Characterization of Adaptive Hyperplasia of the Intestines Following Roux-En-Y Gastric Bypass and Vertical Sleeve Gastrectomy in Rats

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CHARACTERIZATION OF ADAPTIVE HYPERPLASIA OF THE INTESTINES FOLLOWING ROUX-EN-Y GASTRIC BYPASS AND VERTICAL SLEEVE GASTRECTOMY IN RATS

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Biological Sciences

by

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ABSTRACT

Bariatric surgery is currently the only treatment for obesity that is effective in both the short and long term. Despite this, little is known about the mechanisms through which these surgeries act. While no single critical system has yet been identified, a number of major hypotheses have been proposed. One hypothesis is that adaptive hyperplasia of the intestines following Roux-en-Y gastric bypass (RYGB) surgery is required in order to receive the beneficial effects of the surgery. In this thesis I characterized the adaptive response of the intestines following RYGB and showed that significant hyperplasia occurs in the intestinal mucosa. In addition, I characterized the proliferation of enteroendocrine cells. I found that there is an increase in the number, but not density, of glucagon-like-peptide 1, neurotensin, and serotonin releasing cells, as well as an increase in both number and density of cholecystokinin releasing cells, in the Roux and common limbs following RYGB. This could link adaptive hyperplasia to increased circulating levels of these hormones, which has been implicated in a reduction of food intake and an improvement in glucose homeostasis.

A second hypothesis that has been proposed states that RYGB and vertical sleeve gastrectomy (VSG), two similar but ultimately different surgeries, work through a common mechanism of action. In contrast to our observations in rats following RYGB, we found that adaptive hyperplasia is absent following VSG. This leads to the conclusion that either RYGB and VSG share a common mechanism that does not involve adaptive hyperplasia of the intestines, or they do not share a common mechanism.
CHAPTER 1: INTRODUCTION

1.1 The Obesity Epidemic

Obesity has increased at an alarming rate over the last 50 years. The first National Health Examination Survey took data from 1960-1962 and showed that 31.5% of US adults over the age of 20 were overweight (BMI 25.0-29.9), and 13.4% were obese (BMI ≥ 30.0)\(^1\). In 2012, the same survey showed that over the previous 50 years the prevalence of overweight remained relatively stable at 33.9%, but the prevalence of obesity in adults had more than doubled to 35.1%\(^2\). In the same series of surveys it was shown that from 1960-1962 the prevalence of obesity in children 6-17 years old ranged from 3.4% to 4.7% depending on subgroup\(^3\). By 2012, the prevalence of obesity in children aged 6-19 years had tripled to 16.9%\(^2\). Due to the numerous health concerns related to overweight and obesity\(^4\), this has become a serious public health crisis, and there is an increasing focus on research into effective ways to combat this epidemic.

While classical interventions such as dieting and counseling do work in the short term to reduce obesity, surgical intervention remains the only treatment option that maintains a significant reduction in obesity and related co-morbidities for longer than 5-10 years\(^5\). Despite this, little is known about how gastric bypass surgeries actually work to reduce body weight and improve glucose homeostasis. In this thesis, I will review Roux-en-Y gastric bypass (RYGB) and vertical sleeve gastrectomy (VSG) procedures, as well as potential mechanisms of action that are currently being investigated. Following that, I will present my own data and relate it to two major hypotheses that have recently been proposed. The first hypothesis states that adaptive hyperplasia of the intestines following RYGB is required for the weight loss and improvement of glucose homeostasis associated with the surgery. The second hypothesis states that RYGB and
VSG share a common mechanism of action. My research will hopefully shed some light on the validity of these hypotheses and push the field closer to the ultimate goal of an effective, knifeless treatment for obesity.

1.2 Roux-en-Y Gastric Bypass

1.2.1 Introduction

Originally, Roux-en-Y gastric bypass (RYGB) was designed as a restrictive and malabsorptive procedure to physically limit the amount of food that a person could ingest and digest \(^6\). While the physical re-arrangement of the gut does lead to some degree of physical restriction, recent evidence suggests that food restriction alone cannot account for all of the effects of RYGB \(^7\). This has led to the investigation of other possible mechanisms through which RYGB could be acting to maintain long term weight loss. Due to the complexity of the systems that regulate energy balance in mammals, potential mechanisms of action are numerous and include: a changed gut hormone secretion profile, changes to the intestinal microbiome, alterations in central nervous system regulation of food intake and food preference, altered bile acid secretions, adaptive hypertrophy of the gut, and increased energy expenditure \(^13\).

1.2.2 History

Bariatric surgeries can be described as either restrictive or malabsorptive, with the former reducing the amount of food one can store in the stomach, and the latter reducing the ability of the intestines to absorb nutrients. Roux-en-Y gastric bypass was first described in 1967 by Mason and Ito as both a restrictive and malabsorptive procedure designed to reverse obesity by bypassing both the stomach and a significant portion of the intestines \(^6\). This procedure was
refined and then described in its current form by Torres in 1983 and slowly began to rise in popularity as an effective treatment for obesity. Confidence in the procedure increased even further in 1994 with the introduction of laparoscopic techniques that reduced mortality rates to 0.1% and improved post-operative conditions for patients. Since then RYGB has continued to increase in prevalence with over 150,000 surgeries being performed every year.

1.2.3 Procedure

RYGB surgical procedures have been outlined in detail previously. Briefly, RYGB aims to alter the flow of nutrients from the esophagus directly into the jejunum, completely bypassing the stomach and duodenum (Figure 1.1). Only a small gastric pouch with a volume of about 5% of the original stomach remains between the esophagus and jejunum. The pyloric sphincter is bypassed and food is therefore no longer able to be stored in the stomach for any significant period of time before passing into the jejunum. The bypassed stomach and duodenum are not removed, but are instead connected to the lower jejunum to maintain the flow of bile acids and pancreatic juices into the intestines. This preserves the ability to digest food in the shortened intestinal tract to prevent excessive malabsorption.

1.2.4 Efficacy

Patients undergoing RYGB have been shown to lose more than 30% body weight in the first year after surgery. There is a slight trend towards weight regain after the first few years,
Figure 1.1 Schematic illustration of the pre- (a) and postoperative anatomy after Roux-en-Y gastric bypass (b) and Vertical Sleeve Gastrectomy (c). Image reproduced with permission.  

but even after 15 years RYGB patients show a 25% body weight decrease on average when compared to pre-surgical levels. These results have led the National Institutes of Health to classify bariatric surgery as the only known effective long term treatment for obesity. Since 1991, no other treatment has obtained this distinction.  

In addition to maintaining long term weight loss, RYGB has been shown to be effective in reducing a large number of obesity related co-morbidities including type 2 diabetes, heart disease, various cancers, sleep apnea, high blood pressure, high cholesterol, and joint problems. Perhaps the most significant of these is the improvement seen in patients with type 2 diabetes. In one study, complete resolution of type 2 diabetes was observed in 76.8% of RYGB patients. While improvements in glycemic control can be expected after weight loss, it has been suggested that resolution of type 2 diabetes occurs rapidly after the procedure before any significant weight loss has occurred. While this idea has since been contested, this was the
first indication that RYGB surgery may not just be purely restrictive and malabsorptive, but may
in fact cause significant changes in the metabolism of patients.

1.2.5 Animal models

Despite the wealth of studies describing the beneficial effects of RYGB, there is little
known about the actual mechanisms through which RYGB acts. In recent years a number of labs
have developed their own RYGB models using obese rats or mice to begin investigating
potential mechanisms of action. These various RYGB models have consistently shown effects
similar to those seen in humans including weight loss, improvement in glycemic control, and
elevated postprandial secretion of gastric hormones such as peptide YY (PYY) and glucagon-like
peptide 1 (GLP-1)\textsuperscript{21}. Unfortunately, there is a severe lack of standardization in the RYGB
models used by different labs, and therefore comparison between groups has been difficult.

One major difference across RYGB models is the size of the gastric pouch. In humans,
the gastric pouch is left as small as possible, with a resulting volume of less than 5% of the
original stomach\textsuperscript{22}. In rats, the size of the gastric pouch ranges from less than 5% to more than
20% of the original volume\textsuperscript{23}. This is the result of the technical difficulties involved in creating
such a small gastric pouch in rats. While a skilled micro-surgeon can successfully create a small
gastric pouch, a review of surgical techniques has shown that many labs use staplers to transect
the stomach, preventing fine control over the size of the gastric pouch and leaving up to 20% of
the original stomach volume\textsuperscript{24}. The effects of a variable gastric pouch size have not been
carefully studied in rat models of RYGB, but a larger pouch could lead to slower transportation
of food to the intestines, which in turn could alter the physiological response to a meal. In
humans it has been shown that gastric pouch size does affect weight loss, with a smaller pouch having the best outcome for patients \(^{25}\).

Another source of variation between RYGB models is in the length of the intestinal limbs. A review of rat models showed that the roux limb ranged from 10 cm to 50 cm, the biliopancreatic limb length ranged from 10 cm to 40 cm, and the common limb ranged from 18 cm to 34 cm \(^{23}\). Unfortunately, in humans there is also a large amount of variation in what is considered to be the optimal limb length, so there is no human equivalent to base a standardized rodent model on \(^{26,27}\). Differences in intestinal length necessarily alter the degree of malabsorption experienced by rats after RYGB. However, malabsorption has not been shown to be a major mechanism through which RYGB acts, so it is unknown how big of an impact limb length has on surgical outcomes. The intestines are also a major endocrine organ, and variations in limb length may lead to variations in the hormonal response to an ingested meal.

In addition to the variation in surgical techniques, there is significant variation in the mortality rates reported across different RYGB models. Mortality rates range from as low as 0.1% to as high as 35% \(^{23}\), but on average mortality rates are extremely high compared to the human mortality rate of 0.1% \(^9\). Excessive mortality rates are concerning in that they call into question surgeon skill, and therefore how healthy experimental animals are even when they survive surgery. Standardization of RYGB surgical techniques will hopefully make it easier to train surgeons and decrease variation among different research groups.

### 1.3 Vertical Sleeve Gastrectomy

Vertical sleeve gastrectomy (VSG) has a more varied history than RYGB, but was also described in its current form as a purely restrictive procedure in 2001 \(^{28}\). VSG aims to reduce the
volume of the stomach while maintaining the normal flow of nutrients through the gut. Compared to RYGB, VSG is relatively simple. 80% of the stomach is removed along the greater curvature, leaving only a small channel of tissue along the lesser curvature. The remaining tissue is sutured closed resulting in a tube like structure called the gastric sleeve (Figure 1.1). This technique was originally developed in 1999 in an attempt to reduce complications in obese patients that were at high risk when undergoing a procedure known as the duodenal switch. Patients would first undergo VSG to induce initial weight loss. About six months later, once their BMI was reduced to acceptable levels, they would be given the more invasive duodenal switch procedure. To the surprise of the surgeons, VSG alone was effective in maintaining long term weight loss without the need for a duodenal switch. VSG has since become a common weight loss surgery due to its less-invasive nature, and has recently surpassed RYGB in popularity.

In addition to maintaining long term weight loss, VSG appears to induce all of the metabolic changes observed after RYGB. Obese patients with type 2 diabetes tend to have rapid improvement in all markers associated with diabetes, possibly independent of weight loss. The hormonal profile of patients with VSG also mirrors RYGB, with postprandial increases in gastric hormones such as GLP-1, PYY, and amylin. The striking similarities in the outcomes of RYGB and VSG have led many researchers to believe that the two surgeries share a similar mechanism of action, and that both can be used interchangeably in rodent models for research. VSG has since become popular among researchers due to the simpler surgical techniques involved. This is especially true in mouse models where the microsurgery involved in performing RYGB is beyond the skill of many surgeons, and VSG is the only reasonable substitution.
1.4 Mechanisms of Action

1.4.1 Physical restriction

Physical restriction of food intake was conventionally believed to be the mechanism through which bariatric surgeries elicit their effects. It was hypothesized that a smaller stomach would fill more quickly and create mechanical pressure that would lead to earlier satiation after a meal. Even as recently as 2008 it was thought that increased gastric pressure was a possible mechanism of action for VSG. However, recent data has shown that neither VSG nor RYGB seem to rely on physical restriction for any of their major effects.

Comparisons with a procedure known as the adjustable gastric band (AGB) have been the most useful in determining the role of physical restriction in bariatric surgery. Unlike VSG and RYGB, AGB reduces stomach volume without inflicting any surgical trauma on the digestive tract of the patient. This is performed by inserting a silicone band that is fitted around the stomach near the esophageal junction (Figure 1.2). The band is filled with saline and becomes enlarged, compressing the stomach and limiting food storage to a small gastric pouch anterior to the band. After AGB, total gastric volume available for food storage is reduced to around 15-20 ml, about the same volume as the remaining gastric pouch after RYGB, and much smaller than the 150-200 ml gastric sleeve left after VSG. Despite this significant physical restriction, studies have shown that AGB cannot achieve the level of weight loss observed after RYGB and VSG in either the short term or long term.
Additional evidence against a restrictive mechanism is that both VSG and RYGB rats have been shown to be able to increase food intake to presurgical levels in order to increase body weight when necessary. After calorie restriction, VSG rats have reduced body weight and will overeat to return to a healthy weight once food is returned. It has also been shown that pregnant and lactating VSG rats are able to increase food intake in response to an increased demand for energy. In a study performed by our own lab, we showed that RYGB rats are also capable of increasing food intake to increase body weight. After intracerebroventricular infusions of the melanocortin-3/4 receptor antagonist SHU9119, RYGB rats increased their daily food intake by 100%, and increased fat mass by 15% over 2 weeks. These studies found that even though meal size may be restricted after VSG and RYGB, rodents maintain the ability to increase food intake by simply increasing meal frequency. It is therefore unlikely that weight loss is due to purely physical restriction.
1.4.2 Gastric Hormones

One alternative to physical restriction is the hypothesis that both RYGB and VSG alter communication between the gut and other organs leading to changes in the regulation of body weight and glucose homeostasis. The gut is one of the most complex systems in mammals and relies heavily on both endocrine and neural signaling to communicate with other organs in the body. The stomach and intestines secrete over 20 different peptide hormones that regulate a number of functions such as appetite control, insulin and glucagon secretion, gut motility, intestinal blood flow, and cell proliferation. Neural signaling in the gut is also complex with both heavy innervation by the autonomic nervous system (ANS) and a large independent network of neurons known as the enteric nervous system (ENS). These systems play a large role in regulating food intake, metabolism, and energy balance, all of which are dysregulated in obesity. Disruption of endocrine and/or neural signaling after gastric bypass may be responsible for the improvements seen in patients after surgery.

The earliest observations involving endocrine changes after gastric bypass involved the hormones ghrelin and GLP-1. Ghrelin is a peptide hormone secreted by cells in the stomach that has been shown to increase food intake and reduce insulin sensitivity when administered exogenously. These effects promote obesity and diabetes, and it was thought that by removing the stomach after gastric bypass, there would be a reduction in ghrelin and therefore a decrease in food intake and an increase in insulin sensitivity. It was indeed found that ghrelin is reduced after VSG. However, this same study found that VSG was fully effective in ghrelin deficient mice, indicating that ghrelin signaling is not required for the full effects of VSG. The same group also found that ghrelin signaling was not significantly altered after RYGB, and is therefore unlikely to play a role in that surgery either.
GLP-1 is another hormone that received early attention when it was found to be elevated postprandially in patients undergoing either RYGB \(^{45-49}\) or VSG \(^{17,50}\). GLP-1 is an incretin hormone secreted by L-cells in the lower intestine that acts to reduce food intake and increase insulin secretion \(^{51,52}\), two effects that are the hallmark of gastric bypass. In addition, a correlation was found between patients who had the most weight loss and patients who had the most elevated GLP-1 following RYGB \(^{53,54}\). Elevated GLP-1 following a meal in RYGB patients was therefore hypothesized to be one of the factors responsible for the beneficial effects of the surgery. Unfortunately, this hypothesis has not been well supported by the data. RYGB was found to be fully effective in animals that were either GLP-1 receptor (GLP-1r) deficient or receiving intracerebroventricular infusions of GLP-1r antagonists \(^{55}\). Additionally, it was shown that VSG is fully effective in GLP-1r deficient mice \(^{56}\). However, these studies have only looked at GLP-1 independently, despite the fact that the gut secretes a number of other hormones. It is still possible that GLP-1 signaling works in concert with other hormones such as cholecystokinin (CCK), PYY, glucagon-like-peptide 2 (GLP-2), neurotensin, somatostatin, gastric inhibitory peptide (GIP), or any of the other gastric hormones.

1.4.3 CNS control of appetite

The central nervous system also plays a role in the regulation of energy balance and metabolism. The gut is heavily innervated and is able to communicate with the brain through the vagus nerve. Through this gut-brain axis, the vagus nerve is able to relay information when stimulated by signaling molecules such as ghrelin \(^{57}\), CCK \(^{58}\), and PYY \(^{59}\), as well as information about gastric distension and pressure \(^{60}\), to help control meal size and appetite. This information is sent to areas of the brain involved in appetite control and feeding behavior, such as the area
postrema (AP), nucleus of the solitary tract (NTS), parabrachial nucleus (PBN), hypothalamus, and the central amygdaloid nucleus (CeA)\(^1\). Specific neural pathways that have been shown to be important in the regulation of appetite and food intake include the melanocortin system, the neuropeptide-Y-pro-opiomelanocortin (NPY-POMC) neural circuit in the hypothalamus, and the NTS-PBN-CeA circuit driven by calcitonin-gene-related peptide (CGRP) neurons. These systems have been shown to be extremely powerful, and when manipulated can induce either severe obesity\(^2\) or complete loss of food intake\(^3\), even to the point of death.

It is unknown exactly how RYGB and VSG interfere with these regulatory systems. There is conflicting evidence regarding melanocortin signaling, with one study showing it to be necessary for mice to receive the beneficial effects of RYGB\(^4\), but another showing it to not be required for VSG in rats\(^5\). It is unclear whether this is a species specific difference, or a difference between RYGB and VSG surgery, but more information will be necessary to determine if melanocortin signaling plays an important role in either surgery. The NTS-PBN-CeA CGRP circuit has not been well studied after RYGB, but has begun to be investigated due to its profound ability to induce anorexia. Our lab has found a correlation between reduced food intake following RYGB and increased signaling through this pathway (data unpublished), but a causative role for this pathway has yet to be shown. Additional evidence supporting CNS involvement in the beneficial effects of gastric bypass can be found in vagotomy studies. It has been shown that preservation of the vagus nerve following RYGB is necessary to receive the full weight loss effects of the surgery\(^6,7\), suggesting that some signal is being sent through the vagus nerve to help reduce appetite and food intake. It remains to be seen how exactly gastric bypass causes altered signals to the CNS.
1.4.4 Bile acids

A change in bile acid (BA) secretion has also been proposed as a potential mechanism of action for RYGB and VSG surgeries. In addition to aiding in the digestion of fatty acids in the small intestines, BAs work in an endocrine fashion to help regulate metabolism and energy expenditure. The most studied receptor for BAs is TGR5, which is expressed in a number of different tissues. In the intestines, TGR5 activation leads to increased secretion of GLP-1 and PYY from L-cells. As mentioned earlier, these are incretin hormones which stimulate the release of insulin and aid in maintaining proper blood glucose levels. In addition, TGR5 is expressed directly on beta cells within pancreatic islets of the pancreas, and TGR5 agonists have been shown to cause an increase in insulin secretion. In brown adipose tissue (BAT), TGR5 activation leads to increased energy expenditure through thermogenesis, an effect which is believed to help protect against obesity. BAs have also been shown to work through the FXR receptor in the intestines to promote the release of FGF19, which is important in the regulation of glucose and lipid disposal. Together, this evidence supports the positive role of BAs in regulating metabolism and glucose homeostasis.

Circulating BA levels were found to be reduced in obese patients when compared to lean, an effect which is normalized by both RYGB and VSG surgeries. This observation has led to the proposal that increased BA secretion following gastric bypass may play a causal role in subsequent weight loss and diabetes resolution. Unfortunately, this claim has not been well supported by the data. One human study showed no significant increases in BA concentrations until a year after surgery, which conflicts with the almost immediate improvements experienced by most patients. More rigorous TGR5 loss of function studies will be needed to elucidate the role of signaling through this receptor following gastric bypass. One study looking
at the role of the FXR receptor showed more promising results. VSG was found to be ineffective at maintaining weight loss in FXR-ko mice compared to wild type. Interestingly, Ryan et al. found genotype specific changes in the microbiota of the mice following VSG, which may suggest that FXR signaling works through the microbiome rather than direct signaling to any specific tissue. More studies will be necessary to determine whether or not BAs actually play a causal role in the beneficial effects of gastric bypass.

1.4.5 Microbiome

Recently, changes to the microbiome have been suggested as another possible mechanism of action for gastric bypass. Early studies showed that the intestinal flora of obese mice differs from lean mice with a significant increase in Firmicutes and decrease in Bacteroidetes. Interestingly, this same effect was later found in humans, and was found to be reversed by weight loss induced by RYGB. It is unknown whether or not this alteration in microbiota is simply due to the increased energy intake of obese subjects, or if it plays a more causative role in the development of obesity. However, some evidence supporting the latter was found in experiments with germ free mice. Fecal transplants from obese mice into germ free lean mice caused obesity even when the germ free mice were given a standard low fat diet, while transplants from lean mice had no significant effect on weight gain.

There are a number of ways that intestinal flora are able to interact with the host to influence metabolism and energy homeostasis. The most obvious method is an increased ability to digest and extract energy from carbohydrates through fermentation. However, an obese microbiota was found even in mice fed a high fat and nearly carbohydrate free diet, suggesting increased energy availability from fermentation is not a major factor. It has since been shown
that the microbiome may interact with the host through other mechanisms. One such mechanism is an increase in the presence of lipopolysaccharide (LPS), a molecule found in the cell wall of gram-negative bacteria. Increased circulating levels of LPS have been found in obese mice fed a high fat diet \(^{82}\), and are associated with metabolic endotoxemia, a state of chronic low grade inflammation of adipose tissue that is thought to be associated with the development of obesity. Changes in intestinal flora have also been linked to changes in gene expression of receptors involved in fatty acid metabolism and storage. Angiopoietin-related protein 4 (Angptl4) is a protein expressed in skeletal muscle and adipose tissue that inhibits the uptake of fatty acids and promotes fatty acid oxidation, promoting a lean phenotype. This protein was found to be decreased in normal mice compared to germ free mice, suggesting that this gene is responsive to the intestinal microbiota \(^{83}\).

While there is a significant amount of corollary data relating specific differences in microbial phenotype between obese and lean subjects, very little evidence has been generated suggesting an actual causative role of the microbiome in the generation or reduction of obesity. Some studies using probiotics to alter the intestinal flora of obese humans showed modest results \(^{84}\), however the actual composition of the microbiome was never investigated in these studies and it is therefore unknown what effect the probiotics actually had. More work with probiotics and antibiotics will be needed to determine if the microbiome can actually be used to prevent or reverse obesity, and whether or not this “reprogramming” of the intestinal flora plays a crucial role in gastric bypass.
1.5 Conclusions

It is clear that the gut is an enormously complex system with numerous overlapping, and probably redundant, regulatory mechanisms. The massive reconstruction of the gut following gastric bypass surgeries interferes with most, if not all, of these mechanisms and is therefore quite difficult to study. While no individual mechanism has been found to be required to obtain the beneficial effects of RYGB or VSG, a number of critical systems have been identified. It is likely that the full effect of gastric bypass does not rely on a single mechanism, but is instead due to multiple systems changing in concert. Identifying these systems is an arduous process, but will hopefully lead to treatments for obesity that are just as effective as gastric bypass without the need for such invasive and dangerous surgery.

1.6 References


CHAPTER 2: ROUX-EN-Y GASTRIC BYPASS SURGERY INCREASES NUMBER BUT NOT DENSITY OF CCK-, GLP-1-, 5-HT-, AND NEUROTENSIN-EXPRESSING ENTEROENDOCRINE CELLS IN RATS

2.1 Introduction

Gastrointestinal hormones play a major role in the control of appetite and can cause either orexigenic or anorexigenic behaviors by acting through the gut-brain axis. These hormones are released by endocrine cells in the stomach and intestines in order to relay nutritional information about recently consumed food to the brain. An increase in the release of anorexigenic hormones, and/or a decrease in the release of orexigenic hormones, has long been considered a mechanism through which RYGB may act to promote weight loss. Support for this idea includes the observation that after RYGB in humans there is an increase in the postprandial circulation of the lower gut hormones GLP-1 and PYY. These are anorexigenic hormones produced by L-cells in the lower gut. However, despite a high level of correlation between weight loss and circulating levels of these hormones, there is still no causative evidence to suggest that this is a major mechanism of action for RYGB.

In addition to GLP-1 and PYY, the lower gut has significant populations of enteroendocrine cells that produce other hormones such as CCK, serotonin (5-HT), and neurotensin. CCK was the first gastrointestinal hormone shown to have an effect on satiation. The role of CCK in mediating weight loss after RYGB has been controversial due to the fact that early studies found no change in circulating levels of CCK in either humans or rodents. However, since then, there have been a number of studies showing clear increases in circulating CCK following RYGB. Despite this contradictory data, we believe that altered CCK levels

have the potential to play some role after RYGB. The data surrounding 5-HT and neurotensin have been even less clear, but as lower gut hormones we suspected that they should be subjected to the same stimuli as GLP-1, PYY, and CCK, and are therefore worth investigating.

There have been no real attempts at determining the mechanism through which RYGB increases circulating levels of the lower gut hormones. It is possible that there is an increase in releasing stimuli, an increase in the number of hormone releasing cells, or a decrease in the rate at which the hormones are broken down in circulation. An increase in the number of hormone releasing cells is of particular interest due to observations made in mouse models of short bowel syndrome (SBS); a procedure that is similar in many ways to RYGB. In one model of SBS, mice undergo massive resection leaving only 25% of the original small intestine intact. Following resection, the remaining small intestines experience significant muscular and mucosal hyperplasia. It has since been shown that this response is caused by GLP-2, an enteric hormone that is released after injury to the small intestines. GLP-2 is thought to promote intestinal adaptation after injury in order to allow the healthy mucosa to increase its ability to absorb nutrients to make up for any lost or damaged tissue. Similar to the mouse model of SBS, rat models of jejuno-ileal bypass and biliopancreatic diversion have been found to experience muscular and mucosal hyperplasia in the intestines following surgery. In actual RYGB, it has been shown that there is an increase in circulating levels of GLP-2, as well as an increase in crypt depth and villus height. Despite this, no studies have been done characterizing the effect of the adaptive response on enteroendocrine cells within the intestinal mucosa. We aim to investigate whether or not the adaptive response occurs in our model of RYGB, and if it does, how it affects enteroendocrine cell populations.
2.2 Methods

2.2.1 Animals

Male Sprague-Dawley rats weighing ~200 g (Harlan Industries, Indianapolis, IN) were housed individually in wire-mesh cages at a constant temperature of 21-23º C with a 12h light-dark cycle (lights on at 07:00, off at 19:00). Food and water were provided ad libitum unless otherwise indicated. Animals were made obese by putting them on a two-choice diet for 14-16 weeks consisting of normal laboratory chow (Kcal%: Carb, 58; Fat, 13.5; Prot, 28.5, # 5001, Purina LabDiet, Richmond IN ) and high-fat diet (sweet HF diet; Kcal%: Carb, 20; Fat, 60; Prot, 20, D12451, Research Diets, New Brunswick, NJ), with each of the diets containing sufficient minerals and vitamins. Small amounts of liquid Ensure diet (Kcal%: Carb, 64; Fat, 21.6; Prot, 14.4, Abbott Laboratories, Columbus, OH) was also provided before surgery. They were then randomly assigned to either RYGB or sham surgery. After surgery, only liquid Ensure was provided as a source of food for the first 10 days, before giving back increasing amounts of regular chow and HF diet. A lean control group without surgery was placed on a regular chow diet throughout the experiment.

All protocols involved in this study were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center in accordance with guidelines established by the National Institutes of Health.

2.2.2 Roux-en-Y gastric bypass and sham surgery

Details of the RYGB surgical procedure have been reported earlier 23,24(11, 28). Briefly, the procedure resulted in a gastric pouch of about 20% of the total gastric volume, connected to a
15 cm-long Roux limb, a 25 cm-long common limb, and a roughly 40 cm-long biliopancreatic limb.

Sham-surgery consisted of the same procedure as for RYGB, except that the transected jejunum was re-anastomosed, one small incision in the jejunum 25 cm from ileocecal valve and one in the gastric fundus were sutured closed, and the cutting stapler was laid over the stomach without firing. Thus, a similar amount of surgical trauma was inflicted, but the normal flow of nutrients was preserved in sham-operated rats. To overcome potential deficits in iron absorption and development of anemia, rats were administered a macromolecular dextran-iron complex (Iron Dextran injectable, catalog # 93963, 5 mg, s.c.; Town and Country, Ashland, OH) once a week for the first two weeks after RYGB surgery. Additional doses were administered to individual anemic animals if indicated.

2.2.3 Tissue preparation and Immunohistochemistry

Ten to eleven months after surgery, rats were euthanized after overnight food deprivation, and the gastrointestinal tract was harvested. This time point after surgery was chosen because the animals were involved in behavioral tests and hormone measurements, the results of which were reported previously. Furthermore, this extended postsurgical period allowed us to assure the long-term effectiveness of the procedure on body weight and adiposity, and most likely captured the final state of adaptive changes. Half the tissue samples from each location were immersion-fixed in 10% buffered formalin for later immunohistochemical processing, and the other half was rapidly frozen in liquid nitrogen for later quantitative PCR or Western immuno-blotting. After washing in saline, the fixed samples were soaked in 18% sucrose, 0.05% sodium azide in 0.1M phosphate buffered saline (PBS) solution for cryoprotection. Tissue samples were then frozen.
and 30 micron-thick sections were cut in a cryostat and separated into five series. For immediate processing, sections were held in PBS (+4°C), whereas for long-term storage (~20°C), a cryoprotectant solution (50% PBS, 30% ethylene glycol, 20% glycerol) was used. Free-floating sections were pre-treated with 0.5% sodium borohydride in PBS to minimize aldehyde cross-linking of the fixative and treated with a blocking solution containing 5% normal goat or donkey serum in PBS containing 0.5% Triton X-100 (PBST). Appropriate washing in PBS followed all incubations. The sections were incubated in primary antibody (CCK-antibody 1: 400, raised in rabbit against synthetic CCK-39, Peninsula, Belmont, CA; GLP-1 mouse monoclonal antibody 1:5000, ImmunDiagnostik, Bensheim, Germany, distributed by ALPCO, Windham, NH; rabbit anti-neurotensin 1:5000, ImmunoStar, Hudson, WI; 5-HT antibody 1:50’000, rabbit anti-serotonin, ImmunoStar, Hudson, WI; goat anti-ghrelin 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA.) diluted in PBST containing 0.1% gelatin and 0.05% sodium azide, overnight at room temperature, or at 4°C for 20h, under gentle agitation by an orbital shaker. After thoroughly rinsing in PBS, they were reacted with Cy-3-conjugated anti-rabbit, anti-goat, or anti-mouse IgG secondary antibody (Jackson Immuno Research, West Grove, PA) before mounting and cover slipping in an aqueous medium. Staining was completely absent in control experiments with omission of the primary antibody or, for CCK-39, with incubation in primary antibody that had been preabsorbed by adding 10 mol sulfated CCK-8 for 4h at room temperature. Further, manufacturer-supplied data indicate that this antibody is more selective for CCK than antibodies raised against CCK-8, since it is not inhibited by preabsorption in 10 nmol gastrin I, Tetrin, and Somatostatin-14.
2.2.4 Immunohistochemical Analysis

Whole sections were mounted onto Superfrost glass slides using Fluoromount G (Southern Biotechnology, Birmingham, AL) as the mounting medium. Sections were viewed using a Zeiss Axioplan fluorescence microscope. Counts were performed visually. Cells that were immunoreactive but did not have clear enteroendocrine cell morphology, such as 5-HT-positive mast cells, were not counted. Counts were based on averages from 2-4 intestinal cross-sections from each limb for each animal. For Ghrelin cell counts, the average of 7 sections from corpus of the stomach was used.

2.2.5 Morphological measurements

Tissue measurements were performed visually using a Zeiss Axioplan fluorescence microscope in conjunction with a 10x10 ocular grid at 20x magnification. A micro slide ruler was used to determine that the 10x10 grid had a 50 micrometer resolution. For sampling purposes, two sections were chosen from each limb of each animal. Measurements of tissue thickness were taken at four points on each section for the longitudinal muscle, circular muscle, sub-mucosa, and mucosa (combined thickness of crypts and villi). If four measurements could not be taken on each of the two sections selected, two additional sections were chosen for that particular limb and counted to provide additional measurements. The average thickness of the tissue was calculated for each limb of each animal, and these averages were used for statistical analysis.

For measurements of total and mucosal cross-sectional areas, darkfield images were generated in a Leitz microscope with a 1x macro lens and areas determined using the paper weighing method. Average cross-sectional areas were calculated from at least 3 representative
sections per rat and limb. Enteroendocrine cell densities were calculated as number of cells per mm$^2$.

2.2.6 Statistical analysis

Morphological measurements and enteroendocrine cell counts were analyzed by two-way ANOVA with gut location as within subject factor and surgical group as between subject factor, followed by Bonferroni-adjusted multiple comparisons. Ghrelin cell counts were analyzed by one-way analysis of variance. All data were expressed as mean ± SEM.

2.3 Results

2.3.1 RYGB-induces hypertrophy of the Roux and common limbs

The diameter of the Roux and common limbs, but not the biliopancreatic limb, were greatly increased compared to the corresponding gut segments of sham-operated rats (Figure 2.1). Similarly, the total and mucosal surface areas of the cross-sectioned Roux and common limbs were significantly increased (Figure 2.2). Both the longitudinal and circular muscle layers of the Roux limb and the circular muscle layer in the common limb were about 2-fold thicker in RYGB rats compared with both other groups (Figure 2.3). No differences in the thickness of the external muscle layers were observed in the biliopancreatic limb. The total mucosa (crypt plus villous height) in the Roux and common limbs was also significantly thicker in RYGB rats compared to both control groups. No significant differences were observed for the submucosa thickness.
Figure 2.1 Representative dark field images of cross-sections of the biliopancreatic (a, d) Roux (b, e), and common (c, f) limbs or their corresponding gut segments of rats at 10-11 months after RYGB (a-c) or sham surgery (d-f), showing hypertrophy of the Roux and common limbs but not the biliopancreatic limb. Surface areas in mm² of the representative cross-sectioned segments are shown in the left bottom corner of each panel. Scale bar in f, 1.0 mm.

Figure 2.2 Average total and mucosal cross-sectional areas of the Roux and common limbs from rats at 10-11 months after Roux-en-Y gastric bypass surgery (n = 4) or sham-operation (n = 4). * p < 0.05 compared with both chow-fed controls and sham-operated rats, based on t-tests.
Figure 2.3  Thickness of intestinal wall layers in the biliopancreatic, Roux, and common limbs (or their equivalent) in non-operated, chow-fed lean rats (n = 4), sham-operated, high-fat fed obese rats (n = 4), and Roux-en-Y gastric bypass rats (n = 4), 10-11 months after surgery. * p < 0.05 compared with both chow-fed controls and sham-operated rats, based on ANOVA followed by Bonferroni-adjusted multiple comparisons.

2.3.2 Increased number, but not density, of enteroendocrine cells in the Roux and common limbs

ANOVA yielded significant effects of surgical treatment (F[2,24] = 20.7, p< 0.001) and gut location (F[2,24] = 9.9, p< 0.001), as well as a significant interaction (F[4,24] = 7.6, p< 0.001).

As expected, in chow fed lean control rats, almost twice as many CCK-IR cells were expressed in the upper small intestine (the area corresponding to the biliopancreatic limb), compared with the lower small intestine (the area corresponding to the Roux and common limbs). (Figure 2.4,
Figure 2.4 Examples of CCK (a,b), GLP-1 (c,d), and 5-HT (e,f), immunoreactive enteroendocrine cells in the Roux limb, neurotensin immunoreactive cells in the common limb (g,h), and ghrelin immunoreactive cells in the gastric antrum of the remnant stomach (i), 10-11 months after Roux-en-Y gastric bypass surgery. Note that different magnifications are used to emphasize shape and distribution of enteroendocrine cells in villi and crypts. Abbreviations: cm, circular muscle; sm, submucosa. Scale bars, 100 µm for all panels.

Figure 2.5). These numbers were not significantly different in high fat diet-induced obese rats with sham operation. However, in RYGB rats, the numbers of CCK-IR cells were significantly higher in the Roux limb ($t = 3.5, p< 0.005$) and common limbs ($t = 7.4, p< 0.001$), but not in the
biliopancreatic limb. Compared to sham-operated rats, the increase was about 2-fold in the Roux limb and about 3-fold in the common limb.

In the Roux limb, the density of CCK-cells was not significantly different after RYGB, compared with sham-operated rats (Figure 2.7), because the cross-sectional area of the mucosa was also increased about 3-fold (Figure 2.2). In the common limb, the density of CCK-cells was significantly higher after RYGB compared with sham-operated rats (Figure 2.7), as the increased cell number was not fully compensated for by increased mucosal area.

ANOVA yielded significant effects of surgical treatment ($F[2,24] = 34.4, p< 0.001$) and gut location ($F[2,24] = 23.2, p< 0.001$), as well as a significant interaction ($F[4,24] = 8.2, p< 0.001$). There was only a slight and non-significant gradient in the expression of GLP-1-IR cells across the small intestine in chow-fed, lean control rats, with the proximal part (biliopancreatic and Roux limbs) expressing about 150 cells per section and the distal part (common limb) expressing about 200 cells per section (Figure 2.4, Figure 2.5). There were no significant differences between chow-fed lean and sham-operated, high fat-fed obese rats. In contrast, after RYGB, there was a significant ($t = 4.2, p< 0.001$) 2-fold increase of GLP-1-IR cell expression in the Roux limb, and a significant ($t = 8.4, p< 0.001$) 2.5-fold increase in the common limb, compared to sham-operated rats. Most of the CCK-IR and GLP-1-IR cells were found in the crypt area, and there were no obvious differences in this distribution pattern between the three groups. The density of GLP-1 cells was, however, not significantly different after RYGB, compared with sham-operated rats (Figure 2.7).
Figure 2.5  Number of CCK (a) and GLP-1 (b) immunoreactive cells in the biliopancreatic, Roux, and common limbs (or their equivalent) in non-operated, chow-fed lean rats (n = 3), sham-operated, high-fat fed obese rats (n = 4), and Roux-en-Y gastric bypass rats (n = 4), 10-11 months after surgery. * p < 0.05 compared with both chow-fed controls and sham-operated rats, based on ANOVA followed by Bonferroni-adjusted multiple comparisons.

The 5-HT antibody stained both enteroendocrine and mast cells, but only enteroendocrine cells were counted based on their distinctive location in the epithelium and elongated shape (Figure 2.4). ANOVA yielded significant effects of surgical treatment (F[2,26] = 23.5, p< 0.001) and gut location (F[2,26] = 13.7, p< 0.001), as well as a significant interaction (F[4,26] = 10.5, p< 0.001). In chow-fed control rats we found similar numbers of 5-HT-IR enteroendocrine cells per cross-section at the locations corresponding to the three surgical limbs, and sham surgery did not result in significant changes (Figure 2.6). After RYGB, however, there was a significant increase in 5-HT-IR enteroendocrine cells in the Roux (t = 7.26, p<0.001) and common limb (t = 4.0, p<0.01), but not in the bypassed biliopancreatic limb. The density of 5-HT-cells was not significantly different after RYGB compared to sham-operated rats (Figure 2.7).
Figure 2.6  Number of 5-HT (a) and neurotensin (b) immunoreactive cells in the biliopancreatic, Roux, and common limbs (or their equivalent) in non-operated, chow-fed lean rats (n = 4), sham-operated, high-fat fed obese rats (n = 4), and Roux-en-Y gastric bypass rats (n =4), 10-11 months after surgery. * p < 0.05 compared with both chow-fed controls and sham-operated rats, based on ANOVA followed by Bonferroni-adjusted multiple comparisons.

ANOVA yielded significant effects of surgical treatment (F[2,23] = 21.7, p< 0.001) and gut location (F[2,23] = 81.4, p< 0.001), as well as a significant interaction (F[4,23] = 6.8, p< 0.001). In chow-fed control rats, there was a proximal-to-distal gradient in the number of neurotensin-IR enteroendocrine cells, with fewer cells at the location corresponding to the biliopancreatic limb and more in the mid-jejunum (corresponding to the Roux limb) and the distal jejunum (corresponding to the common limb) (Figure 2.4, Figure 2.6). Furthermore, as determined for the biliopancreatic limb and unlike the other types of cells, the majority of neurotensin-IR cells were located in the villi (29 ± 6 cells) and a minority in the crypts (13 ± 1 cells; p < 0.05). The number of cells was not much different in sham-operated rats, but in RYGB rats, there were significantly more neurotensin-IR cells in the Roux-limb (t = 5.8, p<0.001) and in the common
limb (t = 6.1, p<0.001), but not in the biliopancreatic limb. However, because the cross-sectional areas of the Roux and common limb mucosa was increased about 2-3-fold (Figure 2.2), the density of neurotensin cells was significantly decreased after RYGB, compared with sham-operated rats (Figure 2.7).

![Graph showing cell densities](image)

Figure 2.7 Enteroendocrine cell densities in the Roux and common limbs of rats at 10-11 months after Roux-en-Y gastric bypass (n = 4) or sham surgery (n =4). * p < 0.05 compared with sham-operated rats, based on ANOVA followed by Bonferroni-adjusted multiple comparisons.

There were very few ghrelin-IR cells in the small intestines and no obvious differences between the treatment groups (data not shown). In the corpus of the bypassed stomach, we found slightly more ghrelin-IR cells in RYGB rats compared to sham-operated rats and chow-fed control rats, but the difference was not statistically significant (p = 0.096) (Figure 2.3, Figure 2.8).
Figure 2.8  Number of ghrelin-immunoreactive cells in the gastric mucosa of non-operated, chow-fed lean rats (n = 4), sham-operated, high-fat fed obese rats (n = 4), and Roux-en-Y gastric bypass rats (n = 4), 10-11 months after surgery.

### 2.4 Discussion

The mechanisms responsible for an increase in the secretion profile of lower gut hormones such as GLP-1, PYY, CCK, neurotensin, and 5-HT following RYGB are not well understood. It is not clear whether this is caused by an increase in releasing stimuli, increased secretory efficiency, increased number of hormone releasing cells, reduced degradation of hormones in circulation, or some other combination of factors. Here, we provide evidence that there is a significant increase in the number of enteroendocrine cells in the Roux and common limbs after RYGB. This proliferation appears to not be a response specific to enteroendocrine cells, but instead is due to generalized hyperplasia of the intestinal mucosa. This is evidenced by the fact that, apart from CCK, there is no significant increase in the density of enteroendocrine cells in the mucosa, implying that other mucosal cells are also proliferating at approximately the same rate. An increase in the number of hormone releasing cells may be responsible for the increase in hormone secretion observed after RYGB. However, our data does not provide causative evidence for this claim, and does not rule out other possible mechanisms.
Our findings both support and extend data reported by other labs showing mucosal hyperplasia after various intestinal surgeries. Previously, Nadreau et al \(^{21}\) showed that intestinal adaptation occurs as early as 7 weeks after biliopancreatic diversion, a procedure very similar to RYGB. Secretory studies have also shown that there is an increase in plasma concentrations of GLP-2 and the growth factor IGF-1 as early as 2 weeks after RYGB \(^{22}\). These reports, along with our data showing significant hyperplasia at 10-11 months post surgery, support the idea that intestinal adaptation happens quickly after surgery and is maintained indefinitely.

We have also extended our understanding of intestinal adaptation by showing that despite a global increase in the circulating levels of GLP-2 after RYGB, only the Roux and common limbs experienced significant levels of tissue growth. It has been previously suggested that exposure of the lower small intestine to nutrients is not required for adaptive hyperplasia to occur \(^{25}\). However, most studies to this point have used animal models with a continuous digestive tract to investigate this phenomenon, and therefore have not reported a differential response in intestinal hyperplasia in the presence of high circulating GLP-2. Our data shows that despite systemic increases in circulating GLP-2, adaptive hyperplasia still only occurs in the intestinal limbs that are exposed to nutrients. One possible explanation for this phenomenon involves the observation that, in the absence of exposure to enteral nutrients, the intestinal mucosa experiences atrophy \(^{26}\). High circulating GLP-2 following RYGB, which induces hyperplasia, may be balancing against the expected atrophy of the biliopancreatic limb, resulting in no net change in the mucosa. In any case, we show clear evidence that the presence of enteral nutrients plays a key role in the adaptive response of the intestines.

Our study paves the way for new experiments looking into enteric hormones as a major mediator of the beneficial effects of RYGB. GLP-1 and PYY are the hormones that have been
most strongly linked to weight loss and improved glucose homeostasis after RYGB. Both act as anorexigenic hormones, and GLP-1 has the additional benefit of acting as an incretin hormone to help normalize blood glucose levels. One study using PYY-deficient mice found that PYY was necessary for RYGB to lower body weight, however their observation period was only 10 days after surgery and therefore may not give a complete picture of PYY’s role in RYGB. Another study blocked GLP-1 receptor activity by systemically infusing the GLP-1 receptor antagonist Exendin(9-39) for 5-10 days after surgery. They found that this partially prevented RYGB associated anorexia, but did not significantly affect weight loss. Again, this study only looked at the early postoperative period and therefore does not give a clear picture of the role of GLP-1 in RYGB mediated weight loss. Other gut hormones, such as CCK, 5-HT, and neurotensin have been even less well studied.

More in depth long term experiments will be needed to determine whether or not GLP-1, PYY, or any of the other enteric hormones are required to mediate the beneficial effects of RYGB. However, experiments using either antagonist infusion or receptor knockouts are generally limited to looking at a single hormone at a time. This is due to the fact that long term infusion of multiple receptor antagonists quickly becomes prohibitively expensive, and generation of double, triple, or quadruple receptor knockout models is equally difficult. With our observation that proliferation of enteroendocrine cells is a likely mechanism leading to the increase in circulating anorexigenic hormones after RYGB, it may be possible to create a rodent model that does not attempt to interfere with individual hormones and their receptors, but instead attempts to interfere with the adaptive hyperplasia of the small intestine. This would prevent RYGB from having any significant effect on circulating levels of these hormones without
affecting their activity anywhere else in the body. It would also allow for the investigation of the enteric hormones as a group rather than trying to interfere with them each individually.

2.5 References


CHAPTER 3: ADAPTIVE HYPERPLASIA AND REPROGRAMMING OF INTESTINAL GLUCOSE METABOLISM ARE NOT PRESENT AFTER VERTICAL SLEEVE GASTRECTOMY IN RATS

3.1 Introduction

In the months following the experiment outlined in the previous chapter, a number of studies were published investigating the role of both GLP-1 and PYY in mediating the effects of RYGB on obese rats and mice. One study performed by our lab found that pharmacological inhibition of central GLP-1 signaling using the GLP-1r antagonist exendin(9-39) reversed the effects of RYGB and caused rats to increase their food intake and return to pre-surgical obese levels. However, this effect was even more pronounced in sham control animals, and is therefore unlikely to be a unique mechanism through which RYGB acts. In the same study we found that central infusion of the Y2-receptor antagonist BIIE0246 had no significant effect on food intake in either the RYGB or control groups receiving sham surgery. Other studies performed around the same time showed that GLP-1 signaling did not seem to be necessary for the effects of RYGB or VSG surgeries. Surprisingly, no evidence to date has been able to support the idea that any single gastric hormone plays a significant role in mediating the weight loss effects of RYGB. It would appear that, except for the possibility of a complex interaction resulting from altered secretion profiles of multiple gastric hormones simultaneously, elevated levels of these hormones do not play a causative role in weight loss after RYGB. This also cast doubt on the mechanistic role of adaptive hyperplasia, which was implicated in the increased release of gastric hormones following RYGB in the previous chapter.

In the wake of these studies, a seminal paper was published describing another mechanism through which adaptive hyperplasia could mediate the beneficial effects of RYGB\(^4\). Saeidi et al found that intestinal hyperplasia after RYGB lead to massively increased glucose uptake by the intestines, as well as significant reprogramming of intestinal glucose metabolism. They found that key proteins in the glycolytic pathway were upregulated, and that there was a decrease in the amount of glucose shunted towards the tricarboxylic acid cycle, suggesting that the intestines were being switched to a more anabolic metabolic profile. They also found that total glucose disposal via the intestines was doubled, causing the intestines to become the largest glucose sink in the body, with glucose disposal per gram of tissue almost reaching the same level as the brain. Their findings support the idea that intestinal hyperplasia following RYGB could still be responsible for the improvements in body weight and glucose homeostasis seen after the surgery by altering the way that glucose is metabolised after consuming a meal.

Here, we further investigated the importance of intestinal hyperplasia following bariatric surgery by characterizing the response after VSG, a similar procedure. VSG has nearly identical effects on both body weight and glucose homeostasis when compared to RYGB\(^5,6\), and is becoming a popular alternative to RYGB due to its less invasive nature. Although there are physical differences between RYGB and VSG surgeries, the effects on obese subjects are so remarkably similar that it is generally thought that the two work through the same mechanism and are interchangeable when generating mouse models to study\(^7,8\). We hypothesized that if the two surgeries do share a common mechanism, and if that mechanism requires intestinal adaptation after RYGB, then we should observe the same response after VSG.
3.2 Methods

3.2.1 Animals and diets

Male Sprague-Dawley rats weighing ~200 g (Harlan Industries, Indianapolis, IN) were housed individually in wire-mesh cages at a constant temperature of 21-23º C with a 12h light-dark cycle (lights on at 07:00, off at 19:00). Food and water were provided ad libitum unless otherwise indicated. Animals were made obese by putting them on a two-choice diet for 14-16 weeks consisting of normal laboratory chow (Kcal%: Carb, 58; Fat, 13.5; Prot, 28.5, # 5001, Purina LabDiet, Richmond IN ) and high-fat diet (Kcal%: Carb, 35; Fat, 45; Prot, 20; D12451, Research Diets, New Brunswick, NJ), with each of the diets containing sufficient vitamins and minerals. They were then randomly assigned to either VSG or sham surgery. A lean control group without surgery was placed on a regular chow diet throughout the experiment.

All protocols involved in this study were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center in accordance with guidelines established by the National Institutes of Health.

3.2.2 Surgery and postoperative care

To remove all solid food particles from the stomach, rats undergoing VSG were fed liquid Ensure (Kcal%: Carb, 64; Fat, 21.6; Prot, 14.4, Abbott Laboratories, Columbus, OH) for 3-5 days. Ensure was removed the night before surgery. Rats were anesthetized with isoflurane and administered atropine (1 mg/kg, s.c.). After laparotomy and removal of the gastric omentum, the stomach was placed on the open tongue of a cutting stapler (Model ATW35 with two straight triple-staple lines; Ethicon, Ithaca, NY), making sure that most of the fundus was below the staple line. The tongues of the stapler were then slowly closed starting from fundus to antrum.
and the knife activated, resulting in the removal of about 80% of the total stomach volume. Any epiploic blood vessels not included in the staple line were ligated and the severed portion of the stomach was removed. The rest of the stomach was put back in place before suturing muscle and skin separately. Sham surgery consisted of laparotomy and removal of the gastric omentum.

RYGB was carried out according to a protocol described in detail earlier. Briefly, a gastric pouch of about 20% of the total gastric volume was anastomosed with a 15-cm-long Roux limb and the 25-cm-long common limb with a roughly 40-cm-long biliopancreatic limb. Sham surgery consisted of the same basic procedure as for RYGB, except that the transected jejunum was reanastomosed and a small incision in the jejunum 25 cm from ileocecal valve and one in the gastric fundus were sutured closed.

After surgery, only liquid Ensure was provided for the first 10 days, before giving back increasing amounts of regular chow and HF diet.

3.2.3 Measurement of body weight, body composition, and food intake

Body weight was monitored daily for the first two weeks, and then was recorded weekly. Body composition was measured before introduction of the high-fat diet (16 weeks before surgery), and monthly after surgery, by using a Minispec LF 90 NMR Analyzer (Bruker Corporation, The Woodlands, TX). This method uses whole body magnetic resonance relaxometry in unanesthetized rodents with excellent linearity and reproducibility.

3.2.4 Immunohistochemical and morphometric analysis

3 months after surgery, rats were euthanized after overnight food deprivation, and the gastrointestinal tract was harvested. Half the tissue samples from each location were immersion-
fixed in 10% buffered formalin for later immunohistochemical processing, and the other half was rapidly frozen in liquid nitrogen for later extraction of protein. Immunohistochemical processing and analysis was carried out as previously reported \(^9\). Briefly, GLP-1 mouse monoclonal antibody 1:5000 (ImmunDiagnostik, Bensheim, Germany, distributed by ALPCO, Windham, NH) was used as primary antibody. Sections were viewed using a Zeiss Axioplan fluorescence microscope. GLP-1 immunoreactive enteroendocrine cells were counted only if the nucleus was clearly visible. Counts were averages from 2-4 intestinal cross-sections from each limb for each animal.

Measurements of cross-sectional areas and mucosal thickness have been described earlier \(^9\). Briefly, average thickness of the mucosa (combined thickness of crypts and villi) was calculated for each limb of each animal, and these averages were used for statistical analysis.

Cross-sectional areas were measured based on darkfield images generated in a Leitz microscope with a 1x macro lens and image analysis software (ImageJ, NIH, Bethesda, MD). Average cross-sectional areas were calculated from at least 3 representative sections per rat and limb.

3.2.5 Western blotting

Frozen intestinal tissues were crushed to powder using liquid nitrogen cooled mortar and pestles. Frozen powder was transferred to conical tubes and 1 ml of ice cold T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Waltham, MA, Cat # 78510) containing 1X Halt Protease Inhibitor Cocktail (complete EDTA-free, Thermo Fisher Scientific Inc., Cat # 87785) was added. Samples were homogenized for 30 s on ice, and then sonicated using a Branson Sonifier S-450 Digital Ultrasonic Cell Disruptor/Homogenizer (Branson.
Ultrasonics Americas, Danbury, CT). Samples were centrifuged at 12,000 rpm for 20 min. Protein concentration of each lysate was determined using BCA Protein Assay Kit (Thermo Fisher). Protein lysates were heated at 95°C for 5 min with Laemmli buffer and separated by polyacrylamide gel electrophoresis through 12% SDS gels. Proteins were transferred to PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA, Cat. # 162-0177) at 100V for 70 min at 4°C, using a wet transfer system (Mini Trans-Blot, Bio-Rad Laboratories, Inc.). Membranes were blocked in 5% milk dissolved in PBS-T (1X PBS + 0.1% Tween20) for 45 min at room temperature. Primary antibodies were diluted in 5% milk or 5% bovine serum albumin in PBS-T according to manufacturer’s recommendations. Anti-beta 2 Microglobulin antibody (B2M, rabbit monoclonal [EP2978Y], ab75835, Abcam, Cambridge, MA) was used at 1:20,000 dilution. Hexokinase II antibody (rabbit monoclonal [C64G5], Cat. # 2867, Cell Signaling Technology, Danvers, MA) was used at 1:1,000 dilution. Blots were incubated with primary antibodies overnight at 4°C. After washing with PBS-T, blots were incubated with goat anti-rabbit IgG HRP-linked (Cat # 7074, Cell Signaling Technology) in 5% milk dissolved in PBS-T for 1 h at room temperature. Membranes were developed using Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (PerkinElmer Inc., Waltham, MA, Cat # NEL104001EA).

3.2.6 Statistical analysis

Body weight, body composition, and food intake across time were analyzed by two-way repeated measures ANOVA, followed by Bonferroni’s post-hoc multiple comparison test. Cross-sectional area, mucosal thickness, and the number of GLP-1-IR cells per section were analyzed by two-way ANOVA, and hexokinase II expression by t-test.
3.3 Results

3.3.1 VSG in high-fat obese rats reduces body weight and fat mass to levels of chow-fed controls

Exposure to the high-fat diet for 14 weeks resulted in significantly increased body weight (Figure 3.1a) and fat mass (Figure 3.1b) compared with age-matched chow-fed controls. Subsequent VSG in the obese rats led to a rapid decrease, followed by a slow and steady regain of body weight, whereas sham surgery resulted in only a small transient decrease (Figure 3.1a). At the end of the 3-month observation period, VSG rats weighed 521 ± 11 g, significantly less than sham-operated (~609 ± 11 g), but not significantly different from chow-fed control rats (537 ± 16 g). Body weight loss at 54 days after VSG was almost entirely due to fat mass loss, with only a small loss of lean mass (Figure 3.1b,e). Body weight gain in sham-operated rats was due

Figure 3.1  Effect of VSG in diet-induced obese rats on body weight and composition. (a) Rats exposed to a two-choice high/low-fat diet were either subjected to VSG (closed circles, n=8) or sham surgery (open circles, n= 9) and were compared to rats exposed to regular low-fat chow (open triangles, n=4). Body weight was monitored before and for 3 months after surgery. *p<0.05, VSG vs. sham; ^p<0.05, VSG and sham vs. chow. (b, c) Total fat mass and relative fat mass (adiposity) measured by NMR whole body imaging, 80 days after surgery. (d) Epididymal and retroperitoneal fat pad weights measured at termination, 90 days after surgery. (e) Lean mass at 80 days after surgery. Bars that do not share the same letters are significantly different from each other (based on ANOVA followed by Bonferroni-corrected multiple comparisons)
to both fat mass and lean mass gain (Figure 3.1b,e). Just before the end of the 3-month observation period, total fat mass and percent fat mass (adiposity) were significantly lower in VSG rats compared with sham-operated rats, but not significantly different from chow-fed controls (Figure 3.1b,c). Both epididymal and retroperitoneal fat pads weighed significantly less in VSG compared with sham-operated rats and were only slightly higher than in chow-fed controls (Figure 3.1d). Lean mass was slightly, but not significantly, lower 80 days after VSG compared with both sham-operated and chow controls (Figure 3.1e).

3.3.2 VSG transiently reduces energy intake

Before surgery, high-fat-fed rats ingested slightly (~10%) but significantly more calories than chow-fed controls (Figure 3.2). VSG initially reduced total energy intake but, it returned to presurgical levels after about 20 days and remained at this level for the rest of the study. Sham operation reduced energy intake only briefly, followed by an overshoot and subsequent stabilization at a level about 15% higher than VSG animals. Average daily energy intake in VSG rats was significantly reduced by about 40% during the first month and by about 15% during the second and third month, compared to sham-operated rats (Figure 3.2 inset).

3.3.3 VSG does not increase cross-sectional area of small intestine and thickness of mucosa

The small intestine of VSG rats does not show the hypertrophic response seen after RYGB (Figure 3.3). Cross-sectional areas (Figure 3.3a, b) and mucosal thickness (Figure 3.3c) of duodenum, jejunum and ileum, were similar in VSG and sham-operated rats. In contrast, cross-sectional areas and mucosal thickness for the corresponding intestinal locations (Roux and common limbs) are significantly larger after RYGB.
Figure 3.2  Effect of VSG in diet-induced obese rats on food intake. Total intake of high and low fat offered as two-choice diet monitored before and 3 months after surgery in rats with VSG (closed circles, n=8) or sham surgery (open circles, n=9), compared with non-surgical rats fed low-fat regular chow (open triangles, n=4). *p<0.05, VSG vs. sham; # p<0.05, VSG vs. chow; ^p<0.05, VSG and sham vs. chow. The inset shows average daily total (from high- and low-fat diet) food intake during the first, second, and third month post-surgery. Bars that do not share the same letters are significantly different from each other (based on ANOVA followed by Bonferroni-corrected multiple comparisons)

Figure 3.3  Effect of VSG and RYGB in rats on cross-sectional area and mucosal thickness at three locations of the small intestine. (a) Representative histological cross sections of the duodenum, jejunum, and ileum after VSG (n= 6) or sham surgery (n=4) and of the corresponding biliopancreatic (BP), Roux (Rx), and common (CM) limbs after Roux-en-Y gastric bypass (n=4). Note the absence of any enlargement of small intestinal diameter and surface area after VSG. Scale bar=1 mm. (b) Quantitative assessment of cross-sectional area of corresponding intestinal segments after VSG and RYGB and their respective sham surgeries. (c) Mucosal thickness is not significantly different between VSG and sham rats, but as reported earlier is significantly higher after RYGB compared to all other groups. *p<0.01, RYGB vs. sham
3.3.4 VSG does not change number of GLP-1 immunoreactive L-cells

Immunohistochemical staining of GLP-1-producing L-cells showed no difference of number and distribution between VSG and sham-operated rats (Figure 3.4). As expected, the number of L-cells per section was much lower in the duodenum than in the jejunum and ileum. This contrasts with our earlier data and observations by others, showing more than doubling of the number of L-cells and other enteroendocrine cells in the Roux and common limbs but not the biliopancreatic limb after gastric bypass.

![Figure 3.4](image.png)

Figure 3.4 Effect of VSG on number of GLP-1 immunoreactive enteroendocrine cells at three locations of the small intestine. (a) Representative images of GLP-1 cells in the jejunal mucosa after VSG and sham surgery. Scale bars=50 μm. (b) Quantitative analysis of the number of GLP-1 cells per section in the duodenum, jejunum, and ileum of rats after VSG (n=6) and sham surgery (n=7).

3.3.5 RYGB, but not VSG increases hexokinase-II protein expression in small intestine

Protein expression of hexokinase II was significantly elevated in the Roux limb of rats 10 months after RYGB (Figure 3.5b), confirming earlier observations by Saeidi et al. However, expression of this key glycolytic enzyme was not increased in the upper small intestine 3 months after VSG (Figure 3.5a).
3.4 Discussion

Adaptive hyperplasia and reprogramming of intestinal glucose metabolism has been identified as a potential mechanism through which RYGB acts to lower body weight and improve glucose homeostasis in obese patients. Studies have shown that these improvements are almost identical in both rodents and humans after VSG. Because both surgeries have such similar outcomes, it is often hypothesized that they share a common mechanism of action.

Supporting this idea is the observation that after VSG there is a similar increase in circulating levels of GLP-2, the hormone responsible for intestinal hyperplasia following RYGB. It is therefore expected that VSG should cause adaptive hyperplasia of the intestines just like RYGB.

Figure 3.5 Effect of VSG (a) or RYGB (b) in rats on protein expression of hexokinase II (HKII) in the jejunum and corresponding Roux limb. Original Western blot images were generated from one single gel for VSG and it’s sham control (a), and one single gel for RYGB and it’s sham control (b), but have been cropped and condensed for clarity. Quantitative densitometric analysis is shown on the right for VSG (n=6) and sham VSG (n= 6) as well as for RYGB (n=5) and sham RYGB (n=5). There was no significant difference between VSG and sham-operated rats, but significantly increased hexokinase II after RYGB compared with sham. *p<0.01, RYGB vs. sham. B2M beta-2-microglobulin
Here, we show that there is no intestinal hyperplasia in rats after VSG. This is especially surprising considering the increased circulating levels of GLP-2 observed after the surgery\textsuperscript{12}. However, growth of the intestinal mucosa is due to the combined effect of an increase in cellular proliferation in the crypts of lieberkuhn and a decrease in apoptosis in the villi\textsuperscript{13}. It was therefore possible that after VSG there was a reprogramming of intestinal tissue to an anabolic state, but a proportional increase in the rate of apoptosis in the villi leading to no net tissue growth. For this reason we also investigated the change in the expression of hexokinase-II, an important enzyme in the glycolytic pathway. We were able to verify an increase in hexokinase-II expression in our RYGB model, supporting the claims made my Saeidi et al\textsuperscript{4}, but were not able to identify any change after VSG. It is therefore unlikely that glucose metabolism in the intestines is significantly altered after VSG.

Our study does have a few limitations. First, we have only looked at one time point approximately 3 months after surgery. It is possible that hyperplasia and altered glucose metabolism exist earlier in the postoperative period. However, in RYGB animals these effects have been observed in both the early postoperative period and as late as 12 months after surgery\textsuperscript{9,14}. Due to the similar long term effects of both RYGB and VSG on reduced body weight, we would expect intestinal hyperplasia to persist after VSG if it was a major mechanism of action. A second limitation is that we did not verify the altered gut hormone secretion and improved glucose homeostasis observed in other rodent models of VSG. However, our model has similar body weight, body composition, and food intake profiles when compared to other VSG models that have been more completely characterized. We therefore find it unlikely that the metabolic profile of our VSG model is significantly different than those that have been reported. Despite these limitations, we have provided significant evidence showing that there is no intestinal
adaptation or altered glucose metabolism in rodents after VSG. It is therefore unlikely that this is the common mechanism shared by RYGB and VSG to improve body weight and glucose homeostasis in obese patients. It remains to be seen if these surgeries work through different mechanisms, or if they share a mechanism that has yet to be identified.

3.5 References


CHAPTER 4: DISCUSSION

4.1 Key Findings

Gastric bypass surgery remains the only weight loss solution that is effective long term 1. Despite this, little is known about the mechanisms through which the surgery elicits its effects. In this thesis, I characterized and compared the adaptive response of the intestines following RYGB and VSG to test two major hypotheses that have been proposed regarding these surgeries. The first major hypothesis is that adaptive hyperplasia of the intestines following RYGB is responsible for improved body weight and glucose homeostasis. How this is achieved is unknown, but it has been suggested that adaptive hyperplasia may reprogram intestinal glucose metabolism leading to an anabolic metabolic profile 2. The intestines then become a glucose sink helping the body to maintain healthy blood glucose levels and aiding in weight loss. It is also possible that an altered gut hormone secretion profile following RYGB leads to complex interactions that ultimately reduce body weight and improve glucose homeostasis; however, this claim has not been well supported in the literature. The second major hypothesis that has emerged is that both RYGB and VSG, similar but ultimately different surgeries, work through a common mechanism of action 3. This has been suggested due to the fact that body weight, food intake, gut hormone secretion profile, glucose metabolism, and long term retention of weight loss are nearly identical in humans and rodents undergoing either surgery 4,5. Because VSG is much less invasive and requires much less surgical skill to perform, a number of labs have begun using animal models of VSG in place of RYGB in order to study the effects of gastric bypass in general 6. However, there has been no mechanistic evidence to this point that has shown that the two surgeries actually share a mechanism of action. Here, we outlined data that shows that these two major hypotheses are mutually exclusive.
We have shown that there is significant intestinal hyperplasia following RYGB, and that this may lead to improvements in body weight and glucose homeostasis through a number of potential mechanisms (Chapter 2). We have also shown that intestinal hyperplasia and a reprogramming of intestinal glucose metabolism is absent in rats following VSG, and that this cannot be a mechanism of action for that surgery (Chapter 3). We are then left with two options: RYGB and VSG share a common mechanism which does not involve intestinal hyperplasia, or the two surgeries do not share a mechanism of action. While neither major hypothesis that was investigated was directly supported or rejected, the realization that both cannot be true will help direct future research into bariatric surgery.

4.2 Future Directions

The idea that RYGB and VSG do not share a common mechanism is hard to support due to the overwhelming similarities in surgical outcomes between the two procedures. However, the two surgeries do have very different effects on the structure and morphology of the digestive tract (Figure 1.1), and therefore could possibly work through separate mechanisms. The evidence presented by Saiedi et al clearly shows that there is a significant alteration in glucose metabolism in the intestines following RYGB, and it is hard to say that this would have no effect on glucose homeostasis in obese or diabetic patients. Fortunately, this is a problem that can be solved relatively easily by experiments that restrict adaptive hyperplasia following RYGB. It has been shown that GLP-2 is the major signal required for the adaptive response of the intestines following injury or disease, and that blocking GLP-2 signaling prevents adaptive hyperplasia. If hyperplasia and reprogramming...
of glucose metabolism is required to receive the beneficial effects of RYGB, then obese GLP-2r/− mice would be expected to lose little to no weight following RYGB compared to those receiving sham surgery. Alternatively, if RYGB surgery is successful in reducing body weight despite the absence of adaptive hyperplasia, it will be clear that some other mechanism is required. While neither outcome would help determine what mechanism is at play after VSG, this experiment would still help elucidate the role of adaptive hyperplasia of the intestines following RYGB, which is currently a major question in the field of gastric bypass surgery.

If RYGB and VSG do share a common mechanism, it will take a bit more work to determine what exactly that mechanism is. Currently, the most likely candidates are changes to the microbiome, bile acid secretion, and/or CNS control of energy balance (reviewed in chapter 1). These three mechanisms do not directly depend on a specific gut morphology, and therefore may be altered in similar ways despite the differences in gut rearrangement after RYGB or VSG surgery. The research being done into these three potential mechanisms is extensive and outside the scope of this thesis, but it will be exciting to see what role they play in bariatric surgery.

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