Helene Johannessen

Translational Research in Obesity: Animal Models of Bariatric Surgery and the Underlying Mechanisms

Thesis for the degree of Philosophiae Doctor

Trondheim, September 2014

Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine

NTNU – Trondheim
Norwegian University of
Science and Technology

NTNU
Norwegian University of Science and Technology

Thesis for the degree of Philosophiae Doctor

Faculty of Medicine
Department of Cancer Research and Molecular Medicine

ISBN 978-82-326-0380-0 (printed ver.)
ISBN 978-82-326-0381-7 (electronic ver.)
ISSN 1503-8181

Doctoral theses at NTNU, 2014:232

Printed by NTNU-trykk

Norsk sammendrag

Translasjonsforskning i fedme: Dyremodeller for fedmekirurgi og underliggende mekanismer

Antallet mennesker med fedme øker raskt og fører til en global fedmeepidemi. Det eneste alternativet som gir langvarig terapeutisk effekt per dags dato er fedmekirurgi og bruken av slike operasjoner er derfor omfattende. Tatt i betraktning det store antallet pasienter med fedme, risikoen for kirurgiske komplikasjoner og høye kirurgiske kostnader, så er behovet for utvikling av nye, mer skånsomme behandlinger påtrengende. Hovedmålet med denne doktorgraden var derfor å øke kunnskapen om de fysiologiske mekanismene bak fedmekirurgi for å utvikle nye, mindre invasive behandlingsalternativer for fedme, ved bruk av dyremodeller.

I denne doktorgraden fant vi at vagusnerve stimulering/blokkering (VNSB) reduserte kroppsvekten med 10% i normalvektige rotter og at dette var assosiert med redusert matinntak, økt uttrykk av anoreksigene nevropeptider i hjernestammen og hippocampus, samt økt uttrykk av oreksigene nevropeptider i hypothalamus. Injeksjon av Botox i magesekken reduserte kroppsvekten med 20-30% i normalvektige rotter, overvektige rotter og overvektige rotter som hadde fått sleeve gastrektomi. Vekttapet var assosiert med redusert matinntak, økt energiforbruk og økt uttrykk av oreksigene nevropeptider i hypothalamus. Muskarinisk acetylkolin M3 reseptor knockout mus hadde redusert kroppsvekt, økt energiforbruk og økt uttrykk av oreksigene nevropeptider i hypothalamus, men uendret totalt matinntak. Ileal interposisjon og sleeve gastrektomi økte uttrykket av glukagon-lignende peptid-1 (GLP-1) i henholdsvis ileum og bukspyttkjertel i normalvektige rotter. Gastrisk bypass økte energiforbruket og reduserte kroppsvekten med 15%, mens duodenal omkobling reduserte matinntaket, endret spisemønsteret, økte energiforbruket, ga malabsorpsjon og førte til et vekttap på 54% i normalvektige rotter.

Hjerne-mage aksen spiller en viktig rolle i regulering av kroppsvekt, noe som gjør den til et ideelt mål i fedmebehandling. Resultatene fra denne doktorgraden viser at blokkering av den gastriske vagusnerven, ved VNSB eller Botox-injeksjon, kan brukes som minimal invasiv fedmebehandling. I tillegg kan antagonister mot M3 reseptor ha potensiale som behandling av fedme. Kombinasjonen av ileal interposisjon og sleeve gastrektomi er gunstig for å maksimere sekresjon av GLP-1 og har potensiale som metabolsk kirurgi. Resultatene fra denne doktorgraden viser i tillegg at duodenal omkobling gir et større vekttap enn gastrisk bypass gjennom ulike mekanismer. Ved bruk av translasjonsforskning har denne doktorgraden økt kunnskapen om fysiologiske mekanismer bak fedmekirurgi og funnet mindre invasive behandlingsalternativer for fedme.

Helene Johannessen
Institutt for kreftforskning og molekylær medisin (IKM)
Veiledere: Duan Chen og Chun-Mei Zhao
Støttet av European Union Seventh Framework Programme (FP7/2007-13, nº266408)

Ovennevnte avhandling er funnet verdig til å forsvares offentlig for graden philosophiae doctor i molekylær medisin. Disputas finner sted i auditoriet MTA, MTFS, fredag 5. september 2014, kl. 1215.

Table of Contents

Acknowledgements

This thesis was carried out at the Research Group of Experimental Surgery and Pharmacology at the Department of Cancer Research and Molecular Medicine, the Faculty of Medicine, Norwegian University of Science and Technology from June 2011 to May 2014. This thesis is part of the EU-project Full4Health funded by the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n°266408.

I wish to express my greatest gratitude to my supervisor Professor Duan Chen. He has taught me so much these last three years; about science, surgeries, efficiency, hard work, collaboration, commitment, and life. In addition to being a supervisor he has also been a great friend. I especially want to thank him for fulfilling my dream to travel.

The multi-talented Dr. Chun-Mei Zhao has been my co-supervisor, "mother", and friend. I want to thank her for teaching me so much these last years; not only about science but also about life in science, efficiency, accuracy, management, how to treat guests, cooking, and life. I especially want to thank her for all the fun and laughter we have had together, both in the lab and at the office. It has been truly great to take breaks from science once in a while to talk about fashion, travelling, and food.

It has been said that in order to create motivation you need a sense of achievement, to be acknowledged, receive responsibility, and feel that what you do is important. I have been lucky enough to feel all of this during my PhD and for this I can thank Duan and Chun-Mei. We have laughed and celebrated in good times and supported each other when things were tough.

I also wish to thank my colleagues, especially Yosuke Kodama, who has taught me almost all I know about animal research, who was the most patient and humble teacher and a good friend. I also wish to thank Magnus Kringstad Olsen, Anders Øverby, Gøran Andersen, Marianne Waldum Furnes, and Bård Kulseng.

I have spent more time in the animal facility than in my office and working there has been a great pleasure. I want to thank Trine Skoglund for always being positive no matter how many emails I have sent her, Anne Åm for being a great veterinarian, Knut Sverre Grøn for always being positive and talkative, Venke-Lill Nygård for caring so much for the animals, Karin Nykkelmo for being the sweet person she is, and Nils Hagen and Erling Wold for being extremely funny in their own unique ways.

"*A happy life is when you look forward to work in the morning and to going home in the afternoon*" − Professor Duan Chen.

I also wish to thank the people who have made it so great to come home from work. My friends; Line, Guro S, Marianne, Yvonne, Siri, Guro B, Ingrid, Camilla, Nathalie, and Marit. You are fantastic people and I am so happy to have you in my life! It has been great support to have such beautiful friends to share the ups and downs of PhD life with.

I want to thank Espen for always being there for me; cheering with me in good times and supporting me in tough times. For buying me flowers when I have been locked in the lab for 3 weeks, for feeding me when my blood glucose levels are dangerously low, and for understanding how all-absorbing writing a PhD thesis can be.

Last, but not least, I want to thank my mother, Tove, for always being just a small phone call away and for her unconditional love and proudness.

Trondheim, May 2014
Helene Johannessen

List of Papers

This thesis is based on the following papers, which are referred to by their Roman numerals in the text (I-IV).

I. **Helene Johannessen,** David Revesz, Yosuke Kodama, Nikki Cassie, Karolina P. Skibicka, Magnus K. Olsen, Robert Lyle, Perry Barrett, Suzanne L. Dickson, Jens J. Holst, Jens F. Rehfeld, Geoffrey van der Plasse, Roger A. H. Adan, Bård Kulseng, Thorleif Thorlin, Elinor Ben-Menachem, Chun-Mei Zhao, Duan Chen. *The Gastric Vagus Nerve as a Target for Obesity Treatment*. (Submitted 2014)

II. **Helene Johannessen,** Nikki Cassie, Yosuke Kodama, Perry Barrett, Koji Takeuchi, Bård Kulseng, Duan Chen, Chun-Mei Zhao. *Lack of Muscarinic M3 Receptor Induces a Lean Phenotype through Increased Energy Expenditure Rather than Reduced Food Intake.* (Submitted 2014)

III. **Helene Johannessen,** Yosuke Kodama, Chun-Mei Zhao, Mirta M. Sousa, Geir Slupphaug, Bård Kulseng, Duan Chen. *Eating Behavior and Glucagon-Like Peptide-1-Producing Cells in Interposed Ileum and Pancreatic Islets in Rats Subjected to Ileal Interposition Associated with Sleeve Gastrectomy.* Obes Surg, 2013, **23**: 39-49.

IV. Yosuke Kodama, **Helene Johannessen,** Marianne W. Furnes, Chun-Mei Zhao, Gjermund Johnsen, Ronald Mårvik, Bård Kulseng, Duan Chen. *Mechanistic Comparison between Gastric Bypass vs. Duodenal Switch with Sleeve Gastrectomy in Rat Models.* PLoS One, 2013, **8**: e72896. doi: 10.1371/journal.pone.0072896.

Abbreviations

3Rs	Replace, refine and reduce
AGB	Adjustable gastric banding
AgRP	Agouti-related protein
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AP	Anterior-posterior
ARC	Arcuate nucleus
BMI	Body mass index
BPD	Bilio-pancreatic diversion
BPD/DS	Bilio-pancreatic diversion/duodenal switch
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CCK-1	Cholecystokinin A/1
CCKBR	Cholecystokinin B/2 receptor
cDNA	Complementary deoxyribonucleic acid
CLAMS	Comprehensive laboratory animal monitoring system
CNS	Central nervous system
DIO	Diet induced obese
DMN	Dorsal motor nucleus of the vagus nerve
DS	Duodenal switch
DXA	Dual energy X-ray absorptiometry
EWL	Excess weight loss
FDA	Food and drug administration
FELASA	Federation of european laboratory animal science association
FGFR3	Fibroblast growth factor receptor 3
fMRI	Functional magnetic resonance imaging
FOXA2	Forkhead box protein A2
FXR	Farsenoid-X receptor
GABA	Gamma-aminobutyric acid

GB	Gastric bypass
GLP-1	Glucagon-like peptide-1
GPCR	G protein-coupled receptor
HC	Heavy chain
HDL	High-density lipoprotein
II	Ileal interposition
II-SG	Ileal interposition + sleeve gastrectomy
IL1β	Interleukin-1 beta
INSR	Insulin receptor
LC	Light chain
LDA	Low density array
LEPR	Leptin receptor
M3KO	Muscarinic acetylcholine M3 receptor knockout
mAChR	Muscarinic acetylcholine receptor
MAP	Multineuron acquisition processor
MC4R	Melanocortin 4 receptor
MCH	Melanin-concentrating hormone
MES	2-(N-morpholino)ethanesulfonic acid
ML	Medial-lateral
mRNA	Messenger ribonucleic acid
NA	Nucleus ambiguous
nAChR	Nicotinic acetylcholine receptor
NaCl	Sodium chloride
NEFA	Non-esterified fatty acid
NPY	Neuropeptide Y
NSF	N-ethylmaleimide sensitive fusion protein
NTS	Nucleus of the solitary tract
PBS-T	Phosphate buffered saline with tween
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PYY	Peptide YY

RCT	Randomized controlled trial
RER	Respiratory exchange ratio
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
RT PCR	Reverse transcription polymerase chain reaction
RYGB	Roux-en-Y gastric bypass
SD	Standard deviation
SEM	Standard error of the mean
SG	Sleeve gastrectomy
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble NSF attachment protein receptor
SPSS	Statistical package for social sciences
SV2	Synaptic vesicle glycoprotein 2
TGFβ1	Transforming growth factor beta 1
TNF	Tumor necrosis factor
VBG	Vertical banded gastroplasty
VNS	Vagus nerve stimulation
VNSB	Vagus nerve stimulation/blocking
μ-VNSB	micro vagus nerve stimulation/blocking
WT	Wild-type

Abstract

Background/Aims: The number of obese individuals is increasing rapidly, leading to a global obesity epidemic. Only bariatric surgery has demonstrated long-term therapeutic effects, and the use of these procedures is extensive. Considering the large number of obese patients, the risk of surgical complications and high surgery-related cost, development of new, minimally invasive procedures to treat obesity is urgently needed. The principal objective of this thesis was to better understand the physiological mechanisms of bariatric surgery in order to develop minimally or non-invasive obesity treatments using experimental animal models. To this end, specific objectives were *i*) to evaluate the vagus nerve and the muscarinic acetylcholine M3 receptor as possible therapeutic targets, *ii*) to investigate the potential of ileal interposition alone and combined with sleeve gastrectomy as bariatric and metabolic surgery, and *iii*) to investigate the underlying mechanisms of gastric bypass and duodenal switch.

Materials and Methods: A total of 229 animals (rats and mice) were used. Seven different surgical procedures or genetic manipulation have been tested, i.e., vagus nerve stimulation/blocking (VNSB), gastric botulinum toxin type A (Botox) injection, gene knockout of the muscarinic acetylcholine M3 receptor (M3KO), ileal interposition, ileal interposition with sleeve gastrectomy, gastric bypass, and duodenal switch. Physiological variables measured included body weight development, body composition, food intake, eating behavior, metabolic parameters, gastric acid secretion, energy-balance regulating genes in the brain, gut hormones, gastric emptying, fecal energy content, pancreatic and ileal glucagon-like peptide-1 (GLP-1), pancreatic insulin, and cytokines. Dual energy X-ray absorptiometry, open circuit indirect calorimeter, gastric fistula model, taqman array, *in situ* hybridization, RNA sequencing, radioimmunoassay, acetaminophen absorption assay, adiabatic bomb calorimeter, western blot, immunohistochemistry, and multiplex cytokine assay were used.

Results: VNSB reduced body weight by 10% in normal chow-fed rats, which was associated with reduced food intake, increased expression of anorexigenic neuropeptides in brainstem and hippocampus, and increased expression of hypothalamic orexigenic neuropeptides. Botox injection reduced body weight by 20-30% in normal chow-fed rats, diet induced obese (DIO) rats

and DIO rats that had underwent sleeve gastrectomy. The body weight loss was associated with reduced food intake, increased energy expenditure, and increased expression of hypothalamic orexigenic neuropeptides. M3KO mice had a lean phenotype and displayed increased energy expenditure, increased expression of hypothalamic orexigenic neuropeptides but unchanged total food intake. Ileal interposition and sleeve gastrectomy did not reduce body weight, but increased GLP-1 expression in the ileum and pancreatic islets, respectively. Gastric bypass increased energy expenditure and reduced body weight by 15%, while duodenal switch reduced food intake, altered eating behavior, increased energy expenditure, and caused malabsorption, leading to a 54% body weight loss.

Conclusions: The brain-gut axis plays an important role in the regulation of body weight, which makes it an ideal target for obesity treatments. Blocking the gastric vagus nerve, by VNSB or Botox injection, can be used as non- or minimally invasive obesity treatment, and muscarinic acetylcholine M3 receptor antagonists might have potential as obesity treatment. Combining ileal interposition and sleeve gastrectomy would be beneficial to maximize GLP-1 secretion, and holds potential as a metabolic surgery. Duodenal switch induces greater body weight loss than gastric bypass through different mechanisms.

1. Background

1.1 Translational Research

Translational research can be defined as the process of applying insights, ideas, and discoveries generated through basic research for treatment or prevention of human disease. However, it also involves taking insights, ideas, and discoveries from the clinic back to the basic experimental settings to identify underlying mechanisms. This can shortly be described as "from-bench-to-bedside-and-back" (Fig. 1) [1]. Translational research can be studies discovering new therapeutic strategies, studies defining the effects of therapeutics in humans, studies providing basis for improving therapeutics, studies identifying clinically relevant biomarkers, and studies developing new drugs and procedures [2].

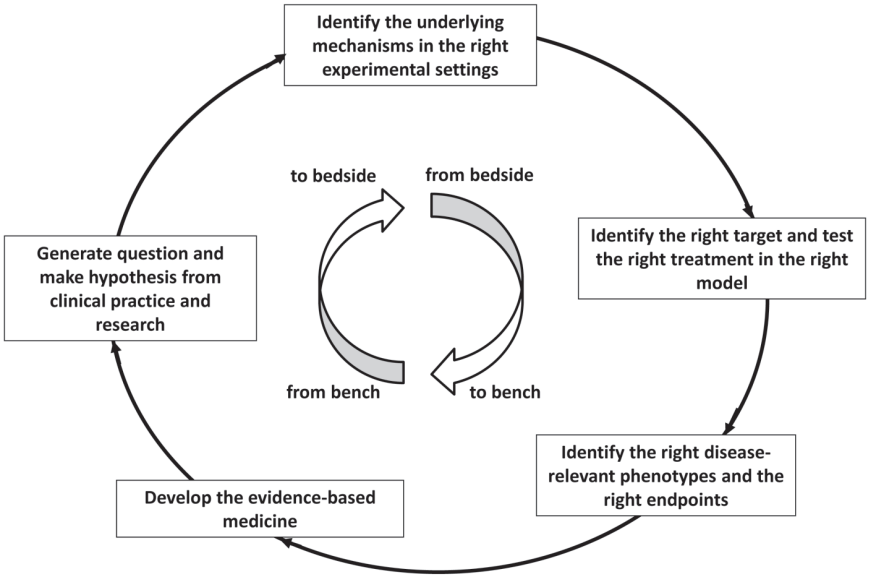


Figure 1 | Model illustrating translational research from-bench-to-bedside-and-back, modified from [1].

In this thesis, the research questions and hypotheses have been generated from clinical practice and research (Table 1).

Table 1 | Hypotheses of translational research investigated in each paper in this thesis.

Clinical practice	Research	Hypothesis	Paper
No significant weight loss in patients after vagotomy or vagal blocking for obesity control (VBLOC)	Role of the vagus nerve in regulation of food intake and energy expenditure	Targeting gastric vagus nerve for new methods in obesity treatment	I – II
Ileal interposition as metabolic surgery?	Ileum as main source of GLP-1 in the circulation	Combination of ileal interposition and sleeve gastrectomy as metabolic and bariatric surgery	III
Greater weight loss after duodenal switch than gastric bypass in morbidly obese	Different postsurgical anatomies	Different physiological mechanisms	IV

This thesis was designed to identify the underlying mechanisms in the right experimental models, i.e. rat models of bariatric surgeries (Papers I, III, and IV) and to identify the right target and test the right treatments (Papers I-IV). The thesis focuses on obesity, therefore biological variables including eating behavior, metabolic parameters, hormones, and neuropeptides in the gut-brain axis have been studied, and the main endpoint has been the body weight loss (Papers I-IV).

The ultimate goal in clinical medicine is to improve patient outcome by maximizing medical efficacy and minimizing adverse effects. To achieve this, evidence-based medicine should be performed wherever it applies. Evidence-based medicine is the integration of clinical expertise, patients' values and research evidence (Fig. 2A). Research evidence includes animal research and preclinical trials, expert opinion, case series/reports, case-controlled studies, cohort studies, and randomized controlled trials (RCTs) (Fig. 2B) [3].

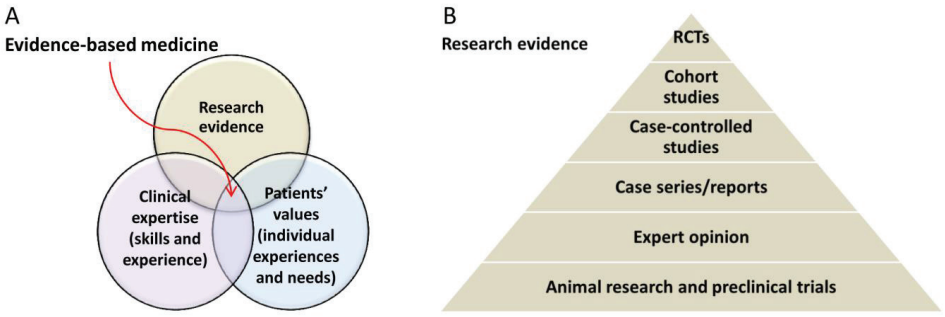


Figure 2 | Illustrations of the components of A) evidence-based medicine and B) research evidence.

However, it is difficult to perform RCTs in the surgical practice where double blinding and placebo controls are both practically challenging and unethical. Therefore, RCTs should not be considered the de facto and sole source of high-level evidence in surgery [4]. There are a number of challenges in evaluating surgical innovation due to the complexity of a surgical intervention, which includes modifications of surgical techniques and procedures, surgical skills, collaborations with medical, nursing, and anaesthesia teams, pre- and post-operative care, and quality of the surgical theatre [5]. Thus, the evidence obtained by animal research and preclinical trials, which have been conducted in this thesis, should be considered a high-level evidence in surgery. Usually, translation of new knowledge into the surgical practice is slow and incomplete, which is frequently due to the animal studies not being timely executed [6]. In this thesis, the animal studies and trials (Papers I, III, and IV) have been conducted either prior or immediately post pilot clinical trials [7-11]. In fact, a clinical phase II trial of gastric antral Botox injection for obesity treatment (REK ref no. 2013/1597, EudraCT no. 2012-004381-18) has been initiated based on and due to the results generated in this thesis (Paper I).

1.2 Obesity

Obesity is a condition where accumulation of excess adipose tissue causes adverse effects on health. In humans, overweight and obesity are assessed by body mass index (BMI), defined as an individual's body mass in kilograms divided by the height in meters squared (kg/m^2). A BMI above 25 kg/m^2 is defined as overweight, while a BMI of more than 30 kg/m^2 is defined as obese. The number of obese individuals is increasing rapidly, leading to a global obesity epidemic [12,13]. Obesity is a major contributor in the development of type 2 diabetes, cardiovascular diseases, certain forms of cancer, overall reduced quality of life, and premature mortality [14,15]. In addition, obesity affects fertility, sexual function, and psychosocial factors such as dissatisfaction with body image and depression [16-19]. Of special concern is the increasing prevalence of obesity in children [20]. Not only is obesity a great health burden, it is also an economic burden to the society as the medical costs for an obese individual is 40% higher than for a normal weight individual [21].

All living organisms, including humans, obey the first law of thermodynamics, stating that the change in internal energy of a closed system is equal to the amount of heat added to the system, minus the amount of work done by the system. This means that imbalance between

energy intake and energy expenditure will result in a change in the body's amount of energy stores (mainly white adipose tissue). It is generally believed that increased consumption of more energy-rich food with high levels of carbohydrate and saturated fats, combined with reduced physical activity, has led to the obesity epidemic [22]. Looking at this from an evolutionary perspective one can explain why this life style is so detrimental. In the ancient hunter-gatherer society food was dependent on physical activity. At this time food supply was never consistent, causing cycles of feast and famine, and of physical activity and rest. To ensure survival during famine, certain genes, the so-called "thrifty genes", evolved to regulate highly efficient intake and utilization of food. For example genes promoting efficient adipose tissue accumulation were beneficial for survival in times of famine. These genes are still a part of our genome, but combining them with today's continuous food supply and physical inactivity can cause metabolic dysfunctions such as obesity [23]. However, in addition to the generally higher energy intake than energy expenditure in today's society, genetics, diseases, psychiatric disorders, medications, environmental factors, and eating disorders are also factors that can cause or contribute to obesity development [16,24-28].

1.3 Obesity Treatment and Bariatric Surgery

Theoretically, treatment of obesity can be achieved by decreasing energy intake and/or increasing energy expenditure. Today, weight loss treatments include diet control, physical exercise, medications, and bariatric surgery [29]. Exercise and diet often fail to induce a long-term body weight reduction [30]. This is because many factors (including the thrifty genes) have evolved to oppose life-threatening weight loss, for example during famine, and will work to attenuate the body weight loss. As energy deficit can threaten survival, pathways increasing food intake and reducing energy expenditure are more powerful than pathways reducing food intake and increasing energy expenditure [31]. In addition, long-term life style changes in diet and physical activity can be difficult to maintain [30]. Pharmacotherapy also have yet limited efficacy [32]. Up to date, only bariatric surgery has been demonstrated to exhibit long-term therapeutic effect [33,34].

The first bariatric surgery, i.e., jejunoileostomy, was performed by Dr. Richard Varco in 1953 and the first gastric bypass was performed by Dr. Edward Mason in 1966. Since then bariatric procedures have gone through many modifications to create the procedures seen in the

clinics today and have been designed to induce restriction and/or malabsorption in the gastrointestinal tract [35]. Restriction is obtained by reducing the gastric volume in order to induce early satiety, reduce food intake, and body weight. Malabsorption is obtained by bypassing the small intestine in order to reduce nutrient absorption and thereby reducing body weight.

Bariatric surgeries are frequently performed worldwide in order to induce weight loss in obese patients (Fig. 3).

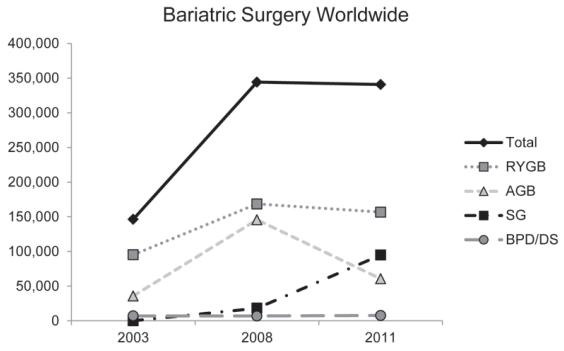


Figure 3 | Number of bariatric surgeries performed worldwide, modified from [36]. RYGB: Roux-en-Y gastric bypass, AGB: adjustable gastric banding, SG: sleeve gastrectomy, BPD/DS: bilio-pancreatic diversion/duodenal switch.

Today, the most frequently performed bariatric surgeries include Roux-en-Y gastric bypass (RYGB), sleeve gastrectomy (SG), and adjustable gastric banding (AGB). In 2011, RYGB, SG, AGB, and duodenal switch (DS) were performed in 47%, 28%, 18%, and 2% of patients, respectively [36]. The RYGB is both a restrictive and malabsorptive procedure where a small gastric pouch is created and a portion of the small intestine is bypassed. SG is a restrictive procedure where 75% of the stomach is removed and AGB is also a restrictive procedure performed by placing an adjustable band around the upper part of the stomach making a small gastric pouch [33]. DS is both restrictive and malabsorptive and consists of a sleeve gastrectomy, an alimentary limb 50% of the total small intestine length, a common channel of 100 cm and a cholecystectomy. While RYGB is most popular, DS seems to have higher efficacy regarding weight loss and improvement of comorbidities, especially in super-obese individuals (BMI $\geq$ 45) [37,38].

Weight loss is often expressed as percent excess weight loss (%EWL), where excess refers to the weight that exceeds "ideal" weight adjusted for height. RYGB, AGB, and DS has been found to induce 62%, 48%, and 70% EWL, respectively [39], while SG has been found to induce 52% EWL one year after surgery [40]. A prospective controlled study showed that bariatric surgery in general induced 23% and 16% weight loss 2 and 10 years after surgery, respectively. After surgery, the patients also recovered from type 2 diabetes, hypertriglyceridemia, low levels of high-density lipoprotein (HDL) cholesterol, and hypertension [41]. Bariatric surgery has also been found to improve mortality [42].

1.4 Need for Minimally Invasive Procedures

There is always a risk of surgical complications, both in general and in particular in bariatric surgery. Even though obesity is in itself a risk factor when surgery is performed perioperative mortality in bariatric surgery is generally seldom and lies around 1% [41,43]. However, a study reported a complication rate up to 40% after bariatric surgery [44]. Such complications can be leaks, infections, small bowel obstruction, anastomotic stenosis, bleeding, pulmonary embolism, vomiting, diarrhea, and respiratory failure [43-45]. Another problem after the malabsorptive procedures is nutritional deficiencies such as calcium, folate, vitamin-B12, vitamin D, and iron-deficiency [46,47]. In addition, many obese patients have diseases that are contraindications for bariatric surgery, and cannot go through today's standard bariatric surgeries. In fact, only 1-2% of the obese people worldwide that are suitable for bariatric surgery undergo these procedures, due to lack of surgical professionals and resources [36,48].

In consideration of the large number of obese patients, the risk of surgical complications, high surgery-related cost, higher demand than capacity for bariatric surgery, and the number of obese patients not qualified for conventional bariatric surgery, development of new, minimally invasive procedures and pharmacological interventions to treat obesity is urgently needed [44].

1.5 The Gut-Brain Axis

The gut-brain axis (communication between the gastrointestinal tract and the central nervous system) plays an important role in the pathogenesis of obesity [49]. Peptide hormones released from the gastrointestinal tract and adipose tissue convey information about the body's energy balance to the brain. This occurs via the vagus nerve or by the hormones acting on brain

regions implicated in energy homeostasis, such as the hypothalamus and brainstem, regulating food intake and energy expenditure. Ingested food interacts with the gut and stimulates or inhibits the release of these peptide hormones [50].

Upon ingestion of food, there is an increase of several anorexigenic peptides in the gastrointestinal tract, such as peptide YY (PYY), cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), amylin, and insulin. Ghrelin is the only known orexigenic peripheral peptide in the gastrointestinal tract, which decrease in concentration when food is ingested and increase in concentration when energy stores are low. The role of ghrelin in regulation of food intake and other physiological functions has been intensively studied since 1999 [50-52]. However, ghrelin knockout mice do not display any phenotype [53]. Secretion of the hormones leptin and insulin is regulated by adiposity, where high levels of adiposity increase the secretion of leptin and insulin, acting on the central nervous system (CNS), reducing appetite. While insulin is secreted by pancreatic β-cells, leptin is secreted by the adipose tissue itself (Fig. 4) [50,51,54].

The vagus nerve is the main neuronal substrate in the gut-brain axis (Fig. 4). In addition to peptide hormones, the vagus nerve responds to mechanical distention of the lumen or gut contraction and to the chemical properties of ingested food, and transmits information about ingestion, absorption, and metabolism of energy to sites in the CNS that regulate ingestive behavior (including the hypothalamus and brainstem) [51,55].

In the brain, the hypothalamus is regarded as a key regulatory component in regulation of food intake and energy expenditure. The arcuate nucleus (ARC) in the hypothalamus acts as a feeding control center, where orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons are thought to play a central role in regulation of food intake and energy expenditure [51]. This nucleus has a modified blood-brain barrier allowing entry of peripheral peptides and proteins. There are extensive reciprocal connections between the hypothalamus and the brainstem, particularly via the nucleus of the solitary tract (NTS) (Fig. 4). The NTS is where the afferent (from periphery to the CNS) vagus nerve signals enter the CNS. Even though the hypothalamus often is regarded as the central feeding organ, the brainstem may have an equally important role in food intake and body weight regulation [56].

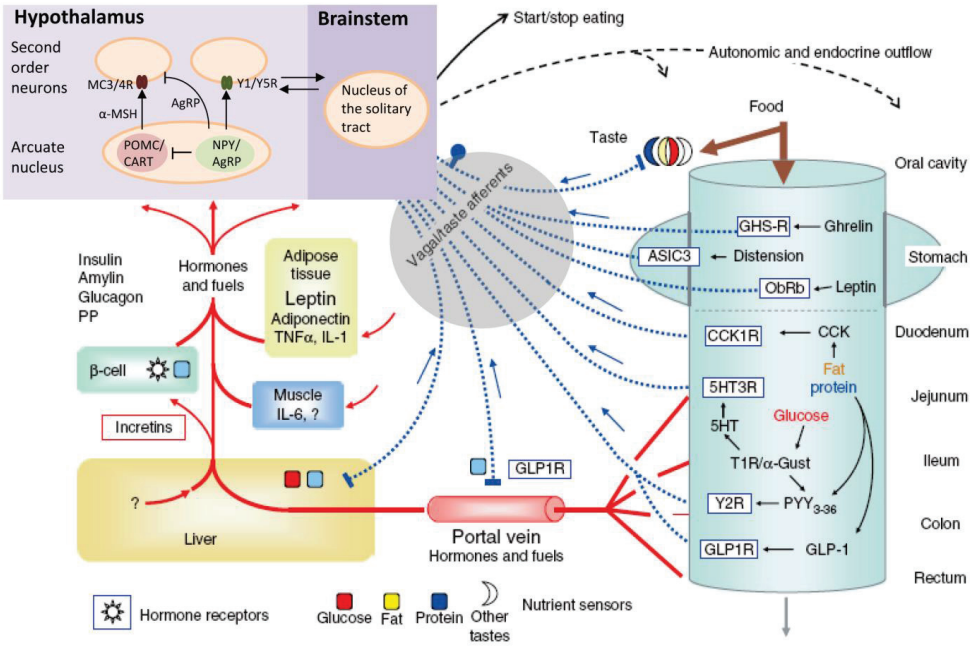


Figure 4 | Illustration of the gut-brain axis, modified from [57,58].

Given that obesity is a long-term disturbance of energy balance, and the central role of the vagus nerve in regulation of food intake and energy expenditure, this nerve could be an ideal target for new, minimally invasive obesity treatments.

1.6 The Vagus Nerve

The latin word vagus means "wandering" and accurately describes the distribution of this nerve, which innervates multiple organs in the neck, thorax, and abdomen [59]. In addition to the regulation of food intake, the vagus nerve is also involved in regulation of cardiac rate, breathing, blood pressure, swallowing, gastric acid, and pancreatic secretions [60-63]. It is the tenth and longest cranial nerve, consisting of 80% afferent and 20% efferent (from CNS to the periphery) nerve fibers [59,64].

Neurons in the vagus nerve has a resting membrane potential of -65 mV, i.e. the voltage inside the cell is 65 mV more negative than outside the cell. Following stimulus from dendrites, sodium ion (Na^+) channels open, causing depolarization and action potentials that propagate

down the length of the neuron unidirectionally. When the action potential reaches the presynaptic nerve terminals it opens voltage gated calcium (Ca^{2+}) channels, causing synaptic vesicles to fuse with the presynaptic membrane and release neurotransmitters into the synaptic cleft through exocytosis. In the synaptic cleft the neurotransmitters diffuse and bind to receptors on the postsynaptic neuron, causing ion channels to open or close, leading to a synaptic potential (Fig. 5). The synaptic potential may further either stimulate or inhibit development of an action potential in the postsynaptic neuron. After generation of an action potiential, Na^+ channels close and potassium ion (K^+) channels open, causing the membrane to repolarize towards its resting potential. However, this repolarization typically overshoots the resting potential to -90 mV and causes a hyperpolarization, which raises the threshold for new stimulus. After hyperpolarization, the Na^+/K^+ pump eventually brings the membrane back to its resting state of -65 mV [65].

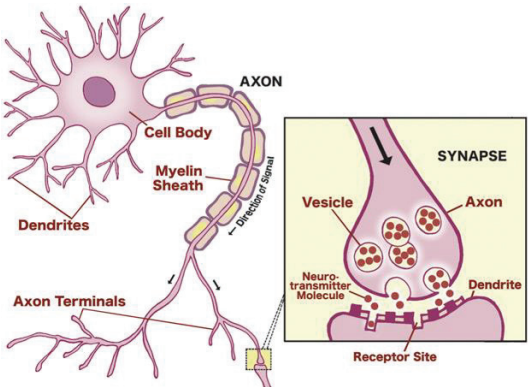


Figure 5 | Illustration of a typical neuron and synapse [66].

The vagus nerve is divided into A, B and C fibers, with the A fibers being the fastest, biggest and most easily activated, followed by B fibers being slower, smaller and somewhat harder to activate, and the C fibers that are slow, small and hard to activate [59,67]. While the A and C fibers contain both afferents and efferents, the B fibers consist only of efferents (Table 2) [68].

Table 2 | Overview of the characteristics of A, B, and C fibers in the vagus nerve [59,67,68].

	A	B	C
Diameter (mm)	5-20	<3	0.40-2
Myelinated	Yes	Also unmyelinated	Mostly unmyelinated
Conductance velocity (ms)	30-90	10-20	0.30-6
Threshold (mA)	0.02-0.20	0.04-0.06	2+
Afferents/efferents	Both	Efferents	Both

The vagus nerve originates from four nuclei in the medulla oblongata; dorsal motor nucleus of the vagus nerve (DMN), nucleus ambiguous (NA), NTS, and spinal nucleus of trigeminal nerve. It exits the cranium through the jugular foramen [69] and as the vagus nerve goes from the medulla to the periphery it divides into different branches along the way, innervating different organs. Two gastric branches innervate the stomach and a portion of the proximal duodenum; the celiac branches innervate the gastrointestinal tract from the duodenum to the transverse colon and the pancreas, while the hepatic branch innervates the distal stomach, liver and proximal duodenum (Fig. 6) [70,71].

The vagus nerve carries afferent signals to the nodose ganglion that relay information to the NTS in the brainstem. Through the NTS, afferent vagal signals reach several parts of the brain including the reticular formation in the medulla, locus coeruleus, parabrachial nucleus, hippocampus, thalamus, dorsal motor nucleus of the vagus, nucleus ambiguous, amygdala, and the hypothalamus (arcuate, paraventricular, and dorsomedial nucleus of hypothalamus) [72-74]. The incoming sensory information is relayed to these regions through autonomic feedback loop, direct projections to the reticular formation, amygdala, and hypothalamus, and ascending projections to the forebrain through the parabrachial nucleus and locus coeruleus [73,75-77]. The dorsal motor nucleus of the vagus nerve is the primary location of gut vagal efferent motor neurons and is found just ventral to the NTS [78].

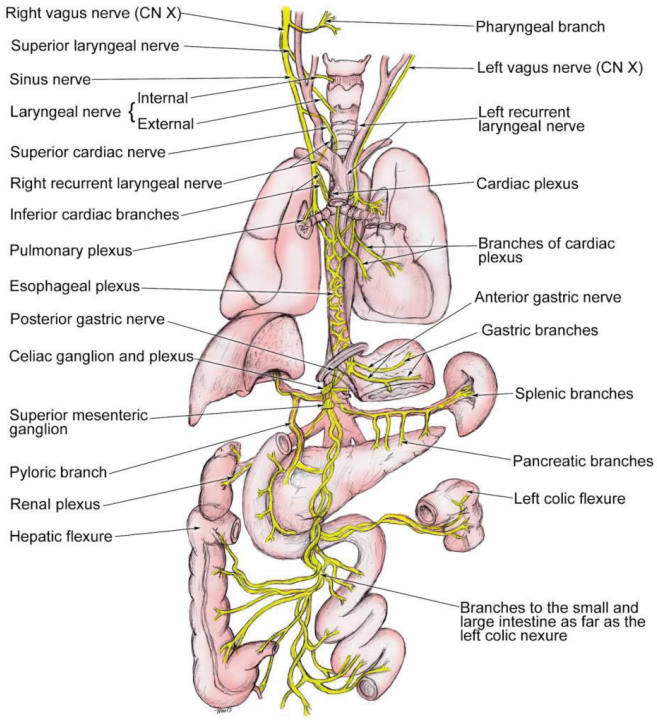


Figure 6 | Distribution of the vagus nerve in the neck, thorax, and abdomen [79].

With so many destinations in both the peripheral and central nervous system the vagus nerve affects a broad range of basic functions, and can be a good target in treatment of different disorders.

1.7 Vagus Nerve Stimulation

Manipulation of the vagus nerve in disease treatment is not a new idea. Vagotomy has been used to treat peptic ulcers with a great success and it was also used for treatment of obesity without success [80-83]. Therefore, it has been replaced by bariatric surgery.

Vagus nerve stimulation (VNS) is a procedure where electrodes are implanted around the vagus nerve, attached to an implanted power source and used to electrically stimulate the nerve. The electrodes used in this thesis are bipolar, i.e. each pole is either a cathode or an anode. As described, the charge in nerve cells is carried by ions, however, the charge in electrical currents is

carried by electrons. The electrical current provided by the electrodes in VNS does not affect the neuronal ion concentrations directly. The anode and cathode generate a negative and positive field respectively and ions with opposite charge are then attracted. At the cathode positive ions are attracted, leaving the outside of the nerve negative. This alters the voltage across the membrane and depolarizes the membrane. When the membrane is sufficiently depolarized this triggers an action potential. The opposite happens at the anode, where the membrane gets hyperpolarized. This hyperpolarization creates an anodic block and the action potential generated by the cathode may be blocked in this direction, resulting in the cathodic pulse only traveling in full extent in one direction (Fig. 7) [84]. Since the cathode is placed proximal and the anode is placed distal in this thesis, the cathodic pulse travels toward the brain (personal communication with Cyberonics, Inc.).

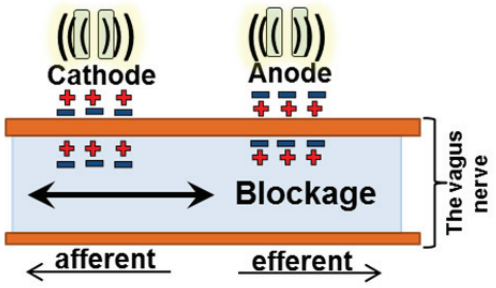


Figure 7 | The mechanism of vagus nerve stimulation.

The first reported use of VNS was in 1883 and in 1938 it was found that VNS induced synchronized activity in the orbital cortex in cats [85,86]. Further animal experiments led to the first human use of VNS for epilepsy treatment in 1988 [87,88]. Several studies have found VNS to be an efficient treatment of epilepsy, reducing seizure frequency by 50% after more than one year of therapy in 50% of patients [87,89,90]. Studies of VNS for epilepsy have indicated improved mood, anxiety, cognitive function, and altered pain threshold and food cravings in patients [91-95], which has led to clinical trials of VNS for depression, anxiety, Alzheimer's disease, and obesity [7,96-98].

Today VNS is an FDA approved treatment of epilepsy and treatment-resistant depression (approved in 1997 and 2005, respectively). During VNS, the electrodes are implanted around the left cervical vagus nerve in order to avoid cardiac complications and to get maximal effect in the

brain, where the effect is thought to occur. To avoid side-effects (hoarseness, coughing, throat pain, paraesthesias, and headache) the stimulation current is generally increased steadily [89,99]. Studies suggest that epilepsy improvement increase steadily over time and that stimulation recruit A and B fibers, while C-fiber activation is not required for epilepsy treatment, since destruction of C fibers does not attenuate anti-seizure response to VNS and electrophysiological studies indicate that typical VNS parameters do not activate C-fibers [59,100,101].

Although the use of VNS is increasing, the precise mechanism is still unknown. Animal models suggest that the anti-seizure effect of VNS may be related to locus coeruleus activation [102,103]. Locus coeruleus is the major site for norepinephrine-producing cells in the brain and has axons extending to the brainstem, thalamus, hippocampus, amygdala, and neocortex. In fact, it has been found that VNS increase norepinephrine concentration in the prefrontal cortex in rats [104]. Effects of VNS has also been found on other neurotransmitters such as serotonin, GABA, and glutamate [72,103,105]. VNS in rats has been found to increase c-fos labeling in amygdala, cingulate, locus coeruleus, hypothalamus, and the brainstem [106], while human studies have found increased cerebral blood flow in bilateral thalamus, hypothalamus, inferior cerebellar hemispheres, and right postcentral gyrus after acute and chronic VNS [107]. In addition, fMRI studies have found VNS to cause changes in hypothalamus, left amygdala, and orbito-frontal, parieto-occipital and left temporal cortex, along with an overall increase in brain activity [108].

Several human studies of VNS for depression have found that VNS reduced body weight and altered appetite, and a study in epileptic patients found that VNS increased energy expenditure [94,109-111]. Some animal studies have also found VNS to induce weight loss [112,113], while others found no effect on body weight [114,115]. These results have inspired more studies of VNS as a possible obesity treatment. For this purpose, VNS has been performed at the subdiaphragmatic level with electrodes attached to vagal branches in proximity to the gastro-esophageal junction [7]. By this method, vagal signals from the periphery can be modified and both branches can be targeted without any detrimental effects on cardiac function. Subdiaphragmatic vagus nerve stimulation is minimally invasive and more studies are needed in order to determine whether this procedure can be a new, minimally invasive obesity treatment.

1.8 Botulinum Toxin Type A Injection

Botulinum neurotoxin is a neurotoxin produced by the gram-positive bacteria *Clostridium botulinum* and can cause the neuroparalytic disease botulism [116]. This neurotoxin can be categorized into seven different serotypes (A-G) with different toxicities, where botulinum neurotoxin type A gives the longest lasting paralysis [116-118]. Botulinum neurotoxin type A is sold under different brand names, such as Botox, Dysport, and Xeomin [119], where Botox is the term and product used in this thesis.

Botox consists of a heavy (HC) and light (LC) chain of 100 kDa and 50 kDa, respectively [116]. The heavy chain of the toxin binds irreversibly and selectively to high affinity receptors at presynaptic neurons. These receptors have been identified as gangliosides, synaptic vesicle glycoprotein 2 (SV2), and possibly fibroblast growth factor receptor 3 (FGFR3) [120,121]. This binding causes the receptor-toxin complex to be taken up by receptor-mediated endocytosis [122,123] and subsequently the disulphide bond that connects the heavy and light chain is cleaved by proteases [116,124]. The light chain is a zinc endopeptidase, and causes cleavage of synaptosomal associated protein 25 (SNAP-25), a SNARE (Soluble NSF attachment protein receptor) protein essential for exocytosis of synaptic vesicles containing neurotransmitters, such as acetylcholine [125,126]. This inhibits the release of acetylcholine from presynaptic terminals, causing a paralysis of the adjacent muscle cells at the neuromuscular junction (Fig. 8) [122]. By this mechanism, Botox leads to paralysis.

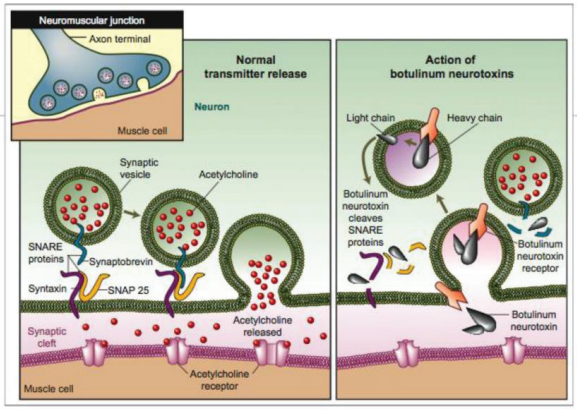


Figure 8 | The mechanism of action of botulinum neurotoxin [127].

Today, Botox is used to treat many conditions and there are few reports of adverse effects [128,129]. Among the conditions treated with Botox are strabismus, headache, hypersalivation, spastic movement disorders, hyperhidrosis, achalasia, anal fissure, spasticity, pain, arthritis, and hemifacial spasm [130-140]. It is also extensively used in the cosmetic and beauty industry [119]. The paralysis caused by Botox can vary in duration, but is estimated to be 3-4 months in patients [141].

The stomach wall is composed of mucosa, submucosa, smooth muscle, and serosa. All these layers are innervated by the gastric vagus nerve [142]. Botox injections here would block the release of neurotransmitters (e.g. acetylcholine), and are thought to paralyze the smooth muscles, thereby inhibiting the gastric propulsive activity and subsequently delaying gastric emptying. This gastroparesis might prolong satiety, reduce food intake, and subsequently induce weight loss. Consequently, injection of Botox into the gastric antral wall has been proposed as a new, minimally invasive treatment of obesity [143]. However, currently there is inconsistency in the literature and unclear results regarding body weight and gastric emptying after Botox injection. More research is needed in order to determine the efficacy and underlying mechanism of this as a minimally invasive obesity treatment.

1.9 Muscarinic Acetylcholine M3 Receptor

After acetylcholine is released into the synaptic cleft it will bind to either nicotinic acetylcholine receptors (nAChR) or muscarinic acetylcholine receptors (mAChR), where nAChR are ligand-gated ion channels and mAChR are G protein-coupled receptors (GPCRs) (Fig. 9). Muscarinic acetylcholine receptors (M1-M5) are expressed throughout the body and have roles in regulation of many physiological functions such as motor control, memory, temperature regulation, glandular secretion, cardiac rate, cardiovascular regulation, and smooth muscle contraction [144].

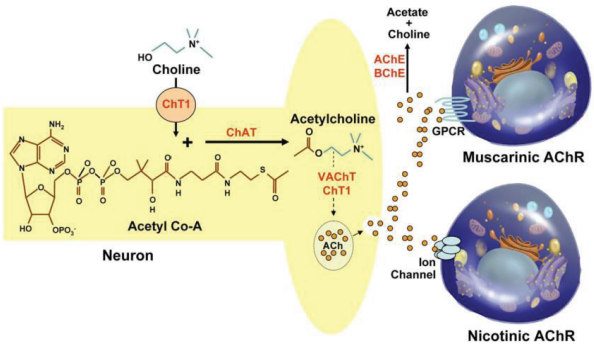


Figure 9 | Illustration of neuronal production and exocytosis of acetylcholine into the synaptic cleft, which subsequently binds to either muscarinic or nicotinic acetylcholine receptors [145].

The muscarinic acetylcholine M3 receptor is distributed in the brain, glands, and smooth muscle [144]. Interestingly, the M3 receptor has been shown to be important in food intake and body weight regulation, as mice deficient of this receptor display a lean phenotype with reduced food intake, total body fat, and serum levels of leptin, insulin, and triglyceride [146,147]. Based on this, a M3 receptor-dependent cholinergic pathway regulating appetite has been suggested [146]. Interestingly, these mice have also been found to be protected against diet induced obesity, obesity-associated glucose intolerance, insulin resistance, hyperinsulinemia, and hyperglycemia. This study also reported increased energy expenditure in these mice and hypothesized that this was caused by enhanced central sympathetic outflow and rate of fatty-acid oxidation [147]. This phenotype has not been observed in mice lacking M1, M2, M4, or M5 receptors [148-151].

Given the role of the M3 receptor in regulation of energy homeostasis, pharmacological targeting of this receptor could have potential for obesity treatment. It has been suggested that antagonists targeting the M3 receptor in the CNS would be preferable since peripheral M3 antagonists could induce significant side effects [152]. However, more research is needed to determine whether the M3 receptor is a potential pharmacological target in obesity treatment.

1.10 Metabolic Surgery

In 2008, 347 million people suffered from diabetes worldwide, 90% of these from type 2 diabetes and the numbers are increasing. Type 2 diabetes is a serious disease developing parallel to the obesity epidemic, as many people suffering from obesity develop type 2 diabetes. In this

disease the body is unable to effectively utilize insulin produced by pancreatic β-cells and since insulin regulates blood glucose levels this leads to hyperglycemia [153,154].

Obesity and type 2 diabetes are closely tied together. In obese individuals, adipose tissue increase lipolysis and secretion of non-esterified fatty acids (NEFAs), which can lead to insulin resistance and reduced β-cell function. Impaired insulin secretion from pancreatic β-cells decrease insulin levels and thereby increase food intake, leading to obesity, which again could give insulin resistance. Decreased insulin secretion also increases glucose production in the liver and reduces the efficacy of glucose uptake in muscle (Fig. 10) [155]. Obesity is associated with a low-grade inflammation, where adipocyte hypertrophy, hypoxia, and endoplasmic reticulum stress may increase the levels of pro-inflammatory cytokines and chemokines. These cytokines, together with NEFAs, can hamper insulin signaling through activation of intracellular serine/threonine kinases [156].

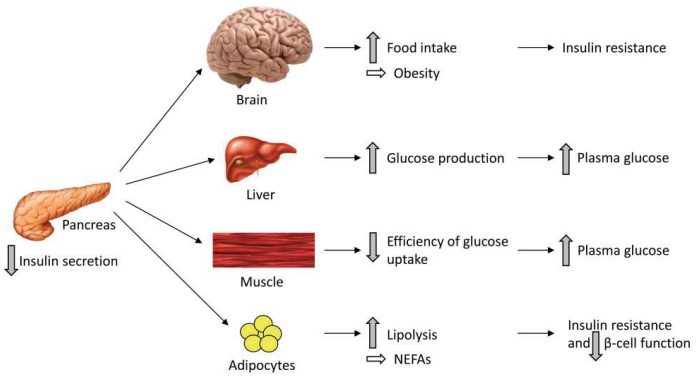


Figure 10 | Model illustrating the link between obesity and type 2 diabetes, modified from [155].

There is an increasing line of evidence of bariatric surgeries having therapeutic effect not only on obesity, but also on type 2 diabetes. Roux-en-Y gastric bypass (RYGB), adjustable gastric banding (AGB), and bilio-pancreatic diversion/duodenal switch (BPD/DS) have given respectively 84%, 48%, and 98% mean ratios of type 2 diabetes remission [39,157]. Multiple studies have shown these metabolic effects to be weight-independent and to be a result of endocrine changes from the surgical alterations of the gastrointestinal tract, as the improved glucose tolerance has been observed before and even without substantial weight loss and in nonobese individuals. The term “metabolic surgery” refers to surgery attempting to achieve

improved glucose homeostasis in both obese and nonobese patients [157]. However, little is known about the exact mechanisms behind these metabolic changes and much investigation is ongoing to identify the source of this diabetes remission. There are several hypothesis regarding these mechanisms, such as the foregut, hindgut, and metabolic reprogramming hypotheses [158,159]. In this thesis the hindgut hypothesis was investigated.

GLP-1 is secreted by the endocrine L-cells located in the distal ileum in response to ingested nutrients [160]. This hormone has several activities, including delaying gastric emptying and reducing food intake [161,162]. In the pancreas GLP-1 works as an incretin, increasing secretion and production of insulin, β-cell proliferation, and neogenesis [163]. It also acts upon the α- and δ-cells in the pancreas, decreasing secretion of glucagon [164,165]. By this mechanism GLP-1 lowers blood glucose levels. GLP-1 has been suggested to be involved in the pathogenesis of type 2 diabetes. This is supported by findings of type 2 diabetes patients having reduced GLP-1 secretion in both basal and postprandial conditions [166-168]. However, GLP-1 administration to diabetic patients is not feasible as the half-life of the native molecule is 60-90 seconds [169]. Several bariatric procedures that improve type 2 diabetes lead to higher ileum stimulation by nutrients, suggesting that the mechanism by which these procedures lead to type 2 diabetes remission may be through an increased secretion of GLP-1 in ileum (the hindgut hypothesis). Interestingly, GLP-1 receptors are distributed on vagal afferents in the gastrointestinal tract, pancreatic β-cells, and on neurons in the CNS. Due to its short half-life, it is likely that the incretin action of GLP-1 is through activation of GLP-1 receptors on vagal afferents in the gastrointestinal tract, leading to vagal efferent signals to pancreatic β-cells, increasing insulin secretion [170].

According to the hindgut hypothesis, ileal interposition (II) has been proposed as a metabolic surgery where a small segment of ileum is surgically interposed to the proximal small intestine. This procedure increases the stimulation of ileum by ingested nutrients and has been found to increase secretion of GLP-1 from L-cells in ileum [171-177]. This may be the mechanism by which ileal interposition has been shown to improve type 2 diabetes in animal studies [173-175,177-180].

Sleeve gastrectomy (SG) was developed as a first step bariatric surgery for high-risk patients intended for a second procedure, however, due to good clinical outcomes the procedure is now performed as an independent bariatric surgery [181]. The procedure has been shown to

reduce body weight and give remission of type 2 diabetes in both animal and human studies [40,182-189]. Some suggest that this is due to reduced ghrelin levels [182-189], while others suggest it to be caused by increased gastric emptying and GLP-1 secretion [190-192]. Interestingly, an animal study showed the effect to be independent of ghrelin [193]. Combining ileal interposition with sleeve gastrectomy was initially proposed to combine increased GLP-1 and reduced ghrelin levels [194]. All clinical studies of ileal interposition involves this combination (II-SG), and the procedure has been found to reduce body weight, resolve type 2 diabetes, increase GLP-1, PYY, and insulin and to decrease glucagon, ghrelin, and leptin levels [195-198].

Although promising, the exact mechanism behind the remission of type 2 diabetes after ileal interposition alone or combined with sleeve gastrectomy is still unknown and more research is needed to determine the potential and mechanisms of these procedures as metabolic and/or bariatric surgeries.

1.11 Mechanisms of Bariatric Surgery: State-of-the-Art

As mentioned, today´s bariatric surgeries efficiently induce weight loss and improve comorbidities, such as type 2 diabetes. However, the underlying physiological mechanisms are unclear, although the procedures are frequently performed worldwide [33,36]. While animal behavior is mostly driven by instincts, human behavior is much more complicated. Especially after bariatric surgery food intake can be strongly affected by postoperative instructions that are designed to induce weight loss and minimize side effects. This does not occur in animal studies and makes it easier to detect physiological effects and underlying mechanisms of surgical procedures in these models, which also provide more opportunity for control and manipulation [199]. Animal studies are also an excellent way of developing bariatric surgical techniques which can be adopted back into the clinics. Most importantly, discovering the underlying mechanisms of bariatric surgery can lead to development of new, minimally invasive procedures and/or pharmacological intervention through the same mechanisms, ultimately increasing quality of patient care [200]. Also, by identifying the different underlying mechanisms of the different procedures, it can be possible to identify personalized treatment.

The classical proposed mechanisms of bariatric surgery are restriction and malabsorption. However, sleeve gastrectomy (SG), which does not give malabsorption, has similar effects as

Roux-en-Y gastric bypass (RYGB) [201], and research indicate that reduction in food intake, rather than malabsorption, is the main reason for weight loss. It has been suggested that this is caused by restriction of the gastric volume, however, the stomach volume after SG is much greater than after adjustable gastric banding (AGB), but SG induces greater weight loss than AGB [202,203]. Also, several studies show that the size of the gastric pouch in RYGB does not correlate with amount of weight loss [204-206]. Thus, it is most likely that the mechanisms of bariatric surgery deal with physiological, rather than mechanical alterations (Fig. 11).

Bariatric surgery leads to altered gut hormone secretion, such as increased levels of GLP-1 and PYY and reduced levels of ghrelin [207-209]. However, GLP-1 receptor and ghrelin knockout mice have shown no change in the effects of bariatric surgeries, indicating that these factors may not be essential [193,210]. Interestingly, weight loss after gastrointestinal bypass was impaired in PYY knockout mice [211]. CCK has also been suggested to have importance, but studies show that RYGB is effective also in CCK-1 deficient rats [212]. Leptin has been suggested to be important after bariatric surgery, as leptin levels are reduced weight-independently [207,213]. However, SG is effective also in rodents that lack the leptin receptor, indicating that the effects of bariatric surgeries do not rely on leptin sensitivity [214].

Several studies have shown that bariatric surgeries increase plasma levels of bile acids [215]. Bile acids are central in lipid absorption, activating the farsenoid-X receptor (FXR), and modulating hepatic lipid metabolism. They have also been found to increase energy expenditure and prevent obesity in mice [216]. In FXR knockout mice SG had no effect, indicating that this could be an important pathway. In addition, SG did not alter gut microbiota in the knockout mice, in contrast to wild-type mice [217]. Several studies have found altered gut microbiota after bariatric surgery [218]. This study indicates a link between bile acids, the FXR, and gut microbiota that could play an important role after bariatric surgery.

Several studies have found RYGB to induce weight loss by increasing energy expenditure [219-221] and therefore, energy expenditure could also be an important mechanism behind bariatric surgeries.

Altered gastric emptying has been proposed to have importance for increased satiety after bariatric surgeries, which might be caused by alterations of the gastrointestinal tract or by response to endocrine and/or neuronal mechanisms. The vagus nerve is also affected by bariatric surgeries as they cause disruption and/or altered pattern of the nerve. Therefore, neuronal changes

affecting gastrointestinal signals to the brain has been proposed as a mechanism of bariatric surgery [222]. It is likely that bariatric surgeries give alterations in the CNS and some studies indicate that after bariatric surgery the homeostatic set-point in hypothalamus is altered, making the body defend a lower body weight [223].

Other mechanisms suggested include altered food preferences, reduced hunger, and reduced food reward [224-228]. In fact, animal models of RYGB and SG select less fat, increase liking of diets of lower calorie density, and reduce liking of sweet food [224,228]. Studies of food reward indicate that intrinsic food reward is not reduced, but that more rapidly achieved satiation reduces motivation after initiation of a meal [224,228].

Several of the mechanisms mentioned above have been suggested to be the underlying mechanisms of RYGB, SG, and duodenal switch (DS). We have previously reported that DS in rats reduce food intake, alter eating behavior, and cause malabsorption [82]. However, other animal studies of this procedure are lacking. SG induces body weight loss in most clinical and animal studies, often comparable to RYGB [189,190,229-231]. However, there are also animal studies finding no significant weight loss [82,187,232,233].

Although much research in this field is ongoing, the underlying mechanisms of bariatric procedures, such as Roux-en-Y gastric bypass, sleeve gastrectomy, and duodenal switch, are still unknown, and more research on these procedures in the right experimental setting is needed.

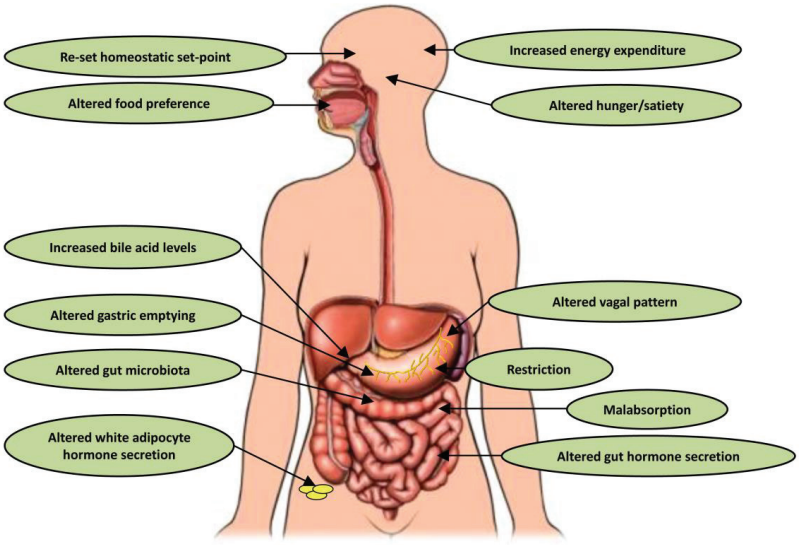


Figure 11 | Illustration of the different proposed underlying mechanisms of bariatric surgeries.

2. Aims of Thesis

2.1 Principal Objective

This thesis aimed to better understand the physiological mechanisms of bariatric surgery in order to develop minimally or non-invasive obesity treatments using experimental animal models.

2.2 Specific Objectives

I. To evaluate the vagus nerve as a possible target in development of new, minimally invasive obesity treatments (Paper I)

II. To evaluate the muscarinic acetylcholine M3 receptor as a possible pharmacological target in obesity treatment (Paper II)

III. To investigate the potential of ileal interposition alone and combined with sleeve gastrectomy as bariatric and metabolic surgery (Paper III)

IV. To investigate the underlying mechanisms of gastric bypass and duodenal switch (Paper IV)

3. Materials and Methods

3.1 Ethics

The use of animals in scientific experiments raises ethical issues, especially when the experiments are likely to cause harm, pain, and/or distress. Therefore, animals should only be used in experiments if the objectives of the study can contribute to animal or human welfare, if the study is likely to meet these objectives, if there are no other alternatives, and if the harm to the animals is not excessive. It is general consensus that the use of animal models is only ethically acceptable when the studies are well designed, efficiently executed, correctly analyzed, clearly presented, and correctly interpreted [234]. In 1959 Russel and Burch defined the 3Rs (replace, refine and reduce) as guidelines for humane use of animals, stating that animals should be *replaced* by other alternatives whenever possible, experiment protocols should be *refined* to minimize adverse effects, and the number of animals should be *reduced* to a minimum in order to obtain the objectives of the study, without using too few as to miss biologically significant effects [235].

In this thesis the animal experiments could not have been replaced by any *in vitro* experiment as the systemic effects of surgical procedures or knockout of receptors cannot be studied by models such as cell cultures or computers. The protocols have been refined, including gas anesthesia, local as well as systemic analgesia, and surgical improvements. Throughout the studies the general condition and behavior of the animals have been closely observed and if there were any abnormalities the veterinarian at the animal facility was contacted. Scoring schemes were used to evaluate whether a humane endpoint was reached. The number of animals in each group was determined based on previous studies and statistical estimation. All studies in this thesis were approved by the Norwegian National Animal Research Authority (Forsøksdyrutvalget).

3.2 Experimental Design and Statistics

The experimental designs in this thesis were made based on the objectives of the individual studies (Papers I-IV). In this thesis confirmatory research (data is collected in order to answer a question) was performed. The “experimental units” were the individual animals, which were assigned to treatment groups randomly with an equal probability (using a random number

table) and the groups were treated equally throughout the experiments. Completely randomized designs like this are widely used due to its simplicity and tolerance for unequal numbers in each group. In this thesis, untreated animals, animals treated with vehicle, animals implanted with sham devices, or sham-operated animals were used as negative controls. To avoid bias, experiments were performed "blindly" with regards to treatments whenever possible. Quantitative data was analyzed, which is preferable as it normally requires a smaller sample size than qualitative data. To avoid random variables, such as circadian rhythms and different people performing experiments, affecting the data, body weight was measured at the same time of day and a limited number of people handled the animals [234].

The results of an experiment should be analyzed using appropriate statistics, reflecting the purpose of the study. In this thesis, all individual studies were performed to test a null hypothesis, H_0, stating that the treatment (either surgery in Papers I, III, and IV or gene knockout in Paper II) had no effect. The results are expressed as means ± SEM. Statistical comparisons were performed using independent *t*-test or Mann Whitney U test between the experimental groups. When suitable, ANOVA with Tukey's or Bonferroni *post hoc* test was performed. Analysis of covariance (ANCOVA) with Sidak test was performed for energy expenditure analysis using fat-free mass or body weight as covariate. A *p*-value of <0.05 (two-tailed) was considered statistically significant. The data analysis was performed in SPSS version 15.0 and 20.0. It is important to note that results can be significantly different using statistics but to be of little or no biological significance [234].

Laboratory animals were used as models for humans in this thesis. Accordingly, the differences between humans and the animal models were taken into consideration in the experimental designs and data analysis in this thesis. It should be noticed that even though a response is found in animals, its true relevance to humans is still unknown [234]. However, if a treatment shows an effect in animals it is more likely to give an effect in humans, than if it does not induce an effect.

3.3 Animals

A total of 229 animals were used in this thesis, including 37 rats in vagus nerve stimulation/blocking (VNSB) studies, 88 rats in Botox studies, 46 mice in muscarinic acetylcholine M3 receptor knockout (M3KO) studies, 24 rats in ileal interposition and sleeve

gastrectomy (II and SG) studies, and 34 rats in gastric bypass (GB) and duodenal switch (DS) studies (Paper I: 125 rats, Paper II: 46 mice, Paper III: 24 rats, and Paper IV: 34 rats).

Rats (Sprague-Dawley, male, adults) were purchased from Taconic, Denmark, Janvier Labs, France, and Harlan™, The Netherlands. Males were mainly used because female rats have food intake coinciding with ovarian hormones and males grow faster than females, making it easier to detect changes in body weight. Long Evans rats (adult, female) were obtained at Norwegian University of Science and Technology, Trondheim, Norway. Muscarinic acetylcholine M3 receptor knockout (M3KO) mice were generated by Prof. Koji Takeuchi's group at Kyoto Pharmaceutical University, Kyoto, Japan, and imported to Norway by Professor Chen. These mice were further bred through sibling matings for two generations. Age-matched control mice, C57BL/6, were purchased from Taconic, Denmark. All animals had free access to tap water and standard rat pellet food (RM1 811004, Scanbur BK AS, Sweden), high-fat-diet in pellets (D12492, Research Diets, Inc., NJ, USA), or standard mice pellet food (RM1 801002, Scanbur BK AS, Sweden). They were housed three-four together in individually ventilated cages on wood chip bedding with a 12-h light/dark cycle, room temperature of 22ºC, and 40-60% relative humidity. The standard housing conditions were specific pathogen free and in agreement with FELASA (Federation of European Laboratory Animal Science Association) recommendations.

3.4 Surgical Models

All surgeries were performed under general anesthesia with isofluran (4% for induction, 2% for maintenance) (Baxter Medical AB, Kista, Sweden). When beneficial the animals were fasted overnight before surgery. Atropin (0.04 mg/kg) (Nycomed Pharma AS, Asker, Norway) was given subcutaneously 20 min before surgeries with duration of more than 45 min. Marcain (1 mg/kg) (Astrazeneca, London, UK) was injected subcutaneously locally at the incision place. Buprenorphine (Schering-Plough Europe, Brussels, Belgium) was injected subcutaneously (0.05 mg/kg) immediately after surgery in all animals, and one day postoperatively when needed. Physiological saline (0.9% NaCl) was given subcutaneously at 10-15 mL after surgeries to keep the animals hydrated.

3.4.1 VNSB and μ-VNSB

The vagus nerve stimulation/blocking (VNSB) implantation was performed through a midline abdominal incision. The subdiaphragmatic truncal vagus nerve was dissected from the esophagus and two electrodes (Lead Model 302, Cyberonics, Houston, TX, USA) were wrapped around the anterior and posterior vagus nerve of the gastric branch together with extra tissue from esophagus (Fig. 12). The wire from the electrodes was attached to fat tissue around the stomach (the greater omentum) and to the muscular layer using 6-0 and 4-0 absorbable sutures (Vicryl, Ethicon Inc., Sommerville, NJ, USA), respectively. On the back of the rat a subcutaneous pocket for the stimulator (Model 102 Pulse Generator, Cyberonics, Houston, TX, USA) was made and here it was connected to the wire. The abdomen was closed in two layers using 4-0 absorbable sutures and the back was closed using the same sutures (Ethicon). The control and VNSB rats received the same procedure. Implantation of the μ-VNSB device followed the same procedure, except that the control animals were implanted with stimulators without electrodes.

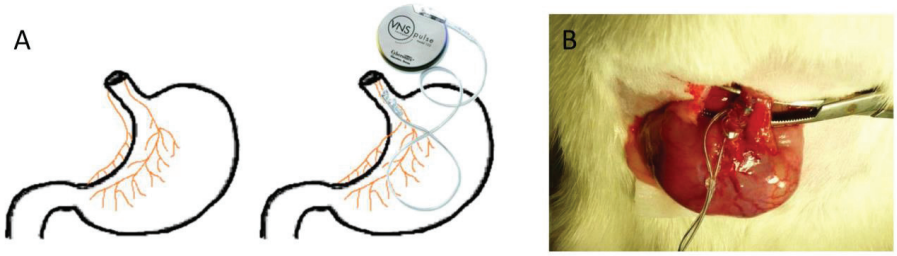


Figure 12 | VNSB model. A) Illustration of the electrode implantation and B) photograph of VNSB electrodes attached to the vagal nerves at the gastro-esophageal junction.

The current of vagus nerve stimulation/blocking can be monophasic or biphasic. In this thesis the current was biphasic, meaning it was first induced in one direction and then in the opposite direction. Stimulation frequency is the number of times a stimulus pulse is applied in a period of time and pulse width is the duration of the stimulus pulse, i.e. the time that the current is applied to the electrode. The duty cycle is defined as the percentage of time during which stimulation occur [236]. In this thesis VNSB was at a current ranging from 0.5 mA to 3.5 mA, a frequency of 30 Hz, a pulse width of 500 μs, and a duty cycle of 30 s ON and 5 min OFF. These settings are regarded as standard, safe, and effective [90,237].

3.4.2 Botulinum Toxin Type A Injection

Botulinum toxin type A 100 U (Botox® Allergan Cooperation, Irvine, CA, USA) was diluted in 0.9% cold saline and 1% methylene blue (Sigma-Aldrich, St. Louis, MO, USA) (for visualization of the injection) achieving a concentration of 40 U Botox/mL. Total volume injected into pyloric antrum of each rat was 0.5 mL (20U/rat). The control rats were injected with 0.5 mL 0.9% saline and 1% methylene blue. Following a short median laparotomy, the stomach was exposed and antrum isolated. Botox was injected to both anterior and posterior side of the antrum with a 30 G needle according to earlier reports [143,238]. In contrast to previous studies the injection was made through one injection on each side and allowed for the fluid to spread over the whole subserosal area of the pyloric antrum (Fig. 13). Abdomen was closed in two layers using 4-0 absorbable sutures (Ethicon).

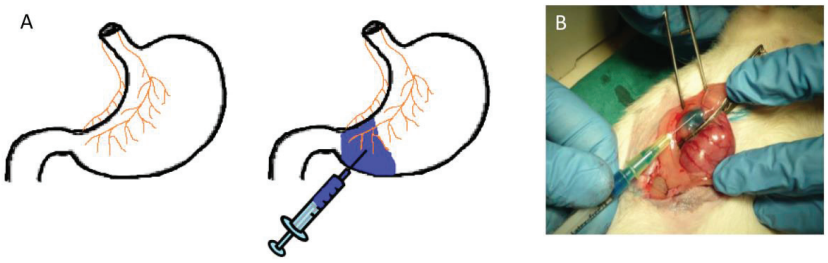


Figure 13 | Botox model. A) Illustration and B) photograph of gastric subserosal Botox injection in the whole area of the pyloric antrum through one injection.

3.4.3 Ileal Interposition

Ileal interposition (II) was performed through a midline abdominal incision. The ligament of Treitz was located and the insertion location for the transposed ileum segment, approximately 3 cm distal to the ligament, was marked with saline-soaked gauze. Cecum was located and a 2-3 cm segment of ileum located 0.5 - 1 cm proximal to the ileocecal valve was isolated and transected. An anastomosis was made with the two open ends of cecum and jejunum. The marked position distal to the ligament of Treitz was found and the isolated ileal segment with intact vascular and nervous supplies was inserted here in the original peristaltic direction by making two end-to-end anastomoses (Fig. 14). All anastomoses were performed with 6-0 absorbable sutures (Ethicon). The abdomen was closed in two layers using 4-0 absorbable sutures (Ethicon).

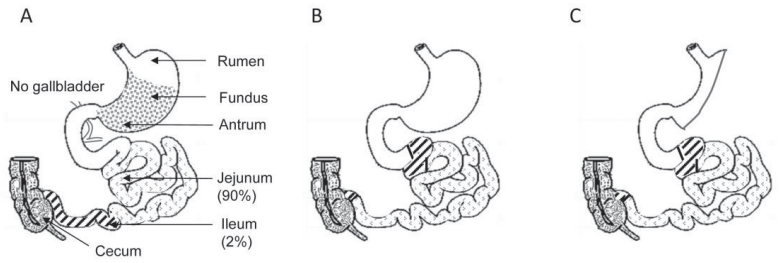


Figure 14 | Ileal interposition model. A) Rat gastrointestinal anatomy, B) ileal interposition, and C) ileal interposition with sleeve gastrectomy.

3.4.4 Sleeve Gastrectomy

Sleeve gastrectomy (SG) was performed through a midline abdominal incision. The stomach was located, blood supply to the spleen and greater omentum was shut down, and the stomach was clamped along the greater curvature from the antrum to the fundus across the forestomach and glandular stomach. A scissor was used along the clamp to excise the greater curvature of the stomach, removing approximately 70% of the stomach. Subsequently, the stomach was closed with 6-0 absorbable sutures (Ethicon). Hemostasis and suture-line integrity were checked, and additional stitches were applied when necessary. The abdominal wall was closed in two layers using 4-0 absorbable sutures (Ethicon). In diet induced obese (DIO) rats the procedure was performed in a similar manner. However, the stomach was clamped using staples (Fig. 15) (Endo GIA, Articulating Reload with Tri-Staple™ Technology, 45 mm, EGIA45AVM, Covidien™, Dublin, Ireland).

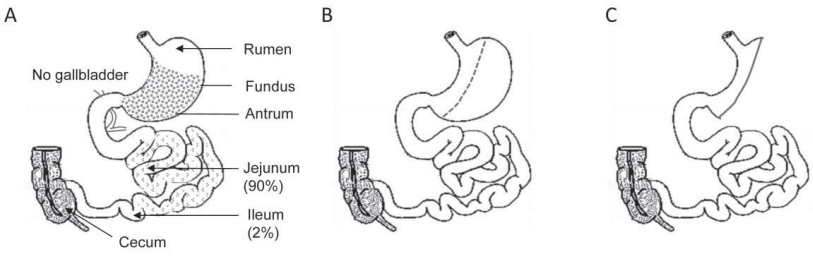


Figure 15 | Sleeve gastrectomy model. A) Rat gastrointestinal anatomy, B) the suture/staple line across the greater curvature of the stomach, and C) sleeve gastrectomy.

3.4.5 Gastric Bypass and Duodenal Switch

Micro-gastric bypass (GB) was performed through a midline abdominal incision by anastomosing the distal esophagus to the proximal jejunum about 2-3 cm distal to the Treitz ligament in an end-to-side manner (Fig. 16B) [221,239]. Duodenal switch (DS) was achieved in two stages. The two-stage procedure has been recommended in patients since the single-stage procedure increases the risk of postoperative complications and staged duodenal switch may avoid biliopancreatic diversion in some patients [240]. Sleeve gastrectomy was performed by resecting approximately 70% of the stomach along the greater curvature using sutures, as described above. Three months later, duodenal switch was achieved by creating a biliopancreatic limb, an alimentary limb (approximately 95% of total small bowel length), and common channel length of 5 cm (Fig. 16C) [82].

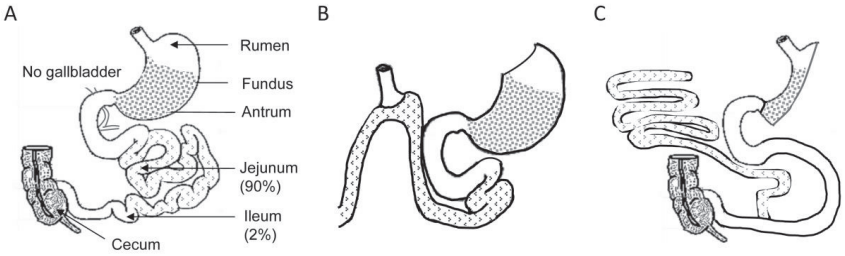


Figure 16 | Gastric bypass and duodenal switch models. A) Rat gastrointestinal anatomy, B) gastric bypass, and C) duodenal switch.

3.4.6 Sham Surgeries

Sham surgery, or laparotomy, was generally performed through a midline abdominal incision, and viscera was gently manipulated. However, in the VNSB studies control animals received the same implantation as experiment animals, in the μ-VNSB study control animals received the stimulator without electrodes, and in the Botox studies control animals received vehicle injection.

3.5 Methods to Study Effects and Mechanisms

3.5.1 Body Weight and Body Composition

Body weight was determined using a standardized balance throughout the studies (one-three times per week). Body composition was determined by dual energy X-ray absorptiometry

(DXA) (Hologic QDR 4500A, Hologic Inc., Bedford, MA, USA). Total fat mass, total fat-free mass, total bone mineral content, and total bone area were measured. From this data bone mineral density and total body mass were calculated.

3.5.2 Food Intake, Eating Behavior, and Metabolic Parameters

Food intake, eating behavior, and metabolic parameters were accurately determined by the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments International, Columbus, OH, USA). This system is composed of a four-chamber open circuit indirect calorimeter designed for continuous monitoring of individual rats or mice in each chamber (Fig. 17 and 18). Parameters that were obtained during daytime (7 am – 7 pm) and nighttime (7 pm – 7 am) for each individual animal were number of meals, meal size (g/meal, kcal/meal), meal duration (min, min/meal), accumulated food and calorie intake (g, g/100g body weight, kcal, kcal/100g body weight), intermeal interval (min), eating rate (g/min), satiety ratio (min/g), drinking activity (mL, mL/100g body weight, mL/time), energy expenditure (kcal/h, kcal/h/100g body weight, kcal/h/cm^2 body surface), and activity. The high-resolution feeding data was generated by monitoring feeding balances every 0.5 sec and air samples were drawn from the cages every 5 min for determination of metabolic parameters.

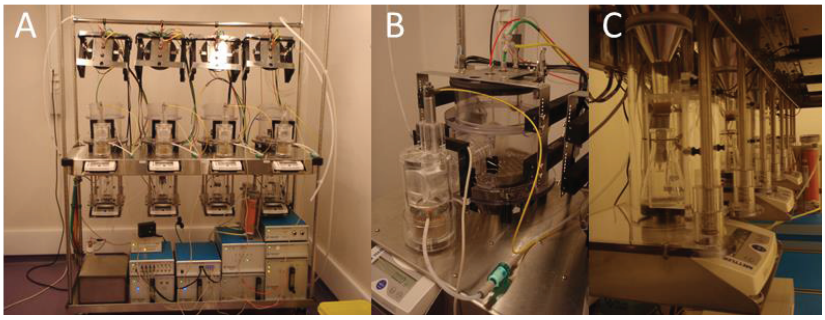


Figure 17 | Photographs of CLAMS. A) The whole CLAMS system, B) a single chamber with feeder, feeder balance, re-circulating tube and the lid with activity sensors and tubes for airflow regulation, and C) urine beakers underneath the chambers and their balances.

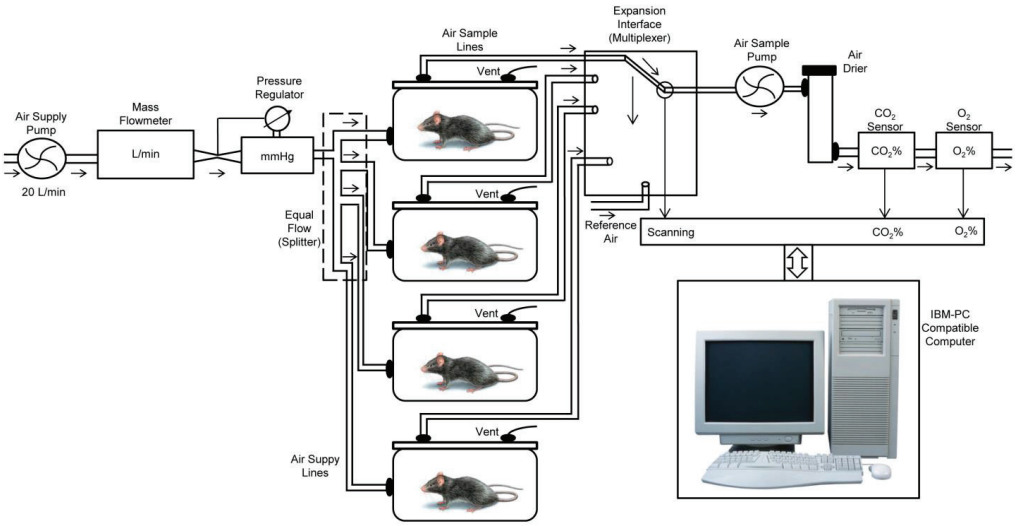


Figure 18 | Illustration of the open circuit indirect calorimeter, CLAMS, and how it monitors O_2 consumption and CO_2 production in laboratory animals for determination of metabolic parameters.

3.5.3 Gastric Acid Secretion

For determination of gastric acid secretion, a gastric fistula was implanted into the stomach and acid was measured at several time points with and without subcutaneous pentagastrin injections (5 µM/kg) (Sigma-Aldrich, St. Louis, MO, USA). Pentagastrin is a peptide that stimulates secretion of gastric acid [241]. The gastric acid was investigated for pH, H^+-secretion, and amount (mL).

3.5.4 Gene Expression in the Brain

Taqman array was performed to analyze expression of specific genes in the brainstem (medulla and pons) and hypothalamus. In this method RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) and reversely transcribed for cDNA synthesis using random hexamers (Applied Biosystems, Sundbyberg, Sweden) and Superscript III reverse transcriptase (Invitrogen Life Technologies, Paisley, UK). This was followed by real-time reverse transcription polymerase chain reaction (RT PCR) using TaqMan®Low Density Array (LDA) custom-made platforms with TaqMan probe and primer sets for target genes chosen from an

online catalogue (Applied Biosystems). Gene expression values were calculated based on the Ct method [242].

In situ hybridization was used to analyze gene expression in ARC, dorsomedial hypothalamic nucleus, and NTS. In this procedure brain samples were snap-frozen and cut in slices that were mounted onto poly-L-lysine-coated slides and fixed. Anti-sense radioactive riboprobes for target mRNA were generated by PCR and applied to the slides. Slides were then dried and exposed to Kodak Biomax MR film, and autoradiographic films were scanned at 600 dpi and analyzed using Image Pro Plus v.7.0 (Media Cybernetics UK, Marlow, Bucks, UK) analysis software (Media Cybernetics UK, Wokingham, UK). Integrated optical density was obtained by reference to the ^{14}C microscale.

RNA sequencing was performed to analyze gene expression in hippocampus. Total RNA was isolated from hippocampus and high-throughput cDNA sequencing (RNA-seq) was performed at Norwegian Sequencing Centre, Dept. of Medical Genetics, Oslo University Hospital, Oslo, Norway, using HiSeq2000 (Illumina, Inc., San Diego, CA, USA). Differential expression analysis on the data was performed, where reads for all samples were aligned to the rat genome using Bowtie2 (http://bowtie-bio.sourceforge.net/index.shtml) and Tophat2 (http://tophat.cbcb.umd.edu) and differential expression was calculated using cuffcompare in the Cufflinks2 (http://cufflinks.cbcb.umd.edu) package.

3.5.5 In Vivo Electrophysiology

For determination of electrical and neuronal signals during VNSB *in vivo* electrophysiology was performed. Following placement of the VNSB the animals were mounted in a stereotaxic frame and the cranium was exposed. A hole was drilled over the hippocampal area (coordinates AP -3.2, ML 2.2) [243], the dura was removed, and a bundle of 4 tetrodes (0.005", Pt/Ir, Fine Wire, California, USA) were lowered into the brain until stable neuronal activity was measured. The drill hole was subsequently filled with mineral oil and a surgical screw was placed into the scull to serve as ground. During VNSB, data was recorded at 40Khz using a Multineuron Acquisition Processor (MAP) recording system (Plexon, Dallas, TX, USA). Signals were passed through a unity-gain amplifier (20×), amplified, and filtered with a Plexon 16-channel preamplifier (PBX3 /16sp-r-G50, 50x amplification, 150-8000 Hz filtered).

3.5.6 Plasma Concentrations of Hormones

For determination of plasma hormone concentrations in-house radioimmunoassays using specific antibodies were performed, except for PYY where a commercial kit was used (Rat/mouse PYY RIA kit, Cat. no. RMPYY-68HK, EMD Millipore Corporation, St. Charles, MO, USA).

3.5.7 Fasting Blood Glucose

Blood glucose levels were measured after 12 h fasting by taking a drop of blood from the tail vein and analyzing with a glucose meter and test strips (FreeStyle Freedom Lite, Abbot Diabetes Care, CA, USA).

3.5.8 Gastric Emptying

For determination of gastric emptying an acetaminophen absorption assay was performed. An oral gavage of 100 mg/kg acetaminophen (Sigma-Aldrich, St. Louis, MO, USA) in 1.5% methyl cellulose (Sigma-Aldrich) was administrated before tail vein blood samples (100 µl) were collected at 15, 30, 45, 60, 90, 120, and 180 min after the oral gavage. Subsequently, plasma concentrations of acetaminophen were measured using an acetaminophen kit (Acetaminophen-sl assay, ref. 505-30, Sekisui Diagnostics, PE Canada) and spectrophotometer (UV-1800, UV-Spectrophotometer, Shimadzu, USA).

3.5.9 Fecal Energy Content

For fecal energy measurement, feces were dried at 60°C for 72 h and subsequently the energy density was measured using an adiabatic bomb calorimeter (IKA-Calorimeter C 5000, IKA-Werke GmbH & Co. KG, Staufen, Germany).

3.5.10 Western Blot Analysis and Immunohistochemistry

Western blot analysis was applied to test GLP-1 antibody specificity. Ileum and pancreatic tissues were homogenized in RIPA Buffer and protein concentrations were determined by BioRad assay and absorbance measurement at 595 nm. 100 µg ileum and pancreatic tissue extracts, 1 µg GLP-1 peptide (code: 028-11, Phoenix Pharmaceuticals, Inc., CA, USA) and M.M (4.0 µL Seeblue + 0.5 µL Magic Marker, Invitrogen, Carlsbad, CA, USA) were subjected to 1DE

separation in 4-12% Novex Bis-Tris gels using MES running buffer (Invitrogen) at 200 V. The tissue extracts, the GLP-1 peptide, and M.M were transferred to a Nitrocellulose membrane (UltraCruz, SantaCruz Biotechnology, Inc., Dallas, TX, USA) (Blot 30 V, 30 min, 220 mA, room temperature). The membrane was dried and blocked in 5% fat-free dry milk in PBS-T and incubated overnight in rabbit monoclonal GLP-1 primary antibody (code: 2914-1, Epitomics, Inc., CA, USA). After washing in PBS-T, membranes were incubated with secondary antibody swine anti-rabbit (1:5000). After washes in PBS-T blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc., DE, USA) and scanned in a Kodak IS4000R imager (Thermo Fisher Scientific).

For determination of expression of GLP-1 and insulin in ileum and pancreas, quantitative immunohistochemistry was performed. The tissues were fixed in formaldehyde, dehydrated and embedded in paraffin. The tissue sections were cut, thawed on superfrost glass slides, deparaffinized and then rehydrated. For immunostaining, the tissue sections were blocked with normal goat serum and incubated with primary antibodies (GLP-1; dilution: 1:3000, code: H-028-13, Phoenix Pharmaceuticals, Inc., CA, USA, insulin; dilution: 1:300, code: A0564, DakoCytomation, Denmark) and subsequently incubated with secondary antibody. Following this the tissue was examined under a microscope (Olympus BX50) with epiflourescence illumination.

3.5.11 Plasma Concentrations of Cytokines

To determine cytokine concentrations in plasma a multiplex cytokine assay was used (Cat no:171-K1002M, Bio-plex Pro Rat Cytokine Th1/Th2 12-plex Panel; Bio-Rad Laboratories, Hercules, CA, USA).

4. Results

4.1 Body Weight Loss

Vagus nerve stimulation/blocking (VNSB) for 48 h or at low current (0.5-1 mA) did not affect the body weight, but when the current was increased to 2 mA a body weight reduction of 10% compared to control was reached ($p>0.05$) (Fig. 19A). However, when VNSB was started at 2 mA a 10% body weight reduction compared to controls was reached instantly ($p<0.05$) (Fig. 19B) (Paper I).

Subserosal gastric injection of Botox into the whole pyloric antrum in normal chow-fed rats induced a body weight reduction of 17% after the 1st injection and a 24% reduction after 2nd injection compared to vehicle-treated animals ($p<0.05$) (Fig. 19C). In diet induced obese (DIO) rats, Botox injection induced a 24% and 33% body weight reduction after 1st and 2nd injection, respectively, compared to vehicle-treated animals ($p<0.05$) (Fig. 19D). Sleeve gastrectomy (SG) in DIO rats induced a 10% weight loss compared to sham-operated animals ($p>0.05$), while a subsequent Botox injection gave a 25% weight loss compared to vehicle-treated sham-operated animals ($p<0.05$) (Fig. 19E) (Paper I).

Muscarinic acetylcholine M3 receptor knockout (M3KO) mice weighed 20-30% less than their age-matched wild-type (WT) mice throughout the lifespan. At 6, 11, and 15 months of age the M3KO mice weighed significantly less than WT ($p<0.01$), but not at 2 months of age ($p>0.05$) (Fig. 19F) (Paper II).

Ileal interposition (II) reduced the body weight by 10% 1 week after surgery compared to sham-operated animals ($p<0.05$) (Fig. 19G). Although the body weight remained lower than the control group throughout the study, this was not significant. Combining ileal interposition with sleeve gastrectomy (II-SG) induced a body weight reduction of about 10% compared to sleeve gastrectomy alone ($p>0.05$) (Fig. 19H). Sleeve gastrectomy alone reduced body weight only transiently at 1 week postoperatively by about 10% compared to before surgery ($p<0.05$) (Fig. 19I) (Paper III).

Gastric bypass (GB) induced a weight loss of about 15% ($p<0.05$), while duodenal switch (DS) caused a weight loss reaching 54% ($p<0.05$), compared to sham-operated animals (Fig. 19J) (Paper IV).

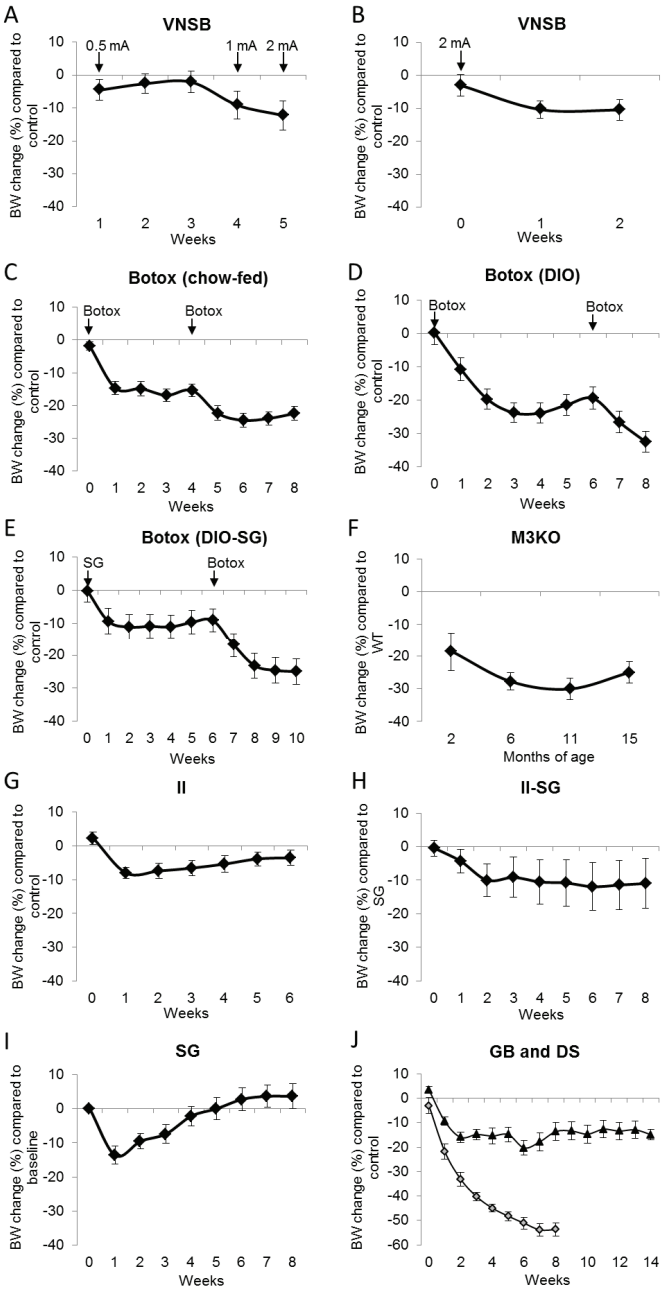


Figure 19 | Body weight loss in animal models established in this thesis. A) VNSB in normal chow-fed rats where current was gradually increased, B) VNSB in normal chow-fed rats when current was started at 2 mA, C) Botox injection in normal chow-fed rats, D) Botox injection in DIO rats, E) Sleeve gastrectomy (SG) in DIO rats and Botox injection 6 weeks later, F) M3KO mice at 2, 6, 11, and 15 months of age, G) Ileal interposition (II) in normal chow-fed rats, H) Sleeve gastrectomy added to ileal interposition (II-SG) in normal chow-fed rats, I) SG in normal chow-fed rats, J) Gastric bypass (GB) (black) and duodenal switch (DS) (grey) in normal chow-fed rats.

4.2 Key Results Regarding Mechanisms

4.2.1 Paper I

VNSB altered hypothalamic gene expression after 48 h and *in vivo* electrophysiology confirmed the parameters used, showing that the stimulation/blocking signals could reach the brain. Basal and pentagastrin-stimulated gastric acid secretions were not affected by VNSB.

Long-term VNSB reduced body weight and food intake at high current, while analysis of gene expression in the hypothalamus showed changes with a drive for increased food intake and reduced energy expenditure (Fig. 20A). Gene expression in brainstem and hippocampus on the other hand were indicative of appetite suppression, compatible with the phenotype of these animals (Fig. 20B and C). Gut hormones were unchanged after VNSB both in short-term and long-term.

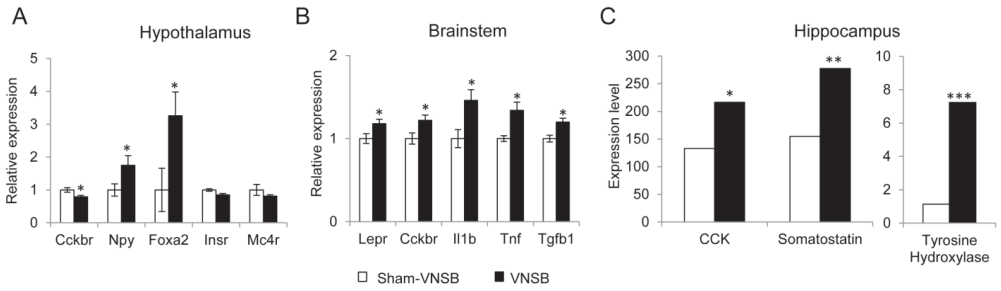


Figure 20 | Gene expression in the brain after long-term VNSB (2 mA). Effect of VNSB on mRNA expression in A) hypothalamus, B) brainstem, and C) hippocampus. *, **, ***:$p<0.05$, 0.01, 0.001 between sham-VNSB and VNSB. Cckbr: CCKB/2 receptor, Foxa2: forkhead box protein A2, Insr: insulin receptor, Mc4r: melanocortin 4 receptor, Lepr: leptin receptor, Il1b: interleukin-1β, Tnf: tumor necrosis factor, Tgfb1: transforming growth factor β1.

Subserosal Botox injection in the whole area of the pyloric antrum efficiently induced weight loss in normal chow-fed, DIO rats, and DIO rats receiving SG (Fig. 19C, D, and E). The weight loss was associated with reduced food intake and blood glucose levels and increased resting (daytime) and active (nighttime) energy expenditure (Fig. 21A). Interestingly, hypothalamic gene expression after injection showed changes with a drive for increased food intake and reduced energy expenditure (Fig. 21B).

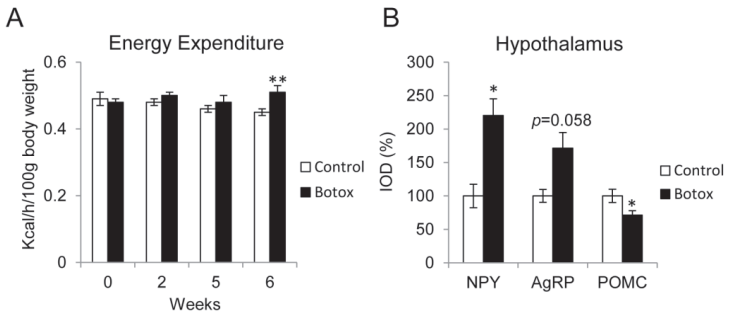


Figure 21 | Effect of Botox injection in normal chow-fed rats on energy expenditure and hypothalamic gene expression. Effect of Botox injection on A) 24 h energy expenditure and B) hypothalamic mRNA expression at 8 weeks. *, **:p<0.05, 0.01 between vehicle (control) and Botox.

Botox injection reduced plasma levels of CCK, gastrin, and PYY. There were no clinical signs of gastroparesis and gastric emptying was not delayed after Botox injection (Fig. 22).

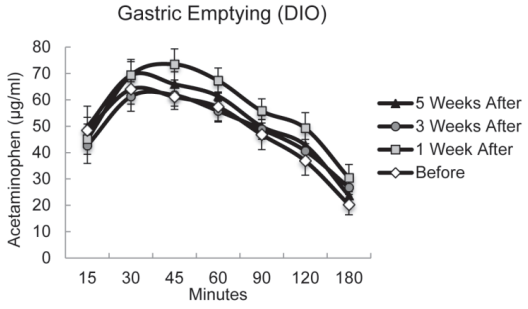


Figure 22 | The effect of Botox injection on gastric emptying in DIO rats. Gastric emptying was measured before and 1, 3, and 5 weeks after Botox injection. Note: no significant difference.

4.2.2 Paper II

Muscarinic acetylcholine M3 receptor knockout (M3KO) mice had a lean phenotype, but unchanged total (24 h) food intake. Lacking the M3 receptor induced an altered eating behavior, with tendency for reduced food intake during daytime and increased food intake during nighttime compared to age-matched wild-type (WT). However, cumulative food intake adjusted for body weight (g/100g body weight) was significantly higher in M3KO mice than WT at 15 months of

age (p<0.01). The M3KO mice had increased resting and active energy expenditure throughout the whole lifespan (Fig. 23A). Hypothalamic mRNA expression showed changes with a drive for increased food intake and reduced energy expenditure (Fig. 23B). Respiratory exchange ratio (RER) was increased until over 1.0 during both daytime and nighttime in M3KO mice.

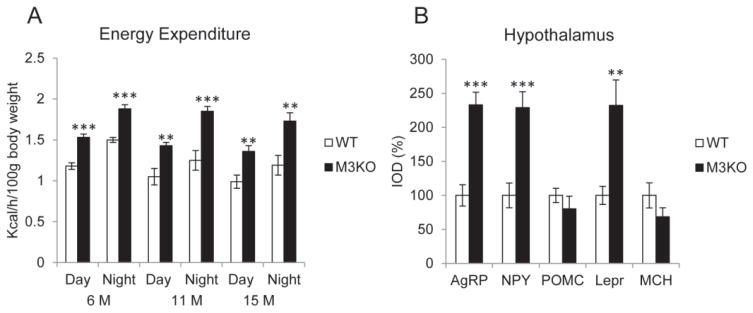


Figure 23 | Energy expenditure and hypothalamic gene expression in WT and M3KO mice. A) Energy expenditure during daytime and nighttime of WT and M3KO mice at 6, 11, and 15 months of age. B) Hypothalamic mRNA expression at 15 months of age. **, ***: p<0.01, 0.001 between WT and M3KO. MCH: melanin-concentrating hormone.

4.2.3 Paper III

Ileal interposition (II) reduced body weight and fat compartment transiently, but did not reduce food intake. II increased food intake and altered the eating cycle, with fewer, but larger (g/meal) and longer lasting meals (min). After II, respiratory exchange ratio (RER) was significantly increased until over 1.0, indicating increased use of carbohydrates as predominant fuel for cellular respiration. Adding sleeve gastrectomy (SG) induced a marginal weight loss, and fat compartment was lower in rats subjected to II–SG than those subjected to SG alone, albeit insignificantly. SG alone reduced body weight transiently (p<0.05 at 1 week) and increased food intake and reduced rate of eating (g/min) compared to baseline. II-SG increased both food intake and meal duration (min), while it reduced rate of eating (g/min), and resting and active energy expenditure. The combination of II and SG increased the expression of GLP-1 in the interposed ileum and in pancreatic α-cells, the latter was also found after SG alone (Fig. 24).

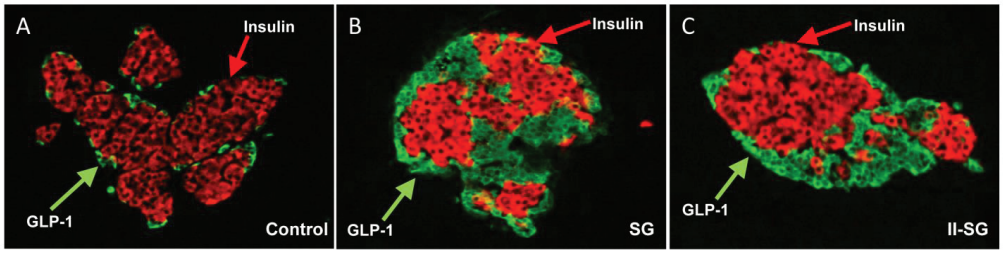


Figure 24 | Immunofluorescence histomicrographs of GLP-1 (green) and insulin (red) in rat pancreatic islets. GLP-1 and insulin in A) non-operated normal rat, B) SG-operated rat nine weeks after SG, and in C) II-SG-operated rats nine weeks after SG with II.

4.2.4 Paper IV

Gastric bypass (GB) reduced body weight, but was not associated with reduced food intake, however with increased active energy expenditure 3 weeks after surgery and increased resting energy expenditure 14 weeks after surgery (Fig. 25A). Duodenal switch (DS) reduced body weight and food intake. After surgery, eating behavior was altered with reduced meal size (g/meal) and rate of eating (g/min). In addition, resting energy expenditure was increased shortly (2 weeks) after surgery and both resting and active energy expenditure were increased 8 weeks after surgery (Fig. 25B). DS also induced diarrhea and malabsorption.

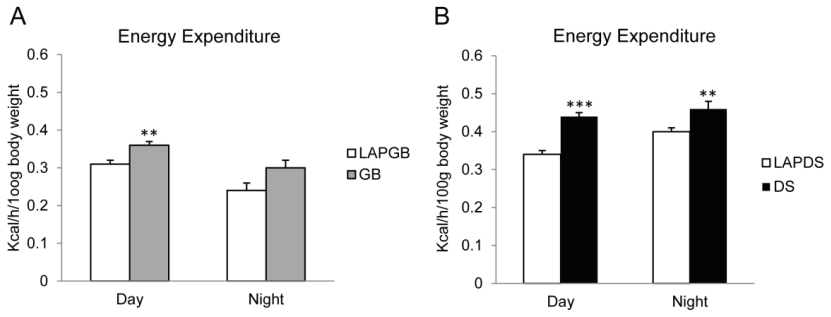


Figure 25 | Energy expenditure after GB and DS and corresponding sham-operated controls (laparotomy, LAP). Daytime and nighttime energy expenditure A) 14 weeks after GB and B) 8 weeks after DS. **, ***:p<0.01, 0.001 between LAPGB vs. GB or LAPDS vs. DS.

5. Discussion

5.1 Animal Models of Bariatric Surgery

In order to achieve the principle objective of the study, it is important to use the right animal models in the right experimental settings (Fig. 1). In this field of research, food intake has often been quantified by measuring left-over chow in feeders. This is highly inaccurate due to spilling and provides no data regarding eating behavior. In this thesis the state-of-the art Comprehensive Laboratory Animal Monitoring System (CLAMS) has been used to accurately determine food intake, eating behavior, and metabolic parameters in the different animal models. Surgical procedures have been performed according to the rat anatomy, where ileal interposition was performed by interposing only the ileum (2.5-3.5 cm) and gastric bypass was performed without the Roux-en-Y reconstruction as this is not necessary in rats.

Several animal studies of subdiaphragmatic vagus nerve stimulation for obesity treatment have been performed. However, in most of these previous studies, the stimulation was applied only to the left vagal nerve with constant voltage stimulation that would ultimately reduce the current at the nerve as adhesion and resistance builds up [244-251]. In this thesis we stimulated/blocked both vagal branches and used constant current stimulation, ensuring correct stimulation of the nerve even as adhesion and resistance were formed around the electrodes. Several of the previous studies reported reduced body weight gain and food intake after vagus nerve stimulation, but these results were of low quality due to big variations, no presentations of standard deviation (SD) or SEM in the presented figures and use of misleading scales [244-251]. However, one report showed reduced weight gain in obese minipigs after subdiaphragmatic vagus nerve stimulation of both vagal branches using constant current stimulation [252]. To our knowledge, our study was the first to show the weight loss after subdiaphragmatic vagus nerve stimulation in animals and to describe the effects on expression of energy balance-regulating genes in hypothalamus, brainstem, and hippocampus. So far, human studies of vagus nerve stimulation for obesity have been inconclusive and they have all been performed by the same financial sponsor who produces the stimulators [7,8,253]. Our data suggest that it is beneficial to start stimulation at high current, rather than increasing it gradually, in order to achieve the body weight loss.

Previously, animal studies of Botox reported that gastric antral Botox injections induced 8-14% weight loss, however the studies were poor due to big variations and no SD or SEM in the presented figures [143,238]. Some clinical studies showed weight loss [254-258], while others observed no effect on body weight after Botox injection into the gastric wall [9,259-262]. In all of these previous studies, Botox injection was performed through point injections in the muscular layer of the antrum. However, in our study we repeatedly showed greater body weight loss (about 20%) with little variations. In our study, subserosal gastric Botox injection into the whole pyloric antrum was highly efficient at reducing body weight and food intake in both normal chow-fed and DIO rats, even more than sleeve gastrectomy (SG). We also showed that Botox injection had higher efficacy than SG at reducing blood glucose levels. Thus, we suggest that Botox injection can be performed in patients who regain weight after SG surgery. These results were accomplished through subserosal injection of Botox into the whole area of pyloric antrum through one injection. As the goal is to induce maximal diffusion of Botox, we added methylene blue (1%) to the solution to verify that we successfully injected Botox to the whole area of antrum. In addition, we found that Botox injection increased energy expenditure. To our knowledge, the effects of Botox injection on energy expenditure and gene expression in hypothalamus and brainstem have not been previously reported.

In several animal and clinical studies of Botox injection for obesity treatment gastric emptying has been investigated. Some found delayed emptying [9,238,256-258], while others found no effect [259,260,262,263]. However, it should be noticed that in the clinical studies there were great differences with regards to selection of patients, doses of Botox, methods of administration, and evaluation of gastric emptying. Unexpectedly, we found no effect of Botox injection on gastric emptying and no gastroparesis after Botox injection, despite of reducing food intake. This suggests that Botox injection-induced weight loss is not due to delayed gastric emptying. This is confirmed by clinical observations where reduced body weight have not been associated with delayed gastric emptying [9,260,262].

Our results indicate that in order to improve the clinical trials of Botox injection for obesity treatment, injection should be performed in subserosa or submucosa in order to allow for maximal diffusion of Botox into the whole area of pyloric antrum. To achieve this, adding methylene blue or other colored solutions to the Botox may be beneficial.

We found that the lean phenotype of M3KO mice was associated with increased energy expenditure, but without effect on total (24 h) food intake. At 2 months of age there was no significant difference between M3KO mice and WT with regards to body weight. This, together with findings of equal body weights at birth and young age in previous studies, suggest that the lean phenotype develops as the mice grow [146,147]. To our knowledge, we were the first to report unchanged total food intake, increased cumulative food intake, altered eating pattern and increased RER in this mouse model. This was in contrast to previous studies where food intake was found to be reduced and RER to be unaltered [146,147]. Taken together, we suggest that the M3 receptor can be a good target in obesity treatments, possibly by a CNS antagonist and that this may affect energy expenditure rather than food intake.

In contrast to humans where ileum constitutes about 50% of the small intestine, the ileum in rats constitutes only 2% of the small intestine, i.e., 2.5 - 3.5 cm in length [264,265]. In many previous studies of ileal interposition (II) using rats, a segment of 8-20 cm in length, dissected 1-20 cm from the cecum was interposed [171-174,176-179,187,266-280]. This means that in those studies, the ileum was in fact not interposed. In this thesis we have used, for the first time, an anatomically correct rat model of ileal interposition. The previous studies of "ileal interposition" in rats were inconclusive with regards to body weight and food intake, with some reporting reduction [176-178,187,267-269,272-275,278] and others reporting no change [172-174,179,266,270,271,276,277,280-282]. This could be due to the wrong models, big variations in surgical procedure (length of "ileal" segment and position), animal strains, inaccurate methods for food intake determination, and differences in follow-up time. Surprisingly, we found no effect of II on body weight, but increased food intake after the procedure. The combination of II and SG (II-SG) did not induce a significant weight loss neither. These results suggest that II is not suitable as a bariatric surgery, neither is II-SG. This was in contrast to previous reports of "ileal interposition", where II-SG in animals and humans have reduced body weight [10,187,195,197,198,282-285]. For the first time, we reported increased GLP-1 in pancreatic α-cells after SG and II-SG. As II-SG increased GLP-1 expression in both the interposed ileum and pancreatic islets we propose this to have more clinical relevance than II or SG alone as metabolic surgery.

Sleeve gastrectomy is increasingly performed due to its safety and efficiency. In contrast to most animal studies [184-192,224,229,286-288] we found no significant body weight or food

intake reduction after SG in neither normal chow-fed or DIO rats, which was in agreement with other studies [82,233]. Several studies reported increased GLP-1 secretion after SG and it was hypothesized to be caused by increased gastric emptying [185-192,286]. In contrast to our findings, a study reported increased GLP-1 in ileum after SG [187]. To our knowledge, we were the first to report increased GLP-1 levels in α-cells in pancreatic islets after SG.

In this thesis gastric bypass (GB) was performed without the Roux-en-y reconstruction according to the rat anatomy [289]. Gastric bypass is believed to restrict food intake and induce malabsorption, however, in our study gastric bypass did not reduce food intake or cause malabsorption. This was in contrast with some studies [225], but supported by others [221,239,290]. We found that gastric bypass reduced body weight due to increased energy expenditure, which was in line with some previous animal and human studies [219,221,229,291-294] but not with others [295,296].

Similar to gastric bypass, duodenal switch is believed to decrease food intake and induce malabsorption, which was confirmed in this thesis. Duodenal switch altered eating behavior by reducing meal size (g/meal) and rate of eating (g/min), as previously described [82]. In addition, duodenal switch increased resting and active energy expenditure. Consistent with clinical reports, we found that duodenal switch induced greater weight reduction than gastric bypass [11,297,298].

From the results of this thesis, the most efficient procedures for weight loss are duodenal switch, subserosal gastric Botox injection and knockout of the muscarinic acetylcholine M3 receptor (Fig. 19C, D, E, F, and J).

5.2 Possible Underlying Mechanisms

In order to provide strong external evidence for the development of evidence-based medicine, animal research on mechanisms and preclinical trials on the efficacy and safety are of importance (Fig. 2).

The results of this thesis suggest that the mechanism behind weight loss and reduced food intake after VNSB is stimulation of vagal afferent nerve fibers and at the same time blocking of vagal efferent nerve fibers. The effects of VNSB after 48 h were seen only in the brain, suggesting that the mechanism is by direct alteration of neuropeptides in the brain and subsequent altered body weight and eating behavior. Furthermore, VNSB activates afferent pathways to the

brainstem and hippocampus leading to increased expression of anorexigenic neuropeptides, further leading to reduced food intake and body weight. The increased expression of orexigenic neuropeptides in hypothalamus is likely to be compensatory to the gene expression in the brainstem and the weight loss. This implies that the brainstem might be more important in eating behavior regulation than hypothalamus after vagal manipulation.

The results of this thesis suggest that the reduced body weight and food intake after gastric antral Botox injection into the whole pyloric antrum is unlikely due to delayed gastric emptying rather than local blockade of efferent vagal fibers, as also seen in VNSB. Since the effects of Botox on food intake and brain gene expression were not seen shortly after injection, the altered expression of neuropeptides in the brain could be secondary to the body weight loss. Thus, either VNSB or Botox injection leads to reduced food intake and body weight, causing hypothalamic neuropeptides and gut hormones to compensate.

Lack of the muscarinic acetylcholine M3 receptor impairs the action of acetylcholine in the stomach as the M3 receptor is the predominant subreceptor in this location. Detailed phenotyping of M3KO mice suggests that the lean phenotype of this animal model is caused by increased energy expenditure rather than reduced food intake. In contrast to the phenotype, the hypothalamic gene expression of M3KO mice showed a drive for increased food intake and reduced energy expenditure. Again, this suggests that the hypothalamic orexigenic neuropeptides probably compensated for the lean phenotype. This was supported by the increased cumulative food intake. It should be noticed that the compensatory mechanism was without effect on energy expenditure. Thus, the net effect of lack of M3 receptor on body weight is a lean phenotype.

The results of this thesis indicate that the metabolic beneficial effects after ileal interposition is caused by increased levels of GLP-1 in ileum and that sleeve gastrectomy has the beneficial effect through increasing GLP-1 in pancreatic α-cells. The combination of these procedures increased GLP-1 at both locations and would therefore be beneficial for glucose homeostasis. Due to the short half-life of GLP-1 it is likely that the action of GLP-1 in the ileum is through activation of GLP-1 receptors on vagal afferents, leading to vagal efferent signals to pancreatic β–cells, increasing insulin secretion [299]. The increased RER after ileal interposition could be caused by increased food intake and more carbohydrates available. However it might also be linked to the increased GLP-1, causing higher insulin, resulting in an increased cellular uptake of glucose and thereby increasing the use of carbohydrates as fuel for cellular respiration.

It should be noticed that ileal interposition did not reduce food intake even though GLP-1 secretion was increased.

The results of this thesis show that gastric bypass reduced body weight by increasing energy expenditure, while duodenal switch induced a greater weight loss by reducing food intake, altering eating behavior, increasing energy expenditure, and causing malabsorption. Obesity is associated with a state of low-grade, chronic inflammation, however neither gastric bypass or duodenal switch altered plasma concentrations of cytokines [300].

Interestingly, while the animal models of VNSB, Botox injection, and knockout of muscarinic acetylcholine M3 receptor reduced food intake and body weight, the expression of energy-balance regulating peptides in the hypothalamus showed a drive for increased food intake. This indicates that gene expression in hypothalamus, although regarded as a key regulatory brain region in food intake regulation, might not be predictive of the phenotype, but could represent a compensatory mechanism. After VNSB, expression of energy-balance regulating peptides in the brainstem and hippocampus was compatible with a drive for reduced food intake, consistent with the phenotype observed. Based on this, we propose that the brainstem may be more important in the regulation of food intake than hypothalamus in the context of the brain-vagus nerve-gut axis.

Interestingly, the bariatric and metabolic surgeries studied in this thesis also affects the vagus nerve by inducing a remodeling of the gastrointestinal tract that alters the pattern and activation of this nerve by hormones and nutrients [301].

6. Conclusions

In this thesis, the research hypotheses have been generated from clinical practice and research, the underlying mechanisms of bariatric surgery have been studied in animal models, and the potential targets for new, minimally invasive obesity treatments have been proposed and tested in animal models (Fig. 1).

Throughout this thesis the brain-vagus nerve-gut axis has been explored as a target for new, minimally invasive obesity treatments, including vagus nerve stimulation/blocking (VNSB), gastric Botox injections, and muscarinic acetylcholine M3 receptor inhibition. The underlying mechanisms behind these new treatments and of currently used bariatric and metabolic surgeries such as gastric bypass, duodenal switch, sleeve gastrectomy, and ileal interposition have been studied. By this manner, the results from the translational research conducted in this thesis has improved our understanding of the physiological mechanisms of bariatric surgery and discovered new, minimally invasive obesity treatments.

In this thesis it was found that blocking the gastric vagus nerve, by VNSB or Botox injection, can be used as non- or minimally invasive obesity treatment, and muscarinic acetylcholine M3 receptor antagonists might have potential as obesity treatment. Also, combining ileal interposition and sleeve gastrectomy holds potential as a metabolic surgery and duodenal switch induces greater body weight loss than gastric bypass through different mechanisms. Figure 26 illustrates the mechanisms and conclusions generated from this thesis.

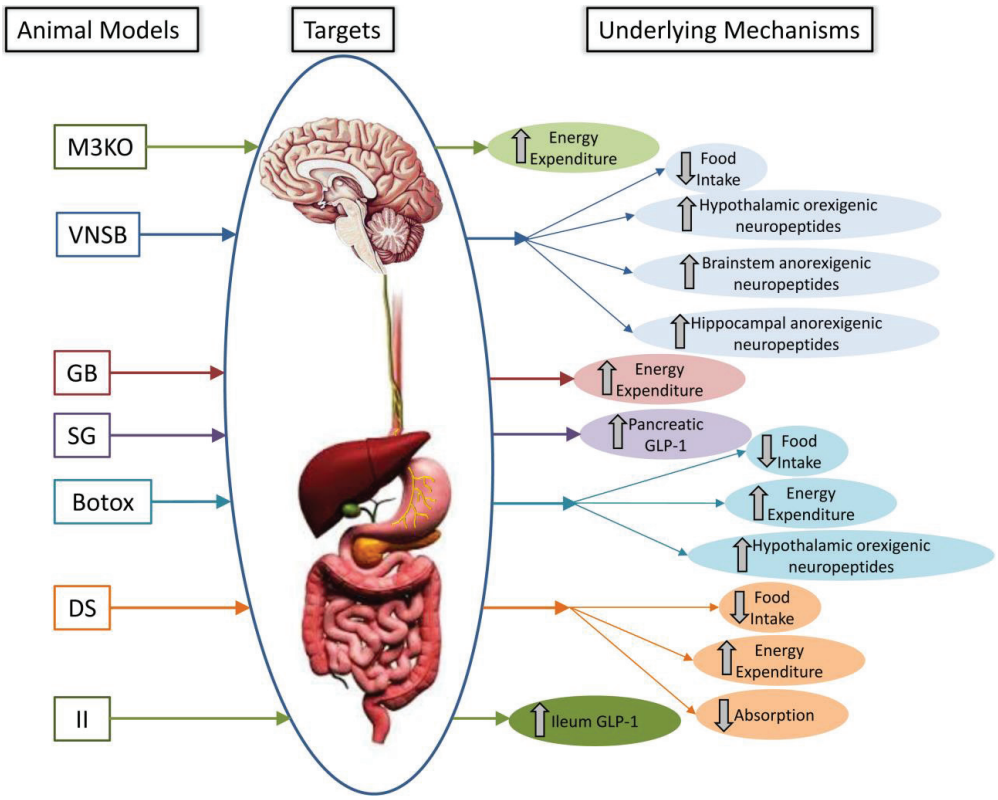


Figure 26 | Summary of the findings of this thesis. The animal models that have been established in this thesis include muscarinic acetylcholine M3 receptor knockout (M3KO) mice, vagus nerve stimulation/blocking (VNSB), gastric bypass (GB), sleeve gastrectomy (SG), Botox injection, duodenal switch (DS), and ileal interposition (II). The targets of the brain-vagus nerve-gut axis have been studied with respect to expression of orexigenic and anorexigenic neuropeptides in hypothalamus, brainstem and hippocampus. The possible underlying physiological mechanisms have been suggested: 1) increased energy expenditure after the ablation of M3 receptor, 2) reduced food intake, increased anorexigenic neuropeptides in brainstem and hippocampus, and increased hypothalamic orexigenic neuropeptides after VNSB, 3) increased energy expenditure after GB, 4) increased pancreatic GLP-1 after SG, 5) reduced food intake, increased energy expenditure and hypothalamic orexigenic neuropeptides after Botox injection, 6) reduced food intake, increased energy expenditure and malabsorption after DS, and 7) increased ileal GLP-1 after II.

7. Future Perspectives

I. This thesis provides strong basis for designing better clinical trials of VNSB (or so-called "VBLOC") and Botox treatment for obesity. In fact, based on the findings in this thesis (Paper I), a clinical phase II trial of gastric antral Botox injection for obesity treatment has been initiated (REK ref no. 2013/1597, EudraCT no. 2012-004381-18).

II. The results of this thesis also suggest that muscarinic acetylcholine M3 receptor antagonists could have potential as obesity treatment. It will be of interest to further investigate whether peripheral or central specific antagonists has the best effect.

III. Based on this thesis, the pancreatic GLP-1 could play an important role in diabetes and diabetes remission, opening up for more research in this area.

IV. The findings that the different bariatric surgeries have different mechanisms highlight the possibility of personalized obesity treatment in the future.

8. References

1. Chen D, Zhao CM (2012) Importance of Translational Research in GI Pharmacology: Lessons Learned from the Successes in History. In: Filaretova LP, Takeuchi K, editors. Cell/Tissue Injury and Cytoprotection/Organoprotection in the Gastrointestinal Tract: Mechanisms, Prevention and Treatment: Front Gastrointest Res. Basel, Karger. pp. 24-31.
2. Horig H, Marincola E, Marincola FM (2005) Obstacles and opportunities in translational research. Nat Med 11: 705-708.
3. Masic I, Miokovic M, Muhamedagic B (2008) Evidence based medicine - new approaches and challenges. Acta Inform Med 16: 219-225.
4. Merkow RP, Ko CY (2011) Evidence-based medicine in surgery: the importance of both experimental and observational study designs. JAMA 306: 436-437.
5. Ergina PL, Cook JA, Blazeby JM, Boutron I, Clavien PA, et al. (2009) Challenges in evaluating surgical innovation. Lancet 374: 1097-1104.
6. Diener MK, Simon T, Buchler MW, Seiler CM (2012) Surgical evaluation and knowledge transfer--methods of clinical research in surgery. Langenbeck Arch Surg 397: 1193-1199.
7. Sarr MG, Billington CJ, Brancatisano R, Brancatisano A, Toouli J, et al. (2012) The EMPOWER study: randomized, prospective, double-blind, multicenter trial of vagal blockade to induce weight loss in morbid obesity. Obes Surg 22: 1771-1782.
8. Shikora S, Toouli J, Herrera MF, Kulseng B, Zulewski H, et al. (2013) Vagal blocking improves glycemic control and elevated blood pressure in obese subjects with type 2 diabetes mellitus. J Obes 245683: 30-38.
9. Topazian M, Camilleri M, Enders FT, Clain JE, Gleeson FC, et al. (2013) Gastric Antral Injections of Botulinum Toxin Delay Gastric Emptying but Do Not Reduce Body Weight. Clin Gastroenterol H 11: 145-150.
10. Kota SK, Ugale S, Gupta N, Naik V, Kumar KV, et al. (2012) Ileal interposition with sleeve gastrectomy for treatment of type 2 diabetes mellitus. Indian J Endocrinol Metab 16: 589-598.
11. Topart P, Becouarn G, Ritz P (2013) Weight loss is more sustained after biliopancreatic diversion with duodenal switch than Roux-en-Y gastric bypass in superobese patients. Surg Obes Relat Dis 9: 526-530.
12. WHO Obesity: preventing and managing the global epidemic. Report of a WHO Consultation presented at: the World Health Organization; June 3-5, 1997; Geneva, Switzerland. Publication WHO/NUT/NCD/98.1.
13. Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, et al. (2011) National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. Lancet 377: 557-567.
14. Burton BT, Foster WR, Hirsch J, Van Itallie TB (1985) Health implications of obesity: an NIH Consensus Development Conference. Int J Obes 9: 155-170.
15. Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, et al. (2009) The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC Public Health 9: 88.
16. Luppino FS, de Wit LM, Bouvy PF, Stijnen T, Cuijpers P, et al. (2010) Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. Arch Gen Psychiatry 67: 220-229.
17. Lash MM, Armstrong A (2009) Impact of obesity on women's health. Fertil Steril 91: 1712-1716.
18. Sermondade N, Faure C, Fezeu L, Shayeb AG, Bonde JP, et al. (2013) BMI in relation to sperm count: an updated systematic review and collaborative meta-analysis. Hum Reprod Update 19: 221-231.

19. Kolotkin RL, Zunker C, Ostbye T (2012) Sexual functioning and obesity: a review. Obesity 20: 2325-2333.
20. Bundred P, Kitchiner D, Buchan I (2001) Prevalence of overweight and obese children between 1989 and 1998: population based series of cross sectional studies. BMJ 322: 326.
21. Finkelstein EA, Trogdon JG, Cohen JW, Dietz W (2009) Annual medical spending attributable to obesity: payer-and service-specific estimates. Health Affair 28: w822-831.
22. Kopelman P, Jebb SA, Butland B (2007) Executive summary: Foresight 'Tackling Obesities: Future Choices' project. Obes Rev 8 Suppl 1: vi-ix.
23. Chakravarthy MV, Booth FW (2004) Eating, exercise, and "thrifty" genotypes: connecting the dots toward an evolutionary understanding of modern chronic diseases. J Appl Physiol 96: 3-10.
24. Bell CG, Walley AJ, Froguel P (2005) The genetics of human obesity. Nat Rev Genet 6: 221-234.
25. Schwartz TL, Nihalani N, Jindal S, Virk S, Jones N (2004) Psychiatric medication-induced obesity: a review. Obes Rev 5: 115-121.
26. Fairburn CG, Cooper Z, Doll HA, Norman P, O'Connor M (2000) The natural course of bulimia nervosa and binge eating disorder in young women. Arch Gen Psychiatry 57: 659-665.
27. Papas MA, Alberg AJ, Ewing R, Helzlsouer KJ, Gary TL, et al. (2007) The Built Environment and Obesity. Epidemiol Rev 29: 129-143.
28. Gulati P, Yeo GS (2013) The biology of FTO: from nucleic acid demethylase to amino acid sensor. Diabetologia 56: 2113-2121.
29. Tsai AG, Wadden TA (2013) In the clinic: obesity. Ann Intern Med 159: ITC3-1.
30. Miller WC (1999) How effective are traditional dietary and exercise interventions for weight loss? Med Sci Sports Exerc 31: 1129-1134.
31. Schwartz MW, Woods SC, Seeley RJ, Barsh GS, Baskin DG, et al. (2003) Is the Energy Homeostasis System Inherently Biased Toward Weight Gain? Diabetes 52: 232-238.
32. Rucker D, Padwal R, Li SK, Curioni C, Lau DC (2007) Long term pharmacotherapy for obesity and overweight: updated meta-analysis. BMJ 335: 1194-1199.
33. Colquitt J, Picot J, Loveman E, Clegg A (2009) Surgery for obesity COCHRANE DB SYST REV.
34. Gloy VL, Briel M, Bhatt DL, Kashyap SR, Schauer PR, et al. (2013) Bariatric surgery versus non-surgical treatment for obesity: a systematic review and meta-analysis of randomised controlled trials. BMJ 22: f5934.
35. Saber AA, Elgamal MH, McLeod MK (2008) Bariatric surgery: the past, present, and future. Obes Surg 18: 121-128.
36. Buchwald H, Oien DM (2013) Metabolic/bariatric surgery worldwide 2011. Obes Surg 23: 427-436.
37. Anthone GJ, Lord RV, DeMeester TR, Crookes PF (2003) The duodenal switch operation for the treatment of morbid obesity. Ann Surg 238: 618-627.
38. Hedberg J, Sundstrom J, Sundbom M (2014) Duodenal switch versus Roux-en-Y gastric bypass for morbid obesity: systematic review and meta-analysis of weight results, diabetes resolution and early complications in single-centre comparisons. Obes Rev.
39. Buchwald H, Avidor Y, Braunwald E, Jensen MD, Pories W, et al. (2004) Bariatric surgery: a systematic review and meta-analysis. JAMA 292: 1724-1737.
40. Wang S, Li P, Sun XF, Ye NY, Xu ZK, et al. (2013) Comparison between laparoscopic sleeve gastrectomy and laparoscopic adjustable gastric banding for morbid obesity: a meta-analysis. Obes Surg 23: 980-986.
41. Sjostrom L, Lindroos AK, Peltonen M, Torgerson J, Bouchard C, et al. (2004) Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery. N Engl J Med 351: 2683-2693.
42. Flum DR, Dellinger EP (2004) Impact of gastric bypass operation on survival: A population-based analysis. J Am Coll Surgeons 199: 543-551.

43. Fernandez AZ, Jr., Demaria EJ, Tichansky DS, Kellum JM, Wolfe LG, et al. (2004) Multivariate analysis of risk factors for death following gastric bypass for treatment of morbid obesity. Ann Surg 239: 698-702.
44. Encinosa WE, Bernard DM, Chen C-C, Steiner CA (2006) Healthcare Utilization and Outcomes after Bariatric Surgery. Med Care 44: 706-712.
45. Podnos YD, Jimenez JC, Wilson SE, Stevens CM, Nguyen NT (2003) Complications after laparoscopic gastric bypass: a review of 3464 cases. Arch Surg 138: 957-961.
46. Xanthakos SA (2009) Nutritional deficiencies in obesity and after bariatric surgery. Pediatr Clin North Am 56: 1105-1121.
47. Vargas-Ruiz AG, Hernandez-Rivera G, Herrera MF (2008) Prevalence of iron, folate, and vitamin B12 deficiency anemia after laparoscopic Roux-en-Y gastric bypass. Obes Surg 18: 288-293.
48. WHO (2013). "Fact sheet - Obesity and overweight." Retrieved 11.05, 2014, from http://www.who.int/mediacentre/factsheets/fs311/en/.
49. Sam AH, Troke RC, Tan TM, Bewick GA (2012) The role of the gut/brain axis in modulating food intake. Neuropharmacology 63: 46-56.
50. Valassi E, Scacchi M, Cavagnini F (2008) Neuroendocrine control of food intake. Nutr Metab Cardiovasc Dis 18: 158-168.
51. Schwartz MW, Woods SC, Porte D, Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. Nature 404: 661-671.
52. Shan X, Yeo GS (2011) Central leptin and ghrelin signalling: comparing and contrasting their mechanisms of action in the brain. Rev Endocr Metab Disord 12: 197-209.
53. Sun Y, Butte NF, Garcia JM, Smith RG (2008) Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. Endocrinology 149: 843-850.
54. Oswal A, Yeo G (2010) Leptin and the control of body weight: a review of its diverse central targets, signaling mechanisms, and role in the pathogenesis of obesity. Obesity 18: 221-229.
55. Dockray GJ (2013) Enteroendocrine cell signalling via the vagus nerve. Curr Opin Pharmacol 13: 954-958.
56. Grill HJ, Hayes MR (2009) The nucleus tractus solitarius: a portal for visceral afferent signal processing, energy status assessment and integration of their combined effects on food intake. Int J Obes 33: S11-15.
57. Zheng H, Berthoud H-R (2008) Neural Systems Controlling the Drive to Eat: Mind Versus Metabolism. Physiology 23: 75-83.
58. Badman MK, Flier JS (2005) The gut and energy balance: visceral allies in the obesity wars. Science 307: 1909-1914.
59. Groves DA, Brown VJ (2005) Vagal nerve stimulation: a review of its applications and potential mechanisms that mediate its clinical effects. Neurosci Biobehav Rev 29: 493-500.
60. Imatake K, Matsui T, Moriyama M (2009) The effect and mechanism of action of capsaicin on gastric acid output. J Gastroenterol 44: 396-404.
61. Brunicardi FC, Shavelle D, Andersen D (1995) Neural regulation of the endocrine pancreas. Int J Pancreatol 18: 177-195.
62. Oberholzer RJ (1963) Afferent Fibers of Cardiovascular and Pulmonary Origins and Their Intrabulbar Connections. Tohoku J Exp Med 80: 288-314.
63. Erman AB, Kejner AE, Hogikyan ND, Feldman EL (2009) Disorders of cranial nerves IX and X. Semin Neurol 29: 85-92.
64. Foley JO, DuBois FS (1937) Quantitative studies of the vagus nerve in the cat. I. The ratio of sensory to motor fibers. J Comp Neurol 67: 49-67.
65. Kandel ER, Schwartz JH, Jessell TM, Siegelbaum SA, Hudspeth AJ (2000) Principles of Neural Science. New York: McGraw Hill.

66. Institute TUC (2010). "Baby's Brain Begins Now: Conception to Age 3." Retrieved 24.03, 2014, from http://www.urbanchildinstitute.org/why-0-3/baby-and-brain.
67. Erlanger J, Gasser ILS (1930) The action potential in fibers of slow conduction in spinal roots and somatic nerves. Am J Physiol 92: 43-81.
68. Nosaka S, Yasunaga K, Tamai S (1982) Vagal cardiac preganglionic neurons: distribution, cell types, and reflex discharges. Am J Physiol 243: R92-98.
69. Ogbonnaya S, Kaliaperumal C (2013) Vagal nerve stimulator: Evolving trends. J Nat Sci Biol Med 4: 8-13.
70. Travagli RA, Hermann GE, Browning KN, Rogers RC (2006) Brainstem circuits regulating gastric function. Annu Rev Physiol 68: 279-305.
71. Berthoud HR, Carlson NR, Powley TL (1991) Topography of efferent vagal innervation of the rat gastrointestinal tract. Am J Physiol 260: R200-207.
72. Ben-Menachem E, Hamberger A, Hedner T, Hammond EJ, Uthman BM, et al. (1995) Effects of vagus nerve stimulation on amino acids and other metabolites in the CSF of patients with partial seizures. Epilepsy Res 20: 221-227.
73. Ricardo JA, Koh ET (1978) Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. Brain Res 153: 1-26.
74. Ruffoli R, Giorgi FS, Pizzanelli C, Murri L, Paparelli A, et al. (2011) The chemical neuroanatomy of vagus nerve stimulation. J Chem Neuroanat 42: 288-296.
75. Ter Horst GJ, de Boer P, Luiten PG, van Willigen JD (1989) Ascending projections from the solitary tract nucleus to the hypothalamus. A Phaseolus vulgaris lectin tracing study in the rat. Neuroscience 31: 785-797.
76. Zardetto-Smith AM, Gray TS (1990) Organization of peptidergic and catecholaminergic efferents from the nucleus of the solitary tract to the rat amygdala. Brain Res Bull 25: 875-887.
77. van der Kooy D, Koda LY, McGinty JF, Gerfen CR, Bloom FE (1984) The organization of projections from the cortex, amygdala, and hypothalamus to the nucleus of the solitary tract in rat. J Comp Neurol 224: 1-24.
78. Schwartz GJ (2000) The role of gastrointestinal vagal afferents in the control of food intake: current prospects. Nutrition 16: 866-873.
79. Tewfik TL (2013). "Gross Anatomy." Retrieved 24.03, 2014, from http://emedicine.medscape.com/article/1875813-overview.
80. Lagoo J, Pappas TN, Perez A (2014) A relic or still relevant: the narrowing role for vagotomy in the treatment of peptic ulcer disease. Am J Surg 207: 120-126.
81. Kral JG (1978) Vagotomy for treatment of severe obesity. Lancet 1: 307-308.
82. Kodama Y, Zhao CM, Kulseng B, Chen D (2010) Eating behavior in rats subjected to vagotomy, sleeve gastrectomy, and duodenal switch. J Gastrointest Surg 14: 1502-1510.
83. Kral JG, Paez W, Wolfe BM (2009) Vagal nerve function in obesity: therapeutic implications. World J Surg 33: 1995-2006.
84. Merrill DR, Bikson M, Jefferys JGR (2005) Electrical stimulation of excitable tissue: design of efficacious and safe protocols. J Neurosci Meth 141: 171-198.
85. Corning JL (1883) Considerations on pathology and therapeutics of epilepsy. J Nerv Ment Dis 10: 243-248.
86. Bailey P, Bremer F (1938) Sensory cortical representation of the Vagus nerve. J Neurophysiol 1: 405-412.
87. Zabara J (1992) Inhibition of experimental seizures in canines by repetitive vagal stimulation. Epilepsia 33: 1005-1012.

88. Penry JK, Dean JC (1990) Prevention of intractable partial seizures by intermittent vagal stimulation in humans: preliminary results. Epilepsia 31: S40-43.
89. Morris GL, 3rd, Mueller WM (1999) Long-term treatment with vagus nerve stimulation in patients with refractory epilepsy. The Vagus Nerve Stimulation Study Group E01-E05. Neurology 53: 1731-1735.
90. Ben-Menachem E, Manon-Espaillat R, Ristanovic R, Wilder BJ, Stefan H, et al. (1994) Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. First International Vagus Nerve Stimulation Study Group. Epilepsia 35: 616-626.
91. Elger G, Hoppe C, Falkai P, Rush AJ, Elger CE (2000) Vagus nerve stimulation is associated with mood improvements in epilepsy patients. Epilepsy Res 42: 203-210.
92. Chavel SM, Westerveld M, Spencer S (2003) Long-term outcome of vagus nerve stimulation for refractory partial epilepsy. Epilepsy Behav 4: 302-309.
93. Clark KB, Naritoku DK, Smith DC, Browning RA, Jensen RA (1999) Enhanced recognition memory following vagus nerve stimulation in human subjects. Nat Neurosci 2: 94-98.
94. Bodenlos JS, Kose S, Borckardt JJ, Nahas Z, Shaw D, et al. (2007) Vagus nerve stimulation acutely alters food craving in adults with depression. Appetite 48: 145-153.
95. Ness TJ, Fillingim RB, Randich A, Backensto EM, Faught E (2000) Low intensity vagal nerve stimulation lowers human thermal pain thresholds. Pain 86: 81-85.
96. Rush AJ, George MS, Sackeim HA, Marangell LB, Husain MM, et al. (2000) Vagus nerve stimulation (VNS) for treatment-resistant depressions: a multicenter study. Biol Psychiatry 47: 276-286.
97. George MS, Ward HE, Jr., Ninan PT, Pollack M, Nahas Z, et al. (2008) A pilot study of vagus nerve stimulation (VNS) for treatment-resistant anxiety disorders. Brain Stimul 1: 112-121.
98. Sjogren MJ, Hellstrom PT, Jonsson MA, Runnerstam M, Silander HC, et al. (2002) Cognition-enhancing effect of vagus nerve stimulation in patients with Alzheimer's disease: a pilot study. J Clin Psychiatry 63: 972-980.
99. Lomarev M, Denslow S, Nahas Z, Chae JH, George MS, et al. (2002) Vagus nerve stimulation (VNS) synchronized BOLD fMRI suggests that VNS in depressed adults has frequency/dose dependent effects. J Psychiatr Res 36: 219-227.
100. Krahl SE, Senanayake SS, Handforth A (2001) Destruction of peripheral C-fibers does not alter subsequent vagus nerve stimulation-induced seizure suppression in rats. Epilepsia 42: 586-589.
101. Koo B, Ham SD, Sood S, Tarver B (2001) Human vagus nerve electrophysiology: a guide to vagus nerve stimulation parameters. J Clin Neurophysiol 18: 429-433.
102. Krahl SE, Clark KB, Smith DC, Browning RA (1998) Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. Epilepsia 39: 709-714.
103. Dorr AE, Debonnel G (2006) Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. J Pharmacol Exp Ther 318: 890-898.
104. Follesa P, Biggio F, Gorini G, Caria S, Talani G, et al. (2007) Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. Brain Res 7: 28-34.
105. Marrosu F, Serra A, Maleci A, Puligheddu M, Biggio G, et al. (2003) Correlation between GABA(A) receptor density and vagus nerve stimulation in individuals with drug-resistant partial epilepsy. Epilepsy Res 55: 59-70.
106. Naritoku DK, Terry WJ, Helfert RH (1995) Regional induction of fos immunoreactivity in the brain by anticonvulsant stimulation of the vagus nerve. Epilepsy Res 22: 53-62.
107. Henry TR, Bakay RA, Pennell PB, Epstein CM, Votaw JR (2004) Brain blood-flow alterations induced by therapeutic vagus nerve stimulation in partial epilepsy: II. prolonged effects at high and low levels of stimulation. Epilepsia 45: 1064-1070.

108. Bohning DE, Lomarev MP, Denslow S, Nahas Z, Shastri A, et al. (2001) Feasibility of vagus nerve stimulation-synchronized blood oxygenation level-dependent functional MRI. Invest Radiol 36: 470-479.
109. Pardo JV, Sheikh SA, Kuskowski MA, Surerus-Johnson C, Hagen MC, et al. (2007) Weight loss during chronic, cervical vagus nerve stimulation in depressed patients with obesity: an observation. Int J Obes 31: 1756-1759.
110. Gibson EL, Mohiyeddini C (2008) Vagus nerve stimulation confuses appetite: comment on Bodenlos et al. (2007). Appetite 51: 223-225.
111. Vijgen GH, Bouvy ND, Leenen L, Rijkers K, Cornips E, et al. (2013) Vagus nerve stimulation increases energy expenditure: relation to brown adipose tissue activity. PLoS One 8: e77221.
112. Roslin M, Kurian M (2001) The Use of Electrical Stimulation of the Vagus Nerve to Treat Morbid Obesity. Epilepsy Behav 2: S11-16.
113. Dedeurwaerdere S, Gilby K, Vonck K, Delbeke J, Boon P, et al. (2006) Vagus nerve stimulation does not affect spatial memory in fast rats, but has both anti-convulsive and pro-convulsive effects on amygdala-kindled seizures. Neuroscience 140: 1443-1451.
114. Koren MS, Holmes MD (2006) Vagus nerve stimulation does not lead to significant changes in body weight in patients with epilepsy. Epilepsy Behav 8: 246-249.
115. Kansagra S, Ataya N, Lewis D, Gallentine W, Mikati MA (2010) The effect of vagus nerve stimulation therapy on body mass index in children. Epilepsy Behav 19: 50-51.
116. Sugiyama H (1980) Clostridium botulinum neurotoxin. Microbiol Rev 44: 419-448.
117. Eleopra R, Tugnoli V, Rossetto O, De Grandis D, Montecucco C (1998) Different time courses of recovery after poisoning with botulinum neurotoxin serotypes A and E in humans. Neurosci Lett 256: 135-138.
118. Sloop RR, Cole BA, Escutin RO (1997) Human response to botulinum toxin injection: type B compared with type A. Neurology 49: 189-194.
119. Flynn TC (2012) Advances in the use of botulinum neurotoxins in facial esthetics. J Cosmet Dermatol 11: 42-50.
120. Montal M (2010) Botulinum neurotoxin: a marvel of protein design. Annu Rev Biochem 79: 591-617.
121. Jacky BP, Garay PE, Dupuy J, Nelson JB, Cai B, et al. (2013) Identification of fibroblast growth factor receptor 3 (FGFR3) as a protein receptor for botulinum neurotoxin serotype A (BoNT/A). PLoS Pathog 9: e1003369.
122. Schiavo G, Matteoli M, Montecucco C (2000) Neurotoxins Affecting Neuroexocytosis. Physiol Rev 80: 717-766.
123. Montal M (2009) Translocation of botulinum neurotoxin light chain protease by the heavy chain protein-conducting channel. Toxicon 54: 565-569.
124. Montecucco C, Schiavo G (1994) Mechanism of action of tetanus and botulinum neurotoxins. Mol Microbiol 13: 1-8.
125. Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, et al. (1993) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature 365: 160-163.
126. Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, et al. (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362: 318-324.
127. Rowland LP (2002) Stroke, spasticity, and botulinum toxin. N Engl J Med 347: 382-383.
128. Bakheit AM, Ward CD, McLellan DL (1997) Generalised botulism-like syndrome after intramuscular injections of botulinum toxin type A: a report of two cases. J Neurol Neurosurg Psychiatry 62: 198.
129. Naumann M, Jankovic J (2004) Safety of botulinum toxin type A: a systematic review and meta-analysis. Curr Med Res Opin 20: 981-990.

130. Scott AB, Magoon EH, McNeer KW, Stager DR (1990) Botulinum treatment of childhood strabismus. Ophthalmology 97: 1434-1438.
131. Anderson TJ, Rivest J, Stell R, Steiger MJ, Cohen H, et al. (1992) Botulinum toxin treatment of spasmodic torticollis. J R Soc Med 85: 524-529.
132. Jitpimolmard S, Tiamkao S, Laopaiboon M (1998) Long term results of botulinum toxin type A (Dysport) in the treatment of hemifacial spasm: a report of 175 cases. J Neurol Neurosurg Psychiatry 64: 751-757.
133. Simpson DM (1997) Clinical trials of botulinum toxin in the treatment of spasticity. Muscle Nerve 6: S169-175.
134. Naumann M, Zellner M, Toyka KV, Reiners K (1997) Treatment of gustatory sweating with botulinum toxin. Ann Neurol 42: 973-975.
135. Vashishta R, Nguyen SA, White DR, Gillespie MB (2013) Botulinum toxin for the treatment of sialorrhea: a meta-analysis. Otolaryngol Head Neck Surg 148: 191-196.
136. Jackson JL, Kuriyama A, Hayashino Y (2012) Botulinum toxin A for prophylactic treatment of migraine and tension headaches in adults: a meta-analysis. JAMA 307: 1736-1745.
137. Langevin P, Peloso PM, Lowcock J, Nolan M, Weber J, et al. (2011) Botulinum toxin for subacute/chronic neck pain. Cochrane Database Syst Rev 6.
138. Mahowald ML, Krug HE, Singh JA, Dykstra D (2009) Intra-articular Botulinum Toxin Type A: a new approach to treat arthritis joint pain. Toxicon 54: 658-667.
139. Maria G, Cassetta E, Gui D, Brisinda G, Bentivoglio AR, et al. (1998) A comparison of botulinum toxin and saline for the treatment of chronic anal fissure. N Engl J Med 338: 217-220.
140. Cuilliere C, Ducrotte P, Zerbib F, Metman EH, de Looze D, et al. (1997) Achalasia: outcome of patients treated with intrasphincteric injection of botulinum toxin. Gut 41: 87-92.
141. Glogau R, Kane M, Beddingfield F, Somogyi C, Lei X, et al. (2012) OnabotulinumtoxinA: a meta-analysis of duration of effect in the treatment of glabellar lines. Dermatol Surg 38: 1794-1803.
142. Olsson C, Holmgren S (2001) The control of gut motility. Comp Biochem Phys A 128: 479-501.
143. Gui D, De Gaetano A, Spada PL, Viggiano A, Cassetta E, et al. (2000) Botulinum toxin injected in the gastric wall reduces body weight and food intake in rats. Aliment Pharmacol Ther 14: 829-834.
144. Caulfield MP, Birdsall NJ (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50: 279-290.
145. Shah N, Khurana S, Cheng K, Raufman J-P (2009) Muscarinic receptors and ligands in cancer. Am J Physiol - Cell Ph 296: C221-C232.
146. Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, et al. (2001) Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. Nature 410: 207-212.
147. Gautam D, Gavrilova O, Jeon J, Pack S, Jou W, et al. (2006) Beneficial metabolic effects of M3 muscarinic acetylcholine receptor deficiency. Cell Metab 4: 363-375.
148. Hamilton SE, Loose MD, Qi M, Levey AI, Hille B, et al. (1997) Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc Natl Acad Sci U S A 94: 13311-13316.
149. Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, et al. (1999) Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 96: 1692-1697.
150. Gomeza J, Zhang L, Kostenis E, Felder C, Bymaster F, et al. (1999) Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M(4) muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 96: 10483-10488.
151. Yamada M, Lamping KG, Duttaroy A, Zhang W, Cui Y, et al. (2001) Cholinergic dilation of cerebral blood vessels is abolished in M(5) muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 98: 14096-14101.

152. Maresca A, Supuran CT (2008) Muscarinic acetylcholine receptors as therapeutic targets for obesity. Expert Opin Ther Targets 12: 1167-1175.
153. Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, et al. (2011) National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. Lancet 378: 31-40.
154. Alberti KG, Zimmet PZ (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15: 539-553.
155. Kahn SE, Hull RL, Utzschneider KM (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444: 840-846.
156. van Greevenbroek MM, Schalkwijk CG, Stehouwer CD (2013) Obesity-associated low-grade inflammation in type 2 diabetes mellitus: causes and consequences. Neth J Med 71: 174-187.
157. Rubino F, R'Bibo S L, del Genio F, Mazumdar M, McGraw TE (2010) Metabolic surgery: the role of the gastrointestinal tract in diabetes mellitus. Nat Rev Endocrinol 6: 102-109.
158. Thomas S, Schauer P (2010) Bariatric surgery and the gut hormone response. Nutr Clin Pract 25: 175-182.
159. Saeidi N, Meoli L, Nestoridi E, Gupta NK, Kvas S, et al. (2013) Reprogramming of intestinal glucose metabolism and glycemic control in rats after gastric bypass. Science 341: 406-410.
160. Herrmann C, Goke R, Richter G, Fehmann HC, Arnold R, et al. (1995) Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients. Digestion 56: 117-126.
161. Willms B, Werner J, Holst JJ, Orskov C, Creutzfeldt W, et al. (1996) Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients. J Clin Endocrinol Metab 81: 327-332.
162. Flint A, Raben A, Astrup A, Holst JJ (1998) Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. J Clin Invest 101: 515-520.
163. Perfetti R, Zhou J, Doyle ME, Egan JM (2000) Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. Endocrinology 141: 4600-4605.
164. Fehmann HC, Habener JF (1991) Functional receptors for the insulinotropic hormone glucagon-like peptide-I(7-37) on a somatostatin secreting cell line. FEBS Lett 279: 335-340.
165. Heller RS, Kieffer TJ, Habener JF (1997) Insulinotropic glucagon-like peptide I receptor expression in glucagon-producing alpha-cells of the rat endocrine pancreas. Diabetes 46: 785-791.
166. Mannucci E, Ognibene A, Cremasco F, Bardini G, Mencucci A, et al. (2000) Glucagon-like peptide (GLP)-1 and leptin concentrations in obese patients with Type 2 diabetes mellitus. Diabet Med 17: 713-719.
167. Vilsboll T, Krarup T, Deacon CF, Madsbad S, Holst JJ (2001) Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. Diabetes 50: 609-613.
168. Lugari R, Dei Cas A, Ugolotti D, Finardi L, Barilli AL, et al. (2002) Evidence for early impairment of glucagon-like peptide 1-induced insulin secretion in human type 2 (non insulin-dependent) diabetes. Horm Metab Res 34: 150-154.
169. Strader AD (2006) Ileal transposition provides insight into the effectiveness of gastric bypass surgery. Physiol Behav 88: 277-282.
170. Hayes MR (2012) Neuronal and intracellular signaling pathways mediating GLP-1 energy balance and glycemic effects. Physiol Behav 106: 413-416.

171. Kohli R, Kirby M, Setchell KD, Jha P, Klustaitis K, et al. (2010) Intestinal adaptation after ileal interposition surgery increases bile acid recycling and protects against obesity-related comorbidities. Am J Physiol Gastrointest Liver Physiol 299: G652-660.
172. Patriti A, Facchiano E, Annetti C, Aisa MC, Galli F, et al. (2005) Early improvement of glucose tolerance after ileal transposition in a non-obese type 2 diabetes rat model. Obes Surg 15: 1258-1264.
173. Patriti A, Aisa MC, Annetti C, Sidoni A, Galli F, et al. (2007) How the hindgut can cure type 2 diabetes. Ileal transposition improves glucose metabolism and beta-cell function in Goto-kakizaki rats through an enhanced Proglucagon gene expression and L-cell number. Surgery 142: 74-85.
174. Cummings BP, Strader AD, Stanhope KL, Graham JL, Lee J, et al. (2010) Ileal interposition surgery improves glucose and lipid metabolism and delays diabetes onset in the UCD-T2DM rat. Gastroenterology 138: 2437-2446.
175. Strader AD, Clausen TR, Goodin SZ, Wendt D (2009) Ileal interposition improves glucose tolerance in low dose streptozotocin-treated diabetic and euglycemic rats. Obes Surg 19: 96-104.
176. Strader AD, Vahl TP, Jandacek RJ, Woods SC, D´Alessio DA, et al. (2005) Weight loss through ileal transposition is accompanied by increased ileal hormone secretion and synthesis in rats. Am J Physiol-Endoc M 288: E447-E453.
177. Wang TT, Hu SY, Gao HD, Zhang GY, Liu CZ, et al. (2008) Ileal Transposition Controls Diabetes as Well as Modified Duodenal Jejunal Bypass With Better Lipid Lowering in a Nonobese Rat Model of Type 2 Diabetes by Increasing GLP-1. Ann Surg 247: 968-975.
178. Sun X, Song M, Bai R, Cheng S, Xing Y, et al. (2013) Ileal interposition surgery-induced improvement of hyperglycemia and insulin resistance in Goto-Kakizaki rats by upregulation of TCF7L2 expression. Exp Ther Med 5: 1511-1515.
179. Ikezawa F, Shibata C, Kikuchi D, Imoto H, Miura K, et al. (2012) Effects of ileal interposition on glucose metabolism in obese rats with diabetes. Surgery 151: 822-830.
180. Culnan DM, Albaugh V, Sun M, Lynch CJ, Lang CH, et al. (2010) Ileal interposition improves glucose tolerance and insulin sensitivity in the obese Zucker rat. Am J Physiol Gastrointest Liver Physiol 299: G751-760.
181. Bohdjalian A, Langer FB, Shakeri-Leidenmühler S, Gfrerer L, Ludvik B, et al. (2010) Sleeve Gastrectomy as Sole and Definitive Bariatric Procedure: 5-Year Results for Weight Loss and Ghrelin. Obes Surg 20: 535-540.
182. Leonetti F, Capoccia D, Coccia F, Casella G, Baglio G, et al. (2012) Obesity, type 2 diabetes mellitus, and other comorbidities: a prospective cohort study of laparoscopic sleeve gastrectomy vs medical treatment. Arch Surg 147: 694-700.
183. Vidal J, Ibarzabal A, Romero F, Delgado S, Momblan D, et al. (2008) Type 2 diabetes mellitus and the metabolic syndrome following sleeve gastrectomy in severely obese subjects. Obes Surg 18: 1077-1082.
184. Li F, Zhang G, Liang J, Ding X, Cheng Z, et al. (2009) Sleeve gastrectomy provides a better control of diabetes by decreasing ghrelin in the diabetic Goto-Kakizaki rats. J Gastrointest Surg 13: 2302-2308.
185. Sun D, Liu S, Zhang G, Colonne P, Hu C, et al. (2014) Sub-sleeve gastrectomy achieves good diabetes control without weight loss in a non-obese diabetic rat model. Surg Endosc 28: 1010-1018.
186. Trung VN, Yamamoto H, Yamaguchi T, Murata S, Akabori H, et al. (2013) Effect of sleeve gastrectomy on body weight, food intake, glucose tolerance, and metabolic hormone level in two different rat models: Goto-Kakizaki and diet-induced obese rat. J Surg Res 185: 159-165.
187. Nausheen S, Shah IH, Pezeshki A, Sigalet DL, Chelikani PK (2013) Effects of sleeve gastrectomy and ileal transposition, alone and in combination, on food intake, body weight, gut hormones, and glucose metabolism in rats. Am J Physiol-Endoc M 305: E507-518.

188. Cummings BP, Bettaieb A, Graham JL, Stanhope KL, Kowala M, et al. (2012) Vertical sleeve gastrectomy improves glucose and lipid metabolism and delays diabetes onset in UCD-T2DM rats. Endocrinology 153: 3620-3632.
189. Chambers AP, Jessen L, Ryan KK, Sisley S, Wilson-Perez HE, et al. (2011) Weight-independent changes in blood glucose homeostasis after gastric bypass or vertical sleeve gastrectomy in rats. Gastroenterology 141: 950-958.
190. Chambers AP, Smith EP, Begg DP, Grayson BE, Sisley S, et al. (2014) Regulation of gastric emptying rate and its role in nutrient-induced GLP-1 secretion in rats after vertical sleeve gastrectomy. Am J Physiol Endocrinol Metab 306: 24.
191. Kawano Y, Ohta M, Hirashita T, Masuda T, Inomata M, et al. (2013) Effects of sleeve gastrectomy on lipid metabolism in an obese diabetic rat model. Obes Surg 23: 1947-1956.
192. Masuda T, Ohta M, Hirashita T, Kawano Y, Eguchi H, et al. (2011) A comparative study of gastric banding and sleeve gastrectomy in an obese diabetic rat model. Obes Surg 21: 1774-1780.
193. Chambers AP, Kirchner H, Wilson-Perez HE, Willency JA, Hale JE, et al. (2013) The effects of vertical sleeve gastrectomy in rodents are ghrelin independent. Gastroenterology 144: 50-52.
194. Gagner M (2011) Surgical treatment of nonseverely obese patients with type 2 diabetes mellitus: sleeve gastrectomy with ileal transposition (SGIT) is the same as the neuroendocrine brake (NEB) procedure or ileal interposition associated with sleeve gastrectomy (II-SG), but ileal interposition with diverted sleeve gastrectomy (II-DSG) is the same as duodenal switch. Surg Endosc 25: 655-656.
195. DePaula AL, Macedo AL, Rassi N, Vencio S, Machado CA, et al. (2008) Laparoscopic treatment of metabolic syndrome in patients with type 2 diabetes mellitus. Surg Endosc 22: 2670-2678.
196. DePaula AL, Stival AR, Halpern A, Vencio S (2011) Surgical treatment of morbid obesity: mid-term outcomes of the laparoscopic ileal interposition associated to a sleeve gastrectomy in 120 patients. Obes Surg 21: 668-675.
197. DePaula AL, Macedo AL, Schraibman V, Mota BR, Vencio S (2009) Hormonal evaluation following laparoscopic treatment of type 2 diabetes mellitus patients with BMI 20-34. Surg Endosc 23: 1724-1732.
198. De Paula A, Stival A, Halpern A, DePaula C, Mari A, et al. (2011) Improvement in Insulin Sensitivity and B-Cell Function Following Ileal Interposition with Sleeve Gastrectomy in Type 2 Diabetic Patients: Potential Mechanisms. J Gastrointest Surg 15: 1344-1353.
199. Mathes CM, Spector AC (2012) Food selection and taste changes in humans after Roux-en-Y gastric bypass surgery: a direct-measures approach. Physiol Behav 107: 476-483.
200. Ashrafian H, Bueter M, Ahmed K, Suliman A, Bloom SR, et al. (2010) Metabolic surgery: an evolution through bariatric animal models. Obes Rev 11: 907-920.
201. Schauer PR, Kashyap SR, Wolski K, Brethauer SA, Kirwan JP, et al. (2012) Bariatric surgery versus intensive medical therapy in obese patients with diabetes. N Engl J Med 366: 1567-1576.
202. Gagner M, Rogula T (2003) Laparoscopic reoperative sleeve gastrectomy for poor weight loss after biliopancreatic diversion with duodenal switch. Obes Surg 13: 649-654.
203. Miller K, Hell E (2003) Laparoscopic surgical concepts of morbid obesity. Langenbeck Arch Surg 388: 375-384.
204. Topart P, Becouarn G, Ritz P (2011) Pouch size after gastric bypass does not correlate with weight loss outcome. Obes Surg 21: 1350-1354.
205. O'Connor EA, Carlin AM (2008) Lack of correlation between variation in small-volume gastric pouch size and weight loss after laparoscopic Roux-en-Y gastric bypass. Surg Obes Relat Dis 4: 399-403.
206. Madan AK, Tichansky DS, Phillips JC (2007) Does pouch size matter? Obes Surg 17: 317-320.

207. Korner J, Bessler M, Cirilo LJ, Conwell IM, Daud A, et al. (2005) Effects of Roux-en-Y gastric bypass surgery on fasting and postprandial concentrations of plasma ghrelin, peptide YY, and insulin. J Clin Endocrinol Metab 90: 359-365.
208. Karamanakos SN, Vagenas K, Kalfarentzos F, Alexandrides TK (2008) Weight loss, appetite suppression, and changes in fasting and postprandial ghrelin and peptide-YY levels after Roux-en-Y gastric bypass and sleeve gastrectomy: a prospective, double blind study. Ann Surg 247: 401-407.
209. Korner J, Bessler M, Inabnet W, Taveras C, Holst JJ (2007) Exaggerated glucagon-like peptide-1 and blunted glucose-dependent insulinotropic peptide secretion are associated with Roux-en-Y gastric bypass but not adjustable gastric banding. Surg Obes Relat Dis 3: 597-601.
210. Wilson-Perez HE, Chambers AP, Ryan KK, Li B, Sandoval DA, et al. (2013) Vertical sleeve gastrectomy is effective in two genetic mouse models of glucagon-like Peptide 1 receptor deficiency. Diabetes 62: 2380-2385.
211. Chandarana K, Gelegen C, Karra E, Choudhury AI, Drew ME, et al. (2011) Diet and gastrointestinal bypass-induced weight loss: the roles of ghrelin and peptide YY. Diabetes 60: 810-818.
212. Hajnal A, Kovacs P, Ahmed T, Meirelles K, Lynch CJ, et al. (2010) Gastric bypass surgery alters behavioral and neural taste functions for sweet taste in obese rats. Am J Physiol Gastrointest Liver Physiol 299: G967-979.
213. Korner J, Inabnet W, Conwell IM, Taveras C, Daud A, et al. (2006) Differential effects of gastric bypass and banding on circulating gut hormone and leptin levels. Obesity 14: 1553-1561.
214. Lopez PP, Nicholson SE, Burkhardt GE, Johnson RA, Johnson FK (2009) Development of a Sleeve Gastrectomy Weight Loss Model in Obese Zucker Rats. J Surg Res 157: 243-250.
215. Patti ME, Houten SM, Bianco AC, Bernier R, Larsen PR, et al. (2009) Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. Obesity 17: 1671-1677.
216. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, et al. (2006) Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 439: 484-489.
217. Ryan KK, Tremaroli V, Clemmensen C, Kovatcheva-Datchary P, Myronovych A, et al. (2014) FXR is a molecular target for the effects of vertical sleeve gastrectomy. Nature 509: 183-188.
218. Liou AP, Paziuk M, Luevano JM, Jr., Machineni S, Turnbaugh PJ, et al. (2013) Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. Sci Transl Med 5: 3005687.
219. Bueter M, Lowenstein C, Olbers T, Wang M, Cluny NL, et al. (2010) Gastric bypass increases energy expenditure in rats. Gastroenterology 138: 1845-1853.
220. Nestoridi E, Kvas S, Kucharczyk J, Stylopoulos N (2012) Resting energy expenditure and energetic cost of feeding are augmented after Roux-en-Y gastric bypass in obese mice. Endocrinology 153: 2234-2244.
221. Furnes M, Tømmerås K, Arum C-J, Zhao C-M, Chen D (2008) Gastric Bypass Surgery Causes Body Weight Loss Without Reducing Food Intake in Rats. Obes Surg 18: 415-422.
222. Stefater MA, Wilson-Perez HE, Chambers AP, Sandoval DA, Seeley RJ (2012) All bariatric surgeries are not created equal: insights from mechanistic comparisons. Endocr Rev 33: 595-622.
223. Tadross JA, le Roux CW (2009) The mechanisms of weight loss after bariatric surgery. Int J Obes 33: S28-32.
224. Wilson-Perez HE, Chambers AP, Sandoval DA, Stefater MA, Woods SC, et al. (2013) The effect of vertical sleeve gastrectomy on food choice in rats. Int J Obes 37: 288-295.
225. Zheng H, Shin AC, Lenard NR, Townsend RL, Patterson LM, et al. (2009) Meal patterns, satiety, and food choice in a rat model of Roux-en-Y gastric bypass surgery. Am J Physiol Regul Integr Comp Physiol 297: 2.

226. Schultes B, Ernst B, Wilms B, Thurnheer M, Hallschmid M (2010) Hedonic hunger is increased in severely obese patients and is reduced after gastric bypass surgery. Am J Clin Nutr 92: 277-283.
227. Delin CR, Watts JM, Saebel JL, Anderson PG (1997) Eating behavior and the experience of hunger following gastric bypass surgery for morbid obesity. Obes Surg 7: 405-413.
228. Shin AC, Zheng H, Pistell PJ, Berthoud HR (2011) Roux-en-Y gastric bypass surgery changes food reward in rats. Int J Obes 35: 642-651.
229. Saeidi N, Nestoridi E, Kucharczyk J, Uygun MK, Yarmush ML, et al. (2012) Sleeve gastrectomy and Roux-en-Y gastric bypass exhibit differential effects on food preferences, nutrient absorption and energy expenditure in obese rats. Int J Obes 36: 1396-1402.
230. Helmio M, Victorzon M, Ovaska J, Leivonen M, Juuti A, et al. (2014) Comparison of short-term outcome of laparoscopic sleeve gastrectomy and gastric bypass in the treatment of morbid obesity: A prospective randomized controlled multicenter SLEEVEPASS study with 6-month follow-up. Scand J Surg 12: 12.
231. Yaghoubian A, Tolan A, Stabile BE, Kaji AH, Belzberg G, et al. (2012) Laparoscopic Roux-en-Y gastric bypass and sleeve gastrectomy achieve comparable weight loss at 1 year. Am Surg 78: 1325-1328.
232. Johannessen H, Kodama Y, Zhao CM, Sousa MM, Slupphaug G, et al. (2013) Eating behavior and glucagon-like peptide-1-producing cells in interposed ileum and pancreatic islets in rats subjected to ileal interposition associated with sleeve gastrectomy. Obes Surg 23: 39-49.
233. Lifante JC, Milone L, Korner J, Kopsombut G, Sebastian M, et al. (2012) Sleeve gastrectomy improves glucose homeostasis in Zucker diabetic fatty rats. Obes Surg 22: 1110-1116.
234. Festing MF, Altman DG (2002) Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J 43: 244-258.
235. Russell WMS, Burch RI (1959) The Principles of Humane Experimental Technique. London: Methuen & Co.
236. Albert GC, Cook CM, Prato FS, Thomas AW (2009) Deep brain stimulation, vagal nerve stimulation and transcranial stimulation: An overview of stimulation parameters and neurotransmitter release. Neurosci Biobehav Rev 33: 1042-1060.
237. Heck C, Helmers SL, DeGiorgio CM (2002) Vagus nerve stimulation therapy, epilepsy, and device parameters: scientific basis and recommendations for use. Neurology 59: S31-37.
238. Coskun H, Duran Y, Dilege E, Mihmanli M, Seymen H, et al. (2005) Effect on gastric emptying and weight reduction of botulinum toxin-A injection into the gastric antral layer: an experimental study in the obese rat model. Obes Surg 15: 1137-1143.
239. Stenstrom B, Furnes MW, Tommeras K, Syversen U, Zhao CM, et al. (2006) Mechanism of gastric bypass-induced body weight loss: one-year follow-up after micro-gastric bypass in rats. J Gastrointest Surg 10: 1384-1391.
240. Dapri G, Cadiere GB, Himpens J (2011) Superobese and super-superobese patients: 2-step laparoscopic duodenal switch. Surg Obes Relat Dis 7: 703-708.
241. Bates S, Sjoden PO, Fellenius J, Nyren O (1989) Blocked and nonblocked acid secretion and reported pain in ulcer, nonulcer dyspepsia, and normal subjects. Gastroenterology 97: 376-383.
242. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
243. Paxinos G, Watson C (2005) The rat brain in stereotaxic coordinates. Amsterdam: Elsevier Academic Press.
244. Gil K, Bugajski A, Kurnik M, Zaraska W, Thor P (2009) Physiological and morphological effects of long-term vagal stimulation in diet induced obesity in rats. J Physiol Pharmacol 3: 61-66.
245. Gil K, Bugajski A, Thor P (2011) Electrical vagus nerve stimulation decreases food consumption and weight gain in rats fed a high-fat diet. J Physiol Pharmacol 62: 637-646.

246. Ziomber A, Juszczak K, Kaszuba-Zwoinska J, Machowska A, Zaraska K, et al. (2009) Magnetically induced vagus nerve stimulation and feeding behavior in rats. J Physiol Pharmacol 60: 71-77.
247. Bugajski AJ, Gil K, Ziomber A, Zurowski D, Zaraska W, et al. (2007) Effect of long-term vagal stimulation on food intake and body weight during diet induced obesity in rats. J Physiol Pharmacol 1: 5-12.
248. Krolczyk G, Zurowski D, Sobocki J, Slowiaczek MP, Laskiewicz J, et al. (2001) Effects of continuous microchip (MC) vagal neuromodulation on gastrointestinal function in rats. J Physiol Pharmacol 52: 705-715.
249. Krolczyk G, Laskiewicz J, Sobocki J, Matyja A, Kolasinska-Kloch W, et al. (2005) The effects of baclofen on the feeding behaviour and body weight of vagally stimulated rats. J Physiol Pharmacol 56: 121-131.
250. Laskiewicz J, Krolczyk G, Zurowski G, Sobocki J, Matyja A, et al. (2003) Effects of vagal neuromodulation and vagotomy on control of food intake and body weight in rats. J Physiol Pharmacol 54: 603-610.
251. Sobocki J, Fourtanier G, Estany J, Otal P (2006) Does vagal nerve stimulation affect body composition and metabolism? Experimental study of a new potential technique in bariatric surgery. Surgery 139: 209-216.
252. Val-Laillet D, Biraben A, Randuineau G, Malbert CH (2010) Chronic vagus nerve stimulation decreased weight gain, food consumption and sweet craving in adult obese minipigs. Appetite 55: 245-252.
253. Camilleri M, Toouli J, Herrera MF, Kulseng B, Kow L, et al. (2008) Intra-abdominal vagal blocking (VBLOC therapy): clinical results with a new implantable medical device. Surgery 143: 723-731.
254. Rollnik JD, Meier PN, Manns MP, Goke M (2003) Antral injections of botulinum a toxin for the treatment of obesity. Ann Intern Med 138: 359-360.
255. Albani G, Petroni ML, Mauro A, Liuzzi A, Lezzi G, et al. (2005) Safety and efficacy of therapy with botulinum toxin in obesity: a pilot study. J Gastroenterol 40: 833-835.
256. Foschi D, Corsi F, Lazzaroni M, Sangaletti O, Riva P, et al. (2007) Treatment of morbid obesity by intraparietogastric administration of botulinum toxin: a randomized, double-blind, controlled study. Int J Obes 31: 707-712.
257. Foschi D, Lazzaroni M, Sangaletti O, Corsi F, Trabucchi E, et al. (2008) Effects of intramural administration of Botulinum Toxin A on gastric emptying and eating capacity in obese patients. Dig Liver Dis 40: 667-672.
258. Li L, Liu QS, Liu WH, Yang YS, Yan D, et al. (2012) Treatment of obesity by endoscopic gastric intramural injection of botulinum toxin A: a randomized clinical trial. Hepato-gastroenterol 59: 2003-2007.
259. Garcia-Compean D, Mendoza-Fuerte E, Martinez JA, Villarreal I, Maldonado H (2005) Endoscopic injection of botulinum toxin in the gastric antrum for the treatment of obesity. Results of a pilot study. Gastroenterol Clin Biol 29: 789-791.
260. Gui D, Mingrone G, Valenza V, Spada PL, Mutignani M, et al. (2006) Effect of botulinum toxin antral injection on gastric emptying and weight reduction in obese patients: a pilot study. Aliment Pharmacol Ther 23: 675-680.
261. Mittermair R, Keller C, Geibel J (2007) Intragastric injection of botulinum toxin A for the treatment of obesity. Obes Surg 17: 732-736.
262. Topazian M, Camilleri M, De La Mora-Levy J, Enders FB, Foxx-Orenstein AE, et al. (2008) Endoscopic ultrasound-guided gastric botulinum toxin injections in obese subjects: a pilot study. Obes Surg 18: 401-407.

263. Junior AC, Savassi-Rocha PR, Coelho LG, Sposito MM, Albuquerque W, et al. (2006) Botulinum A toxin injected into the gastric wall for the treatment of class III obesity: a pilot study. Obes Surg 16: 335-343.
264. Kararli TT (1995) Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm Drug Dispos 16: 351-380.
265. DeSesso JM, Jacobson CF (2001) Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. Food Chem Toxicol 39: 209-228.
266. Atkinson RL, Whipple JH, Atkinson SH, Stewart CC (1982) Role of the small bowel in regulating food intake in rats. Am J Physiol-Reg I 11: R429-R433.
267. Koopmans HS, Sclafani A, Fichtner C, Aravich PF (1982) The effects of ileal transposition on food intake and body weight loss in VMH-obese rats. Am J Clin Nutr 35: 284-293.
268. Kotler DP, Koopmans HS (1984) Preservation of Intestinal Structure and Function Despite Weight Loss Produced by Ileal Transposition in Rats. Physiol Behav 32: 423-427.
269. Chen DC, Stern JS, Atkinson RL (1990) Effects of ileal transposition on food intake, dietary preference, and weight gain in Zucker obese rats. Am J Physiol-Reg I 27: R269-R273.
270. Tsuchiya T, Kalogeris TJ, Tso P (1996) Ileal transposition into the upper jejunum affects lipid and bile salt absorption in rats. Am J Physiol-Gastr L 34: G681-G691.
271. Kindel TL, Yoder SM, Seeley RJ, D´Alessio DA, Tso P (2009) Duodenal-Jejunal Exclusion Improves Glucose Tolerance in the Diabetic, Goto-Kakizaki Rat by a GLP-1 Receptor-Mediated Mechanism. J Gastrointest Surg 13: 1762-1777.
272. Chelikani PK, Shah IH, Taqi E, Sigalet DL, Koopmans HH (2010) Comparison of the Effects of Roux-en-Y Gastric Bypass and Ileal Transposition Surgeries on Food Intake, Body Weight, and Circulating Peptide YY Concentrations in Rats. Obes Surg 20: 1281-1288.
273. Metcalf SA, Washington MC, Brown TA, Williams CS, Strader AD, et al. (2011) Ileal interposition attenuates the satiety responses evoked by cholecystokinin-8 and -33. Peptides 32: 1296-1302.
274. Zhang GY, Wang TT, Cheng ZQ, Feng JB, Hu SY (2011) Resolution of diabetes mellitus by ileal transposition compared with biliopancreatic diversion in a nonobese animal model of type 2 diabetes. Can J Surg 54: 243-251.
275. Chen W, Yan Z, Liu S, Zhang G, Sun D, et al. (2011) The Changes of Pro-opiomelanocortin Neurons in Type 2 Diabetes Mellitus Rats After Ileal Transposition: The Role of POMC Neurons. J Gastrointest Surg 15: 1618-1624.
276. Gaitonde S, Kohli R, Seeley R (2012) The role of the gut hormone GLP-1 in the metabolic improvements caused by ileal transposition. J Surg Res 178: 33-39.
277. Grueneberger JM, Fritz T, Zhou C, Meyer S, Karcz-Socha I, et al. (2013) Long segment ileal transposition leads to early amelioration of glucose control in the diabetic obese Zucker rat. Wideochirurgia Tec M 8: 130-138.
278. Ramzy AR, Nausheen S, Chelikani PK (2014) Ileal transposition surgery produces ileal length-dependent changes in food intake, body weight, gut hormones and glucose metabolism in rats. Int J Obes 38: 379-387.
279. Araujo-Filho I, Rego AC, Azevedo IM, Carvalho MD, Medeiros AC (2013) Ileal Interposition and Viability of Pancreatic Islets Transplanted into Intramuscular Site of Diabetic Rats. J Invest Surg 30: 30.
280. Cummings BP, Bettaieb A, Graham JL, Kim J, Ma F, et al. (2013) Bile-acid-mediated decrease in endoplasmic reticulum stress: a potential contributor to the metabolic benefits of ileal interposition surgery in UCD-T2DM rats. Dis Model Mech 6: 443-456.
281. Smithy WB, Cuadros CL, Johnson H, Kral JG (1986) Effects of ileal transposition on body weight and intestinal morphology in dogs. Int J Obesity 10: 453-460.

282. Boza C, Gagner M, Devaud N, Escalona A, Munoz R, et al. (2008) Laparoscopic sleeve gastrectomy with ileal transposition (SGIT): A new surgical procedure as effective as gastric bypass for weight control in a porcine model. Surg Endosc 22: 1029-1034.
283. Boza C, Munoz R, Yung E, Milone L, Gagner M (2011) Sleeve gastrectomy with ileal transposition (SGIT) induces a significant weight loss and diabetes improvement without exclusion of the proximal intestine. J Gastrointest Surg 15: 928-934.
284. Kumar KV, Ugale S, Gupta N, Naik V, Kumar P, et al. (2009) Ileal interposition with sleeve gastrectomy for control of type 2 diabetes. Diabetes Technol Ther 11: 785-789.
285. Tinoco A, El-Kadre L, Aquiar L, Tinoco R, Savassi-Rocha P (2011) Short-term and mid-term control of type 2 diabetes mellitus by laparoscopic sleeve gastrectomy with ileal interposition. World J Surg 35: 2238-2244.
286. Al-Sabah S, Alasfar F, Al-Khaledi G, Dinesh R, Al-Saleh M, et al. (2014) Incretin response to a standard test meal in a rat model of sleeve gastrectomy with diet-induced obesity. Obes Surg 24: 95-101.
287. Rodriguez A, Becerril S, Valenti V, Ramirez B, Martin M, et al. (2012) Sleeve gastrectomy reduces blood pressure in obese (fa/fa) Zucker rats. Obes Surg 22: 309-315.
288. Stefater MA, Perez-Tilve D, Chambers AP, Wilson-Perez HE, Sandoval DA, et al. (2010) Sleeve gastrectomy induces loss of weight and fat mass in obese rats, but does not affect leptin sensitivity. Gastroenterology 138: 2426-2436.
289. Noun R, Skaff J, Riachi E, Daher R, Antoun NA, et al. (2012) One thousand consecutive mini-gastric bypass: short- and long-term outcome. Obes Surg 22: 697-703.
290. Furnes MW, Stenstrom B, Tommeras K, Skoglund T, Dickson SL, et al. (2008) Feeding behavior in rats subjected to gastrectomy or gastric bypass surgery. Eur Surg Res 40: 279-288.
291. Werling M, Olbers T, Fandriks L, Bueter M, Lonroth H, et al. (2013) Increased postprandial energy expenditure may explain superior long term weight loss after Roux-en-Y gastric bypass compared to vertical banded gastroplasty. PLoS One 8: 3.
292. Stylopoulos N, Hoppin AG, Kaplan LM (2009) Roux-en-Y gastric bypass enhances energy expenditure and extends lifespan in diet-induced obese rats. Obesity 17: 1839-1847.
293. Faria SL, Faria OP, Cardeal Mde A, Ito MK, Buffington C (2014) Diet-induced thermogenesis and respiratory quotient after Roux-en-Y gastric bypass surgery: A prospective study. Surg Obes Relat Dis 10: 138-143.
294. Faria SL, Faria OP, Buffington C, de Almeida Cardeal M, Rodrigues de Gouvea H (2012) Energy expenditure before and after Roux-en-Y gastric bypass. Obes Surg 22: 1450-1455.
295. Shin AC, Zheng H, Townsend RL, Patterson LM, Holmes GM, et al. (2013) Longitudinal assessment of food intake, fecal energy loss, and energy expenditure after Roux-en-Y gastric bypass surgery in high-fat-fed obese rats. Obes Surg 23: 531-540.
296. Tamboli RA, Hossain HA, Marks PA, Eckhauser AW, Rathmacher JA, et al. (2010) Body composition and energy metabolism following Roux-en-Y gastric bypass surgery. Obesity 18: 1718-1724.
297. Sovik TT, Aasheim ET, Taha O, Engstrom M, Fagerland MW, et al. (2011) Weight loss, cardiovascular risk factors, and quality of life after gastric bypass and duodenal switch: a randomized trial. Ann Intern Med 155: 281-291.
298. Sovik TT, Taha O, Aasheim ET, Engstrom M, Kristinsson J, et al. (2010) Randomized clinical trial of laparoscopic gastric bypass versus laparoscopic duodenal switch for superobesity. Br J Surg 97: 160-166.
299. Hayes MR, De Jonghe BC, Kanoski SE (2010) Role of the glucagon-like-peptide-1 receptor in the control of energy balance. Physiol Behav 100: 503-510.
300. Wellen KE, Hotamisligil GS (2005) Inflammation, stress, and diabetes. J Clin Invest 115: 1111-1119.

301. Powley TL, Chi MM, Schier LA, Phillips RJ (2005) Obesity: should treatments target visceral afferents? Physiol Behav 86: 698-708.

9. Appendices

Papers I-IV

Paper I

The Gastric Vagus Nerve as a Target for Obesity Treatment

Helene Johannessen[1], David Revesz[2], Yosuke Kodama[1], Nikki Cassie[3], Karolina P Skibicka[4], Magnus K Olsen[1], Robert Lyle[5], Perry Barrett[3], Suzanne L Dickson[4], Jens J Holst[6], Jens F Rehfeld[7], Geoffrey van der Plasse[8], Roger AH Adan[8], Bård Kulseng[9], Thorleif Thorlin[2], Elinor Ben-Menachem[2], Chun-Mei Zhao[1], Duan Chen[1,9]

[1]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; [2]Department of Clinical Neuroscience and Rehabilitation, The Sahlgrenska Academy at Gothenburg University, Gothenburg, Sweden; [3]Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, Scotland; [4]Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden; [5]Department of Medical Genetics, Oslo University Hospital, Oslo, Norway; [6]Department of Biomedical Sciences, the Panum Institute, University of Copenhagen, Copenhagen, Denmark; [7]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark; [8]Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, Netherlands; [9]Department of Surgery, St. Olav's University Hospital, Trondheim, Norway

Keywords*:* Body weight, Botulinum toxin type A, Food intake, Gut-brain axis, Rats, Vagus nerve stimulation

Running title: Vagus Nerve for Obesity Treatment

Corresponding author: Professor Duan Chen, MD, PhD, Erling Skjalgssons Gate 1, Eastside 3rd Floor, Laboratory Centre of St. Olav's Hospital, 7006 Trondheim, Norway. Tel: +47 725 73320. Fax: +47 725 76612. Mob: +47 984 09 675. E-mail: duan.chen@ntnu.no.

Abstract

Objective: The vagus nerve has a central role in the regulation of energy intake and expenditure, which makes it an ideal target for development of new obesity treatments. The aim of the present study was to explore whether blocking of the gastric vagus nerve can be a new minimally invasive obesity treatment. **Methods:** Blocking of the gastric vagus nerve was achieved either by an electrical device surgically implanted around gastric vagal trunks (vagus nerve stimulation/blocking, VNSB) or by botulinum toxin type A (Botox) injection into the gastric wall in the region of antrum in rats. The effect on body weight, food intake, eating behavior, metabolic parameters, gut hormone concentrations, gastric acid secretion, and expression of candidate energy balance-regulating genes in the hypothalamus and brainstem were determined. **Results:** VNSB reduced body weight and food intake by 10% and 30%, respectively. Botox injection reduced body weight and food intake by 17% and 15% in normal chow-fed rats and by 24% and 50% in diet induced obese rats, respectively. Both procedures affected appetite-regulating neuropeptides mainly in the brainstem. **Conclusion:** Blocking the gastric vagus nerve by VNSB or Botox injection can be new, non-invasive obesity treatments.

Introduction

The gut-brain axis plays an important role in the pathogenesis of obesity. Nutritional and metabolically relevant information is conveyed to the brain by gut peptide hormones and the vagus nerve (1). The vagus nerve responds to peptides and hormones, as well as mechanical and chemical stimuli from the gastrointestinal tract and transmits satiety signals to sites in the central nervous system that regulate ingestive behavior (2, 3). In addition, the vagus nerve regulates gastric acid secretion and pancreatic endocrine function (4, 5). Through the nucleus of the solitary tract (NTS) in the brainstem, afferent vagal signals may reach several parts of the brain including the parabrachial nucleus, reticular formation, hippocampus, amygdala, and hypothalamus (6). The hypothalamus is regarded as a key regulatory component of a central network for food intake where orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons are thought to play a central role (2). Nevertheless, the brainstem is strongly involved in the gut-brain axis, particularly via the vagus nerve in regulation of food intake and body weight (7). The central role of the vagus in the regulation of food intake and energy expenditure makes it an ideal target for the development of new less or non-invasive procedures to treat obesity.

The aim of this study was to target the gastric vagus nerve in order to create less or non-invasive obesity therapies. To this end, we tested whether blocking the vagus nerve by an electrical device (vagus nerve stimulation/blocking, VNSB) (Figure 1A) or Botox injection (Figure 3A) affected body weight, food intake, eating behavior, metabolic parameters, gut hormone concentrations, gastric acid secretion, and the expression profile of candidate energy balance-regulating genes in the hypothalamus and brainstem.

Materials and Methods

Animals and experimental designs

Adult male Sprague-Dawley rats were purchased from Taconic (Ejby, Denmark) and Janvier Labs (Le Genest-Saint-Isle, France). They had free access to tap water and standard rat pellet food (RM1 811004, Scanbur BK AS, Sweden) or pelleted high fat diet (D12492, Research Diets, Inc., NJ, USA). The standard rat diet consisted of 2.57 kcal/g containing 75% carbohydrates, 7.5% fat and 17.5% protein, while the high fat diet consisted of 5.24 kcal/g containing 20% carbohydrates, 60% fat and 20% protein in terms of kcal. For diet induced obesity, rats were fed a mixed normal and high fat diet (50:50) for two weeks, starting when they were 5 weeks old. Subsequently, the normal diet was removed and rats continued to feed on high fat diet until euthanization. Adult female Long Evan rats were obtained at Norwegian University of Science and Technology, Trondheim, Norway. In the present study, all animals were housed three to four rats per individually ventilated cage on a wood chip bedding with a 12-hour light/dark cycle, a room temperature of 22ºC, and 40-60% relative humidity. The standard housing conditions were specific pathogen free and in agreement with Federation of European Laboratory Animal Science Association recommendations. The study was approved by the Norwegian National Animal Research Authority.

In all studies regarding eating behaviour the animals were acclimatized to the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments International, Columbus, OH, USA) for 24h before data collection. At data collection the animals were kept in CLAMS for 48h and data from the last 24h were used for analysis. Before CLAMS the rats were habituated to their normal food as powder for three days, as the food in CLAMS is in powder form. Body weight development was followed three times per week throughout the study period.

VNSB

In the short-term VNSB study, the animals had implanted gastric fistula and were acclimatized to Bollman cages for 3 hours on three separate occasions before and after VNSB and gastric fistula implantation. After gastric acid output measurement the stimulation was turned off. Three days after, rats received VNSB (2.0 mA, 30 Hz, 500 μs, 30 s ON and 5 min OFF) for 48h while eating behavior and metabolic parameters were measured in CLAMS. The control group had the same implantations without stimulation. Immediately after 48h of VNSB the rats were euthanized and brain samples were collected for *in situ* hybridization, and plasma was collected for radioimmunoassay.

In the long-term VNSB study, the stimulation was started 4 weeks after VNSB implantation and was constantly ON while the current (mA) was gradually increased. Each rat was placed in CLAMS at four time points for measurement of eating behavior and metabolic parameters; 3 weeks after VNSB implantation (before stimulation, baseline), at 0.5 mA, 1.0 mA and 2.0 mA stimulation (30 Hz, 500 μs, 30 s ON and 5 min OFF). The control group had the same implantation without stimulation. The duration of stimulation for each rat was 6-8 weeks. At euthanization brain samples were taken for Taqman array analysis and RNA sequencing, and plasma was collected for radioimmunoassay.

Smaller VNSB devices, the so-called μ-VNSB, were developed for use in Long Evan rats. The stimulation was continuously on at 2.0 mA, 30 Hz, 500 μs, 30 s ON, and 5 min OFF for 2 weeks.

In vivo electrophysiology

In vivo electrophysiology in the hippocampus during VNSB (2.0 mA, 30 Hz, 500 μs, 30 s ON and 5 min OFF) was performed as an acute experiment.

Botulinum toxin type A injection

Normal chow-fed rats received Botox or saline injection on three occasions, and were subjected to CLAMS before injection, 2 weeks after 1st injection, and 1 and 2 weeks after 2nd injection. A separate set of rats received one Botox/saline injection and were euthanized after 48h. Brain samples from both long-term (8 weeks) and short-term (48h) Botox treatment were collected for *in situ* hybridization, and plasma collected for radioimmunoassay.

High fat diet induced obese (DIO) rats received Botox/saline injection on two occasions and were subjected to CLAMS before injection, 1 and 3 weeks after 1st injection, and 1 week after 2nd injection. Fasting blood glucose was measured 8 weeks after 1st injection.

DIO rats received sleeve gastrectomy (DIO-SG) or sham surgery. The rats were subjected to CLAMS before and 1 week after surgery. Fasting blood glucose levels were measured 3 weeks after surgery. Six weeks after SG the rats were treated with Botox/saline injection. The rats were then subjected to CLAMS 1 week after injection and fasting blood glucose levels were measured 2 weeks after injection.

Gastric emptying in normal chow-fed and DIO rats after botulinum toxin type A

Both normal chow-fed and DIO rats received Botox/saline injection in antrum. Gastric emptying was measured by an acetaminophen absorption assay before injection, 3 days, and 1, 2, 3, and 5 weeks after injection.

Animal surgery

All surgeries were performed under general anesthesia with isoflurane. Atropin (0.04 mg/kg) (Nycomed Pharma AS, Asker, Norway) was given subcutaneously 20 min before anesthesia for VNSB and μ-VNSB implantation. Buprenorphine (0.05 mg/kg) (Schering-Plough Europe, Brussels, Belgium) was injected subcutaneously immediately after surgery in

all animals, and one day postoperatively when needed. Physiological saline (10 mL of 0.9% NaCl) was given subcutaneously postoperatively.

VNSB

VNSB implantation was performed through a midline abdominal incision. The subdiaphragmatic truncal vagus nerve was dissected from the esophagus and two electrodes (Lead Model 302, Cyberonics, Houston, TX, USA) were wrapped around anterior and posterior vagus nerve of the gastric branch. The cathode was placed proximally and the anode distally. During stimulation the cathode induced depolarization (stimulation of the afferent fibers), while the distal anode hyperpolarized the membrane and imposed an anodic block on the efferent fibers (Figure 1B) (8). The wire from the electrodes was attached to fat tissue around the stomach and to the muscular layer using 6-0 and 4-0 absorbable sutures (Vicryl, Ethicon Inc., Sommerville, NJ, USA), respectively. On the back of the rat a subcutaneous pocket for the stimulator (model 102 Pulse Generator, Cyberonics, Houston, TX) was made and here it was connected to the wire. The abdomen was closed in two layers using 4-0 absorbable sutures and the back was closed using the same sutures (Ethicon). The control and VNSB rats received the same procedure. Implantation of the μ-VNSB device followed the same procedure. In the gastric acid secretion study a gastric fistula was in addition implanted in the stomach. Before this procedure the animals were fasted overnight.

Botulinum toxin type A injection

Botulinum toxin type A 100 U (Botox® Allergan Cooperation, Irvine, CA, USA) was diluted in 0.9% cold saline and 1% methylene blue (Sigma-Aldrich, St. Louis, MO, USA) (for visualization) achieving a concentration of 40 U Botox/mL. Total volume injected into antrum of each rat was 0.5 mL (20U/rat). The control rats were injected with 0.5 mL 0.9% saline and

1% methylene blue. Following a short median laparotomy, the stomach was exposed, and antrum isolated. Botox was injected subserosally to both anterior and posterior side of the antrum with a 30 G needle. In contrast to previous studies, this was performed through one injection and allowed for the fluid to spread over the whole area of the pyloric antrum. Abdomen was closed in two layers using 4-0 absorbable sutures (Ethicon).

Sleeve gastrectomy

Animals were fasted overnight before surgery. The surgery was performed through a midline abdominal incision. The stomach was clamped along the greater curvature from the antrum to the fundus across the forestomach and glandular stomach using staples (Endo GIA, Articulating Reload with Tri-Staple™ Technology, 45 mm, EGIA45AVM, Covidien™, Dublin, Ireland) and 70% of the stomach was removed. Hemostasis and staple-line integrity were checked, and additional stitches were applied when necessary. The abdominal wall was closed in two layers with 4-0 absorbable sutures (Ethicon).

Supplementary Information

Determinations of eating behaviour and metabolic parameters, gastric acid secretion, taqman array, *in situ* hybridization, RNA sequencing, *in vivo* electrophysiology, radioimmunoassay, blood glucose levels and gastric emptying were performed using standard procedures described in the Supplementary Information.

Statistical Analysis

The results are expressed as mean ± SEM. Statistical comparisons were performed using independent *t*-test between the surgical groups. ANCOVA with Sidak test was performed for energy expenditure statistics in the VNSB studies. ANOVA with Tukey's test

was performed in the VNSB study and the DIO-SG study to determine eating behavior and metabolic parameters. A *P*-value of <0.05 (two tailed) was considered statistically significant. The data analysis was performed in SPSS version 15.0 and 20.0.

Results

In vivo electrophysiology tetrodes recorded stimulation-induced activity in the brain, confirming the stimulation parameters used by VNSB (Figure 1C). In response to short-term VNSB (48h at 2.0 mA) gene expression of NPY and AgRP in the arcuate nucleus (ARC) of hypothalamus, as well as NPY gene expression in dorsomedial hypothalamic nucleus (DMH) were up-regulated. However, there were no changes in body weight, food intake, eating behavior, energy expenditure, gene expression in the brainstem, plasma concentrations of gut hormones, or gastric acid secretion (Figure S1).

Next, we performed a long-term VNSB (6-8 weeks) experiment in which the electrical stimulation current was gradually increased (0.5 mA - 2.0 mA) over the time period. Initially, the body weight was unaffected, but as the current was increased, the body weight and food intake (g) were reduced, eventually reaching reductions of 10% ($P>0.05$) and 30% ($P<0.05$), respectively (Figure 2A, B). Energy expenditure (kcal/h/100g body weight) was reduced compared to baseline (Figure S2B). In another study, where the stimulation was started at 2.0 mA, a 10% body weight reduction was achieved within 1 week ($P<0.05$) (Figure 2A). In the long-term VNSB experiment there was significant increase in hypothalamic gene expression of NPY and forkhead box protein a2 (Foxa2), a decrease for CCKb receptor and a tendency for decreased expression of the melanocortin 4 receptor (MC4R), and insulin receptor (IR) (Figure 2C). Plasma concentrations of cholecystokinin (CCK), gastrin, glucagon, glucagon-like peptide-1 (GLP-1), and peptide YY (PYY) were unchanged (Figure 2D). Since the brainstem is the first target for vagal afferent fibers in the central nervous system, we

examined this brain region and found that gene expression of leptin- and CCKb -receptors, and interleukin-1β, tumor necrosis factor, and transforming growth factor β1 was increased (P<0.05) (Figure 2E), changes compatible with an anorexigenic drive in response to VNSB. Given that afferent signals from NTS to hippocampus could be activated by VNSB (9), we examined gene expression in the hippocampus and found increased expression of CCK, somatostatin, and tyrosine hydroxylase (crucial for production of dopamine) (P<0.05) (Figure 2F).

We next examined the effects of blocking the gastric vagus nerve using Botox injection into the gastric wall in the region of the antrum. This blocks the release of acetylcholine, by cleaving the SNARE protein SNAP-25 (10). At 48h post injection, body weight, expression of energy balance-linked genes in hypothalamus and brainstem, and concentrations of gut hormones were unchanged (Figure S3). However, in a long-term study of Botox, body weight was reduced by 17% at 3 weeks post injection, which was associated with reduced food intake, and increased satiety ratio (min/g) (P<0.05). When the rats started to regain weight after the 1st Botox injection (as the effectiveness of Botox declined), a second injection of Botox was performed 4 weeks after 1st injection. A further body weight loss (25%) and reduction in food intake were obtained (P<0.05) (Figure 3B, C). Furthermore, energy expenditure was increased (P<0.01) (Figure 3D). Hypothalamic gene expression for NPY was increased, POMC was decreased, and AgRP had a tendency to be increased (Figure 3E), and plasma concentrations of CCK, gastrin, and PYY were reduced (Figure 3F).

To further validate the efficiency and potential of gastric Botox injection as a non-invasive therapy for obesity, we treated high fat diet induced obese (DIO) rats with Botox injection. At injection DIO rats had a 22% higher body weight than age-matched normal chow-fed rats. The Botox treatment, injected 6 weeks apart, successfully led to body weight loss, i.e., 24% and 33% reduction after first and second administration, respectively (P<0.05)

(Figure 4A). This was associated with decreased food intake (Figure 4B and Figure S4B) and increased energy expenditure. Fasting blood glucose levels were reduced 8 weeks after Botox injection ($P<0.001$) (Figure S5A).

Sleeve gastrectomy (SG) is a commonly used bariatric surgery (11). We treated DIO rats with SG and found that the body weight and food intake were reduced by 10% and 20%, respectively ($P>0.05$). Fasting blood glucose levels were not altered ($P>0.05$) (Figure S5B). Subsequently we performed Botox injections in the DIO rats that had SG 6 weeks earlier and found that body weight was reduced by 25% and food intake was reduced by 31% ($P<0.05$) (Figure 4C, D). Also fasting blood glucose levels were reduced ($P=0.002$) (Figure S5C). We did not observe any sign of gastroparesis, reflected not only by the general condition of animals, but also the shape and size of the solid food-containing stomach. Furthermore, we assessed gastric emptying in both normal and DIO rats and found that gastric emptying was not delayed by Botox injection, neither in the short-term (3 days) nor long-term (5 weeks) post-injection (Figure 5).

There was no mortality, adverse effects, or pathological changes in rats subjected either to VNSB or Botox injection.

Discussion

By using two different procedures, i.e., VNSB and Botox injection, we have demonstrated that targeting the gastric vagus nerve can be a new approach for obesity treatment. Furthermore, our results suggest that gastric Botox injection is more efficient than sleeve gastrectomy at reducing body weight, food intake, and blood glucose levels, and can be performed in case of postsurgical body weight regain, e.g. after sleeve gastrectomy.

Previously, several animal studies reported reduced body weight gain and food intake in response to vagus nerve stimulation which was applied mostly at the left vagal branch.

However, the results reported were of low quality due to large variations, and no SD or SEM in the figures (12-17). A study using obese minipigs showed that subdiaphragmatic vagus nerve stimulation attenuated weight gain (18). Three clinical trials of vagus nerve stimulation for obesity treatment have been performed by the same financial sponsor who produces the instrument (19-21). One of the trials was a randomized, prospective, double-blind, multicenter trial that showed no significant body weight reduction. The present study shows, for the first time, that constant high current stimulation should be applied at the beginning in order to achieve body weight loss.

Botox injection into the gastric antrum has been proposed as an obesity treatment, based on the dogma that this will cause gastroparesis and delayed gastric emptying (22). Previously, several animal studies reported reduced body weight (by 8-14%) after Botox injection which was given by "point injections" into the muscular layers. Again, the results were poorly reported; large variations and no SD or SEM in figures (22, 23). Clinical trials of Botox injection are controversial; some reporting weight loss (24-27), and others reporting no effect (28-31), and some reporting delayed gastric emptying (23, 25, 26, 31, 32), while others report no change (28, 30). In the present study, Botox injection was applied to block the vagal nerve terminals as did VNSB rather than to delay gastric emptying. Therefore, it was given by "area injection" into the subserosal layer of the pyloric antrum without delaying gastric emptying, leading to a reduced body weight by 20-25%. We have shown that repeated injection of Botox resulted in increased weight loss. The present study shows, for the first time, that the area injection of Botox into subserosal layer in the region of pyloric antrum should be used in order to achieve body weight loss. Conceivably, it could be injected into submucosal layer in patients via gastroscopy.

Furthermore, the present study shows that gene expression in brainstem and hippocampus was altered by VNSB, indicative of appetite suppression. In relation to energy

balance, it is noteworthy that the hippocampus has a role in incentive motivation (e.g. for food), in processing of hormonal signals (CCK, ghrelin, and, motilin), and that damage to this region causes hyperphagia (33-37). The results of the present study suggest that VNSB activates afferent pathways to the brainstem and hippocampus leading to reduced food intake. On the other hand, both VNSB and Botox injection increased hypothalamic expression of orexigenic neuropeptides, probably suggesting compensatory changes. For instance, while long-term VNSB reduced body weight and food intake, gene expression in the hypothalamus changed with a drive for increased food intake and reduction of energy expenditure (2, 38-40). Gut hormones are thought to be central in appetite regulation in the gut-brain axis, and hormones such as CCK, glucagon, GLP-1, and PYY are known to reduce food intake (1). However, these were unaltered after VNSB neither in short-term or long-term. Thus, the profile of gene expression in the hypothalamus indicative of appetite stimulation was not associated with changes in gut hormones, and was inconsistent with the effects of VNSB to reduce food intake and body weight, representing a failed compensatory mechanism to counteract the weight loss.

Clinical trials of VNSB and Botox injection for weight loss have so far been inconclusive (19, 20, 31), which is most likely due to improper methods. The present study provides evidence that the vagus nerve is a potential target for obesity therapy. VNSB at high current has certain potential as a less-invasive therapy for obesity, and gastric Botox area injection (via gastroscopy) can be performed as non-invasive therapy for obesity in general, as well as in those patients who are not suitable for bariatric surgery, or have failed to respond or regained body weight after sleeve gastrectomy. This study could help design better clinical trials in the future, particularly in terms of technique and the regime of VNSB and the methods of Botox injection.

Conflict of interest: All authors declare no conflict of interest.

Acknowledgements: The research leading to these results has received funding from European Union Seventh Framework Programme (FP7/2007-2013, n°266408 to DC and n°607310 to SLD), from the Joint Programme of the Medical Faculty of Norwegian University of Science and Technology (NTNU) and St. Olav's University Hospital, the Liaison Committee between the Central Norway Regional Health Authority and NTNU (to DC and CMZ), the Swedish Research Council for Medicine (VR 2011-3054 to KPS and 2012-1758 to SLD), the Novo Nordisk Fonden Excellence Award (KPS) and Forskning och Utvecklingsarbete/Avtal om Läkarutbildning och Forskning Göteborg (ALFGBG-138741 to SLD).

Author contributions: HJ contributed to the design of the experiments, performance of experiments, and collection and analysis of data. DR, EB-M and TT contributed to the establishment of VNSB animal model. YK contributed in performance of animal experiments, and collection and analysis of data. NC and PB performed *in situ* hybridization. KPS and SLD performed taqman array. MKO performed part of animal experiments. RL analyzed RNA-sequencing data, JJH and JFR performed radioimmunoassay on plasma samples. GP and RAHA performed *in vivo* electrophysiology. BK contributed with ideas and discussions. C-MZ performed part of animal experiments and contributed with ideas and discussions. DC was senior author, contributed to the concepts and development of the methods (particularly Botox injection), study supervision, performance of experiments and coordination. All authors contributed to the discussion of results and the preparation of the final manuscript. Also, Lars Lyse Moen at NTNU was hired to help with the development of µ-VNSB.

References

1 Sam AH, Troke RC, Tan TM, Bewick GA. The role of the gut/brain axis in modulating food intake. *Neuropharmacology* 2012; **63**: 46-56.

2 Schwartz MW, Woods SC, Porte D, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000; **404**: 661-71.
3 Dockray GJ. Enteroendocrine cell signalling via the vagus nerve. *Curr Opin Pharmacol* 2013; **13**: 954-8.
4 Brunicardi FC, Shavelle D, Andersen D. Neural regulation of the endocrine pancreas. *Int J Pancreatol* 1995; **18**: 177-95.
5 Imatake K, Matsui T, Moriyama M. The effect and mechanism of action of capsaicin on gastric acid output. *J Gastroenterol* 2009; **44**: 396-404.
6 Ben-Menachem E, Hamberger A, Hedner T, Hammond EJ, Uthman BM, Slater J, et al. Effects of vagus nerve stimulation on amino acids and other metabolites in the CSF of patients with partial seizures. *Epilepsy Res* 1995; **20**: 221-7.
7 Grill HJ, Hayes MR. The nucleus tractus solitarius: a portal for visceral afferent signal processing, energy status assessment and integration of their combined effects on food intake. *Int J Obes* 2009; **33**: S11-5.
8 Merrill DR, Bikson M, Jefferys JGR. Electrical stimulation of excitable tissue: design of efficacious and safe protocols. *J Neurosci Meth* 2005; **141**: 171-98.
9 Revesz D, Tjernstrom M, Ben-Menachem E, Thorlin T. Effects of vagus nerve stimulation on rat hippocampal progenitor proliferation. *Exp Neurol* 2008; **214**: 259-65.
10 Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, Camilli PD, et al. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature* 1993; **365**: 160-3.
11 Brethauer SA, Hammel JP, Schauer PR. Systematic review of sleeve gastrectomy as staging and primary bariatric procedure. *Surg Obes Relat Dis* 2009; **5**: 469-75.
12 Gil K, Bugajski A, Thor P. Electrical vagus nerve stimulation decreases food consumption and weight gain in rats fed a high-fat diet. *J Physiol Pharmacol* 2011; **62**: 637-46.
13 Ziomber A, Juszczak K, Kaszuba-Zwoinska J, Machowska A, Zaraska K, Gil K, et al. Magnetically induced vagus nerve stimulation and feeding behavior in rats. *J Physiol Pharmacol* 2009; **60**: 71-7.
14 Bugajski AJ, Gil K, Ziomber A, Zurowski D, Zaraska W, Thor PJ. Effect of long-term vagal stimulation on food intake and body weight during diet induced obesity in rats. *J Physiol Pharmacol* 2007; **1**: 5-12.
15 Krolczyk G, Laskiewicz J, Sobocki J, Matyja A, Kolasinska-Kloch W, Thor PJ. The effects of baclofen on the feeding behaviour and body weight of vagally stimulated rats. *J Physiol Pharmacol* 2005; **56**: 121-31.
16 Laskiewicz J, Krolczyk G, Zurowski G, Sobocki J, Matyja A, Thor PJ. Effects of vagal neuromodulation and vagotomy on control of food intake and body weight in rats. *J Physiol Pharmacol* 2003; **54**: 603-10.
17 Sobocki J, Fourtanier G, Estany J, Otal P. Does vagal nerve stimulation affect body composition and metabolism? Experimental study of a new potential technique in bariatric surgery. *Surgery* 2006; **139**: 209-16.
18 Val-Laillet D, Biraben A, Randuineau G, Malbert CH. Chronic vagus nerve stimulation decreased weight gain, food consumption and sweet craving in adult obese minipigs. *Appetite* 2010; **55**: 245-52.
19 Camilleri M, Toouli J, Herrera MF, Kulseng B, Kow L, Pantoja JP, et al. Intra-abdominal vagal blocking (VBLOC therapy): clinical results with a new implantable medical device. *Surgery* 2008; **143**: 723-31.
20 Sarr MG, Billington CJ, Brancatisano R, Brancatisano A, Toouli J, Kow L, et al. The EMPOWER study: randomized, prospective, double-blind, multicenter trial of vagal blockade to induce weight loss in morbid obesity. *Obes Surg* 2012; **22**: 1771-82.
21 Shikora S, Toouli J, Herrera MF, Kulseng B, Zulewski H, Brancatisano R, et al. Vagal blocking improves glycemic control and elevated blood pressure in obese subjects with type 2 diabetes mellitus. *J Obes* 2013; **245683**: 30-8.

22 Gui D, De Gaetano A, Spada PL, Viggiano A, Cassetta E, Albanese A. Botulinum toxin injected in the gastric wall reduces body weight and food intake in rats. *Aliment Pharmacol Ther* 2000; **14**: 829-34.
23 Coskun H, Duran Y, Dilege E, Mihmanli M, Seymen H, Demirkol MO. Effect on gastric emptying and weight reduction of botulinum toxin-A injection into the gastric antral layer: an experimental study in the obese rat model. *Obes Surg* 2005; **15**: 1137-43.
24 Albani G, Petroni ML, Mauro A, Liuzzi A, Lezzi G, Verti B, et al. Safety and efficacy of therapy with botulinum toxin in obesity: a pilot study. *J Gastroenterol* 2005; **40**: 833-5.
25 Foschi D, Corsi F, Lazzaroni M, Sangaletti O, Riva P, La Tartara G, et al. Treatment of morbid obesity by intraparietogastric administration of botulinum toxin: a randomized, double-blind, controlled study. *Int J Obes* 2007; **31**: 707-12.
26 Foschi D, Lazzaroni M, Sangaletti O, Corsi F, Trabucchi E, Bianchi Porro G. Effects of intramural administration of Botulinum Toxin A on gastric emptying and eating capacity in obese patients. *Dig Liver Dis* 2008; **40**: 667-72.
27 Li L, Liu QS, Liu WH, Yang YS, Yan D, Peng LH, et al. Treatment of obesity by endoscopic gastric intramural injection of botulinum toxin A: a randomized clinical trial. *Hepato-gastroenterol* 2012; **59**: 2003-7.
28 Gui D, Mingrone G, Valenza V, Spada PL, Mutignani M, Runfola M, et al. Effect of botulinum toxin antral injection on gastric emptying and weight reduction in obese patients: a pilot study. *Aliment Pharmacol Ther* 2006; **23**: 675-80.
29 Mittermair R, Keller C, Geibel J. Intragastric injection of botulinum toxin A for the treatment of obesity. *Obes Surg* 2007; **17**: 732-6.
30 Topazian M, Camilleri M, De La Mora-Levy J, Enders FB, Foxx-Orenstein AE, Levy MJ, et al. Endoscopic ultrasound-guided gastric botulinum toxin injections in obese subjects: a pilot study. *Obes Surg* 2008; **18**: 401-7.
31 Topazian M, Camilleri M, Enders FT, Clain JE, Gleeson FC, Levy MJ, et al. Gastric Antral Injections of Botulinum Toxin Delay Gastric Emptying but Do Not Reduce Body Weight. *Clin Gastroenterol H* 2013; **11**: 145-50.
32 Li L, Liu QS, Liu WH, Yang YS, Yan D, Peng LH, et al. Treatment of obesity by endoscopic gastric intramural injection of botulinum toxin A: a randomized clinical trial. *Hepatogastroenterology* 2012; **59**: 2003-7.
33 Guan Y, Tang M, Jiang Z, Peeters TL. Excitatory effects of motilin in the hippocampus on gastric motility in rats. *Brain Res* 2003; **984**: 33-41.
34 Diano S, Farr SA, Benoit SC, McNay EC, da Silva I, Horvath B, et al. Ghrelin controls hippocampal spine synapse density and memory performance. *Nat Neurosci* 2006; **9**: 381-8.
35 Davidson TL, Kanoski SE, Walls EK, Jarrard LE. Memory inhibition and energy regulation. *Physiol Behav* 2005; **86**: 731-46.
36 Forloni G, Fisone G, Guaitani A, Ladinsky H, Consolo S. Role of the hippocampus in the sex-dependent regulation of eating behavior: studies with kainic acid. *Physiol Behav* 1986; **38**: 321-6.
37 Tracy AL, Jarrard LE, Davidson TL. The hippocampus and motivation revisited: appetite and activity. *Behav Brain Res* 2001; **127**: 13-23.
38 Silva JP, von Meyenn F, Howell J, Thorens B, Wolfrum C, Stoffel M. Regulation of adaptive behaviour during fasting by hypothalamic Foxa2. *Nature* 2009; **462**: 646-50.
39 Clerc P, Coll Constans MG, Lulka H, Broussaud S, Guigne C, Leung-Theung-Long S, et al. Involvement of cholecystokinin 2 receptor in food intake regulation: hyperphagia and increased fat deposition in cholecystokinin 2 receptor-deficient mice. *Endocrinology* 2007; **148**: 1039-49.
40 Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, et al. Targeted Disruption of the Melanocortin-4 Receptor Results in Obesity in Mice. *Cell* 1997; **88**: 131-41.

Figure Legends

Figure 1: Vagus nerve stimulation/blocking (VNSB). (**A**) Photograph showing the implanted VNSB electrodes around the gastric vagus nerve. (**B**) Illustration of how the cathode induces depolarization and stimulation of the afferent fibers, while the anode hyperpolarizes the membrane and imposes an anodic block on the efferent fibers. (**C**) *In vivo* electrophysiological recordings of stimulation-induced activity in the brain. The graph depicts the average waveform of measured activity, the insert show the raw data from which the average waveform was constructed.

Figure 2: VNSB reduced body weight and food intake, and altered gene expression in hypothalamus, brainstem, and hippocampus. Mean ± SEM (n=4-9). (**A**) Effect of different currents of VNSB on body weight (black column). Note: when current was started at 2.0 mA a weight reduction was accomplished instantly ($P<0.05$) (white column). (**B**) Effect of VNSB on food intake (g/24h). *: $P<0.05$ between sham-VNSB (white column) and VNSB (black column). (**C**) Effect of VNSB (2.0 mA) on hypothalamic mRNA expression of CCKB/2 receptor (Cckbr), NPY, Foxa2, insulin receptor (Insr), and melanocortin 4 receptor (Mc4r). Note: $P=0.067$ on Insr and $P=0.086$ on Mc4r between sham-VNSB (white column) and VNSB (black column). (**D**) Effect of VNSB (2.0 mA) on plasma concentrations of gut hormones. Note: no significant difference between sham-VNSB (white column) and VNSB (black column). (**E**) Effect of VNSB (2.0 mA) on mRNA expression of leptin receptor (Lepr), Cckbr, interleukin-1β (IL1b), tumor necrosis factor (Tnf), and transforming growth factor β1 (Tgfb1) in brainstem. *: $P<0.05$ between sham-VNSB (white column) and VNSB (black column). (**F**) Effect of VNSB (2.0 mA) on mRNA expression of CCK, somatostatin, and tyrosine hydroxylase in hippocampus. *, **, ***: $P<0.05$, 0.01, 0.001 between sham-VNSB (white column) and VNSB (black column).

Figure 3: Gastric Botox injections reduced body weight and food intake, increased energy expenditure and altered gene expression in the hypothalamus and gut hormones in plasma in normal chow-fed rats. Mean ± SEM (n=6-12). (**A**) Photograph showing the site of Botox injection. (**B**) Effect of Botox injection on body weight in normal chow-fed rats. Note: Botox was injected at 0 and 4 weeks. (**C**) Effect of Botox injection on food intake (g/24h) in normal chow-fed rats. *,***: $P<0.05$, 0.001 between vehicle (white column) and Botox (black column). (**D**) Effect of Botox injection on energy expenditure in normal chow-fed rats. **: $P<0.01$ between vehicle (white column) and Botox (black column). (**E**) Effect of Botox injection on hypothalamic mRNA expression of NPY, AgRP, and POMC in chow-fed rats at 8 weeks. *: $P<0.05$, or $P=0.058$ between vehicle (white column) and Botox (black column). (**F**) Effect of Botox injection on plasma concentrations of gastrin, glucagon, GLP-1, PYY, and CCK in chow-fed rats at 8 weeks. **, ***: $P<0.01$, 0.001 between vehicle (white column) and Botox (black column) injection.

Figure 4: Gastric Botox injections reduced body weight and food intake in high-fat diet-induced obese (DIO) rats and DIO rats with sleeve gastrectomy (DIO-SG). Mean ± SEM (n=6-12). (**A**) Effect of Botox injection on body weight in DIO rats. Note: Botox was injected at 0 and 6 weeks. (**B**) Effect of Botox injection on food intake in DIO rats. *, ***: $P<0.05$, 0.001 between vehicle (white column) and Botox (black column). (**C**) Effect of sleeve gastrectomy (SG) and Botox injection in DIO rats (DIO-SG) on body weight. Note: Botox was injected 6 weeks post SG. (**D**) Effect of Botox injection in DIO-SG rats on food intake. Note: white column is vehicle and black column is Botox. *: $P<0.05$ between values before and after SG+Botox.

Figure 5: Gastric Botox injections did not delay gastric emptying or cause gastroparesis in normal chow-fed or high fat diet induced obese (DIO) rats. Means ± SEM (n=6-10). (**A**) Gastric emptying 2 weeks after Botox injection in normal chow fed rats. (**B**) Gastric emptying

before and 1, 3 and 5 weeks after Botox injection in DIO rats. Note: no significant difference between before and after injection. (**C**) Gastric emptying in normal chow-fed (ND) and DIO rats. Note: no significant difference. In (**D**) and (**E**), photographs showing the stomachs after vehicle injection (control) and Botox injection in normal chow-fed rats.

Figure 1

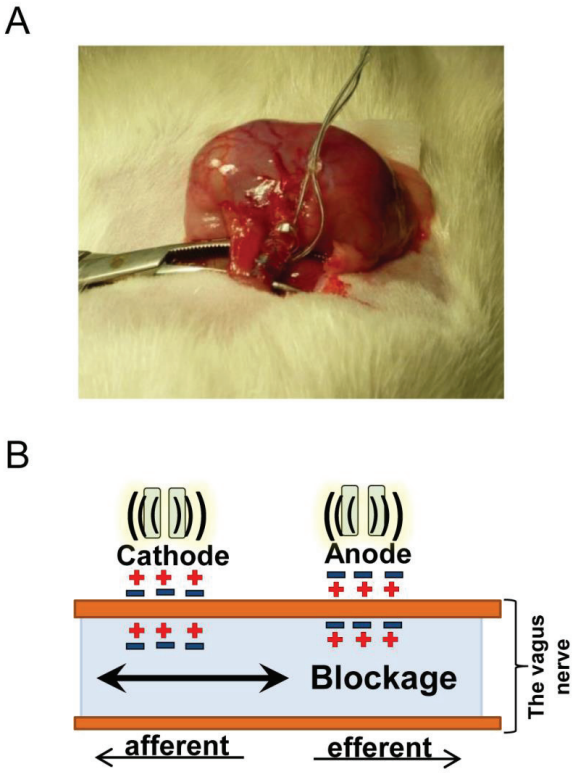

A
B
Cathode
Anode
Blockage
The vagus nerve
afferent
efferent
C
300 μV
0 μV
-200 μV
100 μV
100 μs

Figure 2

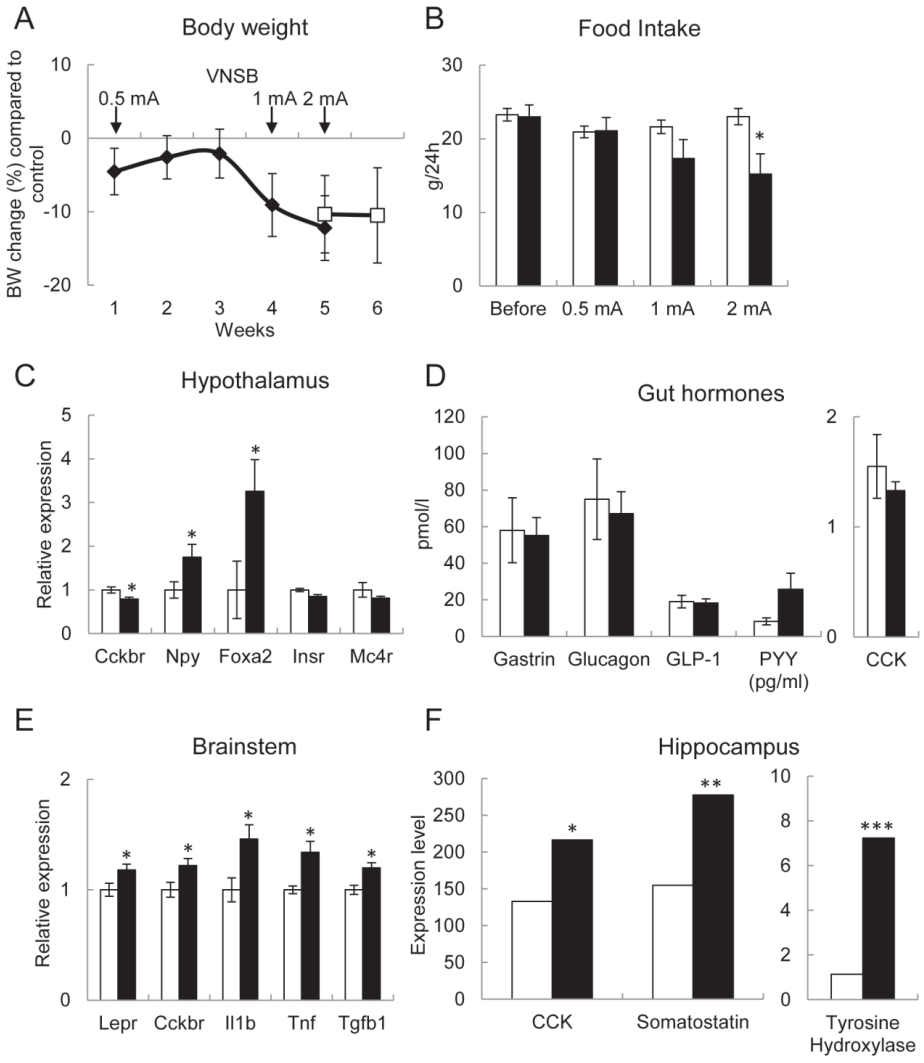

A
Body weight
BW change (%) compared to control
VNSB
0.5 mA
1 mA 2 mA
Weeks
B
Food Intake
g/24h
Before
0.5 mA
1 mA
2 mA
C
Hypothalamus
Relative expression
Cckbr
Npy
Foxa2
Insr
Mc4r
D
Gut hormones
pmol/l
Gastrin
Glucagon
GLP-1
PYY (pg/ml)
CCK
E
Brainstem
Relative expression
Lepr
Cckbr
Il1b
Tnf
Tgfb1
F
Hippocampus
Expression level
CCK
Somatostatin
Tyrosine Hydroxylase

Figure 3

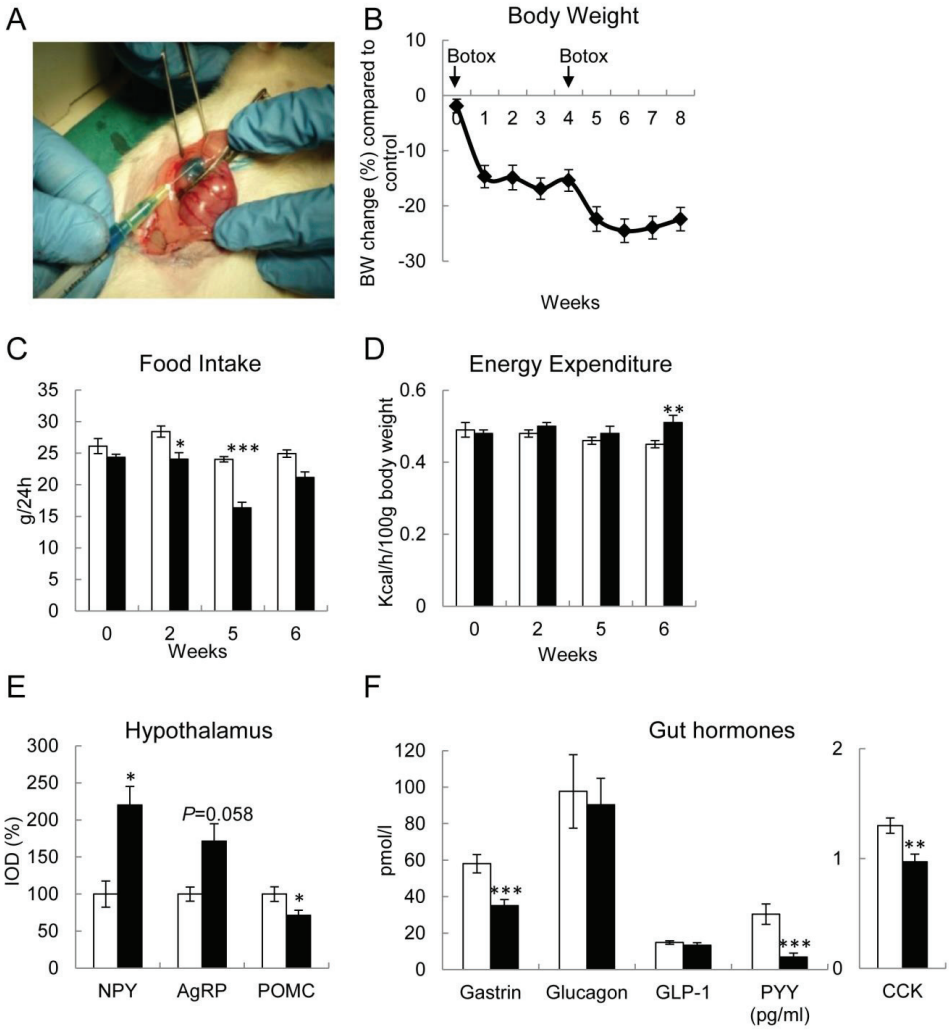

A
B
Body Weight
Botox
Botox
BW change (%) compared to control
Weeks
C
Food Intake
g/24h
Weeks
D
Energy Expenditure
Kcal/h/100g body weight
Weeks
E
Hypothalamus
IOD (%)
P=0.058
NPY
AgRP
POMC
F
Gut hormones
pmol/l
Gastrin
Glucagon
GLP-1
PYY (pg/ml)
CCK

Figure 4

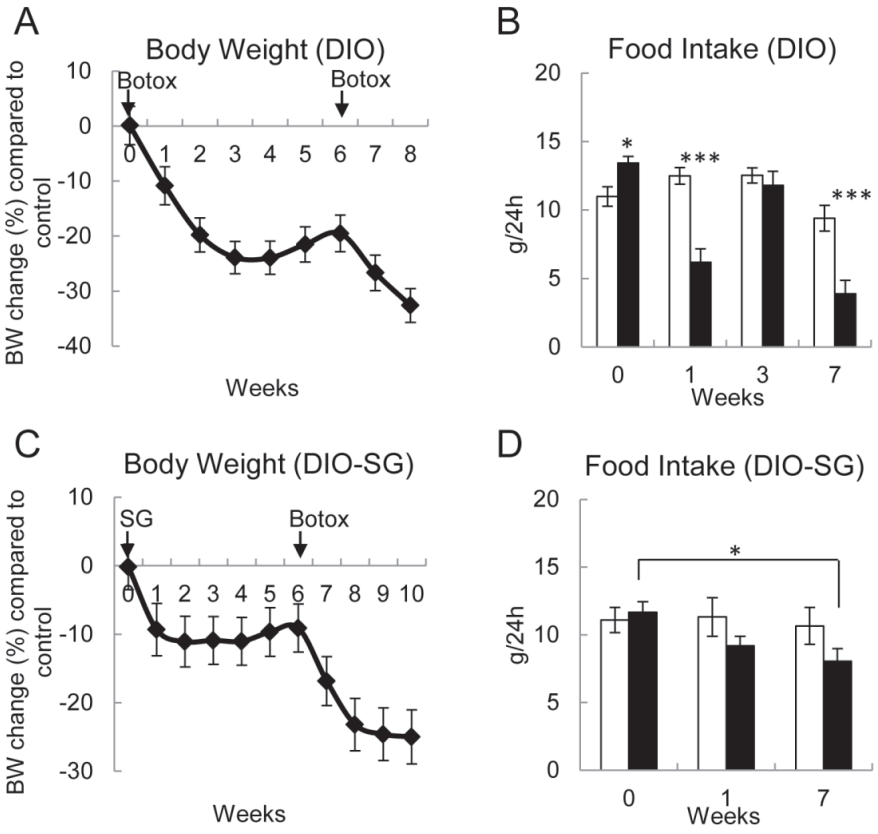
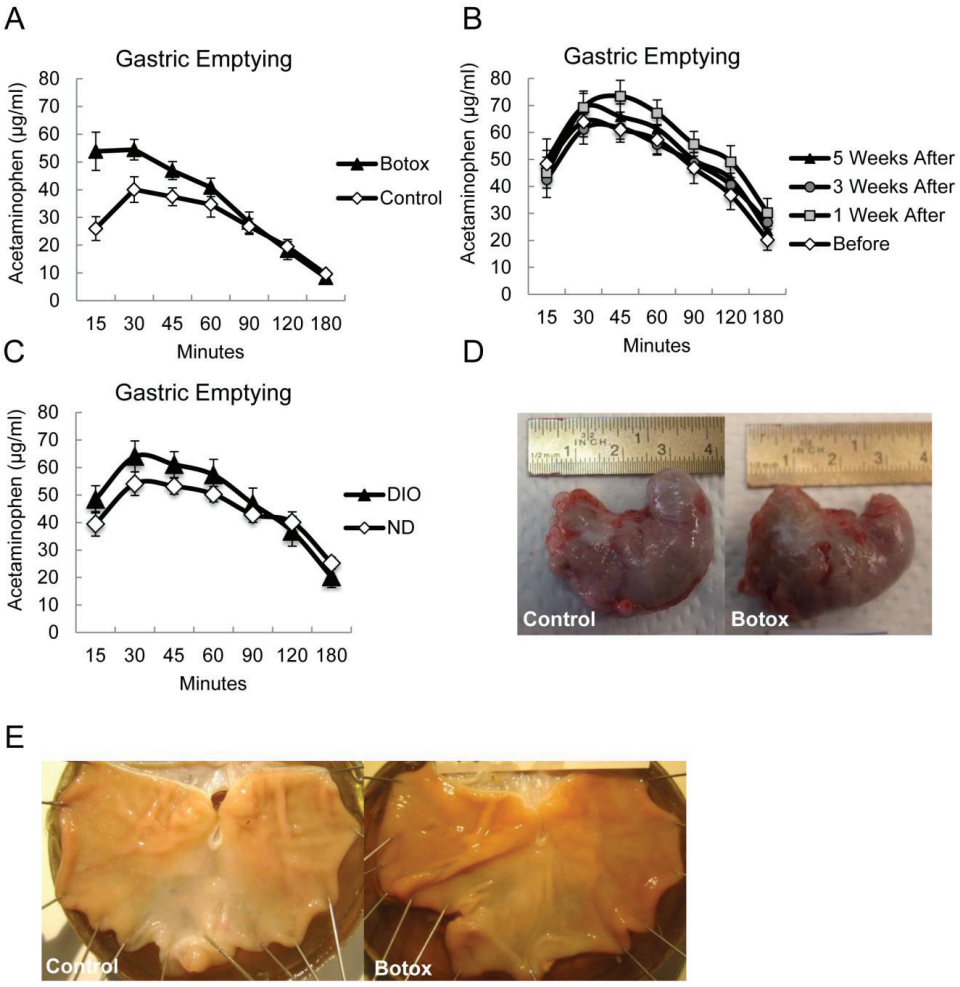

A
Body Weight (DIO)
Botox
Botox
BW change (%) compared to control
Weeks
B
Food Intake (DIO)
g/24h
Weeks
C
Body Weight (DIO-SG)
SG
Botox
BW change (%) compared to control
Weeks
D
Food Intake (DIO-SG)
g/24h
Weeks

Figure 5


A
Gastric Emptying
Acetaminophen (µg/ml)
80
70
60
50
40
30
20
10
0
15 30 45 60 90 120 180
Minutes
Botox
Control
B
Gastric Emptying
Acetaminophen (µg/ml)
15 30 45 60 90 120 180
Minutes
5 Weeks After
3 Weeks After
1 Week After
Before
C
Gastric Emptying
Acetaminophen (µg/ml)
15 30 45 60 90 120 180
Minutes
DIO
ND
D
Control
Botox
E
Control
Botox

Supplementary Information

The Gastric Vagus Nerve as a Target for Obesity Treatment

Helene Johannessen[1], David Revesz[2], Yosuke Kodama[1], Nikki Cassie[3], Karolina P Skibicka[4], Magnus K Olsen[1], Robert Lyle[5], Perry Barrett[3], Suzanne L Dickson[4], Jens J Holst[6], Jens F Rehfeld[7], Geoffrey van der Plasse[8], Roger AH Adan[8], Bård Kulseng[9], Thorleif Thorlin[2], Elinor Ben-Menachem[2], Chun-Mei Zhao[1], Duan Chen[1,9]

[1]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; [2]Department of Clinical Neuroscience and Rehabilitation, The Sahlgrenska Academy at Gothenburg University, Gothenburg, Sweden; [3]Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, Scotland; [4]Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden; [5]Department of Medical Genetics, Oslo University Hospital, Oslo, Norway; [6]Department of Biomedical Sciences, the Panum Institute, University of Copenhagen, Copenhagen, Denmark; [7]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark; [8]Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, Netherlands; [9]Department of Surgery, St. Olav's University Hospital, Trondheim, Norway

Corresponding author: Professor Duan Chen, MD, PhD, Erling Skjalgssons Gate 1, Eastside 3rd Floor, Laboratory Centre of St. Olav's Hospital, 7006 Trondheim, Norway. Tel: +47 725 73320. Fax: +47 725 76612. Mob: +47 984 09 675. E-mail: duan.chen@ntnu.no.

Supplementary Materials and Methods

Determination of Eating Behavior and Metabolic Parameters

Rats were placed in the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments International, Columbus, OH, USA) with free access to standard rat powder food (RM1 811004, Scanbur BK AS, Sweden) or high fat diet powder food (D12492, Research Diets, Inc., NJ, USA) and tap water. Energy expenditure (EE) (kcal/h) was calculated according to this equation: (3.815+1.232 RER) × VO_2, where RER (respiratory exchange ratio) was the volume of CO_2 produced per volume of O_2 consumed. VO_2 was the volume of O_2 consumed per h per kilogram of mass of the animal. Parameters that were obtained during daytime (7 am –7 pm) and nighttime (7 pm –7 am) for each individual rat included number of meals, meal size, meal duration, accumulated food intake, intermeal interval, rate of eating, satiety ratio, drinking activity, energy expenditure, and ambulatory activity. Satiety ratio, an index of the non-eating time produced by each gram of food consumed, was calculated by dividing the average intermeal interval by the average meal size.

Gastric acid secretion measurement

The rats were fasted for 24h before measurement. Gastric acid was collected for 30 min at baseline, 0.5 mA, 1.0 mA, 2.0 mA, 3.5 mA, and at the same settings with pentagastrin subcutaneous injection. Between each collection the stimulation was turned off and there was a break of 90 min. The rats received 1 mL physiological saline subcutaneously every second hour. The gastric acid was expressed as pH and H^+ content/30 min.

Taqman array

Brainstem (medulla and pons) and hypothalamus were dissected at euthanization and stored in RNAlater (Qiagen, Hilden, Germany) at -80°C. Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen), and reversed transcribed using random hexamers (Applied Biosystems, Sundbyberg, Sweden), and Superscript III reverse transcriptase (Invitrogen Life Technologies, Paisley, UK). Real-time RT PCR was performed using TaqMan®Low Density Array (LDA) custom-made platforms. Target genes as well as the primer sets were Hmbs, Foxa2, Npy, Lepr, Insr, Cckbr, Mc4r, Il1b, Tnf, and Tgfb1 (catalogue number Rn00565886_m1, Rn01415600_m1, Rn01410145_m1, Rn01433205_m1, Rn00567070_m1, Rn00565867_m1, Rn01491866_s1, Rn00580432_m1, Rn01525859_g1 and Rn00572010_m1, respectively) (Applied Biosystems).

***In situ* hybridization**

Brain samples were taken at euthanization and snap-frozen and cut (14 μm) in the region spanning the hypothalamus between Bregma –0.10 to –2.54 mm and brainstem (NTS). Primers for the amplification of AgRP spanned were based on Genbank sequence U89484 to amplify the sequence between bases 113-341 (forward primer 5'-TGTTCCCAGAGTTCCCAGGTC-3', reverse primer 5'-GCATTGAAGAAGCGGCAGTAGCAC-3'). Primers for the amplification of POMC were based on Genbank sequence J00162 to amplify the sequence between bases 263-665 (forward primer 5'-GGGCAAGCGCTCCTACTCCAT-3', reverse primer 5'-GCCCTTCTTGTRSRCGTTCTTGA-3'). The DNA sequence for NPY was a full-length cloned rat NPY gene sequence. Radioactive ^{35}S antisense probes were made using 150ng amplified fragment by T7(AgRP), T3(NPY), or SP6(POMC) RNA polymerase as stated. Slides were dried, exposed to Kodak Biomax MR film, and Autoradiographic films were

scanned at 600 d.p.i. and analysed using Image Pro Plus v.7.0 (Media Cybernetics UK, Marlow, Bucks, UK), analysis software (Media Cybernetics UK, Wokingham, UK). Integrated optical density was obtained by reference to the ^{14}C microscale.

RNA sequencing

Hippocampus was dissected at euthanization and stored in RNAlater (Qiagen) before total RNA was isolated. High-throughput cDNA sequencing (RNA-seq) was performed at Norwegian Sequencing Centre, Dept. of Medical Genetics Ullevål, Oslo University Hospital, using HiSeq2000. Differential expression analysis on the data was performed, where reads for all samples were aligned to the rat genome using Bowtie2 (http://bowtie-bio.sourceforge.net/index.shtml) and Tophat2 (http://tophat.cbcb.umd.edu), and differential expression was calculated using cuffcompare in the Cufflinks2 (http://cufflinks.cbcb.umd.edu) package.

***In vivo* electrophysiology**

The rats were anaesthetized with 0.1ml/100gr i.m. Hypnorm and 0.05ml/100gr i.p. Midazolam. Following placement of VNSB the animals were mounted in a stereotaxic frame (Kopf Instruments) and the cranium was exposed. A hole was drilled over the hippocampal area (coordinates AP -3.2, ML 2.2), dura was removed, and a bundle 4 tetrodes (0.005", Pt/Ir, Fine Wire, California) were lowered into the brain (DV -3.2) until stable neuronal activity was measured. The drill hole was subsequently filled with mineral oil (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and a surgical screw was placed into the scull to serve as ground. During VNSB (2.0 mA, 30 Hz, 500 μs, 30 s ON and 5 min OFF) data was recorded at 40Khz using a Multineuron Acquisition Processor (MAP) recording system (Plexon, Dallas, USA). Signals were passed through a unity-gain amplifier (20×), amplified and filtered with a

Plexon 16-channel preamplifier (PBX3 /16sp-r-G50, 50x amplification, 150-8000 Hz filtered). Subsequently, a 1200μs digitized data sample was stored by the MAP system whenever the signal crossed a preset voltage threshold. The data were subsequently analyzed with offline cluster cutting procedures based on the average waveforms across the four leads of each tetrode (Offline Sorter x64 V3; Plexon).

Radioimmunoassay

Plasma samples were collected at euthanization and kept at -80°C. In-house radioimmunoassay using specific antibodies was performed to analyze GLP-1, gastrin, CCK, and glucagon concentrations, while measurement of PYY concentrations was performed using a commercial kit (Rat/mouse PYY RIA kit, Cat. no. RMPYY-68HK, EMD Millipore Corporation, St.Charles, MO, USA). Results were reported as pmol/l for GLP-1, gastrin, CCK, glucagon and pg/ml for PYY.

Fasting blood glucose

Blood glucose levels were measured after 12h fasting by taking a drop of blood from the tail vein and analyzing with a glucose meter and test strips (FreeStyle Freedom Lite, Abbot Diabetes Care, California).

Gastric emptying

Rats were fasted for 16h before an oral gavage of 100 mg/kg acetaminophen (Sigma-Aldrich Chemie GmbH, Germany) in 1.5% methyl cellulose (Sigma-Aldrich Chemie GmbH, Germany) was administrated. 100 μl blood samples were collected from the tail at 15, 30, 45, 60, 90, 120, and 180 min after oral gavage. Plasma levels of acetaminophen were subsequently measured using an acetaminophen kit (Acetaminophen-sl assay, ref. 505-30,

Sekisui Diagnostics, PE Canada) and spectrophotometer (UV-1800, UV-Spectrophotometer, Shimadzu).

Supplementary Figures

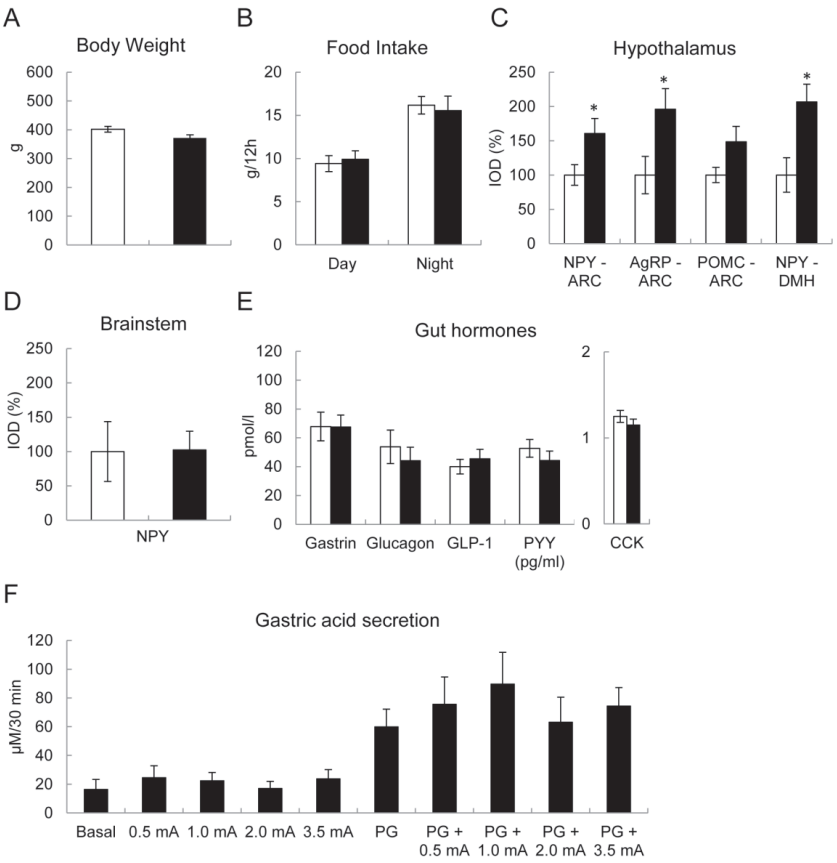


Figure S1: Effects of short-term VNSB (48h at 2.0 mA) on (**A**) body weight, (**B**) food intake, (**C**) gene expression of neuropeptides in hypothalamus and (**D**) brainstem, (**E**) plasma concentrations of gut hormones, and effects of VNSB on (**F**) gastric acid secretion. Mean ± SEM (n=6-8). Note: no significant difference between sham-VNSB (white column) and VNSB (black column), except some hypothalamic genes in (**C**) (*: $P<0.05$). In (**F**), electrical currents from 0.5 mA to 3.5 mA; PG: pentagastrin.

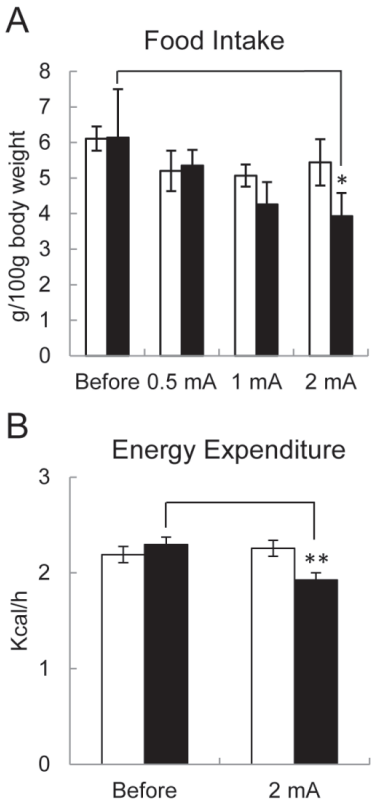


Figure S2: Effects of long-term VNSB (6-8 weeks) on (**A**) food intake (g/100g body weight) and (**B**) energy expenditure. Mean ± SEM (n=4-9). In (**A**), different currents of VNSB. White columns: sham-VNSB, black columns: VNSB. *, **:*P*<0.05, 0.01.

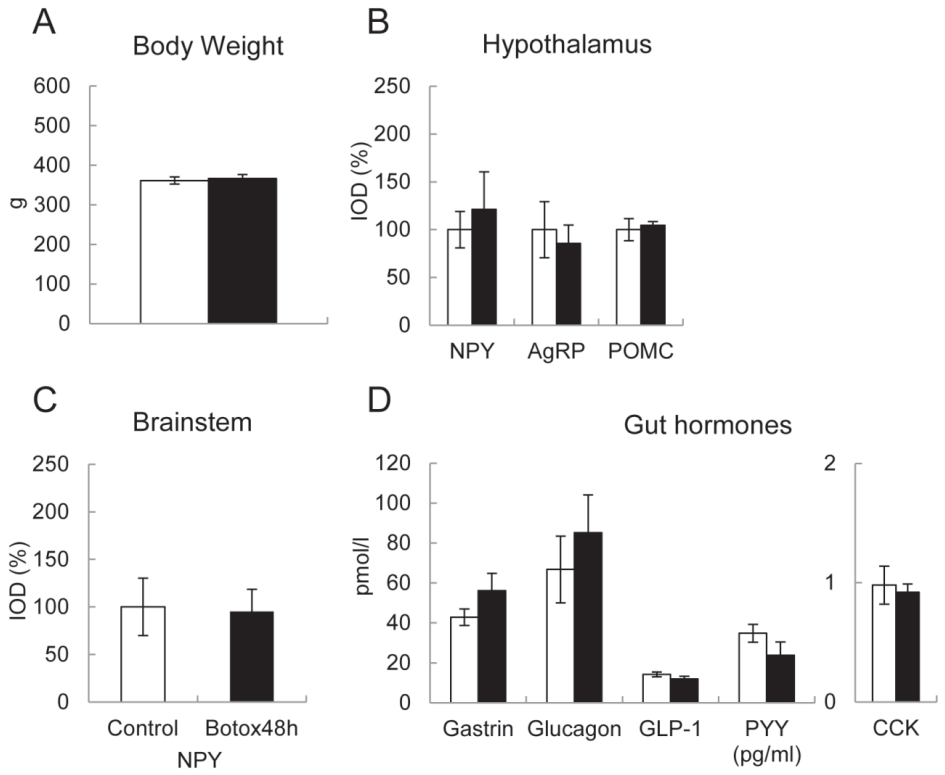


Figure S3: Effects of Botox (48h post injection) on (**A**) body weight, (**B**) gene expression in hypothalamus and (**C**) brainstem, and (**D**) plasma concentrations of gut hormones. Mean ± SEM (n=5). Note: no significant difference between vehicle (white column) and Botox (black column).

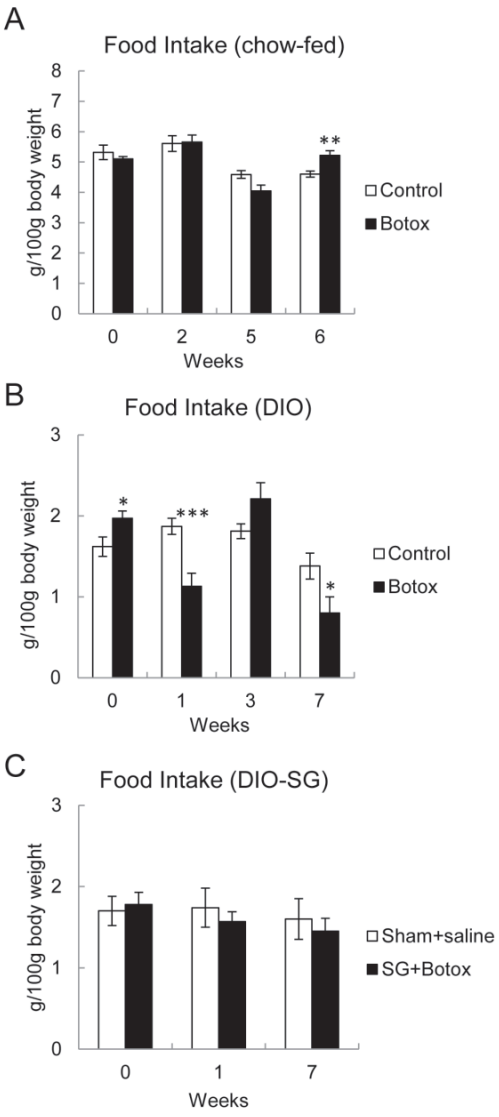


Figure S4: Effects of Botox injection on food intake (g/100g body weight) in (**A**) normal chow-fed rats, (**B**) high fat diet induced obese (DIO) rats and (**C**) DIO rats with sleeve gastrectomy (DIO-SG). Mean ± SEM (n=6-12). *, **, ***: P<0.05, 0.01, 0.001. Note: 0 week: before injection; 1 week: 1 week after the 1st Botox injection (**B**) or SG (**C**); 2 weeks: 2 weeks after the 1st Botox injection (**A**); 3 weeks: 3 weeks after the 1st Botox injection (**B**); 5

weeks: 1 weeks after the 2nd Botox injection; 6 weeks: 2 weeks after the 2nd Botox injection; and 7 weeks: 1 week after the 2nd Botox injection (**B**) and 1 week after Botox injection (**C**).

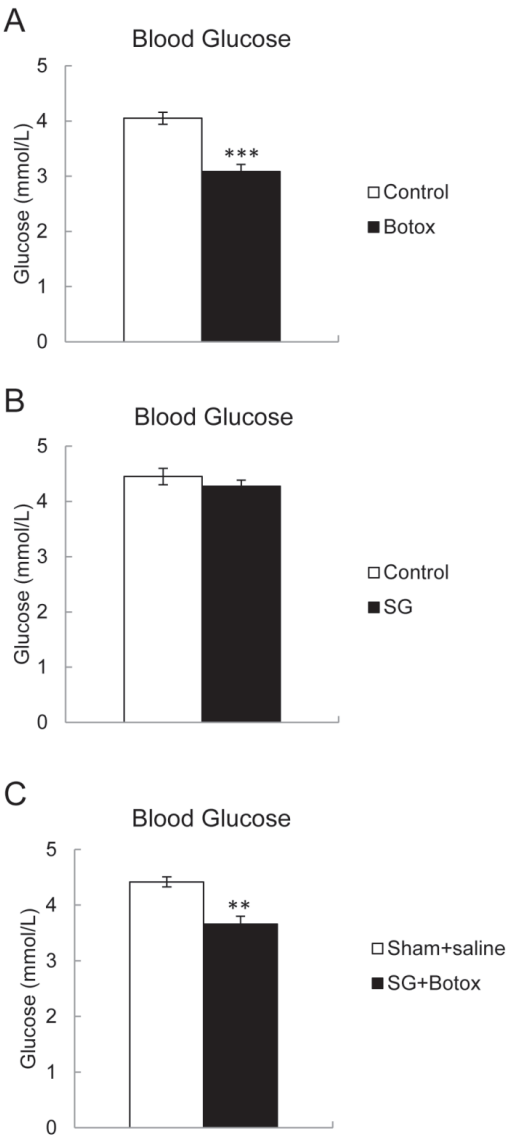


Figure S5: Fasting blood glucose levels in DIO rats after (**A**) Botox, (**B**) sleeve gastrectomy (SG) and (**C**) the combination (SG+Botox). Mean ± SEM (n=6-14). **, ***: P<0.01, 0.001.

Paper II

Lack of Muscarinic Acetylcholine M3 Receptor Induces a Lean Phenotype through Increased Energy Expenditure Rather than Reduced Food Intake

Short title: Lack of Muscarinic M3 Receptor does not Reduce Food Intake

Helene Johannessen[1], Nikki Cassie[2], Yosuke Kodama[1], Perry Barrett[2], Koji Takeuchi[3], Bård Kulseng[1,4], Duan Chen[1,4], Chun-Mei Zhao[1]

[1]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; [2]Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, Scotland; [3]Department of Pharmacology and Experimental Therapeutics, Kyoto Pharmaceutical University, Kyoto, Japan; [4]Department of Surgery, St. Olav's University Hospital, Trondheim, Norway

Corresponding author: Dr. Chun-Mei Zhao, Erling Skjalgssons Gate 1, Eastside 3rd Floor, Laboratory Centre of St. Olav's Hospital, 7006 Trondheim, Norway. E-mail: chun-mei.zhao@ntnu.no. Tel: +47 725 73080. Mob: +47 92077604.

Abstract

Obesity has become a worldwide problem, affecting both rich and poor countries. Up to date, bariatric surgery is the only obesity treatment with long-term effect. Due to high surgical cost and risk of complications, there is an urgent need for drug discovery against obesity. Mice lacking the muscarinic acetylcholine M3 receptor display a lean phenotype. To assess this receptor as a potential target for obesity treatment, we performed detailed phenotyping of M3 receptor knockout (M3KO) mice with respect to food intake, eating behavior, energy expenditure and hypothalamic gene expression by open circuit indirect calorimeter and *in situ* hybridization throughout the lifespan of the mice. In comparison with wild-type mice, the M3KO mice had a lean phenotype with increased energy expenditure. Surprisingly, these mice had increased cumulative food intake (g/100g body weight) and gene expression of orexigenic peptides in the hypothalamus. Thus, we suggest that targeting the muscarinic acetylcholine M3 receptor and/or increasing energy expenditure may have great potential to streamline the development of anti-obesity drugs.

Introduction

The number of obese individuals is increasing, leading to a global obesity epidemic [1,2]. Obesity is a major contributor in the development of type 2 diabetes, cardiovascular diseases, certain forms of cancer, overall reduced quality of life and premature mortality [3,4]. Not only is obesity a great health burden, it is also an economic burden to society as the medical cost for an obese individual is 40% higher than for a normal weight individual [5]. Weight loss treatments include diet control, physical training, pharmaceutical drugs, and bariatric surgery [6]. To date, only bariatric surgery has demonstrated long-term therapeutic effects [7,8]. In consideration of the large number of obese patients, risk of surgical complications, high surgery-related cost, and those who are not eligible for conventional bariatric surgery, development of new effective pharmacological interventions to treat obesity is urgently needed [9].

Currently, anti-obesity drugs on the market mainly target food intake by acting on noradrenergic or serotonergic receptors, or reduce dietary fat absorption (e.g. Orlistat). By average, the pharmacological interventions reduce body weight by 4 kg, relative to placebo [10]. Although this can be beneficial for health it is not comparable to the effects of bariatric surgery [8]. Therefore it is important to explore new targets in drug design for obesity treatment.

The muscarinic acetylcholine M3 receptor is distributed both in the peripheral and central nervous system and may play an important role in the regulation of food intake and body weight. It has been reported that mice deficient of this receptor (M3KO mice) have reduced food intake, body weight, total body fat, and serum leptin, insulin and triglyceride levels [11,12]. This phenotype has not been observed in mice lacking M1, M2, M4 or M5 muscarinic receptors [13-16]. Apparently, the M3 receptor is a new target in treatment of obesity.

In the report by Yamada and coworkers, M3KO mice had reduced food intake on one hand, but increased hypothalamic gene expression of the orexigenic neuropeptide AgRP and reduced expression of the anorexigenic neuropeptide POMC on the other hand [11]. According to common paradigm, the changes in the hypothalamus indicate a drive for increased food intake, which does not coincide with a phenotype of reduced food intake [17]. Therefore we performed a detailed phenotyping of the M3KO mice with respect to eating behavior, metabolic parameters and hypothalamic gene expression using a more advanced method (Comprehensive Laboratory Animal Monitoring System) and *in situ* hybridization.

Materials and Methods

Animals and Experimental Design

M3KO mice were generated [18] and backcrossed 14 generations onto C57BL/6 background at Prof. Takeuchi's laboratory, Kyoto Pharmaceutical University and imported to Norway by Prof. D. Chen for this study. The M3KO mice were further bred through sibling matings for 2 generations. Age-matched wild-type (WT) mice on the same background (C57BL/6) were purchased from Taconic (Ejby, Denmark). All the mice including M3KO and WT mice were fed in-house together during the study period. The mice had free access to tap water and standard pellet food (RM1 801002, Scanbur BK AS, Sweden). All the mice were housed three-four together in individually ventilated cages on wood chip bedding with a 12 h light/dark cycle, room temperature of 22ºC and 40-60% relative humidity. The standard housing conditions were specific pathogen free and in agreement with FELASA (Federation of European Laboratory Animal Science Association) recommendations. Animal experiments were performed according to the guidelines for the design and statistical analysis of experiments using laboratory animals after being approved by the Norwegian National Animal Research Authority (Forsøksdyrutvalget, FDU, permit number: 4764).

The mice were acclimatized to the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments International, Columbus, OH USA) for 24 h before data collection. At data collection the mice were kept in CLAMS for 48 h and data from the last 24 h were used for analysis. Before CLAMS the mice were habituated to their normal food as powder for three days, as the food in CLAMS is in powder form.

M3KO and age-matched WT mice were placed in CLAMS at 6, 11 and 15 months of age for determination of food intake, eating behaviour and metabolic parameters. Body weight was measured at 2, 6, 11 and 15 months of age. At 2 months of age there were 7 mice in each group, 3:4 male:female ratio in M3KO group and 4:3 male:female ratio in WT group. At the subsequent time points there were 8 mice in each group, with male:female ratio of 4:4 ratio in the WT group, and 4:4, 5:3 and 6:2 in the M3KO group at 6, 11 and 15 months, respectively. Brain tissue from 15 months old mice were collected for *in situ* hybridization.

Determination of Eating Behavior and Metabolic Parameters

Mice were placed in the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments International, Columbus, OH, USA) with free access to standard mice powder food (RM1 801002, Scanbur BK AS, Sweden) and tap water. This system is composed of a four-chamber open circuit indirect calorimeter designed for continuous monitoring of individual mice. Eating behavior and metabolic parameters were recorded automatically. High-resolution feeding data was generated by monitoring all feeder balances every 0.5 seconds. The end of an eating event (meal) was determined when the balances were stable for more than 10 seconds and a minimum of 0.05 g of food were eaten. An air sample was withdrawn every 5 min. The energy expenditure (EE) (kcal/h) was calculated according to this equation: $(3.815+1.232\ RER) \times VO_2$, where RER (respiratory exchange ratio) was the volume of CO_2 produced per volume of O_2 consumed. VO_2 was the

volume of O_2 consumed per h per kilogram of mass of the animal. Parameters that were obtained during daytime (7 am–7 pm) and nighttime (7 pm–7 am) for each individual mouse included number of meals, meal size, meal duration, accumulated food intake, intermeal interval, rate of eating, satiety ratio, drinking activity, energy expenditure and ambulatory activity. The intermeal interval was defined as the interval in minutes between two meals. Rate of eating was calculated by dividing the average meal size by the average duration of a meal, and satiety ratio, an index of the non-eating time produced by each gram of food consumed, was calculated by dividing the average intermeal interval by the average meal size.

Food intake was higher and satiety ratio lower during nighttime than daytime for all animals at every timepoint.

In Situ Hybridization

Brain samples were taken at euthanization and snap-frozen in isopentane on dry ice before stored at -80°C wrapped in aluminum foil. The frozen brains were cut (14 μm) in the region spanning the hypothalamus between Bregma –0.10 to –2.54 mm according to the Mouse Brain Atlas of Franklin & Paxinos 1997 and sections were mounted onto poly-L-lysine-coated slides. Briefly, sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), washed in 0.1 M PB, acetylated in 0.25% acetic anhydrate in 0.1 M triethanolamine, and washed again in PB. Sections were dehydrated using graded ethanol. DNA templates for the generation of anti-sense riboprobes for AgRP and POMC were generated by PCR. Briefly, primers for the amplification of AgRP spanned were based on Genbank sequence U89484 to amplify the sequence between bases 113-341 (forward primer 5'-TGTTCCCAGAGTTCCCAGGTC-3', reverse primer 5'-GCATTGAAGAAGCGGCAGTAGCAC-3'). Primers for the amplification of POMC were based on Genbank sequence J00162 to amplify the sequence between bases 263-665 (forward

primer 5’-GGGCAAGCGCTCCTACTCCAT-3’, reverse primer 5’-GCCCTTCTTGTRSRCGTTCTTGA-3’). The DNA sequence for NPY was a full-length cloned rat NPY gene sequence. Primers for the amplification of Ob-Rb (leptin receptor) were based on Genbank sequence U49107 to amplify the sequence between bases 5'-GTGTGAGCATCTCTCCTGGAG-3' (+2829 to +2849) and 5'-ACCACACCAGACCCTGAAAG-3' (+3362 to +3343). Primers for the amplification of MCH were based on Genbank sequence M29712 to amplify the sequence between bases 99-403 (forward primer bases 99-117 5’-ACGGCATTTTACTTTCGGC-3’; reverse primer bases 384-403 5’-CTGAACTCCATTCTCAGCTGG- 3’). ^{35}S antisense probes were made using 150ng amplified fragment by T7(AgRP), T3(NPY), or SP6(POMC, Ob-Rb, and MCH) RNA polymerase as stated. The radioactive probes were applied to the slides in 70 µl hybridization mixture (0.3 M NaCl, 10 mM Tris-HCL (pH 8), 1 mM EDTA, 0.05% transfer RNA, 10 mM dithiothreitol, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA and 10% dextran sulphate) and hybridized overnight at 58°C. Post-hybridization, slides were rinsed in 4x SSC and treated with ribonuclease A (20 µg/µl) at 37°C before being washed in decreasing concentrations of SSC and dehydrated using graded ethanol. Slides were dried and exposed to Kodak Biomax MR film for various lengths of time. Autoradiographic films were scanned at 600 d.p.i. and analysed using Image Pro Plus v.7.0 (Media Cybernetics UK, Marlow, Bucks, UK), analysis software (Media Cybernetics UK, Wokingham, UK). Integrated optical density was obtained by reference to the 14C microscale. NPY, POMC, AgRP, leptin receptor and MCH mRNA expression was measured in three to four sections. Values were averaged for each animal.

Statistical Analysis

The results are expressed as means ± SEM. Statistical comparisons were performed using independent *t*-test between the groups. A *p*-value of <0.05 (two tailed) was considered statistically significant. The data analysis was performed in SPSS version 15.0 and 20.0.

Results

Body Weight

At 2 months of age the body weight did not differ between M3KO and WT mice when comparing all mice (both sexes) (p=0.065) (Fig. 1A). However, the male M3KO mice weighed less than male WT mice at this time point (p=0.000) (Fig. 1B). At 6, 11 and 15 months of age the M3KO mice weighed significantly less than WT mice when comparing all mice (both sexes) (p<0.001) (Fig. 1A). When we compared age-matched males, the M3KO mice weighed significantly less than WT mice at all time points (p<0.001) (Fig. 1B). The same was true for females (p<0.05), except for 2 and 15 months of age when there were few female M3KO mice (Fig. 1C). The M3KO mice had a continuous growth throughout the lifespan.

Food Intake, Eating Behavior and Metabolic Parameters

At 6 months of age the M3KO mice had unaltered total (24 h) food intake, but there was a trend of reduced food intake (g) during daytime (p=0.054) (Fig. 2A and 2C). At 11 months of age the M3KO mice had unaltered total (24 h) food intake, but food intake (g/100g body weight) during nighttime was increased (p=0.014) (Fig. 2A and 2B). At 15 months of age the M3KO mice had unaltered total (24 h) food intake, but food intake (g) during daytime was reduced, while food intake (g/100g body weight) during nighttime was increased (p=0.043 and p=0.034, respectively) (Fig. 2A, 2B and 2C).

Cumulative food intake, expressed as g per mouse, was unchanged at the three time points examined. When adjusted for body weight (g/100g body weight), it was significantly higher at 15 months of age in M3KO mice compared to WT mice ($p<0.01$) (Fig. 3A and 3B).

At 6 months of age the M3KO mice ate significantly fewer, but bigger meals than WT mice (g/meal) ($p=0.000$ and $p=0.005$, respectively). The time between each meal was longer (min), but the rate of eating (g/min) was faster ($p=0.008$ and $p=0.002$, respectively). These mice had significantly higher water intake (ml/100g body weight) than WT mice ($p=0.050$). Interestingly, the mice also had significantly higher energy expenditure (kcal/h/100g body weight) and respiratory exchange ratio (RER) (VCO_2/VO_2) ($p<0.001$) (Fig. 4A and 4B) (Table 1).

At 11 months of age water intake (ml/100g body weight) was increased, as was energy expenditure (kcal/h/100g body weight) and RER in M3KO mice compared to WT mice ($p=0.001$, $p=0.003$ and $p=0.000$, respectively) (Fig. 4A and 4B) (Table 2).

At 15 months of age water intake (ml/100g body weight) was increased, as was energy expenditure (kcal/h/100g body weight) and RER in M3KO mice compared to WT mice ($p=0.032$, $p=0.003$ and $p=0.000$, respectively) (Fig. 4A and 4B) (Table 3).

Number of meals was reduced at all ages at nighttime in M3KO mice compared WT mice ($p<0.05$) (Tables 1, 2 and 3).

In Situ Hybridization

At 15 months of age there were great differences in the phenotype between M3KO and WT, therefore brain tissue was collected at this age for analysis. The 15 months old M3KO mice had significantly higher mRNA expression of neuropeptide Y (NPY), agouti-related peptide (AgRP) and leptin receptor in the arcuate nucleus (ARC) in the hypothalamus compared to WT ($p=0.001$, $p=0.000$ and $p=0.003$, respectively). Interestingly, mRNA

expression of pro-opiomelanocortin (POMC) in the ARC and melanin-concentrating hormone (MCH) in ventromedial hypothalamus was unaltered (p>0.05) (Fig. 4C).

Discussion

In contrast to previous studies [11,12], we found, for the first time to our best knowledge, that M3KO mice do not have significantly reduced 24h food intake by using state-of-the art methods for determination of food intake, eating behavior and metabolic parameters. Interestingly, we found that M3KO mice had altered eating behavior, with tendency for reduced food intake during daytime and increased food intake during nighttime compared to WT mice and that cumulative food intake (g/100g body weight) was increased in M3KO mice compared to WT mice at 15 months of age. Our results suggest that the lean phenotype of M3KO mice is due to increased active (nighttime) and resting (daytime) energy expenditure. This was reproducible throughout the whole lifespan of the mice. In contrast to the previous studies, where respiratory exchange ratio (RER) was unaltered, we found that RER was increased in M3KO mice [11,12]. This implies an increased use of carbohydrates as predominant fuel for cellular respiration. Interestingly, this was also found during daytime, when the M3KO mice had tendency of reduced food intake compared to WT mice.

By comparing all the mice (both sexes), the body weight at 2 months of age did not differ between M3KO and WT mice. Taking together with the findings of equal body weights at birth and at young age in the previous studies [11,12], we suggest that the lean phenotype of M3KO mice is not caused by prenatal factors, but develops as the mice grow.

Hypothalamic mRNA expression in the M3KO mice was indicative of changes with a drive for increased food intake and reduced energy expenditure, as reported previously [11]. This could represent a compensatory mechanism, which is supported by the increased cumulative food intake in these mice at 15 months of age (Fig. 3B). However, the

compensatory mechanism was without effect on energy expenditure which was greater than the food intake, resulting in a lean phenotype. In the previous studies it was suggested that reduced levels of MCH could play an important role in development of the lean phenotype [11]. However, we found no significant reduction of MCH expression in the 15-month old M3KO mice. To compensate for the low fat compartment and leptin serum levels, as reported previously, expression of leptin receptor in hypothalamus was increased [11,12].

Our results suggest that antagonists of the muscarinic acetylcholine M3 receptor may have potential in obesity treatment. However, our pilot study showed that there were severe side effects, high mortality and little effect on body weight when peripheral M3 antagonist Darifenacin was given continuously for more than two months in mice (Andersen G and Zhao C-M, unpublished data). Indeed, it has been suggested that a central nervous system (CNS) specific antagonist would be beneficial, due to side effects of peripheral M3 receptor antagonists [19]. This is supported by findings of a lean phenotype in brain-specific M3KO mice [20]. However, development of CNS specific M3 antagonists is still highly challenging [19].

According to our results, we also suggest that pharmacological interventions aiming to increase energy expenditure may have more potential than those aiming to reduce food intake in obesity treatment. In fact, there are several possible pharmacological treatments for obesity targeting energy expenditure, such as thyroid hormone receptor β agonists (GC-1 and GC-24) and NADPH:quinone reductase 1 (NQO1) activators (MB12066) [21,22]. Some of these compounds have shown promising results as obesity treatment in animal studies and MB12066 has completed Phase I clinical trial [23-25].

In conclusion, the results of the present study shows that the lean phenotype of M3KO mice is associated with increased energy expenditure rather than reduced food intake. CNS

antagonists of the muscarinic acetylcholine M3 receptor may have potential in obesity treatment by increasing energy expenditure.

References

1. WHO Obesity: preventing and managing the global epidemic. Report of a WHO Consultation presented at: the World Health Organization; June 3-5, 1997; Geneva, Switzerland. Publication WHO/NUT/NCD/98.1.
2. Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, et al. (2011) National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. Lancet 377: 557-567.
3. Burton BT, Foster WR (1985) Health implications of obesity: an NIH Consensus Development Conference. J Am Diet Assoc 85: 1117-1121.
4. Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, et al. (2009) The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC Public Health 9: 88.
5. Finkelstein EA, Trogdon JG, Cohen JW, Dietz W (2009) Annual medical spending attributable to obesity: payer-and service-specific estimates. Health Affair 28: w822-831.
6. Tsai AG, Wadden TA (2013) Obesity. Ann Intern Med 159: ITC3-1.
7. Colquitt J, Picot J, Loveman E, Clegg A (2009) Surgery for obesity COCHRANE DB SYST REV.
8. Gloy VL, Briel M, Bhatt DL, Kashyap SR, Schauer PR, et al. (2013) Bariatric surgery versus non-surgical treatment for obesity: a systematic review and meta-analysis of randomised controlled trials. BMJ 22: f5934.
9. Encinosa WE, Bernard DM, Chen C-C, Steiner CA (2006) Healthcare Utilization and Outcomes after Bariatric Surgery. Med Care 44: 706-712.
10. Yanovski SZ, Yanovski JA (2014) Long-term drug treatment for obesity: A systematic and clinical review. JAMA 311: 74-86.
11. Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, et al. (2001) Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. Nature 410: 207-212.
12. Gautam D, Gavrilova O, Jeon J, Pack S, Jou W, et al. (2006) Beneficial metabolic effects of M3 muscarinic acetylcholine receptor deficiency. Cell Metab 4: 363-375.
13. Hamilton SE, Loose MD, Qi M, Levey AI, Hille B, et al. (1997) Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc Natl Acad Sci U S A 94: 13311-13316.
14. Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, et al. (1999) Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 96: 1692-1697.
15. Gomeza J, Zhang L, Kostenis E, Felder C, Bymaster F, et al. (1999) Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M(4) muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 96: 10483-10488.
16. Yamada M, Lamping KG, Duttaroy A, Zhang W, Cui Y, et al. (2001) Cholinergic dilation of cerebral blood vessels is abolished in M(5) muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 98: 14096-14101.
17. Schwartz MW, Woods SC, Porte D, Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. Nature 404: 661-671.

18. Matsui M, Motomura D, Karasawa H, Fujikawa T, Jiang J, et al. (2000) Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M3 subtype. Proc Natl Acad Sci U S A 97: 9579-9584.
19. Maresca A, Supuran CT (2008) Muscarinic acetylcholine receptors as therapeutic targets for obesity. Expert Opin Ther Targets 12: 1167-1175.
20. Gautam D, Jeon J, Starost MF, Han SJ, Hamdan FF, et al. (2009) Neuronal M3 muscarinic acetylcholine receptors are essential for somatotroph proliferation and normal somatic growth. Proc Natl Acad Sci U S A 106: 6398-6403.
21. Chugh PK, Sharma S (2012) Recent advances in the pathophysiology and pharmacological treatment of obesity. J Clin Pharm Ther 37: 525-535.
22. Amorim BS, Ueta CB, Freitas BC, Nassif RJ, Gouveia CH, et al. (2009) A TRbeta-selective agonist confers resistance to diet-induced obesity. J Endocrinol 203: 291-299.
23. Hwang JH, Kim DW, Jo EJ, Kim YK, Jo YS, et al. (2009) Pharmacological stimulation of NADH oxidation ameliorates obesity and related phenotypes in mice. Diabetes 58: 965-974.
24. Villicev CM, Freitas FR, Aoki MS, Taffarel C, Scanlan TS, et al. (2007) Thyroid hormone receptor beta-specific agonist GC-1 increases energy expenditure and prevents fat-mass accumulation in rats. J Endocrinol 193: 21-29.
25. Corp KGLS (2011) Safety, Tolerability and Pharmacokinetic Study of MB12066 in Healthy Volunteers. ClinicalTrials.gov.

Figure Legends

Figure 1: **Body weight of M3KO and WT mice at 2, 6, 11 and 15 months of age.** (A) Body weight of both male and female wild-type (WT) and M3KO mice. (B) Body weight of male WT and M3KO mice. (C) Body weight of female WT and M3KO mice. Mean ± SEM (n=7-8). ***: $p<0.001$ between WT and KO.

Figure 2: **Food intake of M3KO and WT mice at 6, 11 and 15 months of age.** (A) Food intake 24 h (g/100g body weight) of wild-type (WT) and M3KO mice. (B) Food intake 12 h (g/100g body weight) during day and night time of WT and M3KO mice. (C) Food intake 12 h (g/mouse) during day and night time of WT and M3KO mice. Mean ± SEM (n=8). *: $p<0.05$ between WT and KO.

Figure 3: **Cumulative food intake in M3KO and WT mice at 6, 11 and 15 months of age.** (A) Cumulative food intake (g/mouse) of wild-type (WT) and M3KO mice. (B) Cumulative food intake (g/100g body weight) of WT and M3KO mice. Mean ± SEM (n=8). **: $p<0.01$ between WT and KO.

Figure 4: **Energy expenditure, respiratory exchange ratio and hypothalamic neuropeptide expression in M3KO and WT mice**. (A) Energy expenditure during day and night time of wild-type (WT) and M3KO mice at 6, 11 and 15 months of age. (B) Respiratory exchange ratio (RER) (24 h) of WT and M3KO mice at 6, 11 and 15 months of age. (C) mRNA expression of AgRP, NPY, POMC and leptin receptor (Lepr) in arcuate nucleus and of MCH in ventromedial hypothalamus of WT and M3KO mice at 15 months of age. Mean ± SEM (n=8). **, ***: $p<0.01$, 0.001 between WT and KO.

Table 1: Food intake, eating behavior and metabolic parameters in WT and M3KO mice at 6 months of age.*,**,***:p<0.05, 0.01, 0.001 between WT and M3KO mice.

		6 months	
	Parameters	WT (n=8)	M3KO (n=8)
Daytime	Food Intake (g)	1.55±0.11	0.93±0.27
	Food Intake (g/100g body weight)	4.78±0.37	3.9±1.08
	Number of meals	28.25±4.14	7.25±2.96**
	Meal size (g/meal)	0.06±0.01	0.31±0.1*
	Intermeal interval (min)	27.23±3.64	196.09±51.27*
	Rate of eating (g/min)	0.11±0.03	0.35±0.12
	Water intake (mL)	0.65±0.09	0.62±0.1
	Water intake (ml/100g body weight)	2.01±0.31	2.71±0.43
	Energy expenditure (kcal/h/100g body weight)	1.18±0.04	1.53±0.04***
	Energy expenditure (kcal/h/cm^2 body surface)	0.004±0	0.005±0***
	RER	0.84±0.01	0.94±0.01***
Nighttime	Food Intake (g)	2.45±0.25	2.56±0.49
	Food Intake (g/100g body weight)	7.55±0.76	10.79±1.78
	Number of meals	52±5.69	16.75±3.44***
	Meal size (g/meal)	0.05±0.01	0.18±0.03**
	Intermeal interval (min)	13.75±1.54	52.23±9.71**
	Rate of eating (g/min)	0.06±0.01	0.18±0.03**
	Water intake (mL)	1.96±0.17	2.84±0.6
	Water intake (ml/100g body weight)	6.02±0.52	12.23±2.53*
	Energy expenditure (kcal/h/100g body weight)	1.5±0.03	1.88±0.05***
	Energy expenditure (kcal/h/cm^2 body surface)	0.005±0	0.006±0**
	RER	0.93±0.02	1.07±0.02***
24 h	Food Intake (g)	4±0.24	3.49±0.51
	Food Intake (g/100g body weight)	12.33±0.8	14.69±1.79
	Number of meals	80.25±8.76	24±6.17***
	Meal size (g/meal)	0.05±0.01	0.19±0.04**
	Intermeal interval (min)	18.28±1.92	86.49±18.76**
	Rate of eating (g/min)	0.07±0.01	0.18±0.03**
	Water intake (mL)	2.61±0.2	3.46±0.68
	Water intake (ml/100g body weight)	8.03±0.63	14.94±2.9*
	Energy expenditure (kcal/h/100g body weight)	1.34±0.03	1.71±0.04***
	Energy expenditure (kcal/h/cm^2 body surface)	0.005±0	0.005±0***
	RER	0.89±0.01	1±0.01***

Table 2: Food intake, eating behavior and metabolic parameters in WT and M3KO mice at 11 months of age.*,**,***:p<0.05, 0.01, 0.001 between WT and M3KO mice.

		11 months	
	Parameters	WT (n=8)	M3KO (n=8)
Daytime	Food Intake (g)	1.19±0.31	0.77±0.17
	Food Intake (g/100g body weight)	3.48±1.06	3.02±0.7
	Number of meals	26.5±5.95	22.25±7.98
	Meal size (g/meal)	0.05±0.01	0.18±0.09
	Intermeal interval (min)	39.93±10.26	151.88±61.13
	Rate of eating (g/min)	0.1±0.03	0.34±0.17
	Water intake (mL)	0.76±0.14	0.78±0.2
	Water intake (ml/100g body weight)	2.14±0.42	2.97±0.72
	Energy expenditure (kcal/h/100g body weight)	1.05±0.1	1.43±0.04**
	Energy expenditure (kcal/h/cm^2 body surface)	0.004±0	0.005±0
	RER	0.83±0.02	0.97±0.03**
Nighttime	Food Intake (g)	1.85±0.27	2.33±0.24
	Food Intake (g/100g body weight)	5.3±0.99	9±0.88*
	Number of meals	46.13±5.85	24.13±8.36*
	Meal size (g/meal)	0.05±0.01	0.52±0.36
	Intermeal interval (min)	16.26±1.8	82.67±40.89
	Rate of eating (g/min)	0.07±0.01	0.49±0.29
	Water intake (mL)	1.52±0.17	2.97±0.42**
	Water intake (ml/100g body weight)	4.34±0.67	11.65±1.9**
	Energy expenditure (kcal/h/100g body weight)	1.25±0.12	1.85±0.06***
	Energy expenditure (kcal/h/cm^2 body surface)	0.005±0	0.006±0**
	RER	0.88±0.02	1.11±0.02***
24 h	Food Intake (g)	3.04±0.56	3.1±0.25
	Food Intake (g/100g body weight)	8.78±1.99	12.03±0.73
	Number of meals	72.63±9.47	46.38±16.07
	Meal size (g/meal)	0.04±0.01	0.32±0.18
	Intermeal interval (min)	21.91±3.23	112.61±56.13
	Rate of eating (g/min)	0.08±0.01	0.4±0.22
	Water intake (mL)	2.28±0.27	3.74±0.41**
	Water intake (ml/100g body weight)	6.48±1	14.62±1.71**
	Energy expenditure (kcal/h/100g body weight)	1.15±0.11	1.64±0.03**
	Energy expenditure (kcal/h/cm^2 body surface)	0.004±0	0.005±0*
	RER	0.85±0.02	1.04±0.02***

Table 3: Food intake, eating behavior and metabolic parameters in WT and M3KO mice at 15 months of age.*,**,***:p<0.05, 0.01, 0.001 between WT and M3KO mice.

		15 months	
	Parameters	WT (n=8)	M3KO (n=8)
Daytime	Food Intake (g)	1.09±0.15	0.66±0.12*
	Food Intake (g/100g body weight)	2.9±0.45	2.39±0.46
	Number of meals	35.75±10.68	19.38±6.89
	Meal size (g/meal)	0.04±0.01	0.12±0.05
	Intermeal interval (min)	30.78±7.92	118.35±45.17
	Rate of eating (g/min)	0.15±0.05	0.07±0.01
	Water intake (mL)	0.82±0.16	0.78±0.28
	Water intake (ml/100g body weight)	2.24±0.51	2.83±1.06
	Energy expenditure (kcal/h/100g body weight)	0.99±0.08	1.36±0.07**
	Energy expenditure (kcal/h/cm^2 body surface)	0.004±0	0.005±0*
	RER	0.82±0.02	0.96±0.02***
Nighttime	Food Intake (g)	1.88±0.46	3.07±0.4
	Food Intake (g/100g body weight)	5.34±1.64	11.02±1.77*
	Number of meals	57.25±11.66	23.5±8.4*
	Meal size (g/meal)	0.03±0.01	0.32±0.15
	Intermeal interval (min)	15.16±2.79	69.65±27.16
	Rate of eating (g/min)	0.06±0.02	0.14±0.03*
	Water intake (mL)	1.56±0.28	3.89±0.72*
	Water intake (ml/100g body weight)	4.24±0.95	14±2.99*
	Energy expenditure (kcal/h/100g body weight)	1.19±0.12	1.73±0.1**
	Energy expenditure (kcal/h/cm^2 body surface)	0.005±0	0.006±0*
	RER	0.82±0.03	1.07±0.02***
24 h	Food Intake (g)	2.98±0.51	3.73±0.5
	Food Intake (g/100g body weight)	8.25±1.85	13.41±2.17
	Number of meals	93±21.96	42.88±13.88
	Meal size (g/meal)	0.04±0.01	0.22±0.1
	Intermeal interval (min)	20.42±4.11	82.2±32.6
	Rate of eating (g/min)	0.07±0.02	0.11±0.03
	Water intake (mL)	2.37±0.38	4.67±0.94*
	Water intake (ml/100g body weight)	6.49±1.35	16.83±3.84*
	Energy expenditure (kcal/h/100g body weight)	1.09±0.1	1.54±0.08**
	Energy expenditure (kcal/h/cm^2 body surface)	0.004±0	0.005±0*
	RER	0.82±0.03	1.01±0.02***

Figure 1

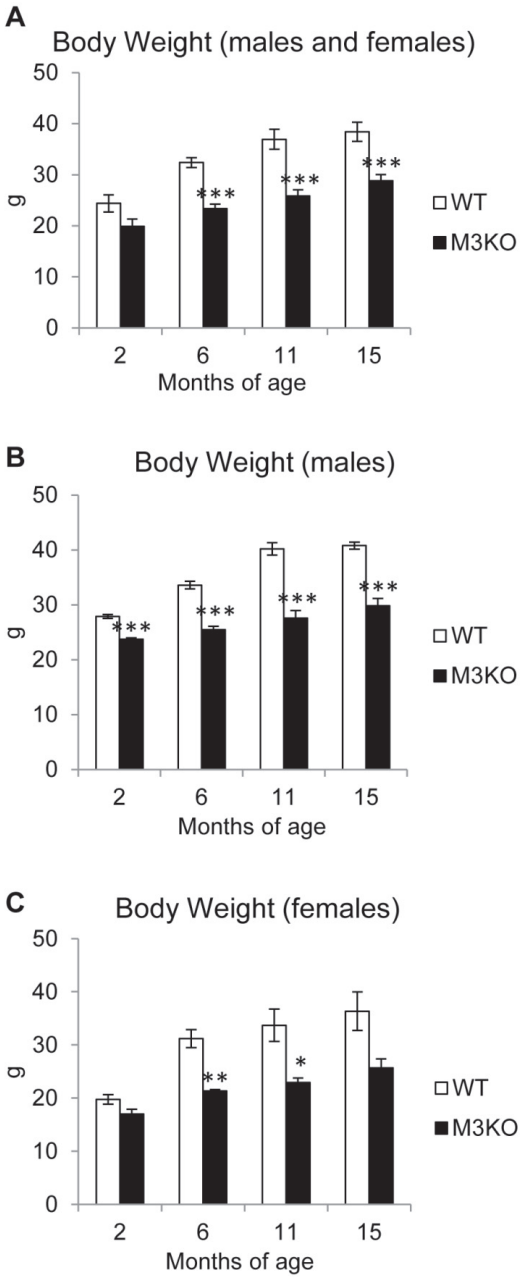

A
Body Weight (males and females)
50
40
30
20
10
0
g
***
***
***
***
2
6
11
15
Months of age
WT
M3KO
B
Body Weight (males)
50
40
30
20
10
0
g
***
***
***
***
2
6
11
15
Months of age
WT
M3KO
C
Body Weight (females)
50
40
30
20
10
0
g
**
*
2
6
11
15
Months of age
WT
M3KO

Figure 2

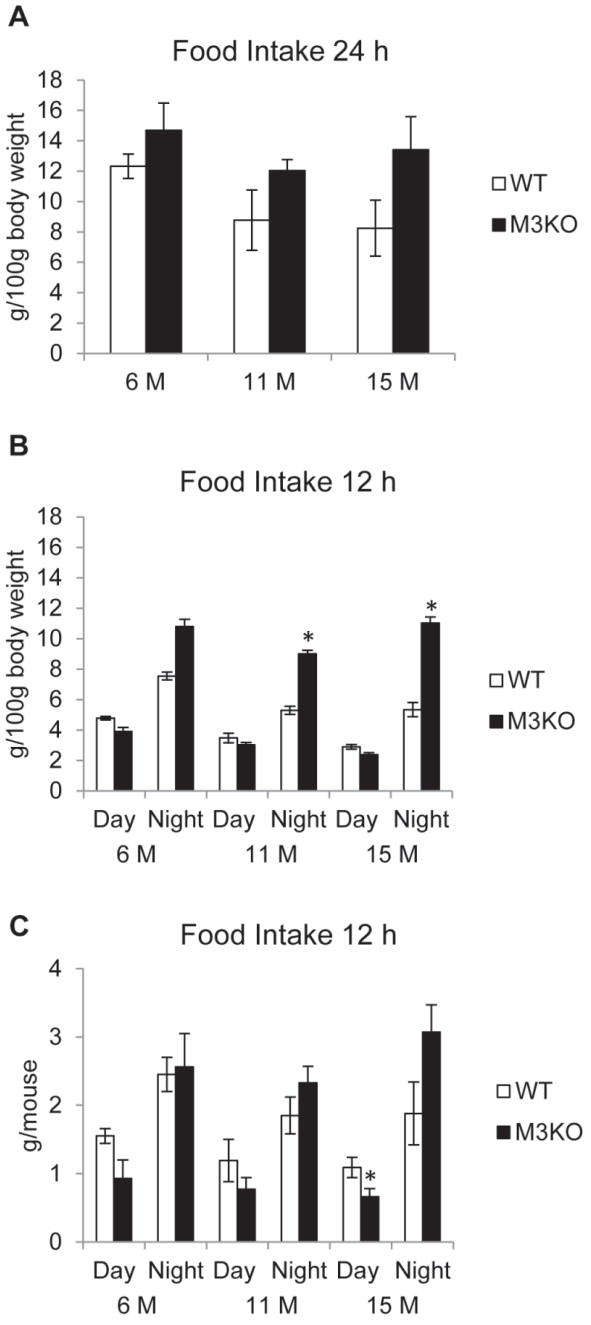

A
Food Intake 24 h
g/100g body weight
18
16
14
12
10
8
6
4
2
0
6 M
11 M
15 M
WT
M3KO
B
Food Intake 12 h
g/100g body weight
*
*
Day Night Day Night Day Night
6 M
11 M
15 M
WT
M3KO
C
Food Intake 12 h
g/mouse
4
3
2
1
0
*
Day Night Day Night Day Night
6 M
11 M
15 M
WT
M3KO

Figure 3

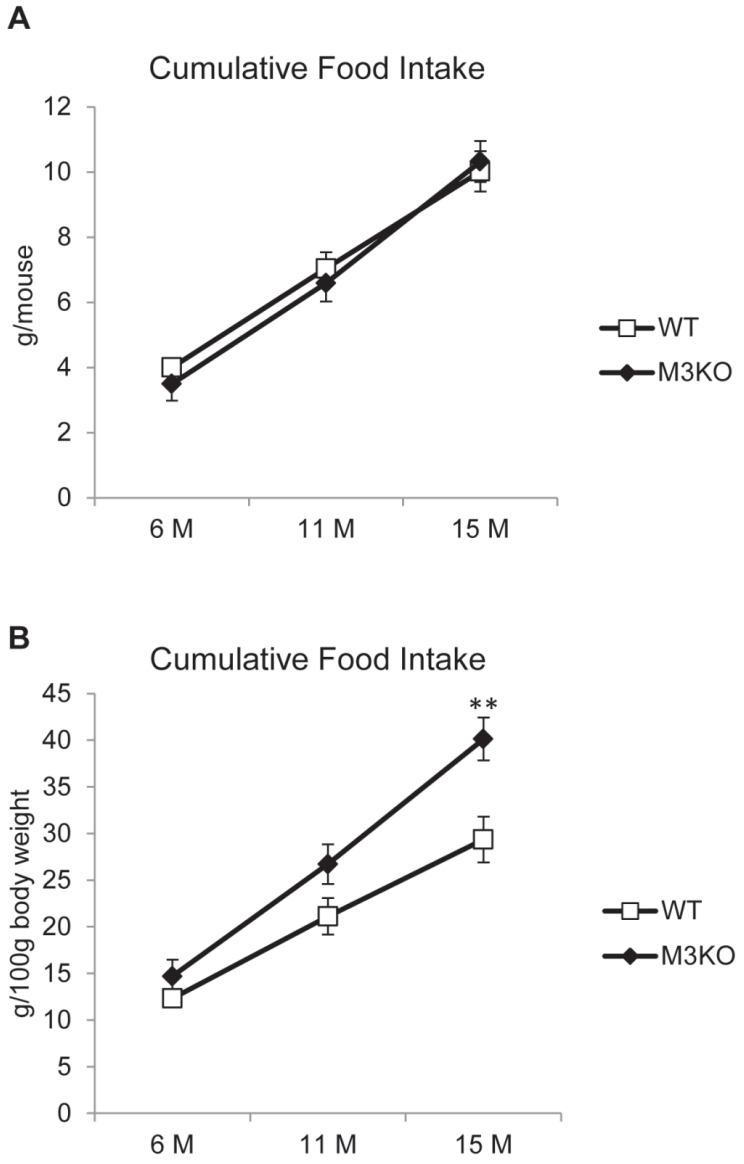
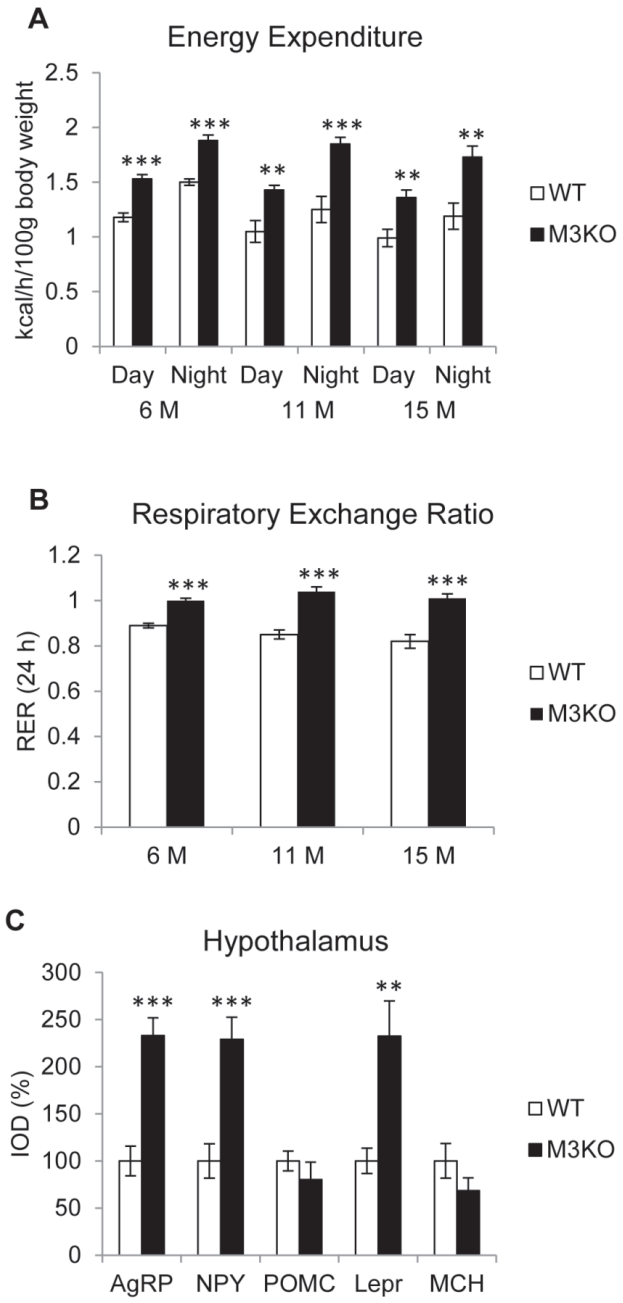

A
Cumulative Food Intake
g/mouse
12
10
8
6
4
2
0
6 M
11 M
15 M
WT
M3KO
B
Cumulative Food Intake
g/100g body weight
45
40
35
30
25
20
15
10
5
0
**
6 M
11 M
15 M
WT
M3KO

Figure 4


A
Energy Expenditure
kcal/h/100g body weight
2.5
2
1.5
1
0.5
0
***
***
**
***
**
**
Day
Night
Day
Night
Day
Night
6 M
11 M
15 M
WT
M3KO
B
Respiratory Exchange Ratio
RER (24 h)
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
6 M
11 M
15 M
WT
M3KO
C
Hypothalamus
IOD (%)
300
250
200
150
100
50
0
***
***
**
AgRP
NPY
POMC
Lepr
MCH
WT
M3KO

Paper III

OBES SURG
DOI 10.1007/s11695-012-0750-9

ANIMAL RESEARCH

Eating Behavior and Glucagon-Like Peptide-1-Producing Cells in Interposed Ileum and Pancreatic Islets in Rats Subjected to Ileal Interposition Associated with Sleeve Gastrectomy

Helene Johannessen · Yosuke Kodama · Chun-Mei Zhao · Mirta M L Sousa · Geir Slupphaug · Bård Kulseng · Duan Chen

Abstract
Background Ileal interposition–sleeve gastrectomy (II–SG) has been developed as a metabolic surgery based on the hindgut hypothesis. The aim of the present study was to test this hypothesis by studying the eating behavior, metabolic changes, and glucagon-like peptide-1 (GLP-1)-producing cells in rat models.
Methods Male Sprague–Dawley rats were subjected to laparotomy, II, SG, or II–SG. Eating behavior and metabolic parameters were monitored by an open-circuit indirect calorimeter designed for a comprehensive laboratory animal monitoring system. GLP-1-producing cells were examined by quantitative immunohistochemistry.
Results After II alone, satiety ratio, i.e., intermeal interval/meal size, was reduced, while calorie intake was increased at 2 and 6 weeks postoperatively. Respiratory exchange ratio, VCO_2/VO_2, was increased to above 1.0 (i.e., carbohydrate metabolism) during both daytime and nighttime at 2 weeks postoperatively. After SG alone, GLP-1-producing cells were increased in the pancreatic islets (in terms of volume density), but not in the ileum (number/mm). After II–SG, the rate of eating was reduced, while meal duration (min) was increased during both daytime and nighttime at 2 weeks postoperatively. GLP-1-producing cells were increased by about 2.5-fold in the interposed ileum and also increased to the same extent in the pancreatic islets as seen after SG alone. The increased GLP-1-producing cells in the pancreatic islets after SG or II–SG were located around the insulin-producing β cells.
Conclusions The present study provides evidence supporting the hindgut hypothesis. II–SG increased GLP-1 production both in the interposed ileum and in the pancreatic islets, leading to metabolic beneficial effects and altered eating behavior.

Keywords Food intake · GLP-1 · Ileal interposition · Ileum · Pancreatic islets · Sleeve gastrectomy · Energy expenditure · Respiratory exchange ratio

H. Johannessen · Y. Kodama · C.-M. Zhao · M. M. L. Sousa · G. Slupphaug · B. Kulseng · D. Chen
Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway

C.-M. Zhao · B. Kulseng · D. Chen (✉)
Department of Surgery, St. Olav's University Hospital, Trondheim, Norway
e-mail: duan.chen@ntnu.no

Introduction

Bariatric surgeries, such as Roux-en-Y gastric bypass, biliopancreatic diversion, or sleeve gastrectomy, exhibit a better therapeutic effect than conventional medical therapy on type 2 diabetes by mechanisms other than weight loss and/or reduced food intake [1–4]. The hindgut hypothesis has been proposed to explain the underlying mechanisms, i.e., early arrival of food in the hindgut suppresses gastrointestinal motility, gastric emptying, and small intestinal transit by neural as well as hormonal pathways, such as GLP-1 in the ileum [5–8]. According to this hypothesis, ileal interposition (II) with or without sleeve gastrectomy (SG) has been suggested as a novel method of metabolic surgery [1]. The aim of the present study was to test this hypothesis by studying the effects of II with or without SG on body weight, body composition, eating behavior, metabolic parameters, and GLP-1-producing cells. Anatomically correct surgical procedure in which only ileum was interposed and accurate determination of eating behavior using Comprehensive Laboratory Animal Monitoring System (CLAMS) were applied. GLP-1-producing cells in the ileum as well as the pancreatic islets were examined by quantitative immunohistochemistry.

Published online: 05 September 2012

Materials and Methods

Animals

Rats (Sprague–Dawley, male, adults) were purchased from Harlan™, Horst, The Netherlands. Males were used because female rats have food intake coinciding with ovarian hormones and males grow faster than females, making it easier to detect changes in body weight. The rats were housed in Makrolon Type-4 individually ventilated cages (four rats per cage) with regulated room temperature and humidity (22± 2 °C, 50±10 %) and 12-h light/dark cycle. They had free access to tap water and standard rat pellet food (RM1 811004, Scanbur BK AS, Sweden). The study was approved by the Norwegian National Animal Research Authority (Forsøksdyrutvalget, FDU).

Experimental Design

The animals were divided into two groups: II (14 animals) and laparotomy (LAP) (six animals). In consideration of the "3Rs" for the human use of animals (i.e., reducing the number of animals while achieving the scientific purposes of the experiment) [9], rats in both groups were re-used and subjected to SG 7 weeks after the first operation. In addition, four non-operated normal rats were used as controls for immunohistochemical analysis of GLP-1.

The body weight was recorded regularly (three times/week) throughout the study period. Each rat was placed in the CLAMS cages five times and kept for 48 h at each time point for measurement of eating behavior and metabolic parameters (records from the second 24 h were used for data analysis). The five time points were 2 weeks before II or LAP, 2 and 6 weeks after II/LAP, and 2 and 6 weeks after SG. At the same time points, the body composition was determined and fecal energy content was measured (for details, see the following discussion). At euthanization, tissue samples from ileum and pancreas were collected for analysis by immunohistochemistry.

Surgeries

Before all surgeries, the animals were fasted overnight but with free access to drinking water. Surgeries were performed under general anesthesia with isofluran (4 % for induction, 2 % for maintenance). Atropin was given to SG rats at a dose of 0.04 mg/kg subcutaneously 20 min before anesthesia. Buprenorphine was injected subcutaneously (0.05 mg/kg) immediately after surgery in all animals and at 1 day postoperatively if needed. Physiological saline (0.9 % NaCl) was given subcutaneously at 10–15 mL after surgeries to keep the animals hydrated. Drinking water (but not food) ad libitum was started 4 h after the surgery and powdered food was provided on the next morning, until it was switched to ordinary pellet food on the 2nd postoperative day.

Ileal Interposition

II was performed through a midline abdominal incision. The ligament of Treitz was located, and the insertion location for the transposed ileum segment was marked approximately 3 cm distal to the ligament with saline-soaked gauze. The ileum segment (2.5–3.5 cm in length) with blood vessels at 0.5–1 cm proximal to the ileocecal valve was isolated and transected. Three anastomoses in an end-to-end fashion were made between cecum–distal jejunum, Treitz-side jejunum–proximal ileum, and distal ileum–jejunum (Fig. 1b, c). All anastomoses were performed in one layer with 6-0 absorbable sutures (Vicryl, Ethicon Inc., Sommerville, NJ, USA). The abdomen was closed in two layers using 4-0 absorbable sutures (Vicryl, Ethicon Inc., Sommerville, NJ, USA).

Sleeve Gastrectomy

SG was performed through a midline abdominal incision. The stomach was clamped along the greater curvature from the antrum to the rumen (forestomach) across the corpus (fundus), approximately 70 % of the stomach was removed, and then the stomach was closed in one layer with 6-0 absorbable sutures (Fig. 1b, c) [10]. The abdominal wall was closed in two layers with 4-0 absorbable sutures.

Laparotomy

LAP was performed by making a midline abdominal incision, locating the ligament of Treitz and ileum, and then closing the abdomen in two layers using 4-0 absorbable sutures.

Eating Behavior and Metabolic Parameters

Rats were placed in the cages of CLAMS (Columbus Instruments International, Columbus, OH, USA) with free access to standard rat powdered food (RM1 811004, total metabolizable energy of 2.57 kcal/g, Scanbur BK AS, Sweden) and tap water. This system is composed of a four-chamber open-circuit indirect calorimeter designed for continuous monitoring of individual rats. In order for rats to acclimate to this system, they were placed in these metabolic cages for 24 h before the first CLAMS monitoring. Food intake, feeding behavior, and metabolic parameters were recorded automatically. High-resolution feeding data were generated by monitoring all feeder balances every 0.5 s. The end of an eating

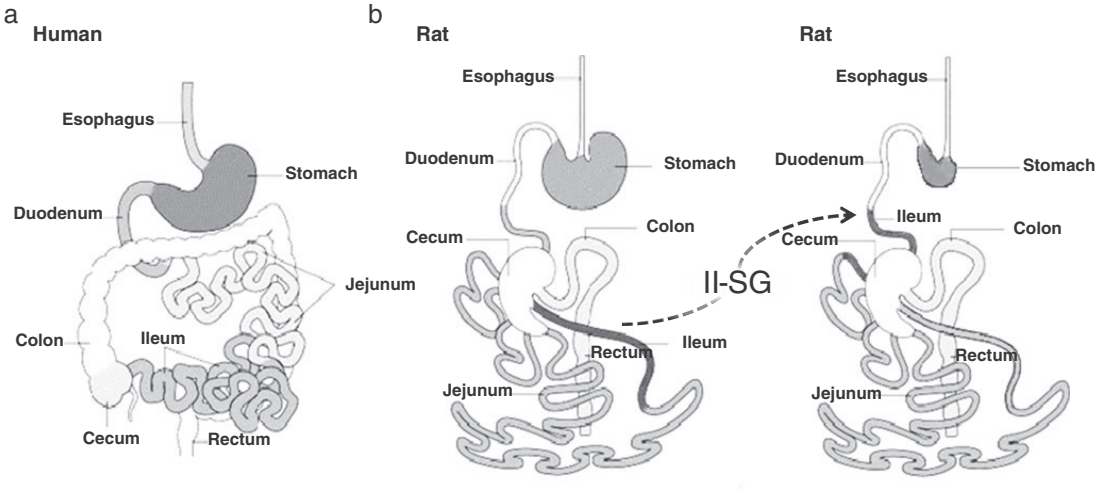


Fig. 1 Anatomy of the gastrointestinal tract of human (**a**) and rat (**b**). Ileal interposition with sleeve gastrectomy (II–SG) is illustrated (**b**) (modified from [26])

event (meal) was determined when the balances were stable for more than 10 s and a minimum of 0.05 g of food was eaten. An air sample was withdrawn every 5 min. The energy expenditure (EE) (kcal/h) was calculated according to this equation: (3.815+1.232 RER) × VO_2, where RER (respiratory exchange ratio) was the volume of CO_2 produced per volume of O_2 consumed. VO_2 was the volume of O_2 consumed per hour per kilogram of mass of the animal.

Parameters that were obtained during daytime (7 am–7 pm) and nighttime (7 pm–7 am) for each individual rat included the number of meals, meal size, meal duration, accumulated food intake, intermeal interval, rate of eating, satiety ratio, drinking activity, energy expenditure, and ambulatory activity. The intermeal interval was defined as the interval in minutes between two meals. Rate of eating was calculated by dividing the average meal size by the average duration of a meal, and satiety ratio, an index of the non-eating time produced by each gram of food consumed, was calculated by dividing the average intermeal interval by the average meal size.

Determination of Body Composition

Body composition was determined with dual-energy X-ray absorptiometry (Hologic QDR 4500A, Hologic Inc., Bedford, MA, USA) under general anesthesia with isofluran (4 % for induction, 2 % for maintenance). Total fat mass, fat-free mass, bone mineral content, and bone area were measured.

Determination of Fecal Energy Content

Feces were collected in CLAMS cages and dried for 72 h at 60 °C. The energy content was determined by using an adiabatic bomb calorimeter (IKA-Calorimeter C 5000, IKA-Werke GmbH & Co. KG, Staufen, Germany).

Western Blot Analysis and Immunohistochemistry

Western blot analysis was applied to test antibody specificity. Ileum and pancreatic tissues were suspended in RIPA buffer and ground on ice until homogenized. Determination of protein concentration was performed using BioRad assay and absorbance was measured at 595 nm. A total of 100 μg ileum and pancreas tissue extracts, 1 μg GLP-1 peptide (code: 028-11, Phoenix Pharmaceuticals, Inc., CA, USA), and M.M (4.0 μL Seeblue + 0.5 μL Magic Marker, Invitrogen) were subjected to 1DE separation in 4–12 % Novex Bis-Tris gels using MES running buffer (Invitrogen) for 25 min at 200 V prior to the Western blot analysis. The tissue extracts, the GLP-1 peptide, and M.M were transferred to a Nitrocellulose membrane (UltraCruz) (Blot 30 V, 30 min, 220 mA, room temperature) as this membrane was superior to PVDF membranes. The membrane was dried and then blocked for 1 h in 5 % fat-free dry milk in PBS-T and incubated overnight in rabbit monoclonal GLP-1 primary antibody (code: 2914-1, Epitomics, Inc., CA, USA) diluted in blocking buffer. After washing 3×10 min in PBS-T, membranes were incubated with secondary antibody swine anti-rabbit (1:5,000) in blocking buffer for 1 h. After 4×10 min washes in PBS-T, blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and scanned in a Kodak IS4000R imager (Fisher Scientific).

Tissue samples of the ileum and the pancreas from control, SG, and II–SG rats were fixed in 4 % formaldehyde,

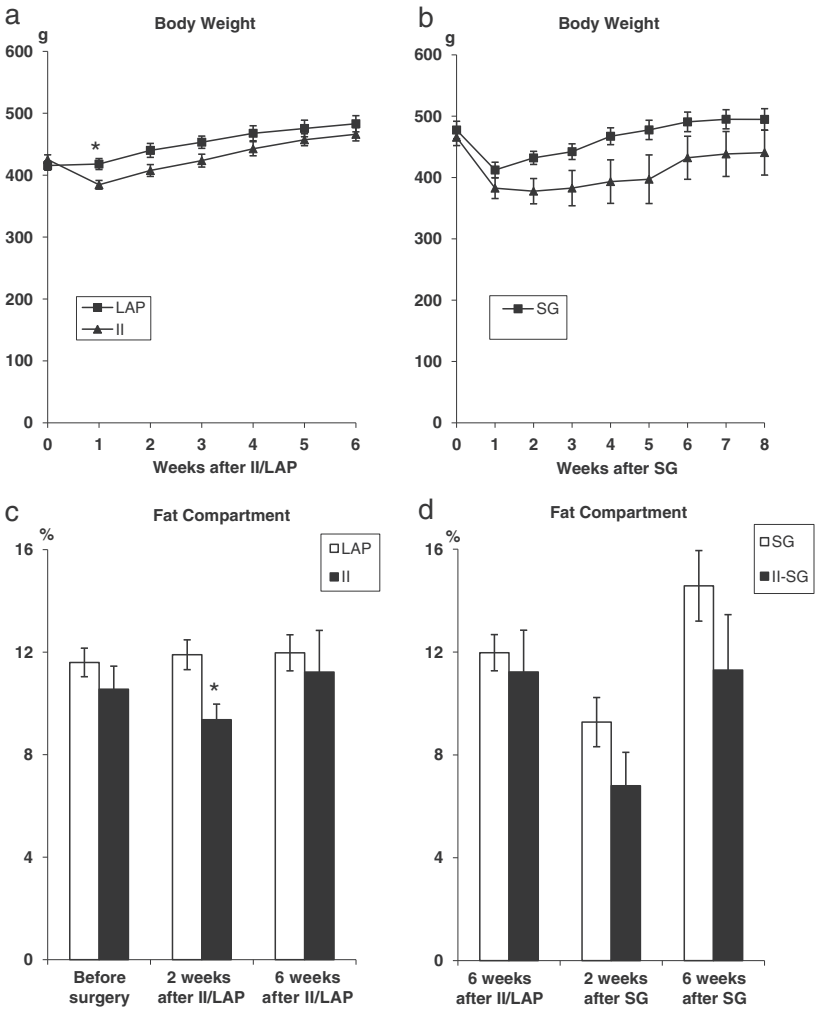


Fig. 2 Body weight development (g) (**a**, **b**) and total fat compartment (%) (**c**, **d**) after ileal interposition (*II*), laparotomy (*LAP*), sleeve gastrectomy (*SG*), or ileal interposition with sleeve gastrectomy (*II–SG*). *$p<0.05$ between LAP vs. II (**a**, **c**)

dehydrated, and embedded in paraffin. Sections were cut at a thickness of 4 μm, thawed on superfrost glass slides, deparaffinized, and rehydrated. For immunostaining with GLP-1 antibody, the tissue sections were blocked with 10 % normal goat serum and incubated with primary antiserum (dilution, 1:1,500; code: H-028-13, Phoenix Pharmaceuticals, Inc., CA, USA, and dilution, 1:250, code: 2914-1, Epitomics, Inc., CA, USA) overnight at 4 °C. After being thoroughly rinsed, the sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG (H + L) (dilution, 1:200, Alexa Fluor 488, Molecular Probes, The Netherlands) for 1 h at room temperature, washed several times in TRIS-buffered saline, mounted in glycerol/TBS buffer (1:1) under glass coverslips, and examined under a microscope (Olympus BX50) equipped with epifluorescence illumination. For double-immunostaining using primary antibodies of GLP-1 and insulin, the sections were blocked with normal sera and then incubated with a mixture of antibodies of GLP-1 and insulin (dilution, 1:800; code: A0564, DakoCytomation, Denmark) for 18 h at 4 °C. After several washes in TRIS-buffered saline, the mixture of Alexa Fluor 488 goat anti-rabbit IgG (H + L) (1:200) and Rhodamine-conjugated donkey anti-guinea pig IgG (dilution, 1:80; Jackson ImmunoResearch Europe) was applied to the sections at room temperature for 1 h. The sections were rinsed and mounted in glycerol/TBS buffer (1:1) under

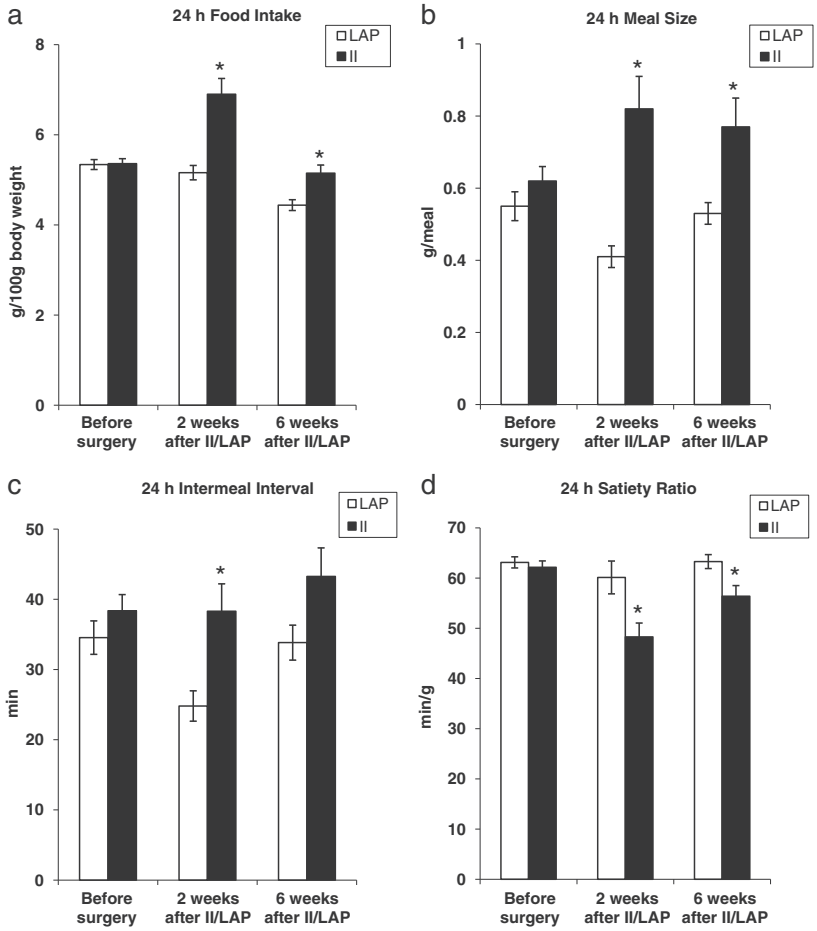


Fig. 3 Eating behavior after ileal interposition (*II*) or laparotomy (*LAP*): 24-h food intake (g/100 g body weight) (**a**), meal size (g/meal) (**b**), intermeal interval (min) (**c**), and satiety ratio min/g (**d**). *$p<0.05$ between LAP vs. II

glass coverslips and examined under a microscope (Olympus BX50) equipped with epifluorescence illumination. GLP-1-immunoreactive cells were counted and expressed as number per millimeter in the mucosa of ileum and as volume density (% of islet) in the pancreas using point-counting technique.

Statistical Analysis

The results are expressed as mean ± SEM. Statistical comparisons were performed using independent *t*-test between the surgical groups and ANOVA with Tukey's test or ANCOVA with Sidak test (the latter for EE analysis) between different time points. A *p*-value of <0.05 (two-tailed) was considered as statistically significant. Data analysis was performed in SPSS version 15.0.

Results

Mortality and Morbidity

Five of 14 II rats, five of 14 SG rats, and one of six LAP rats were killed before the end of study due to surgical trauma and complications, including opening of sutures, pica behavior, and partial obstruction of pylorus and infection. Otherwise, the surviving rats had no morbidity. The impact of learning curve on the mortality rate should also be considered (HJ was under micro-surgical training). Based on inspection at euthanization, all surgeries were successfully performed with the exception of two SG that led to partial obstruction of pylorus, probably because of damage of the vagal nerves. After II, there was hypertrophy of the transposed ileal segment as reported in previous studies [11–14].

Table 1 Eating behavior at 2 and 6 weeks after LAP or II

		2 weeks after LAP/II		6 weeks after LAP/II	
	Parameters	LAP (n=5)	II (n=9)	LAP (n=5)	II (n=9)
Daytime	Food intake (g)	6.54±0.93	9.45±0.96	5.54±0.39	6.07±0.57
	Food intake (g/100 g body weight)	1.48±0.2	2.34±0.26 *	1.14±0.07	1.31±0.13
	Number of meals	16.2±2.75	15.67±1.77	13.2±1.02	11.67±1.46
	Meal size (g/meal)	0.42±0.04	0.66±0.09*	0.42±0.02	0.55±0.05*
	Meal size (kcal/meal)	1.09±0.09	1.7±0.23*	1.08±0.04	1.41±0.13*
	Meal duration (min)	13.31±2.1	24.19±4.19	11.68±0.82	13.21±1.72
	Meal duration (min/meal)	0.85±0.05	1.61±0.24*	0.89±0.04	1.18±0.13
	Intermeal interval (min)	47.55±10.71	46.01±5.23	50.82±3.3	61.01±5.87
	Satiety ratio (min/g)	109.29±15.66	75.74±9.24	120.72±7.45	113.7±10.24
	Rate of eating (g/min)	0.5±0.03	0.42±0.03	0.47±0.01	0.47±0.02
	RER	0.99±0.02	1.11±0.02**	1.04±0.01	1.04±0.02
	Ambulatory activity	2,307.2±419.87	1,925.89±288.24	2,280.4±330.23	1,814.67±260.68
Nighttime	Food Intake (g)	16.36±1.38	18.76±1.34	15.97±0.76	17.94±0.68
	Food Intake (g/100 g body weight)	3.68±0.26	4.56±0.24*	3.3±0.17	3.84±0.13*
	Number of meals	40.4±5.22	22.11±3**	27.8±2.87	21.44±2.14
	Meal size (g/meal)	0.42±0.04	0.94±0.11**	0.59±0.05	0.94±0.14
	Meal size (kcal/meal)	1.09±0.12	2.41±0.27**	1.52±0.12	2.41±0.36
	Meal duration (min)	40.7±3.21	51.64±4.29	36.08±0.97	43.52±3.06
	Meal duration (min/meal)	1.05±0.12	2.55±0.27**	1.35±0.12	2.2±0.28*
	Intermeal interval (min)	17.47±2.15	32.11±3.18**	24.73±2.49	33.35±4.27
	Satiety ratio (min/g)	41.66±3.59	35.14±2.19	41.64±1.89	36.31±1.57
	Rate of eating (g/min)	0.4±0.02	0.38±0.03	0.44±0.01	0.42±0.02
	RER	1.02±0.04	1.12±0.02*	1.09±0.02	1.11±0.02
	Ambulatory activity	6,298.6±970.17	5,450.56±835.2	5,877.2±593.57	5,900.22±750.46

*p<0.05; **p<0.01 (LAP vs. II)

Body Weight and Body Composition

Body weight was lower in II rats than LAP rats (at week 1, p<0.05) until 6 weeks (p>0.05) postoperatively. Compared with LAP, II rats had a lower fat compartment at 2 weeks after surgery (p=0.018) but no longer after 6 weeks (p=0.687) (Fig. 2a, c). The body weight and fat compartment tended to be lower in II–SG rats than in SG rats at every time point examined, albeit insignificantly (Fig. 2b, d).

Food Intake and Eating Behavior

Effects by II

Food intake (in terms of g/100 g body weight) was higher in II rats than in LAP rats at 2 weeks after surgery both during day- and nighttime (p=0.046 and p=0.036, respectively). Compared with LAP, II rats had fewer meals (p=0.006) and higher intermeal interval (p=0.008) during nighttime and also had larger meal size (g/meal) and longer meal duration (min/meal) both during day- and nighttime (p<0.05). At 6 weeks after surgery, II rats still had higher food intake (g/100 g body weight), particularly during nighttime (p=0.027), larger meal size (g/meal) (p=0.034) during daytime, and longer meal duration (min/meal) (p=0.049) during nighttime in comparison with LAP rats. At both 2 and 6 weeks after surgery, II rats had lower 24-h satiety ratio compared with LAP rats (p=0.021 and p=0.043, respectively) (Fig. 3; Table 1).

Effects by SG or II–SG

At 2 weeks after SG surgery in LAP or II rats, II–SG rats had a lower rate of eating (g/min) at both day- and nighttime (p=0.017 and p=0.006, respectively), and longer meal duration (min) (p=0.018) during nighttime compared with SG rats. At 6 weeks after SG surgery, the changes became non-significant (Fig. 4b, c). However, II–SG rats had lower intermeal interval (min) during daytime (p=0.042) and higher food intake (g/100 g body weight) during nighttime (p=0.033) than SG rats (Fig. 4a).

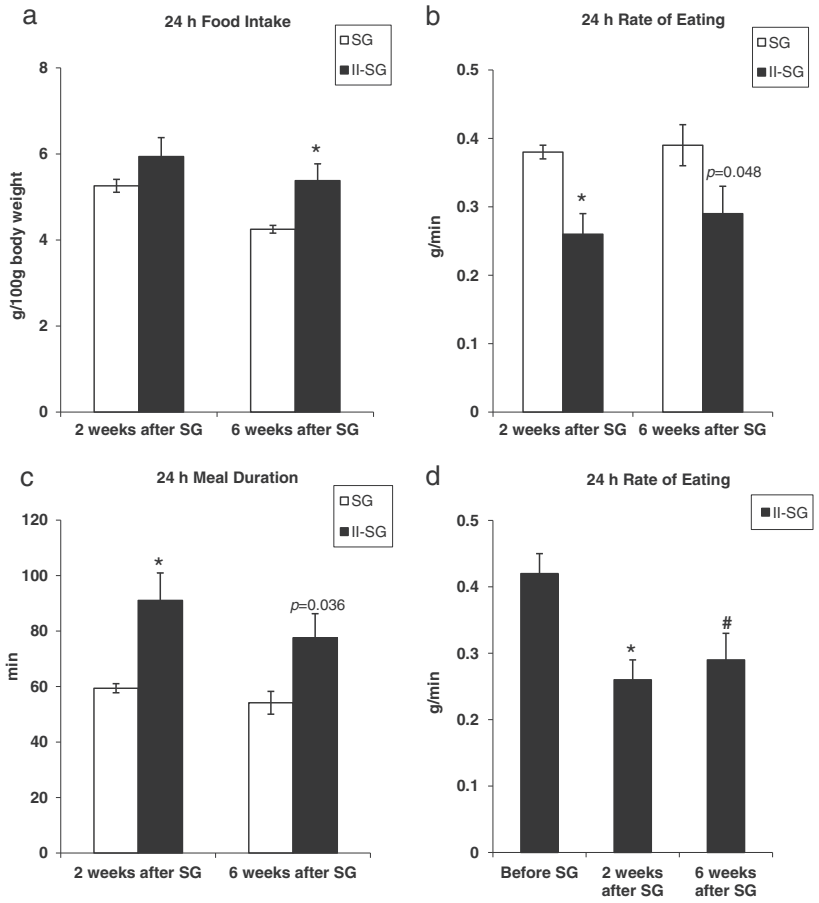


Fig. 4 Eating behavior after sleeve gastrectomy (*SG*) with or without ileal interposition (*II*): 24-h food intake (g/100 g body weight) (**a**), rate of eating (g/min) (**b**, **d**), and meal duration (min) (**c**). *$p<0.05$ (two-tailed) or p-values (one-tailed) (**a**–**c**). *, #$p<0.05$ (before vs. 2 or 6 weeks after SG) (**d**)

ANOVA with Tukey's test showed that at 2 and 6 weeks after SG surgery, II–SG rats had a lower rate of eating both during day- and nighttime compared with the level before operation (Fig. 4d). SG rats had increased food intake (g/100 g body weight) both during day- and nighttime at 2 weeks postoperatively when compared with the level before surgery and reduced rate of eating (g/min) at both 2 and 6 weeks after surgery during daytime (Table 2).

Energy Expenditure and Fecal Energy Content

ANCOVA with Sidak test (using fat-free mass as covariate), which is recommended for EE analysis [15], showed no change in EE after II or LAP. Compared to LAP rats, II rats had higher RER at 2 weeks after surgery both during day- and nighttime ($p=0.009$ and $p=0.036$, respectively), which was caused by increased CO_2 production. At 6 weeks after surgery, there was no longer difference in RER between II and LAP, but II rats had higher RER compared to the level before surgery (Fig. 5). After II–SG, EE was reduced at 2 weeks postoperatively both during day- ($p=0.004$) and nighttime ($p=0.030$) (Table 2).

Neither LAP, II, SG, nor II–SG led to changes in fecal energy content, indicating that none of the procedures caused malabsorption (data not shown).

GLP-1-Producing Cells

Western blot analysis showed a strong signal in the MW range corresponding to synthetic GLP-1 peptide (about 3 kDa) and very faint signals that precluded direct quantitative analysis of ileum and pancreatic tissue extracts

Table 2 Eating behavior at 2 and 6 weeks after II–SG and SG

		II–SG		SG	
	Parameters	2 weeks after SG	6 weeks after SG	2 weeks after SG	6 weeks after SG
Daytime	Food intake (g)	7.41±1.04	6.44±0.78	6.77±0.29	5.36±0.89
	Food intake (g/100 g body weight)	2.02±0.35	1.71±0.29	1.56±0.07§*	1.09±0.17
	Satiety ratio (min/g)	96.3±12.92	108.74±13.87	94.47±4.62	128.49±24.2
	Rate of eating (g/min)	0.3±0.04*	0.33±0.05#	0.43±0.01*	0.4±0.01#
	RER	1.06±0.04	1.03±0.02	0.99±0.01	1.07±0.04
	Energy expenditure (kcal/h)	1.77±0.06*	1.96±0.06	1.98±0.06	2.07±0.06
	Ambulatory activity	1,471.33±231.2	1,860.33±413.77	1,598.5±765.15	1,779.25±392.34
Nighttime	Food intake (g)	14.86±1.19	14.57±1.35	16±0.25	15.5±0.6
	Food intake (g/100 g body weight)	3.92±0.2	3.67±0.14	3.7±0.12§	3.16±0.11
	Satiety ratio (min/g)	43.7±3.55	46.24±5.46	40.68±0.82	42.08±1.4
	Rate of eating (g/min)	0.24±0.03*	0.27±0.04#	0.37±0.02*	0.38±0.03
	RER	1.12±0.03	1.09±0.03	1.07±0.009§	1.17±0.02#
	Energy expenditure (kcal/h)	1.91±0.09*	2.15±0.09	2.26±0.09	2.38±0.09
	Ambulatory activity	4,314.5±824.13	4,939.5±818.59	3,868.25±1,406.08	5,673.5±1,402.69

*$p<0.05$ between before and 2 weeks after SG; §$p<0.05$ between 2 and 6 weeks after SG; #$p<0.05$ between before and 6 weeks after SG

(Fig. 6f). This likely reflects that only a minor fraction of the cells in the tissues expressed GLP-1. GLP-1 was detected in the mucosa of ileum and the α-cells of pancreatic islets in control rats as well as SG and II–SG rats by immunohistochemistry using either of the two antibodies without any difference.

GLP-1-immunoreactive cells were increased (in terms of number per millimeter) in the interposed ileum in II–SG rats compared with SG rats ($p=0.011$) or control rats ($p<0.001$). There was no difference between SG rats and control rats (Fig. 6b, d, h). The volume density of GLP-1-immunoreactive cells in the pancreatic islets was increased in both II-SG rats and SG rats compared with control rats ($p=0.010$ and $p=0.023$, respectively). There was no difference between II–SG and SG rats (Fig. 6a, c, e, g).

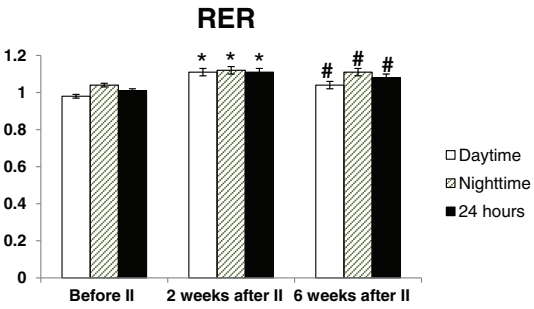


Fig. 5 Respiratory exchange ratio (*RER*) before and at 2 and 6 weeks after ileal interposition (*II*) during daytime, nighttime, and 24 h. *, #$p<0.05$ between before and after II

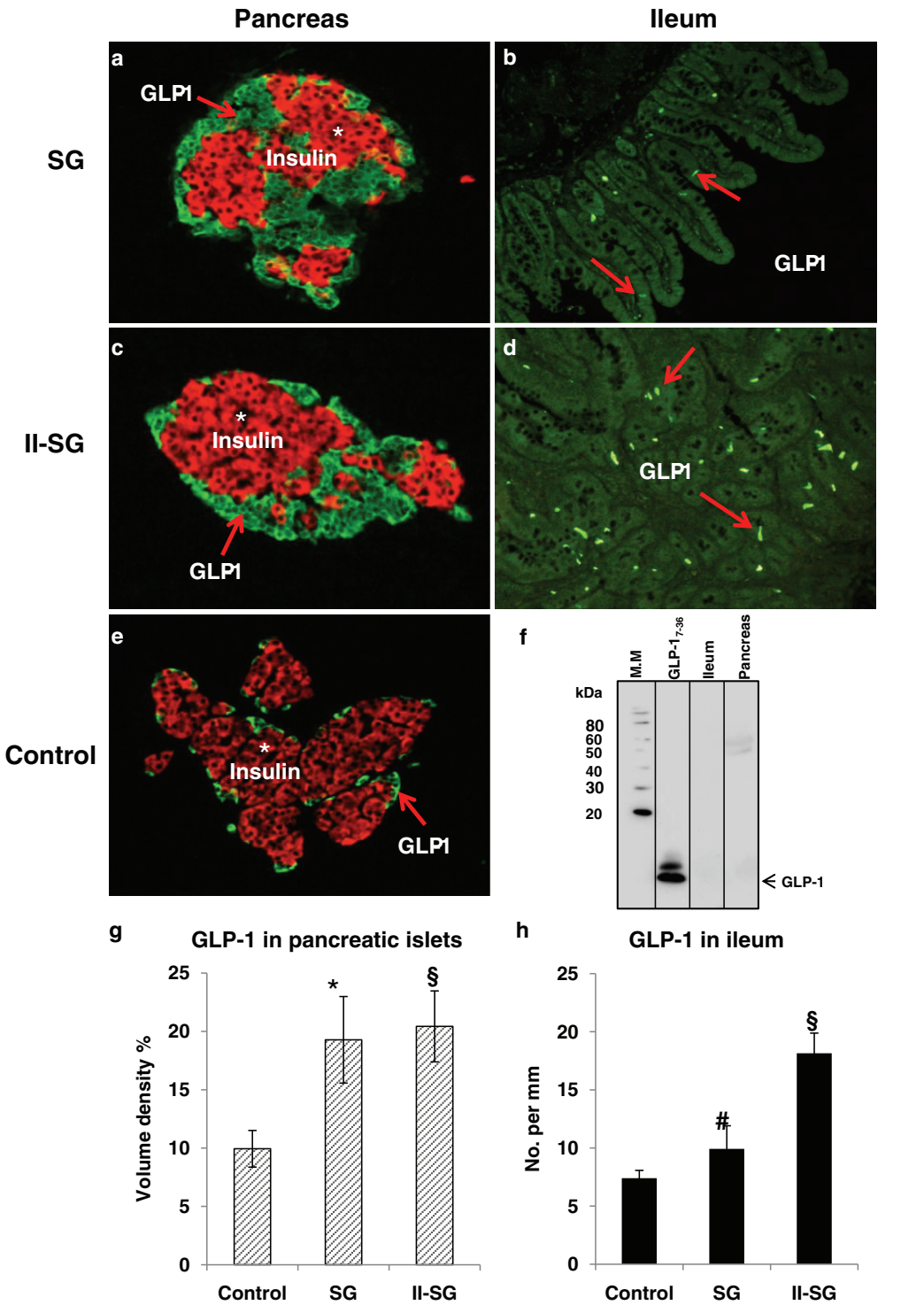
Discussion

Surgical Models

Previously, eating behavior data obtained in rats subjected to II are confusing; food intake and body weight were reduced in some reports [11–14, 16–20], but unchanged in others [7, 8, 21–24]. There are at least two possible explanations for these controversial reports: imprecise monitoring of eating behavior and different lengths and positions of transposed ileal segment. In most studies, an ileal segment of approximately 10 cm was transected from different positions [7, 8, 11–13, 16, 19, 21, 23–25]. There are substantial differences in the intestinal anatomy between humans and rats; whereas ileum is approximately 400 cm (58 % of small intestine) in human, it is only 2.5–3.5 cm (2 %) in rats (Fig. 1a, b) [26]. In many II studies using rat models, not only the entire ileum (2.5–3.5 cm) but also part of the distal jejunum (7–8 cm) were transposed [7, 8, 11–14, 16–19, 21, 27], and II was often performed without SG [7, 8, 11–14, 16, 17, 21, 22, 27].

In patients that undergo II, about 40 % (150–170 cm) of ileum is transposed to 30–50 cm distal to the ligament of

Fig. 6 Representative immunofluorescence micrographs showing GLP-1 (indicated by *arrows*) and insulin (*asterisks*) in the pancreatic islets (**a**, **c**, **e**) and GLP-1 (*arrows*) in the ileum (**b**, **d**) in rats subjected to sleeve gastrectomy (*SG*), ileal interposition with sleeve gastrectomy (*II–SG*), or sham operation (*Control*). Western blot analysis showing specificity of GLP-1 antibody used to GLP-1 (7-36) and faint signals in ileum and pancreatic tissue after SG (**f**). Quantitative values of GLP-1 (%*v*/*v*) in the pancreatic islets (**g**) and the ileum (no./gland) (**h**). *$p<0.05$ between control vs. SG; §$p<0.05$ between control vs. II–SG; #$p<0.05$ between SG vs. II–SG


Pancreas
Ileum
SG
II-SG
Control
a
GLP1
Insulin
b
GLP1
c
Insulin
GLP1
d
GLP1
e
Insulin
GLP1
f
M.M
GLP-1 7-36
Ileum
Pancreas
kDa
80
60
50
40
30
20
GLP-1
g
GLP-1 in pancreatic islets
Volume density %
25
20
15
10
5
0
*
§
Control
SG
II-SG
h
GLP-1 in ileum
No. per mm
25
20
15
10
5
0
#
§
Control
SG
II-SG

Treitz [28–31], and II is usually performed in combination with SG in an attempt to reduce body weight [5, 28–30, 32–34]. In the present study, only the ileum was transposed to 3 cm distal to the Treitz ligament and II with and without SG was performed in order to maximally mimic the clinical procedure and to detect postoperative changes caused by each procedure [26, 31].

Evidence Supporting the Hindgut Hypothesis

Previously, we and others have shown that gastric bypass-induced weight loss was associated neither with reduced food intake nor malabsorption but with increased EE, whereas weight loss induced by duodenal switch with sleeve gastrectomy was associated with reduced food intake, malabsorption, and increased EE in rodent models [35–38]. Using the same method of monitoring the eating behavior and metabolic parameters, the results of the present study showed that II alone did not reduce daily food intake. This is at odds with other reports [11–14, 16], probably because of different lengths of transposed intestine (3 cm in this study vs. 10 cm in others) and different methods of monitoring food intake (CLAMS vs. leftover chow weighing). CLAMS analysis further showed normal eating cycle before surgery, namely, more frequent meals during nighttime than daytime and altered eating cycle and behavior after II, as manifested by similar frequency through 24 h but eating slowly. It should also be noticed that EE was unchanged and that RER was above 1.0 after II, indicating an increased use of carbohydrates as predominant fuel for cellular respiration. Analysis of fecal energy content by bomb calorimetry did not indicate malabsorption after II. Taken together, it is not surprising that II alone did not cause a significant weight loss, and the initial drop in body weight was likely due to surgical stress.

We have also reported that SG alone resulted in a marginal weight loss (10 %) for about 6 weeks, which was reproduced in the present study [35]. Body weight appeared to be lower at the time points examined in rats subjected to II–SG than those subjected to SG alone, although the differences at each time point did not reach statistical significance. It should be noticed that the meal duration was increased, the rate of eating per 24 h was reduced, but food intake (g/100 g body weight/24 h) was increased eventually after II–SG.

Interestingly, the results of the present study showed the increased GLP-1-producing cells in the interposed ileum after II, which is in line with a report showing that ileal transposition procedure increased circulating GLP-1 levels in Goto–Kakizaki rats [17]. Since about 90 % of GLP-1 secreted from L-cells is supposed to be degraded before it reaches the circulation [39], the physiological significance of increased GLP-1 in the ileum needs to be further investigated. Even more interestingly, the present study showed increased GLP-1 in α cells in the pancreatic islets after SG alone or II–SG. It is likely that GLP-1 acts on β cells via paracrine mechanism. In a separate study, we will examine GLP-1 in rats subjected to gastric bypass, which also causes the remission of diabetes.

Acknowledgments The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under Grant Agreement No. 266408, the Faculty of Medicine, Norwegian University of Science and Technology, and the Central Norway Regional Health Authority. The authors thank Erik Langørgen and Morten Grønli at the Department of Energy and Process Engineering for assistance with the bomb calorimeter, Shalini Rao at Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, for assistance with Western blot, and Dr. Gjermund Johnsen and Dr. Ronald Mårvik at the Department of Surgery, St. Olav's University Hospital, for valuable discussions.

Conflict of Interest Statement Helene Johannessen, Yosuke Kodama, Chun-Mei Zhao, Mirta ML Sousa, Geir Slupphaug, Bård Kulseng and Duan Chen have no conflict of interest.

References

1. Rubino F, R'bibo SL, del Genio F, et al. Metabolic surgery: the role of the gastrointestinal tract in diabetes mellitus. Nat Rev Endocrinol. 2010;6:102–9.
2. Buchwald H, Avidor Y, Braunwald E, et al. Bariatric surgery: a systematic review and meta-analysis. JAMA. 2004;292:1724–37.
3. Mingrone G, Panunzi S, De Gaetano A, et al. Bariatric surgery versus conventional medical therapy for type 2 diabetes. N Engl J Med. 2012;366:1577–85.
4. Schauer PR, Kashyap SR, Wolski K, et al. Bariatric surgery versus intensive medical therapy in obese patients with diabetes. N Engl J Med. 2012;366:1567–76.
5. Mason EE. Ilial transposition and enteroglucagon/GLP-1 in obesity (and diabetic?) Surgery. Obes Surg. 1999;9:223–8.
6. Patriti A, Facchiano E, Sanna A, et al. The enteroinsular axis and the recovery from type 2 diabetes after bariatric surgery. Obes Surg. 2004;14:840–8.
7. Patriti A, Facchiano E, Annetti C, et al. Early improvement of glucose tolerance after ileal transposition in a non-obese type 2 diabetes rat model. Obes Surg. 2005;15:1258–64.
8. Cummings BP, Strader AD, Stanhope KL, et al. Ileal interposition surgery improves glucose and lipid metabolism and delays diabetes onset in the UCD-T2DM rat. Gastroenterology. 2010;138:2437–46.
9. Russell W, Burch R. The principles of humane experimental technique. London: Methuen; 1959.
10. Lopez PP, Nicholson SE, Burkhardt GE, et al. Development of a sleeve gastrectomy weight loss model in obese Zucker rats. J Surg Res. 2009;157:243–50.
11. Kotler DP, Koopmans HS. Preservation of intestinal structure and function despite weight loss produced by ileal transposition in rats. Physiol Behav. 1984;32:423–7.
12. Chen DC, Stern JS, Atkinson RL. Effects of ileal transposition on food intake, dietary preference, and weight gain in Zucker obese rats. Am J Physiol-Reg I. 1990;27:R269–73.
13. Atkinson RL, Whipple JH, Atkinson SH, et al. Role of the small bowel in regulating food intake in rats. Am J Physiol-Reg I. 1982;11:R429–33.

14. Koopmans HS, Sclafani A, Fichtner C, et al. The effects of ileal transposition on food intake and body weight loss in VMH-obese rats. Am J Clin Nutr. 1982;35:284–93.
15. Arch J, Hislop D, Wang S, et al. Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. Int J Obesity. 2006;30:1322–31.
16. Strader AD, Vahl TP, Jandacek RJ, et al. Weight loss through ileal transposition is accompanied by increased ileal hormone secretion and synthesis in rats. Am J Physiol-Endoc M. 2004;288: E447–53.
17. Wang TT, Hu SY, Gao HD, et al. Ileal transposition controls diabetes as well as modified duodenal jejunal bypass with better lipid lowering in a nonobese rat model of type 2 diabetes by increasing GLP-1. Ann Surg. 2008;247:968–75.
18. Yan Z, Chen W, Liu S, et al. Myocardial insulin signaling and glucose transport are up-regulated in Goto–Kakizaki type 2 diabetic rats after ileal transposition. Obes Surg. 2012;22:493–501.
19. Chen W, Yan Z, Liu S, et al. The changes of pro-opiomelanocortin neurons in type 2 diabetes mellitus rats after ileal transposition: the role of POMC neurons. J Gastrointest Surg. 2011;15:1618–24.
20. Chelikani PK, Shah IH, Taqi E, et al. Comparison of the effects of Roux-en-Y gastric bypass and ileal transposition surgeries on food intake, body weight, and circulating peptide YY concentrations in rats. Obes Surg. 2010;20:1281–8.
21. Patriti A, Aisa MC, Annetti C, et al. How the hindgut can cure type 2 diabetes. Ileal transposition improves glucose metabolism and beta-cell function in Goto–Kakizaki rats through an enhanced Proglucagon gene expression and L-cell number. Surgery. 2007;142:74–85.
22. Kindel TL, Yoder SM, Seeley RJ, et al. Duodenal–jejunal exclusion improves glucose tolerance in the diabetic, Goto–Kakizaki rat by a GLP-1 receptor-mediated mechanism. J Gastrointest Surg. 2009;13:1762–77.
23. Ikezawa F, Shibata C, Kikuchi D, et al. Effects of ileal interposition on glucose metabolism in obese rats with diabetes. Surgery. 2012;151:822–30.
24. Culnan DM, Albaugh V, Sun M, et al. Ileal interposition improves glucose tolerance and insulin sensitivity in the obese Zucker rat. Am J Physiol Gastrointest Liver Physiol. 2010;299: G751–60.
25. Zhang GY, Wang TT, Cheng ZQ, et al. Resolution of diabetes mellitus by ileal transposition compared with biliopancreatic diversion in a nonobese animal model of type 2 diabetes. Can J Surg. 2011;54:243–51.
26. DeSesso JM, Jacobson CF. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. Food Chem Toxicol. 2001;39:209–28.
27. Tsuchiya T, Kalogeris TJ, Tso P. Ileal transposition into the upper jejunum affects lipid and bile salt absorption in rats. Am J Physiol-Gastr L. 1996;34:G681–91.
28. DePaula AL, Macedo ALV, Rassi N, et al. Laparoscopic treatment of metabolic syndrome in patients with type 2 diabetes mellitus. Surg Endosc. 2008;22:2670–8.
29. DePaula AL, Stival A, Halpern A, et al. Thirty-day morbidity and mortality of the laparoscopic ileal interposition associated with sleeve gastrectomy for the treatment of type 2 diabetic patients with BMI <35: an analysis of 454 consecutive patients. World J Surg. 2011;35:102–8.
30. DePaula AL, Stival A, Halpern A, Vencio S. Surgical treatment of morbid obesity: mid-term outcomes of the laparoscopic ileal interposition associated to a sleeve gastrectomy in 120 patients. Obes Surg 2010.
31. Kararli TT. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm Drug Dispos. 1995;16:351–80.
32. Gagner M. La transposition ileale avec ou sans gastrectomie par laparoscopie chez l'homme (TIG): la troisieme generation de chirurgie bariatrique. J Coeliochirurgie 2005:4-10.
33. DePaula AL, Macedo ALV, Schraibman V, et al. Hormonal evaluation following laparoscopic treatment of type 2 diabetes mellitus patients with BMI 20–34. Surg Endosc. 2009;23:1724–32.
34. De Paula A, Stival A, Halpern A, et al. Improvement in insulin sensitivity and B-cell function following ileal interposition with sleeve gastrectomy in type 2 diabetic patients: potential mechanisms. J Gastrointest Surg. 2011;15:1344–53.
35. Kodama Y, Zhao CM, Kulseng B, et al. Eating behavior in rats subjected to vagotomy, sleeve gastrectomy, and duodenal switch. J Gastrointest Surg. 2010;14:1502–10.
36. Furnes M, Tømmerås K, Arum CJ, et al. Gastric bypass surgery causes body weight loss without reducing food intake in rats. Obes Surg. 2008;18:415–22.
37. Bueter M, Löwenstein C, Olbers T, et al. Gastric bypass increases energy expenditure in rats. Gastroenterology. 2010;138:1845–53. e1.
38. Stylopoulos N, Hoppin AG, Kaplan LM. Roux-en-Y gastric bypass enhances energy expenditure and extends lifespan in diet-induced obese rats. Obesity (Silver Spring). 2009;17:1839–47.
39. Hansen L, Deacon CF, Ørskov C, et al. Glucagon-like peptide-1-(7–36)amide is transformed to glucagon-like peptide-1-(9–36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. Endocrinology. 1999;140:5356–63.

Paper IV

OPEN ACCESS Freely available online

PLOS ONE

Mechanistic Comparison between Gastric Bypass vs. Duodenal Switch with Sleeve Gastrectomy in Rat Models

Yosuke Kodama[1], Helene Johannessen[1], Marianne W. Furnes[1], Chun-Mei Zhao[1], Gjermund Johnsen[2], Ronald Mårvik[2], Bård Kulseng[1,2], Duan Chen[1,2]*

1 Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway, **2** Department of Surgery, Saint Olav's University Hospital, Trondheim, Norway

Abstract

Background: Both gastric bypass (GB) and duodenal switch with sleeve gastrectomy (DS) have been widely used as bariatric surgeries, and DS appears to be superior to GB. The aim of this study was to better understand the mechanisms leading to body weight loss by comparing these two procedures in experimental models of rats.

Methods: Animals were subjected to GB, DS or laparotomy (controls), and monitored by an open-circuit indirect calorimeter composed of comprehensive laboratory animal monitoring system and adiabatic bomb calorimeter.

Results: Body weight loss was greater after DS than GB. Food intake was reduced after DS but not GB. Energy expenditure was increased after either GB or DS. Fecal energy content was increased after DS but not GB.

Conclusion: GB induced body weight loss by increasing energy expenditure, whereas DS induced greater body weight loss by reducing food intake, increasing energy expenditure and causing malabsorption in rat models.

Citation: Kodama Y, Johannessen H, Furnes MW, Zhao C-M, Johnsen G, et al. (2013) Mechanistic Comparison between Gastric Bypass vs. Duodenal Switch with Sleeve Gastrectomy in Rat Models. PLoS ONE 8(9): e72896. doi:10.1371/journal.pone.0072896

Editor: Yvette Tache, University of California, Los Angeles, United States of America

Received April 9, 2013; **Accepted** July 13, 2013; **Published** September 9, 2013

Funding: The present research has been supported by funding from the Central Norway Regional Health Authority, and the European Union Seventh Framework Programme (FP7/2007–2013) under grant agreement n 266408. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: duan.chen@ntnu.no

Introduction

Various bariatric surgical procedures, such as gastric banding, gastric bypass (GB) and duodenal switch with sleeve gastrectomy (DS), have been developed in order to reduce food intake and/or lead to malabsorption [1]. GB was invented by Dr. Edward Mason as a bariatric surgery in 1965, and later it was converted to Roux-en-Y procedure which was created by Dr. Cesar Roux already in 1897. Recently, a laparoscopic mini-GB procedure has been shown to be regarded as a simpler and safer alternative to laparoscopic Roux-en-Y with similar efficacy at 5 or 10 year experience [2]. However, the different procedures have shown different efficacy in individual patients, and the underlying mechanisms are not yet clear. Therefore, it is a challenge to select the most effective bariatric procedure for individual patients.

Various rat models of bariatric surgery have been developed in order to understand the underlying physiological mechanisms of different surgical procedures. There have been many studies in the literature reporting the surgical procedures (such as Roux-en-Y) in rats that are made as same as they are used in humans [3,4]. However, there is a significant difference in the anatomy and physiology of the gastrointestinal tract between rats and humans, which should be kept in mind when creating the surgical models in rats and translating findings from animals and humans. For instance, the rat stomach consists of antrum, fundus (also called corpus) and rumen (forestomach), while the human stomach is divided into antrum, body and fundus (Fig 1A, E). The rat jejunum represents almost 90% of the small intestine, the human jejunum about 40% [5]. Unlike humans, rats are nonemetic (not vomiting) and has no gallbladder. The Roux-en-Y reconstruction was initially created to prevent post-gastrectomy bile vomiting in patients [6]. Apparently, it is not necessary to create the Roux-en-Y reconstruction in rats that are subjected to GB [7–9]. The duodenal switch procedure was originally created as a surgical solution for primary bile reflux gastritis and/or to decrease postoperative symptoms after distal gastrectomy and gastroduodenostomy [10]. In patients, the operation usually consists of a 75% longitudinal gastrectomy (the so-called sleeve gastrectomy), creation of an alimentary limb approximately 50% of total small bowel length (i.e. bypassing jejunum), a common channel length of 100 cm, and cholecystectomy. In the present study using rats, GB was performed without the Roux-en-Y reconstruction and the postsurgical anatomy was similar to mini-GB on humans, and DS was performed according to the rat anatomy (Fig 1A–H) [11].

GB is the most common procedure because of relatively high efficacy and safety, whereas DS seems to be even more effective, particularly in super-obese patients [12]. Both GB and DS are believed to cause restriction in food intake and malabsorption by decreasing stomach size and bypassing part of the small intestine. In patients, DS is superior to GB in body weight loss as well as in

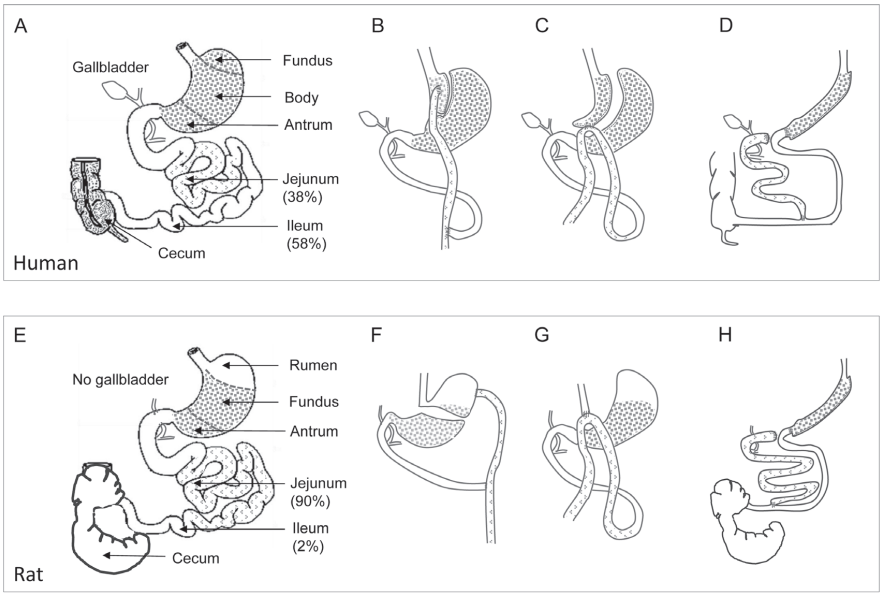


Figure 1. Schematic drawing of anatomy. The gastrointestinal tract of human (**A–D**) and rat (**E–H**) before (**A, E**) and after Roux-en-Y gastric bypass (GB) (**B, F**), mini-GB (**C, G**), and duodenal switch (**D, H**). Glandular stomach is indicated by grid gray and jejunum by light grid gray. The rumen of rat stomach is non-glandular (white area). Note: In **A, E**, percentages mean % of small intestine, e.g. in **E**, jejunum is 90% of total small intestine in rats based on [11]; in **F**, rat Roux-en-Y GB [3], and in **G**, Mini-GB used in the present study.
doi:10.1371/journal.pone.0072896.g001

improvement of comorbidities such as diabetes, hypertension and dyslipidemia [12–18]. Mechanisms underlying the postoperative weight loss and possible regain remain unclear. Whether this is due to biological or behavioral factors is one of the major debates [19]. The aim of the present study was to compare the postoperative effects of GB *vs.*DS on eating behavior and energy expenditure in rat models.

Materials and Methods

Animals and Experimental Design

Adult rats (male, Sprague-Dawley, 6–12 months of age) were purchased from Taconic M&B, Skensved, Denmark and housed in ventilated cages in a specific pathogen free environment with room temperature of 22°C, 40–60% relative humidity and 12 hr day/night cycle with 1 hr dusk/dawn. The rats had free access to tap water and standard rat pellet food (RM1 801002, Scanbur BK AS, Sweden). In our previous studies, we have reported that the male rats gained body weight mainly as a result of continuous expansion of the fat compartment after puberty (8 weeks of age with 200 g body weight), and that the male rats that were fed a high-fat diet starting at 5 weeks of age gained body weight up to ~650 g at 40 weeks of age as a result of increased fat mass [7,8]. In the present study, normal adult male rats (~600 g body weight) were chosen after considerations of the small difference in body weight (~650 g *vs.* ~600 g induced by high-fat diet) and the experimental efforts in terms of time-consuming and financial expense (Fig 2A). Furthermore, the body weight development of naïve rats reaches a plateau (580±20 g) at 40 weeks of age, and laparotomy performed at 13 weeks of age did not affect the development of body weight (Fig 2A).

Thirty-four rats, at 587.0±8.1 g body weight, were randomly divided into experimental (GB and DS) as well as control groups (laparotomy, LAP): GB (14 rats), DS (7 rats), and LAP (13 rats). The body weight was not different between the groups before surgery ($p = 0.276$). Because of markedly loss of body weight after DS, the group of DS rats, together with age-matched group of laparotomized rats (LAP_{DS}, 7 rats), were followed up only for 8 weeks, while GB rats and the rest of laparotomized rats (LAP_{GB}, 6 rats) were followed up for 14 weeks. In consideration of the "3Rs" for the human use of animals (i.e., reducing the number of animals while achieving the scientific purposes of the experiment), rats that had been used for studies of the effects of individual surgical procedures were re-used [8,20]. The study was approved by the Norwegian National Animal Research Authority (Forsøksdyrutvalget, FDU).

Surgery

All operations were performed under general anesthesia with isofluran (4% for induction and 2% for maintenance) (Baxter Medical AB, Kista, Sweden). Buprenorphine (0.05 mg/kg) (Schering-Plough Europe, Brussels, Belgium) was administrated as an analgesic agent subcutaneously immediately during surgery. LAP was performed through a middle-line incision with gentle manipulation of viscera. A rat model of Roux-en-Y GB procedure has been described by Stylopoulos and his colleagues [3]. Gastric pouch in that rat model was created at the site of rumen which does not exist in humans (Fig 1F). In the present study, GB was

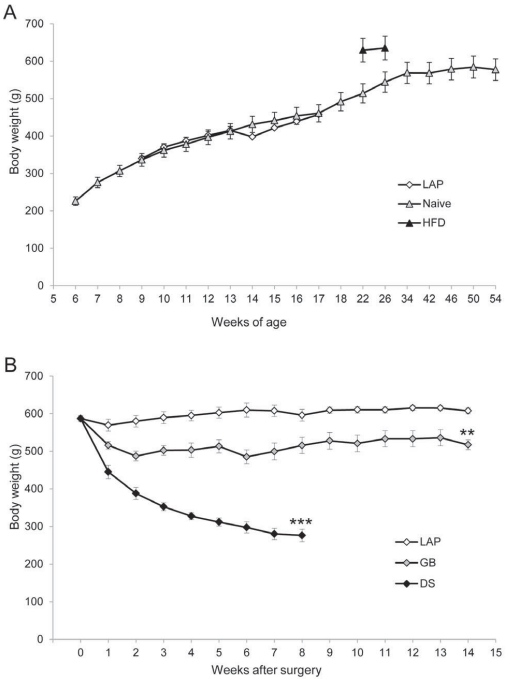


Figure 2. Body weight. Naïve rats (data from Taconic), rats that underwent laparotomy (LAP) at 13 weeks (LAP) and rats that have had high-fat since 5 weeks of age (data from [31]) (**A**)**.** Rats after gastric bypass (GB), duodenal switch (DS) and laparotomy (LAP) (**B**). Data are expressed as means ± SEM. **: $p<0.01$, ***: $p<0.001$ between LAP *vs.* GB or DS.
doi:10.1371/journal.pone.0072896.g002

performed by anastomosing the distal esophagus to the proximal jejunum about 2–3 cm distal to the Treitz ligament in an end-to-side manner (Fig 1G) as described previously [7,8]. DS was achieved in two stages. The two-stage procedure has been recommended in patients because the single-stage procedure increases the risk of postoperative complications and staged DS may avoid biliopancreatic diversion in some patients [21]. In the present study, sleeve gastrectomy was performed by resecting approximately 90% of the rumen and 70% of the glandular stomach along the greater curvature. Three months later, duodenal switch was achieved by creating biliopancreatic limb, alimentary limb (bypassing jejunum) and common channel length of 5 cm (Fig 1H). The duration of surgical time was 30–60 min for GB or DS. In all surgeries performed in the present study, proper aseptic surgical techniques were applied, and therefore, neither prophylactic nor postoperative antibiotics were used. This was done according to the guidelines and recommendations by the Federation of European Laboratory Animal Science Associations (FELASA 2008) and the guide for the care and use of laboratory animals by the Committee of USA National Research Council (2010). After recovering from anesthesia, the animals were placed 2–4 per cage throughout the study period.

Measurements of Eating Behavior and Energy Expenditure Parameters

These were monitored by the Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments International, Columbus, OH, USA) 2–3 weeks after GB, DS or LAP and 14 weeks after GB, 8 weeks after DS or 8–14 weeks after LAP. The CLAMS is composed of a 4-chamber indirect calorimeter designed for the continuous monitoring of individual rats from each chamber. The eating data was generated by monitoring all feed balances every 0.5 s. In CLAMS program used in the present study, the end of an eating event (meal) was when the balance was stable for more than 10 s and a minimum of 0.05 g was eaten. The eating parameters during daytime and nighttime (12 hr each time) for each rat included: accumulated food intake (g or kcal), number of meals, meal size (g/meal), meal duration (min/meal), intermeal interval (min), rate of eating (g/min), and satiety ratio (min/g). The intermeal interval was defined as the interval in minutes between two meals. The rate of eating was calculated by dividing meal size by meal duration. The satiety ratio, an index of non-eating time produced by each gram of food consumed, was calculated as intermeal interval divided by meal size [22]. The volume of O_2 consumption (VO_2 mL/kg/hr) and the volume of CO_2 production (VCO_2 mL/kg/hr) were measured by an air sample withdrawn every 5 min from each chamber through the gas dryer. The energy expenditure (kcal/hr) was calculated according to equation: $(3.815+1.232\ \mathrm{RER}) \times VO_2$, where the respiratory exchange ratio (RER) was obtained by VCO_2 divided by VO_2. In order for rats to acclimate to CLAMS, they were placed in these metabolic chambers for 24 hr one week before the first CLAMS monitoring. For the measurement of eating and metabolic parameters, the rats were placed in the CLAMS for 48 hr. In order to minimalize possible effect of stress, only data from the last 24 hr in CLAMS were used for the analysis. An analysis of eating pattern in control rats over a time period from day 1 and 21 showed no significant differences in any parameters, indicating that the animals had acclimated to CLAMS (Table S1). The rats have had free access to standard rat powder food (RM1 811004, Scanbur BK AS, Sweden) and tap water while they were in CLAMS. The total metabolizable energy was 2.57 kcal/g for both the pellet food (RM1 811002) and the powder food (RM1 811004).

Determination of Fecal Energy Density

Feces were collected while the rats were placed in CLAMS chambers and dried for 72 hr at 60°C. The energy density was determined by means of an adiabatic bomb calorimeter (IKA-Calorimeter C 5000, IKA-Werke GmbH & Co. KG, Staufen, Germany).

Determination of Plasma Levels of Cytokines

Blood was drawn from the abdominal aorta under the anesthesia just before the animals were killed, and plasma was stored at −80°C until determination of levels of cytokines. The multiplex cytokine assay was used (Cat no:171-K1002M, Bio-plex Pro Rat Cytokine Th1/Th2 12-plex Panel; Bio-Rad Laboratories, Hercules, CA, USA). It contained the following analytics: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα).

Statistical Analysis

The values were expressed as means ± SEM. Two-tailed independent-samples *t*-test or Mann Whitney U test was

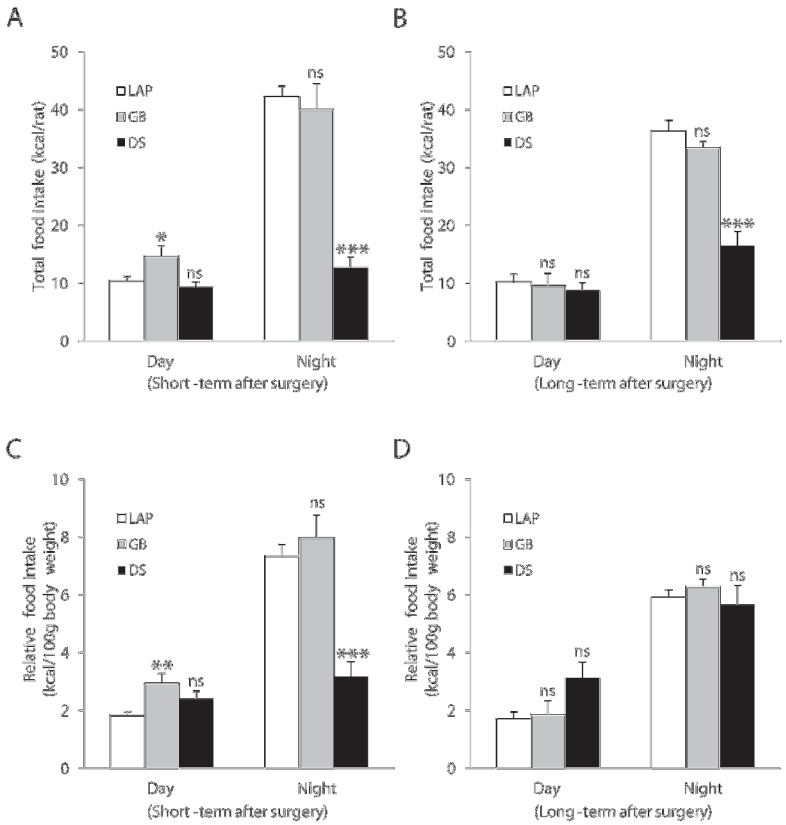


Figure 3. Food intake. Total food intake (kcal/rat) (**A,B**) and relative food intake (kcal/100 g body weight) (**C,D**) during day- and night-time. Short-term after surgery: 3 weeks after gastric bypass (GB), 2 weeks after duodenal switch (DS) or 2–3 weeks after lapatoromy (LAP). Long-term after surgery: 14 weeks after GB, 8 weeks after DS or 8–14 weeks after LAP. Data are expressed as means ± SEM. *: $p<0.05$, **: $p<0.01$, ns: not significant between LAP (n = 13) *vs.* GB (n = 8) or DS (n = 5).
doi:10.1371/journal.pone.0072896.g003

performed for two-group comparisons. ANOVA followed by Bonferroni test was performed for multiple comparisons. Homogeneity of regression assumption test and ANCOVA were performed for analysis of energy expenditure. SPSS version 19.0 (SPSS Inc. Chicago, IL, USA) was used. A p-value of <0.05 was considered statistically significant.

Results

Mortality

No one died after LAP alone, 6 after GB, and 2 after DS due to surgical complications, trauma and learning curve factors.

Body Weight

LAP alone did not reduce body weight during the study period (maximum 14 weeks). GB caused approximately 20% weight loss throughout the study period (14 weeks). DS induced approximately 50% weight loss within 8 weeks (Fig 2B).

Food Intake and Eating Behavior

In comparison with LAP, GB increased daytime (but not nighttime) food intake (expressed as either kcal/rat or kcal/100 g body weight) at 3 weeks, and had no effects afterwards (14 weeks postoperatively). In contrast, DS reduced nighttime (but not daytime) food intake (kcal/rat at both 2 and 8 weeks or kcal/100 g body weight at 2 weeks). The food intake (kcal/100 g) at 8 weeks was not reduced because of markedly loss of the body weight after DS (Fig 3).

GB was without effects neither on satiety ratio (min/g) nor rate of eating (g/min), whereas DS increased satiety ratio during nighttime, and decreased rate of eating during both daytime and nighttime at 2 weeks and 8 weeks postoperatively (Fig 4) (Tables 1,2).

Energy Expenditure

Age-matched control rats that underwent LAP only were included for comparisons because metabolic parameters are age-dependent [23,24]. GB increased nighttime energy expenditure

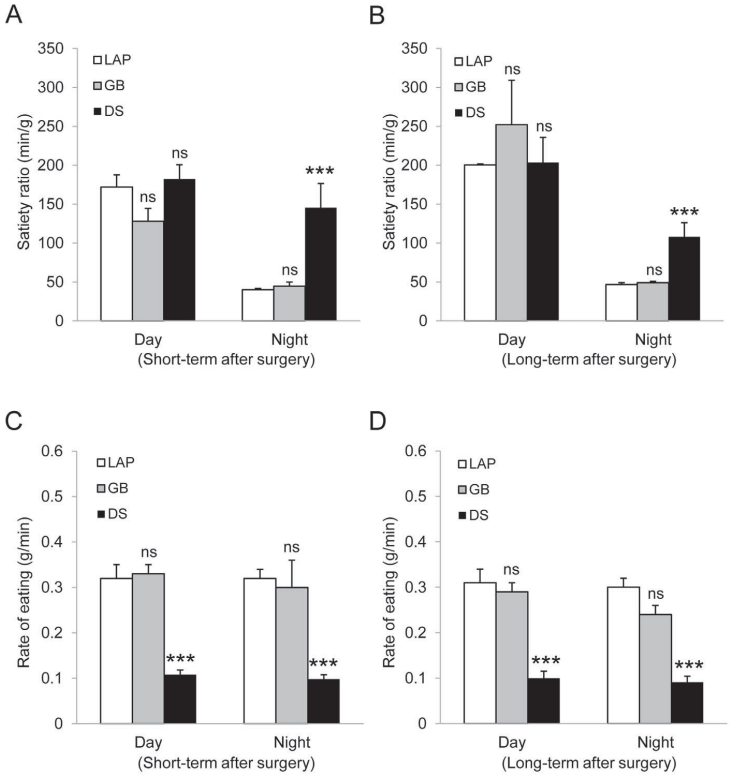


Figure 4. Eating behavior. Satiety ratio (min/g) (**A,B**) and rate of eating (g/min) (**C,D**) during day- and night-time**.** Short-term after surgery: 3 weeks after gastric bypass (GB), 2 weeks after duodenal switch (DS) or 2–3 weeks after lapatoromy (LAP). Long-term after surgery: 14 weeks after GB, 8 weeks after DS or 8–14 weeks after LAP. Data are expressed as means ± SEM. ***: $p<0.001$, ns: not significant between LAP (n = 13) *vs.* GB (n = 8) or DS (n = 5).
doi:10.1371/journal.pone.0072896.g004

(kcal/hr/100 g body weight) at 3 weeks and daytime energy expenditure at 14 weeks postoperatively (Fig 5A, C) (Tables 3, 4). RER was unchanged after GB. DS increased daytime energy expenditure both at 2 and 8 weeks as well as nighttime energy expenditure at 8 weeks postoperatively (Fig 5B, D) (Tables 3, 4). RER tended to be reduced during nighttime at 2 weeks after DS ($p = 0.051$) (Table 3).

Analysis of the homogeneity of regression slopes indicated that there was positive correlation between the body weight and energy expenditure (kcal/hr) particularly in LAP_{DS} rats and similar regression slopes between LAP and GB or DS ($p>0.05$) (Fig S1). ANCOVA showed that there was no significant difference in adjusted energy expenditure between LAP and GB or DS ($p>0.05$) (Fig S2).

Fecal Energy Density

There was no change in the fecal energy density after GB. DS had severe diarrhea within 2 weeks postoperatively, so that it was difficult to collect the fecal samples. At 2 months, the solid feces were collected and the energy density was increased (Fig 6).

Plasma Levels of Cytokines

There was no difference between LAP *vs.* GB or DS in the plasma levels of the 11 cytokines measured (Table S2).

Discussion

The present study shows that the rat models provide results that are in accordance with results from clinical series in patients , i.e. greater weight loss by DS than GB [17,18,25]. Furthermore, the results of the present study show different postsurgical effects of GB *vs.* DS in terms of food intake, eating rate, energy expenditure and absorption.

It is a common dogma that to reduce size of stomach by surgery would lead to early satiety and consequently reduce food intake. Regardless of difference in surgical procedure, either GB or DS reduces stomach size and bypasses part of the small intestine (duodenum and most of the jejunum). Previously, we have found that the food intake was independent on the size of stomach by comparing gastrectomy and GB in rat models [9]. In clinical studies, the size of pouch after GB was found not to correlate with weight loss outcome in patients [25,26]. In our previous and the

Table 1. Eating behavior.

	Parameter	LAP	GB	DS
Day	Food Intake (g)	4.08±0.3	5.69±0.68*	3.67±0.31†
	Food Intake (g/100g body weight)	0.7±0.06	1.14±0.13**	0.93±0.11
	Food intake (kcal)	10.5±0.76	14.62±1.74*	9.42±0.80†
	Food intake (kcal/100g body weight)	1.83±0.14	2.93±0.33**	2.39±0.28
	Number of meals	12.15±0.97	18.29±2.48*	16.6±0.92
	Meal size (g/meal)	0.35±0.03	0.32±0.02	0.22±0.021*
	Meal size (kcal/meal)	0.91±0.08	0.82±0.05	0.57±0.05*
	Meal duration (min)	13.5±1.39	17.96±2.82	34.47±3.29***,†††
	Meal duration (min/meal)	1.14±0.1	0.98±0.08	2.13±0.29***,†††
	Intermeal interval (min)	57.5±4.42	40.37±5.38*	39.38±2.04
	Satiety ratio (min/g)	171.89±15.81	127.93±16.63	182.34±18.24
	Rate of eating (g/min)	0.32±0.03	0.33±0.02	0.11±0.01***,†††
Night	Food Intake (g)	16.45±0.69	15.58±1.72	4.96±0.70***,†††
	Food Intake (g/100g body weight)	2.86±0.15	3.11±0.30	1.24±0.19***,†††
	Food intake (kcal)	42.28±1.77	40.05±4.41	12.76±1.81***,†††
	Food intake (kcal/100g body weight)	7.35±0.39	7.98±0.78	3.19±0.50***,†††
	Number of meals	34.38±3.92	31.71±3.01	24.40±3.85
	Meal size (g/meal)	0.57±0.09	0.51±0.07	0.21±0.03*
	Meal size (kcal/meal)	1.47±0.22	1.30±0.17	0.55±0.08*
	Meal duration (min)	54.21±4.54	56.25±5.13	53.21±9.39
	Meal duration (min/meal)	1.88±0.36	1.82±0.18	2.20±0.28
	Intermeal interval (min)	22.6±3.08	21.36±1.97	29.43±5.57
	Satiety ratio (min/g)	40±1.86	44.64±5.41	145.51±31.17***,†††
	Rate of eating (g/min)	0.32±0.02	0.30±0.06	0.10±0.01***,††

Parameters during day- and night-time at 3 weeks after gastric bypass (GB), 2 weeks after duodenal switch (DS) and 2–3 weeks after laparotomy (LAP). Data are expressed as means ± SEM. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ between LAP *vs.* GB or DS. †: $p<0.05$, ††: $p<0.01$, †††: $p<0.001$ between GB *vs.* DS.
doi:10.1371/journal.pone.0072896.t001

present studies, GB did reduce body weight but not food intake in rats [7–9]. Behavior of rats is mostly driven on instincts, while behavior of humans is much more complicated. In fact, there is still an open question: "Does GB reduce food intake in humans?". A recent review shows that large and persistent alterations in macronutrient intake after GB have not generally been reported, and when the changes do occur, they are either transient or relatively modest. The authors argue for more direct measures of food intake in human studies that are similar to those used in animal studies [27]. Food intake in patients is also affected by following the "postoperative instruction" to achieve the best possible conditions for weight reduction and to minimize side-effects like gastro-esophageal reflux and dumping syndrome which unlikely occur in rats. Recently, a human study of eating behavior and meal pattern following GB was still performed by manually weighing differences to determine food and water intake and by the Three-Factor Eating Questionnaire to evaluate eating behavior [28]. However, in that human study, the food intake was not reported, but *ad libitum* meal size was reduced while number of meals per day was increased, and hunger and satiety scores did not change after GB, which are in line with our findings in rats following GB [8]. Methods with more direct measures of food intake (and food-selection and taste-related behavior) for humans are needed in order to facilitate translation between findings from animal models and clinical research [27].

Unlike GB, DS does reduce the food intake. Previously, we have shown that food intake was reduced by duodenal switch alone but not by sleeve gastrectomy alone by comparing sleeve gastrectomy only *vs.* duodenal switch without sleeve gastrectomy in rats [20]. In the present study, DS markedly reduced food intake and increased satiety ratio particularly during nighttime. The rate of eating has also impacts on body weight. It has been reported that there is a correlation between rate of eating and body weight or body mass index (BMI) [29,30]. Previously, we have shown that high-fat-diet-induced obesity was associated with increased rate of eating, increased size of meals, but not with daily calories intake [31]. In the present study, DS decreased the eating rate during both day- and night-time.

Mechanisms underlying postoperative weight loss and possible regain remain unclear. A major point of controversy is whether this is due to biological or behavioral factors [19,32]. We and others have shown that GB increased the energy expenditure in rats and mice, which could be one of the mechanisms explaining the physiologic basis of weight loss after this procedure [8,33,34]. The increased resting energy expenditure in the animal models after GB is in accordance with some, but not all, reports in humans. The discrepancies in the clinical studies may include the heterogeneity of patient populations and measurements of energy expenditure for a limited time using portable metabolic carts under artificial rather than "free-living" conditions [35]. Nerve-less, resting energy expenditure has been suggested to be a

Table 2. Eating behavior.

	Parameter	LAP	GB	DS
Day	Food Intake (g)	3.97±0.51	3.71±0.85	3.42±0.50
	Food Intake (g/100g body weight)	0.66±0.09	0.72±0.19	1.22±0.21
	Food intake (kcal)	10.19±1.32	9.54±2.18	8.78±1.29
	Food intake (kcal/100g body weight)	1.71±0.24	1.85±0.48	3.14±0.54
	Number of meals	10.92±1.29	11.25±1.81	18.60±4.50
	Meal size (g/meal)	0.4±0.06	0.34±0.06	0.23±0.05
	Meal size (kcal/meal)	1.03±0.17	0.86±0.15	0.59±0.12
	Meal duration (min)	13.54±1.74	13.89±3.86	37.17±6.92***,†††
	Meal duration (min/meal)	1.37±0.22	1.23±0.22	2.34±0.45
	Intermeal interval (min)	68.39±7.92	73.68±16.91	50.16±17.90
	Satiety ratio (min/g)	200.34±27.9	252.16±57.01	203.50±32.22
	Rate of eating (g/min)	0.31±0.03	0.29±0.02	0.10±0.01***
Night	Food Intake (g)	14.15±0.71	13.00±0.38	6.43±0.96***,†††
	Food Intake (g/100g body weight)	2.3±0.1	2.45±0.10	2.20±0.26
	Food intake (kcal)	36.35±1.82	33.42±0.98	16.52±2.46***,†††
	Food intake (kcal/100g body weight)	5.92±0.25	6.29±0.26	5.65±0.68
	Number of meals	26.92±2.76	26.50±3.69	34.20±7.13
	Meal size (g/meal)	0.59±0.06	0.58±0.09	0.24±0.07*,†
	Meal size (kcal/meal)	1.52±0.15	1.48±0.23	0.61±0.19*,†
	Meal duration (min)	50.66±4.57	56.36±2.96	70.58±5.38*
	Meal duration (min/meal)	2.01±0.18	2.53±0.47	2.39±0.44
	Intermeal interval (min)	27.78±3.84	27.91±4.11	21.49±4.03
	Satiety ratio (min/g)	46.78±2.4	49.20±1.56	108.18±17.98***,†††
	Rate of eating (g/min)	0.3±0.02	0.24±0.02	0.09±0.01***,††

Parameters during day- and night-time at 14 weeks after gastric bypass (GB), 8 weeks after duodenal switch (DS) and 8–14 weeks after laparotomy (LAP). Data are expressed as mean ± SEM. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ between LAP *vs.* GB or DS. †: $p<0.05$, ††: $p<0.01$, †††: $p<0.001$ between GB *vs.* DS.

doi:10.1371/journal.pone.0072896.t002

therapeutic target for obesity [32,36]. In the present study, we further showed that the increased energy expenditure took place only during nighttime (relevant to active energy expenditure) shortly after GB (weeks) and switched to daytime (resting energy expenditure) after months, whereas the energy expenditure was increased during daytime shortly after DS and during both day- and night-time months after DS.

The most extensively used method for calculation of energy expenditure is dividing O_2 consumption by body weight or body surface area [37]. In the present study, the energy expenditure was calculated by taking into account both O_2 consumption and CO_2 production, and expressed as kcal/hr/rat, kcal/hr/100 g body weight, and kcal/hr/cm^2 body surface. Dividing the energy expenditure by body weight does not take into account differences in body composition, and therefore, the fat-free mass or lean body mass (as denominator) has often been used in both human and mouse studies. However, this could be inappropriate because brown fat can be the most metabolically active tissue in the body [37]. ANCOVA has been suggested to be appropriate method for analysis of the mouse energy expenditure, but it cannot be used when the samples sizes are small [37,38]. In rat studies, ANCOVA has not been used with the exception of a few reports including our previous study of ileal interposition associated with sleeve gastrectomy [5,39]. The reasons for not widely use of ANCOVA than ANOVA in rat studies might be less statistical power when sample size is small and nonlinear relationship between covariate (s) and dependent variable. Another reason may be that ANCOVA is best used with quasi-experimental data, such as genetically-modified mice [37] or humans [40,41]. The results of the present study showed that there were highly correlation between the body weight and the energy expenditure (kcal/hr/rat) in control LAP rats, and significant increases in the energy expenditure (kcal/hr and/or kcal/hr/100 g body weight) after GB or DS (by ANOVA). However, ANCOVA showed no significant difference in the energy expenditure (kcal/hr) between LAP and GB or DS. The difference in terms of p values by ANOVA *vs.* ANCOVA (i.e. testing the body-weight independent differences) can be interpreted as that GB or DS increases the energy expenditure (possible cause) while reducing the body weight (effect), which is at odds with the positive correlation between the body weight and the energy expenditure in control animals (LAP).

Both GB and DS are designed for restriction and malabsorption by creating the alimentary limb. However, DS, but not GB, caused diarrhea shortly after surgery (2 weeks) and malabsorption (measured at 2 months postoperatively) in rats, which is in line with observations in patients [42].

It should be noticed that in the present study, neither prophylactic nor postoperative antibiotics were used and none of the 11 plasmas cytokines measured was changed after surgery, indicating no or little impact of microflora and inflammation on the eating behavior and the body weight changes. Recently, a mouse study showed that specific alterations in the gut microbiota

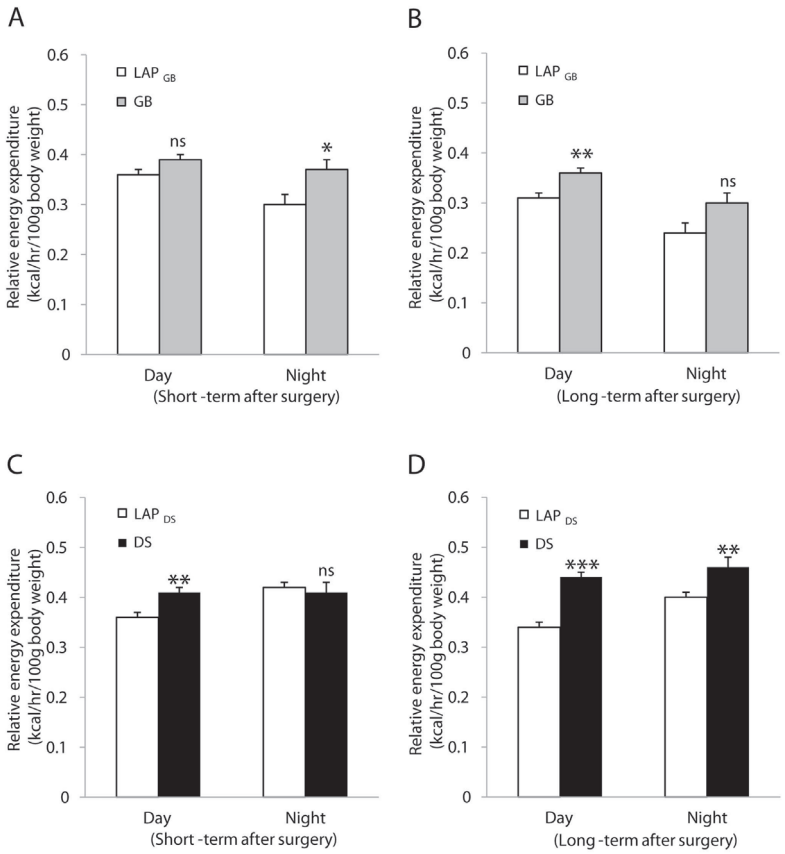


Figure 5. Energy expenditure during day- and night-time. Short-term after surgery: 3 weeks after gastric bypass (GB), 2 weeks after duodenal switch (DS) or 2–3 weeks after laparotomy (LAP). Long-term after surgery: 14 weeks after GB, 8 weeks after DS or 8–14 weeks after LAP. Data are expressed as means ± SEM. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: not significant between LAP_{GB} (n = 7) *vs.* GB (n = 8) or LAP_{DS} (n = 6) *vs.* DS (n = 5).
doi:10.1371/journal.pone.0072896.g005

contributed to the beneficial effect of bariatric surgery on energy balance [43]. Whether GB or DS alters the gut microbiota and consequently leads to the weight loss via same or different pathways might be of interest for future study.

There are several limitations of the present study. 1) The rats used were not obese. Whether or not the postsurgical effects of these two procedures are different between normal and obese rats that are induced by high-fat diet or developed spontaneously (e.g. Zucker, Otsuka Long-Evans Tokushima Fatty, Obese SHR, or Wistar Ottawa Karlsburg W rats), and which animal model of obesity best mimics the obese humans in response to the bariatric surgery could be the subjects for further research. 2) GB procedure used in rats was not exactly the same as it was applied in humans. Fig 1 shows different procedures of GB. A laparoscopic mini-gastric bypass procedure (which is similar with one used in the present study) has been shown to be regarded as a simpler and safer alternative to laparoscopic Roux-en-Y procedure with similar efficacy at 5 or 10 year experience [2,44]. It may be of interest to compare different GB procedures in the future, if there is any clinical relevancy. 3) Although the size of gastric pouch after GB does not correlate with weight loss outcome in patients [25,26], it cannot be excluded whether lack of the pouch in GB has impact on food intake, satiety and eating behavior. 4) The differences between rats and humans are not only in terms of the GI anatomy but also the responses to surgery. For instance, sleeve gastrectomy only (without duodenal switch) works in some patients but not in rats [20,45]. It may be of interest to directly compare the effects of sleeve only *vs.* sleeve with duodenal switch (one or two-staged) in the future.

In general, research in patients is directly clinical relevant. However, studies in animals provide much greater latitude in control and experimental manipulation of the system, and ultimately help to reveal the underlying mechanisms and to adopt the protocols and methods that are tested in animals to humans [46]. Research using animal models is an excellent way of developing and learning bariatric surgical techniques as well as understanding the postsurgical physiology [47]. Taken the data from the previous and the present studies together, the

Table 3. Metabolism.

		3 weeks after surgery		2 weeks after surgery	
	Parameter	**LAP_{GB}**	**GB**	**LAP_{DS}**	**DS**
Day	Energy expenditure (kcal/hr)	2.08±0.03	1.93±0.06	2.08±0.07	1.66±0.12**
	Energy expenditure (kcal/hr/100g body weight)	0.36±0.01	0.39±0.01	0.36±0.01	0.41±0.01**
	Energy expenditure (kcal/hr/cm^2 body surface)	0.003±0.0001	0.004±0.0001	0.003±0.0001	0.004±0.0002
	RER	0.94±0.01	0.93±0.02	1.02±0.03	0.96±0.03
	VO_2	726.68±15.56	779.91±23.26	705.18±18.00	823.91±31.49**
	VCO_2	682.50±12.08	727.85±23.54	717.08±26.45	791.69±26.59
Night	Energy expenditure (kcal/hr)	1.75±0.14	1.85±0.10	2.45±0.08	1.66±0.10***
	Energy expenditure (kcal/hr/100g body weight)	0.30±0.02	0.37±0.02*	0.42±0.01	0.41±0.02
	Energy expenditure (kcal/hr/cm^2 body surface)	0.003±0.0002	0.003±0.0002	0.004±0.0001	0.004±0.0002*
	RER	1.00±0.01	1.01±0.03	1.07±0.03	0.97±0.04
	VO_2	600.02±36.70	737.88±43.20*	820.65±20.88	823.94±35.51
	VCO_2	594.19±37.54	735.07±35.39*	877.73±35.71	803.06±49.36

Parameters during day- and night-time at 3 weeks after gastric bypass (GB) and the age-matched laparotomy-operated group (LAP_{GB}), and at 2 weeks after duodenal switch (DS) and the age-matched laparotomy-operated group (LAP_{DS}). Data are expressed as means ± SEM. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ between LAP_{GB} *vs.* GB or LAP_{DS} *vs.* DS.

doi:10.1371/journal.pone.0072896.t003

appropriately designed rat models provide significant insights into the mechanisms of bariatric surgery which explain well the clinical observations, e.g. that DS is superior to GB in body weight loss. The results of the present study may suggest further that GB induces body weight loss by increasing energy expenditure, whereas DS induces greater body weight loss by reducing food intake, increasing energy expenditure and causing malabsorption.

Table 4. Metabolism.

		14 weeks after surgery		8 weeks after surgery	
	Parameter	**LAP_{GB}**	**GB**	**LAP_{DS}**	**DS**
Day	Energy expenditure (kcal/hr)	1.92±0.08	1.90±0.05	2.10±0.06	1.27±0.07***
	Energy expenditure (kcal/hr/100g body weight)	0.31±0.01	0.36±0.01**	0.34±0.01	0.44±0.01***
	Energy expenditure (kcal/hr/cm^2 body surface)	0.003±0.0001	0.003±0.0000*	0.003±0.0000	0.003±0.0001
	RER	0.98±0.03	0.91±0.05	0.99±0.03	0.98±0.01
	VO_2	622.25±27.61	720.95±14.41**	681.99±13.62	873.32±25.35***
	VCO_2	605.31±17.73	652.48±25.93	675.60±23.89	853.15±25.17***
Night	Energy expenditure (kcal/hr)	1.48±0.11	1.60±0.10	2.45±0.12	1.34±0.01***
	Energy expenditure (kcal/hr/100g body weight)	0.24±0.02	0.30±0.02	0.40±0.01	0.46±0.02***
	Energy expenditure (kcal/hr/cm^2 body surface)	0.002±0.0002	0.003±0.0002	0.004±0.0001	0.004±0.0001*
	RER	0.97±0.06	1.04±0.05	1.06±0.03	0.99±0.03
	VO_2	475.14±36.56	598.36±45.29	778.37±16.51	919.50±28.99**
	VCO_2	487.63±35.15	609.64±55.95	825.43±27.97	906.74±39.86

Parameters during day- and nighttime at 14 weeks after gastric bypass (GB) and the age-matched laparotomy-operated group (LAP_{GB}), and at 8 weeks after duodenal switch (DS) and the age-matched laparotomy-operated group (LAP_{DS}). Data are expressed as means ± SEM. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ between LAP_{GB} *vs.* GB or LAP_{DS} *vs.* DS.

doi:10.1371/journal.pone.0072896.t004

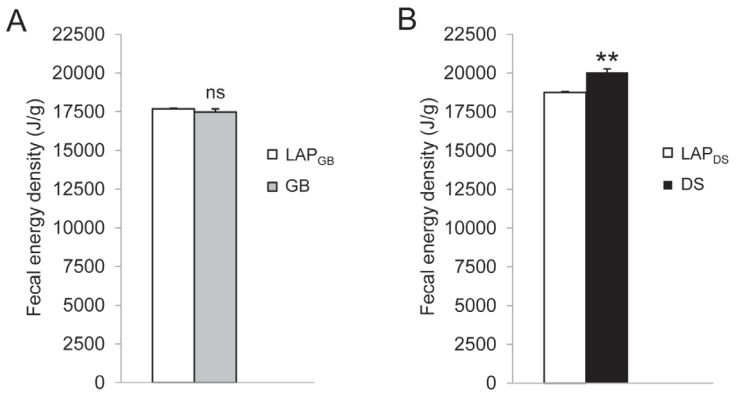


Figure 6. Fecal energy density. Three weeks after gastric bypass (GB) or laparotomy (LAP_{GB}) (**A**) and eight weeks after duodenal switch (DS) or laparotomy (LAP_{DS}) (**B**). Data are expressed as mean ± SEM. **: $p<0.01$, ns: not significant between LAP_{GB} (n = 7) *vs.* GB (n = 8) or LAP_{DS} (n = 6) *vs.* DS (n = 5).
doi:10.1371/journal.pone.0072896.g006

Supporting Information

Figure S1 Scatterplot of energy expenditure against body weight. $LAP_{GB\ or\ DS}$: laparotomy as control for GB or DS; GS: gastric bypass; DS: Duodenal switch.
(TIF)

Figure S2 Adjust energy expenditure by ANCOVA. $LAP_{GB\ or\ DS}$: laparotomy as control for GB or DS; GS: gastric bypass; DS: Duodenal switch. Means ± SEM.
(TIF)

Table S1 CLAMS measurements of normal rats. Data at day 1 and 21 one week after 24 hours training with CLAMS cage are expressed as means ± SEM. ns: not significant between day 1 vs. day 21.
(DOC)

Table S2 Plasma levels of cytokines. Data of rats after gastric bypass (GB) and duodenal switch (DS) compared with the age-matched laparotomy-operated groups (LAP_{GB} or LAP_{DS}, respectively) are expressed as means ± SEM. ns: not significant between LAP_{GB} vs. GB or LAP_{DS} vs. DS.
(DOC)

Acknowledgments

The authors thank Morten Grønli and Erik Langørgen for assistance of bomb calorimeter at Department of Energy and Process Engineering, and Terje Espevik and Liv Ryan for analysis of cytokines at Centre of Molecular Inflammation Research, Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology.

Author Contributions

Conceived and designed the experiments: YK HJ MWF C-MZ GJ RM BK DC. Performed the experiments: YK HJ DC. Analyzed the data: YK HJ DC. Contributed reagents/materials/analysis tools: MWF C-MZ BK DC. Wrote the paper: YK HJ C-MZ GJ BK DC.

References

1. Ward M, Prachand V (2009) Surgical treatment of obesity. Gastrointest Endosc 70: 985–990.
2. Noun R, Skaff J, Riachi E, Daher R, Antoun NA, et al. (2012) One thousand consecutive mini-gastric bypass: short- and long-term outcome. Obes Surg 22: 697–703.
3. Stylopoulos N, Hoppin AG, Kaplan LM (2009) Roux-en-Y gastric bypass enhances energy expenditure and extends lifespan in diet-induced obese rats. Obesity (Silver Spring) 17: 1839–1847.
4. Sabench Pereferrer F, Hernandez Gonzalez M, Del Castillo Dejardin D (2011) Experimental metabolic surgery: justification and technical aspects. Obes Surg 21: 1617–1628.
5. Johannessen H, Kodama Y, Zhao CM, Sousa MM, Slupphaug G, et al. (2013) Eating behavior and glucagon-like peptide-1-producing cells in interposed ileum and pancreatic islets in rats subjected to ileal interposition associated with sleeve gastrectomy. Obes Surg 23: 39–49.
6. Earlam R (1983) Bile reflux and the Roux en Y anastomosis. Br J Surg 70: 393–397.
7. Stenstrom B, Furnes MW, Tommeras K, Syversen U, Zhao CM, et al. (2006) Mechanism of gastric bypass-induced body weight loss: one-year follow-up after micro-gastric bypass in rats. J Gastrointest Surg 10: 1384–1391.
8. Furnes MW, Tømmerås K, Arum CJ, Zhao CM, Chen D (2008) Gastric bypass surgery causes body weight loss without reducing food intake in rats. Obes Surg 18: 415–422.
9. Furnes MW, Stenström B, Tømmerås K, Skoglund T, Dickson SL, et al. (2008) Feeding behavior in rats subjected to gastrectomy or gastric bypass surgery. Eur Surg Res 40: 279–288.
10. DeMeester TR, Fuchs KH, Ball CS, Albertucci M, Smyrk TC, et al. (1987) Experimental and clinical results with proximal end-to-end duodenojejunostomy for pathologic duodenogastric reflux. Ann Surg 206: 414–426.
11. DeSesso JM, Jacobson CF (2001) Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. Food Chem Toxicol 39: 209–228.
12. Anthone GJ, Lord RV, DeMeester TR, Crookes PF (2003) The duodenal switch operation for the treatment of morbid obesity. Ann Surg 238: 618–627; discussion 627–618.
13. Laurenius A, Taha O, Maleckas A, Lonroth H, Olbers T (2010) Laparoscopic biliopancreatic diversion/duodenal switch or laparoscopic Roux-en-Y gastric bypass for super-obesity-weight loss versus side effects. Surg Obes Relat Dis 6: 408–414.
14. Strain GW, Gagner M, Inabnet WB, Dakin G, Pomp A (2007) Comparison of effects of gastric bypass and biliopancreatic diversion with duodenal switch on weight loss and body composition 1–2 years after surgery. Surg Obes Relat Dis 3: 31–36.
15. Prachand VN, Davee RT, Alverdy JC (2006) Duodenal switch provides superior weight loss in the super-obese (BMI > or = 50 kg/m2) compared with gastric bypass. Ann Surg 244: 611–619.

16. Prachand VN, Ward M, Alverdy JC (2010) Duodenal switch provides superior resolution of metabolic comorbidities independent of weight loss in the super-obese (BMI > or = 50 kg/m2) compared with gastric bypass. J Gastrointest Surg 14: 211–220.
17. Sovik TT, Taha O, Aasheim ET, Engstrom M, Kristinsson J, et al. (2010) Randomized clinical trial of laparoscopic gastric bypass versus laparoscopic duodenal switch for superobesity. Br J Surg 97: 160–166.
18. Sovik TT, Aasheim ET, Taha O, Engstrom M, Fagerland MW, et al. (2011) Weight loss, cardiovascular risk factors, and quality of life after gastric bypass and duodenal switch: a randomized trial. Ann Intern Med 155: 281–291.
19. Hill JO, Wyatt HR (1999) Relapse in obesity treatment: biology or behavior? Am J Clin Nutr 69: 1064–1065.
20. Kodama Y, Zhao CM, Kulseng B, Chen D (2010) Eating behavior in rats subjected to vagotomy, sleeve gastrectomy, and duodenal switch. J Gastrointest Surg 14: 1502–1510.
21. Dapri G, Cadiere GB, Himpens J (2011) Superobese and super-superobese patients: 2-step laparoscopic duodenal switch. Surg Obes Relat Dis 7: 703–708.
22. Zorrilla EP, Inoue K, Fekete EM, Tabarin A, Valdez GR, et al. (2005) Measuring meals: structure of prandial food and water intake of rats. Am J Physiol Regul Integr Comp Physiol 288: R1450–1467.
23. Iossa S, Lionetti L, Mollica MP, Barletta A, Liverini G (1999) Energy intake and utilization vary during development in rats. J Nutr 129: 1593–1596.
24. Roberts SB, Dallal GE (2005) Energy requirements and aging. Public Health Nutr 8: 1028–1036.
25. Topart P, Becouarn G, Ritz P (2012) Weight loss is more sustained after biliopancreatic diversion with duodenal switch than Roux-en-Y gastric bypass in superobese patients. Surg Obes Relat Dis.
26. MacLean LD, Rhode BM, Nohr CW (2000) Late outcome of isolated gastric bypass. Ann Surg 231: 524–528.
27. Mathes CM, Spector AC (2012) Food selection and taste changes in humans after Roux-en-Y gastric bypass surgery: a direct-measures approach. Physiol Behav 107: 476–483.
28. Laurenius A, Larsson I, Bueter M, Melanson KJ, Bosaeus I, et al. (2012) Changes in eating behaviour and meal pattern following Roux-en-Y gastric bypass. Int J Obes (Lond) 36: 348–355.
29. Otsuka R, Tamakoshi K, Yatsuya H, Murata C, Sekiya A, et al. (2006) Eating fast leads to obesity: findings based on self-administered questionnaires among middle-aged Japanese men and women. J Epidemiol 16: 117–124.
30. Takayama S, Akamine Y, Okabe T, Koya Y, Haraguchi M, et al. (2002) Rate of eating and body weight in patients with type 2 diabetes or hyperlipidaemia. J Int Med Res 30: 442–444.
31. Furnes MW, Zhao CM, Chen D (2009) Development of obesity is associated with increased calories per meal rather than per day. A study of high-fat diet-induced obesity in young rats. Obes Surg 19: 1430–1438.
32. Astrup A, Gotzsche PC, van de Werken K, Ranneries C, Toubro S, et al. (1999) Meta-analysis of resting metabolic rate in formerly obese subjects. Am J Clin Nutr 69: 1117–1122.
33. Bueter M, Lowenstein C, Olbers T, Wang M, Cluny NL, et al. (2010) Gastric bypass increases energy expenditure in rats. Gastroenterology 138: 1845–1853.
34. Nestoridi E, Kvas S, Kucharczyk J, Stylopoulos N (2012) Resting Energy Expenditure and Energetic Cost of Feeding Are Augmented after Roux-en-Y Gastric Bypass in Obese Mice. Endocrinology.
35. Bueter M, le Roux CW (2011) Gastrointestinal hormones, energy balance and bariatric surgery. Int J Obes (Lond) 35 Suppl 3: S35–39.
36. Bays HE (2004) Current and investigational antiobesity agents and obesity therapeutic treatment targets. Obes Res 12: 1197–1211.
37. Tschop MH, Speakman JR, Arch JR, Auwerx J, Bruning JC, et al. (2012) A guide to analysis of mouse energy metabolism. Nat Methods 9: 57–63.
38. Butler AA, Kozak LP (2010) A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. Diabetes 59: 323–329.
39. Zelova J, Sumbera R, Okrouhlik J, Skliba J, Lovy M, et al. (2011) A seasonal difference of daily energy expenditure in a free-living subterranean rodent, the silvery mole-rat (Heliophobius argenteocinereus; Bathyergidae). Comp Biochem Physiol A Mol Integr Physiol 158: 17–21.
40. Miller GA, Chapman JP (2001) Misunderstanding analysis of covariance. J Abnorm Psychol 110: 40–48.
41. Owen SV, Froman RD (1998) Uses and abuses of the analysis of covariance. Res Nurs Health 21: 557–562.
42. Leff DR, Heath D (2009) Surgery for obesity in adulthood. BMJ 339: b3402.
43. Liou AP, Paziuk M, Luevano JM, Jr., Machineni S, Turnbaugh PJ, et al. (2013) Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. Sci Transl Med 5: 178ra141.
44. Lee WJ, Ser KH, Lee YC, Tsou JJ, Chen SC, et al. (2012) Laparoscopic Roux-en-Y Vs. Mini-gastric Bypass for the Treatment of Morbid Obesity: a 10-Year Experience. Obes Surg.
45. Victorzon M (2012) An update on sleeve gastrectomy. Minerva Chir 67: 153–163.
46. Mathes CM, Spector AC (2012) Food selection and taste changes in humans after Roux-en-Y gastric bypass surgery: A direct-measures approach. Physiol Behav.
47. Ashrafian H, Bueter M, Ahmed K, Suliman A, Bloom SR, et al. (2010) Metabolic surgery: an evolution through bariatric animal models. Obes Rev 11: 907–920.

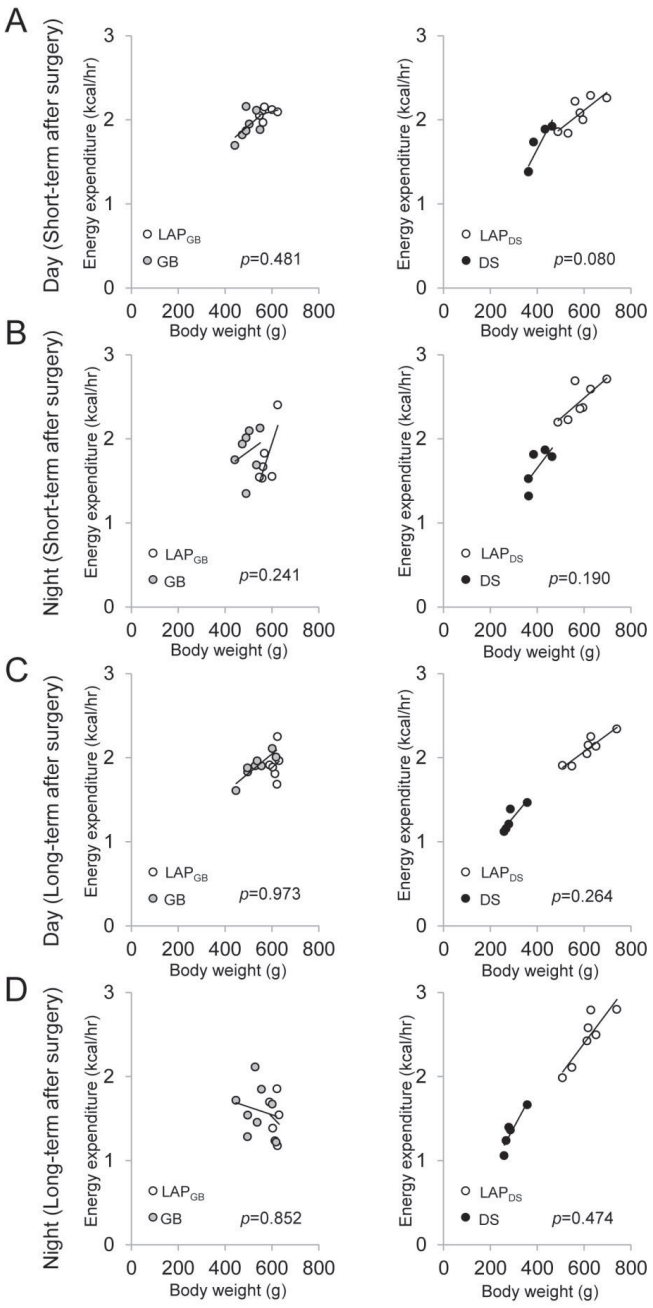
Supplementary Information

Mechanistic Comparison between Gastric Bypass vs. Duodenal Switch with Sleeve Gastrectomy in Rat Models

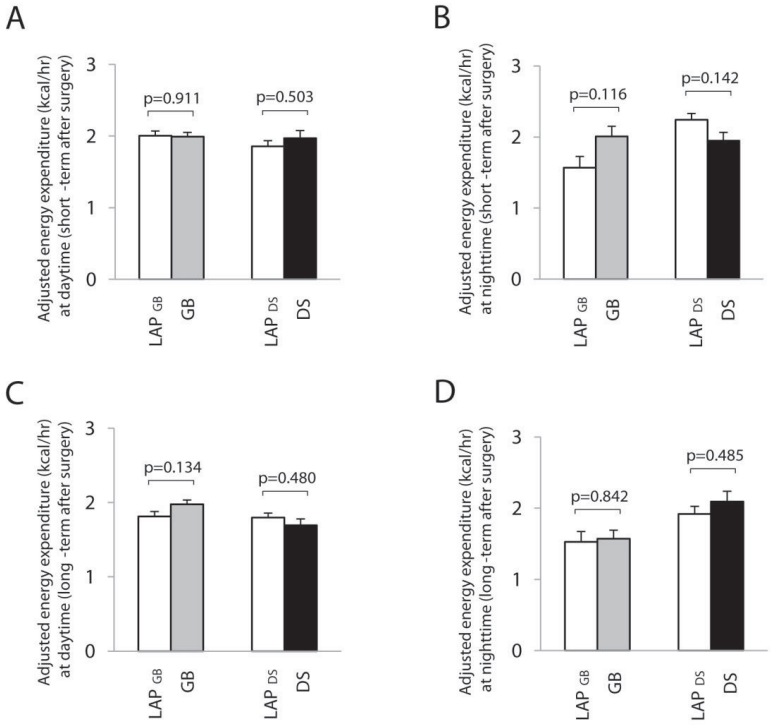
Yosuke Kodama[1], Helene Johannessen[1], Marianne W. Furnes[1], Chun-Mei Zhao[1], Gjermund Johnsen[2], Ronald Mårvik[2], Bård Kulseng[1,2], Duan Chen[1,2]

[1]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; [2]Department of Surgery, Saint Olav's University Hospital, Trondheim, Norway

Corresponding author: Professor Duan Chen, MD, PhD, Erling Skjalgssons Gate 1, Eastside 3rd Floor, Laboratory Centre of St. Olav's Hospital, 7006 Trondheim, Norway. Tel: +47 725 73320. Fax: +47 725 76612. Mob: +47 984 09 675. E-mail: duan.chen@ntnu.no.



Supplementary Fig. 1: Scatterplot of energy expenditure against body weight for each of the experimental groups. LAP $_{GB\ or\ DS}$: laparotomy as control for GB or DS; GS: gastric bypass; DS: Duodenal switch.



Supplementary Fig. 2: Adjust energy expenditure (kcal/hr) by ANCOVA (data from Figure. 5). LAP $_{GB\ or\ DS}$: laparotomy as control for GB or DS; GS: gastric bypass; DS: Duodenal switch. Means ± SEM

Supplementary table 1. CLAMS measurements of normal rats at day 1 and 21 one week after 24 hours training with CLAMS cage. Data are expressed as means ± SEM. ns: not significant between day 1 *vs.* day 21.

	Parameter	Day 1	Day 21
Day	Food Intake (g)	6.76±0.67	7.36±0.60 ^{ns}
	Food Intake (g/100g body weight)	1.38±0.14	1.46±0.14 ^{ns}
	Calories intake (kcal)	17.36±1.72	18.90±1.53 ^{ns}
	Calories intake (kcal/100g body weight)	3.54±0.35	3.74±0.36 ^{ns}
	Number of meals	14.17±1.66	13.50±1.48 ^{ns}
	Meal size (g/meal)	0.52±0.10	0.56±0.05 ^{ns}
	Meal size (kcal/meal)	1.34±0.25	1.45±0.12 ^{ns}
	Meal duration (min)	18.98±2.07	21.10±1.95 ^{ns}
	Meal duration (min/meal)	1.45±0.27	1.61±0.15 ^{ns}
	Intermeal interval (min)	49.44±5.95	50.43±4.48 ^{ns}
	Satiety ratio (min/g)	101.03±9.37	90.73±6.44 ^{ns}
	Water intake (mL)	2.17±0.64	2.24±0.42 ^{ns}
	Water intake (mL/100g body weight)	0.45±0.13	0.45±0.09 ^{ns}
	Water intake during one interval (mL/time)	0.25±0.07	0.28±0.05 ^{ns}
	Ambulatory activity	1305.17±318.30	1451.17±326.57 ^{ns}
Night	Food Intake (g)	19.38±0.72	21.07±0.78 ^{ns}
	Food Intake (g/100g body weight)	3.94±0.13	4.15±0.19 ^{ns}
	Calories intake (kcal)	49.80±1.85	54.16±2.00 ^{ns}
	Calories intake (kcal/100g body weight)	10.13±0.33	10.67±0.50 ^{ns}
	Number of meals	29.17±2.65	28.83±3.89 ^{ns}
	Meal size (g/meal)	0.68±0.04	0.79±0.09 ^{ns}
	Meal size (kcal/meal)	1.75±0.11	2.02±0.24 ^{ns}
	Meal duration (min)	62.62±3.01	63.66±2.10 ^{ns}
	Meal duration (min/meal)	2.21±0.15	2.36±0.24 ^{ns}
	Intermeal interval (min)	22.57±1.80	23.88±2.95 ^{ns}
	Satiety ratio (min/g)	32.98±1.22	30.20±1.05 ^{ns}
	Water intake (mL)	16.14±0.74	17.70±0.60 ^{ns}
	Water intake (mL/100g body weight)	3.28±0.12	3.48±0.13 ^{ns}
	Water intake during one interval (mL/time)	0.87±0.15	0.89±0.09 ^{ns}
	Ambulatory activity	5292.00±1166.98	4484.00±833.95 ^{ns}

Supplementary table 2. Plasma levels of cytokines in rats after gastric bypass (GB) and duodenal switch (DS) compared with the age-matched laparotomy-operated groups (LAP_{GB} or LAP_{DS}, respectively). Data are expressed as means ± SEM. ns: not significant between LAP_{GB} *vs.* GB or LAP_{DS} *vs.* DS.

Cytokine	LAP_{GB}	GB	LAP_{DS}	DS
IL1α (pg/mL)	66.20±21.31	33.48±21.01 ^{ns}	94.34±33.31	124.84±19.20 ^{ns}
IL-1β (pg/mL)	74.06±22.89	32.63±11.51 ^{ns}	310.22±175.16	215.53±87.70 ^{ns}
IL-2 (pg/mL)	201.62±59.23	112.19±46.99 ^{ns}	190.21±40.10	373.21±69.13 ^{ns}
IL-4 (pg/mL)	10.62±2.48	27.33±22.06 ^{ns}	39.37±17.56	41.69±9.90 ^{ns}
IL-5 (pg/mL)	38.08±8.80	41.19±10.48 ^{ns}	129.45±26.03	106.05±18.71 ^{ns}
IL-6 (pg/mL)	111.53±51.69	208.33±176.17 ^{ns}	216.85±82.11	158.36±49.23 ^{ns}
IL-10 (pg/mL)	213.33±29.71	145.88±31.72 ^{ns}	534.60±139.86	588.50±74.00 ^{ns}
IL-13 (pg/mL)	19.52±8.16	4.47±1.23 ^{ns}	61.05±33.76	55.41±19.65 ^{ns}
GM-CSF (pg/mL)	9.42±4.59	1.23±0.41 ^{ns}	41.06±24.68	30.81±12.69 ^{ns}
IFNγ (pg/mL)	53.90±25.31	106.47±105.90 ^{ns}	76.90±38.39	75.45±24.11 ^{ns}
TNFα (pg/mL)	9.25±6.98	3.72±0.35 ^{ns}	65.66±46.06	56.94±29.70 ^{ns}