tracer; left panels – retrograde tracer; middle panels – GLP-1r immunoreactivity; right panels – merged images; white arrows indicate neurons that double label; 20x
2.4. DISCUSSION

The portal vein is proposed to be a site of sensors that detect changes in the concentration of circulating glucose and hormones in order to initiate neural responses (Burcelin et al., 2001; Vahl et al., 2007). We have previously demonstrated that the GLP-1r is expressed in nodose neurons and portal neural fibers, and that blockade of this receptor in the portal circulation, specifically, causes glucose intolerance (Vahl, 2007). These findings suggest a model of GLP-1 action whereby peptide in the portal plasma, the highest concentrations in the circulation, initiate neural signals that eventuated in regulatory responses. However, it has never been demonstrated that neurons in the portal vein respond to circulating constituents, as would be necessary for our proposed model of GLP-1 signaling to work. In the studies reported here, we show that small molecules are capable of interacting with sensory neurons specifically in the hepatic portal circulation, that these are likely to be vagal afferent fibers, and that some of these neurons express the GLP-1r. These findings support the hypothesis that the portal vein is a site for detecting changes in plasma GLP-1 and that this could be a major site of GLP-1r mediation of metabolic effects (Saberi et al., 2008).

By using IHC, we were able to identify nodose ganglion neurons that produce the GLP-1r (Figure 2.4). Additionally, we were able to co-localize some of these neurons with uptake of tracer (Figure 2.5). By identifying neurons that both produce the GLP-1r and were able to pick up tracer from circulation, this finding supports the hypothesis that the nerves that can access circulation are ones that express the GLP-1r and thus could mediate GLP-1 signaling. Previously, our lab has demonstrated that the GLP-1r is
expressed on nerves in the portal vein (Vahl et al., 2007). Additionally, other groups have identified the importance of the portal vein sensing with GLP-1 and GP-1r, finding that the ability of the portal vein to be a glucose sensor requires an active GLP-1r (Brucelin et al., 2001). In combination, these results indicate that there is a subpopulation of hepatic vagal afferent nerves that innervate the portal vein and are accessible by constituents of the circulation and thus could mediate neural signaling by GLP-1 to produce insulinotropic effects.
CHAPTER 3

Assessment of methods for

retrograde labeling of vagal afferents
3.1. INTRODUCTION

Retrograde tracing is a popular method to label nerves in the periphery, such as the nerves supplying the duodenum, the bladder, the pancreas and the stomach (Kreier et al., 2006; Wild et al., 1991; Salin et al., 2008; Schmued et al., 1986; Coolen et al., 1999; Plato et al., 2005; Li et al., 2007; Raybould et al., 2002; Ambaavanar et al., 2003; Wan et al., 1982). We sought to use this method to label the vagal afferent nerves of the portal vein, which we have previously shown to express the GLP-1r (Vahl, et al., 2007). The commonly used method for application of tracer for retrograde labeling is that of injection. Since our goal was to label the portal vein, injection into this structure is not possible, due to risk of bleeding and risk of injecting the tracer into circulation. We chose to apply the tracer topically and developed a painting method to accomplish this. Our data raised important caveats during our attempts to specifically label the portal vein vagal afferents and this chapter will describe these and other issues we faced.
3.2. METHODS

Animals

All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Male Long Evans rats from Harlan, 450-650g, were housed in individual cages at 23°C on a 12 hour light/dark cycle.

Retrograde tracers used in this study

Dextran Amines conjugated to Texas Red, Alexa 680; cholera toxin subunit B (CTB) conjugated to Alexa 555 (Invitrogen, California); Fluorogold (Fluorochrome, LLC, Colorado).

Application of Retrograde Tracer

The tracers were applied via two methods, injection and topical application, which was called the painting method. For either method employed, the rat was anesthetized with isofluorane and an abdominal laparotomy performed to expose the visceral organs. Either the portal vein or the small intestine (duodenum) was isolated and surrounded with warm saline soaked gauze to prevent tracer leakage into the peritoneum. For the painting method, a fine detail paintbrush was used to apply the tracer solution to the surface of the organ, approximately a 1cm x 2cm area. For the injection method, an insulin syringe was used to inject 100 µL of tracer into the duodenum, 2.5cm distal to the pylorus. After application of the tracer was complete, the gauze was removed and in the case of the painted animals, GelFoam was placed over the site of painting to ensure no transfer of the tracer from the painted surface. The
animals were sutured, stapled and allowed to recover for the allotted time period. 0.3mL of Buprenex was administered subcutaneously for pain management. A number of doses and incubation periods was tested; see Table 3.1 for an outline.

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Application</th>
<th>Source</th>
<th>Dose</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorogold</td>
<td>Painted</td>
<td>Fluorochrome, LLC</td>
<td>5%</td>
<td>7 Days</td>
</tr>
<tr>
<td>CTB-555</td>
<td>Injection</td>
<td>Invitrogen</td>
<td>0.2%, 2%</td>
<td>1, 3, 7 Days</td>
</tr>
<tr>
<td>CTB-555</td>
<td>Painted</td>
<td>Invitrogen</td>
<td>0.2%, 1%</td>
<td>1, 3 Days</td>
</tr>
<tr>
<td>BDA-680</td>
<td>Painted</td>
<td>Invitrogen</td>
<td>10mg/mL</td>
<td>7 Days</td>
</tr>
</tbody>
</table>

Table 3.1 – List of tracers used in experiments and their conditions; n=4 per group, per dose, and per incubation time. Fluorogold and CTB-555 stock = 50 µg/mL and the doses were: 5% = 5µL/100 µL, 0.2% = 2 µL/1000 µL, 2% = 2 µL/100 µL, 1% = 1 µL/100 µL.

**Tissue preparation for immunohistochemistry**

Rats were euthanized by pentobarbital injection and perfused intracardially with 300 mL of normal saline followed by 300 mL of 4% buffered paraformaldehyde. Tissues were post-fixed in 4% paraformaldehyde for 4 h and then placed in 30% sucrose in PBS at 4°C for at least 24 h. Tissues removed included the nodose ganglia, trigeminal ganglia, small intestine, and brain. Freezing media was added and the tissue blocs were frozen and subsequently cut at 20 µm, 20 µm, 10 µm, and 16 µm, respectively, on
a cryostat and then stored directly on slides at -20°C.

Copper Sulfate Treatment

Lipofuscin autofluorescence can be a confounding factor in tracing studies using tracers with fluorescent detection methods. CuSO4 treatment was used to eliminate autofluorescence from lipofuscin (method described in Schnell et al., 1999). Briefly, slides were dried at RT for 1hr and washed in KPBS for 30 min. They were then dipped briefly in ddH20 and placed into the desired concentration of CuSO4 (in Ammonium Acetate + EDTA) for 10-90min. The slides were washed briefly in ddH20 and allowed to dry. Finally, the slides were mounted with Gelvatol or dehydrated through graded ethanols, cleared in xylene, and mounted with DPX.

Microscopy/Image Analysis

To view the retrograde tracer, the frozen slides were dried at room temperature and coverslipped with Gelvatol. Then the fluorogold slides were viewed under the DAPI filter, the CTB-555 slides were viewed under the Cy3 filter and the CTB-555 slides were also viewed under the Cy5 filter to look for non-specific fluorescence on a fluorescence microscope (Zeiss Imager.Z1 with Apotome).
3.3. RESULTS

The nodose ganglia (NG) contain the cell bodies of the vagal afferent nerves. When the retrograde tracer is applied topically (i.e., painted), the afferent nerve endings pick up the tracer and actively transport it back to their cell bodies where it can be detected. Figure 3.1 illustrates the presence of the retrograde tracer CTB-555 in the nodose ganglia after being painted on the portal vein.

Figure 3.1 – Nodose Ganglia from ctb-555 painted portal veins, 20x; Top panels = 1 day incubation, Bottom panels = 3 day incubation, Left panels = 0.2%, Right panels = 1%. White arrows indicate neurons positive for the ctb-555 tracer.
The negative control tissue for these experiments was the trigeminal ganglion (TG). This organ was selected as a negative control because it has some similarities with the nodose ganglion, but does include minimal numbers of neurons that access blood vessels and none that access the portal vein. Thus, neurons in the TG should not come in contact with tracer painted onto the portal vein. Figure 3.2 illustrates the trigeminal ganglia from the portal painted animals in Figure 3.1. Unexpectedly, they are positive.

Figure 3.2 – Trigeminal ganglia from ctb-555 painted portal veins, 20x; Top panels = 1 day incubation, Bottom panels = 3 day incubation, Left panels = 0.2%, Right panels = 1%. White arrows indicate neurons positive for the ctb-555 tracer.
To address the issue of the negative control being positive, we next looked at the dose of tracer administered, with the thought that the amount we are used was excessive causing an increased circulatory load that resulted in nonspecific neural uptake. Comparing the right and left panels of Figures 3.1 (NG) and 3.2 (TG), we find that a range of doses shows positive neuronal cells, in both the NG and TG.

Finally, we addressed the issue of incubation time with the thought that prolonged exposure to the tracer caused nonspecific uptake into neurons. Comparing the top and bottom panels of Figures 3.1 (NG) and 3.2 (TG) we find that there are positive cells at both 1 day and 3 days post application.

This phenomenon is not limited to topical application of retrograde tracers. This time the animals received an injection of CTB-Alexa 555 into the duodenum, an area of the small intestine known to be highly innervated by vagal afferents, at two different doses. Figures 3.3 and 3.4, respectively, illustrate the NG’s and TG’s with these two doses and the 3 different incubation times with the result that they are all positive.
Figure 3.3 - Nodose ganglia from CTB-555 Intestinal Injected animals; representative sections, 20x; Top panels = 2%, Bottom panels = 0.2%, Left panels = 1 day incubation, Middle panels = 3 day incubation, Right panels = 7 day incubation. White arrows indicate neurons positive for the ctb-555 tracer.
Figure 3.4 - Trigeminal ganglia from CTB-555 Intestinal Injected animals; representative sections, 20x; Top panels = 2%, Bottom panels = 0.2%, Left panels = 1 day incubation, Middle panels = 3 day incubation, Right panels = 7 day incubation. White arrows indicate neurons positive for the ctb-555 tracer.
This phenomenon is also not limited to the tracer CTB-555. We tested both BDA-680 and Fluorogold to see if the same effect would be observed. Figure 3.5 illustrates TG from this experiment showing no difference.

![Image of Trigeminal ganglia from 3 different tracers painted on the portal vein, From left to right – 2% ctb-555, BDA-Alexa 680, and 5% Fluorogold; 20x. White arrows indicate neurons positive for the ctb-555 tracer.]

We quantified the number of positive neuronal cells in the NG and found there was no difference between neither the dose nor the incubation time of various tracers. Representative images found in Figure 3.6 and quantification in Figure 3.7.
Figure 3.6 – Nodose ganglion from animals receiving various retrograde tracer concentrations at 3 different incubation periods: A) 0.2% CTB, 1 day; B) 0.2% CTB, 3 days; C) 1% CTB, 1 day; D) 1% CTB, 3 days; E) 5% FG, 7 days; 20x. White arrows indicate neurons positive for the ctb-555 tracer.

[51]
Figure 3.7 – Quantification of nodose ganglia neurons positive for tracer; neurons were counted by a blind observer (both positive and negative) and counts are represented as the percentage of positive neurons; representative sections found in Fig 3.6. Bars indicate standard error; P value = not significant.
3.4. DISCUSSION

Neuronal tracing is a commonly used method in many laboratories, employing both retrograde and anterograde transported tracers applied to trace neural pathways (Kreier et al., 2006; Wild et al., 1991; Salin et al., 2008; Schmued et al., 1986; Coolen et al., 1999; Plato et al., 2005; Li et al., 2007; Raybould et al., 2002; Ambaavanar et al., 2003; Wan et al., 1982). We set out to specifically label vagal afferent nerves in the portal vein using retrograde tracers. Since we wanted to label the nerves of only the wall of the vein without tracer leakage into circulation or damage the wall of the vein, we developed a technique for topically applying the tracer to the surface of the portal vein using a fine paintbrush, thus calling it the painting method. A very small amount of liquid was used, to minimize the amount of leakage into the peritoneum, along with applying Gel-foam over top of the painted area to conceal the tracer to the portal vein.

We initially used the supraoptic nucleus (SON) as our control tissue. The SON is a circumventricular organ with a decreased blood brain barrier, and where brain cells have more direct access to the circulation. Our reasoning was that uptake of tracer into the SON would reflect transport through the circulation providing a positive control for leakage of material into the peritoneum from our painting procedure. However, the SON was not consistent, as it was sometimes positive and sometimes negative when different animals were painted under the same conditions. The SON was always positive when the animals received an IP injection of the tracer (data not shown). To provide a more direct comparison to the nodose ganglia, we felt that the trigeminal ganglion (TG) would be a good negative control, being a peripheral, skull based...
ganglion like the nodose and without having access to circulation. However, in all of our tracing experiments, we found the TG consistently positive across animals and experiments. This led us to question our methodology and the specificity of the tracers. We presume that retrograde tracers are designed to make them very efficient at being transported, and we have observed exactly that at various concentrations and incubation times (Figures 3.1 & 3.2), even much lower doses and recommended incubation times than reported in current literature and product information sheets.

We decided to include a group of animals that received an injection of the same tracer, with a varied dose and incubation time, to confirm that this phenomenon was not unique to our topical application method. As demonstrated in Figures 3.3 and 3.4, injection of tracer into the wall of the intestine caused trigeminal neurons to fluoresce identically to painted neurons. At both a 0.2% and a 2% dose, when injected, both the nodose and the trigeminal ganglia were positive (Figures 3.3 & 3.4). Also, looking at both doses at 3 different incubation times, 1, 3 and 7 days, the same result was observed (Figures 3.3 & 3.4). This confirmed that the apparent promiscuity of the retrograde tracer was not limited to our painting technique. We also tested different tracers, to see if the phenomenon was related to the CTB-555. Figure 3.5 shows that the trigeminal ganglia are also positive and show a similar staining pattern with BDA and FG as with CTB. Additionally, looking at the nodose ganglia, quantifying the number of neuronal cells which took up the tracer showed no difference between the dose nor the incubation time (Figures 3.6 & 3.7).

Examination of the distinct staining pattern in the trigeminal ganglion revealed
distinct features consistent with lipofuscin, an autofluorescent pigment that accumulates with age in mammalian cells, including neurons (Schnell et al. 1999; Koike et al., 2000). The presence of lipofuscin can be confirmed with detection of fluorescence under the Cy5 filter set and persistence of fluorescence after a period of photobleaching. To test if our tissues did indeed have lipofuscin, we performed both of these methods. Figure 3.8 demonstrates that there was significant fluorescence detectable at Cy5, under a variety of experimental conditions. Additionally, this fluorescence could not be eliminated with a 20 or 30 minute photobleaching using the Cy3 excitation laser (Figures 3.9 and 3.10). This confirmed the presence of lipofuscin in our tissue samples.
Figure 3.8 – Confirmation of lipofuscin in trigeminal ganglion (top panels, 40x) and nodose ganglion (bottom panels, 20x); Left panels = Cy3 filter, Right Panels = Cy5 filter. White arrows indicate neurons positive for the ctb-555 tracer and lipofuscin.
Figure 3.9 – Photobleached trigeminal ganglion; Left Panel = Cy3 unbleached, Middle panel = Cy3 with 30 minutes photobleaching, Right panel = Cy5 with 30 minutes of photobleaching; 20x. White arrows indicate neurons positive for lipofuscin.
Figure 3.10 – Photobleached nodose ganglion; Top panels = Cy3, Bottom panels = Cy5, Left panels = unbleached, Right Panels = 30 minutes of photobleaching; 20x. White arrows indicate neurons positive for lipofuscin.
To try and eliminate the fluorescence from the lipofuscin, we employed a copper sulfate treatment to the slides (Schnell et al., 1999). This treatment, though widely used, was not successful in the tissues from our particular experimental design/setup (Figures 3.11 and 3.12).

Figure 3.11 – CuSO4 treatment on trigeminal ganglia; Top panels = Cy3, Bottom panels = Cy5, From left to right = Untreated, 5mM, 20mM, 100mM CuSO4; 20x.
Figure 3.12 - CuSO4 treatment on nodose ganglia; Top panels = Cy3, Bottom panels = Cy5, From left to right = Untreated, 50mM, 100mM CuSO4; 20x.
To get around the problem of autofluorescence due to the presence of the lipofuscin, for the purposes of our primary research question (to specifically label the vagal afferent nerves of the portal vein) we have abandoned the retrograde labeling method and moved to an anterograde labeling method of injecting the tracer into the NTS to label the vagal afferents transganglionically (Powley, 2008). Unfortunately, we will be unable to identify the nodose neurons that specifically innervate the portal vein and co-localize those with GLP-1r production at the level of the nodose ganglion, but we may be able to accomplish this at the peripheral level.

3.5. CONCLUSION

Retrograde labeling of the vagal afferents of the portal vein was unsuccessful in our paradigm. Switching to anterograde labeling methods will circumvent the problems described and allow us to examine the co-localization of the GLP-1 system and vagal afferents in the periphery.
CHAPTER 4

Identification and characterization of the Glucagon-Like Peptide-1 Receptor in the small intestine of rat and mouse

*Portions of the chapter are being prepared for submission to the Journal of Histotchemistry and Cytochemistry*
4.1. INTRODUCTION

The small intestine is the major site of nutrient absorption, digestion, and endocrine secretion. It begins at the stomach, ends at the cecal valve and is approximately 5 meters long in the human. The small intestine consists of 3 different segments: the duodenum, the jejunum and the ileum. The layers of the small intestine are (from outside in): the serosa, the muscularis (containing the longitudinal muscle layer, the myenteric plexus and the circular muscle layer), the submucosa (containing the lymphatic vessel, the arteries and the veins) and the mucosa (containing the villi, the lamina propria, the capillaries and the lacteal) (Figure 4.1). The villi are 0.5 to 1.5mm in length and project into the lumen of the small intestine. They range from leaf-like in the duodenum, to fingerlike in the jejunum and the ileum. Between the villi are the crypts, which contain the intestinal glands. (Jenqueira et al., 2003).
The vili of the small intestine contain a plethora of specialized cells that perform the functions of the small intestine. There are absorptive cells, which are tall columnar cells with the nucleus in the basal part of the cell. The absorptive cells have a brush border at the apex side which is packed with microvilli. Microvilli increase the surface area of the intestinal lining to increase absorption. The absorptive cells absorb the nutrient molecules produced by digestion. Goblet cells are interspersed throughout the digestive tract, being more abundant with progression from the duodenum to the ileum. They produce mucins that are hydrated and cross-linked to form the mucus that
protects and lubricates the intestine. Paneth cells are exocrine cells that contain lysozyme, an enzyme that has antibacterial activity, likely important in maintaining the intestinal flora. M (microfold) cells are specialized endothelial cells overlying the Peyer’s patches which can endocytose antigens and present them to the antigen presenting cells for transport to the lymph nodes. (Jenqueira et al., 2003).

Another important cell population of the small intestine is the enteroendocrine cells (EEC). EEC’s are positioned in the gut in such a way as to sense the luminal contents and release their cellular contents accordingly to initiate responses to incoming nutrients, some of which act through the nervous system. EEC’s are known to be innervated, but how they are controlled neutrally is still unknown. (Sjolund et al., 1983; Furnes, 2000). There are two types of endocrine cells, open and closed. Open endocrine cells have microvilli on the apical side and have direct contact with the lumen. Closed endocrine cells have the apical side covered by other epithelial cells. There are many specific EEC’s, which include: D-cells that secrete somatostatin to inhibit other endocrine cells and are located primarily in the pylorus and the duodenum; I-cells that secrete CCK to stimulate pancreatic enzyme secretion and contraction of the gall bladder and are found throughout the small intestine; K-cells that secrete GIP to inhibit gastric acid secretion and are found throughout the small intestine; L-cells that secrete GLP-1 to promote satiety, inhibit gastric emptying, induce insulin secretion, and inhibit hepatic glucose production, and are found throughout the small intestine; G-cells that secrete gastrin to stimulate gastric acid secretion and are found in the pylorus; S-cells that secrete secretin to promote pancreatic and biliary bicarbonate and acid secretion, and are found throughout the small intestine; Mo-cells that secrete motlin to increase

[65]
gut motility found throughout the small intestine; and Enterochromaffin cells which secrete serotonin and substance P for neurotransmission and to increase gut motility. (Jenqueira et al., 2003).

Another important component of the gastrointestinal system is the enteric nervous system (ENS) which extends from the esophagus to the anus and includes the associated organs, such as the pancreas, liver, gall bladder and salivary glands. The ENS is part of the autonomic nervous system and functions to regulate gastrointestinal motility, absorption, gastric emptying, secretions, blood flow and immune responses (Powley et al., 2002). The ENS consists of various ganglia, fibers and effector cells to carry out its regulatory processes. Of the approximate 100 million neurons (in the human ENS), there are four types of functional neurons: sensory neurons, interneurons, muscle motor neurons, and secretomotor neurons, found in an approximate ratio of 2:1:1(Weidmann et al., 2007; Hansen, 2003a and b). The sensory neurons are both extrinsic and intrinsic. The extrinsic sensory neurons are the vagal and spinal afferents with their cell bodies located outside of the wall of the gut. The intrinsic sensory neurons are IPAN’s and have their cell bodies located within the wall of the gut. The extrinsic and intrinsic neurons work together and communicate with each other and the brain to survey the gut (Hansen, 2003a and b).

The ENS has three primary ganglionated plexuses: the myenteric plexus, the submucosal plexus, and the mucous plexus. The myenteric plexus is found between the longitudinal and circular muscle layers, the submucosal plexus is found between the muscularis mucosa and the circular muscle layer, and the mucous plexus is found in the
mucous layer (Hansen, 2003a and b) (Figure 1.8). The myenteric plexus also contains the intraganglionic laminar vagal afferent endings (IGLEs) which respond to muscle tension and are found in great numbers throughout the GI tract (Berthoud, 2008).

Since GLP-1 is secreted from intestinal L-cells, and is degraded so rapidly, we hypothesize that there is a population of GLP-1 receptors in the small intestine that GLP-1 could bind to elicit responses to incoming nutrients, since this is the where the concentration of GLP-1 is highest. It has been published that the GLP-1r mRNA is found there (Dunphy et al., 2008; Korner et al., 2007), but to date the expression of the GLP-1r protein has not been characterized in the small intestine. In this study, we demonstrate the expression of the GLP-1r in the small intestine of rat and mouse.
4.2. MATERIALS AND METHODS

Animals

All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Male Long-Evans rats from Harlan, 300-350g, were housed in individual cages at 23 C on a 12 hour light-dark cycle. Male FVB/N and C57Bl/6 mice were housed in individual cages at the same conditions.

Tissue preparation for immunohistochemistry

The animals were euthanized by pentobarbital injection (rats) or CO$_2$ asphyxiation (mice) and perfused intracardially with saline followed by 4% paraformaldehyde. For both the rats and mice, the small intestine was removed and divided into the three sections: the first 6cm distal from the stomach was removed for the duodenal sample, the last 6cm proximal to the cecal valve was removed for the ileal sample, and the middle 6cm of the remaining length was removed for the jejunal sample. Additionally, the right and left nodose ganglia were also removed. Tissues were postfixed in 4% paraformaldehyde for 4 h and then placed in 30% sucrose in PBS at 4ºC for at least 24 h. Freezing media (Tissue Tek) was added and the tissue blocs were frozen and subsequently cut at 10µm (intestinal samples) or 20µm (nodose ganglia) on a cryostat and then stored directly on slides at -20ºC.
**Immunohistochemistry**

To assess for the GLP-1r, standard immunohistochemical procedures were followed. In brief, the slides were allowed to dry at room temperature for one hour. The slides were then washed in 50mM KPBS for 30 min, in 0.3% H₂O₂ for 15 min and 50mM KPBS for 30 min, before incubation in a humidity chamber with blocking serum for 30 min at RT. The primary antibodies were then added and the sections incubated in a humidity chamber overnight at 4C. The primary antibodies and dilutions were: anti-GLP-1 Primary Antibody 1:300 (Zhang et al, 2005), and anti-GLP-1r Primary Antibody 1:300 (LS-A1206, MBL, Massachusetts). The following morning sections were washed in 50mM KPBS for 30 min, incubated in a humidity chamber with Goat Anti-Rabbit Alexa 555 or 660 (1:200), or Goat Anti-Mouse Alexa 555 or 660 (1:200), (both Invitrogen, California) for 60 min at RT, washed in 50mM KPBS for 30min and in Millipore H₂O for 5 min; dried at RT (in dark); coverslipped with Gelvatol; viewed with the Cy3 (Alexa 555), the Cy5 (Alexa 660) filters on a fluorescence microscope (Zeiss Imager.Z1 with Apotome).

**Tissue Collection for RNA Isolation**

Six wild type FVB/N male mice and six Long Evans male rats were placed into a CO₂ chamber (separately, one at a time) until respiration ceased and the eyes were cloudy. The animals were also cervically dislocated. The entire length of the small intestine was removed from the animal (including the cecal valve). The first 6cm of the duodenum proximal to the stomach was removed, the last 6cm descending to the cecal valve was removed for the ileum sample, and the middle 6cm of the remaining length...
was removed for the jejunum sample. Additionally, a piece of lung was removed to include as a positive control. The tissue samples were placed in aluminum foil and rapidly frozen on dry ice followed by storage at -80°C until further processing.

**RNA Isolation**

A one centimeter piece of sample tissue was homogenized with 1.9mL Tri-Reagent then stored at room temperature for 5min followed by storage on ice. Next the homogenates were transferred to a 2.5mL eppendorf tube, 200µL of BCP was added to each tube, vigorously shaken for 15 seconds, then allowed to sit at RT for 10 minutes. The tubes were then centrifuged at a speed of 12,000xg at 4°C for 15 minutes. Next, 500 µL of the aqueous phase was removed to a 1.5mL eppendorf tube, 1.0mL of isopropanol was added vigorously shaken for 15 seconds, then stored overnight at -20°C. The next day the samples were centrifuged at a speed of 12,000xg at 4°C for 10 minutes and the aqueous phase aspirated off. The pellets were then washed with 500µL of 75% ethanol and centrifuged at a speed of 8,000xg for 5 minutes. The ethanol was aspirated off of the pellet and this step was repeated with 100% ethanol. After aspirating the 100% ethanol, the pellets were dissolved in 20µL of DEPC water and stored at -20°C. RNA concentrations and A260/280 ratios were determined with the NanoVue system (GE Healthcare, Chalfont St. Giles, UK).

**Q-PCR: Primers**

L-32 (L32-2-STEP-95-98) F5>CAT CGT AGA AAG AGC AGC AC (Source ID: IDT10178853), R5>GCA CAC AAG CCA TCT ATT CA (Source ID: IDT10178860);
ProG1 (ProG1-3-STEP-95-97) R5>GAC GTT TGG CAA TGT TGT TC (Source ID: IDT10178839), L5>GCA ATT ACC TAG ACT CCC GC (Source ID: IDT10178836);
GLP-1R (GIP-3-STEP-95-98) R5>CAG CCG TGC TAT ACA TCC AC (Source ID: IDT22608731), L5> CAT CCA CCT GAA CCT GTT TG (Source ID: IDT22608730).
cDNA was synthesized using Superscript III First Strand Synthesis System for RT-PCR (18080-051, Invitrogen, CA). Q-PCR was performed using 2µL of cDNA and iQ SYBR-Green (170 8882, BioRad, CA). Amplifications were performed using the default cycling parameters for 40 cycles. Lung (positive control for the GLP-1r), duodenum, jejunum, and ileum from both rat and mouse were analyzed in triplicate.
4.3. RESULTS

To assess for the presence of GLP-1r mRNA, Q-PCR was performed on lung (positive control), duodenum, jejunum and ileum from both rat and mouse. Figures 4.2a and 4.2b show detectable levels of GLP-1r mRNA were expressed in all three regions of the small intestine in both species. Additionally, we assessed for the presence of Proglucagon and detected a gradient of expression, as expected, in both species (data not shown).

Figure 4.2a – mRNA expression levels in the lung, duodenum, jejunum and ileum of rat. Values are normalized to L32 and compared to expression in the lung (positive control); n=6; bars represent SE.
Figure 4.2b – mRNA expression levels in the lung, duodenum, jejunum and ileum of mouse. Values are normalized to L32 and compared to expression in the lung (positive control); n=6; bars represent SE.
After determining that there was GLP-1r mRNA present, we decided to look for the GLP-1r protein using Immunohistochemistry. To confirm antibody specificity, we performed immunohistochemistry on mouse pancreas sections with no primary, no secondary and pre-absorbed antibody controls (Figure 4.3). Additionally, this antibody has also been tested by another group in a GLP-1r KO mouse and showed no immunoreactivity (Tornehave et al., 2008). We also assessed an untreated section of mouse duodenum for autofluorescence and found that serotonin filled enterochromaffin cells displayed green autofluorescence under the FITC filter (data not shown). An untreated rat duodenal section showed red and green autofluorescence in the enterocytes under both the Cy3 and FITC filter, making double immunohistochemistry not possible on these sections (data not shown). Using our antibody against the GLP-1r protein we were able to detect enterocytes that stained positive in all 3 sections of both the mouse (Figure 4.4) and the rat (Figure 4.5).
Figure 4.3 – No primary antibody, no secondary antibody and pre-absorption with blocking peptide GLP-1r immunohistochemistry controls; positive cells are seen as red (white arrows); 20x.
Figure 4.4 – H&E (20x) and GLP-1r immunoreactivity (20x and 40x) in the mouse small intestine; Positive cells are seen as red in the middle and right panels (white arrows).
Figure 4.5 – H&E (20x) and GLP-1r (40x) immunoreactivity in the rat small intestine; Positive cell are seen as blue in the right panels (white arrows).
After confirming that the GLP-1r was present in the intestinal sections, we tried to identify the type of enterocyte that was expressing the GLP-1r. To see if the GLP-1r is expressed on three known enteroendocrine cells, GLP-1 producing L-cells, serotonin filled enterochromaffin cells, and somatostatin secreting D-cells, we performed double IHC and serial section single IHC. The GLP-1r (red) is not found to be co-localized with L-cells (purple) (Figure 4.6), serotonin containing enterochromaffin cells (green) (Figure 4.6), or somatostatin cells (data not shown), though frequently they can all be found on the same villi (Figure 4.6a and b).
Figure 4.6a – Autofluorescence of serotonin in enterochromaffin cells, GLP-1r, and GLP-1 immunoreactivity in the mouse small intestine; 20x.
Figure 4.6b – Autofluorescence of serotonin in enterochromaffin cells, GLP-1r, and GLP-1 immunoreactivity in the mouse small intestine; 20x image cropped and zoomed in.
Finally, to see if there was a difference in the protein expression levels of the GLP-1r in the different sections of the small intestine, we quantified the number of GLP-1r immunoreactive enterocytes in the duodenum, jejunum and ileum. We observed that the greatest number of GLP-1r positive enterocytes was found in the jejunum, with fewer and somewhat equal numbers in the duodenum and ileum (Table 4.1).

<table>
<thead>
<tr>
<th>Section of Intestine</th>
<th>Average GLP-1r Positive Cells</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>23.6</td>
<td>6.04</td>
</tr>
<tr>
<td>Jejunum</td>
<td>72.5</td>
<td>23.41</td>
</tr>
<tr>
<td>Ileum</td>
<td>15.0</td>
<td>2.39</td>
</tr>
</tbody>
</table>

Table 4.1 – Average GLP-1r positive enterocytes per 10 um cross-section of duodenum, jejunum and ileum in the mouse. Average is based on counts from 24 sections of each; P=0.036 one-way Anova.
4.4. DISCUSSION

Glucagon-Like Peptide-1 or GLP-1 is a protein secreted from the L-cells of the intestine that is necessary for normal glucose tolerance. It is widely accepted that GLP-1 stimulates insulin secretion by traveling through the circulation and binding to its receptor on the B-cells of the pancreas (Burcelin, 2005; Drucker 2006). However, since GLP-1 is rendered inactive by a protease present in the walls of the vasculature, it has a short half life. Additionally, after one pass through the liver, a large percentage of the active GLP-1 is broken down (Kieffer et al., 1999). This leads us to believe that GLP-1 acts at a pre-hepatic site to mediate its actions on glucose tolerance. It has been previously shown that the GLP-1r mRNA is found in the stomach and parts of the duodenum (Dunphy et al., 2008; Korner et al., 2007), however to date the expression profile of the protein has been unknown. We investigated if and where the GLP-1r is expressed in the small intestine.

We were able to confirm the previously published results of the detection of GLP-1r mRNA in the duodenum, as well as in the jejunum and the ileum of both the rat and the mouse. Since nutrient absorption occurs throughout the entire length of the small intestine, and GLP-1 is known to be a glucose homeostasis hormone, it seems reasonable that GLP-1 could exert its effects throughout all three sections of the small intestine.

Presence of mRNA in a particular location does not necessarily mean that the protein will be expressed and functional. This is why we performed immunohistochemistry with an antibody directed against the GLP-1r. We wanted to see
if the protein was expressed in the same sections of the small intestine as we detected the mRNA. As demonstrated by the red and blue intestinal cells in Figures 4.3 and 4.4, we did find that the GLP-1r protein is expressed in all 3 sections of the small intestine. In fact, similar to what we see with the mRNA expression, there is a differential expression of the GLP-1r throughout the length of the small intestine. The greatest number of receptors was observed in the jejunum, with fewer but somewhat equal numbers in the duodenum and the ileum (Table 4.1). The jejunum is the longest section of the small intestine where the majority of nutrient absorption and endocrine secretion takes place. Finding the GLP-1r protein expressed in greater numbers in this particular section supports its role as a glucose homeostasis hormone, since there are a great number of different enteroendocrine cells that are expressed here for GLP-1 to signal to resulting in the promotion or inhibition of other hormones necessary for digestion, absorption, and glucose tolerance.

Additional support for an important role of GLP-1r in the intestine is that collaborative work done with Rohit Kholi, M.D., showed that the number of GLP-1r positive enterocytes significantly increased after the ileal transposition (IT) surgery. IT surgery is used to improve diabetes and body weight by moving a section of the ileum up to the beginning of the jejunum, thus causing nutrients to be exposed to the ileum sooner. Since the greatest number of L-cells is present in the ileum, causing the L-cells to see a greater amount of nutrients earlier in the digestive process would lead to an increased secretion of GLP-1 and improved glucose tolerance. We observed on the histological sections of pre- and post- IT animals that the number of enterocytes expressing the GLP-1r was increased (Kholi et al., 2009). This could also point to the
importance of GLP-1r in the morphological changes in the ileum as a result of earlier exposure to more nutrients and also allowing the transposed ileum to handle the nutrient load, further supporting GLP-1’s role as a global glucose homeostasis hormone.

We attempted to identify which enterocytes were expressing the GLP-1r by performing double immunohistochemistry or when the two antibodies were in the same species and were highly cross-reactive, single immunohistochemistry of serial sections to eliminate cross-reactivity. The first enterocyte we wanted to look at for co-localization was the GLP-1 producing L-cells. There is some evidence in the literature that GLP-1 can regulate its own secretion from studies that administered exenatide and measured an increase in endogenous GLP-1 levels (Schirra et al., 2006; Salehi et al., 2008), so we thought it was likely that the L-cells would express the GLP-1r supporting the self-regulation hypothesis. In Figure 4.5, we showed that this was not the case. We did not find any L-cells (purple fluorescence) that also stained positive for the GLP-1r (red fluorescence). Additionally, we had a good antibody for the somatostatin secreting D-cells and performed single IHC on serial sections to see if perhaps GLP-1 was regulating the secretion of somatostatin. In data not shown, we were unable to find any D-cells that also stained positive for the GLP-1r, with the two proteins frequently not even found on the same field of vision on the microscope of the serial sections. We were also able to see if GLP-1r were expressed on serotonin filled enterochromaffin cells. Since serotonin is a potent neurotransmitter, perhaps GLP-1 could also signal the release of serotonin to illicit some of its glucose homeostasis effects. Serotonin has an indoleamine chemical structure that typically has an emission peak around 350nM, but is shifted to longer wavelengths when in vesicles because of interactions with other
molecules (Kaushalya et al., 2007), allowing for autofluorescence to occur at the FITC wavelength. As demonstrated in Figure 4.5, the enterochromaffin cells autofluoresce green and we were unable to find any enterochromaffin cells (green) that also stained positive for the GLP-1r (red). There are other enteroendocrine cells we could look at in the future for the expression of the GLP-1r, such as CCK secreting I-cells and GIP secreting K-cells. Another method to identify which enterocytes are expressing the GLP-1r would be to use laser capture microdissection (LCM) to collect the GLP-1r expressing enterocytes for further analysis. LCM is used to rapidly and reliably obtain pure populations of cells from tissue sections under direct microscopic visualization (Emmert-Buck et al., 1996; Curran et al., 2000). It works by applying a transparent ethylene vinyl acetate layer over the section of interest, applying a low power infrared laser beam at the cells of interest causing the ethylene vinyl layer to melt allowing the removal of the cells of interest (Emmert-Buck et al., 1996; Curran et al., 2000). The cells can be identified based on morphology, immunohistochemical phenotype, or genotype using ISH. In our case, we could use our GLP-1r antibody to identify the cells for capturing (Curran et al., 2000).

Finding the GLP-1r expressed on enterocytes has some interesting implications for emerging diabetes treatments. Amylin Pharmaceuticals is developing alternative delivery methods for exenatide, a long lasting form of GLP-1 (Gedulin, et al., 2008). One of the methods they are developing is an oral administration of the drug, versus the injection method currently used (Gedulin, et al., 2008). They were able to show an increase in plasma GLP-1 levels after intraduodenal administration of exenatide, though glucose and insulin levels were not measured. If there are GLP-1r present in the
gastrointestinal tract, then the orally administered exendin-4 could bind directly to these receptors, without having to be absorbed and distributed through the bloodstream. A second group is exploring the implantation of intestinal bacteria that have been engineered to secrete GLP-1 (Duan et al., 2008). This group engineered an over-the-counter probiotic strain of bacteria, *Escherichia coli* Nissle 1917, to secrete GLP-1[7-36] that was capable and successful of inducing insulin secretion (Duan et al., 2008). As is with the oral exendin-4 case, if there are GLP-1r in the gastrointestinal tract that this secreted GLP-1 could bind to, it would be beneficial for the newly secreted GLP-1 to bind to receptors in close proximity, versus battling degradation by DPP-IV to reach the target tissue of the pancreas.

To summarize, we confirmed the presence of GLP-1r mRNA in the small intestine of both the rat and the mouse, we identified the GLP-1r protein in all 3 sections of the small intestine of both the rat and the mouse, and we found that the GLP-1r is not expressed on L-cells, enterochromaffin cells, nor somatostatin cells. We also identified differential expression of the GLP-1r in the three sections of the mouse intestine. These findings support the hypothesis that GLP-1 released from L-cells could bind to GLP-1r present on enterocytes to initiate insulinotropic effects to incoming nutrients.
CHAPTER 5

Identification of the Glucagon-Like Peptide-1

Receptor on enteric nerves (?)
5.1. INTRODUCTION

Recent studies have identified an interaction between GLP-1 and the peripheral nervous system, particularly afferent nerves in the portal vein. In 2007 Vahl et al. demonstrated that GLP-1 receptors are expressed on nerves in the wall of the portal vein and that these receptors are important for glucose tolerance (Vahl et al., 2007). In 1996 the group of Nakabayashi et al. demonstrated that intraportal GLP-1 increases the firing rate in the afferent branches of the hepatic vagus nerve (Nakabayashi et al., 1996). In 2000 Balkan et al found that intraportal GLP-1 augments insulin release in response to intraportal glucose and that this response could be inhibited by a ganglionic blockade (Balkan et al., 2000). These studies, along with others, suggest that the vagal nerves are involved in sensing of glucose in the portal bed.

It is also known that visceral afferent nerves in the mucosa of the intestine mediate the effects of some GI peptides. One example is that of secretin. Secretin regulates pancreatic exocrine secretion, gastric acid secretion, and gastric motility (Li et al., 2005). Li et al has demonstrated the importance of the vagal afferent nerve in the functioning of secretin, showing that physiological doses of secretin act on vagal afferent pathways originating in the gastrointestinal mucosa for gastric motility regulation and gastric acid and pancreatic secretion (Li et al., 1995 and 1998). Secretin has secretin receptors present on vagal afferent fibers and it has been shown that axonal transport of secretin receptors occurs within the abdominal vagal branches (Wang et al., 1995).

Another example of peptides mediating their effects through the vagal afferent
nerves is that of cholecystokinin (CCK). CCK is a major mediator of pancreatic exocrine secretion, acts in part via an afferent vagal pathway originating in the gut mucosa, which is sensitive to capsaicin (Li et al., 1996). CCK is produced and secreted from intestinal I-cells in response to incoming nutrients, particularly fat and protein (Sartor et al., 2008). CCK works by binding to its receptors on target tissues. In the small intestine, it binds primarily to CCK-1 receptors present on vagal afferent nerves signaling to the NTS (Konturek et al., 2003; Rogers et al., 2008). CCK can also signal via an endocrine mechanism to the pancreas and gall bladder (Sartor et al., 2008). Recent studies have identified the importance of the vagal afferents for CCK signaling, demonstrating that the effects of CCK to promote satiety (Li et al., 1997), reduce gastric motility (Moran et al., 1994), and induce pancreatic exocrine secretion are the result of direct interactions of CCK with its receptors on vagal afferent nerves. Other studies have shown that CCK can signal directly to the CNS to mediate digestion and feeding behavior, by demonstrating via in vitro electrophysiological studies that CCK can activate neurons in the NTS and activate efferent neurons in the DMN (Appleyard et al., 2005; Baptista et al., 2005; Peters et al., 2004 and 2006; Simasko et al., 2002).

Since both secretin and CCK have been shown to signal neurally, we believe that GLP-1 could signal this way as well. In the previous chapter, we identified a population of enterocytes that express the GLP-1r. Since CCK signals through receptors present on vagal afferents, we set out to see if GLP-1r were also present on the vagal afferents. In Chapter 3, we encountered confounding with our retrograde tracing experiments. This time, to specifically label the vagal afferents, we used a transganglionic labeling method, described by Powley et al (Powley et al., 2008). The nodose ganglion neurons
are bipolar, sending projections to the NTS and also to the periphery. We targeted a region of the NTS that projects to the small intestine and injected a bi-directional tracer to label the peripheral vagal afferents in the small intestine and then looked for co-localization with the GLP-1r. We hypothesize that GLP-1, secreted from intestinal L-cells, can act directly on GLP-1r located on sensory vagal nerves in the wall of the small intestine to initiate neural responses to incoming nutrients.
5.2. METHODS

Animals:

All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Male Sprague-Dawley rats from Harlan, 300-350g, were housed in individual cages at 23 C on a 12 hour light-dark cycle. Male FVB/N mice were housed in individual cages at the same conditions. Female GLP-1r KO mice (C57/Bl6 background) and female WT littermates were also housed in individual cages at the same housing conditions.

Tracers:

The animals received an NTS injection of cholera toxin subunit B conjugated with Alexa 555 (Invitrogen, CA).

Anterograde Labeling of Visceral Afferents:

NTS Tracer Injection: Procedure (notes taken while watching): Anesthetic used was 87% ketamine/13% xylamine i.p. 1µl/g; The tracer was loaded into a 26 gauge Hamilton syringe (3µl loaded to inject 1µl per side – bilateral injections); The coordinates used for our NTS injections were AP 13.28, DR 7.8 Bregma – Between figures 72 and 73, targeting the regions projecting to the gut. Half the animals were sacrificed at Day 1 post-op to assess for tracer in the nodose ganglion, the other half at Day 3 post-op to asses for tracer in the small intestine.
**Tissue preparation for immunohistochemistry**

Rats were euthanized by pentobarbital injection and perfused intracardially with 300 mL of normal saline followed by 300 mL of 4% paraformaldehyde. Tissues were postfixed in 4% paraformaldehyde for 4 h and then placed in 30% sucrose in PBS at 4°C for at least 24 h. The portal vein was removed en bloc with the liver hilus and the mesentery attached and placed together in tissue boats, leaving the surrounding anatomy of the hepatoportal region intact. Freezing media was added and the tissue blocs were frozen and subsequently cut at 20 µm on a cryostat and then stored directly on slides at -20°C. The right and left nodose ganglia, and the trigeminal ganglia were removed, processed, and sectioned in the same manner. The brains were also removed and processed similarly, however they were sectioned at 16 µm.

**Immunohistochemistry**

To view the retrograde tracer, the frozen slides were dried at room temperature and coverslipped with Gelvatol. Then the slides were viewed under the Cy3 filter on a standard fluorescence microscope (Zeiss) to look for Alexa 555 fluorescence.

To assess for the GLP-1r, standard immunohistochemical procedure was followed. Briefly: the frozen slides were allowed to dry at room temperature for one hour; washed in 50mM KPBS for 30 min; washed in 0.3% H2O2 for 15 min; washed in 50mM KPBS for 30 min; incubated in a humidity chamber with blocking serum for 30 min at RT; incubated in a humidity chamber with anti-GLP-1r Primary Antibody 1:500 (LS-A1206, MBL, Massachusetts) overnight at 4C; washed in 50mM KPBS for 30 min;
incubated in a humidity chamber with Goat Anti-Rabbit Alexa 488 1:200 (Invitrogen, California) for 60 min at RT; washed in 50mM KPBS for 30min; washed in Millipore H20 for 5 min; dried at RT (in dark); coverslipped with Gelvatol; viewed with FITC filter on a standard fluorescence microscope (Zeiss).
5.3. RESULTS

To indentify the vagal afferent nerves in the small intestine, the vagal afferents were labeled transganglionically with cholera toxin subunit beta conjugated with Alexa 555 injected into the NTS. Since CTB is known to act as both a retrograde and an anterograde tracer, the tracer traveled retrogradely from the NTS to the nodose ganglion then anterogradely from the NG to the visceral vagal afferents in the small intestine. Figure 5.1 shows the presence of the tracer in the nodose ganglion at day 1 post-injection and the small intestine at day 3 post injection (red granules in the cytoplasm of the neurons in the left panel, punctuate red dots in the lumen of the viili in the right panel).

Fig 5.1 – Presence of the transganglionic tracer ctb in the nodose ganglion (left panel, 40x) and the small intestine (right panel, 63x).
After confirming transport of the tracer to the target tissue, we first used IHC staining of the sections for calretinin, a marker for vagal afferent nerves (Figure 5.2). The tracer appeared to co-localize with or be found in close proximity to the calretinin, confirming that the tracer is on the vagal afferent nerves. Additionally, we co-localized the tracer with another neuronal marker synaptophysin (Figure 5.3) and found some co-localization. This confirmed that the transganglionic tracer was present on afferent nerves and some presynaptic nerves in the small intestine.
Fig 5.2 – Co-localization of the transganglionic tracer (red) with calretinin (green), a marker for vagal afferent nerves; 20x and 40x. White arrows indicate potential co-localization.
Figure 5.3 – Co-localization of the transganglionic tracer with synaptophysin; clockwise from top left: 40x, 63x, 20x and 20x. White arrows indicate synaptophysin positive fibers and synaptophysin immunoreactivity in myenteric plexuses.
Next, we stained the sections for the GLP-1r and looked for co-localization with the transganglionic tracer (Figures 5.4a, 5.4b, and 5.4c). As you can see, there did not seem to be any co-localization in the enterocytes (Figure 5.4a), however there was some co-localization in the wall of the small intestine, in the myenteric plexus (Figure 5.4b) and also significant co-localization in the lumen of the villi (Figure 5.4c).
Figure 5.4a – Co-localization of the transganglionic tracer with the GLP-1r in the enterocytes; 40x.
Fig 5.4b - Co-localization of the transganglionic tracer with the GLP-1r in the wall of the small intestine; 40x.
We were unable to perform double IHC to co-localize the GLP-1 receptor and calretinin, however we were able to perform double IHC with the GLP-1r and synaptophysin, demonstrating that the GLP-1 receptor is expressed on some neural elements in the small intestine (Figure 5.5).
Figure 5.5 – Double IHC of synaptophysin (blue) and the GLP-1r (red) in the duodenum, jejunum and ileum of mouse; 20x.
We were observing a great amount of co-localization of the transganglionic tracer, particularly within the lumen of the small intestine, more than we expected. We checked the sections of the nodose ganglion and small intestine for non-specific fluorescence of the tracer under the Cy5 and FITC filter, since the tracer is tagged with Alexa 555 and should fluoresce only under the Cy3 filter. As you can see in Figure 5.6a and 5.6b, there was a significant amount of non-specific fluorescence resulting in a false positive for our co-localization studies.

Figure 5.6a – Non-specific fluorescence of the transganglionic tracer in the nodose ganglion; 40x. The tracer is tagged with Alexa 555 (Cy3).
Figure 5.6b - Non-specific fluorescence of the transganglionic tracer in the small intestine; 40x. The tracer is tagged with Alexa 555 (Cy3).
5.4. DISCUSSION

Recent studies have identified an interaction between GLP-1 and the peripheral nervous system, particularly afferent nerves in the portal vein (Vahl et al., 2007; Nakabayashi et al., 1996; Balkan et al., 2000). It is also known that visceral afferent nerves in the mucosa of the intestine mediate the effects of other GI peptides. The enteric system is highly innervated, consisting of both extrinsic and intrinsic neuronal sources. The sensory neurons are both extrinsic and intrinsic in origin. The extrinsic sensory neurons are the vagal and spinal afferents with their cell bodies located outside of the wall of the gut. The intrinsic sensory neurons have their cell bodies located within the wall of the gut (Hansen, 2003a and b).

Cholecystokinin (CCK) is a major mediator of pancreatic exocrine secretion, gallbladder contractions, gastrointestinal motility, and a satiety factor (Rogers et al., 2008). CCK receptors are found in abundance on peripheral vagal afferent fibers and in the cell bodies in the nodose ganglion (Broberger et al., 2001; Corp et al., 1993; Lankisch et al., 2002; Simasko et al., 2002). CCK signals via the extrinsic neural pathway, through the vagal afferent pathway. Vagal afferents have their peripheral terminals within the wall of the gastrointestinal tract in both the mucosal and muscle layers, where they terminate within the lamina propria close to the basolateral membranes of EEC’s (Raybould et al., 2007). This puts the receptors in close proximity to cells that release CCK supporting the hypothesis that CCK can signal directly to receptors on vagal afferents to initiate its responses to intestinal contents.

Since CCK is able to signal neurally, we believe that GLP-1 could signal this way
as well. In the previous chapter, we identified a population of enterocytes that express the GLP-1r. We hypothesized that GLP-1, secreted from intestinal L-cells, can act directly on GLP-1r located on sensory vagal nerves in the wall of the small intestine to initiate neural responses to incoming nutrients.

To look at this, we first wanted to specifically label the vagal afferent nerves projecting to the gut and we accomplished this by transganglionic labeling. We injected a tracer conjugated with a fluorescent tag (CTB-Alexa 555) into the NTS of rats and looked for the tracer in the nodose ganglion and the small intestine. We chose to use CTB because it has been shown to have both retrograde and anterograde properties. We chose an Alexa 555 fluorescent tag on the tracer for easy detection and because in our previous studies using Alexa 555 as a secondary antibody showed little to no bleedthrough into other channels. Unfortunately for us, the CTB-Alexa 555 combination did show promiscuity and non-specific fluorescence under the Cy3, Cy5, and FITC filters, unlike the Alexa 555 secondary antibody which is specific. The non-specific fluorescence confounds our co-localization observations. We also injected a group of animals with a different transganglionic tracer with a colorimetric detection method, that of wheat germ agglutinin tagged with horseradish peroxidase (WG-HRP). This combination was used in a transganglionic tracing of the vagal afferents of the small intestine with whole-mount en bloc processing and visualization of the nerves (Powley et al., 2008). Upon examination of the small intestine of these animals, the HRP attached to the tracer could not be distinguished from the peroxidase activity on remaining red blood cells. When the nodose ganglia of these animals was observed, no tracer was detected in the nodose neurons. When the injection sites of these animals
were observed, a large amount of tracer was still present in the NTS, suggesting that it was not successfully transported from the NTS to the NG, and subsequently not transported to the peripheral vagal afferents.

Despite the non-specific fluorescence of the transganglionic tracer, we did detect the GLP-1r in the small intestine sections, confirming the results from Chapter 4. Not only did we see the GLP-1r expressed in enterocytes (Figure 5.4a), but we also detected some GLP-1r immunoreactivity in neurons of the myenteric plexus (Figure 5.4b). Because of the non-specific CTB fluorescence, we cannot be certain that the GLP-1r staining we observe in the lumen of the villi (Figure 5.4c) is real co-localization.

We were able to perform double immunohistochemistry for the GLP-1r and synaptophysin, a presynaptic marker, and we did observe some co-localization with that population. Synaptophysin stained a distinct population of enterocytes and almost every cell in that population also stained positive for the GLP-1r. We did not observe any co-localization in the nerve fibers of the villi lumen. Even though we cannot answer the question of whether or not the GLP-1r co-localizes with vagal afferent nerves, we were able to detect the GLP-1r on some neural elements of the small intestine. The vagal afferent nerve population is not the only source of enteric innervation, being that the vagal nerves are part of the extrinsic neural system and the possibility that the GLP-1r is found on elements of the intrinsic neural system is still open. By finding the GLP-1r on neural elements in the enteric system, it leaves open the possibility that GLP-1 can exert its effect neurally through a vagal independent mechanism.

Another way to examine if the GLP-1r is co-localized on vagal afferent nerves, is
to perform double immunohistochemistry with a known vagal afferent nerve marker. Calretinin is such a marker and when we tried this double stain, we ran into problems with antibody specificity because the two antibodies are both raised in rabbit and were particularly cross-reactive. We attempted to obtain a calretinin antibody raised in the mouse, but due to problems beyond our control we were unable to receive the antibody. Future studies will involve finding and employing methods to perform double immunohistochemistry with two highly cross-reactive antibodies from the same species.

Even though the tracing approach used for co-localization in this study turned out to be inconclusive, we did identify neural elements that were positive for the GLP-1r. Since GLP-1 elicits a wide range of effects, ranging from insulin secretion to inhibition of gastric emptying, it is still possible that GLP-1 binds to GLP-1rs that are present on nerves to induce its effects. CCK is a well understood system that does produce effects both through the vagal afferent system and direct effects on the pancreas which supports the hypothesis that GLP-1 could also signal both ways. Neural signaling is much faster than endocrine signaling, and since GLP-1 is necessary for normal glucose tolerance and glucose homeostasis is a multi-tier regulated system, it is logical that GLP-1 could signal in multiple ways to affect glucose homeostasis. There is evidence suggesting the importance of an intact nervous system for the action of GLP-1, but it is yet to be physically co-localized with elements of the sensory nervous system to further support this hypothesis.

In conclusion, we were able to identify the GLP-1r on neural elements in the small intestine, both in the wall neurons of the myenteric plexus and with synaptophysin
positive enterocytes. We cannot confirm that the GLP-1r co-localizes with the transganglionic tracer in the vagal afferents due to non-specific fluorescence, nor with calretinin due to antibody specificity issues. Future work will involve better tracing choices (method of tracing, tracer, and the type of tag for detection), co-localization methods, and detection methods to confirm the co-localization of the GLP-1 receptor with vagal afferent nerves in the small intestine.
CHAPTER 6

Conclusions and future directions
6.1. Concluding Remarks

The aim of this dissertation research was to investigate how Glucagon-Like Peptide-1 activated the central nervous system to illicit changes in blood glucose. Understanding how the body regulates blood glucose levels is important for not only understanding the disease diabetes, but also in developing treatments for the disease.

In Chapter 1, the necessary background was presented to understand the major components of not just the GLP-1 system, but also the nervous system and relevant anatomy and physiology. To understand how a system works, it's necessary to understand its components. GLP-1 is made in L-cells of the intestine, secreted into circulation, and binds to the GLP-1r on peripheral tissues to illicit its effector functions. Since GLP-1 is degraded rapidly and has a short half-life, an emerging hypothesis is that it can signal on GLP-1r present on nerves to send signals to the CNS about the glucose state of the body.

Two likely places for this signaling to take place are the portal vein and the small intestine. GLP-1r have been previously identified on nerves in the wall of the portal vein (Vahl et al., 2007) so this is the location we started investigating in Chapter 2. The first step was to see if the nerves in the wall of the portal vein could indeed access circulation and whether or not the GLP-1r expressed there were capable of interacting with constituents in the circulation. By using retrograde tracing and immunohistochemistry techniques, we were able to identify sensory afferent nerves that were capable of accessing circulation that expressed the GLP-1r.
Next we attempted to further characterize the sensory vagal afferent neurons again utilizing retrograde tracing and immunohistochemical techniques, but we ran into a plethora of problems that did not allow us to make any conclusions from the body of work in Chapter 3. The only conclusion we can make is that controls are incredibly important and by having our controls confounded, it calls into question the use of the particular retrograde tracers tested and published without the appropriate controls.

After a visiting student demonstrated that when GLP-1 was infused into the portal vein no resulting increase in insulin was observed, we decided to investigate the GLP-1/GLP-1r signaling system in the other likely place for it to occur – the small intestine. In Chapter 4 we were able to identify a population of enterocytes that express the GLP-1r in the duodenum, jejunum and ileum. We attempted to identify which enterocytes were expressing the GLP-1r, but were only able to rule out expression on L-cells, D-cells, and enterochromaffin cells. Further work is required to identify this unique population of enterocytes, perhaps by additional double immunohistochemical staining or laser capture microdissection.

The next step was to investigate whether the GLP-1r was present on vagal afferent nerves in the small intestine. In Chapter 5, we attempted this experiment, but once again ran into problems similar to Chapter 3 with autofluorescence and additional promiscuity of our anterograde tracer showing non-specific fluorescence under filters it shouldn’t. We were able to identify the GLP-1r on neural elements in the small intestine, however we cannot definitively say whether or not they are present on vagal afferent nerves. The small intestine receives innervation from a variety of sources, both
intrinsic and extrinsic, so further work is required to identify the nerve populations in the small intestine that express the GLP-1r.

Finally, a further direction that can be investigated, once the neural population of GLP-1r and the enterocyte population are identified, is to see if the GLP-1r are functional and important for glucose homeostasis. Our lab is in the process of developing methods to answer these questions, including finding a way to deliver GLP-1r antagonists or long-lasting GLP-1 directly to the site of action (determined prior to the study) and developing methods for measuring the GLP-1r antagonists/agonists in the resulting plasma.

Discovering a population of enterocytes that express the GLP-1r is an important finding in developing administration of pharmaceuticals that are directed at the GLP-1 system, either by administering a DPP-IV resistant form of GLP-1, by blockage of DPP-IV, or by agonism of the GLP-1r. The small intestine is the site of the greatest concentration of GLP-1 and if the concentration of GLP-1 can be preserved by inhibiting DPP-IV in this location, then a greater amount of GLP-1 can reach general circulation and ultimately the beta cell to stimulate insulin secretion. Additionally, perhaps a greater concentration of GLP-1 can interact with GLP-1r present on vagal afferent nerves in the small intestine to neutrally regulate insulin secretion.

Pharmaceuticals are not the only treatment for diabetes. Bariatric surgery is also becoming a more common procedure, with the obesity epidemic in full force. One type of bariatric surgery is the Roux-en-Y gastric bypass. In this procedure, the duodenum and jejunum are bypassed and nutrients are delivered directly to the ileum. This causes
the large population of L-cells in the ileum to see nutrients much sooner than they
normally would and as a result, increases in GLP-1 secretion are observed. Another
procedure, the Ileal Transposition, leaves the stomach intact and transposes the ileum
to the beginning of the jejunum segment. This results in the ileum becoming more like
the jejunum, changing its morphology as such. Additionally, we found that the number
of GLP-1r enterocyte population increased. It is becoming more evident that a better
understanding of the GLP-1 system is required as these procedures become more
popular and necessary with the growing obesity and diabetes trends.

In conclusion, we identified that sensory neurons in the portal vein can access
circulation, that careful controls need to be included when employing neuronal tracing
methods, that there is a population of enterocytes in the small intestine that express the
GLP-1r and that the GLP-1r is can also be found on neural elements in the small
intestine. These findings are important for the understanding of GLP-1’s multiple effects
and sites of action to elicit changes in glucose homeostasis. Further work will continue
to expand on our knowledge of this important global glucose homeostasis hormone and
implications for the treatment of obesity and diabetes.

[114]


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