

Obesity Induced Dysfunction of Gastric Vagal Afferent Signalling

Stephen James Kentish

Discipline of Medicine

School of Medicine

The University of Adelaide

July 2013



This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Stephen James Kentish and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis (as listed below*) resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Stephen James Kentish

BSc. (Biomed) (Honours, First Class)

July 11th 2013

TABLE OF CONTENTS

Acknowledgements	10
Conference Proceedings	12
Abbreviations	14
Abstract	17
CHAPTER 1: GENERAL INTRODUCTION	20
1.1 OBESITY	21
1.1.1 Causes	25
1.1.1.1 Environmental	25
1.1.1.2 Genetic	29
1.2 REGULATION OF FOOD INTAKE	32
1.2.1 Central mechanisms	37
1.2.1.1 Hypothalamic control of feeding	37
1.2.1.2 Reward pathway involvement in feeding	44
1.2.2 Peripheral regulation of food intake	48
1.2.2.1 Adipose tissue	51
1.2.2.2 Proximal gastrointestinal tract	56
1.2.2.2.1 Nutrient sensing	57
1.2.2.2.1.1 Small intestine	57
1.2.2.2.1.2 Stomach	58
1.2.2.2.2 Innervation of the gastrointestinal tract	60
1.2.2.2.2.1 Enteric nervous system	63
1.2.2.2.2.2 Spinal afferents	65
1.2.2.2.2.3 Vagal afferents	67
1.2.2.2.2.3.1 Tension receptors	69

1.2.2.2.2.3.2 Mucosal receptors	70
1.2.2.2.2.4 Vagal afferent mediators	78
1.2.2.2.2.4.1 Gastric mediators	78
Leptin	78
Neuropeptide W	84
Ghrelin	85
1.2.2.2.2.4.2 Intestinal mediators	87
CCK	87
GLP-1	89
PYY	91
1.2.2.2.2.5 The vagus in obesity	93
1.3 HYPOTHESES	95
CHAPTER 2: DIET INDUCED ADAPTATION OF VAGAL AFFERENT FUNCTION	96
2.1 ABSTRACT	101
2.2 INTRODUCTION	102
2.3 MATERIALS AND METHODS	105
2.3.1 Ethical approval	105
2.3.2 Short term restriction of food intake	105
2.3.3 High fat diet model	105
2.3.4 <i>In vitro</i> mouse gastro-oesophageal afferent preparation	106
2.3.5 Characterisation of gastro-oesophageal vagal afferent properties	106
2.3.6 Effect of ghrelin on the mechanosensitivity of vagal afferents	108

2.3.7 Drugs	109
2.3.8 Quantitative reverse-transcription polymerase chain reaction	109
2.3.9 Tracing studies	111
2.4 RESULTS	113
2.4.1 Short term restriction of food intake reduces mechanosensitivity of vagal afferents	113
2.4.2 Effects of long term alterations in diet	113
2.4.3 Anatomy of vagal afferent endings and ghrelin containing cells in the gastric mucosa	114
2.4.4 Ghrelin receptor expression in vagal afferent pathways	115
2.4.5 Vagal afferent responses to ghrelin are altered by changes in food intake	115
2.5 DISCUSSION	118
Figure 2.1	123
Figure 2.2	125
Figure 2.3	127
Figure 2.4	129
Figure 2.5	131
Figure 2.6	133
Figure 2.7	135
CHAPTER 3: GASTRIC VAGAL AFFERENT MODULATION BY LEPTIN IS INFLUENCED BY FOOD INTAKE STATUS	137
3.1 ABSTRACT	142
3.2 INTRODUCTION	144

3.3 MATERIALS AND METHODS	146
3.3.1 Ethical approval	146
3.3.2 Mice	146
3.3.3 <i>In vitro</i> mouse gastro-oesophageal afferent preparation	147
3.3.4 Characterization of gastric vagal afferent properties	147
3.3.5 Single unit vagal afferent recordings	148
3.3.6 Drugs	149
3.3.7 Nodose ganglia quantitative RT-PCR	149
3.3.8 Anterograde tracing	150
3.3.9 Retrograde tracing	151
3.3.10 Laser capture microdissection	152
3.3.11 Immunohistochemistry	153
3.3.12 Statistical analysis	154
3.4 RESULTS	155
3.4.1 Effect of leptin on gastric vagal afferent mechanosensitivity in fed and fasted mice	155
3.4.2 Effect of leptin in diet-induced obese mice	156
3.4.3 Leptin receptor expression	157
3.4.4 Leptin localization	157
3.4.5 The second messaging systems utilized by leptin	158
3.5 DISCUSSION	160
Figure 3.1	166
Figure 3.2	168
Figure 3.3	170
Figure 3.4	172

Figure 3.5	174
Figure 3.6	176
Figure 3.7	178
Supplementary Figure 3.1	180
Supplementary Figure 3.2	182
Supplementary Figure 3.3	184
CHAPTER 4: A CHRONIC HIGH FAT DIET ALTERS THE HOMOLOGOUS AND HETEROLOGOUS CONTROL OF SATIETY PEPTIDE RECEPTOR EXPRESSION	186
4.1 ABSTRACT	189
4.2 INTRODUCTION	190
4.3 MATERIALS AND METHODS	192
4.3.1 Ethical approval	192
4.3.2 High fat diet model	192
4.3.3 Cell culture	192
4.3.4 RNA isolation	194
4.3.5 Quantitative RT-PCR	194
4.3.6 Quantification of plasma concentrations of peptides	195
4.3.7 Data analysis	196
4.4 RESULTS	197
4.4.1 Effect of high fat diet on body weight and plasma hormones	197
4.4.2 Effect of leptin on expression of Ob-R, GHS-R, GPR7 and CCK1R in SLD and HFD vagal cell bodies	197

4.4.3 Effect of ghrelin on expression of Ob-R, GHS-R, GPR7 and CCK1R in SLD and HFD vagal cell bodies	198
4.4.4 Effect of NPW on expression of Ob-R, GHS-R, GPR7 and CCK1R in SLD and HFD vagal cell bodies	199
4.5 DISCUSSION	201
Figure 4.1	206
Figure 4.2	208
Figure 4.3	210
Figure 4.4	212
CHAPTER 5: ALTERED GASTRIC VAGAL MECHANOSENSITIVITY IN DIET INDUCED OBESITY PERSISTS ON RETURN TO NORMAL CHOW AND IS ACCOMPANIED BY INCREASED FOOD INTAKE	214
5.1 ABSTRACT	217
5.2 INTRODUCTION	219
5.3 MATERIALS AND METHODS	222
5.3.1 Ethical approval	222
5.3.2 Animals	222
5.3.3 <i>In vitro</i> mouse gastro-oesophageal afferent preparation	222
5.3.4 Characterization of gastric vagal afferent properties	223
5.3.5 Effect of leptin on the mechanosensitivity of vagal afferents	224
5.3.6 Data recording and analysis	224

5.3.7 Nodose ganglia quantitative RT-PCR	224
5.3.8 Retrograde tracing	225
5.3.9 Laser capture microdissection	226
5.3.10 Plasma hormone measurements	226
5.3.11 Statistical analysis	227
5.4 RESULTS	228
5.4.1 Diet induced changes to mouse weight, fat mass, food consumption and plasma peptide levels	228
5.4.2 High fat diet induced changes in gastric mechanosensitivity are not altered by reverting to 'normal' chow feed	229
5.4.3 Leptin's effects on gastric vagal afferent mechanosensitivity are dependent on diet	229
5.4.4 Diet induced changes in the expression of leptin receptor in vagal afferents	230
5.5 DISCUSSION	231
Figure 5.1	235
Figure 5.2	237
Figure 5.3	239
Figure 5.4	241
Figure 5.5	243
CHAPTER 6: CONCLUSIONS	245
REFERENCES	253

ACKNOWLEDGEMENTS

I would like to sincerely thank my primary supervisor Associate Professor Amanda Page for the tireless supervision she has provided me over the past three years. Without the support she provided I doubt that I would have been able to complete this thesis. I am truly thankful for her guidance and support in allowing me to develop research ideas and implement them. I am furthermore grateful for the opportunities provided to me to attend a number of local and international scientific meetings.

I am also grateful to my co-supervisor, Professor Ashley Blackshaw. Whilst for the majority of my candidature he was not in the country; the advice and guidance he provided both remotely and in person were well appreciated.

I would also like to offer my thanks to a Tracey O'Donnell for her contributions in caring for the obese mice and Gary Wittert for his excellent advice at various stages of my candidature as well as acting as a pseudo supervisor. I would also like to thank every member of the Nerve Gut Laboratory for their support. My time in this lab has made me realise just how specialised the work is and how much effort is put in. I wish every member of the lab every success imaginable.

On a more personal note I wish to thank my parents for seeing me through the initial stages of my education and encourage me to take my desire to learn as far as I have (Pete I think I may have beaten your PhD completion record by just a tad).

I would also like to give very special endless heartfelt thanks to my partner of 6 years, Alice. She who has had to endure the mountains of paper, the ridiculous hours, the stress that shaves years off your life and all with a smile on her face and a kind word to be said.

“Far and away the best prize that life offers is the chance to work hard at work worth doing.”- Thomas Jefferson

CONFERENCE PROCEEDINGS

Kentish S.J., O'Donnell T., Wittert G., Page A.J. (2012) Obesity induced suppression of gastric satiety signals are only partially reversed by dietary change. Presented at NGM 2012, Bologna, Italy.

Kentish S.J., O'Donnell T., Wittert G., Page A.J. (2012) Obesity induced suppression of gastric satiety signals are not reversed by dietary change. Presented at SSIB 2012, Zurich, Switzerland.

Kentish S.J., Wittert G., Blackshaw L.A., Page A.J. (2012) A chronic high fat diet alters the homologous and heterologous control of satiety peptide receptor expression. Presented at DDW 2012, San Diego, United States of America.

Kentish S.J., O'Donnell T.A., Wittert G., Blackshaw L.A., Page A.J. (2011) High fat diet feeding switches the second messenger system activated by leptin on vagal afferents. Presented at ANZOS 2011, Adelaide, Australia.

Kentish S., O'Donnell T., Wittert G., Blackshaw L.A. & Page A. (2011) The "leptin switch" mechanism in gastric vagal afferents. Presented at Digestive Disease Week 2011, Chicago, United States of America.

Kentish S., O'Donnell T., Li H., Wittert G., Blackshaw L.A. & Page A. (2011) Obesity switches the effect of leptin on vagal afferent mechanosensitivity. Presented at Digestive Disease Week 2011, Chicago, United States of America .

Kentish S., T., Wittert G., Blackshaw L.A. & Page A. (2010) Increased Ghrelin Inhibition of Vagal Afferents in an Obese Mouse Model. Presented at Digestive Disease Week 2010, New Orleans, United States of America.

ABBREVIATIONS

ACh; Acetylcholine

AgRP; Agouti-related peptide

α -MSH; α -Melanocyte-stimulating hormone

ANOVA; Analysis of variance

AP; Area postrema

ARC; Arcuate nucleus

AT; Adaptive thermogenesis

BBB; Blood brain barrier

BKCa; Large conductance calcium activated potassium channel

CART; Cocaine- and amphetamine-regulated transcript

CB1; Cannabinoid receptor 1

CCK; Cholecystokinin

CCK1R; CCK receptor 1

ChAT; Choline acetyltransferase

CNS; Central nervous system

CT; Cycle threshold

db/db; Leptin receptor knockout mouse

DMH; Dorsal medial hypothalamus

DMV; Dorsal motor nucleus of the vagus

DRG; Dorsal root ganglion

ENS; Enteric nervous system

GABA; γ -Amino butyric acid

GHS-R; Growth hormone secretagogue receptor

GPCRs; G-protein coupled receptors

GPR7; G-Protein coupled receptor 7 (endogenous receptor for neuropeptide W)

IGLE; Intraganglionic laminar endings

IL-; Interleukin

IMA; Intramuscular array

IP; Intraperitoneal

IRS; Insulin receptor substrate

JAK; Janus kinase

LDL; low density lipoprotein

LepR; Leptin receptor

LH; Lateral hypothalamus

MCR; Melanocortin receptor

mRNA; Messenger RNA

NANC; Non-adrenergic non-cholinergic

NPW; Neuropeptide W

NPY; Neuropeptide Y

NTS; Nucleus tractus solitarii

Ob/Ob; Leptin knockout mouse

PDE; Phosphodiesterase

PI3K; Phosphatidylinositide 3-kinases

PLC; Phospholipase C

POMC; Pro-opiomelanocortin

PYY; Peptide YY

PVN; Paraventricular nucleus

QRT-PCR; Quantative reverse transcription polymerase chain reaction

RMR; Resting metabolic rate

RNA; Ribonucleic acid

RT; Reverse transcription

RYGB; Roux-en-Y gastric bypass

5-HT; 5-hydroxytryptamine

SEM; Standard error of the mean

SGLT1; Sodium-glucose transporter 1

SOCS; Suppressor of cytokine signalling

STAT; Signal transducer and activator of transcription

TRPC; Transient receptor potential: Canocial subtype

TRPV; Transient receptor potential: Vannilloid subtype

UCP; Uncoupling protein

VTA; Ventral tegmental area

ABSTRACT

Background: The stomach has the ability to respond to chemical and mechanical stimuli to mediate satiety through vagal pathways. Within the stomach specialised endocrine and epithelial cells synthesise and secrete leptin and ghrelin, which influence food intake through vagal afferent pathways. However, it remains to be determined if mechanosensitive gastric vagal afferent signalling is disrupted in obesity and whether this may play a role in the overconsumption of energy required for the maintenance of diet-induced obesity. Furthermore, whether leptin can modulate mechanically sensitive gastric vagal afferents and whether any ability of leptin and ghrelin to modulate mechanically sensitive endings is altered in obesity has not been conclusively determined.

Aims: To determine in lean mice and in high fat diet induced obese mice:

- 1) The effect of gastric peptides ghrelin and leptin on gastric vagal afferent mechanosensitivity.
- 2) The effect of gastric peptides on the expression of their own and other peptide receptors.
- 3) The reversibility of diet-induced obesity.

Methods: Lean and diet-induced obese mice were created by feeding 8 week old female C57BL/6 mice a standard chow diet (N=4-20; 7% energy from fat) or a high-fat diet (N=4-20; 60% of energy from fat) respectively. An *in vitro* gastro-oesophageal vagal flat sheet preparation was utilised to determine the

mechanosensitivity of vagal afferent endings and the effect of leptin, ghrelin and diet-induced obesity on this mechanosensitivity. Messenger RNA (mRNA) content in nodose ganglia was measured by QRT-PCR. Specific gastric vagal afferent cell bodies were identified by retrograde labelling and this technique was combined with QRT-PCR to determine mRNA content in specific gastric cell bodies. Anterograde tracing by injection of tracer into the nodose ganglia allowed visualisation of the distribution of gastric vagal afferents in relation to leptin and ghrelin positive cells. Nodose ganglia were cultured overnight in medium containing leptin, ghrelin or neuropeptide W (NPW) followed by QRT-PCR to determine any homologous or heterologous receptor expression regulation.

Results: Diet-induced obesity caused a reduction in the mechanosensitivity of gastric tension receptors. Furthermore, it increased the inhibitory effect of ghrelin on gastric vagal afferent mechanosensitivity and resulted in a switch in the effect of leptin from potentiating to inhibitory. The gut peptides leptin, ghrelin and NPW modified the mRNA content of their own and each other's receptors in a manner that was dependent on dietary group. Placing obese mice back on a chow diet resulted in an initial weight loss but subsequent increased food consumption and weight gain. The decrease in mechanosensitivity caused by the high fat diet was not reversible by placing diet-induced obese mice back on a chow diet and the effects of leptin were only partially reversed.

Conclusions: Vagal afferent function is altered in diet-induced obesity to the extent that both the baseline response and the effects of leptin and ghrelin may act to facilitate increased food intake. Given the lack of reversibility of changes observed in diet-induced obesity this suggests that gastric vagal afferents may play a role in the maintenance of obesity and may act to oppose weight loss.

CHAPTER 1: GENERAL INTRODUCTION

Control of food intake is critical to maintain a healthy body weight. If left uncontrolled too much or too little food can be consumed resulting in obesity or emaciation respectively. A number of mechanisms act to sense and control food intake and energy expenditure to maintain a stable body weight. The gastrointestinal tract, in particular, is uniquely placed to be able to respond to food intake to control gastrointestinal function and food intake through neural and hormonal mechanisms. The following is an overview of obesity and the central and peripheral neural and hormonal mechanisms that play a part in the control of food intake.

1.1 OBESITY

Obesity is a major health concern for the majority of the developed world. In 2008 there were an estimated 1.5 billion people worldwide who were overweight (BMI 25-29.9) and a further 500 million that were obese (BMI >30) [1]. Global obesity rates have more than doubled since 1980 [2]. Obesity has been thought to cause upwards of 3 million premature deaths each year. Whilst originally believed to be a problem in the more developed nations of late there has been an alarming increase in obesity in developing countries [2]. It is suggested that by 2020 close to 80% of the adult population in some countries will be considered overweight or obese [3]. Such an increase would lead to a substantial increase of the burden on health systems, economies and personal lives.

Whilst increased adiposity that occurs in obesity is the most obvious and common symptom of obesity the consequences of this are far more reaching, with numerous conditions believed to be partially caused by the development of obesity. From Figure 1.1 it is very apparent that obesity affects nearly every facet of the body. This indicates why it is so important to understand why obesity develops and possible ways of effectively intervening and reversing the detrimental weight gain in hopes of alleviating some of the more dangerous co- morbidities it can cause.

NOTE:

This figure/table/image has been removed
to comply with copyright regulations.
It is included in the print copy of the thesis
held by the University of Adelaide Library.

Figure 1.1: Medical conditions associated with obesity. As displayed the development of obesity can have a detrimental effect on almost all tissue types within the body. Figure compiled from [4].

1.1.1 Causes

Simplistically obesity is caused by an excess of energy being consumed. However, evidence suggests an array of factors including both genetic and environmental contribute to the development of obesity. The following is an overview of just some of the evidence that supports the involvement of environmental and genetic factors in the development of obesity.

1.1.1.1 Environment

Food Environment

The increase in obesity prevalence has been observed to be parallel with changing of diets to incorporate more processed foods, which are typically high in saturated fat and sugar [5, 6]. The impact of changing the food available for consumption has been demonstrated previously. For example, Aboriginal Australian populations display a higher prevalence of obesity when they adopt a western style diet compared to non-Aboriginal Australians consuming the same style diet and Aboriginal Australians still participating in a traditional hunting and gathering lifestyle [7]. This is not an isolated example as similar findings have been observed in Samoans [8], Indians [9], Chinese [10], Native Americans [11], Japanese [12] and Mexican Pima Indians [13].

The modern western diet has a number of facets which makes it a likely major factor in the development of obesity. The first is portion size. Portion size of meals has been reported to be increasing since the 1970's [14]. It has been suggested that the increase in portion size leads to increased energy consumption [15, 16] and also encourages the overconsumption of food [17].

In terms of increasing portion size, fast food appear to be leading the way with some items available at fast food restaurants increasing in size by 2 to 5 times over the last 2 decades [18]. However, some home prepared items including french fries and hamburgers have been shown to be served in larger portions at home than at a restaurant [19]. Interestingly there are regional differences in the portion size of food available for consumption with comparable items in France 25% smaller than in the U.S. [20]. Coincidentally, France also has a lower level of obesity relative to the U.S. [21]. However, this connection is anecdotal and there would undoubtedly be other factors involved in this difference.

Secondly, there is a tendency towards consumption of high-energy dense foods in a western diet [22, 23]. Consumption of food with a high energy density has been shown to lead to overconsumption of energy, which is independent of macronutrient content [24] and portion size [25]. High fat foods have been demonstrated to cause damage to regions of the brain which ironically regulate food intake and cause metabolic disruptions including insulin resistance [26-28]. This suggests that not only are many modern western foods energy dense, but also metabolically toxic.

Energy Expenditure

Given the balance between consumption of food and energy expenditure form the basis for weight regulation it is important to address the role changes in energy expenditure may play in obesity.

Principally, energy expenditure can be divided into three categories:

- 1) *Resting metabolic rate (RMR)*: The energy required to perform the basic physiological processes to sustain life. This component of energy expenditure accounts for the most energy spent at around 50-70%. Body composition has been shown to effect RMR with fat free mass accounting for 60-70% of RMR and fat mass being responsible for as little as 5-7% [29]. Weight loss has been associated with a reduction in RMR of 3-5% [30, 31].

- 2) *Adaptive thermogenesis (AT)*: Accounts for 10% of total energy expenditure. Brown adipose tissue is the site for AT in both rodents and humans [32] and causes the production of heat instead of ATP. Similar to RMR weight loss has been associated with substantial reductions in adaptive thermogenesis which has been postulated to be a mechanism which creates a predisposition to weight regain [33].

- 3) *Activity related energy expenditure*: This component encapsulates the physical activity performed and is the category which can be most easily altered. It accounts for 10-30% of total energy expenditure. Evolution of society has had dramatic impacts on the level of physical activity performed. With increased prevalence of car ownership there has been a decreased amount of travel by foot or bicycle [34]. Modern appliances have reduced the need for physical activity in everyday tasks such as using an electric toothbrush or washing machine compared to their manual counterparts [35]. Along with the reduction in active energy expenditure there has also been an increase in time spent in sedentary activities relative to physical activity. There

are reports that people spend six times more time watching television than exercising or playing sport [34].

Given that weight gain is predicated on energy expenditure being less than intake a sustained elevation of any of the 3 components that make up total energy expenditure should result in weight loss. However, it appears that all three facets of energy expenditure are compromised in a modern society and adapt to prevent successful weight loss.

Sleep

Incidence of partial sleep deprivation has increased to the point where more than 28% of adults in the U.S. sleep for less than 6 hours a night [36]. It has been postulated that reductions in sleep duration may be partially responsible for increased prevalence of obesity [37, 38].

Sleep is a state that requires very little energy with a 15-30% reduction in resting metabolic rate. However, partial sleep deprivation is associated with a reduction in resting metabolic rate [39], increased periods participating in sedentary activity and decreased moderate intensity activity [40, 41]. Shortened sleep duration has also been shown to cause an increase in snacking [42] and preferential increase in consumption of fat, specifically saturated fat [41, 43]. Sleep deprivation has also been reported to cause increased feelings of hunger [44, 45] and decreased satiety [46], which may play a role in the overconsumption of food required to gain weight.

Sleep deprivation is also associated with a poorer metabolic status exhibiting decreased glucose tolerance [47] and insulin sensitivity [48, 49]. Thus sleep deprivation is identified as a risk factor for the development of metabolic conditions including type 2 diabetes [50].

There are increased levels of cortisol in partially sleep deprived individuals [47, 48]. This may, at least in part, be responsible for the development of obesity as elevated cortisol levels have been linked to increased visceral adiposity [51].

Shortened sleep duration whilst attempting to lose weight through caloric restriction resulted in a 55% reduction in adiposity loss and 60% increase in fat free mass loss [39]. This indicates that changes that occur as a result in sleep deprivation act to preserve adipose mass.

1.1.1.2 Genetics

It has been suggested that the genetic factors account for between 30% and 70% of obesity [52, 53]. The case for genetic control over predisposition to obesity was supported by a study in monozygotic twins who were overfed by 1000kcal/day [54]. There was variation in the amounts of total weight, body fat and muscle mass gained within the study, but there was little difference between siblings in a pair of twins, demonstrating the importance of genetic factors on weight gain [54]. Further evidence of the importance of inherited genetic factors in controlling body weight is from the findings of a study that looked at the body weight of adopted children [55]. The study found the body

weight classification of the adopted children as adults was closely related to the BMI of their biological parents, but had no correlation with the BMI of their adoptive parents. However, the infant weight (first 2 years of life) is unrelated to either the maternal or paternal BMI, which suggests genetic programming of body weight may not become active until later in life.

The involvement of genetics in regulating weight has been demonstrated in rodent models exhibiting monogenetic mutations which result in obesity. However, whilst they may have the same end point there are a number of different processes they can affect. Firstly, disruptions to proteins involved in the regulation of feeding such as melanocortin-4 receptor (MCR4) [56] or leptin [57] result in altered eating behaviour resulting in hyperphagia and obesity. Secondly, alterations to the genes that regulate adaptive thermogenesis in brown adipose tissue including members of the uncoupling protein (UCP) family can compromise energy expenditure and thus lead to weight gain as previously demonstrated [58]. This indicates that disruption to either the food intake side or the energy expenditure side of the energy balance equation is sufficient to drive the development of obesity.

Whilst very uncommon, some monogenetic mutations modelled in rodents have also been observed in humans including loss of functional leptin [59, 60], leptin receptor [61], pro-opiomelanocortin (POMC) [62] and MCR4 [63]. However, there are additionally about 30 pleiotropic syndromes that result in obesity along with other traits including Prader-Willi [64], Bardet-Biedl [65], Alström [66] and Borjesson-Forssman-Lehman [67]. These conditions are still

relatively uncommon compared to the overwhelming prevalence of obesity, but illustrate that there are multiple genes whose normal function allows for regulation of body weight.

However, whilst there is ample evidence arguing that genetic predispositions play an important role in the development of obesity the very laws of thermodynamics means that there must be an excess amount of energy to enable accumulation of fat which highlights diet as a major cause of obesity. For the most part dietary intervention as a sole treatment for obesity is ineffective as weight loss is seldom maintained [68]. This suggests that there are alterations in food intake regulatory mechanisms that essentially act to combat reduced levels of adiposity after weight gain has occurred.

1.2 REGULATION OF FOOD INTAKE

Obviously, it is fundamentally important to ensure that only the required amount of food is consumed to produce the appropriate amount of energy, thus limiting any excess being stored as fat. In addition to needing to meet energy requirements there are nutritional needs, which need to be met in order to maintain healthy body composition. Thus, it is important to consume a diet with appropriate levels of carbohydrate, fat and protein as well as vital micronutrients in the form of vitamins and minerals to maintain vital processes. There is some evidence to support the notion that the regulation of food intake and energy expenditure adjusts to ensure an adequate protein intake. The protein leverage hypothesis proposes that the amount of food consumed is determined by the protein requirement [69, 70]. Thus, consumption of a modern western style diet which is relatively high in carbohydrate and fats, results in an increase in energy consumption in an attempt to achieve the required consumption of protein [71] (Figure 1.2). Therefore, it is important to recognise that appetite regulation involves both energy and specific nutrient sensing mechanisms.

For most people the fact that their body weight changes very little over long periods of time is testament to the body's ability to balance consumption and expenditure of energy [72]. Models have been proposed that explain the maintenance of stable body weight. The first was dubbed the set point regulation model [73]. When this model was originally suggested no adipokines had been isolated, yet there were theories that fat may be

producing a signal that the brain picked up and compared it to the ideal level of fatness for the body [73, 74]. It was believed that any difference between the current level of fat and the ideal level would be compensated for by a suitable change in food consumption and/or energy expenditure in order to restore the target level of adiposity [75].

With the discovery of leptin extensive research was conducted to determine whether leptin was acting as this set point signal and indeed strong relationships were found between the degree of adiposity and leptin levels [76]. This concept has often been used to explain why after acute weight loss there is a common re-gaining of all the lost weight, as leptin levels decrease with weight loss [77], which would cause an increase in food consumption, due to a reduction in leptin's anorexigenic effect. However, this would suggest that increased leptin would limit the ability for weight gain to occur, which is obviously not occurring in obesity. A possible explanation is, whilst decreased adiposity is readily corrected through increased hunger and food intake [78-80], increases in adiposity may be allowed, as it is evolutionarily advantageous to allow storage of energy when food is readily available and plentiful in preparation for potential reductions in food availability.

This staunch defence of the lower limit of adiposity indicates why once a high level of adiposity is obtained it can be hard to correct through simple dietary measures, as there are food intake regulatory mechanisms that are actively trying to restore fat mass to the previously attained level. The following is an outline of mechanisms that are involved in controlling food intake and

highlights the multiple interacting pathways both centrally and peripherally responsible for controlling food intake.

NOTE:

This figure/table/image has been removed
to comply with copyright regulations.
It is included in the print copy of the thesis
held by the University of Adelaide Library.

Figure 1.2: The protein leverage hypothesis. The protein leverage hypothesis proposes that the food consumption is occurring to reach a target amount of protein. Thus a balanced diet (dashed line) with a relatively high proportion of protein will lead to lower energy consumption before this target is reached compared to a high carbohydrate and fat diet which is typical of many western diets. Figure based on results of [71].

1.2.1 Central mechanisms

The body utilises a number of mechanisms to communicate its needs for energy and nutrients which in terms of food intake culminates in a conscious feeling of hunger [81]. Subsequently, when sufficient food has been consumed, feelings of satiety and fullness are conveyed. These feelings are controlled centrally, largely within specific regions of the hypothalamus [82, 83]. The hypothalamus acts as the site for integration of circulating hormonal, nutrient and neuronal afferent signals to maintain energy homeostasis. On top of energy homeostatic processes there are also regions of the CNS which control motivation for seeking out of food and hedonic reward pathways. These centres are activated by food intake to provide a feeling of pleasure and reinforcement for consuming specific foods. Below is an overview of the hypothalamic and reward pathway anatomy and how they work together to control feeding behaviour.

1.2.1.1 Hypothalamic control of feeding

The hypothalamus consists of a number of nuclei organised around the third ventricle, which are critical in energy homeostasis. The arcuate nucleus (ARC; located in the mediobasal hypothalamus) is vital in limiting food intake as lesions within this region result in hyperphagia and obesity [84]. Neighbouring the arcuate nucleus are a series of smaller nuclei including the paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH) and the lateral hypothalamic area (LHA) (Figure 1.3). Previous work has suggested that specific nuclei in the hypothalamus are responsible for increasing food intake, 'feeding centres', whilst others are

responsible for reducing it, 'satiety centres' [85]. This was supported by lesions of the LHA and DMH causing decreased food intake which in the case of the LHA lesions progressed to starvation and death [86, 87], on the contrary injury to the VMH and ARC resulted in hyperphagia and obesity [88].

The ARC is believed to play a pivotal role in the integration of signals coming from a variety of locations. The ARC lies in close apposition to the median eminence, which is unique as it lacks a complete blood brain barrier (BBB) [89]. This would allow the ARC to be directly affected by gut hormones and signalling molecules circulating in the blood that are unable to cross the BBB. Some hormones do have the ability to cross the BBB, such as peptide YY (PYY) [90] and glucagon like peptide 1 (GLP-1) [91], whilst others require saturable transport systems to be actively transported across the BBB, such as leptin [92], in order to act on targets usually protected by the BBB.

Within the ARC there are at least two unique sub-populations of neurons that regulate appetite [93, 94]. One of the populations in the medial ARC expresses the orexigenic neuropeptides, neuropeptide Y (NPY) and agouti-related peptide (AgRP) [95]. These neurons project mainly to the ipsilateral PVN [96, 97], but also have endings projecting locally within the ARC [98], and to mesolimbic, midbrain and pontine structures. They can also release γ -aminobutyric acid (GABA) locally to inhibit another sub-population of neurons known as POMC neurons [99]. POMC neurons express the anorexigenic peptides POMC and cocaine and amphetamine regulated transcript (CART) [100]. The POMC is processed into α -melanocyte-stimulating hormone (α -

MSH) [101], which exerts a potent anorexigenic effect via MC4R [102]. This sub-population of neurons signals more diversely through the CNS [103, 104] indicating that this population of neurons may play a role in linking feeding behaviour to other actions that are controlled elsewhere in the CNS. It has been shown that the POMC and NPY neurons can be activated by leptin and ghrelin (both will be detailed later) respectively and simultaneously inhibit the other sub-population enhancing their own response (Figure1.4) [105].

The hypothalamus receives afferent input from the periphery, including the gastrointestinal tract via vagal and spinal afferents. Peripheral vagal afferents terminate in the NTS within the brainstem. From here second order neurons synapse onto multiple hypothalamic nuclei including the ARC [106, 107], LHA [108], DMH [109] and PVN [110, 111] as well as locally onto other nuclei in the brainstem. This anatomical organisation highlights the vagus as a neural link between the periphery and CNS by which peripheral signals can initiate changes in feeding behaviour (the mechanism involved will be detailed later). There is also reciprocal input from the hypothalamus onto NTS neurons which would allow central input to the control of gastrointestinal function [112, 113].

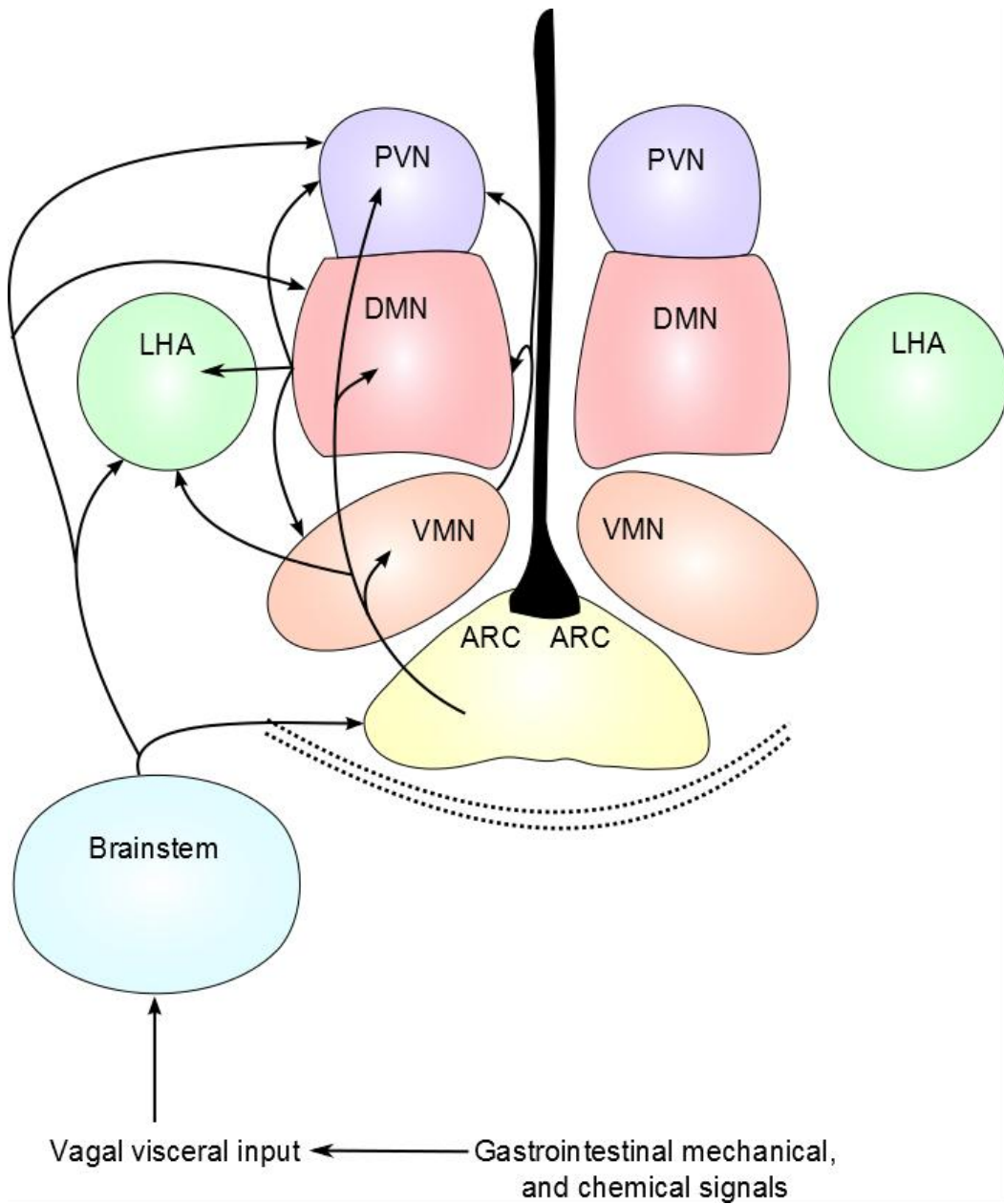


Figure 1.3: The organisation of hypothalamic nuclei involved in food intake regulation. Peripheral signals from the gastrointestinal tract are integrated into the hypothalamus via vagal and spinal afferents travelling through brainstem structures which have synaptic inputs into most of the major food intake regulatory nuclei. ARC, arcuate nucleus; VMN, ventromedial nucleus; LHA, lateral hypothalamic area; DMH, dorsomedial hypothalamus; PVN, paraventricular nucleus.

NOTE:

This figure/table/image has been removed
to comply with copyright regulations.
It is included in the print copy of the thesis
held by the University of Adelaide Library.

Figure 1.4: A schematic illustrating the interaction of leptin and ghrelin with different population of neurons within the arcuate nucleus. Leptin causes simultaneous activation of anorexigenic pro-opiomelanocortin (POMC)/ cocaine and amphetamine regulated transcript (CART) neurons and inhibition of orexigenic agouti-related peptide (AgRP)/ neuropeptide Y (NPY) neurons, whilst ghrelin activates the AgRP/NPY neurons which can inhibit POMC/CART neurons which allows for co-ordinated control of food intake and energy expenditure. LepR; leptin receptor, Ghsr; ghrelin receptor, MC3R; Melanocortin receptor 3, MC4R Melanocortin receptor 4, Y1R; neuropeptide Y receptor 1. Adapted from [114].

1.2.1.2 Reward pathway involvement in feeding

The anatomy of the reward pathway is largely contained within the ventral tegmental area (VTA) and has inputs to a number of other nuclei, most critically into the amygdala, the limbic cortex and the nucleus accumbens. A number of electrophysiological studies have shown that dopaminergic neurons within the VTA are activated by food intake [115-117] and also cues that are predictive of reward. Food consumption leads to activation of the same pathways that are associated with addiction to psychoactive drugs [118]. Highlighting the possibility that addiction to food is possible. The VTA receives inputs from the NTS (within the brainstem) and the hypothalamus [119, 120] and is modulated by vagal nerve stimulation [121, 122]. Furthermore, gastric distension, which is signalled via vagal afferents, causes activation of the amygdala and reduced activity in the anterior insula, neither of which occur in obese humans [123]. Therefore, peripheral gastric signals lead to reward pathway modulation and obesity may be associated with altered reward activation, which may lead to a blunted awareness of body status. However, this conclusion still remains to be proved.

The pleasure that is perceived by eating reinforces the behaviour, which evolutionarily speaking is a desirable attribute, as it promotes the accumulation of energy stores [124]. Constant consumption of palatable food by rats has been shown to create a learned behaviour exhibited by a preference for those palatable foods [125]. As a lot of highly palatable foods have a high energy density this could create a learned behaviour to seek out and consume high energy dense food resulting in hyperphagia and obesity.

Obese humans show a much stronger preference for highly palatable foods than lean individuals [126]. This finding has been attributed to a potential desensitization of the reward response upon attaining highly palatable foods, thus requiring a greater level of activation in order to cause the desirable reward pathway activation [127-129]. Alternatively, there may be a potential genetic predisposition to preferentially consume energy dense palatable food, which may create a predisposition to obesity.

Dopamine is the main neurotransmitter of the reward pathways and lesioning of these neurons leads to a reduction in reward induced food intake [130], whilst electrical stimulation of the same population of neurons actually drives an increase in food intake [131]. There is a reduction in the activation of these neurons in high fat diet fed mice that exists before the actual development of obesity [132]. This suggests that desensitisation of reward pathway activation is a potential mechanism that may drive increased energy intake in order to attain the desired level of food driven reward pathway activation. The importance of this pathway in feeding is highlighted by mice lacking dopamine signalling reducing their food consumption to the point of death from starvation [133, 134]. This indicates that the drive to eat is mediated through activation of dopaminergic neurons activating reward pathways.

As previously described there are receptors within the ARC for gastrointestinal peptides. However, the expression of these receptors within the CNS is not limited to the ARC. For example, the receptor for the peripheral orexigenic peptide, ghrelin, whilst densely expressed in the ARC, is also expressed

throughout the VTA and the laterodorsal tegmental areas [135]. Administration of ghrelin has also been shown to cause an increase in dopamine release via a cholinergic dependant mechanism [136]. Such an action has been credited with playing a role in addiction caused by reward pathway activation in response to alcohol [137], cocaine, amphetamines [138] and also highly palatable food [139]. Direct administration of ghrelin into the VTA has been shown to cause an increase in food intake [140], specifically the intake of palatable foods. This suggests that the effect of ghrelin on food preference may be directly mediated through cholinergic reward pathways, as any food intake preference is lost in the presence of nicotinic or ghrelin receptor antagonists [139, 141]. Together the data on ghrelin's action within the reward pathways suggests that in addition to its effects on appetite, ghrelin appears to have a role in increasing the motivation for seeking out rewarding stimuli, including particular foods based on taste.

Leptin has a well-documented role in the control of food intake (which will be specifically described later) largely attributed to its effect within the ARC. The absence of leptin (ob/ob mice) produces mice that are overweight and hyperphagic. However, if such mice are also crossed with dopamine deficient mice then the resulting offspring are not just lean, but they preferentially do not feed at all [142]. Thus it appears that the hyperphagia and body weight increase caused by leptin deficiency are at least partially dependent on mesolimbic signalling of dopamine. Leptin has also been shown to reduce the preference for stimuli associated with palatable food, suggesting that leptin attenuates the rewarding properties of food [143]. Administration of leptin

directly into the VTA causes a reduction in food intake indicating that in addition to its actions within the hypothalamus leptin is able to act directly within the VTA to modulate feeding behaviour [144]. However, it has also been suggested that leptin can indirectly inhibit VTA activity by acting on lateral hypothalamic neurons, which project onto the VTA to inhibit the release of dopamine [145].

As described above both the hedonic and homeostatic facets to food intake control are influenced by gastrointestinal endocrine and neural regulation. It is therefore of paramount importance when considering the role of central pathways in mediating food intake to consider peripheral appetite regulatory mechanisms.

1.2.2 Peripheral Regulation of Food Intake

Within the periphery a number of organs including the pancreas, liver, adipose and gastrointestinal tract detect and monitor the current metabolic status. Changes to the metabolic state i.e. food deprivation is met with changes in neural and hormonal signals which can act peripherally or centrally to correct the disturbance. The peripheral signals involved in the regulation of food intake can be broadly classified into either long-term or short-term control. Long term control tends to be largely mediated by levels of circulating hormones, some of which are released by adipose tissue. The short term control of food intake is largely controlled by neural and endocrine mediators released by the gastrointestinal tract, usually in response to food, to control factors such as meal termination and initiation (Figure 1.5). The following is a description of how adipose tissue and the gastrointestinal tract can influence food intake and exert their respective roles in mediating long and short term energy balance.

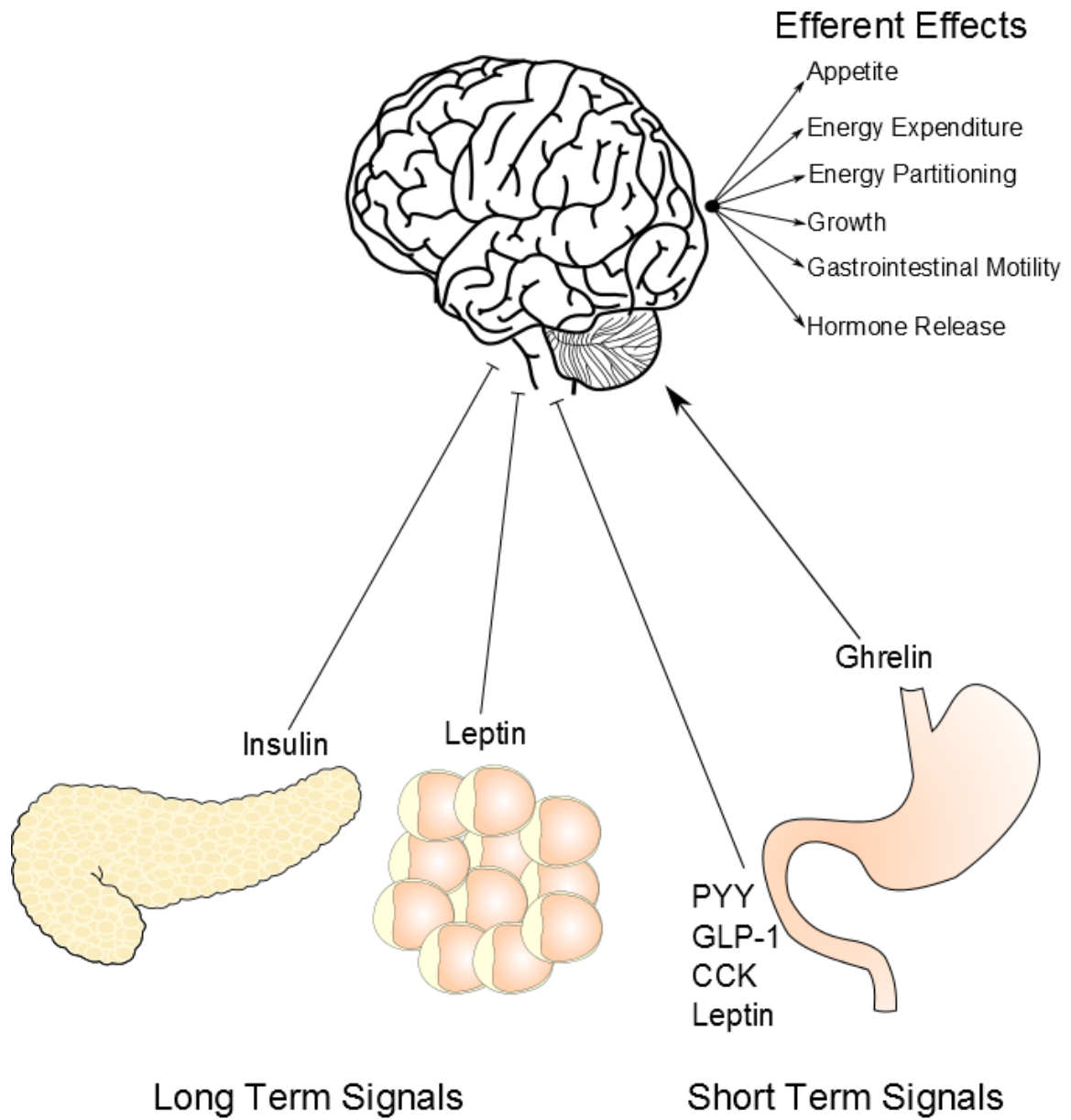


Figure 1.5: Sites of release of long term and short term mediators on energy balance. The pancreas and adipose tissue release insulin and leptin respectively, which provide information to the CNS regarding the metabolic status of the body with perturbations in their levels reflecting increased or decreased energy stores. The gastrointestinal tract releases ghrelin as a short term signal to initiate a meal and increase food consumption. After meal consumption an array of peptides including PYY, GLP-1, CCK and leptin are released from cells within the gastrointestinal tract to signal satiety. On top of mediating appetite both the long term and short term mediators also play a role in a number of efferent pathways including regulating energy expenditure, growth, release of other hormones, gastrointestinal motility and partitioning available energy. PYY; peptide YY, GLP-1; glucagon-like peptide 1, CCK; cholecystokinin. Figure adapted from [146]

1.2.2.1 Adipose tissue

Historically adipose tissue was viewed as a store for excess energy that the body could mobilize to sustain body processes when food was not readily available for consumption [147]. However, subsequently adipose tissue was found to be capable of releasing endocrine mediators capable of mediating feeding behaviour [148], with leptin being the first identified [149]. Currently, at least 30 peptides have been discovered to be released from adipocytes with many of these adipokines having roles in regulating energy homeostasis and metabolic processes [150].

From an evolutionary perspective, adipose tissue allows animals to consume more food than they need for their immediate use to store for later use, when food is less abundant due to seasonal variations in food availability [151, 152]. A similar phenomenon has been observed in native and rural human populations [153, 154]. This suggests that there must be an endogenous mechanism that permits accumulation of fat above and beyond what could be considered necessary for immediate survival. Studies have presented evidence suggesting the body is aware of current and desired levels of adiposity and this led to the paradigm of an adipostat (Figure 1.6), a signal that would effectively defend the level of adiposity within the body [73]. This is consistent with food restriction induced fat loss in rats being restored after re-feeding to levels comparable with continuously *ad libitum* feed rats [155]. Animal models have revealed that surgically removing one fat deposit leads to a compensatory increase in fat being stored in the remaining intact fat deposits [156]. The same phenomenon has been observed in humans

undergoing lipectomy, with the removal of the subcutaneous fat leading to an initial weight loss, however this lost weight is soon replaced with an increase in visceral fat [157]. This indicates that the simple removal of fat is not a viable approach for weight loss. The adipostat paradigm does not, however, explain obesity. The adipostat model would predict that an increase in adipose mass would increase the adiposity signal, which should result in a reduction in food intake and a decrease in fat mass. In obesity this obviously is not occurring. However, in accordance with the necessity to store energy when available in an environment with an uncertain food supply, the purpose of an adipostat may be to permit this to occur to a genetically defined upper limit, and defend a decrease from any level reached. Unfortunately, in a modern society where food is readily available such an adaptation would be a liability rather than advantageous.

Adipose tissue has also been shown to anatomically and functionally change with metabolic state. Adipocyte size has been shown to be linked to the state of energy balance; with hypocaloric diets reducing adipocyte size but not the number of adipocytes [158, 159]. This is important to keep in mind because there is a strong positive relationship between adipocyte size and the release of adipokines. For example, increased adipocyte size is associated with increased leptin, interleukin (IL)-6 and IL-8 and a decrease in the anti-inflammatory mediator, IL-10 [160]. Increased adipose size in obesity is also known to increase adipocyte death [161]. This in turn results in increased macrophage infiltration into the adipose tissue producing the chronic low grade inflammation of adipose tissue in obesity [161-163]. However, obesity

can occur in the absence of adipose tissue inflammation [164]. The inflammation occurring within adipose tissue has been proposed to be a mechanism for insulin resistance that often occurs in obesity [165, 166]. This in turn could be a mechanism to protect from obesity as insulin plays a role in adipocyte differentiation [167], lipogenesis [168] and inhibition of lipolysis [169].

There are at least 24 reported adipokines/cytokines which have elevated circulating levels in human obesity, with their plasma levels closely associated to visceral adiposity [170]. However, the actual source of the increased adipokines/cytokines is uncertain, as in addition to the adipocytes they could also be released from the increased number of macrophages in obese adipose tissue. Macrophages are a source of many cytokines and as such both the adipocytes and macrophages may be responsible for altered levels of adipokines/cytokines observed in obesity [171].

As well as subcutaneous and visceral adipose tissue stores there is also sub-serosal and sub-mucosal adipocytes within the stomach [172]. It has been suggested that adipokines released from similar deposits in the colon could act in a paracrine manner to play a role in mediating intestinal barrier function [173]. This raises the hypothesis that sub-mucosal and sub-serosal gastric adipocytes could release adipokines that may act in a paracrine fashion to mediate gastric function or modulate intrinsic and extrinsic gastric afferents. It still remains to be determined if such a mechanism exists and if so whether it is altered by the adipose hypertrophy observed in obesity.

NOTE:

This figure/table/image has been removed
to comply with copyright regulations.
It is included in the print copy of the thesis
held by the University of Adelaide Library.

Figure 1.6: The adipostat theory for the control of body weight. This model proposes that the size of adipose tissue controls the level of circulating hormones which in turn act centrally to modulate food intake and energy expenditure. So when adipose tissue is increased (left) there is an increase in circulating hormones such as leptin that act centrally to cause an increase in energy expenditure whilst decreasing food intake, which results in a negative energy balance and subsequent weight loss. Alternatively weight loss leads to decreased adipose tissue and reduced circulating anorexigenic signals and causes an increase in food intake and a decrease in energy expenditure leading to a positive energy balance and weight gain. Figure adapted from [174].

1.2.2.2 Proximal gastrointestinal tract

Consisting of the stomach and small intestine, the proximal gastrointestinal tract is the site for the digestion of food and absorption of nutrients. This system relies on co-ordinated mechanical and chemical processes to achieve its tasks. Upon food entering the stomach relaxation of the gastric muscle allows accommodation of the food to occur [175]. This phenomenon was dubbed adaptive relaxation and occurs within seconds of food entering the stomach [176]. This allows for the maintenance of intragastric pressure whilst increasing the volume available for occupation by food [177]. The ability of the stomach to increase its volume has been shown to be relevant to the control of food intake, as disorders of the reservoir function of the stomach are associated with early satiety [178]. The relaxation occurs through chemical and neural mechanisms [179, 180], which lead to activation of inhibitory enteric motor neurons, causing relaxation of the gastric smooth muscle. However, the role of the smooth muscle within the gastrointestinal tract is not limited to allowing food to be accommodated. It is also vital for the transit of the food through the entire gastrointestinal tract by rhythmically contracting and relaxing in the form of peristaltic waves [181]. The muscle within the walls of the stomach contract to mix the food with digestive enzymes and acid released from epithelial and endocrine cells that line the gastric lumen [182, 183]. Peristaltic contractions propel the stomach contents toward the pylorus where movement into the duodenum is prevented for all but the smallest particles and liquids [183].

Once in the duodenum the digestive process continues to occur due to release of digestive enzymes from the pancreas and gall bladder [184]. Whilst originally thought of as reservoirs for the digestive process to occur within, the small intestine and stomach have a profound ability to sense food [185, 186] and cause changes in food intake behaviour [187, 188] as well as control the digestive progress [183, 189]. As such the roles of the small intestine and stomach have been expanded by an ever growing literature base to highlight that they are involved in the regulation of local gastrointestinal function as well as the regulation of food intake and satiety.

Appetite regulation by the gastrointestinal tract is mediated by sensing both volume and composition of meals. This can occur through gastrointestinal secretion of peptides as well as activation of afferent endings located within the wall of the gastrointestinal tract. Below is an overview of the abilities of the small intestine and stomach to sense nutrients and signal through multiple pathways, including neuronal and hormonal pathways, to regulate appetite.

1.2.2.2.1 Nutrient sensing

1.2.2.2.1.1 Small intestine

The small intestine can initiate satiety in response to luminal nutrients [190]. The ability for this to occur has been eluded to be due to nutrient driven release of cholecystikinin (CCK), which is an intestinal peptide released from I cells within the proximal small intestine [191] when exposed to nutrients (predominately protein and lipids) [192-195]. Evidence has shown that CCK causes satiety through activating local vagal afferent endings [196-198].

Furthermore, nutrients in the small intestine have been shown to induce an increase in thermogenesis (a major source of energy expenditure) in brown adipose tissue via CCK mediated activation of local vagal afferent endings [199]. This suggests that as well as being involved in controlling food intake, nutrient sensing within the small intestine is a regulator of energy expenditure.

Specialised intestinal epithelial cells have been shown to express nutrient receptors identical to those found on the surface of the tongue [200, 201], suggesting that the intestine has the ability to 'taste' nutrients in the gut. Some cells expressing these taste receptors are co-localised with intestinal satiety peptides [200-202] and nutrient absorption proteins like sodium-glucose transporter 1 (SGLT1) [203]. These findings together led to the hypothesis that specialised intestinal epithelial cells can sense specific nutrients and respond by releasing appetite modulatory peptides to control food intake and gastrointestinal motor function [204]. This is supported by small intestinal lipid inducing gastric relaxation [205, 206]. Furthermore, high level of Intestinal fat induces a feeling of nausea during gastric distension [207]. This suggests that intestinal nutrient sensing modulates sensory signals from the stomach as well.

1.2.2.2.1.2 Stomach

The involvement of the stomach in nutrient sensing is less clear. Early studies found that infusion of saline elicited the same meal terminating effect as infusions of sucrose, fructose and glucose [208]. This indicated that the stomach was simply detecting a mechanical stimuli not a chemical one.

However, there is also evidence that the stomach can respond to nutrients to control food intake [209-211] and that changes in intragastric pressure only result in increased satiety after intragastric infusion of nutrients [212], which casts doubts on the paradigm that gastric satiety is purely mechanical.

Further evidence for a nutrient sensing ability of the stomach is the expression of α -gustducin [213] and the taste receptor component, T1R3 [214, 215] in cells within epithelium brush border of the stomach. Furthermore, these taste receptor signalling components are located on ghrelin positive cells [214, 216] and have been show to trigger the release of ghrelin in an α -gustducin dependant manner [216], which further contradicts the theory that the stomach's role in the regulation of food intake is purely mechanical. Gastric nutritional sensing has also been demonstrated in response to amino acids [217] . This further strengthened the argument that taste sensing occurs within the stomach. However, this still remains to be conclusively demonstrated.

There is evidence that suggests that gastric nutrient sensing may be comprised in obesity. Gastric mucosal expression of the fatty acid receptor GPR120 is increased and sweet taste receptor component T1R3 has been shown to be decreased in morbidly obese humans [218]. This led the authors to conclude that alterations in gastric nutrient sensing ability may play a role in the increased food consumption seen in obesity. However, their evidence is very preliminary and the conclusions are based on immunohistochemical and mRNA expression levels and thus the actual phenotypic effect of such changes needs to be determined. However, these results do show that gastric

nutrient sensing is likely to be dynamic and that obesity is capable of potentially disrupting gastric satiety signals. This warrants further investigation.

It has been demonstrated that the suppression of food intake by nutrient infusion into the stomach with an open pyloric cuff is greater than the suppression in food intake caused by gastric distension or intestinal nutrient exposure alone [208]. This indicates that both locations work together to cause a larger net reduction in food intake than either could on their own. An example of such a mechanism is CCK, which is released from the small intestine and has been shown to activate the same vagal afferents activated by gastric distension [198]. This indicates the neuronal innervation of the gastrointestinal tract is a site for convergence of both gastric and intestinal satiety signals.

1.2.2.2 Innervation of the proximal gastrointestinal tract

Within the stomach and small intestine there are discrete populations of neurons which represent extrinsic innervation (through splanchnic and vagal nerves) and also intrinsic innervation (through the ENS; Figure 1.7). Whilst each is able to act independently there are an abundance of synapses between the intrinsic and extrinsic neurons highlighting the functional relationship that exists between them. Below is an outline of the roles of these pathways, focusing on food intake.

NOTE:

This figure/table/image has been removed
to comply with copyright regulations.
It is included in the print copy of the thesis
held by the University of Adelaide Library.

Figure 1.7: Anatomical organisation of the layers of the gastrointestinal wall and intrinsic and extrinsic innervation. Within the wall of the gastrointestinal tract there are vagal and spinal afferents which have endings within both the muscular and mucosal layers enabling detection of multiple stimuli. Enteric neurons are also present within the myenteric and submucosal plexus. Whilst capable of autonomous action they can also communicate with extrinsic neurons. Figure adapted from [185].

1.2.2.2.1 Enteric nervous system (ENS)

The gastrointestinal tract is unique due to its extensive intrinsic nervous system, the ENS. This intrinsic innervation allows continuation of multiple processes even after separation from the CNS; however there is substantial penetration of vagal neurons into the ENS [219], suggesting communication between the two systems. The ENS is organised into two plexuses. One is located between the circular and longitudinal layers of muscle, called the myenteric plexus. The other exists within the submucosal layer called the submucosal plexus. Within these plexuses there are primary afferent neurons, interneurons and motor neurons that are able to act on a variety of targets including smooth muscle, blood glands, epithelium, pace maker cells, mucosal glands and extrinsic neurons. The ENS has multiple roles within the gastrointestinal tract including regulating gastric acid secretion [220], epithelium proliferation [221], immune function [222], endocrine release and determining the patterns of movement along the gastrointestinal tract [223]. Given its involvement in multiple processes that occur within the gastrointestinal tract it is not surprising that dysfunction of the ENS is associated with a number of detrimental conditions [224].

In terms of involvement in the regulation of food intake, enteric neurons have a couple of desirable properties. Firstly, they express receptors for appetite modulatory peptides [225]. Secondly, the ENS contains first order neurons that are able to sense and respond to food within the lumen of the gastrointestinal tract and are in direct contact with enteroendocrine cells such as CCK secreting I cells [226]. The physiological relevance of this has been

highlighted by the finding that CCK administration causes a dose dependent increase in c-Fos activation in both small intestinal myenteric and submucosal neurons [227]. This effect is abolished when administered in the presence of a CCK1R antagonist [228] and is not mediated via a vagal pathway or hindbrain activation [229]. CCK has been shown to activate choline acetyltransferase (ChAT) immunoreactive neurons within the myenteric plexus which project to the sphincter located at the duodenal orifice of the bile duct [230]. Activation of these neurons causes a relaxation of the sphincter and increase bile release from the gall bladder into the duodenum [230]. This suggests that digestion and the subsequent absorption of nutrients could be modulated by activation of enteric neurons.

Enteric neurons also express 5-hydroxytryptamine (5-HT)₃ receptors within both the myenteric plexus and submucosal plexus [231]. Given that the majority of 5-HT exists within enterochromaffin cells of the gastrointestinal tract [232] and that enteric neurons themselves have been shown to be a major site of 5-HT release it seems logical that the ENS is involved in the regulation of gastrointestinal function and is modulated by locally released modulators including 5-HT [233].

The function and responsiveness of the ENS to chemical mediators including CCK and ghrelin has been demonstrated to be altered by feeding status [234]. Like extrinsic afferents there is also evidence that enteric neurons are able to sense and modulate their activity in response to changes in glucose levels [235, 236]. Such modulation may explain, at least in part, why acute changes

to glycaemia can substantially alter gastric motor function [237], which in turn would alter the availability for nutrient absorption. Consistent with this is a state of hyperexcitability in enteric neurons and increased frequency of peristaltic activity in fasted mice that have been re-fed [234]. There has also been demonstrations of nutrient specific adaptations of enteric neuron function with chronic high fat diet feeding associated with reduced enteric neuron response to intestinal oleate [238]. This reduction in enteric activation was associated with a reduced level of satiety induced by intestinal oleate [238]. However, it was not possible to determine what, if any, the role the reduced enteric response to oleate played in the reduced level of satiety.

Therefore, although there is a lack of evidence to suggest that the ENS can directly influence food intake, by controlling gastrointestinal motility it is reasonable to suggest the ENS plays an indirect role in the control of food intake by responding to nutrient and chemical stimuli to mediate the amount of nutrient available for absorption at any one time.

1.2.2.2.2 Spinal Afferents

Whilst the majority of peripheral food intake regulatory signals are believed to be conveyed through vagal pathways, it is important to recognise that there are still significant levels of spinal afferents within the upper gastrointestinal tract, including the stomach [239] and small intestine [240]. The purpose of these spinal afferents is believed to be to convey noxious stimuli such as pain [241], however there is evidence that these pathways are also involved in food intake regulation and gastric function [242, 243]. For instance, the

peptide bombesin has been shown to have receptors located throughout the CNS and also within the gastrointestinal tract [244, 245]. Bombesin causes release of gastrin [246] and also causes a reduction in food intake and meal size [247, 248]. This bombesin induced reduction in food intake is abolished by severing the spinal and vagal nerves, indicating that the food intake modulatory effect is mediated through extrinsic gastrointestinal neuronal pathways [249]. However, bombesin was still able to reduce food intake by virtually the same amount with only the vagal or spinal innervation disrupted compared to both being intact [249]. This indicates that bombesin can reduce food intake through independent spinal and vagal afferents.

Furthermore, dorsal root ganglia (DRG; location of the spinal afferent cell bodies) have receptors for ghrelin [250] and CCK [251]. Spinal afferents have also been implicated in the control of motor functions of the upper gastrointestinal tract with ablation of the spinal pathways causing a reduction in gastric motility [252, 253]. Gastric electrical stimulation, which has previously been shown to reduce food intake [254], elicits an overall excitatory effect on spinal afferent pathways, both in the presence and absence of intact vagal nerves [255]. This provides extra support to the notion that spinal afferents may respond to gastric stimuli to modulate feeding behaviour, but this remains to be conclusively determined.

Both chemosensitive and mechanosensitive afferent endings have been identified in spinal afferents [256, 257]. These mechanosensitive endings have been very well characterised, however the majority of the investigation

has occurred within the colon [258], where vagal innervation is sparse. Spinal mucosal, muscular and serosal mechanosensitive afferents have been identified in the colon [258], with the latter being responsive to high intensity stimuli and suggested to be involved in detecting noxious stimuli [259]. Similarly, in the stomach of rats there are populations of high threshold spinal afferents, which only respond to distension within the noxious range [260]. Thus, it is possible that gastric distension may activate specific populations of spinal afferents to signal pain in the event of over distension of the stomach, as well as providing input regarding normal distension of the stomach [261].

1.2.2.2.2.3 Vagal afferents

Tracing studies have revealed the vagus is predominately comprised of afferent fibres, which originate from much of the viscera, including the gastrointestinal tract [262]. The vagus has its cell bodies located within the nodose ganglia, from which the vagus projects centrally to the NTS [263]. In addition to the vagus the NTS receives inputs from the facial and glossopharyngeal nerves [263] with the latter two being involved in oral gustatory signalling [264-266]. Therefore the NTS appears to be a 'hub' for the convergence of neuronal activity resulting from food intake. This is supported by stimulation of the vagus increasing activation of c-Fos within the NTS [267] and suppressing subsequent food intake [268].

In addition to having central projections (described earlier), the neurons within the NTS that are activated by vagal afferents are able to activate neurons within the dorsal motor nucleus of the vagus (DMV; Figure 1.8) [269, 270].

Activated DMV neurons can induce gastric relaxation via activation of a non-adrenergic, non-cholinergic (NANC) pathway, leading to the release of nitric oxide or vasoactive intestinal peptide from intragastric neurons [271]. Alternatively, a cholinergic excitatory pathway can be activated, resulting in muscarinic receptor dependent increases in gastric motility and tone [272].

Much of the past research into the role of the vagus in controlling food intake involved some form of vagotomy to remove all vagal innervation. This crude method involves severing the vagal nerves by hand. Such an approach could lead to different effects based on the location the nerves severed. This may explain why reports on the effects of vagotomy on body weight vary considerably, with some reporting a decrease in weight [273, 274], whilst others report no difference [275] or even an increase [276]. Perivagal application of capsaicin has been used to selectively destroy vagal afferent fibres [277]. However, evidence suggests that perivagal capsaicin does not necessarily only destroy afferent fibres [278]. These approaches allow no specific evidence to be extracted from the results in regards to the role of gastric innervation in the control of food intake [279]. Furthermore, a vagotomy also reduces gastric relaxation [280], motility [281] and secretions i.e. acid and gastrin [282]. All of these can alter the rate that nutrient absorption can occur. This adds further difficulty in identifying mechanisms behind changes in food intake behaviour caused by a vagotomy.

Closure of the pyloric sphincter (to prevent any intestinal response to eating) showed an attenuation of food intake following gastric loads [283]. This

suggests that there is a distinct gastric mechanism that is likely being conveyed through vagal pathways to trigger satiety. This concept was further strengthened by findings that gastric vagal electrical stimulation caused a reduction in food intake and a subsequent reduction of body weight in rats [284], with a similar technique being implemented in humans as a treatment for obesity [285]. This suggests that not only are vagal afferents involved in regulation of food intake, but they also present a possible target for weight reducing therapies.

Whilst it was previously discussed that nutrient sensing within the stomach is a likely possibility; there are also specialised vagal afferent endings that are responsive to mechanical stimuli. Upon entering the stomach, food is detected by vagal afferent fibre endings in the mucosa that are sensitive to mucosal touch [286, 287]. The volume of food is also sensed by afferent endings located in the deeper muscular layers of the stomach, which are sensitive to stretch and tension [288]. Activation of the latter type of afferent has been shown to trigger satiation [289]. Gastric mechanosensitive vagal afferents have previously been investigated in depth and below is a brief overview of their anatomical and functional characteristics.

1.2.2.2.3.1 Tension receptors

There have been two populations of vagal neurons identified in the stomach which are thought to respond to stretch. The first are intraganglionic laminar endings (IGLEs). These are vagal afferent endings located within the connective tissue capsule of the myenteric plexus ganglia between the outer longitudinal and inner circular muscle layers [290]. They respond to muscle

tension generated by passive stretch of the stomach as well as active contractions of the stomach [291] and are believed to be the mechanotransduction sites of tension sensitive vagal afferents [291]. This particular type of afferent ending is found in very large numbers throughout the oesophagus [292] and stomach [293] and are believed to have roles in generating and maintaining vagal tone.

Intramuscular arrays (IMAs) are the second class of tension sensitive afferent ending, however they are exclusively located within the circular and longitudinal muscle layers [294]. They have been postulated to detect changes in muscle length [295] and respond to stretching of the stomach walls with a sustained high rate of action potentials which is still present after muscular tension is reduced through gastric accommodation [296]. If correct, such a trait suggests that they may be a prime candidate for providing feedback regarding stomach fullness, which could help signal satiety. However, to date most speculations are based on anatomical evidence with a distinct lack of morphological evidence linking IMAs with a mechanotransduction site [291]. Therefore, it still remains to be confirmed whether IMAs do in fact play a role in detecting gastric stretch and satiation.

1.2.2.2.3.2 Mucosal receptors

Vagal afferent fibres also have specialised endings in the mucosa [287]. These endings are commonly found to be closely associated to endocrine and epithelial cells containing peptides with known effects on food intake. This suggests they may be readily modulated by locally released peptides *in vivo*.

These endings have also been shown to have mechanical sensitivity, although unlike IMAs and IGLEs are not sensitive to tension [287]. However, when stroked over they respond with a burst of activity. These afferents have been suggested to be important in detecting food particle size and have been linked to delaying of gastric emptying. This was supported by an increase in food particle size being passed into the small intestine of dogs which had neuronal innervation of the gastric mucosa destroyed [297].

Using a flat sheet *in vitro* electrophysiological preparation (Figure 1.9) it is possible to record the responses of both classes of afferents in mice (Figure 1.10). This approach has led to significant information being discovered regarding the effects of different endogenous modulators on the response of these afferents. For example nitric oxide [298] and GABA [299] have been shown to have inhibitory effects on the mechanosensitivity of gastric vagal afferent endings. Furthermore, peptides such as galanin [300] and ghrelin [301] have also been shown to have modulatory effects on these endings. These studies have provided important mechanistic information about how the stomach may regulate peripheral satiety signalling as well as other visceral signals through peripherally acting mediators acting on afferent pathways. However, no information exists in regards to whether the sensitivity of vagal afferents to mechanical stimuli or gut peptides such as leptin and ghrelin are altered under states of energy deprivation or excess, which given the role of the vagus in mediating food intake is worthy of investigation and forms part of the current thesis.

Inputs to higher CNS centres
i.e. Hypothalamus, Ventral Tegmental Area

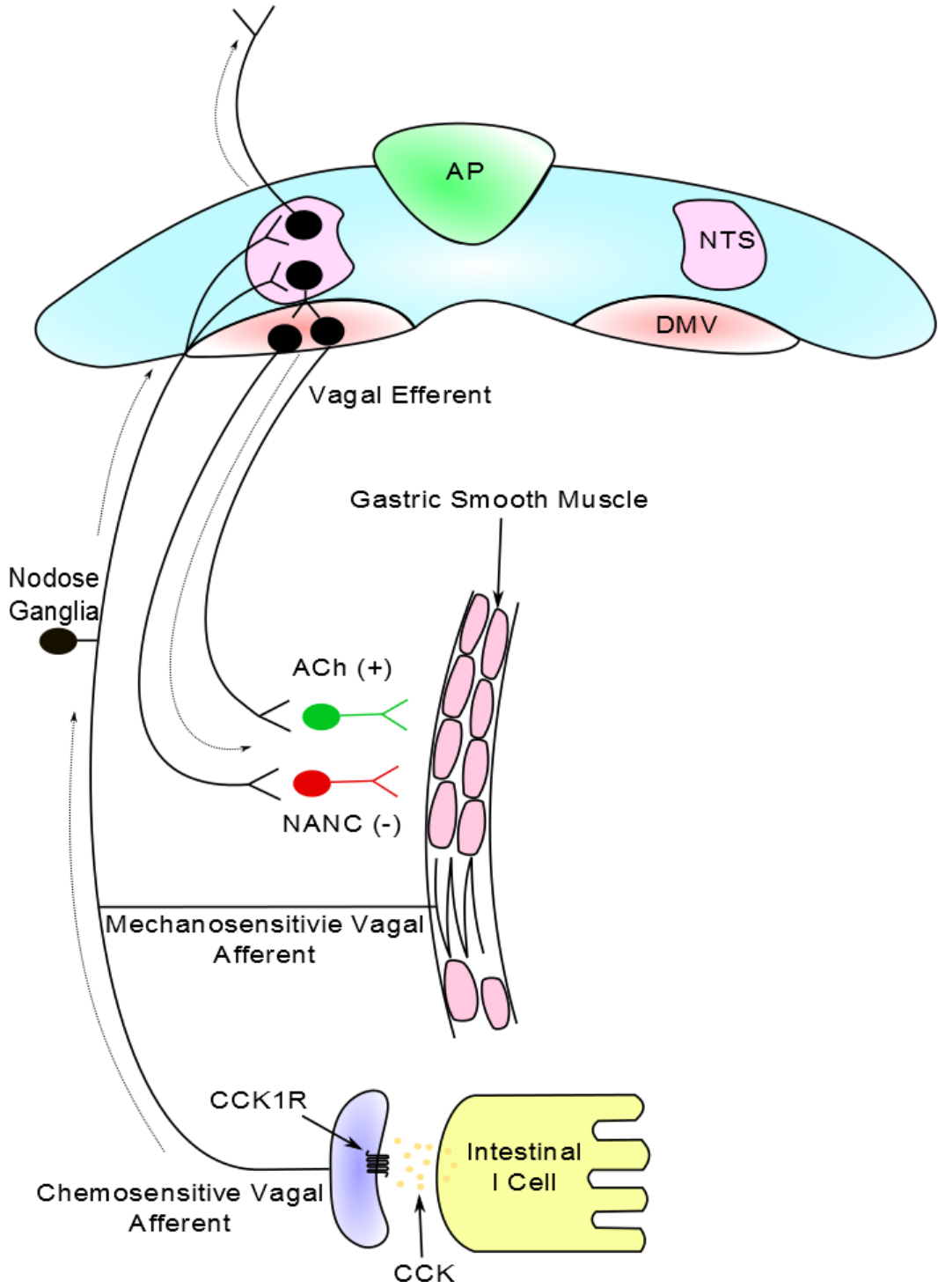


Figure 1.8: Vagal afferent connection within the brainstem. Activation of mechanosensitive or chemosensitive vagal afferents in the gastrointestinal tract leads to activation of second order neurons within the nucleus tractus solitarius (NTS). The second order neurons can activate high regions of the central nervous system (CNS) where they can mediate satiety and energy expenditure. Alternatively they can activate neurons within the dorsal motor nucleus of the vagus (DMV) which can cause contraction or relaxation of gastric smooth muscle through cholinergic (ACh) or non-adrenergic non-cholinergic (NANC) pathways respectively. Descending input into the NTS occurs but is not shown. Dashed lines with arrows indicate the direction signal is travelling. AP; area postrema, CCK; cholecystokinin, CCK1R; CCK receptor 1.

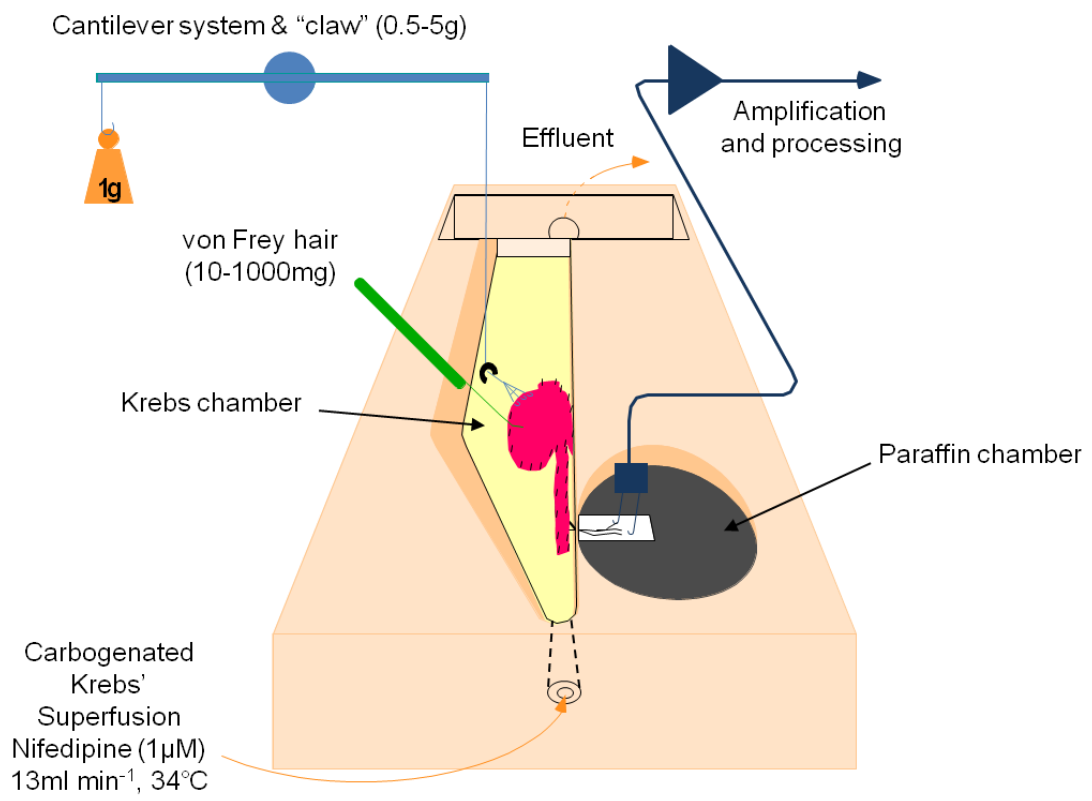
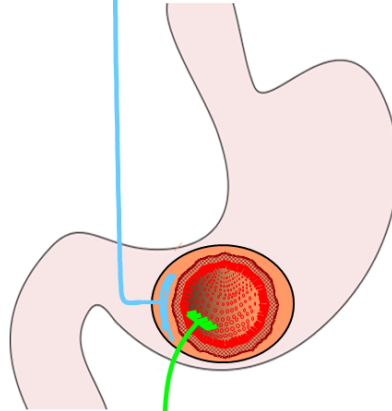
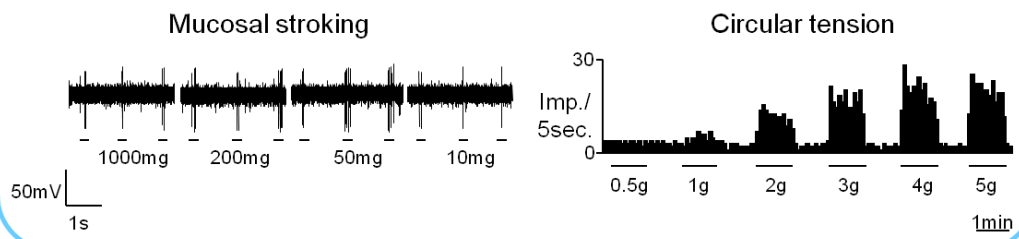


Figure 1.9: Schematic representation of gastro-oesophageal vagal afferent recording flat sheet preparation. This setup is used to record gastric vagal afferent responses to mechanical stimulation. Drugs are applied via the krebs solution which is superfused over the tissue. .

Tension Receptors



Mucosal Receptors

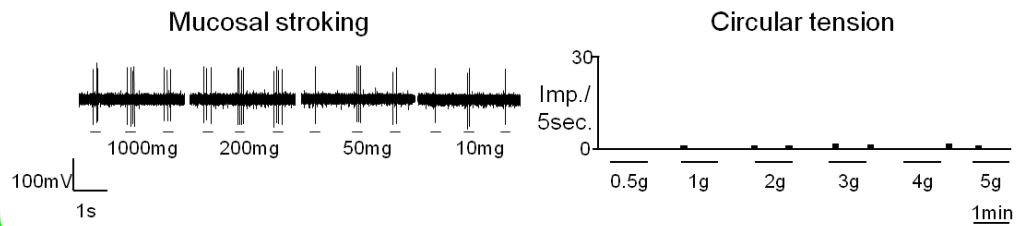


Figure 1.10: Original electrophysiological traces showing the two types of mechanically sensitive gastric vagal afferent endings that have been identified in the mouse. The first class of afferent are tension receptors (top). These afferents exist in the muscular layers of the stomach wall and respond to mucosal stroking with a burst of action potentials, but also respond to tension with a sustained graded burst of action potentials in proportion to the size of the stimuli. The second class are mucosal receptors (bottom) which respond to mucosal stroking with calibrated von Frey hairs with a burst of action potentials each time the receptive field is stroked. However, unlike tension receptors, these afferents do not respond to stretch.

1.2.2.2.4 Vagal Afferent Mediators

In addition to having mechanosensitive afferents that can be activated by the presence of food, the gastrointestinal tract also contains a great amount of endocrine and epithelial cells which have been shown to release peptides in response to feeding [302-304]. Below is a summary of a selection of mediators that are released from these cells and have been suggested to have roles in regulating food intake through vagal pathways. This is by no means a full list of peptides/hormones released from the gastrointestinal tract but is simply an illustration of the important role gastrointestinal peptides play in control of food intake.

1.2.2.2.4.1 Gastric mediators

Leptin: Classically described to be released from adipose tissue into the blood, leptin plasma concentrations are proportional to the amount of adipose tissue [305]. This suggests that leptin's primary role is to provide information to the CNS in regards to whether energy storage is sufficient. It has been described that leptin, upon entering the circulation, travels to the brain where it crosses the BBB in a saturable receptor mediated fashion [92]. Upon crossing the BBB leptin can bind to its receptor, which is present on a number of crucial brain centres, including the hippocampus [306] and, more relevant to food intake, the hypothalamus [307]. Here it causes modulation of neural activity, specifically inhibiting the NPY [308] and activating POMC [99] neurons within the arcuate nucleus, which results in a reduction in food consumption, an increase in energy expenditure and a preferential oxidation of fat [309-311].

There are six isoforms of the leptin receptor (LepRa-f) [312], which are closely related to the class 1 cytokine receptor family. LepRa-d and LepRf all have the same extracellular and transmembrane domains. LepRe represents a soluble form of the leptin receptor and does not have a known role in leptin signalling [312]. However, it has been shown to protect leptin from degradation in the gastrointestinal tract [313]. Therefore, it is likely to be involved in controlling the circulating level of leptin [314], as the relative quantities of free and receptor bound leptin are likely to impact on the level of leptin signalling that can occur [315]. Of LepRa-d and LepRf only LepRb has a long intracellular domain [312]. Truncation of the LepRb isoform such that it possesses an intracellular domain similar to that exhibited by LepRa results in an obese phenotype similar to that observed in leptin receptor knockout mice (db/db mice) [312]. This suggests that the long intracellular domain is responsible for the anorexigenic effect of leptin.

The process of leptin signalling begins with receptor dimerisation [316, 317]. Without this occurring leptin cannot cause any intracellular changes. However, the short receptor isoforms also form dimers [316]. Given the short receptors do not have leptin signalling capability indicates that receptor dimerisation, whilst vital for signalling to occur, has no effect on the activity of the receptor itself. Upon binding to the long form of its receptor leptin causes Janus kinase (JAK) mediated phosphorylation of signal transducer and activator of transcription (STAT) 3 [318], which forms a dimer [319] and translocates to the nucleus. From here it acts as a transcription factor [320] for a number of

genes including suppressor of cytokine signalling (SOCS) 3, which in turn acts to negatively regulate the activity of the leptin receptor [321] (Figure 1.11). On top of transcriptional activation, leptin has been demonstrated to inhibit neuronal activity by activating ion channels such as ATP activated potassium (KATP) [322] and large conductance calcium activated potassium (BKCa) channels [323]. In addition to neuronal inhibition, leptin has been shown to activate canonical transient receptor potential (TRPC) channels [324] via a phosphatidylinositide 3-kinase (PI3K) mediated mechanism increasing neuronal excitability. Activation of a cation channel has been suggested to be the mechanism by which leptin can induce meal termination [325]. Given the evidence that leptin can reduce food intake via a vagal mechanism [326], this thesis will investigate whether leptin activates modulates mechanosensitive vagal afferents via TRPC dependent pathway.

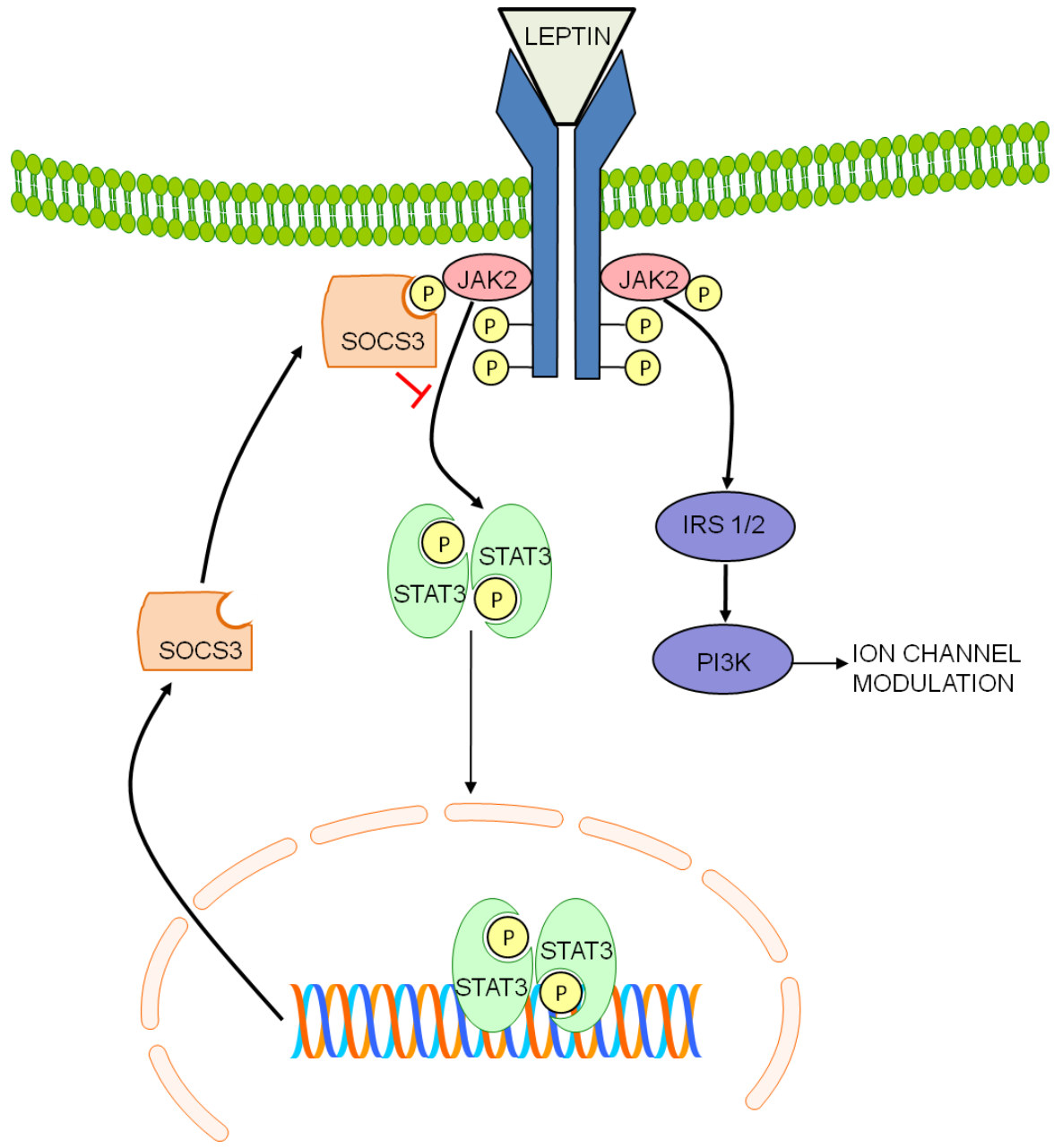


Figure 1.11: Schematic representation of leptin receptor signalling. Upon binding to the long form of its receptor (LepRb), leptin causes phosphorylation of signal transducer and activator of transcription 3 (STAT3) molecules, which dimerise and translocate to the nucleus where they act as a transcription factor. This causes an increase in suppressor of cytokine signalling 3 (SOCS3) expression, which inhibits the phosphorylation of STAT3 by Janus kinase 2 (JAK2). JAK2 also causes activation of phosphatidylinositide 3-kinases (PI3K) through insulin receptor substrate (IRS)1/2 which has been linked with the modulation of ion channel activity in a variety of neurons.

However, in addition to leptin's role as an adipokine, it has been shown to be a gut peptide with the ability to interact with local vagal afferent endings [326]. Leptin is synthesized by gastric chief cells [327], which can release leptin into the gastric lumen along with the digestive enzyme pepsinogen, indicating release from these stores is triggered by food intake. It has also been suggested that gastric leptin release may in fact also occur during the cephalic phase and act to modulate intestinal function before food is even consumed [328]. Furthermore, leptin is also located within gastric parietal cells [329] which can release leptin into the blood. The release of leptin from these gastric stores has been shown to be mediated by feeding [330] (however, whether this is triggered by specific nutrients has yet to be determined), small intestine peptides (such as CCK) [304] and acetylcholine released from the vagus nerve [328]. It is also suggested that this gastric source of leptin is responsible for the fluctuations in plasma levels of leptin as adipocyte derived leptin is constitutively released [304, 331].

The long form of the leptin receptor (LepRb) has been identified on vagal endings innervating the gastrointestinal tract, with the highest level of expression being in the stomach and duodenum [332], suggesting that leptin may have a vagal modulatory role. This was confirmed by a series of studies which identified that leptin was able to activate cultured duodenal [333] and gastric [334] nodose neurons on its own, but also had a profound synergistic effect with CCK. Leptin infused directly into the gastric blood supply had the ability to cause a substantial reduction in food intake, an effect that was lost in

vagotomised animals [326]. This suggested that the effect of leptin on acute food intake is mediated through vagal afferent pathways.

The discovery of leptin was expected to lead to the production of a 'cure' for obesity. However, these hopes were diminished after the discovery that the majority of obese humans have sustained hyperleptinemia and are unable to respond to leptin administration with the expected anorexigenic effects. Indeed animals and humans who have defects in either the leptin or leptin receptor gene exhibit early onset morbid obesity, which highlighted just how important the biological activity of this peptide is. Of particular interest is that obesity has now been shown to be linked with leptin resistance in vagal afferent neurons [335, 336]. This indicates that the dysfunction in leptin signalling associated with obesity is not localised to the CNS. However, this leptin resistance in vagal afferents was concluded based on the whole nodose ganglia [336]. As such, it is difficult to conclude more specifically whether gastric mechanically sensitive vagal afferents exhibit this leptin resistance. Investigating whether leptin modulation of gastric vagal afferent mechanosensitivity is altered under diet-induced obesity forms a major focus of this thesis.

Neuropeptide W (NPW): NPW is a recently discovered peptide that activates the orphan G-protein coupled receptors GPR7 and GPR8 [337]. NPW was originally believed to exert a modulatory role on food intake centrally since central administration of NPW into the rat brain stimulated food intake [338, 339]. However, on top of the central targets NPW has also been shown to be

present in antral G cells of rodent and human stomach where it is co-localized with gastrin [340]. The expression of NPW in these G cells varies with fed status with fasted animals having a reduction in NPW content further indicating a role in modulating food intake [341]. In addition GPR7 knockout mice (the only NPW receptor subtype in mice) develop hyperphagia and obesity [342]. This suggests an anorectic role for NPW, however there is evidence which suggests that NPW in fact increases food intake [338] and has the ability to inhibit the responses of vagal afferent to mechanical stimuli [343].

Ghrelin: Ghrelin is a 28-amino acid peptide secreted from X/A cells within the gastric fundus [344]. It acts as an endogenous mediator of growth hormone release [345]. Ghrelin has also been shown to be expressed along the length of the gastrointestinal tract; however the level of expression diminishes distally from the stomach [346]. Ghrelin has a unique structure with an octanyl group present, which is vital for activation of its receptor (the growth hormone secretagogue receptor- GHS-R). Cleavage of this group causes instant deactivation of ghrelin and as the enzyme responsible for this cleavage freely circulates ghrelin has a very short active half life in blood.

Post transcriptional processing of GHS-R leads to the production of two splice variants, GHS-R1a and GHS-R1b [347, 348]. The GHS-R1a consists of 366 amino acids and forms a receptor with seven transmembrane domains linked with alternating intracellular and extracellular loops [347]. Alternatively, GHS-R1b is truncated at the carboxyl end resulting in only five transmembrane

domains [347]. GHS-R1a is potently activated by ghrelin and other growth hormone secretagogues. GHS-R1b is not activated by either and there is limited documentation related to the physiological effects of GHS-R1b. The GHS-R1a is unique as it exhibits an abnormally high level of constitutive activity ($\approx 50\%$ of its maximal signalling ability) when compared to other G-protein coupled receptors (GPCRs) [349-351].

Ghrelin receptors are present in central neurons and of particular interest are the receptors on NPY neurons [352], which act in direct opposition to the POMC neurons activated by leptin. However, the GHS-R has also been detected in many other tissues and neurons including the nodose ganglia and gastric vagal afferents [353]. It has been previously shown that ghrelin causes an inhibition of vagal afferent firing [301, 354]. However, the consequence of this inhibition is still being debated with some studies showing the orexigenic effect of ghrelin is mediated by vagal pathways [355], whereas others show the opposite [356]. Neuroimaging studies have revealed that ghrelin levels below the threshold for causing an increase in the perception of hunger are able to inhibit the satiating effect of CCK [357]. This suggests that ghrelin influences appetite through multiple pathways targeting potentiation of its own signalling, as well as inhibiting opposing signals.

Ghrelin to date is the only peripheral orexigenic peptide [358]. Given that obesity is a state which requires consumption of excess amounts of energy to achieve the required weight gain; further investigation into whether ghrelin plays a part in permitting the increased energy consumption is necessary.

Currently, there is some evidence suggesting that within the ARC ghrelin resistance occurs in obesity [359]. However, there is no information regarding potential changes to the peripheral orexigenic effect of ghrelin in obesity. The fact that ghrelin still increases energy intake in obese humans [360] suggests that ghrelin must still be able to activate an orexigenic mechanism. As stated previously, it is already established that ghrelin reduces the mechanosensitivity of gastric vagal tension receptors [301]. As part of this thesis I will determine if these effects on gastric vagal afferent mechanosensitivity are altered under conditions of diet restriction and excess.

1.2.2.2.4.2 Intestinal mediators

CCK: CCK is released from I-cells located largely within the proximal small intestine. The release of CCK is mediated by the presence of luminal nutrients, with a preference for the digestion products of amino acids and fatty acids rather than those of carbohydrates [193, 194, 361]. Fatty acids are required to have an acyl chain length greater than 12 carbon atoms in order to trigger the release of and elicit the well documented meal termination effects of CCK [362, 363]. The link between luminal fatty acid and CCK release is believed to be mediated through the fatty acid receptor GPR40. The GPR40 ligand, linoleic acid, causes release of CCK from I-cell populations with but not without GPR40 [202].

There is an abundance of evidence for CCK having a food intake modulatory effect, with its exogenous administration causing a reduction in food intake as well as a slowing of gastric emptying, both of which are lost after vagotomy

[364, 365]. The same loss of effect of exogenous CCK administration is also seen in mice with CCK1R blockade [366, 367]. The expression of CCK1R does not appear to be effected by acute nutritional changes (i.e. fasting) [368], but there are examples of upregulation in response to high fat diet feeding [369] although this is not consistently reported [370]. The ability for CCK to activate CCK1 receptors on vagal afferents is inhibited by the orexigenic peptides ghrelin, orexin A and anandamide [353, 371-373]. In addition to its own anorexigenic effect on food intake, CCK also appears limit ghrelin induced drive to eat [374]. Therefore, CCK seems to both activate anorexigenic and suppress orexigenic signals in order to control food intake. However, CCK is more than just a satiety signal as it appears to be a crucial component in the absorption of fats in the intestine. This is highlighted by CCK knockout mice having higher levels of lipid in their faeces [375]. These mice have a reduced body weight even though they are on the high fat diet and consuming equal amounts of food [375].

It is believed that CCK acts predominately as a short term modulator of food intake as the main effect of CCK appears to be meal termination. With chronic use there is no change in weight, as the shorter meals are merely offset by an increase in the number of meals [376, 377]. However, CCK exists in a number of different lengths with the short form, CCK-8 being the most commonly studied. There is evidence to suggest that whilst this shorter and more studied isoform is indeed involved in limiting meal size [378] the longer forms of CCK may contribute to determining meal to meal intervals [379].

The ability for CCK to reduce food intake appears to be compromised in response to chronic high fat diet feeding with the absence of increased weight [380]. This suggests that poor nutrition may cause changes that create a susceptibility to obesity before any weight gain is observed. This may be due to a reduced ability of CCK to activate intestinal vagal afferents as previously described in diet-induced obese mice [381].

GLP-1: GLP-1 is an incretin hormone released from intestinal L-cells [382], which have the ability to respond and broadly detect the digestion products of carbohydrates, fats and proteins [383, 384]. GLP-1 immunoreactivity is found throughout the gastrointestinal tract; however there is substantially greater density within the distal small intestine [385, 386]. The release of GLP-1 has been shown to be tightly controlled and is released in proportion to caloric load [387] and also to the amount of small intestine that is being exposed to the nutrient [388]. Its release causes a decrease in food intake [389], stimulation of insulin release [390], reduction of glucagon secretion [391] and a reduction in gastric emptying [392]. GLP-1 positive cells have also been shown to be co-localised with GPCRs activated by sweet stimulants [201]. Therefore it has been hypothesised that the presence of sugars within the intestinal lumen causes activation of these GPCRs which triggers the release of GLP-1 from the L-cells. However, given that glucose does not normally reach the distal parts of the small intestine in high concentrations and fatty acids do it is likely that fatty acids have a more physiologically significant effect on the release of GLP-1 from the distal intestinal L-cells. Supporting this view is the finding that fatty acid receptors are present on the surface of L-

cells and the administration of agonists to these receptors causes an increase in the release of GLP-1 [393].

GLP-1 can activate vagal afferents [394] and this is believed to be the mechanism responsible for its effects on insulin release and food intake [395]. However, there is some debate over whether the vagal pathway is the main effector pathway by which GLP-1 signals to the CNS. Subdiaphragmatic vagotomy has been shown to have no effect on the ability of GLP-1 to reduce food intake when administered into the hepatic portal vein, but when administered intraperitoneally, subdiaphragmatic vagotomy abolishes the ability for GLP-1 to reduce food intake [396]. This has led to the suggestion that local and circulating GLP-1 may have different effector locations. GLP-1 receptor has been localised on the endings of vagal afferents [397], within the nodose ganglia [398] and on neurons within the dorsal vagal complex within the brainstem [399].

Originally it was believed that the satiation effect of GLP-1 was mediated through an endocrine effect with intestinally released GLP-1 acting on central receptors. However, this paradigm is unlikely to be true due to the fact that GLP-1 is rapidly degraded within the systemic circulation [400] to the point that systemic levels of GLP-1 do not remain elevated after consumption of a solid mixed nutrient meal [401]. Therefore it would be highly unlikely that systemic GLP-1 would be able to have a significant effect on the observed termination of meals [401]. However, there has been central release of GLP-1 documented [402, 403] indicating the possibility of a direct activation of GLP-1

receptors within the CNS. The importance of this direct CNS mechanism is in doubt as blockade of central GLP-1 receptors does not inhibit intraperitoneal (IP) GLP-1 induced anorexia, whereas a peripheral GLP-1 receptor blockade induced an increase in food intake [404]. This further supports the concept that the role of GLP-1 in reducing food intake is largely mediated through a peripheral vagal mechanism.

Obese mice have been reported to have reduced plasma GLP-1 and reduced release of GLP-1 in response to oral glucose [405]. Similarly in humans obesity has been associated with attenuated GLP-1 release in response to oral carbohydrate but not lipid [406]. This finding was later suggested to be due to altered gastric emptying by a subsequent study demonstrating no difference in GLP-1 release in response to carbohydrate or lipid in obese men [407]. It therefore appears that GLP-1 signalling is likely to be maintained in obesity, which is supported by GLP-1 induced suppression of hunger and food intake being unchanged in obesity [408]. This has led to GLP-1 analogues being developed as potential treatments for diabetes and obesity [409, 410].

PYY: Just like GLP-1, PYY is released from intestinal L-cells [411]. There is PYY present throughout the intestines, with very low levels in the proximal small intestine, increasing substantially in the ileum and even more into the colon [412]. This is reflected by a marked attenuation of PYY release after the removal of the colon [413, 414]. The mechanisms regulating the release of PYY appear to involve both direct contact with luminal nutrients and also

through CCK release in response to more proximal exposure to fat [415]. PYY release is also related to caloric load and is triggered by carbohydrate, fatty acid and to a lesser extent amino acid presence in the lumen [416]. It acts to slow gastric emptying as well as promote satiation [417, 418]. There are two endogenous forms of PYY [419]. Initially PYY is released as PYY₁₋₃₆, however once in the circulation the first two amino acids are cleaved to form PYY₃₋₃₆, the major circulating type [420]. PYY binds to specific GPCRs of the Y receptor family, Y1, 2, 4, 5 and 6 with PYY₃₋₃₆ showing a preferential affinity for Y2 and to a lesser extent Y1 and Y5 [411].

IP administration of PYY₃₋₃₆ has been shown to have an anorectic effect in rodents [421], which is completely abolished by sub-diaphragmatic vagotomy [422, 423]. The Y2 receptor has been identified on both intestinal vagal afferents [424] as well as neurons within the arcuate nucleus [425], indicating that PYY₃₋₃₆ may elicit its anorectic effects through either a central, peripheral or a combination of pathways.

A decrease in Y2 receptor expression in nodose ganglia of fasted rats and an increase in re-fed rats or in fasted rats that have received an infusion of CCK has previously been reported [424]. This upregulation of the Y2 receptor is believed to be due to increased CART expression in response to CCK, with CART acting in an autocrine fashion to upregulate Y2 expression [371]. A blockade of the Y2 receptor has been shown to abolish the anorectic effects of PYY₃₋₃₆ [426] and Y2 knockout mice exhibit hyperphagia and obesity

highlighting the importance of this pathway in the long term control of body weight [427].

In human obesity the postprandial release of PYY is impaired [428] and diet-induced obese rats exhibit reduced plasma PYY [429]. There is also reduced expression of Y2 receptors in vagal afferent neurons [335], which together suggests that in obesity there may be blunted intestinal satiety signals conveyed via PYY₃₋₃₆. However, exogenous PYY₃₋₃₆ still reduced food intake in obese mice [428]. It still remains to be determined whether obesity affects the ability for endogenous PYY to modulate food intake and whether the effect of PYY on vagal pathways is altered.

1.2.2.2.5 The vagus in obesity

Given the ability of the vagus to mediate homeostatic food intake processes, it is logical to consider that the vagal innervation of the gastrointestinal tract is an important site for control of food intake. As such it may be a site where adaptations occur to allow for the consumption of the higher level of food required to maintain an obese state

Obese people have been shown to have altered eating habits [430] and this is also seen in rodents with obese rats consuming larger meals [431]. This suggests that obesity may be associated with an altered perception of stomach distension to accommodate larger meals. Consistent with this is findings that obese humans can tolerate an increased level of intragastric volume before discomfort is felt [432]. This would suggest that there is an

altered sensitivity of gastric mechanoreceptors in obesity which allow for greater distension, but currently there is no evidence which conclusively demonstrates such a vagal adaptation.

It has also been demonstrated that in high fat diet-induced obesity the previously described activation of satiety circuits in response to intestinal luminal nutrients is decreased, suggesting a possible inability to detect when adequate nutrients have been consumed [433]. It is also reported that the mechanosensitivity of small intestinal vagal afferents is reduced in obesity [381]. Together this raises the possibility that diet-induced obesity may disturb normal vagal afferent signals from the gastrointestinal tract in a way which could promote excess energy consumption to maintain an obese state. However, currently there is no evidence whether gastric vagal mechanosensitive mechanisms are altered by diet-induced obesity and as such forms a major focus of this thesis.

In obesity some of the mechanisms that would normally act to maintain a healthy weight by limiting meal size and frequency are not doing so. Considering the ability of the vagus to respond to a wide array of meal related stimuli (both mechanical and chemical) and that it has central inputs into regions that are critical to multiple facets of food consumption regulation; it is a logical step to investigate the role of peripheral vagal afferent endings in food intake regulation in health and obesity.

1.3 HYPOTHESES

Given the substantial evidence implicating vagal afferents as important mediators in regulating food intake at a variety of levels, this thesis is designed to investigate the possibility that gastric vagal afferents act as a site of adaptation in obesity that act to protect a higher level of adiposity and perpetuates an obese phenotype. As such experiments were setup to address a number of hypotheses, which were:

1. Food restriction and diet-induced obesity will reduce the response of mechanosensitive gastric vagal afferent fibres to mechanical stimuli.
2. The ability of the gut peptides ghrelin and leptin to modulate vagal afferent mechanosensitivity will be altered by food restriction and obesity to permit increased food intake.
3. The gut peptides leptin, ghrelin and NPW regulate the expression of their own and other appetite peptide receptors and these effects are altered by diet-induced obesity to increase the potential for orexigenic signalling.
4. The effects of diet-induced obesity on vagal afferent mechanosensitivity are not reversible upon return to a 'normal' diet.

Addressing these hypotheses will provide vital information on the changes that are occurring in neuronal circuitry upon the development of obesity, highlight possible new and novel targets for the treatment of obesity and also explain why obesity can be such a troublesome condition to rid oneself of.

CHAPTER 2

DIET INDUCED ADAPTATION OF VAGAL AFFERENT FUNCTION

**Kentish S, Li H, Philp LK, O'Donnell TA, Isaacs
NJ, Young RL, Wittert GA, Blackshaw LA,**

Page AJ.

**Nerve Gut Research Laboratory, University of
Adelaide, Adelaide, SA 5005, Australia**

Published in The Journal of Physiology

2012 Jan; 590(1):209-221

STATEMENT OF AUTHORSHIP

Title of Paper: Diet Induced Adaptation of Vagal Afferent Function

Journal of Physiology – 2012, 590, 209-221

Stephen Kentish (Candidate)

Performed majority of electrophysiology recordings, RNA extraction and PCR experiments, husbandry of the mice, analysed and interpreted data and wrote the manuscript

I hereby certify that the statement of contribution is accurate.

SignedDate...21/02/2013

Hui Li

Aided in the husbandry of animal.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate...21/2/13

Lisa Philp

Performed feeding and blood glucose/insulin analysis of the SLD and HFD mice.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date.....7/2/13.....

Tracey O'Donnell

Assisted in the husbandry of the high fat diet mice.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date.....21/2/2013.....

Nicole Isaacs

Performed immunohistochemical and neuronal tracing data.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date.....7/9/13.....

Richard Young

Performed anterograde tracing procedure.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate 1.3.2013

Gary Wittert

Assisted in the construction and presentation of the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate 07/03/2013

Ashley Blackshaw

Assisted in the conceptualisation of experiments and construction of manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate 25/3/13

Amanda Page

Assisted in conceptualisation of studies, provided some electrophysiological data, data analysis and interpretations and manuscript construction .

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed

.....Date.....21/2/13

2.1 ABSTRACT

Afferent signals from the stomach play an important role in inhibition of food intake during a meal. The gastric hormone ghrelin can influence gastric satiety signalling by altering the sensitivity of gastric vagal afferents. Changes in diet, including food restriction and high fat diet (HFD) induced obesity, alter satiety signalling. We hypothesised that the function of gastric vagal afferent endings are affected by both a period of food restriction and a high fat diet, and that the inhibitory effect of ghrelin on vagal afferents is influenced by the different feeding conditions. We found that both fasting and HFD reduced the responses of gastric vagal tension receptors to distension, but not responses of mucosal receptors to mucosal contact. We traced vagal afferents anterogradely to their terminals in the mucosa where we found they were in close apposition to ghrelin containing cells. Ghrelin receptor mRNA was expressed in vagal afferent cell bodies of the nodose ganglia, and increased in response to caloric restriction, but decreased in HFD mice. In control mice, ghrelin decreased the sensitivity of tension but not mucosal receptors. After caloric restriction or high fat diet, ghrelin inhibited mucosal receptors, and the inhibition of mechanosensitive tension receptors was enhanced. Therefore, both caloric restriction and HFD decrease mechanosensory vagal afferent signals, and augment the inhibitory effect of ghrelin on vagal afferents, but different mechanisms mediate the short- and longer-term changes.

2.2 INTRODUCTION

It is well established that signals generated in the gastrointestinal tract are able to influence food intake [434]. One of these signals is mechanical distension of the stomach which inhibits feeding via stimulation of vagal afferents [435]. However, there are two distinct subtypes of vagal afferent mechanoreceptors, mucosal receptors and tension receptors [287, 436, 437] which are classified according to their response to specific types of mechanical stimulation. Mucosal receptors respond to fine tactile stimulation whereas tension receptors respond to stretch [436]. The role of gastric distension in the control of food intake is clear but that of mucosal contact is less obvious. In addition to generating sensations of nausea and vomiting, in response to chemical stimuli, [438] mucosal receptors may be important in detecting particle density, a factor which influences the rate of gastric emptying, which in turn relates inversely to satiety [439, 440]. In response to nutrients in the small intestine, enteroendocrine cells release peptides that activate adjacent mucosal vagal afferents to elicit satiation [193]. There are also enteroendocrine cells in the gastric mucosa that contain and release peptides known to affect appetite [304, 327, 441]. The afferents innervating the gastric mucosa are in an ideal position to detect both mechanical stimuli and locally released hormones such as ghrelin.

Ghrelin is a 28 amino acid orexigenic peptide released from exocrine X/A cells in the stomach [442-444]. The acute effect of ghrelin administration is a rapid increase in food intake [345, 445-449] and chronic administration of ghrelin

increases body weight in rodents [448]. The data suggest that ghrelin is involved in both the initiation of food intake and the signalling of energy deficit and thus plays an important role in regulation of energy homeostasis [445, 448, 449]. At least part of the signalling mechanism for ghrelin involves the vagus nerve and brain stem nuclei that ultimately signal to the hypothalamus [355, 445]. There is controversy about the role of the vagus nerves in the orexic effects of ghrelin. [355] showed that either truncal or selective gastric vagotomy or perivagal capsaicin abolished its action when given intravenously in rats. Asakawa et al. showed that ghrelin inhibited the resting discharge in whole vagal nerve recordings and that lesion of vagal afferent fibres inhibited the appetite-stimulating actions of ghrelin [445], whereas others have shown no effect of vagotomy on the acute effects of ghrelin administered intraperitoneally . The receptor for ghrelin, growth hormone secretagogue (GHS) 1 receptor, is found in vagal afferents that project to the stomach [450] and is expressed in mouse nodose ganglia [301]. We have reported that ghrelin selectively inhibited a subpopulation of mechanosensitive gastric vagal afferents [301]. Therefore there is an integral relationship between ghrelin and gastric vagal afferent satiety signals.

In addition to the acute effect of gastrointestinal peptides on vagal afferent satiety signals it is becoming apparent that they undergo longer-term changes in neurochemical phenotype with changes in nutritional status [451]. For example, it has been shown that withdrawal of food is responsible for changes in expression of G-protein coupled receptors such as melanin-concentrating hormone receptor-1 in vagal afferent neurons projecting to the gastrointestinal

tract [452]. High fat diets have also been reported to change the expression in nodose ganglia of transcripts encoding receptors known to be involved in appetite regulation, such as cholecystokinin (CCK1) and cannabinoid (CB1) receptors [369, 453]. We have shown that ghrelin modulates the mechanosensitivity of gastric vagal afferents *in vitro* [301] but it has not been established whether feeding state affects this relationship between ghrelin and gastric vagal afferents. Ghrelin release and plasma ghrelin levels depend significantly on the feeding state; fasting causes increased plasma ghrelin levels, whereas a high fat diet reduces plasma ghrelin levels in mice [441].

Using an *in vitro* gastro-oesophageal vagal afferent preparation to accurately evaluate different populations of mechanosensitive vagal afferent fibres, we investigated the effect of feeding state on the mechanosensitivity of gastric vagal afferents. In addition, we determined the effect of three feeding states on the neuromodulatory action of ghrelin on gastric vagal afferents. To establish if any changes in function of ghrelin could be the result of changes in receptor expression we determined the relative expression of ghrelin receptor in the vagal afferent cell bodies in the nodose ganglia.

2.3 MATERIALS AND METHODS

2.3.1 Ethical approval

All experimental studies were performed with the approval of the animal ethics committee of the Institute of Medical and Veterinary Science and the University of Adelaide, and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.3.2 Short term restriction of food intake

Eight week old female C57/BL6 mice were randomly assigned to one of two groups. The first group (controls) were fed *ad libitum* and allowed free access to a standard laboratory diet. The second group were fasted for 14 hours (fasted). Food was removed but they still had free access to water.

2.3.3 High fat diet model

Eight week old female C57/BL6 mice were randomly assigned to one of two groups. The first group (controls) were fed *ad libitum* and allowed free access to a standard laboratory diet (SLD) comprised of 7% energy from fat. The second group were fed *ad libitum* and allowed free access to a high fat diet (HFD; Specialty foods, Glen Forrest, Western Australia) comprised of 60% energy from fat. Water was freely available for both SLD and HFD fed mice. The mice were kept on either the SLD or the HFD for a period of 12 weeks during which time food intake was measured. The weight of the mice was monitored weekly. Blood was collected from a subset of these mice at the 12 week point, for determination of plasma glucose and insulin concentration.

2.3.4 *In vitro* mouse gastro-oesophageal afferent preparation

C57/BL6 mice were humanely killed via CO₂ inhalation and the thorax was opened by a midline incision. The stomach and oesophagus with attached vagal nerves were removed and placed in a modified Krebs solution containing (mM): NaCl 118.1, KCl 4.7, NaHCO₃ 25.1, NaH₂PO₄ 1.3, MgSO₄·7H₂O 1.2, CaCl₂ 1.5, citric acid 1.0 and glucose 11.1, bubbled with 95% O₂ – 5% CO₂ at 4°C during dissection to prevent metabolic degradation. After further dissection, the preparation was opened out longitudinally along the oesophagus and greater curve of the stomach. The preparation was then placed mucosal side up in the organ bath. This preparation has been described in detail previously [300, 436]. Nifedipine (1 µM) was also added to the Krebs solution to prevent smooth muscle contraction. In a previous preliminary study we have shown that nifedipine has no effect on the mechanical sensitivity of gastro-oesophageal vagal afferents [454].

2.3.5 Characterisation of gastro-oesophageal vagal afferent properties

Two types of mechanosensitive afferent fibre were studied, those responding to mucosal stroking but not circular tension (mucosal receptors) and those responding to mucosal stroking and circular tension (tension receptors) as reported previously [436].

Location of receptive fields of all types of afferent fibre was determined by mechanical stimulation throughout the preparation with a brush. Accurate quantification of mechanical responses was performed differently according to

the primary adequate stimulus for the type of fibre. Mechanical thresholds of both types of fibre were determined using calibrated von Frey hairs. The most reproducible, stimulus-dependent responses of these afferents to mucosal stroking were evoked when the probe was moved at a rate of 5 mm s^{-1} across the receptive field rather than being kept static. Due to the fact that the receptive fields were small ($< 1 \text{ mm}^2$), a single test at each intensity is prone to missing the centre of the receptive field on some occasions. Therefore, we minimised experimenter error by measuring the mean response to the middle eight of ten standard strokes given at 1 s intervals. Because the von Frey hair was bent throughout the stroking stimulus, the receptive field was subjected to an even force as the hair passed over it. Tension-response curves were also obtained and used in combination with von Frey thresholds to determine whether the receptive fields of fibres were located in the mucosa or the muscle layer. Tension stimuli were applied via fine suture silk attached via a hook to an unpinned point adjacent to the mechanoreceptive fields. The thread was attached to a cantilever via a pulley close to the preparation. Reference standard weights were then placed on the opposite end of the cantilever. Each weight was applied as a step and maintained for 1 min, and the response was measured as the mean discharge evoked over this period. Due to the fact that all responses to tension adapted slowly, this method of assessment was considered representative of physiological responsiveness. The tension-response curves were produced by applying weights to the cantilever system in the range of 1-5 g. A recovery period of at least 1 min was allowed between each tension stimulus. The effect of diet and mechanical stimuli on responses of tension and mucosal receptors was

determined using a two-way ANOVA. For some animals the mechanosensitivity of multiple receptors were determined. For analysis these were averaged to provide one replicate per receptor type per animal.

2.3.6 Effect of ghrelin on the mechanosensitivity of vagal afferents

After mechanical sensitivity of the gastric and oesophageal vagal afferents had been established, the effect of ghrelin on mechanical sensitivity was determined. Ghrelin (1 nM) was added to the superfusing solution and allowed to equilibrate for 20 min after which time the tension-response and stroke-response curves were re-determined. This equilibration period was observed so as to ensure penetration of the drug into all layers of the tissue. After this time the tension-response and stroke-response curves were re-determined. This procedure was repeated for ghrelin at increasingly higher doses (3-10 nM). Time-controlled experiments were performed in which there was no significant change in the mechanical responses over a comparable duration. The effect of ghrelin and mechanical stimuli on the mechanosensitivity of tension and mucosal receptors was determined using a two-way ANOVA. The effect of diet on the modulatory action of ghrelin on responses to mechanical stimulation was determined by accessing the response to either stroking (50mg von Frey hair; mucosal receptors) or tension (3g; tension receptors) at different concentrations of ghrelin (0, 1, 3, & 10nM). Significant differences between diets and ghrelin concentration were assessed using a two-way ANOVA.

2.3.7 Drugs

Stock solution of the peptide ghrelin (0.1mM; Sigma, Australia) was kept frozen (-80°C) and diluted to its final concentration in Krebs solution on the day of the experiment.

2.3.8 Quantitative reverse transcription polymerase chain reaction

Nodose ganglia were removed bilaterally from the same animals used for the *in vitro* mouse gastro-oesophageal vagal afferent preparation. RNA was isolated using an RNeasy Micro RNA extraction kit as per the manufacturer's instructions (Qiagen, Sydney, Australia). This process utilised RNeasy MinElute SpinElute Columns, allowing adsorption of RNA to the silica membrane, an on-column DNase digestion treatment, removal of contaminants with simple wash steps and elution of the RNA with RNase-free water. RNA quantification was determined by measuring the absorbance at 260 nm (A260) via a spectrophotometer (Bio-Rad, Regents Park, New South Wales, Australia). RNA quality was estimated by the A260 and A280 nm ratio.

Quantitative RT-PCR (QRT-PCR) reactions were performed as described in detail previously [455]. Briefly, QRT-PCR reactions were performed by using a Chromo4 (MJ Research, Bio-Rad) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and analysed with Opticon Monitor software (MJ Research). QRT-PCR reactions were performed with a Qiagen QuantiTect SYBRgreen RT-PCR one-step RT-PCR kit (Qiagen) according to the manufacturer's specifications with specific Quantitect Primer Assays (Qiagen) optimised for the detection of the known sequence of mouse ghrelin

receptor and β -tubulin transcripts contained in the NCBI reference sequence database (www.ncbi.nlm.nih.gov/RefSeq). These primer assays were used under the following conditions: reverse transcription, 50°C for 30 min; initial PCR activation, 95°C for 15 min; PCR cycles 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s repeated for 50 cycles. A melting curve program verified the specificity and identity of the RT-PCR products and no primer dimers were observed. Confirmation of the amplified products was resolved by 3% agarose gel electrophoresis and visualised via ethidium bromide staining. Each assay was run in at least triplicate in separate experiments. Control PCRs were performed by substituting RNA template with distilled RNase-free water. All assays were validated for linearity of amplification efficiency and quantitative standard curves obtained by serial dilutions of RNA. Calculations for relative mRNA transcripts expression were performed by the comparative C_T method, comparing to the internal reference gene β -tubulin using the equation ΔC_T (C_T of target transcript - C_T of β -tubulin). To determine the relative expression of these transcripts in whole nodose ganglia from high fat fed mice compared to standard laboratory fed mice and fasted mice compared to mice fed ad libitum, the $\Delta\Delta C_T$ was calculated by using the formula $\Delta\Delta C_T = \Delta C_T$ (ghrelin receptor in HFD or fasted mice) - ΔC_T (ghrelin receptor in SLD or fed ad libitum mice respectively). As such fold differences were calculated relative to the levels of β -tubulin RNA in the same sample. The relative fold differences were calculated by using the modified version of $2^{-\Delta\Delta C_T}$ [301, 456] correcting for PCR efficiencies. Quantitative data are expressed as means \pm SEM, and significant differences in transcript expression were determined using a nonparametric Mann-Whitney test at a significance level of $p < 0.05$.

2.3.9 Tracing studies

Adult (8 weeks old) female C57BL/6 mice had *ad libitum* access to water and a SLD and were fasted overnight before experimentation.

Anterograde tracing

The mice were anaesthetised with isoflurane (1-1.5% in oxygen). The left nodose was exposed and 0.5µL of wheat-germ agglutinin conjugated horseradish peroxidase [WGA-HRP (4mg.ml⁻¹); Vector Laboratories] was pressure injected into the nodose ganglia via a glass micropipette (ID=25µm). The injection site was then dried, the skin incision closed and antibiotics (Terramycin; 10mg/kg) and analgesic (butorphanol; 5mg/kg) administered subcutaneously. Two days following injection, mice were anaesthetised with pentobarbitone (60mg/kg, i.p.) and transcardially perfused with heparinised saline at 40°C, then 4% paraformaldehyde in 0.1M PBS (PFA-PBS) at 4°C. The stomach was then removed and cryoprotected with 30% sucrose for a minimum of 18 hours. The stomach was then frozen and 10µm transverse sections cut. Permanent visualisation of WGA-HRP was achieved using tyramide signal amplification (TSA). Briefly, the tissue sections were rinsed in TNT buffer (0.05% Tween 20, 0.15M NaCl, and 0.1M Tris-HCl, pH 7.5), blocked for 30 min in TNB (prepared as per the directions provided with the Perkin-Elmer TSA biotin reagent kit), and then incubated for 20 min with tyramide-biotin, diluted 1:50 in amplification diluent. The tissue was then rinsed in TNT buffer and reacted with streptavidin conjugated to Alexa Fluor® 647 (Invitrogen) for 2 hr at room temperature. Following ghrelin

immunohistochemistry (below) sections were coverslipped using ProLong antifade mounting media (Invitrogen).

Immunohistochemistry

Immunoreactivity for ghrelin was detected in anterograde traced stomach sections using a chicken polyclonal antibody to ghrelin (Abcam). Primary antibody was visualised using goat anti-chicken secondary antibody conjugated to Alexa Fluor®488 (Invitrogen). Briefly, sections were air dried at room temperature and rinsed in PBS + 0.2% Triton X-100 (Sigma-Aldrich; PBS-T, pH7.4) to facilitate antibody penetration. Primary antibody was diluted 1:1000 in PBS-T and incubated at 4°C overnight. Unbound antibody was then removed with PBS-T washes and sections were incubated for 1hr at room temperature with secondary antibody (1:200 in PBS-T). The sections were given final PBS-T washes, drained and mounted with ProLong Antifade (Invitrogen). Slides where the primary antibody was omitted showed no labelling and served as negative controls.

Visualisation

Slide sections were visualised using an epifluorescence microscope (BX-51, Olympus) equipped with filters for AF647 and AF488, with images acquired by a Cool-Snapfx monochrome digital camera (Roper Scientific). Individual fluorochromes were pseudo-coloured using Olympus imaging analySis software: however the luminance of images was not adjusted.

2.4 RESULTS

2.4.1 Short term restriction of food intake reduces mechanosensitivity of vagal afferents

The effect of mechanical stimulation on gastric and oesophageal vagal afferents from control and fasted mice is illustrated in Figure 2.1. The mechanosensitivity of mucosal receptors was similar in both groups of mice (Figure 2.1Ai and ii respectively). In contrast, the responses of gastric tension receptors from fasted mice were significantly smaller than those in control mice ($p < 0.0001$: diet effect, two-way ANOVA; Figure 2.1Bi). Oesophageal tension receptors were more variably affected, and although there appears to be a similar reduction in the responses of oesophageal tension receptors from fasted mice the difference in response did not differ significantly from those in control mice (Figure 2.1Bii: $p > 0.05$: diet effect, two-way ANOVA).

2.4.2 Effects of long term alterations in diet

Mice on the HFD increased in weight by $6.57 \pm 0.3\text{g}$ ($N=51$) over the 12 week period that they were on the diet ($N=51$) and gained significantly more weight ($p < 0.001$; unpaired t-test) than SLD mice who gained $5.22 \pm 0.12\text{g}$ ($N=52$). This is likely to be due to the significant increase ($p < 0.0001$; unpaired t-test) in energy intake, over the 12 week period of the HFD mice ($4798.97 \pm 149.49\text{MJ}$; $N=11$) compared to the SLD mice ($3434.82 \pm 99.72\text{MJ}$; $N=12$) in accord with previous reports [457]. The gonadal fat pad weight was significantly heavier ($p < 0.01$; unpaired t-test) in HFD mice ($0.55 \pm 0.07\text{g}$; $N=17$) compared to SLD mice ($0.32 \pm 0.03\text{g}$; $N=16$). Blood glucose and insulin levels

were not significantly different between SLD (7.34 ± 0.64 mmol/L and 0.41 ± 0.08 pmol/L respectively; N=11) and HFD (5.96 ± 0.48 mmol/L and 0.66 ± 0.15 pmol/L respectively; N=11) indicating that the HFD group did not developed diabetes over the 12 week diet period.

The effect of mechanical stimulation on gastric and oesophageal vagal afferents from both groups of mice is illustrated in Figure 2.2. The mechanosensitivity of mucosal receptors was not significantly different between SLD and HFD mice (Figure 2.2Ai and ii). However, the response of gastric and oesophageal tension receptors of mice fed a HFD was significantly less than the responses of mice fed a SLD ($p < 0.001$: diet effect, two-way ANOVA; Figure 2.2Bi and ii respectively). Thus the effect of a HFD on the mechanosensitivity of gastro-oesophageal tension and mucosal receptors appears similar to the effect of fasting (Figure 2.1).

2.4.3 Anatomy of vagal afferent endings and ghrelin containing cells in the gastric mucosa

We determined the anatomical relationship of ghrelin immunopositive cells in the gastric mucosa with identified vagal afferent endings. Ghrelin-like immunoreactivity was restricted to isolated cells at the base of submucosal glands with apical surfaces in the lumen (Figure 2.3Aii) which had morphology consistent with enteroendocrine X/A cells. Anterograde tracing of vagal afferents from the nodose ganglion revealed intraepithelial fibres within the mucosal layer of the stomach (Figure 2.3Ai), as previously described in the small intestine [458]. These were closely apposed (within a few micrometres)

to ghrelin containing cells (Figure 2.3Aiii). Identified vagal endings also surrounded myenteric ganglia, consistent with previously characterised intraganglionic laminar endings [288]. These endings were located at a distance from ghrelin-containing cells separated by the lamina propria, submucosa, muscularis mucosae and circular smooth muscle (data not shown).

2.4.4 Ghrelin receptor expression in vagal afferent pathways

QRT-PCR was used to determine expression of ghrelin receptor in vagal afferent neurons in the nodose ganglia. Quantitative analysis revealed that ghrelin receptor mRNA levels in the nodose ganglia of mice fasted for 14 hours were 1.53-fold higher than in fed mice ($p < 0.05$; Mann-Whitney test; Figure 2.3B). There was no significant difference in the expression of ghrelin receptor mRNA detected in the nodose ganglia of HFD mice compared to SLD mice (Figure 2.3B). Agarose gel electrophoresis confirmed amplification of housekeeper (β -tubulin) and ghrelin receptor with product sizes of 118 and 121bp respectively (Figure 2.3C).

2.4.5 Vagal afferent responses to ghrelin are altered by changes in food intake

An overnight fast had major effects on the ghrelin sensitivity of gastric mucosal receptors (Figure 2.5). The response to mucosal stroking of gastric mucosal receptors was significantly reduced in fed mice, but only at the highest dose of ghrelin (10nM; Figure 2.4Ai; $p < 0.05$: ghrelin effect, two-way ANOVA). In fasted mice ghrelin (1-10nM) significantly reduced the responses

of gastric mucosal receptors to mucosal stroking with calibrated von Frey hairs (10-1000mg; $p < 0.01$: ghrelin effect, two-way ANOVA; Figure 2.5Aii). When the response to 50mg von Frey hair at varying concentrations of ghrelin (0-10nM) was plotted (Figure 2.4B) it was evident that diet significantly altered the response to mechanical stimulation in the presence of ghrelin ($P < 0.01$; diet effect, two-way ANOVA). Therefore gastric mucosal receptor sensitivity to ghrelin was significantly increased following an overnight fast.

Ghrelin (3-10nM) significantly and dose-dependently reduced the response of gastric (Figure 2.5Ai & Figure, 2.5Bi) tension receptors to circular tension ($p < 0.05$: ghrelin effect, two-way ANOVA; 1-5g). In fasted mice ghrelin (1-10nM) also significantly reduced the response of gastric tension receptors (Figure 2.5Aii & 2.5Bii) to circular tension even at the lowest dose tested of 1nM ($p < 0.001$: ghrelin effect, two-way ANOVA). When response to a 3g load was plotted against increasing concentrations of ghrelin there was no significant difference between the fed and fasted curves (data not shown). Therefore the sensitivity of gastric tension receptors to ghrelin was unaffected by a short term restriction of food intake.

The effect of longer term changes in food intake on ghrelin's neuromodulatory action on vagal afferent responses to mechanical stimulation is illustrated in figures 2.6 and 2.7. Ghrelin (1-10nM) did not significantly affect the response of gastric (Figure 2.6Ai & 2.6Ci) mucosal receptors to mucosal stroking in SLD mice. In contrast, ghrelin significantly reduced the response to mechanical stimulation of gastric (Figure 2.6Aii & 2.6Cii) mucosal receptors ($p < 0.05$:

ghrelin effect, two-way ANOVA) in HFD mice, thus mimicking responses seen in the fasted state. When the response to stroking with a 50mg von Frey hair was plotted against varying concentrations of ghrelin (0-10nM; Figure 2.6B) it was evident that diet significantly altered the response to mechanical stimulation in the presence of ghrelin ($P < 0.01$: diet effect, two-way ANOVA). Therefore gastric mucosal receptor sensitivity to ghrelin was significantly increased after a HFD.

Ghrelin significantly reduced the response of gastric tension receptors to circular stretch (1-5g) in SLD and HFD mice (Figure 2.7i & ii respectively; $p < 0.05$: ghrelin effect, two-way ANOVA). When the response to a 3g load was plotted against increasing concentrations of ghrelin there was no significant difference between the SLD and HFD curves (data not shown). Therefore the sensitivity of gastric tension receptors to ghrelin was unaffected by a HFD.

2.5 DISCUSSION

These data provide the first evidence that signalling of gastric distension by vagal afferents is significantly reduced by both food withdrawal and a high fat diet. Moreover, ghrelin inhibits mucosal vagal afferents that are normally insensitive to it. Thus, not only is the gastric vagal afferent satiety signal reduced by food withdrawal and high fat feeding, but the orexigenic effect of ghrelin is also enhanced.

Short-term restriction of food intake selectively reduced the mechanosensitivity of tension receptors and in particular gastric tension receptors. As these afferents detect distension of the stomach [288, 290, 296] their reduced sensitivity to stretch may delay satiety signalling, leading to increased food intake of an individual in order to achieve satiation; this would constitute a natural adaptive response after a fast. Importantly we found that in mice chronically fed a high fat diet there was also a reduction in the mechanosensitivity of tension receptors suggesting that the satiety signal would also be attenuated in response to a high fat diet. Such a mechanism may have evolved to maximise assimilation of energy from calorie-rich foods in anticipation of famine. A number of studies have shown that obese humans have increased gastric capacity [432, 459] which results in increased energy intake. This could occur due to a reduction in the gastric distension-stimulated vagal afferent satiety signal. A recent publication has revealed that the sensitivity of jejunal afferents to satiety stimuli is reduced after a HFD [381]. Further, the major mechanism for this decrease in sensitivity was due to a

reduction in excitability of the neuronal cell membrane [381]. This reduced excitability of all vagal afferent cell membranes in HFD mice may explain the reduced mechanosensitivity of oesophageal afferents observed in the present study.

The mechanisms leading to reduced mechanosensitivity of vagal afferents are currently unknown both in terms of its precise causes and how it is manifested at the molecular level. It has been shown that fasting alters the neurochemical phenotype of vagal afferent neurons that project to the gastrointestinal tract in terms of the G-protein coupled receptors and peptide neurotransmitters they express [368, 424, 452, 460]. High fat diets have also been reported to modify vagal afferents transcript expression of specific G-protein coupled receptors and growth factors [369, 453, 461]. Together with changes in the levels of plasma satiety hormones these adaptations are likely to profoundly alter vagal satiety signals during a high fat diet. For example, the plasma levels of leptin are significantly elevated in mice fed a high fat diet [462] and this adipokine may directly influence vagal afferent mechanosensitivity while also acting to modulate their responsiveness to ghrelin. Our data now adds new knowledge that diet induces changes in the fundamental mechanosensory properties of gastric vagal afferents. Now that we have demonstrated that reduced mechanosensitivity is a robust outcome in response to a HFD, we are in a good position to investigate the mechanism of this change, which may reveal more empirical targets for the treatment of obesity.

In the present study we confirmed our earlier findings [301] that ghrelin selectively and dose-dependently reduces the response of tension receptors in normal control mice fed *ad libitum* on a SLD. This effect of ghrelin on tension receptors was maintained after food withdrawal and after a HFD. The effect of ghrelin was not selective for gastric vagal afferents as it also reduced the mechanosensitivity of oesophageal tension sensitive afferents which are distant from the site of ghrelin release (data not shown), suggesting this would be a systemic action *in vivo*. The concentration of ghrelin used in our *in vitro* organ bath preparation is comparable with the concentration of ghrelin in mouse plasma which ranges from 1 – 3 nM depending on the feeding state [441]. Plasma ghrelin levels are significantly higher after withdrawal of food for 18 hours and significantly reduced in mice fed a HFD [441]. Therefore, based on plasma levels and the fact that sensitivity of gastric vagal tension receptors to ghrelin did not significantly change in both conditions, it could be predicted that ghrelin inhibition of tension receptors would be increased after food withdrawal, but decreased in mice fed a high fat diet.

The response of mucosal receptors to mucosal stroking was unaffected by both a short-term restriction in food intake and a high fat diet. However, where these particular vagal afferent fibres become relevant is their innervation of the gastric mucosa, where they are also likely to detect locally released hormones such as ghrelin. Indeed, we observed that vagal afferents innervating the gastric mucosa are in close apposition to ghrelin containing epithelial cells. However, in agreement with our earlier observations [301], mucosal receptors were unaffected by ghrelin in control mice other than at the

highest dose used. After food withdrawal ghrelin potently reduced the response of gastric vagal mucosal receptors to mucosal stroking. This effect was seen also after a high fat diet, so mucosal receptors acquire ghrelin sensitivity. Gastric mucosal receptors are considered to be important in detecting particle size, which reduces gastric emptying and food intake [439, 440], so ghrelin could act to reduce this signal in fasting and obesity. In fasted healthy volunteers, ghrelin administration has been shown to increase the rate of gastric emptying in addition to elevating hunger ratings [463]; this may be a consequence of the decreased mechanosensitivity of gastric mucosal receptors by ghrelin.

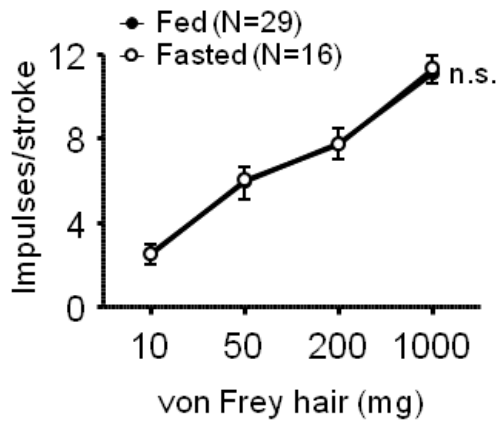
The expression of ghrelin receptor transcript in the nodose ganglia was significantly increased in mice that were fasted for 14 hours. An increase in ghrelin receptor expression may explain the increase in effect of ghrelin on gastric mucosal receptors. Correspondingly it has been established that growth hormone secretagogue (GHS) receptor containing vagal neurons project to the stomach [450] although reports vary on changes in ghrelin receptor expression after food withdrawal. One group has reported that the transcript levels of ghrelin receptor in the rat nodose ganglia are unaltered [353] while another group has shown a doubling of transcript expression in fasted compared to fed rats [464]. Previously we reported that ghrelin reduced the mechanosensitivity of mucosal receptors in the ferret and concluded that this was a possible species difference [301]. The difference is most likely due to the fact that ferrets were fasted prior to experimentation. The expression of ghrelin receptor in the nodose ganglia of mice fed a HFD was not significantly

different from mice fed a SLD, and therefore the increased effect of ghrelin on gastric mucosal receptors cannot be explained by an increase in ghrelin receptor expression unlike the effect of fasting. It is possible that in the longer term, changes occur in the coupling of ghrelin receptors to specific intracellular pathways, rather than in absolute levels of ghrelin receptor expression.

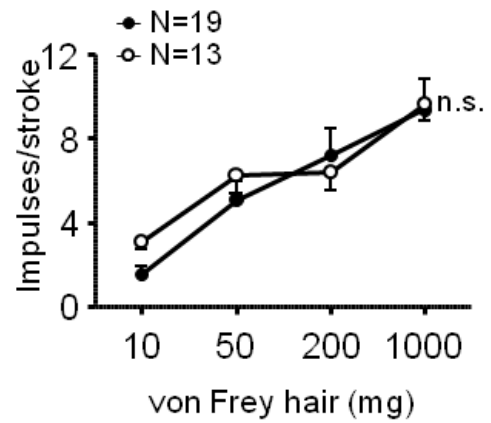
In conclusion, we have established that the basic mechanosensitivity of gastro-oesophageal vagal afferents, particularly gastric afferents, is significantly reduced by both restriction and excess of energy intake. A consequence of this decreased mechanosensitivity would be an undesirable dampening of the satiety signal in obesity. In addition, the inhibitory effect of ghrelin on vagal afferents becomes less selective after food withdrawal and a high fat diet and is therefore amplified. The additional inhibitory effect of ghrelin on gastric mucosal receptors would reinforce the inhibitory effect of ghrelin on tension receptors and enhance its orexigenic effect. This data indicates the plasticity in the mechanism of action of ghrelin under different feeding states, and the complex role of gastric mechanisms in food intake regulation.

A Mucosal receptors

i Gastric

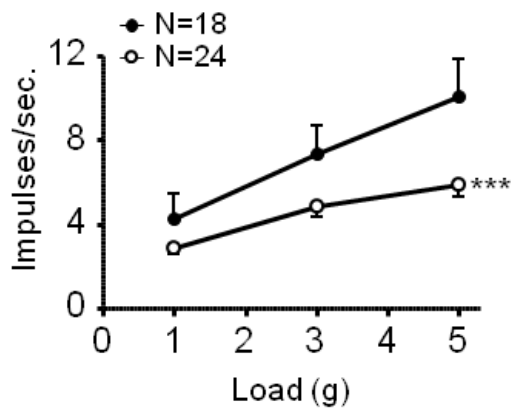


ii Oesophageal



B Tension receptors

i Gastric



ii Oesophageal

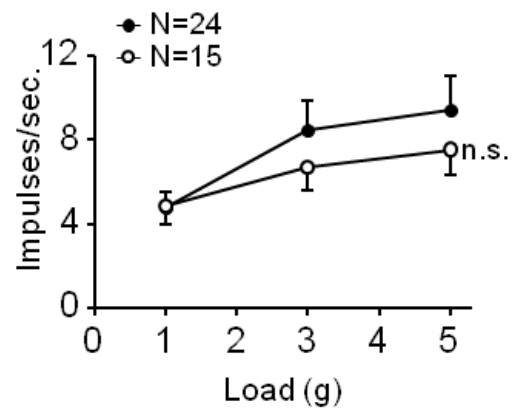
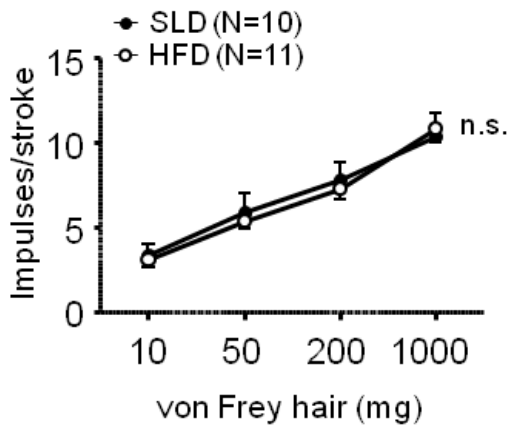


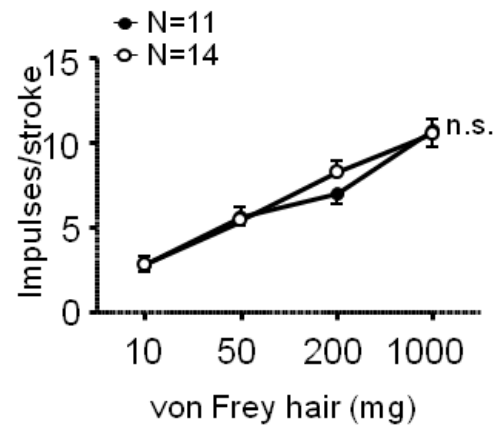
Figure 2.1: The effect of short term restriction in food intake on the response of gastric and oesophageal mechanosensitive vagal afferents to mechanical stimulation. Stimulus-response functions of mucosal (A) and tension sensitive (B) gastric (i) and oesophageal (ii) vagal afferents from mice fed ad libitum (●) and mice fasted for 14 hours (○). ***P<0.001 compared with fed mice (Diet effect, two-way ANOVA).

A Mucosal receptors

i Gastric

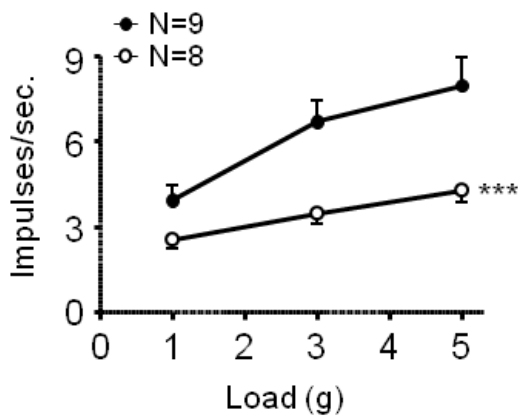


ii Oesophageal



B Tension receptors

i Gastric



ii Oesophageal

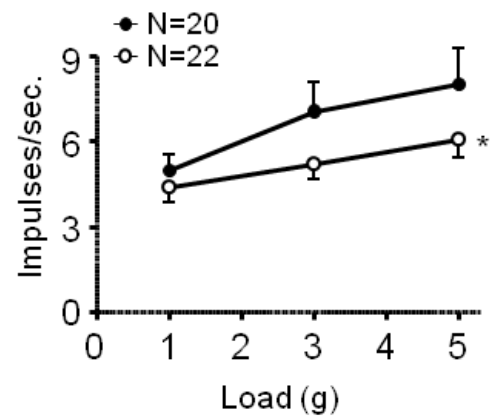


Figure 2.2: The effect of a high fat diet on the response of gastric and oesophageal mechanosensitive vagal afferents to mechanical stimulation. Stimulus-response functions of mucosal (A) and tension sensitive (B) gastric (i) and oesophageal (ii) vagal afferents from mice fed a standard laboratory diet (SLD; ●) and mice fed a high fat diet (HFD; ○). *P<0.05, ***P<0.001 compared with mice fed a standard laboratory diet (Diet effect, two-way ANOVA).

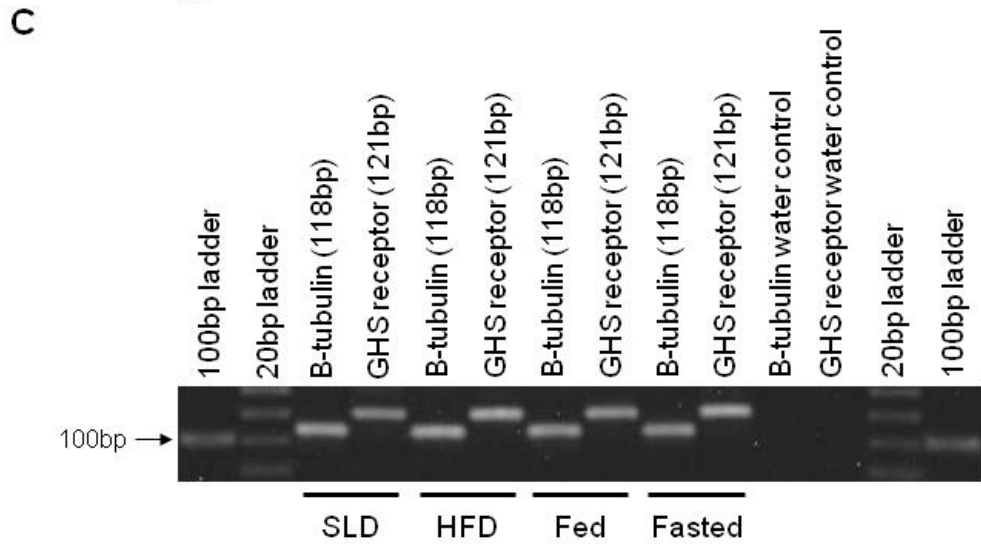
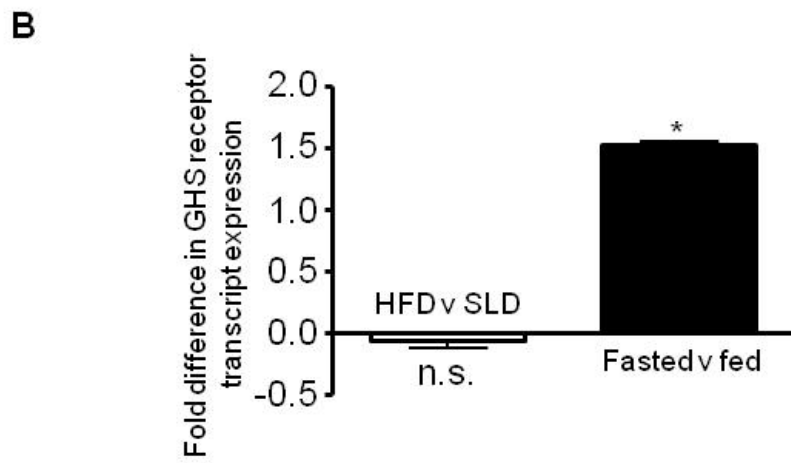
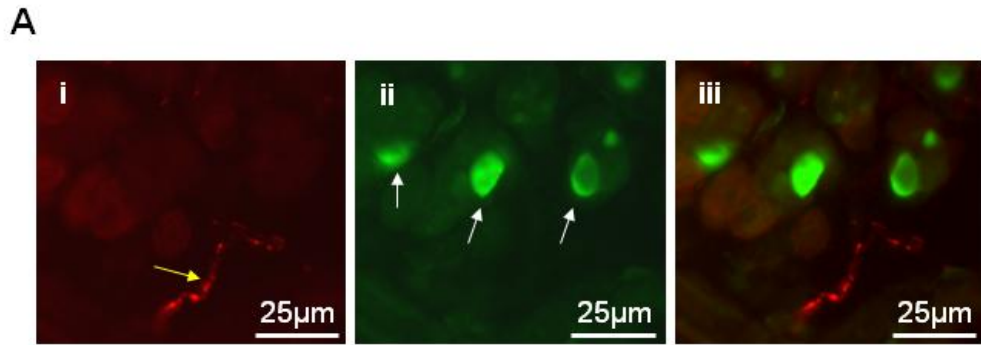


Figure 2.3: Relationship between vagal afferents and ghrelin containing cells in the gastric mucosa. Combined ghrelin immunohistochemistry and anterogradely traced vagal afferent fibres in the mouse stomach (Ai-iii). The stomach sections show a cross section through a gland. (Ai) Anterogradely traced (WGA-HRP) vagal afferent fibre (yellow arrow). (Aii) Epithelial cells immunopositive for ghrelin in stomach villi (white arrows). (Aiii) Overlay of i and ii showing close proximity of ghrelin-containing epithelial cells and anterograde labeled vagal afferent fibres. (B) Relative expression of growth hormone secretagogue 1 (GHS) receptor mRNA in nodose from HFD mice compared with SLD mice (open bar) and fasted mice compared with fed mice (closed bar). The relative expression values were calculated using $\Delta\Delta CT$ ($\Delta CT_{HFD} - \Delta CT_{SLD}$ or $\Delta CT_{fasted} - \Delta CT_{fed \textit{ ad libitum}}$) of ghrelin receptor transcript. The fold difference in transcript expression in HFD nodose relative to SLD and nodose from fasted mice compared to mice fed *ad libitum* was calculated using the formula $2^{-\Delta\Delta CT}$. In nodose from mice fasted for 14hr there is a 1.53 fold increase in transcript compared to fed mice (closed bar, *P<0.05; Mann Whitney test). In nodose from HFD mice there is a small but not significant 0.06 fold decrease in transcript compared to SLD mice (open bar). (C) Agarose gel electrophoresis confirmed the amplified product sizes.

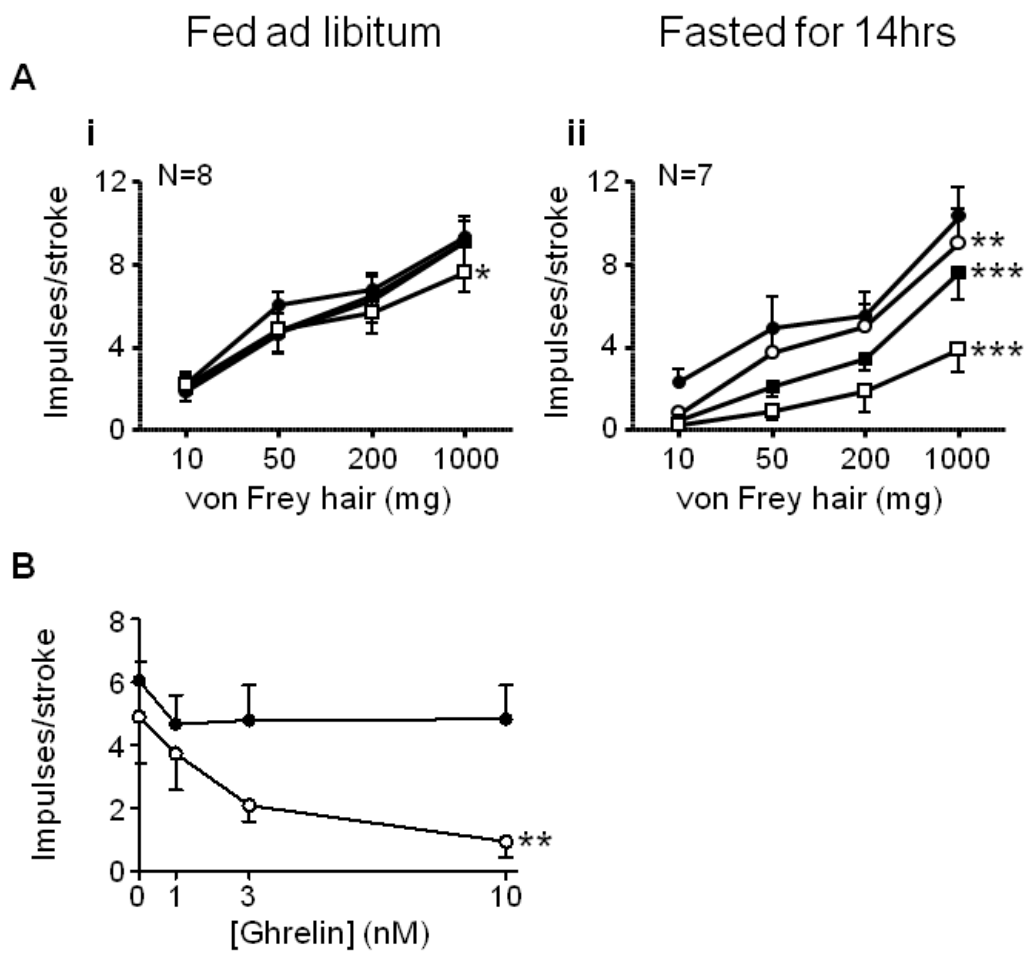


Figure 2.4: Fasting increases the effect of ghrelin on the response of gastric mucosal receptors. (A) The responses of gastric mucosal receptors to mucosal stroking with calibrated von Frey hairs (10-1000mg) in the absence (●) and presence of ghrelin (1 (○), 3 (■) and 10nM (□). These recordings were taken from fed mice (i) or fasted mice (ii) prior to the experiment. *P<0.05, **P<0.01, ***P<0.001 compared with control (●; Ghrelin effect, two-way ANOVA). (B) The effect of ghrelin on the responses of mucosal receptors to stroking with a 50mg von Frey hair in mice fed ad libitum (●) and mice fasted (○) for 14 hours. Diet significantly enhanced the inhibitory action of ghrelin (**P<0.01; diet effect, two-way ANOVA).

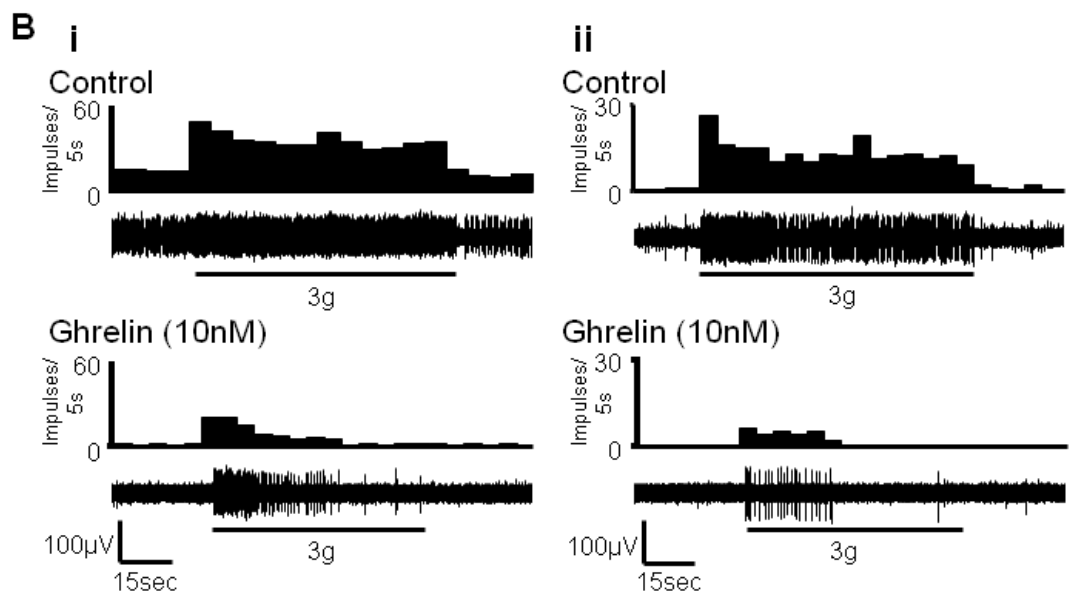
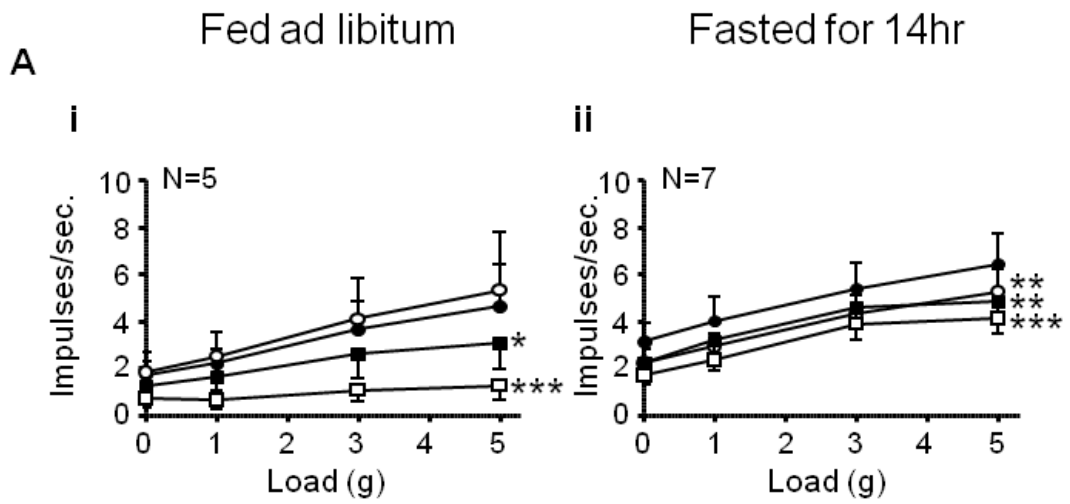


Figure 2.5: The effect of ghrelin on the response of gastric tension receptors. (A) The responses of gastric tension sensitive receptor to circumferential stretch (1-5g) in the absence (●) and presence of ghrelin (1 (○), 3 (■) and 10nM (□). These recordings were taken from fed (i) or fasted mice (ii). *p<0.05, **P<0.01, ***P<0.001 compared with control (●; ghrelin effect, two-way ANOVA). There was no significant difference in the inhibitory effect of ghrelin between the two diets. (B) Typical traces of tension receptors from a fed (i) and a fasted (ii) mouse.

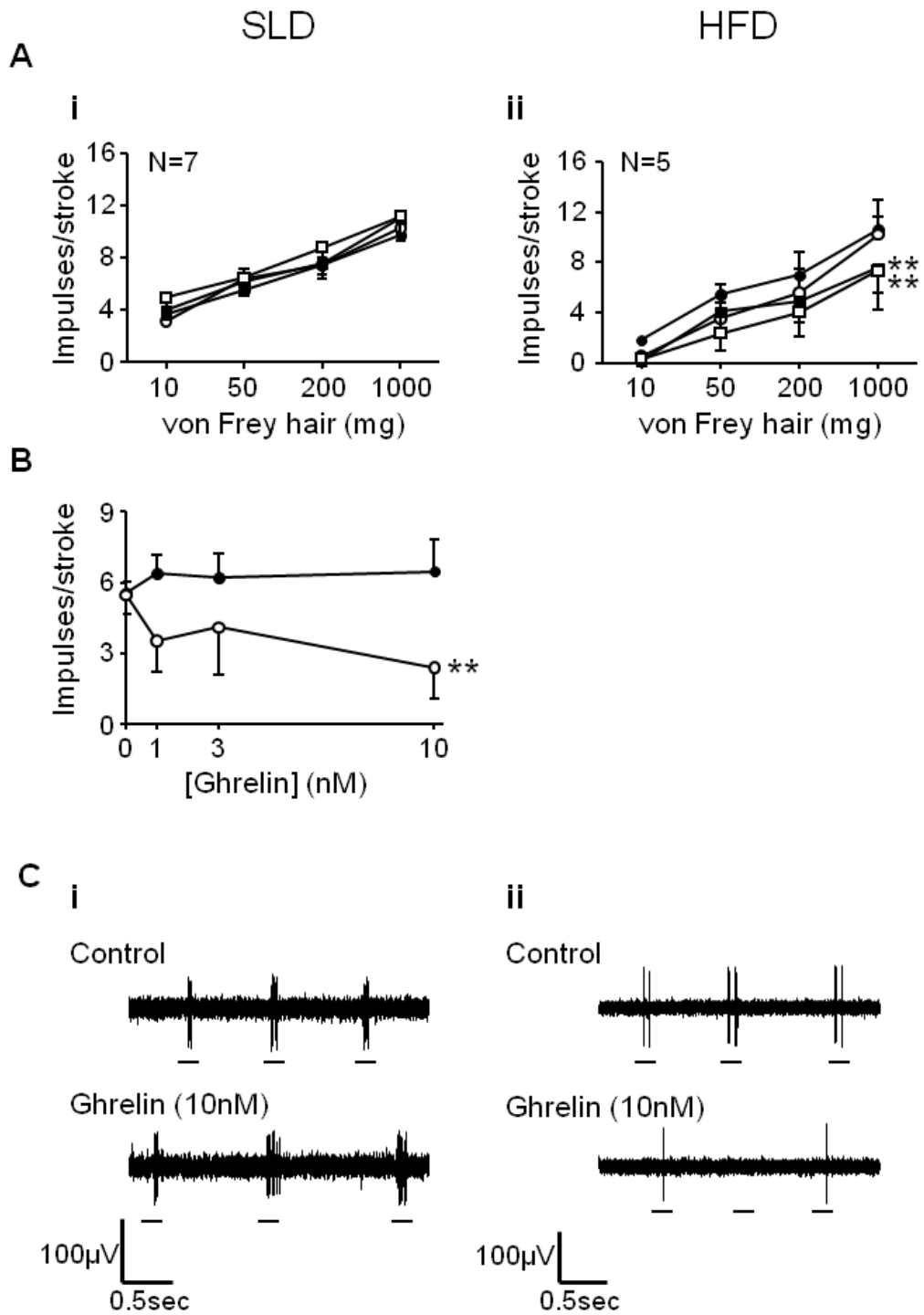


Figure 2.6: High fat diet increases the effect of ghrelin on the response of gastric (A & C) mucosal receptors. (A & C) The responses of gastric mucosal receptors to mucosal stroking with calibrated von Frey hairs (10-1000mg) in the absence (●) and presence of ghrelin (1 (○), 3 (■) and 10nM (□). These recordings were taken from mice fed a standard laboratory diet (Ai & Ci) or mice fed a high fat diet (Aii & Cii) for 12 weeks prior to the experiment. **P<0.01 compared with control (●; ghrelin effect, two-way ANOVA). (B) The effect of ghrelin on the response of mucosal receptors to stroking with a 50mg von Frey hair in mice fed a SLD (●) and mice fed a HFD (○). Diet significantly enhanced the inhibitory action of ghrelin (**P<0.01; diet effect, two-way ANOVA).

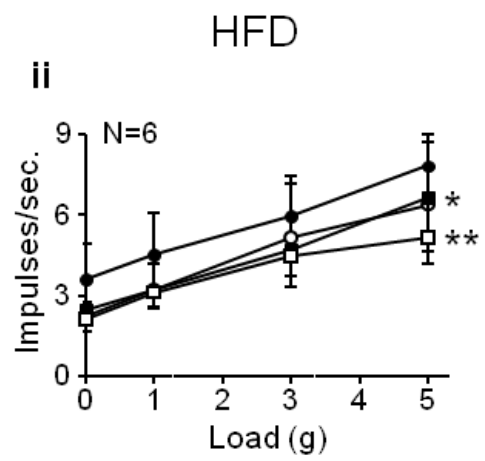
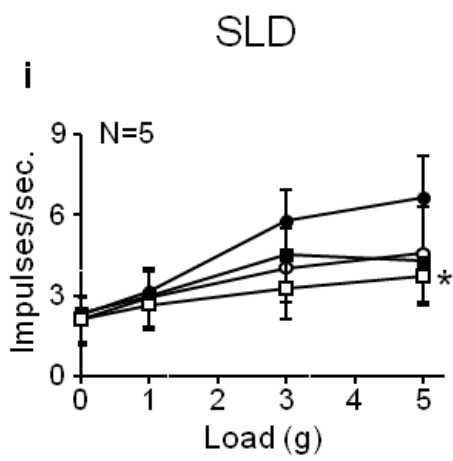


Figure 2.7: The effect of ghrelin on the response of gastric tension receptors. The responses of gastric tension receptors to circumferential stretch (1-5g) in the absence (●) and presence of ghrelin (1 (○), 3 (■) and 10nM (□)). These recordings were taken from mice fed a standard laboratory diet (i) or mice fed a high fat diet (ii) for 12 weeks prior to the experiment. *p<0.05, **P<0.01 compared with control (●; ghrelin effect, two-way ANOVA). There was no significant difference in the inhibitory effect of ghrelin between the two diets.

CHAPTER 3

GASTRIC VAGAL AFFERENT MODULATION BY LEPTIN IS INFLUENCED BY FOOD INTAKE STATUS.

**Kentish SJ, O'Donnell TA, Isaacs NJ, Young RL,
Li H, Harrington AM, Brierley SM, Wittert GA,
Blackshaw LA, Page AJ.**

**Nerve Gut Research Laboratory, University of
Adelaide, Adelaide, SA 5005, Australia**

Published in The Journal of Physiology

2013 April; 591(7):1921-1934

STATEMENT OF AUTHORSHIP

Title of Paper: Gastric vagal afferent modulation by leptin is influenced by food intake status.

Journal of Physiology – 2013, 591(7): 1921-1934

Stephen Kentish (Candidate)

Performed majority of electrophysiology recordings, RNA extraction and PCR experiments, husbandry of the mice, analysed and interpreted data and wrote the manuscript .

I hereby certify that the statement of contribution is accurate.

SignedDate...21/02/2013

Tracey O'Donnell

Performed weekly feeding and weighing of the mice.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate...21/2/2013

Nicole Isaacs

Performed immunohistochemical and neuronal tracing data.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate.....7/3/13.

Richard Young

Performed anterograde tracing procedure.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate.....1-3-2013

Hui Li

Performed retrograde tracing procedure.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate.....21/2/13.

Andrea Harrington

Developed laser capture microdissection procedure.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate..06/03/2013

Stuart Brierley

Developed laser capture microdissection procedure.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate..21/2/2013

Gary Wittert

Assisted in the construction and presentation of the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate..07/03/2013

Ashley Blackshaw

Assisted in the conceptualisation of experiments and construction of manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date..... 25/3/13

Amanda Page

Assisted in conceptualisation of studies, data analysis and interpretations and manuscript construction

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date..... 21/2/13

3.1 ABSTRACT

Energy intake is strongly influenced by vagal afferent signals from the stomach, and is also modulated by leptin. Leptin may be secreted from gastric epithelial cells, so we aimed to determine the direct effect of leptin on gastric vagal afferents under different feeding conditions. Female C57BL/6 mice were fed standard laboratory diet, high fat diet or were food restricted. The expression of leptin receptor and its signal transduction molecules in vagal afferents was determined by retrograde tracing and reverse-transcription polymerase chain reaction (RT-PCR), and the relationship between leptin immunopositive cells and gastric vagal afferent endings determined by anterograde tracing and leptin immunohistochemistry. An *in vitro* preparation was used to determine the functional effects of leptin on gastric vagal afferents and the second messenger pathways involved. Leptin potentiated vagal mucosal afferent responses to tactile stimuli, and epithelial cells expressing leptin were found close to vagal mucosal endings. After fasting or diet-induced obesity, potentiation of mucosal afferents by leptin was lost and leptin receptor expression reduced in the cell bodies of gastric mucosal afferents. These effects in diet-induced obese mice were accompanied by a reduction in anatomical vagal innervation of the gastric mucosa. In striking contrast, after fasting or diet-induced obesity, leptin actually inhibited responses to distension in tension receptors. The inhibitory effect on gastric tension receptors was mediated through PI3K-dependent activation of BKCa channels. The excitatory effect of leptin on gastric mucosal vagal afferents was mediated by PLC-dependent activation of TRPC1 channels. These data

suggest the effect of leptin on gastric vagal afferent excitability is dynamic and related to the feeding state. Paradoxically, in obesity, leptin may reduce responses to gastric distension following food intake.

3.2 INTRODUCTION

The satiety hormone, leptin, which is secreted from adipose tissue provides a signal to the hypothalamus reflecting the amount of fat stored [465, 466] and serves as a long-term regulator of nutrient intake, adiposity and body weight [467]. Levels of circulating leptin reflect the degree of adiposity [466]. However, despite elevated leptin levels obese individuals do not show diminished appetite, suggesting a relative resistance to leptin [305]. An alternative explanation may be that leptin permits fat stores to be maintained at or around a set point, that is genetically determined but, susceptible to environmental modification [174].

Leptin is also expressed in, and secreted by, chief cells [304] and parietal (P) cells [468] in the gastric mucosa. Leptin released from this site could act on adjacent gastric vagal afferent endings to modulate peripheral signals in response to food intake. Indeed, leptin has been shown to directly activate cultured gastric and duodenal vagal afferents from rats [333, 334] and the leptin receptor (Lep-R) is expressed in rat vagal afferent neurons [469, 470], many of which terminate in the stomach. A vagal action of gastric leptin may serve to augment the acute appetite suppressant effects of circulating leptin [326]. However, recent evidence shows that the effects of leptin on vagal afferents are lost with chronic high fat diet feeding [335, 336].

Within the stomach wall there are two classes of mechanically sensitive gastric vagal afferents. In the muscular layer, tension receptors detect fullness

by responding to distension and contraction of the stomach wall [471, 472]. In contrast, mucosal receptors are excited by mechanical contact of larger food particles with the epithelium, and may contribute to the discrimination of particle size. The net effect of activating mucosal afferents is to trigger vagal reflexes which slow gastric emptying and facilitate mechanical digestion in the stomach [297]. It is currently unknown whether leptin has differential effects on gastric vagal afferent subclasses or whether any such effects can be modified by caloric restriction or excess. Furthermore, the mechanism by which Lep-Rs trigger changes in the electrical activity of vagal afferent endings has not been established.

We therefore determined the effect of leptin on the mechanosensitivity of gastric vagal afferents under different feeding conditions, including a short term restriction in food intake and long term consumption of a high fat diet (HFD), and examined the second messenger signalling pathways activated by leptin. In addition, we established the relationship between leptin containing cells in the gastric mucosa and vagal afferent endings.

3.3 MATERIALS AND METHODS

3.3.1 Ethical approval

All studies were approved and performed in accordance with the guidelines of the Animal Ethics Committees of the University of Adelaide and SA Pathology, Adelaide, Australia.

3.3.2 Mice

All mice used for this study were housed with littermates in groups of four, unless otherwise stated. For the fed and fasted treatment groups, 8wk old female C57BL/6 mice, housed individually, were fed *ad libitum* or fasted for 14 hours prior to experimentation. In diet studies, 7 week old female C57BL/6 mice were allowed to acclimatize for a period of one week after which control mice were continued on a standard laboratory mouse diet (SLD (7% energy from fat), n=52), whilst the HFD mice (n=51) were placed on a diet high in fat (60% of energy from fat, Specialty Feeds, Glen Forrest, WA, Australia) for a further 12 weeks. During this period the mice were weighed weekly. We have previously determined that female C57BL/6 mice fed a HFD have increased total body weight and fat mass over a 12 week feeding period compared to SLD mice [473]. Reduced mechanosensitivity of gastric afferents observed in female HFD mice [473] has also been observed in intestinal afferents of male mice [381] suggesting that diet induced modulation of vagal afferent satiety signals are independent of gender.

3.3.3 *In vitro* mouse gastro-oesophageal afferent preparation

This preparation has been described in detail previously [436]. In short, C57BL/6 mice were killed via CO₂ inhalation and the thorax opened by a midline incision. The stomach and oesophagus, with intact vagal nerves, were removed. The stomach and oesophagus were opened out longitudinally mid way between the two main vagal branches, either side of the oesophagus, and along the greater curvature of the stomach. The tissue was then pinned down mucosa side up in an organ bath containing a modified Krebs solution composing of (in mM): 118.1 NaCl, 4.7 KCl, 25.1 NaHCO₃, 1.3 NaH₂PO₄, 1.2 MgSO₄·7H₂O, 1.5 CaCl₂, 1.0 citric acid, 11.1 glucose and 0.001 Nifedipine, bubbled with 95% O₂- 5% CO₂. The dissection process was carried out at 4°C.

3.3.4 Characterization of gastric vagal afferent properties

Receptive fields of mechanically sensitive vagal afferents, described earlier, were initially located using mechanical stimulation by brushing gently along the mouse gastro-oesophageal preparation. Once located specific stimuli were then applied. Mucosal stroking was performed using calibrated von Frey hairs (10-1000mg) which were stroked across the mucosa at a rate of 5mm/s. Each receptive field was stroked ten times and mechanical responses from the middle eight strokes were taken for analysis. Circular tension was applied using a threaded hook attached to an underpinned point adjacent to the receptive field. The threaded hook was attached to a cantilever via a pulley close to the preparation. Standard weights (0.5-5g) were then placed on the opposite end of the cantilever. Each weight was applied for one minute with a

break of another minute between removing one weight and applying the next. After analysing the two stimulus response curves, we classified a receptive field as either a mucosal or tension receptor.

3.3.5 Single unit vagal afferent recordings

Single units were discriminated on the basis of action potential shape, duration, and amplitude by use of Spike 2 software (Cambridge Electronic Design, Cambridge, UK). After mechanosensitivity of a receptive field was established the effect of leptin on this mechanosensitivity was assessed by adding leptin (0.1nM) to the Krebs and allowing it to equilibrate for 20 minutes. This equilibration period was observed so as to ensure penetration of the drug into all layers of the tissue. After this time the tension-response and stroke-response curves were re-determined. This procedure was repeated for leptin at increasingly higher doses (1-10nM). Time-controlled experiments were performed in which there was no significant change in the mechanical responses over a comparable duration. The second messenger system used by leptin to elicit its effects on mechanosensitivity was established using inhibitors of Janus kinase 2 (JAK2) - AG490 (5 μ M) [474], phosphatidylinositol 3-kinases (PI3K) - wortmannin (5nM) [475], phosphodiesterase 3 (PDE3) - cilostamide (5 μ M) [476], phospholipase C (PLC) - U73122 (10 μ M) [477], large conductance calcium activated potassium channels (BKCa) - iberiotoxin (100nM) [478] and canonical transient receptor potential cation channels (TRPC) channels - 2-APB (30 μ M) [479], at concentrations previously reported in the literature before and after the addition of leptin (10nM) in a separate series of experiments to the leptin dose-response experiments.

3.3.6 Drugs

Stock solutions of all drugs were kept frozen at -80°C and diluted to their final concentration in Krebs immediately prior to their use in the experiment. Leptin was obtained from Sigma-Aldrich (Sydney, Australia). Iberiotoxin, wortmannin, cilostamide, U73122, 2-APB and AG490 were obtained from Tocris Bioscience (Bristol, UK).

3.3.7 Nodose ganglia quantitative RT-PCR

Nodose ganglia were removed bilaterally from mice from all 4 experimental groups. Total RNA was extracted using an RNeasy Micro Kit (Qiagen, Doncaster, Australia) according to the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260nm (A₂₆₀) using a NanoDrop™ ND 1000 spectrophotometer (Thermo Scientific) and RNA purity was estimated via the 260/280 absorbance ratio. Quantitative RT-PCR reactions were performed as described in detail previously [455]. Primers targeting Lep-R, TRPC1, TRPC5 and β -tubulin were pre-designed Quantitect Primer assays (Qiagen). The primer pairs for KCa1.1 were designed using Primer 3.0 software (Applied Biosystems; Foster City, USA). Forward primer (TCTACTTTGGCTTGCGGTTT) and reverse primer (CAAGCCAAGCCAAGTTCTGT) for KCa1.1 were supplied by Geneworks (Adelaide, Australia). RT-PCR reactions were carried out using 10ng of RNA under the following conditions: reverse transcription, 50°C for 30min; initial PCR activation, 95°C for 15min: PCR cycles 94°C for 15s, 55°C for 30s and 72°C for 30s repeated for 50 cycles. A melt curve was obtained to confirm the

specificity of the products produced and products were visualised using 3% agarose gel electrophoresis with ethidium bromide staining. Relative RNA levels were calculated using the comparative CT method as described previously [456]. Quantitative data are expressed as mean \pm SEM.

3.3.8 Anterograde tracing

This procedure has been previously documented [298]. In brief, mice from the SLD (n=7) and HFD (n=7) groups were anaesthetised with isoflurane (1-1.5% in oxygen). The left nodose ganglion was exposed, and 0.5 μ l of a horseradish peroxidase conjugate of wheat-germ agglutinin (WGA-HRP, 4 mg/ml; Vector Laboratories) was pressure injected into the nodose ganglion via a glass micropipette (ID 25 μ m). The injection site was dried and skin incision closed. Antibiotic (terramycin; 10 mg/kg) and analgesic (butorphanol; 5 mg/kg) were administered subcutaneously. Two days following injection of tracer (WGA-HRP), into the left nodose, the stomach was removed and dissected into ventral and dorsal flat sheets along the greater and lesser curvature. Frozen serial transverse sections of ventral flat sheet (10 μ M) were then cut for immunohistochemistry. The dorsal flat sheet had submucosa and mucosa gently removed leaving the smooth muscle layers and myenteric plexus intact.

Permanent visualization of WGA-HRP was carried out using tyramide signal amplification (Perkin Elmer, USA) [298]. Briefly, dorsal sheet whole mounts and on-slide sections were rinsed in TNT buffer (0.05% Tween 20, 0.15 M NaCl, and 0.1 M Tris-HCl, pH 7.5), blocked for 30min in TNB (supplied with

Perkin-Elmer TSA reagent kit), and then reacted for 20min with tyramide-biotin, diluted 1:50 in amplification diluent. The tissue was then rinsed in TNT buffer and reacted with streptavidin conjugated to Alexa Fluor® 647 (1:200, Invitrogen) for 1-2hrs at room temperature. Frozen sections were subsequently processed for leptin immunohistochemistry, then both sections and wholemount specimens were mounted on slides and cover slipped using ProLong antifade (Invitrogen).

3.3.9 Retrograde tracing

Cell bodies of gastric vagal afferents innervating specific stomach layers were identified using differential tracing from the stomach as previously documented [298].

Gastric muscle: SLD (n = 5) and HFD (n=5) mice were anaesthetised with isoflurane (1-1.5% in oxygen), a laparotomy performed, and an Alexa Fluor® 555 conjugate of cholera toxin β -subunit (CTB-AF555 (0.5%); Invitrogen) injected subserosally into the muscularis externa of the proximal stomach using a 30 ga Hamilton syringe. Multiple equally spaced injections of 2 μ l were made parallel to and 1–2 mm from the lesser curvature on both dorsal and ventral surfaces (total volume 10 μ l). The injection sites were dried, the laparotomy incision was closed, and antibiotic and analgesic were administered as above.

Gastric mucosa: SLD (n = 5) and HFD (n=5) mice were anaesthetised with isoflurane (1-1.5% in oxygen), a laparotomy performed and a mucolytic (10%

N-acetylcysteine; 200 µl) was injected into the stomach lumen, then removed via syringe after 5min, followed by two saline rinses (200 µl each). Subsequently, 10µl of 0.5% CTB-AF555 was injected into the proximal gastric lumen via a 30 ga Hamilton syringe and the proximal stomach walls gently opposed to expose the dorsal and ventral surfaces to tracer. The laparotomy incision was then closed and antibiotic and analgesic administered as above. Food and water were withheld for 2hrs postoperatively to maximize exposure of tracer.

After two days, traced mice were anaesthetised and perfused as indicated above. The left and right nodose ganglia were then removed and fixed in 4% PFA/PB at room temperature for 4hrs and subsequently cryoprotected in 30% sucrose at 4°C for 24hrs. Frozen serial transverse sections (10 µM) were then cut for immunohistochemistry.

Due to the ethical consideration of restricting food intake in mice a few days after surgery, these studies were only performed on SLD and HFD mice. We use the tracer WGA-HRP because WGA is widely accepted as one of the optimal anterograde tracers for filling of fine varicosity endings in vagal afferent pathways, while HRP allows for tyramide signal amplification to resolve fine anatomical structures [480].

3.3.10 Laser capture microdissection

Retrogradely traced nodose ganglion (NDG) were removed and dissociated before being cultured on a duplex dish for 2hrs at 37°C in 5% CO₂. Cells were

then subject to laser-capture microdissection, performed on a P.A.L.M.[®] microbeam microdissection system (Carl Zeiss, Jena, Germany). Fluorescent labelled nodose neurons were microdissected and catapulted directly into a lysis and stabilization buffer (Buffer RLT, RNeasy Micro RNA extraction Kit, Qiagen) containing 0.14M β -Mercaptoethanol (Sigma-Aldrich, Australia). The number of neurons captured varied with diet so that the maximum neurons possible were captured in order to maximise the RNA yield. RNA was extracted from these cells using the same protocol as for whole NDG [455]. There was no significant difference in RNA yield between the diet groups.

3.3.11 Immunohistochemistry

Immunohistochemistry was carried out in anterograde-traced stomach sections as described previously [298]. The primary anti-leptin antibody (rabbit polyclonal, Santa Cruz) was diluted 1:200 and a chicken anti-rabbit Alexa Fluor[®] 488 secondary antibody (1:200; Invitrogen) was used. Slide sections and whole mounts were visualized using an epifluorescence microscope (BX-51, Olympus, Australia) equipped with filters for Alexa Fluor[®] 488 and 647, with images acquired by a CoolSnapfx monochrome digital camera (Roper Scientific, Tuscon, AZ). Pseudocoloured fluorescence images were overlaid and fluorescence imaging software (Analysis LifeScience, Olympus) used to calculate the percentage of complete traced NDG neurons and density of traced vagal afferent endings in the stomach. Luminance of images was not adjusted. Counts were performed on a minimum of 10 sections per animal.

3.3.12 Statistical analysis

All data in graphs are expressed as mean \pm SEM with n= the number of individual animals used. Vagal afferent stimulus-response curves and weight change were analysed using two-way analysis of variance (two-way ANOVA) and Bonferroni post hoc tests. We compared the effects of leptin and mechanical stimuli on the response of tension and mucosal receptors using a two-way ANOVA, to establish if leptin affected the response to mechanical stimulation. The effect of diet on the modulatory action of leptin on responses to mechanical stimulation was determined by assessing the response to either stroking (50mg von Frey hair; mucosal receptors) or tension (3g; tension receptors) at different concentrations of leptin (0.1, 1 and 10nM). Significant difference between diets and leptin concentration were assessed using two-way ANOVA to determine if diet caused a change in leptin effect. RNA levels, fat mass and vagal afferent profiles were analysed using unpaired t-tests. Significance was defined at $P < 0.05$.

3.4 RESULTS

3.4.1 Effect of leptin on gastric vagal afferent mechanosensitivity in fed and fasted mice

As previously reported [473], short-term restriction of food intake consistently decreased the mechanosensitivity of tension receptors to circumferential stretch ($p < 0.05$; two-way ANOVA: data not shown), but not mucosal afferent responses to stroking.

In normal fed mice, leptin (0.1-10nM) potentiated the mechanosensitivity of mucosal receptors ($p < 0.01$; leptin effect: two-way ANOVA, Figure 3.1Ai&C). This effect was completely lost in fasted mice (Figure 3.1Aii). When the response to mucosal stroking (50mg) at varying concentrations of leptin was plotted (Figure 3.1Aiii) it was evident that fasting reduces mucosal receptor sensitivity to leptin ($p < 0.001$; diet effect; two-way ANOVA). Leptin had no effect on tension receptor mechanosensitivity in fed mice (Figure 3.1Bi), however in fasted mice the mechanosensitivity of these receptors was reduced by leptin ($p < 0.05$; leptin effect: two-way ANOVA, Figure 3.1Bii&D). When the percentage change in response to a 3g load was plotted against varying concentrations of leptin (Figure 3.1Biii) it was evident that a restricted diet increased sensitivity of tension receptors to inhibition by leptin ($p < 0.01$; diet effect; two-way ANOVA). Recordings were also made from oesophageal receptors which showed effects of leptin similar to those in gastric afferents (Supplementary Figure 3.1A&B).

3.4.2 Effect of leptin in diet-induced obese mice

Mice fed the HFD gained a greater amount of body weight ($p < 0.001$; two-way ANOVA) and fat mass ($p < 0.01$; unpaired t-test) than age-matched controls (Supplementary Figure 3.2).

As previously reported [473], the mechanosensitivity of tension receptors was reduced in HFD compared to SLD mice ($p < 0.05$, two-way ANOVA) and there was no difference in the mechanosensitivity of gastric mucosal receptors between HFD and SLD mice (data not shown).

In SLD mice, leptin (0.1-10nM) increased the mechanosensitivity of mucosal receptors ($p < 0.001$; leptin effect: two-way ANOVA, Figure 3.2Ai&C), a result similar to that reported in 8wk old fed mice (above), indicating no change in leptin effect with age. Leptin failed to increase the mechanosensitivity of mucosal receptors in HFD mice (Figure 3.2Aii). When the response to mucosal stroking (50mg) was plotted against leptin concentration (Figure 3.2Aiii) it was evident that a HFD diet reduced the sensitivity of gastric mucosal receptors to leptin ($p < 0.05$; diet effect; two-way ANOVA). Correspondingly, the effect of leptin on tension receptor mechanosensitivity was switched from no effect in SLD (Figure 3.2Bi) to inhibition in HFD mice ($p < 0.001$; leptin effect: two-way ANOVA, Figure 3.2Bii&D). When the percentage change in response to 3g load was plotted against concentration of leptin (Figure 3.2Biii) it was evident that a HFD diet increased the sensitivity of tension receptors to leptin ($p < 0.01$; diet effect; two-way ANOVA).

Oesophageal vagal afferents were also recorded, and their modulation by leptin closely mirrored that reported for gastric afferents (Supplementary Figure 3.1C&D).

3.4.3 Leptin receptor expression

To determine if the differential effects of leptin were due to changes in Lep-R expression we first compared mRNA levels of Lep-R in whole NDG. We found there was no difference in Lep-R expression in the fed and fasted mice (Figure 3.3Ai), or SLD and HFD mice (Figure 3.3Aii). However, following differential retrograde tracing to identify muscular or mucosal gastric afferents [298], we observed a 94% reduction in Lep-R expression in mucosal afferents from HFD compared to SLD mice ($p < 0.001$; unpaired t-test: Figure 3.3Bi). In contrast, Lep-R expression in muscular afferents was similar in mice fed a HFD or SLD ($p > 0.05$: Figure 3.3Bii). It appears, therefore, that Lep-R expression is regulated by feeding state only in mucosal afferents. QRT-PCR products were confirmed to be of appropriate size by agarose gel electrophoresis (Figure 3.3C).

3.4.4 Leptin localization

To determine if the effects of leptin on mechanosensitivity of mucosal afferents can occur locally, we examined the relationship between leptin immunopositive cells in the gastric mucosa and vagal afferent endings. Anterogradely traced mucosal vagal afferents were abundant in SLD mice and were closely associated with leptin immunopositive cells (Figure 3.3Di). In contrast, the number of mucosal afferents traced in HFD mice was reduced by

80% ($p < 0.001$; unpaired t-test: Figure 3.3Diii) and they were located at an increased distance from leptin immunopositive cells (Figure 3.3Dii). In retrograde labelling experiments, the number of mucosal afferents that could be traced to the NDG was 50% lower in HFD compared to SLD mice ($p < 0.01$; unpaired t-test: Figure 3.3Ei-iii), presumably due to the increased distance of the afferent endings from the lumen of the stomach where the tracer is added. In contrast, there was no change in the location or number of muscular afferents in either the stomach or NDG (Supplementary Figure 3.3).

3.4.5 The second messaging systems utilized by leptin

The potentiating effect of leptin on mucosal afferents (Fed (8wk old), SLD (20wk old) mice) and the inhibitory effect of leptin on tension sensitive afferents (Fasted (8wk old), HFD (20wk old) mice) suggests that the Lep-R is coupled to different second messenger systems. Evidence from previous work indicates that JAK2, PI3K, PDE3, PLC and TRPC channels may be involved in the potentiating effects of leptin on hypothalamic neuronal function [318, 324, 325, 481]. Using a range of blockers for these molecules, we show that the potentiating effect of leptin on afferent mechanosensitivity is abolished when any of these targets were inhibited individually (Fed-Figure 3.4Ai-v; SLD-Figure 3.5Ai-v: indicating no age effect). However, the potentiating effect of leptin was not affected by the BKCa blocker iberiotoxin (Fed-Figure 3.4Avi; SLD-Figure 3.5Avi). This suggests that the potentiating effect of leptin may involve in-series activation of signal transduction molecules illustrated in Figure 3.5B. The presence of TRPC channels in vagal afferents was corroborated by QRT-PCR on whole NDG. We assessed levels of TRPC1 and

TRPC5 previously shown to be expressed in NDG [482] and suggested to be activated by leptin in hypothalamic POMC neurons [324]. There was no difference in mRNA transcript expression of TRPC5 in whole NDG of fed and fasted mice ($p > 0.05$; Figure 3.4B). However, transcript expression of TRPC1 was higher in fed compared to fasted mice ($p < 0.05$; unpaired t-test: Figure 3.4C) and SLD compared to HFD mice ($p < 0.01$; unpaired t-test: data not shown), suggesting this may be the channel subtype activated by leptin to potentiate function of mucosal afferents.

The inhibitory effect of leptin on mechanosensitivity of tension receptors (fasted, HFD mice) was eliminated by inhibitors targeting JAK2, PI3K and BKCa (Fasted-Figure 3.6Ai-iii; HFD-Figure 3.7Ai-iii), but was unaffected by inhibitors targeting PDE3, PLC and TRPC (Fasted-Figure 3.6Aiv-vi; HFD-Figure 3.7Aiv-vi). These data demonstrate a divergence in the signalling pathway prior to the effector channel as illustrated in Figure 3.7B. Performing QRT-PCR targeting the KCa1.1 subunit of BKCa we found that transcript levels were higher in the whole NDG of fasted compared to fed mice ($p < 0.05$; unpaired t-test: Figure 3.6B) and HFD compared to SLD mice ($p < 0.05$; unpaired t-test: data not shown).

3.5 DISCUSSION

We have shown that the appetite suppressant peptide leptin, has a potent potentiating effect on mucosal vagal afferents during feeding, which is lost in response to fasting or HFD-induced obesity. In contrast, the mechanosensitivity of muscular vagal afferents was not affected by leptin during feeding but was reduced by leptin after fasting or HFD-induced obesity. The varying effect of leptin on specific vagal afferent subtypes is due to coupling of the Lep-R to alternative second messenger systems. The changes in effect of leptin with feeding status is due to alterations in the coupling of Lep-R to these alternate second messenger systems and partly through reduced Lep-R expression. The data strongly suggest that leptin has important but opposite effects on vagal afferent signalling in normal and energy imbalanced states.

The observation that leptin increases mechanosensitivity of mucosal receptors in fed mice, but not in fasted or HFD fed mice, indicates that at least in part, the anorexigenic effect of leptin may be peripherally mediated. Consistent with this notion is the observation that leptin acts synergistically with cholecystinin (CCK) on peripheral intestinal vagal afferent neurons to potentiate the effect of CCK on satiety [483, 484]. In our preparation, leptin potentiates the mechanosensitivity of gastric vagal mucosal afferents. This mechanism of action makes sense, since it is well described that the tactile sensitivity of mucosal receptors facilitates detection of food particle size in the gastric lumen and can initiate sensory feedback to delay gastric emptying and

food intake via a vagal pathway [197, 297]. It has been reported that only 15 percent of vagal afferent cell bodies projecting to the rat fundus are leptin receptor immunoreactive [485] whereas, in the current study, all mucosal afferents from fed and SLD mice were potentiated by leptin. We speculate that, other than the possibility of species differences, leptin receptor protein is rapidly trafficked to vagal peripheral endings, upon which leptin released from the gastric mucosa elicits modulation, and that the residual low levels of Lep-R in vagal cell bodies are difficult to detect with current immunohistochemical techniques.

The loss of effect of leptin on mucosal receptors in HFD mice is consistent with existing evidence of leptin resistance in obesity [305, 335, 336] which we extend here to show is due to a specific reduction in Lep-R expression in mucosal vagal afferents, which may be paired with reduced expression of the putative effector channel, TRPC1. Furthermore, we identified a marked reduction in the close apposition of mucosal afferents to leptin secreting cells in HFD mice. Such re-organisation of peripheral afferent endings would reduce the likelihood of gastric leptin-vagal signalling per se, further contributing to the net loss of signal from vagal afferents due to reduced leptin potentiation of individual mucosal afferents. We observed that Lep-R transcript expression in mouse whole NDG was unaffected by a short-term restriction in food intake or a HFD. In contrast, it has previously been demonstrated that Lep-R protein expression in the rat whole NDG is increased after fasting [470]. These differences may be species specific, or differences between transcript and protein regulation. In our traced vagal

afferents we observed a quantitative reduction in Lep-R expression in mucosal afferents from HFD mice, which is in accord with the lack of effect of leptin on these afferents.

Evidence here of an inhibitory effect of leptin on tension receptors in fasting and HFD mice contrasts significantly with the extensive literature on leptin resistance in obesity [305, 335, 486]. There is evidence that leptin can inhibit neurons, for example, leptin has been shown to inhibit hypothalamic appetite stimulatory NPY neurons in rats, whilst also activating appetite suppressing POMC neurons [99, 308, 324]. The novel observation in the current study is that the peripheral inhibitory effect of leptin on muscular afferents appears to occur in mutual exclusion to its excitatory effect on mucosal afferents, dependent on feeding status. However, due to the limited time points studied it is not possible to determine whether there is a point where both the potentiating and inhibiting effects of leptin are present. We have previously shown that mechanosensitivity of tension receptors is reduced after fasting or HFD [473]. Our current findings reveal that leptin causes a further decrease in the mechanosensitivity of tension receptors in both fasted and HFD mice. Tension receptors in the stomach detect distension [188] following the ingestion of food. Gastric distension has been shown to activate satiety circuitry in the human brain and increase feelings of fullness [471]. Therefore, reduced mechanosensitivity of these afferents by leptin may permit ingestion of more food before satiation is reached, and although we have yet to undertake the behavioural studies to confirm this, rodents with diet induced obesity have been shown to consume larger meals [487]. This may be an

adaptive function to allow an increase in energy intake to occur when food is readily available and perceived as “abundant”. The exact triggers for this ‘switch’ in effect of leptin requires further investigation but lead candidates are specific nutrients and appetite regulating hormones/peptides.

It is well established that the Lep-R is linked to JAK2 [318] signalling through a PI3K pathway [325]. Our data indicate that the modulatory effect of leptin on gastric afferent mechanosensitivity is, likewise mediated through JAK2 and PI3K. There appears, however, to be a divergence in the second messenger pathways utilised by tension and mucosal afferents after this point. Importantly, our study suggests that the excitatory effect of leptin on gastric mucosal afferents may be mediated through PLC activation of a TRP channel, possibly TRPC1, which has been shown to be preferentially expressed in capsaicin sensitive vagal afferents [482]. This view is further supported by studies showing that the short-term appetite suppressive effect of leptin is abolished upon capsaicin pre-treatment, and by reports that TRPC1 is highly expressed in POMC neurons which are leptin activated [324]. TRPC1 knockout mice are also known to gain more weight than their wild-type counterparts, indicating a role for TRPC1 in appetite regulation [488]. The potentiating effect of leptin on gastric mucosal afferents also appears to rely on activity of PDE3. This could be due to an indirect activation of TRPC1, as cAMP and PKA can inhibit PLC [489, 490] and would therefore prevent PLC activation of TRP channels. Accordingly, PDE3 may assist in mediating excitation by inhibiting activity of molecules which oppose excitatory responses to leptin.

Leptin can activate ATP-activated potassium channels, via a PDE3 mediated pathway [322, 491], which represents a candidate mechanism for the inhibitory action of leptin on tension receptors in HFD and fasted mice. However, in the present study, PDE3 inhibition did not alter the inhibitory effect of leptin and therefore this pathway is less likely to be involved. Another report demonstrated that leptin can cause neuronal inhibition via BKCa [492]. Consistent with this we found that the inhibitory effect of leptin on gastric tension sensitive afferents was abolished by blockade of BKCa. This was further supported by an increase in KCa1.1 expression seen in the whole NDG of fasted and HFD mice, suggesting that the channel responsible for signalling the inhibitory effect of leptin is up-regulated by food intake.

The *in vitro* effects of leptin on gastric vagal afferents were also observed in oesophageal afferents indicating that changes in afferent sensitivity following a HFD extend more generally to gastrointestinal afferent populations. However, the close apposition of vagal afferent endings and leptin containing P-cells in the stomach mean that only the gastric afferent populations would be likely to undergo *in vivo* signalling changes based on leptin sensitivity. This selectivity, however, may be reduced in obesity where the plasma levels of leptin are significantly increased [305] and needs to be considered in any potential targeted pharmacotherapy for obesity.

In conclusion, we have established that leptin has an excitatory effect on gastric mucosal vagal afferents which is abolished after both food restriction

and an excess of energy intake. In contrast, leptin has an inhibitory effect on gastric tension sensitive vagal afferents, evident only after food restriction or energy excess. This data highlights the plasticity in the mechanism of action of leptin under different feeding conditions and provides potential new targets for novel peripherally acting pharmacotherapies to modify food intake in obesity.

Fed ad libitum

Fasted for 14hr

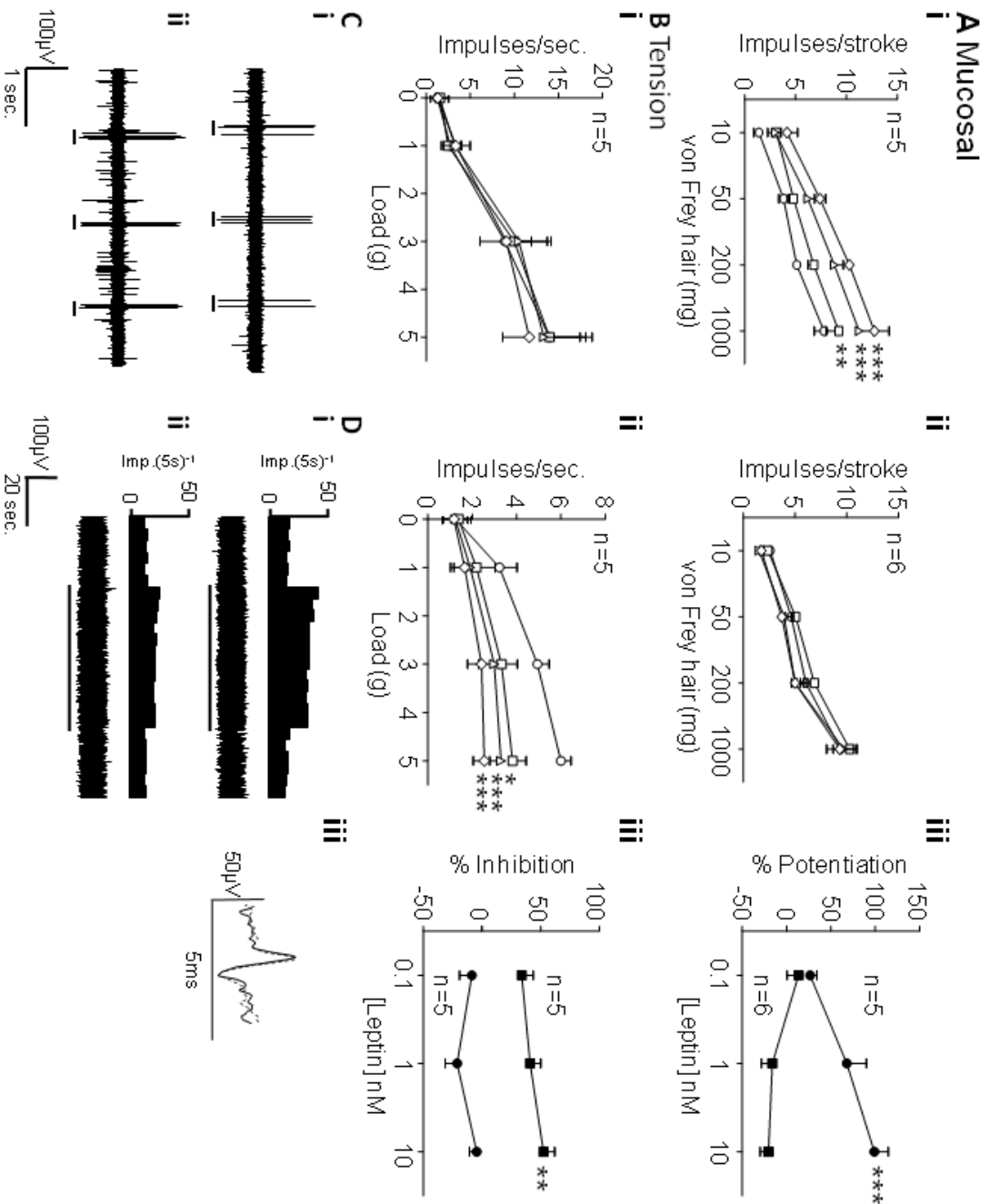


Figure 3.1: Fasting changes the effects of leptin on gastric vagal afferent mechanosensitivity. Single fibre recording stimulus response curves of mucosal (Ai, n=5 & Aii, n=6) and tension (Bi, n= 5 & Bii, n=5) receptors to mucosal stroking and circular tension respectively in fed (Ai&Bi) and fasted mice (Aii&Bii) before ○ and after exposure to leptin 0.1nM □, 1nM Δ and 10nM ◇. Aiii&Biii: Percentage change in response to a 50mg von Frey hair and 3g tension respectively compared to control at varying concentrations of leptin in fed (●) and fasted (■) mice. *p<0.05, **p<0.01, ***p<0.001 vs control (two-way ANOVA). Data expressed as mean ± SEM. (C) Original recording, from a fed mouse, of a mucosal receptor response to mucosal stroking with a 50mg von Frey hair (i) prior to leptin and (ii) after addition of leptin(10nM). (D) Original recording, from a fasted mouse, of a tension receptor response to 3g circular tension (i) prior to leptin, (ii) after addition of leptin (10nM) and (iii) the average spike shape of the tension receptor prior to leptin (solid line) and after addition of leptin (dashed line) illustrating that both responses were obtained from the same unit.

Figure 3.2: Leptin's effect on gastric vagal afferent mechanosensitivity is modified by high fat diet-induced obesity. Single fibre recording stimulus response curves of mucosal (Ai, n=5 & Aii, n=6) and tension (Bi, n=5 & Bii, n=5) receptors to mucosal stroking and circular tension respectively in SLD (Ai&Bi) and HFD fed mice (Aii&Bii) before ○ and after exposure to leptin 0.1nM □, 1nM Δ and 10nM ◇. Aiii&Biii: Percentage change in response to 50mg von Frey hair and 3g tension respectively compared to control at varying concentrations of leptin in SLD (●) and HFD (■) mice. *p<0.05, **p<0.01, ***p<0.001 vs control (two-way ANOVA). Data are expressed as mean ± SEM. (C) Original recording, from a SLD mouse, of a mucosal receptor response to mucosal stroking with a 50mg von Frey hair (i) prior to leptin and (ii) after addition of leptin(10nM). (D) Original recording, from a HFD mouse, of a tension receptor response to 3g circular tension (i) prior to leptin, (ii) after addition of leptin(10nM) and (iii) the average spike shape of the tension receptor prior to leptin (solid line) and after addition of leptin (dashed line) illustrating that both responses were obtained from the same unit.

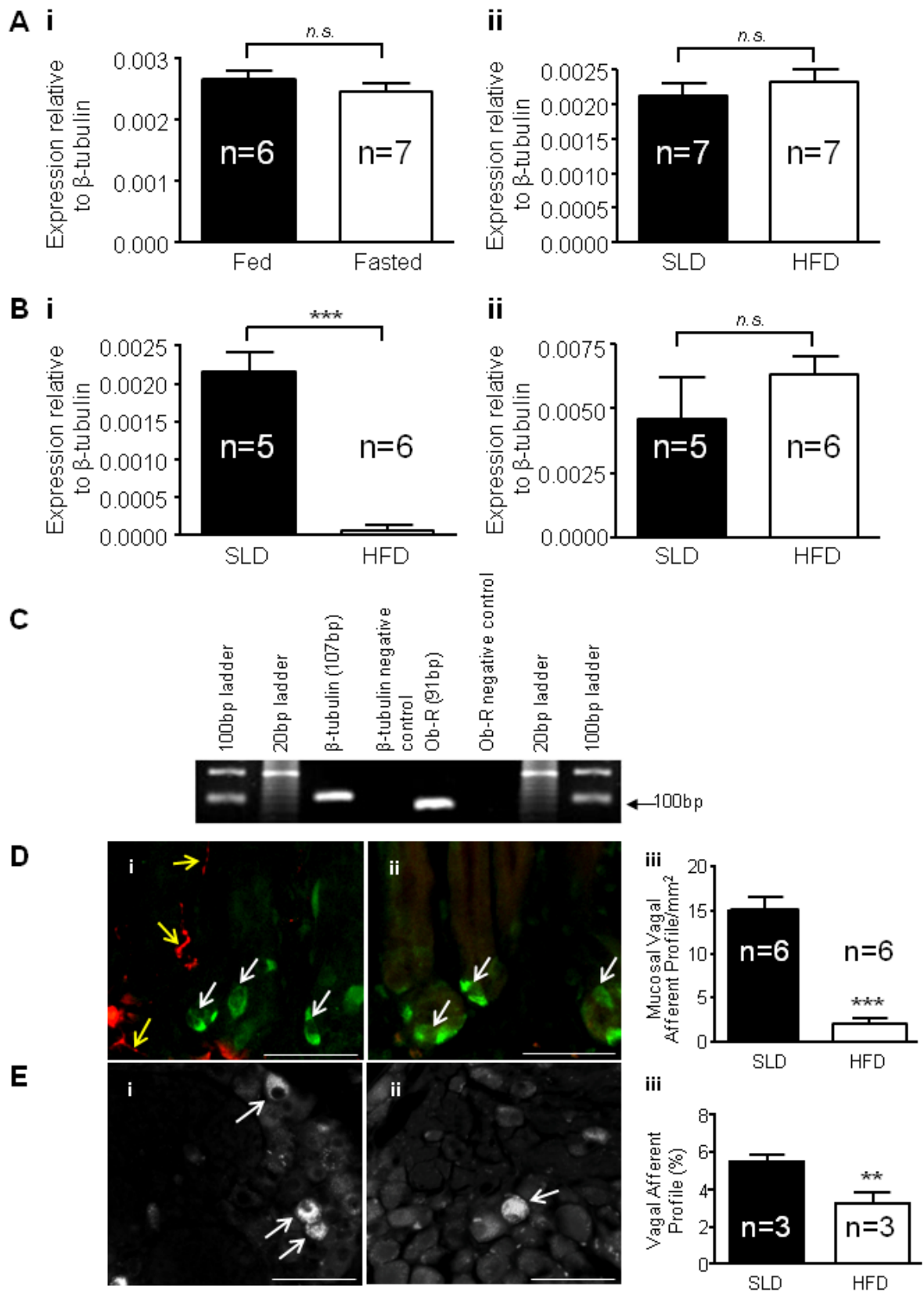


Figure 3.3: Changes in leptin receptor expression and neuronal innervation of the mucosa induced by fasting and diet induced obesity.

(Ai) Expression of Lep-R mRNA in whole NDG of fed (n=6) or fasted (n=7) 8wk old female C57BL/6 mice. (Aii) Expression of Lep-R mRNA in SLD (n=7) or HFD (n=7) fed mice. (Bi&ii) Expression of Lep-R mRNA in gastric mucosal and tension afferent neurons respectively in SLD (n=5) or HFD (n=6) mice. ***p<0.001 vs SLD (unpaired t-test). (C) Agarose gel electrophoresis confirming QRT-PCR product size from the mucosal traced cells. (D) Combined leptin immunohistochemistry and anterogradely traced vagal afferent fibres in the mouse stomach (i-SLD; ii-HFD), showing the relationship between leptin-containing epithelial cells (white arrows) and anterograde labeled vagal afferent fibres (yellow arrows). The stomach sections show a cross section through glands at the base of villi. (Diii) Proportion of vagal afferent profiles in gastric mucosa of SLD (n=6) or HFD (n=6) mice following anterograde tracing (***p<0.001; unpaired t-test). (E) Vagal afferent cell bodies (white arrows) traced from the gastric mucosa in the nodose ganglia of SLD (i) or HFD (ii) mice. (Eiii) Proportion of vagal afferent cell bodies labelled in SLD (n=3) or HFD (n=3) mice (**p<0.01; unpaired t-test). Scale 50 μ m in all instances. Graphed data expressed as mean \pm SEM.

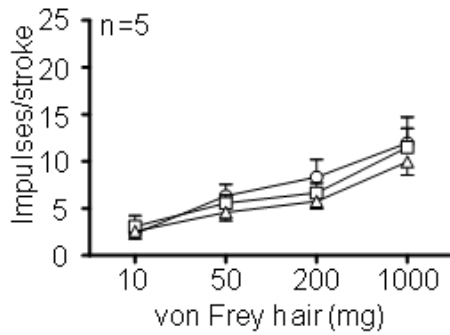
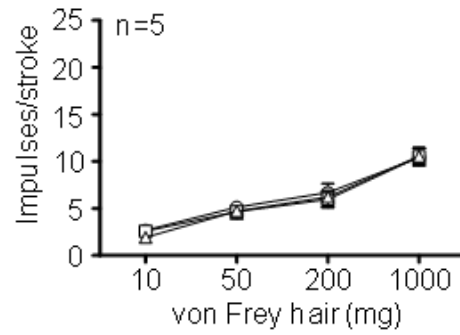
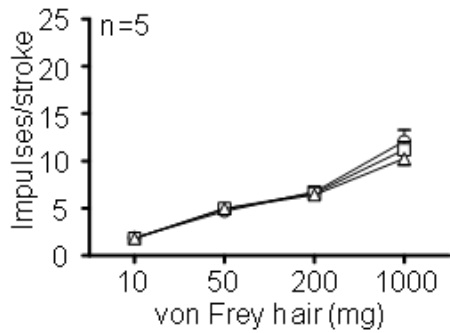
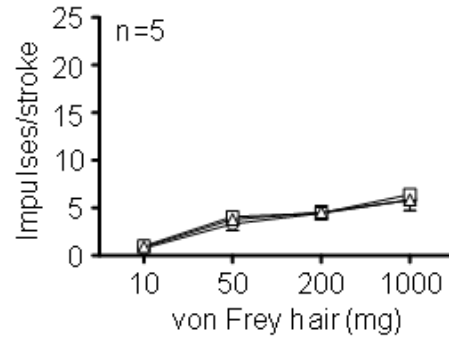
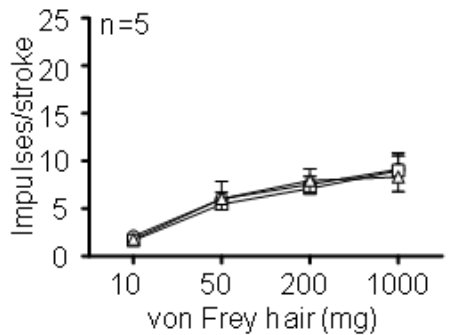
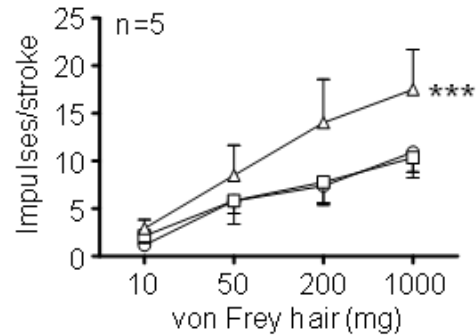
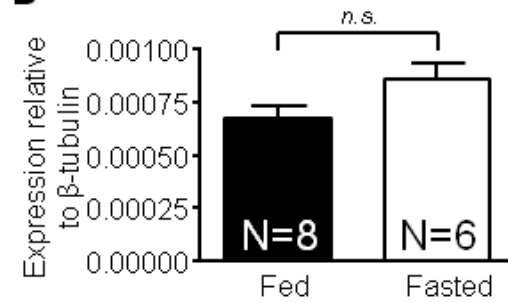
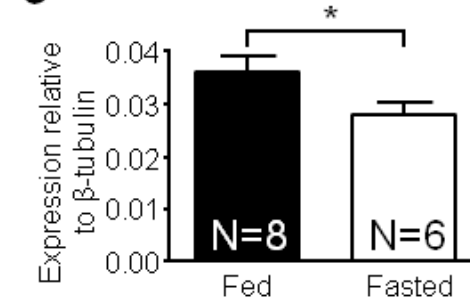
A i JAK2 - AG490**ii** PI3K - Wortmannin**iii** PDE3 - Cilostamide**iv** TRPC - 2-APB**v** PLC - U73122**vi** BKCa - Iberiotoxin**B****C**

Figure 3.4: The excitatory effect of leptin on mucosal afferents from fed mice is conveyed through PLC mediated activation of a TRPC channel.

Single fibre recording stimulus response curves of mucosal receptors to mucosal stroking in fed mice (Ai-vi, n=5) before ○ and after exposure to second messenger inhibitors ((i) AG490-5μM, (ii) wortmannin-5nM, (iii) cilostamide-5μM, (iv) 2-APB-30μM, (v) U73122-10μM, (vi) iberiotoxin-100nM) □ and in the presence of the respective second messenger inhibitor and leptin 10nM Δ, ***p<0.001 vs control (leptin effect: two-way ANOVA). (B&C) Expression of TRPC5&C1 mRNA respectively in whole NDG of fed and fasted mice (*p<0.05; unpaired t-test). Data expressed as mean ± SEM.

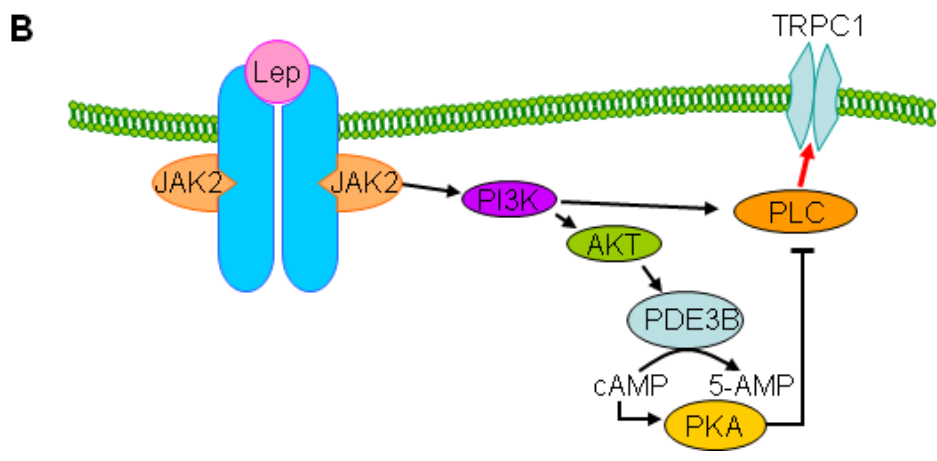
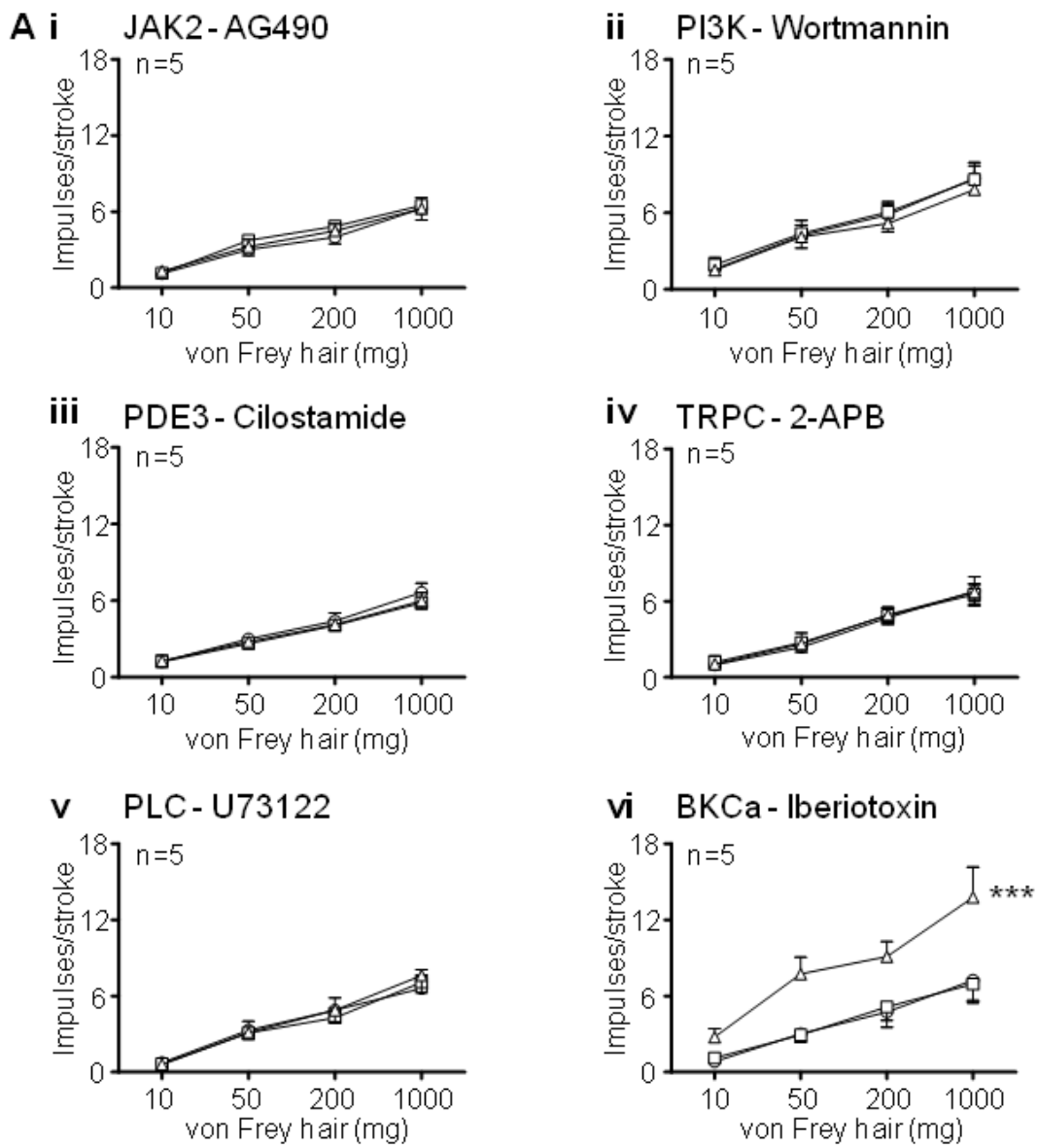
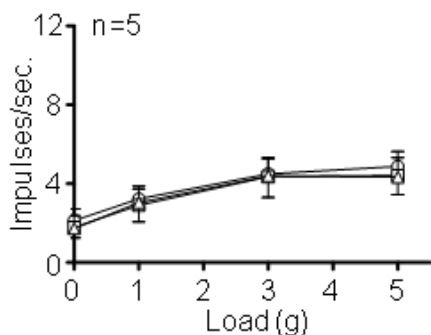
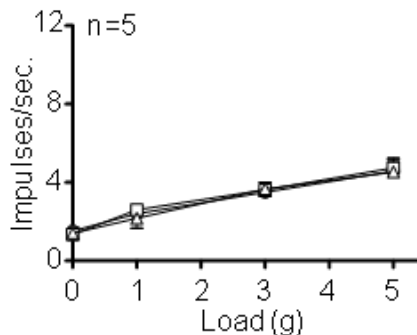


Figure 3.5: The excitatory effect of leptin on mucosal afferents from 20 week old SLD mice is conveyed through PLC mediated activation of a TRPC channel and therefore not affected by age. (A) Single fibre recording stimulus response curves of mucosal receptors to mucosal stroking in SLD (Ai-vi, n=5) before \circ and after exposure to second messenger inhibitors ((i) AG490-5 μ M, (ii) wortmannin-5nM, (iii) cilostamide-5 μ M, (iv) 2-APB-30 μ M, (v) U73122-10 μ M, (vi) iberiotoxin-100nM) \square and in the presence of the respective second messenger inhibitor and leptin 10nM Δ . *** p <0.001 vs control (leptin effect: two-way ANOVA). Data expressed as mean \pm SEM. (B) Schematic showing proposed signal transduction used by leptin to exert potentiation.

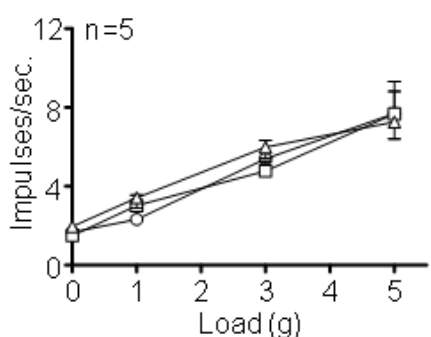
A i JAK2 - AG490



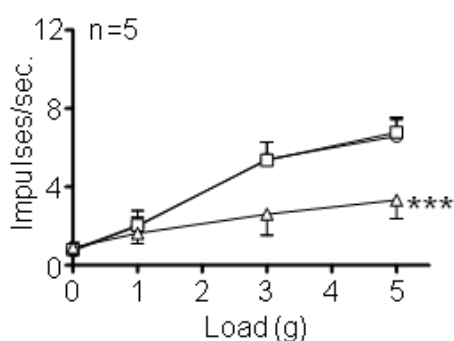
ii PI3K - Wortmannin



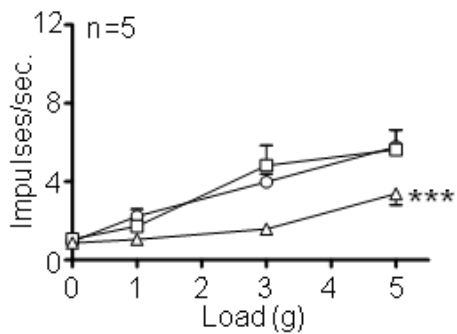
iii BKCa - Iberiotoxin



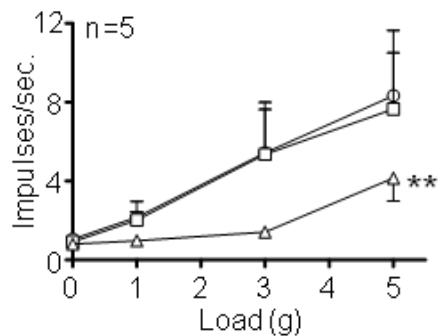
iv PDE3 - Cilostamide



v PLC - U73122



vi TRPC - 2-APB



B

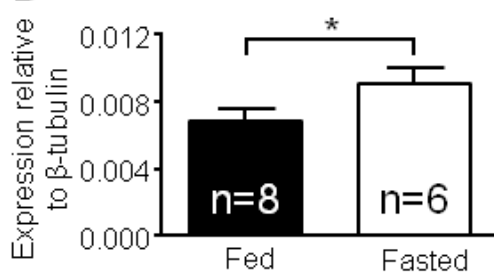
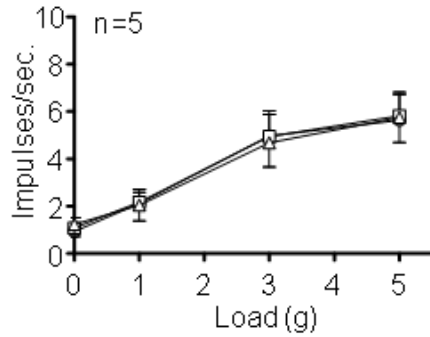
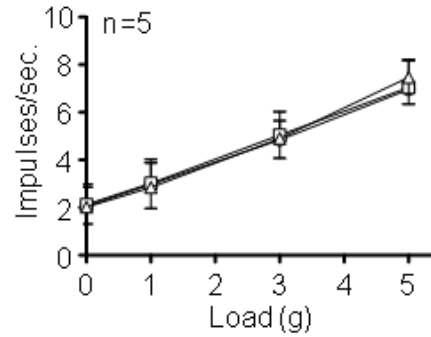


Figure 3.6: Leptin inhibits gastric tension receptors through PI3K activation of BKCa in fasted mice. Single fibre recording stimulus response curves of tension receptors to circular tension in fasted mice (Ai-vi, n=5) before ○ and after exposure to the second messenger inhibitors ((i) AG490-5μM, (ii) wortmannin-5nM, (iii) iberiotoxin-100nM, (iv) cilostamide-5μM, (v) U73122-10μM, (vi) 2-APB-30μM) □ and in the presence of the respective second messenger inhibitor and leptin 10nM Δ, **p<0.01, ***p<0.001 vs control (leptin effect: two-way ANOVA). (B) Expression of KCa1.1 mRNA in whole NDG of fed and fasted mice (*p<0.05; unpaired t-test). Data expressed as mean ± SEM.

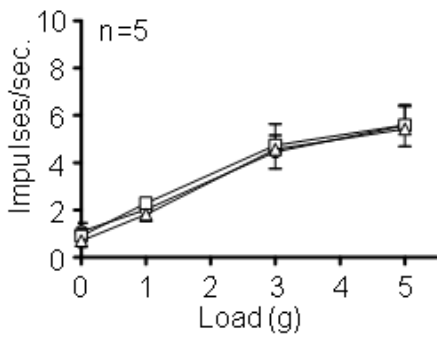
A i JAK2-AG490



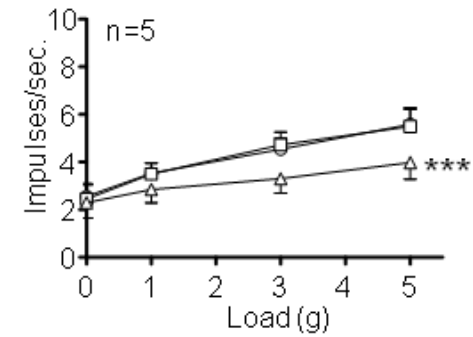
ii PI3K- Wortmannin



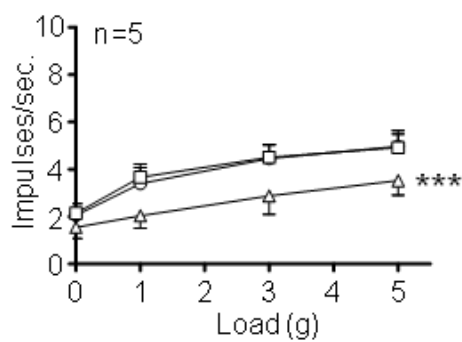
iii BKCa- Iberiotoxin



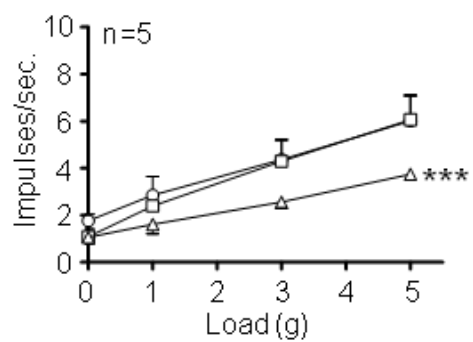
iv PDE3- Cilostamide



v PLC- U73122



vi TRPC- 2-APB



B

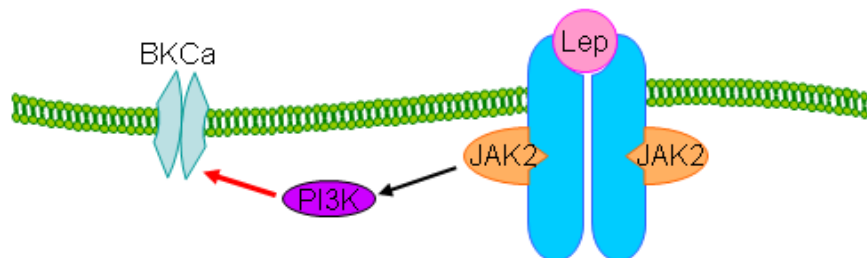
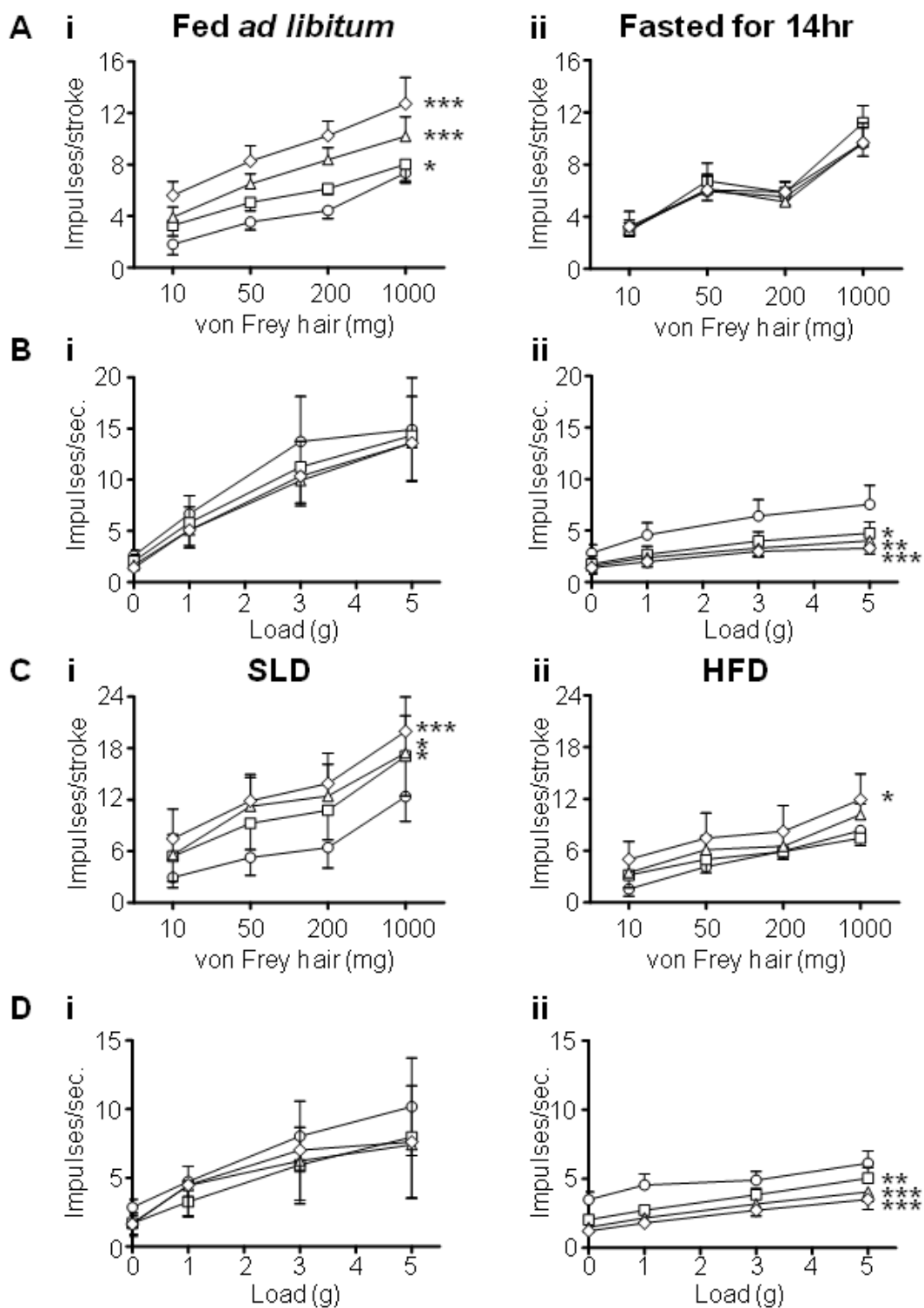
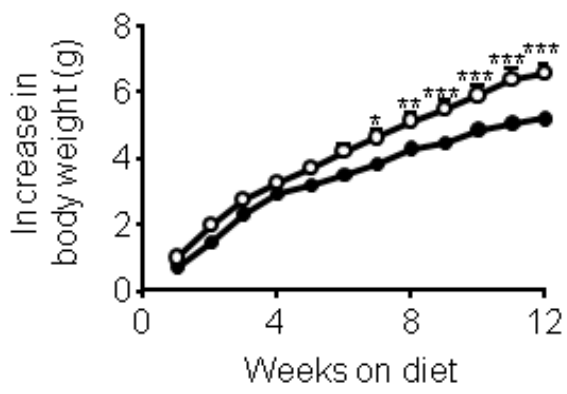
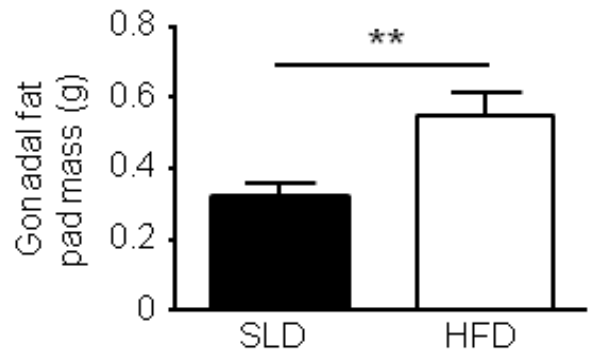


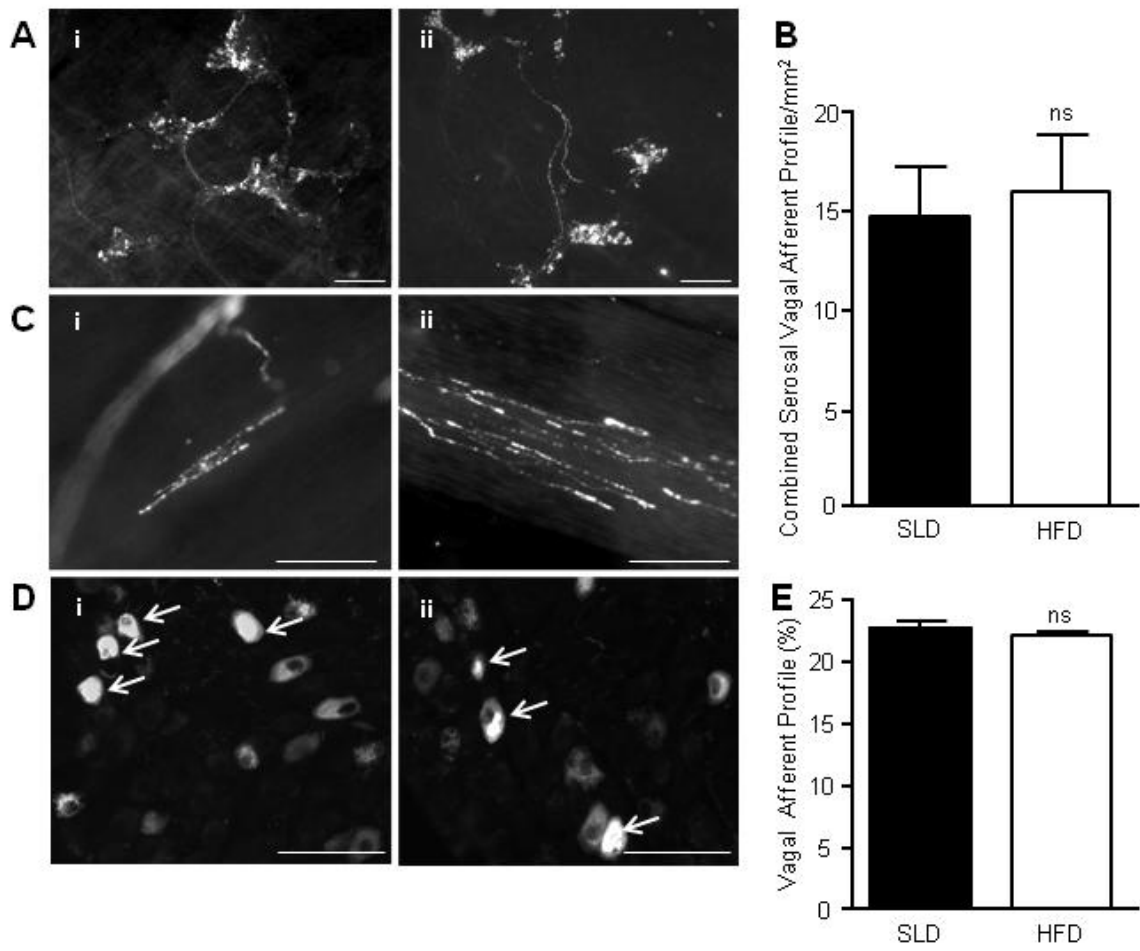
Figure 3.7: Leptin induced inhibition of tension receptor mechanosensitivity in mice fed a HFD is mediated by activation of BKCa channels. (A) Single fibre recording stimulus response curves of tension receptors to circular tension in HFD mice (Ai-vi, n=5) before ○ and after exposure to second messenger inhibitors ((i)AG490-5μM, (ii)wortmannin-5nM, (iii) iberiotoxin-100nM, (iv) cilostamide-5μM, (v)U73122-10μM, (vi)2-APB-30μM) □ and in the presence of the respective second messenger inhibitor and leptin 10nM Δ, ***p<0.001 vs control (leptin effect: two-way ANOVA). Data expressed as mean ± SEM. (B) Schematic showing proposed signal transduction used by leptin to exert inhibition.



Supplementary Figure 3.1: Leptin modulates esophageal vagal afferent mechanosensitivity in a similar fashion to gastric afferents. (A) Stimulus-response curves of oesophageal mucosal afferents from fed (i, n=6) and fasted mice (ii, n=6) before ○ and after exposure to leptin (0.1nM □, 1nM Δ, 10nM ◇). (B) Stimulus-response curves of oesophageal tension receptors from fed (i, n=5) and fasted mice (ii, n=6) before ○ and after exposure to leptin (0.1nM □, 1nM Δ, 10nM ◇). (C) Stimulus-response curves of oesophageal mucosal receptors from mice fed a SLD (i, n=5) and a HFD (ii, n=5) before ○ and after exposure to leptin (0.1nM □, 1nM Δ, 10nM ◇). (D) Stimulus-response curves of oesophageal tension receptors from mice fed a SLD (i, n=7) and a HFD (ii, n=13) before ○ and after exposure to leptin (0.1nM □, 1nM Δ, 10nM ◇). * p<0.05, **p<0.01, ***p<0.001. Data are expressed as mean±SEM.

A**B**

Supplementary Figure 3.2: HFD feeding caused an increase in body weight and fat mass. (A) Change in body weight over 12 week period on either SLD (●) (n=52) or HFD (○) (n=51). (B) Gonadal fat pad mass in mice fed either SLD (n=16) or a HFD (n=17). *p<0.05, **p<0.01, ***p<0.001 compared with mice fed a standard laboratory diet. Data are expressed as mean±SEM.



Supplementary Figure 3.3: A HFD does not change muscular afferent profile in the stomach or nodose ganglia. (A) Anterograde traced vagal afferent fibres and intraganglionic laminar endings in wholemount stomach of the mouse (i, SLD; ii, HFD). (B) Number of vagal afferent profiles identified in muscular layers of the stomach in mice fed a SLD (n=6) or HFD (n=6). (C) Anterograde traced vagal afferent fibres and intramuscular arrays in the wholemount stomach of the mouse (i, SLD; ii, HFD). (D) Vagal afferents (white arrows) traced from gastric muscle (serosa) in the nodose ganglia of mice fed a SLD (i) or HFD (ii). (E) Proportion of vagal afferents traced from gastric muscle in nodose ganglia of mice fed a SLD or HFD. Scale 50µm in all instances. Graphed data are expressed as mean±SEM.

CHAPTER 4

A CHRONIC HIGH FAT DIET ALTERS THE HOMOLOGOUS AND HETEROLOGOUS CONTROL OF SATIETY PEPTIDE RECEPTOR EXPRESSION

**Kentish SJ, Wittert GA, Blackshaw LA and Page
AJ.**

**Nerve Gut Research Laboratory, University of
Adelaide, Adelaide, SA 5005, Australia**

Published in Peptides 2013 Aug; 46: 150–158

STATEMENT OF AUTHORSHIP

Title of Paper: A chronic high fat diet alters the homologous and heterologous control of satiety peptide receptor expression

Peptides – 2013, 46: 150–158

Stephen Kentish (Candidate)

Performed cell culture protocol, RNA extraction and PCR experiments, husbandry of the mice, analysed and interpreted data and wrote the manuscript .

I hereby certify that the statement of contribution is accurate.

Signed Date..... 7/3/2013

Gary Wittert

Assisted in the construction and presentation of the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date..... 7/3/2013

Ashley Blackshaw

Assisted in the conceptualisation of experiments and construction of manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date 25/3/13

Amanda Page

Assisted in conceptualisation of studies and manuscript construction.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date 7/3/13

4.1 ABSTRACT

Leptin, ghrelin and neuropeptide W (NPW) modulate vagal afferent activity, which may underlie their appetite regulatory actions. High fat diet (HFD) induced obesity induces changes in the plasma levels of these peptides and alters the expression of receptors on vagal afferents. We investigated homologous and heterologous receptor regulation by leptin, ghrelin and NPW. Mice were fed (12-weeks) a standard laboratory diet (SLD) or HFD. Nodose ganglia were cultured overnight in the presence or absence of each peptide. Leptin (LepR), ghrelin (GHS-R), NPW (GPR7) and cholecystinin type-1 (CCK1R) receptor mRNA, and plasma peptide levels were measured. SLD: leptin reduced LepR, GPR7, increased GHS-R and CCK1R mRNA; ghrelin increased LepR, GPR7, CCK1R, and decreased GHS-R. HFD: leptin decreased GHS-R and GPR7, ghrelin increased GHS-R and GPR7. NPW decreased all receptors except GPR7 which increased with HFD. Plasma leptin was higher and NPW lower in HFD. Thus, HFD-induced obesity disrupts inter-regulation of appetite regulatory receptors in vagal afferents.

4.2 INTRODUCTION

Vagal nerves innervate the gastrointestinal tract where they have both chemosensitive and mechanosensitive endings [436]. In the stomach these endings respond to mechanical stimuli such as distension [471], and activate neurons within the nucleus tractus solitarius [268] and regions of the brain involved in the regulation of food intake such as the arcuate nucleus [493] and paraventricular nucleus [494]. The activity of gastric vagal nerve endings can be modulated by locally released peptides, such as leptin, ghrelin and neuropeptide W (NPW), which are secreted by gastric endocrine and epithelial cells [340, 473, 495]. These three peptides have well documented effects on appetite, food intake and vagal afferent mechanosensitivity. Leptin potentiates vagal activity [333, 496] and reduces appetite [326], whilst ghrelin inhibits vagal afferent firing [301] and promotes food intake [497]. NPW reduces vagal afferent activity (Li et al., 2011), but its effects on food intake remain unresolved, with evidence for both anorexigenic [340] and orexigenic effects [498].

Previously it has been shown that the ability of the stomach to respond to mechanical stimuli through vagal pathways is altered by the induction of high fat diet (HFD) induced obesity [473] and obese humans have diminished perception of gastric distension [432]. Furthermore, the anorexigenic response of vagal afferents to leptin and cholecystinin (CCK) has also been shown to be abrogated under diet induced obese conditions [336].

The receptors for leptin [496], ghrelin [473],NPW [499] and CCK [369] are synthesized in the cell bodies of the vagal afferents located in the nodose ganglia and transported to the neuron endings. The expression of the receptors in vagal afferents appears modified by feeding status [369]. For instance, ghrelin receptor expression is increased in fasted mice [473] and obese rats [369]. These changes in receptor expression are associated with decreased (fasted) or increased (obese) plasma leptin levels and increased (fasted) or decreased (obese) plasma ghrelin levels [462, 500]. It has not been established whether the change in circulating peptides is linked to the change in their receptor expression in the vagal afferent neurons. This led us to hypothesise that a change in peptide exposure may drive a change in the expression of receptors involved in the regulation of appetite. This notion is supported indirectly by leptin's ability to cause suppression of ghrelin [501] and NPW [498] release, whilst increasing the release of the anorexigenic vagal afferent modulator CCK [502], which together may augment the anorexigenic effect of leptin. Similarly, appetite hormones, including leptin and ghrelin can regulate expression and receptor abundance of their cognate and other receptors in a variety of tissues, including vagal afferents [353, 424, 503]. Accordingly we sought to determine (i) the effects of leptin, ghrelin and NPW on their own and each other's receptor expression in cultured nodose ganglia, and (ii) whether any homologous or heterologous regulation of receptor expression is altered by diet-induced obesity.

4.3 MATERIALS AND METHODS

4.3.1 Ethical approval

All experimental studies were performed with the approval of the animal ethics committee of the Institute of Medical and Veterinary Science and the University of Adelaide, and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.3.2 High fat diet model

Eight week old female C57BL/6 mice (N=12/group) were randomly assigned to consume, for 12 weeks, either a standard laboratory diet (SLD) with 7% energy from fat, or HFD (Specialty foods, Glen Forrest, Western Australia) with 60% energy from fat . Water and food was available ad libitum for both groups. Mice were housed under 12 hour light dark conditions with lights on at 06:00. Weights of mice were monitored weekly and gonadal fat pads collected and weighed after euthanasia. All tissue was collected at 08:00.

4.3.3 Cell culture

After 12 weeks on their respective diet the mice were anesthetized with isoflurane (1-1.5% in oxygen) and decapitated after exsanguination via the abdominal aorta. Both nodose ganglia from each animal were harvested and placed immediately together, into a tube containing F12 complete nutrient medium (+ foetal calf serum and penicillin/streptomycin) (Invitrogen, Mulgrave, Australia) and stored on ice. Nodose ganglia were dissociated by incubating at 37°C in a 3mg/ml solution of dispase and collagenase II made

up in Hanks Balanced Salt Solution (HBSS) (Invitrogen) with agitation at 5 minute intervals. After 30 minutes the dispase/collagenase solution was removed, and the ganglia were incubated in a 3mg/ml solution of collagenase II in HBSS for 15 minutes. After this incubation the cells were rinsed in cold HBSS and F12 and then further dissociated by passing them through a fire polished Pasteur pipette until no cell clumps were visible. The cells were then pelleted, rinsed in HBSS, and resuspended in Neurobasal A medium (NBA) containing B-27 supplement (Invitrogen). A cell count was performed using Tryphan blue exclusion. An equal number of cells were plated as a dot in the centre of six wells in a 48 well cell culture plate with the wells coated with poly-D-lysine and laminin (Sigma, Castel Hill, Australia), to promote cell adhesion. The plate was placed in a 5% CO₂ incubator set at 37°C for two hours to allow cell adherence. After two hours the wells were filled with warm (37°C) NBA medium. Two wells were left with just NBA in them as a control and leptin (0.1nM and 1nM) was added to the other wells (two wells for each concentration). The plate was then returned to the incubator for a 14 hour incubation which has previously been shown to be of sufficient length to see a change in receptor mRNA expression in nodose ganglia in vivo [473].

The above process was repeated using 4 SLD and 4 HFD mice with ghrelin (1 and 3nM) and neuropeptide W (1 and 3nM) in the place of leptin (N=4 for each peptide and each diet). Leptin was obtained from Sigma and ghrelin and NPW were obtained from Tocris (Bristol, UK). The concentrations used were based on values found within previous literature that have been shown to cause modulation of vagal afferent activity [343, 441, 462, 473].

4.3.4 RNA isolation

After 14 hours the plate was removed and culture medium drawn off. The wells were washed using sterile PBS. Cells were lysed in the wells by addition of buffer RLT+ β Me (Qiagen, Doncaster, Australia). Cells lysate was processed and RNA isolated using an RNeasy Micro kit (Qiagen) following the manufacturer's instructions. This process utilized RNeasy MinElute SpinElute Columns, allowing adsorption of RNA to the silica membrane, an on-column DNase digestion treatment, removal of contaminants with wash steps and elution of the RNA with RNase-free water. RNA quantification was determined by measuring the absorbance at 260 nm (A260) via a NanoDrop™ ND 1000 spectrophotometer (Thermo Scientific, Australia). RNA quality was estimated by the A260 and A280 nm ratio.

4.3.5 Quantitative RT-PCR

Expression of leptin receptor (LepR), ghrelin receptor (GHS-R), neuropeptide W receptor (GPR7) and CCK receptor (CCK1R) was carried out using Quantitative RT-PCR (QRT-PCR) reactions performed as described in detail previously [455]. Briefly, QRT-PCR reactions were performed by using a Chromo4 (MJ Research, Bio-Rad) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and analysed with Opticon Monitor software (MJ Research). QRT-PCR reactions were performed with a Qiagen QuantiTect SYBRgreen RT-PCR one-step RT-PCR kit (Qiagen) according to the manufacturer's specifications with specific Quantitect Primer Assays (Qiagen) optimized for the detection of the known sequence of mouse LepR,

GHS-R, GPR7, CCK1R and β -tubulin transcripts contained in the NCBI reference sequence database (www.ncbi.nlm.nih.gov/RefSeq). These primer assays were used under the following conditions: reverse transcription, 50°C for 30 min; initial PCR activation, 95°C for 15 min; PCR cycles 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec repeated for 50 cycles. A melting curve program verified the specificity and identity of the RT-PCR products and no primer dimers were observed. Each assay was run in triplicate in two separate experiments. Control PCRs were performed by substituting RNA template with distilled RNase-free water. All assays were validated for linearity of amplification efficiency and quantitative standard curves obtained by serial dilutions of RNA. Calculations for relative mRNA transcripts expression were performed by the comparative C_T method, comparing to the internal reference gene β -tubulin using the equation ΔC_T (C_T of target transcript - C_T of β -tubulin). The relative fold differences were calculated by using the modified version of $2^{-\Delta\Delta C_T}$ [456] correcting for PCR efficiencies.

4.3.6 Quantification of plasma concentrations of peptides

Blood withdrawn from the aorta during euthanasia was placed into pre-chilled K_2EDTA coated tubes and spun for 15 minutes at 4°C 1000xg to separate the plasma from the cells. The plasma concentrations of ghrelin and leptin were determined by ELISA using kits supplied by Millipore (Billerica, USA) and NPW-23 was quantified using a kit supplied by Phoenix Pharmaceuticals (Burlingame, USA) according to the manufacturers' instruction. The leptin and ghrelin assays have previously been used for plasma quantification by other groups [504, 505]. The sensitivities of the assays were 0.05ng/ml (leptin), 0.04

ng/ml (ghrelin) and 0.2ng/ml (NPW), respectively. The intra-assay variations were 1.4% (leptin), 1.4% (ghrelin) and 3.7% (NPW) and the inter-assay variations were 3.8% (leptin), 2.6% (ghrelin) and 5.3% (NPW).

4.3.7 Data analysis

Change in mouse weight were analysed by two-way ANOVA with Bonferroni post hoc test. Gonadal fat weights and plasma peptide levels were analysed with unpaired t-test. The sample expression levels are expressed as either expression relative to β -tubulin \pm SEM to analyse the effect of peptide exposure and significance determined using a one-way ANOVA with Tukey post hoc test or as n-fold difference relative to the control non-exposed cell expression level \pm SEM. Significant differences in transcript expression were determined by a two-way ANOVA with Bonferroni post hoc test to determine whether the effect of the peptide was altered by high fat diet feeding. Differences were considered significant if $p < 0.05$ vs. untreated cells or SLD.

4.4 RESULTS

4.4.1 Effect of high fat diet on body weight and plasma hormones

Body weight (Figure 4.1A) and body fat (Figure 4.1B) increased more in the HFD than SLD mice (both $p < 0.001$). Plasma leptin levels were 3.906 ± 0.2928 ng/ml and 2.286 ± 0.1826 ng/ml in the HFD and SLD mice ($N=12/\text{group}$) respectively ($p < 0.01$) (Figure 4.1C). Plasma ghrelin levels were 3.961 ± 0.3600 ng/ml and 4.234 ± 0.3188 ng/ml in the SLD and HFD mice respectively ($p > 0.05$) (Figure 4.1D). Plasma NPW-23 concentrations were 9.68 ± 0.6947 ng/ml and 5.867 ± 0.5069 ng/ml in the SLD and HFD mice respectively ($p < 0.001$) (Figure 4.1E).

4.4.2 Effect of leptin on expression of LepR, GHS-R, GPR7 and CCK1R in SLD and HFD vagal cell bodies

Compared to control, leptin (0.1nM, 1nM) caused a 0.45 and 0.73 fold reduction in expression of LepR mRNA (both $p < 0.05$) in neurons from SLD mice but was without effect in those from HFD mice (Figure 4.2Ai; $p > 0.05$). Receptor expression in SLD and HFD neurons under control conditions for some peptides was significantly different. Therefore, in order to determine an effect of diet itself, receptor expression was plotted and analysed relative to control. A HFD abolished the effect of leptin on LepR expression in vagal afferent cell bodies (Figure 4.2Aii; $p < 0.001$). GHS-R expression was up regulated by the addition of leptin only at the 1nM concentration in the SLD mice with a 2.04 fold increase in GHS-R mRNA being present in the leptin (1nM) exposed cells than the control cells (Figure 4.2Bi; $p < 0.001$). In contrast,

exposure of leptin (0.1nM, 1nM) to vagal afferent cell bodies from HFD mice resulted in a 0.42 and 0.70 fold decrease in ghrelin receptor expression (Figure 4.2Bi; $p < 0.05$ and $p < 0.001$ respectively). Thus a HFD switched the effect of leptin on GHS-R mRNA expression from an increase, observed in SLD nodose neurons, to a decrease (Figure 4.2Bii; $p < 0.01$). Expression of the NPW receptor, GPR7 was almost completely abolished when leptin was added to the culture medium with 0.1nM and 1nM of leptin both causing a 0.99 fold reduction in the expression of GPR7 (Figure 4.2Ci; $p < 0.001$). In the vagal afferent cell bodies of HFD fed mice leptin (0.1nM and 1nM) still caused a significant reduction (0.90 and 0.97 fold respectively) in the expression of GPR7 (Figure 4.2Ci; $p < 0.001$). However, a HFD significantly attenuated the inhibitory effect of leptin on GPR7 mRNA expression (Figure 4.2Cii; $p < 0.01$). Exposure to the highest dose of leptin (1nM) caused a 2.61 fold increase in the expression of CCK1R in vagal afferent cell bodies from SLD mice (Figure 4.2Di; $p < 0.001$), however in vagal afferent cell bodies from HFD mice leptin failed to have any effect on CCK1R expression (Figure 4.2Di; $p > 0.05$). Therefore a HFD abolished the excitatory effect of leptin on CCK1R expression (Figure 4.2Dii; $p < 0.05$).

4.4.3 Effect of ghrelin on expression of LepR, GHS-R, GPR7 and CCK1R in SLD and HFD vagal cell bodies

Ghrelin (3nM) caused a significant increase in LepR expression of 1.64 fold (Figure 4.3Ai; $p < 0.01$). In the HFD fed mice cells ghrelin 1nM no longer had an effect on expression of LepR ($p > 0.05$), but ghrelin 3nM caused a 0.44 fold reduction in expression (Figure 4.3Ai; $p < 0.001$). Therefore a HFD switched

the effect of ghrelin on LepR mRNA expression from an increase observed in SLD nodose neurons to a decrease (Figure 4.3Aii; $p < 0.01$). Ghrelin (3nM) caused a 0.70 fold reduction in GHS-R expression (Figure 4.3Bi; $p < 0.05$) in the cells from SLD mice, whilst exposure to ghrelin (1nM, 3nM) caused an increase of GHS-R expression (1.86 and 2.50 fold respectively) in HFD vagal cell bodies (Figure 4.3Bi; $p < 0.05$ and $p < 0.001$ respectively). Thus the HFD switched the effect of ghrelin on GHS-R mRNA transcript expression from a decrease as seen in SLD nodose neurons to an increase in the HFD neurons (Figure 4.3Bii; $p < 0.001$). Ghrelin (3nM) caused a 3.00 fold increase in GPR7 expression (Figure 4.3Ci; $p < 0.05$). In HFD vagal cell bodies ghrelin caused a 6.66 (1nM) and 23.00 fold (3nM) increase in expression of GPR7 (Figure 4.3Ci; $p < 0.01$ and $p < 0.001$ respectively). Demonstrating that HFD feeding causes a potentiation of the increase in GPR7 mRNA transcript expression caused by ghrelin (Figure 4.3Cii; $p < 0.001$). Ghrelin (3nM) caused a 2.02 fold increase in CCK1R expression in SLD vagal cell bodies ($p < 0.05$), however when HFD cell bodies were exposed to ghrelin (1nM and 3nM) there was a significant reduction in the expression of CCK1R (0.67 and 0.64 fold reduction respectively) (Figure 4.3Di; both $p < 0.01$). Therefore a HFD causes ghrelin to switch its effect from causing an increase in CCK1R mRNA expression in SLD to a decrease in HFD vagal cell bodies (Figure 4.3Dii; $p < 0.001$).

4.4.4 Effect of NPW on expression of LepR, GHS-R, GPR7 and CCK1R in SLD and HFD vagal cell bodies

When NPW (1nM, 3nM) was added to the cell culture medium it caused a reduction in LepR in cultured cells from both SLD (1nM - 0.63, 3nM - 0.89 fold

reduction; both $p < 0.001$) and HFD (1nM - 0.60, 3nM - 0.75 fold reduction; $p < 0.05$ and $p < 0.01$ respectively) mice (Figure 4.4Ai). Thus a HFD did not alter the effect of NPW on LepR mRNA expression (Figure 4.4Aii; $p > 0.05$). NPW (3nM) caused a significant 0.85 fold reduction in GHS-R expression in SLD cultured cells (Figure 4.4Bi; $p < 0.01$). In the vagal cell bodies from HFD mice NPW still caused a decrease in expression of GHS-R, however the reduction caused by NPW (3nM) was now only 0.36 fold (Figure 4.4Bi; $p < 0.05$). This indicates that a HFD significantly ablated the down regulatory effect of NPW on GHS-R mRNA expression (Figure 4.4Bii; $p < 0.001$). NPW (3nM) caused a 0.70 fold reduction in expression of GPR7, in SLD cell bodies ($p < 0.05$; Figure 4.4Ci). In the HFD cells NPW (1nM, 3nM) caused an increase in GPR7 expression of 1.87 and 2.05 fold respectively (Figure 4.4Ci; both $p < 0.05$). This demonstrated that a HFD caused the effect of NPW on GPR7 mRNA expression in vagal cell bodies to be altered from a decrease to an increase (Figure 4.4Cii; $p < 0.05$). NPW (1nM, 3nM) caused a significant reduction in CCK1R expression of 0.36 and 0.40 fold in SLD vagal cell bodies respectively (Figure 4.4Di; both $p < 0.05$) and 0.52 and 0.47 fold reduction in HFD cell bodies respectively (Figure 4.4Di; both $p < 0.01$). Therefore the effect of NPW on CCK1R mRNA expression was unaffected by HFD feeding (Figure 4.4Dii; $p > 0.05$).

4.5. DISCUSSION

These data show that the appetite regulating peptides, leptin, ghrelin and NPW modify the expression of their own, and each other's receptors. Chronic high fat diet feeding alters the magnitude and direction of these effects, which may alter effects mediated by gastric vagal afferent signalling, including satiety.

Leptin has previously been shown to decrease the expression of its cognate receptor [506, 507]. The observation that this is abrogated in nodose ganglia in response to HFD induced obesity is consistent with previous data [369, 370, 496]. In contrast to what occurs in vagal afferents, leptin induces an increase in LepR mRNA in the arcuate nucleus of lean mice [508-510], an effect that is also lost in HFD induced obesity [511]. In obese mice leptin increases SOCS3 [512], but the phosphorylation of STAT3 is reduced [335, 513, 514]. Therefore it seems likely that receptor binding occurs, but there is a reduction in downstream signalling. However, we have not differentiated between the isoforms of LepR, which have been shown to follow different patterns of regulation [515]. Therefore, although the full length isoform of the receptor is considered the functional receptor [516], further investigation into the possible contributions of all LepR isoforms is required.

In addition to its central effects, leptin can decrease food intake by acting alone or in concert with CCK to activate gastrointestinal vagal afferents [326, 333, 334, 517]. The finding in this study that leptin increases the expression of

CCK1R provides additional support for the notion that leptin may reduce food intake in part via a CCK-mediated vagal mechanism of action [334, 335]. Our finding that leptin no longer increased CCK1R expression in vagal afferent cell bodies from HFD fed mice may contribute to the dampened vagally mediated CCK satiety signal that is observed in obese rodents [335, 518].

Leptin has previously been shown to increase GHS-R expression in human T-cells [503]. We now show that leptin increases GHS-R in vagal afferent cell bodies. Leptin reducing GHS-R mRNA in HFD fed mice may represent a mechanism of suppressing orexigenic signals. Similarly in obese mice [359] and rats [519] hypothalamic GHS-R is decreased. However, in isolated pituitary cells from obese leptin knockout mice (*ob/ob*) GHS-R mRNA expression increases in response to increasing concentrations of leptin [520]. This may reflect tissue or culture specific effects or alternatively, a nutrient driven change in receptor expression regulation, as the *ob/ob* mice used were maintained on a chow diet.

The observations that leptin decreased mRNA expression for its own receptor at a concentration lower than that required to change CCK1R and GHS-R mRNA expression and that leptin has no effect on CCK1R or LepR mRNA, whilst still effecting GHS-R and GPR7 in the HFD vagal afferents suggests that leptin alters transcriptional regulation through multiple pathways. However, this possibility requires further investigation.

Ghrelin is orexigenic and believed to be primarily involved in the initiation of meals; increasing plasma concentrations precede food intake and fall dramatically thereafter [521]. It has previously been shown that obese male and female mice have reduced plasma ghrelin [359, 441], which is independent of leptin levels [522]. In contrast, the current study showed no difference in plasma ghrelin levels in lean and obese mice, which has also been shown previously [504]. There are a number of possible explanations for this, including feeding status and sampling differences. As plasma ghrelin levels decrease after eating, plasma level measurements depend on the timing of blood collection in relation to meal intake. Plasma ghrelin levels have also been shown to oscillate in a circadian manner [523]. The degree to which plasma ghrelin levels change over the course of a day is altered in obesity [523]. Our plasma samples were collected at 08:00, which has previously been shown to be a time when there is no difference in plasma ghrelin between lean and obese mice [523]. Previous studies have not provided a measure of recent food intake, or detailed the time plasma samples were collected which makes comparisons difficult.

In lean mice the decreased GHS-R mRNA in response to ghrelin may decrease ghrelin signalling after meal initiation. The concomitant increase of LepR and CCK1R mRNA, may serve to enhance meal termination signals. The increase in LepR and CCK1R mRNA is consistent with previous reports showing an increased proportion of vagal neurons expressing LepR and CCK1R in nodose ganglia from fasted mice [424, 470], with elevated plasma ghrelin [524, 525]. Given the opposing effect of leptin and ghrelin on food

intake, it seems contradictory that leptin and ghrelin have the same effect on CCK1R mRNA in vagal cell bodies from SLD mice. This may be due to the predominately short term role of ghrelin and long term role for leptin in the regulation of food intake [526]. Thus we could speculate that ghrelin increases CCK1R expression to ensure that vagal afferents are always primed to respond to CCK release in response to nutrients and that leptin released from adipose tissue increases CCK1R expression when there is sufficient energy stores.

In vagal cell bodies from HFD mice, the increase in GHS-R mRNA in response to ghrelin, may serve to maintain ghrelin signalling in obesity, where levels have been found to be decreased [359]. Plasma ghrelin continues to show preprandial rises in obesity [527]. This suggests ghrelin is still involved in the initiation of meals in obesity. The switch in the effect of ghrelin on regulation of GHS-R mRNA in mice with diet induced obesity may be mediated by activation of an alternate signalling pathway, as we have previously described for leptin [496], that results in different mRNA transcript levels. The ghrelin induced decrease in LepR and CCK1R in the HFD vagal afferent cell bodies may blunt meal termination in obesity, which is consistent with previous findings that obese mice eat longer meals [377] and have reduced satiation in response to leptin and CCK [335, 336, 380, 518]. However, this is speculative and the relationship between altered receptor regulation and reduced CCK and leptin mediated satiety signalling needs to be clarified with more specific experiments.

In the current study, NPW suppressed receptor mRNA expression for anorexigenic and orexigenic peptides. Similarly in obese cell bodies NPW decreased expression of all receptors with the exception of its own receptor. However, the regulation of GPR7 in vivo in lean and obese mice still needs to be determined. Thus it remains unclear as to what the physiological role of the peptinergetic regulation of GPR7 in lean and obese mice is. We also reported a decrease in the plasma concentration of NPW-23 in obese mice. Comparison with previous values was not possible due to conflicting models and methodological approaches.

The static cultures used do not reflect the oscillations in plasma levels of leptin and ghrelin that occur over a 24 hour period [528, 529], or the combined effects of changes in plasma nutrients and other gut peptides that occur in vivo. Therefore, although we have established that the gut peptides studied have direct regulatory effect on appetite receptor regulation in vagal afferent cell bodies that are modified by diet induced obesity; the physiological relevance in vivo remains to be determined.

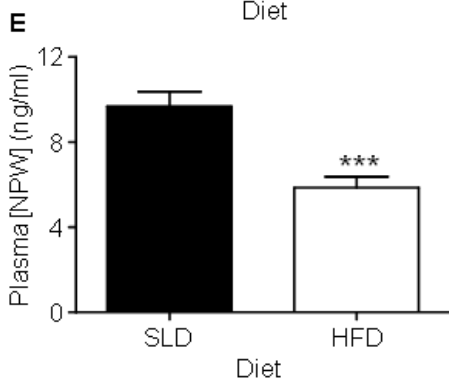
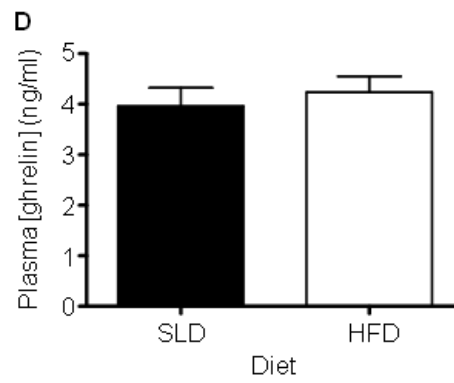
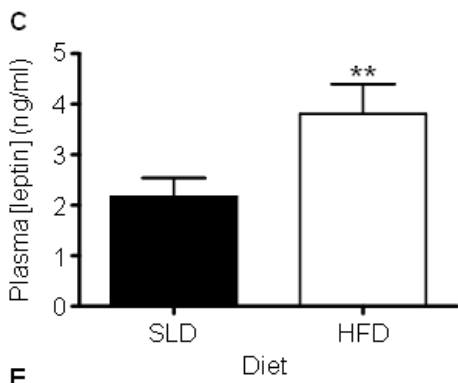
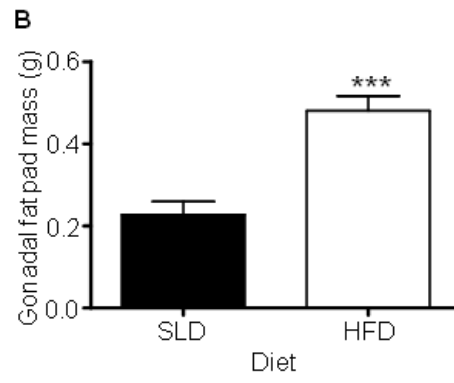
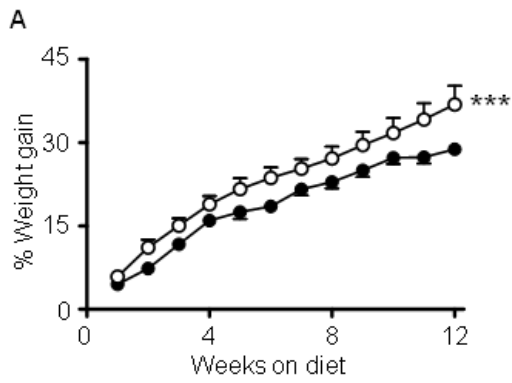


Figure 4.1: High fat diet induced changes in weight and plasma peptides.

(A) Mice on a HFD (○ N=12) gained a significantly greater proportion of body weight over the 12 week diet period compared to the SLD mice (● N=12). (B) The HFD fed mice also had significantly heavier gonadal fat pads. (C) HFD mice had elevated plasma leptin levels, (D) no difference in plasma ghrelin and (E) decreased plasma NPW. Weight analysed using two-way ANOVA with Bonferroni post hoc test, fat mass and plasma peptide levels compared using unpaired t-tests. **p<0.01, ***p<0.001.

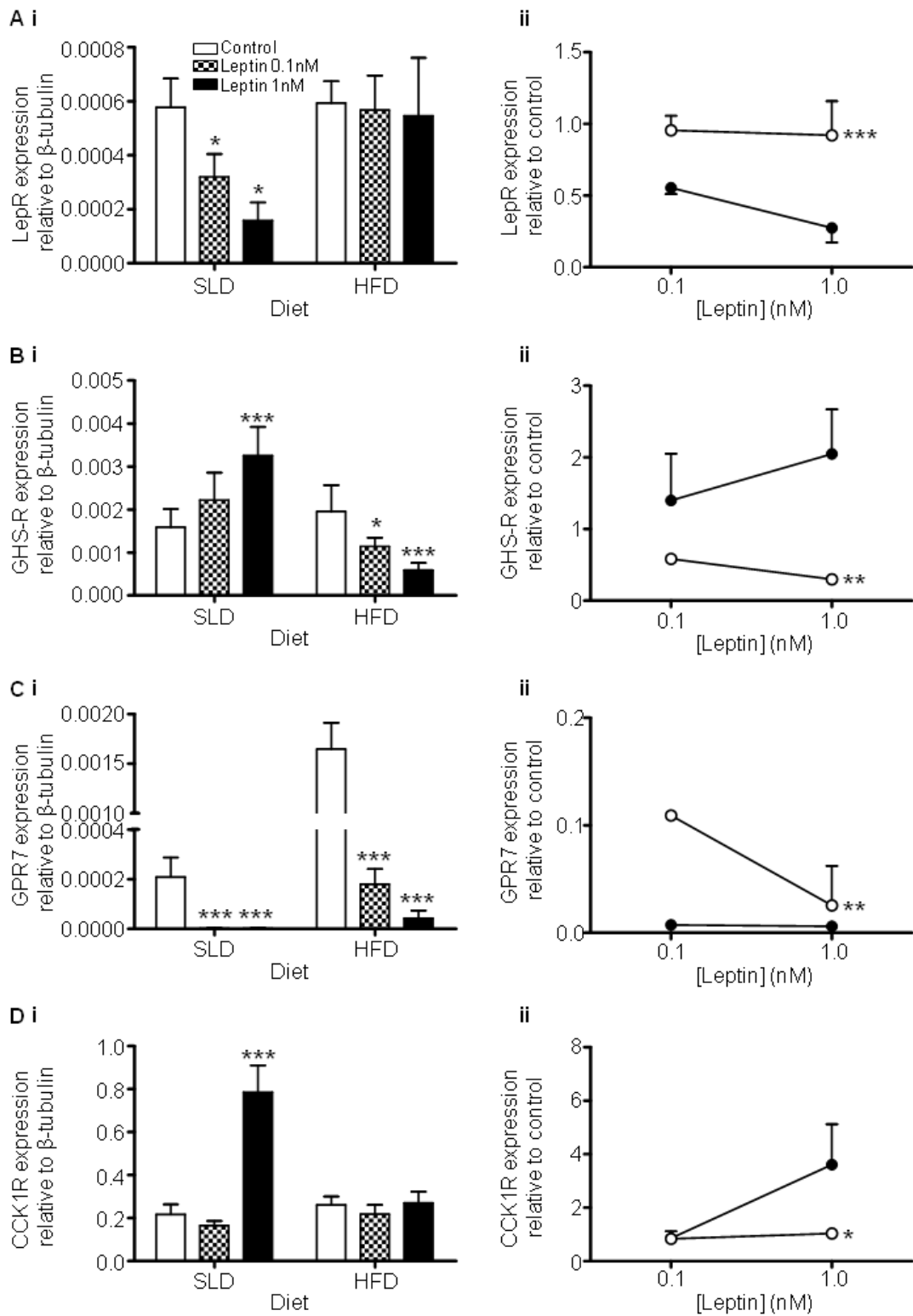


Figure 4.2: Expression of appetite regulatory peptide receptors is altered by leptin exposure. Changes in expression of LepR (Ai), GHS-R (Bi), GPR7 (Ci) and CCK1R (Di) relative to β -tubulin in the absence and presence of leptin (0.1, 1nM). Changes in expression of LepR (Aii), GHS-R (Bii), GPR7 (Cii) and CCK1R (Dii) mRNA relative to untreated cells from SLD (●) and HFD (○) mice. Data are means \pm SEM (N=4 in all instances), Leptin effect (i) within group analysed using one-way ANOVA with Tukey post hoc test with significance being determined if $p < 0.05$. Diet effect (ii) analysed with two-way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

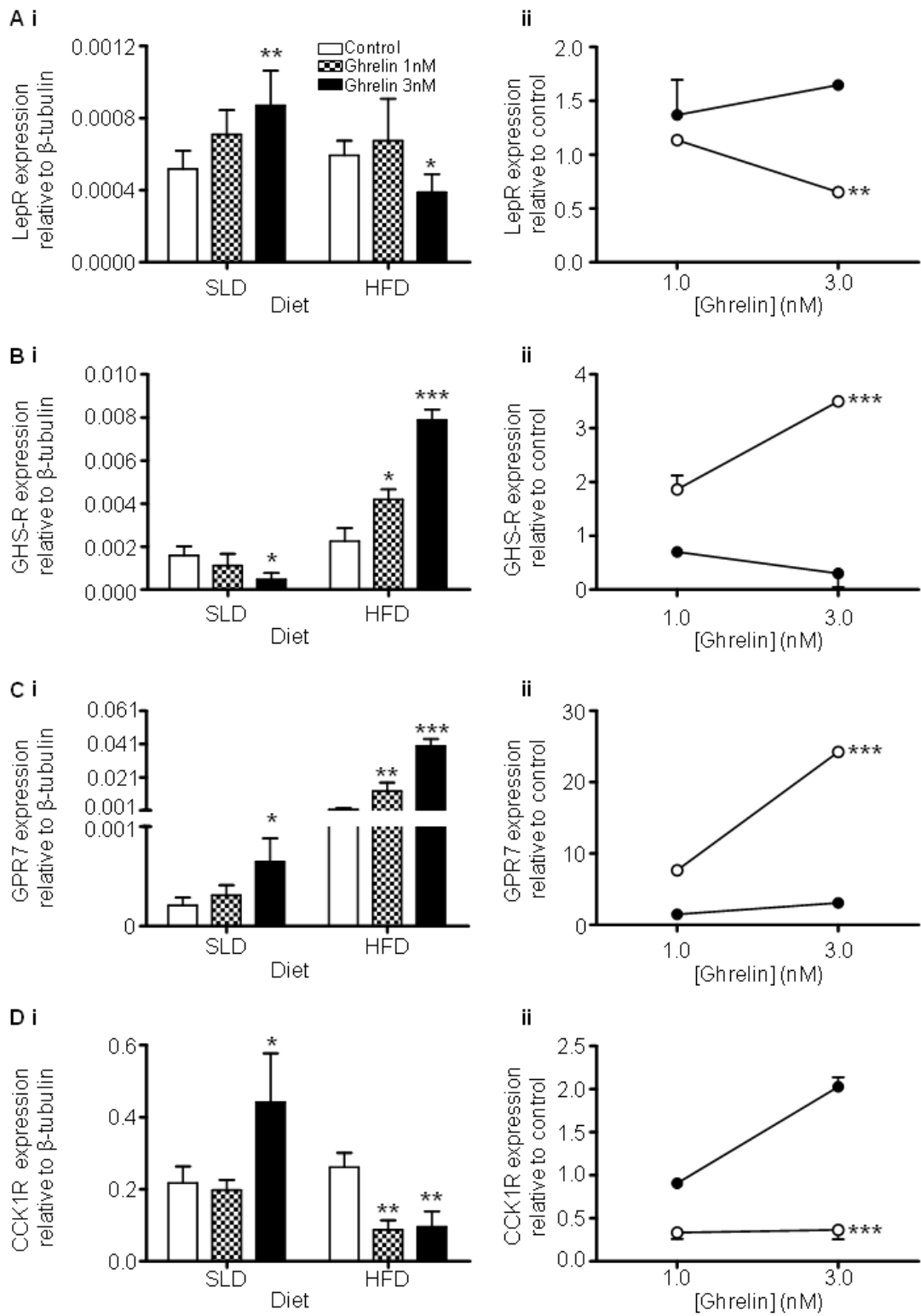


Figure 4.3: Effect of ghrelin exposure of expression of appetite modulator peptide receptor expression. Ghrelin (1nM, 3nM) induced changes in expression of LepR (Ai), GHS-R (Bi), GPR7 (Ci) and CCK1R (Di) in SLD and HFD cell bodies relative to β -tubulin. Changes in expression of LepR (Aii), GHS-R (Bii), GPR7 (Cii) and CCK1R (Dii) mRNA relative to untreated cells from SLD (●) and HFD (○) mice. Data are means \pm SEM (N=4 in all instances), Ghrelin effect (i) within group analysed using one-way ANOVA with Tukey post hoc test with significance being determined if $p < 0.05$. Diet effect (ii) analysed with two-way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

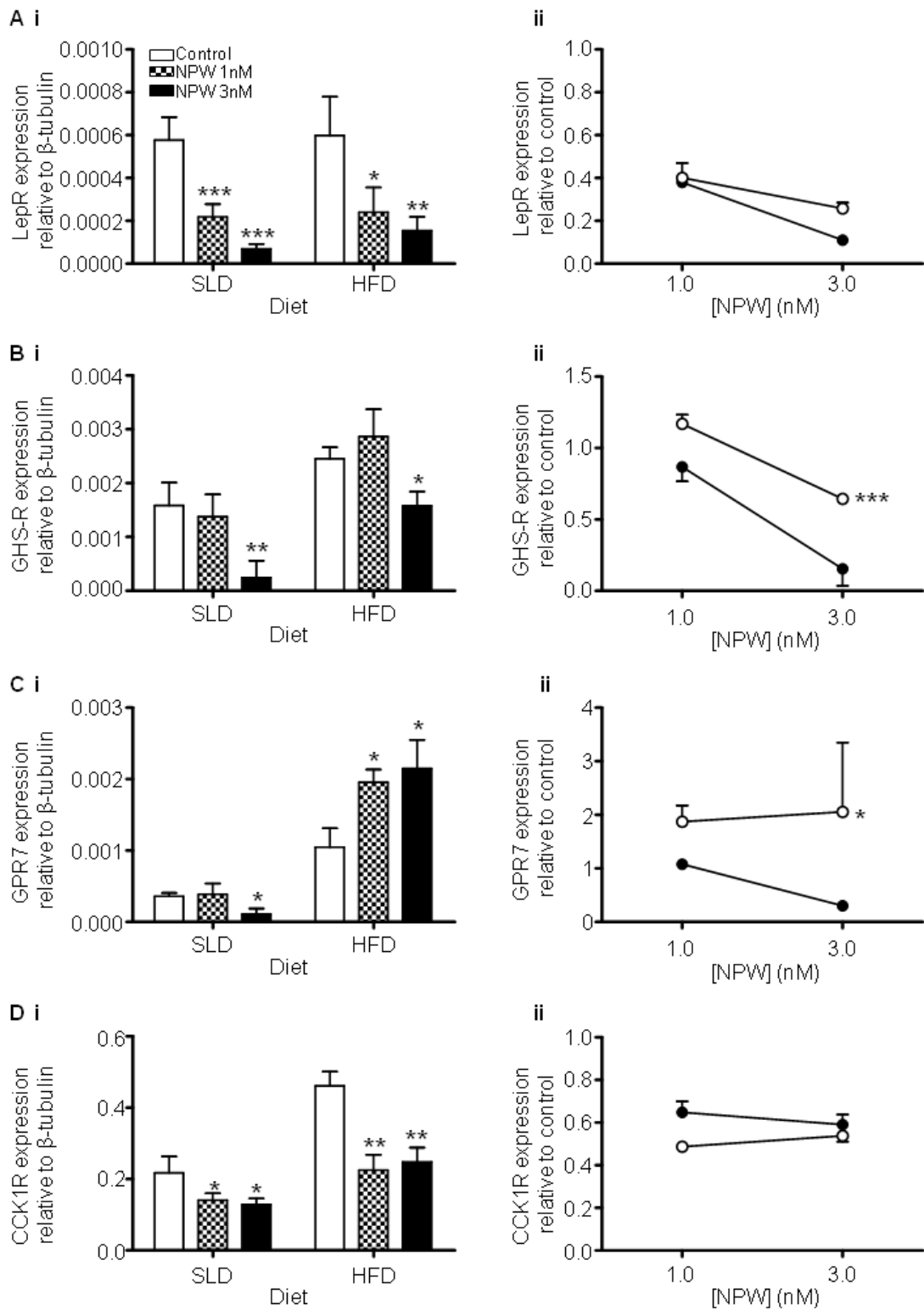


Figure 4.4: NPW changes the expression of appetite regulatory receptors. NPW (1nM, 3nM) induced changes in expression of LepR (Ai), GHS-R (Bi), GPR7 (Ci) and CCK1R (Di) in SLD and HFD cell bodies relative to β -tubulin. Changes in expression of LepR (Aii), GHS-R (Bii), GPR7 (Cii) and CCK1R (Dii) mRNA relative to untreated cells from SLD (●) and HFD (○) mice. Data are means \pm SEM (N=4 in all instances), NPW effect (i) within group analysed using one-way ANOVA with Tukey post hoc test with significance being determined if $p < 0.05$. Diet effect (ii) analysed with two-way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CHAPTER 5

ALTERED GASTRIC VAGAL MECHANOSENSITIVITY IN DIET-INDUCED OBESITY PERSISTS ON RETURN TO NORMAL CHOW AND IS ACCOMPANIED BY INCREASED FOOD INTAKE

Kentish SJ, O'Donnell TA, Li H, Wittert GA,

Page AJ.

Nerve Gut Research Laboratory, University of

Adelaide, Adelaide, SA 5005, Australia

Submitted to The International Journal of

Obesity

STATEMENT OF AUTHORSHIP

Title of Paper: Altered gastric vagal mechanosensitivity in diet-induced obesity persists on return to normal chow and is accompanied by increased food intake.

Submitted to: The International Journal of Obesity (28/03/2013)

Stephen Kentish (Candidate)

Performed electrophysiology experiments, ELISA assays, RNA extraction and PCR experiments, husbandry of the mice, analysed and interpreted data and wrote the manuscript.

I hereby certify that the statement of contribution is accurate.

SignedDate...28/03/2013

Tracey O'Donnell

Assisted with mouse weight and food intake measurements.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

SignedDate...28/3/2013

Hui Li

Performed retrograde tracing procedure.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date 7/3/2013

Gary Wittert

Assisted in the construction and presentation of the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date 07/03/2013

Amanda Page

Assisted in conceptualisation of studies and manuscript construction

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed Date 7/3/13

5.1 ABSTRACT

Background and aims: Gastric vagal afferents convey satiety signals in response to mechanical stimuli. The sensitivity of these afferents is decreased in diet induced obesity. Leptin, secreted from gastric epithelial cells, potentiates the response of vagal afferents to mechanical stimuli in lean mice, but has an inhibitory effect in high fat diet (HFD) induced obese mice. We sought to determine whether changes in vagal afferent function and response to leptin in obesity were reversible by returning obese mice consuming a HFD to standard laboratory chow diet (SLD).

Methods: 8wk old female C57BL/6 mice were either fed a SLD (N=20) or HFD (N=20) for 24wks. A third group was fed a HFD for 12wks and then a SLD for a further 12wks (RFD, N=18). An *in vitro* gastro-oesophageal vagal afferent preparation was used to determine the mechanosensitivity of gastric vagal afferents and the modulatory effect of leptin (0.1-10nM) was examined. Retrograde tracing and quantitative RT-PCR were used to determine the expression of leptin receptor (LepR) mRNA in whole nodose and specific cell bodies traced from the stomach.

Results: After 24wks both the HFD and RFD mice had increased body weight, gonadal fat mass, plasma leptin, plasma insulin and daily energy consumption compared to the SLD mice. The HFD and RFD mice had reduced tension receptor mechanosensitivity and leptin further inhibited responses to tension in HFD, RFD but not SLD mice. Mucosal receptors from both the SLD and RFD mice were potentiated by leptin, an effect not seen in HFD mice. LepR

expression was unchanged in the whole nodose, but was reduced in the mucosal afferents of the HFD and RFD mice.

Conclusions: Disruption to gastric vagal afferent function by HFD induced obesity is only partially reversible by dietary change, which provides a potential mechanism preventing maintenance of weight loss.

5.2 INTRODUCTION

The experience of most obese people is that following diet induced weight loss, they return at least to their previous weight within two years [68, 530-532]. There is evidence from both animal [73] and human studies [533] that fat mass is permitted to increase to what is presumably a genetically determined upper limit. At any particular point along that trajectory, the fat mass reached is defended such that if it decreases, mechanisms are activated to ensure that it is restored. The mechanism responsible for this defence of body fat has been suggested to be mediated by prolonged changes in the level of appetite regulating gastrointestinal hormones, known to be altered following weight loss and changed in such a way as to promote weight regain [78].

Neurally mediated signals from the gastrointestinal tract play a role in the regulation of food intake [534-536]. For example, cognitive perception of fullness following food intake is reliant on two vagally mediated mechanisms. One pathway relies on the presence of nutrients, which triggers gastrointestinal hormone endocrine and paracrine secretions from the stomach and small intestine [537, 538]. The other pathway is via mechanical distension of the stomach, which in conjunction with small intestinal nutrient signals can induce satiety [472, 539]. Two classes of vagal afferent mechanoreceptors have been identified in the stomach [436]. The first are tension receptors which respond to distension and contraction of the stomach.

The second class are mucosal receptors, which are located close to the lumen, are responsive to contact by food particles with the mucosa and are believed to participate in the control of gastric motor function [281, 297, 436, 471]. Previously it has been shown that diet induced obesity caused a reduction in the ability of gastric and intestinal vagal afferents to respond to stretch/distension, suggesting in obesity there is reduced gastrointestinal signalling in response to food intake [381, 473].

Leptin is a peptide released from both adipocytes[540] and gastric epithelial cells [304]. Ordinarily leptin reduces food intake largely attributed to actions within the arcuate nucleus[541]. However, leptin receptor (LepR) is also present in gastric vagal afferent neurons [470] and leptin has been shown to play a role in regulating food intake through a vagally mediated mechanism[326]. In diet-induced obesity this effect is lost [335, 336]. Previously, we have demonstrated that leptin has a potentiating effect on gastric vagal mucosal receptors [496]. Furthermore, we have shown that obesity causes leptin to lose its potentiating effect and instead have an inhibitory effect on vagal tension receptors [496]. This indicates that leptin may act within the stomach to modulate peripheral appetite signals and its ability to do so is disrupted by obesity to a degree that defends the obese state. We sought to determine the effect of returning these mice to a standard laboratory diet (SLD) for 12 weeks on weight, food intake, gastric mechanosensitivity, and the vagal afferent response to leptin. To establish whether any change in the effect of leptin was associated with receptor

expression changes we determined the expression of LepR in both whole nodose and mucosal traced gastric vagal afferents.

5.3 MATERIALS AND METHODS

5.3.1 Ethical approval

All studies were approved and performed in accordance with the guidelines of the Animal Ethics Committees of the University of Adelaide and Institute for Medical and Veterinary Science, Adelaide, Australia. Every attempt was made to limit the number of animals used and minimize their suffering.

5.3.2 Animals

All mice in these studies were obtained from Animal Resource Centre (Canning Vale, Australia) and group housed under a 12 hour light: dark cycle (lights on at 06:00) with free access to food and water. Female C57BL/6 mice were obtained at 7 weeks of age and assigned randomly to either a 24 week SLD group (7% energy from fat; N=20), a 24 week high fat diet (HFD) group (60% energy from fat; N=20) or a group that was fed a HFD for 12 weeks and then returned to a SLD for a further 12 weeks (RFD; N=18). All mice were allowed to acclimatize for one week before being started on their respective diet. The mice were weighed weekly and had food intake monitored over the 24 week diet period. Blood glucose level and gonadal fat pad mass were determined from all mice on the day they were used for the *in vitro* mouse gastro-oesophageal afferent preparation.

5.3.3 *In vitro* mouse gastro-oesophageal afferent preparation

This preparation has been described in detail previously [436]. In short, female C57BL/6 mice on the SLD, HFD or RFD diets were anaesthetised with

isoflurane (1-1.5% in oxygen) and killed via exsanguination. The stomach and oesophagus, with intact vagal nerves, were removed and placed mucosa side up in an organ bath containing a modified Krebs solution comprised of (in mM): 118.1 NaCl, 4.7 KCl, 25.1 NaHCO₃, 1.3 NaH₂PO₄, 1.2 MgSO₄·7H₂O, 1.5 CaCl₂, 1.0 citric acid, 11.1 glucose and 0.001 nifedipine, bubbled with 95% O₂-5% CO₂. The dissection process was carried out at 4°C to prevent metabolic breakdown.

5.3.4 Characterization of gastric vagal afferent properties

In mice two types of mechanosensitive gastric vagal afferent have been reported [436], those that respond to mucosal stroking, but not to circular tension (mucosal receptors) and those that respond to both mucosal stroking and circular tension (tension receptors). Receptive fields of these receptors were first located using mechanical stimulation with a brush in the mouse gastro-oesophageal preparation. Once located specific stimuli were then applied. Mucosal stroking was performed using calibrated von Frey hairs (10-1000mg) which were stroked across the mucosa at a rate of 5mms⁻¹. Each receptive field was stroked ten times and mechanical responses from the middle eight strokes taken for analysis. Circular tension was applied using a threaded hook attached to an underpinned point adjacent to the receptive field. The threaded hook was attached to a cantilever via a pulley close to the preparation. Standard weights (0.5-5g) were then placed on the opposite end of the cantilever. Each weight was applied for one minute with a break of another minute between removing one weight and applying the next. After

analysing the two stimulus response curves, we would classify a receptive field as either a mucosal or tension receptor.

5.3.5 Effect of leptin on the mechanosensitivity of vagal afferents

After the mechanosensitivity of a receptive field was determined, the effect of leptin was assessed. Leptin (0.1nM, Sigma-Aldrich, Castle Hill, Australia) was added to the superfusing Krebs solution and allowed to equilibrate for 20 min after which the stimulus response curves were re-determined. This procedure was repeated for leptin 1 and 10nM.

5.3.6 Data recording

Afferent impulses were amplified with a biological amplifier (DAM 50, World Precision Instruments, Sarasota, FL), and filtered (band-pass filter 932, CWE, Ardmore, PA, USA). Single units were discriminated on the basis of action potential shape, duration, and amplitude by use of Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

5.3.7 Nodose ganglia quantitative RT-PCR

Nodose ganglia were removed bilaterally from mice from all 3 experimental groups. Total RNA was extracted using an RNeasy Micro Kit (Qiagen, Doncaster, Australia) according to the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260nm (A₂₆₀) using a NanoDrop™ ND 1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, Australia) and RNA purity was estimated via the 260/280 absorbance ratio. Quantitative RT-PCR (QRT-PCR) reactions were performed

as described in detail previously [455]. In short, reactions were performed using a Chromo4 (MJ Research, Bio-Rad, Gladesville, Australia) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and analysed with Opticon Monitor software (MJ Research). QRT-PCR reactions were performed using a QuantiTect SYBRgreen RT-PCR one-step RT-PCR kit (Qiagen) according to the manufacturer's instructions. All primers used were pre-designed validated Quantitect Primer assays (Qiagen) targeting leptin receptor and β -tubulin. RT-PCR reactions were carried out under the following conditions: reverse transcription, 50°C for 30min; initial PCR activation, 95°C for 15min; PCR cycles 94°C for 15sec, 55°C for 30sec and 72°C for 30sec repeated for 50 cycles. A melt curve was obtained to confirm the specificity of the products produced. Each assay was run in triplicate and repeated on separate days. Control PCRs were carried out substituting RNase-free water for template RNA. Relative RNA levels were calculated using the comparative CT method as described previously [456].

5.3.8 Retrograde tracing

This procedure has been detailed previously [473]. In short; to identify specific gastric mucosal afferents SLD (N = 4), HFD (N=4) and RFD (N=4) mice were anesthetized with isoflurane (1-1.5% in oxygen), a laparotomy performed, and a mucolytic (10% N-acetylcysteine; 200 μ l) was injected into the stomach lumen then removed via syringe after 5 min, this was followed by two saline rinses (200 μ l each). Subsequently, 10 μ l of 0.5% Alexa Fluor® 555 conjugate of cholera toxin β -subunit (CTB-AF555); Invitrogen, Mulgrave, Australia) was injected into the proximal gastric lumen via a 30 ga Hamilton syringe and the

proximal stomach walls gently opposed to expose the dorsal and ventral surfaces to the tracer. The injection site was dried and skin incision closed. Antibiotic (Baytril; 50µl of 50mg/ml) and analgesic (butorphanol; 5mg/kg) were administered subcutaneously. Food and water were withheld for 2 hours postoperatively to maximize exposure of tracer. All mice recovered well from surgery and were routinely monitored. Isolation of tension afferents is possible, however we have previously determined that diet induced obesity has no effect on the expression of LepR mRNA in this sub-population [496].

5.3.9 Laser capture microdissection

After 2 days, retrogradely traced mice were anesthetized with pentobarbitone (60 mg/kg, i.p.) their left and right nodose ganglion was then removed and dissociated before being cultured on a duplex dish for 2 hours at 37°C in 5% CO₂ [496]. Cells were then subject to laser-capture microdissection, performed on a P.A.L.M.[®] microbeam microdissection system (Carl Zeiss, Jena, Germany). Fluorescent labelled nodose neurons were microdissected and catapulted directly into a lysis and stabilization buffer (Buffer RLT, RNeasy Micro RNA extraction Kit, Qiagen) containing 0.14M β-Mercaptoethanol (Sigma-Aldrich). RNA was extracted and QRT-PCR was performed on these cells using the same protocol as for whole nodose ganglia.

5.3.10 Plasma hormone measurements

Blood samples from all groups of mice were collected on the day of experiment from the abdominal aorta, under anaesthesia. Blood was placed

into K₂EDTA coated tubes and spun for 15min at 1000xg to separate the plasma which was stored at -80°C until needed. Plasma leptin and insulin were determined using commercially available ELISA assay kits (Millipore, Billerica, MA, USA) according to manufacturers' instructions. The sensitivities of the assays were 0.05ng/ml (leptin) and 0.2ng/ml (insulin); the intra-assay variations were 1.4% (leptin), 4.65% (insulin).

5.3.11 Statistical analysis

All data in graphs are expressed as mean values \pm SEM with N= number of animals studied and n= number of individual afferents analysed. Vagal afferent stimulus-response curves and weight change were analysed using two-way analysis of variance (2-way ANOVA) and Bonferroni post hoc tests. RNA levels, fat mass, food/energy intake and plasma peptide levels were analysed using one-way ANOVA's with Tukey post hoc tests. Significance was defined at $p < 0.05$.

5.4 RESULTS

5.4.1 Diet induced changes to mouse weight, fat mass, food consumption and plasma peptide levels

The HFD mice gained more weight than both the SLD and RFD mice (Figure 5.1A, both $p < 0.001$). The RFD mice initially lost some weight upon return to the SLD, but by the end of the 24th week they had gained back all lost weight and on average weighed more than at the point they were removed from the HFD (Figure 5.1A, $p < 0.001$ SLD vs. RFD). Gonadal fat mass of the HFD ($2.71 \pm 0.37\text{g}$) mice was greater than that of both the RFD ($0.62 \pm 0.10\text{g}$) and SLD ($0.34 \pm 0.02\text{g}$) mice (Figure 5.1B, both $p < 0.001$), however the RFD fat mass was also greater than the SLD mice (Figure 5.1B, $p < 0.01$ SLD vs. RFD). Blood glucose levels were higher in both the HFD and RFD mice ($12.78 \pm 0.82\text{mM}$ and $11.07 \pm 0.79\text{mM}$ respectively) compared to the SLD mice ($6.45 \pm 0.73\text{mM}$) (Figure 5.1C, both $p < 0.01$ vs. SLD). The average 24-hour food consumption of the RFD mice ($4.8 \pm 0.43\text{g}$) was significantly greater than that of the SLD ($2.78 \pm 0.23\text{g}$) and HFD ($3.14 \pm 0.19\text{g}$) mice (Figure 5.1D, $p < 0.001$ vs. SLD, $p < 0.01$ vs. HFD) at the end of the 24 week period. The energy intake of the RFD mice ($16.23 \pm 1.41\text{kcal}$) was similar to the HFD: ($16.42 \pm 0.99\text{kcal}$), and both were greater than the SLD mice ($9.29 \pm 0.77\text{kcal}$) (Figure 5.1E, HFD vs. SLD $p < 0.001$, RFD vs. SLD $p < 0.01$). Plasma leptin levels (Figure 5.1F) were higher in the HFD ($23.09 \pm 1.27\text{ng/ml}$, $p < 0.001$ vs. SLD and RFD) and RFD ($16.33 \pm 1.83\text{ng/ml}$, $p < 0.001$ vs. SLD) mice compared to the SLD mice ($5.01 \pm 0.31\text{ng/ml}$). Plasma insulin (Figure 5.1G) was also

elevated in the HFD ($0.97 \pm 0.08 \text{ ng/ml}$ $p < 0.001$ vs. SLD $p < 0.01$ vs. RFD) and RFD ($0.69 \pm 0.03 \text{ ng/ml}$ $p < 0.01$ vs. SLD) relative to SLD mice ($0.55 \pm 0.13 \text{ ng/ml}$).

5.4.2 High fat diet induced changes in gastric mechanosensitivity are not altered by reverting to 'normal' chow feed

Gastric tension mechanosensitivity of the HFD and RFD mice was reduced by 50% (at 5g) compared to the SLD mice (Figure 5.2A, both $p < 0.001$ vs. SLD). There was no significant difference between the responses of the gastric tension receptors of the HFD mice compared to the RFD mice ($p > 0.05$). There was no difference in the response to mucosal stroking by mucosal receptors between any of the groups of mice (Figure 5.2B, $p > 0.05$).

5.4.3 Leptin's effects on gastric vagal afferent mechanosensitivity are dependent on diet

Leptin (0.1-10nM) potentiated gastric mucosal mechanosensitivity (Figure 5.3A & E $p < 0.001$ vs. Control) in SLD mice, but had no effect in HFD mice (Figure 5.3B & F, $p > 0.05$). In the RFD mice leptin increased mucosal receptor mechanosensitivity (Figure 5.3C & G, $p < 0.001$ vs. Control). The level of potentiation induced by leptin (0.1-10nM) in response to 50mg stroking was different in all three groups of mice (Figure 5.3D, $p < 0.001$ SLD vs. HFD, $p < 0.01$ RFD vs. HFD and SLD).

Leptin had no effect on the mechanosensitivity of gastric tension receptors (1-5g) in SLD mice (Figure 5.4A & E, $p>0.05$), however caused a reduction in HFD (Figure 5.4B & F, $p<0.001$ vs. Control) and RFD (Figure 5.4C & G, $p<0.001$ vs. Control) mice. The level of inhibition caused by leptin in response to 3g stretch was highest in the HFD mice followed by the RFD and SLD mice (Figure 5.4D, $p<0.05$ HFD vs. RFD, $p<0.001$ HFD and RFD vs. SLD).

5.4.4 Diet induced changes in the expression of leptin receptor in vagal afferents

There was no difference in LepR expression in whole nodose ganglia between any of the groups of mice (Figure 5.5A, $p>0.05$). However, when LepR mRNA expression was quantified specifically in mucosal afferents there was a 99% reduction in LepR mRNA in the HFD mice compared to the SLD mice (Figure 5.5B, $p<0.001$ SLD vs. HFD). The RFD mucosal afferents had a 68% reduction in LepR mRNA relative to the SLD mice ($p<0.001$ SLD vs. RFD). The RFD LepR mRNA content was greater than that observed in the HFD mice ($p<0.05$ HFD vs. RFD).

5.5 DISCUSSION

These data show when HFD induced obese mice received a standard chow diet; brief weight loss was followed by a return to their previously established weight, associated with an increase in food intake. The reduced mechanosensitivity of gastric vagal afferent tension receptors, in HFD obesity, was maintained. However, the switch in effect of leptin, from excitatory on gastric mucosal receptors in SLD mice to inhibitory on gastric tension receptors in HFD mice, is partially reversed upon return to a standard chow diet.

The reduction in tension receptor mechanosensitivity is consistent with the reduced neural activation seen in the hypothalamus of obese humans in response to gastric distension [123]. Its persistence on return to the standard chow diet is presumably part of the mechanism that facilitates the increased food intake required to maintain the higher body weight. Although the amount of food consumed increased, the absolute caloric intake matched that consumed on the HFD. It is well established that chronic feeding of a palatable, high fat, energy dense diet, induces obesity [542], which once obtained, is defended against perturbations in body weight [543, 544]. These data demonstrate that after 12 weeks on a high fat diet, there appears be changes in gastric mechanosensitivity which serve to protect an increased body weight, but it remains to be determined whether the increased food intake will see the RFD mice obtain the same level of adiposity and weight as the HFD mice or settle at a point between the HFD and SLD mice.

The partial restoration of leptin sensitivity, on mucosal receptors, is of interest since it may preclude the ongoing increase in weight occurring in mice that continue on the HFD, and facilitate maintenance of the weight reached at the time the switch occurred. Leptin sensitivity has previously been shown to be restored both centrally and peripherally in diet induced obese rats resuming a chow diet [545]. However, we only observed a partial restoration in the potentiating effect of leptin. This may represent either a new level of leptin sensitivity or a transient effect with restoration to normal after a longer period back on the standard chow diet. The mechanisms responsible for the varying levels of potentiation of mucosal receptors by leptin still need to be elucidated, but we speculate that it may, at least, involve differential expression of the LepR as we observed a 99% reduction in LepR mRNA in mucosal afferents from HFD mice, and a 68% reduction of LepR mRNA in the RFD mice. It remains to be determined whether these changes at the transcript level, reflect changes in functional protein at the cell surface.

Similar to the effect of leptin on mucosal receptors, the RFD mice exhibited a partial restoration of the effect of leptin on tension receptors to the lean phenotype. Previously we have shown that LepR expression in muscular vagal afferents is unchanged in obese mice [496], however changes in LepR protein levels have been observed in cultured Caco-2 cells in the absence of any changes in mRNA [515] and it remains to be determined whether such a change in LepR protein exists in these afferents. Alternatively there may be a change in the ability for leptin to activate a downstream signalling protein,

which previously has been suggested to involve activation of the large conductance calcium activated potassium channel [496].

It has been demonstrated in humans that following diet-induced weight loss there are persistent long term changes to gut peptides, with increased levels of the orexigenic peptide ghrelin, and decreased levels of the anorexigenic peptides, cholecystokinin, glucagon-like peptide 1 and peptide YY present one year later [78]. The lack of adaptation of gastric tension receptors upon return to a normal diet could therefore exacerbate the situation making it more difficult to lose weight. Whether the reduced mechanosensitivity of gastric tension receptors are also part of the mechanism favouring weight regain after diet-induced weight loss in obese humans and the extent to which the remarkable efficacy of bariatric surgery, even in individuals with hypothalamic obesity [546, 547], depends on circumventing this decrease, remains to be determined.

In conclusion we have established that changes in regards to leptin's vagal afferent modulatory action are not fully reversible by placing obese mice on a standard chow diet for a period of 12 weeks. Furthermore, they continue to exhibit decreased response to stretch, which could indicate a mechanism that promotes the over consumption of food in an attempt to maintain the obese state and thus combat successful weight loss. Further studies including a longer time course and pair-feeding experiments are required to determine whether the observed changes are driven by the macronutrient content of the

diet, the inducement of obesity, or a combination of the two, as well the timing of the onset of the irreversibility, and potential for reversibility over time.

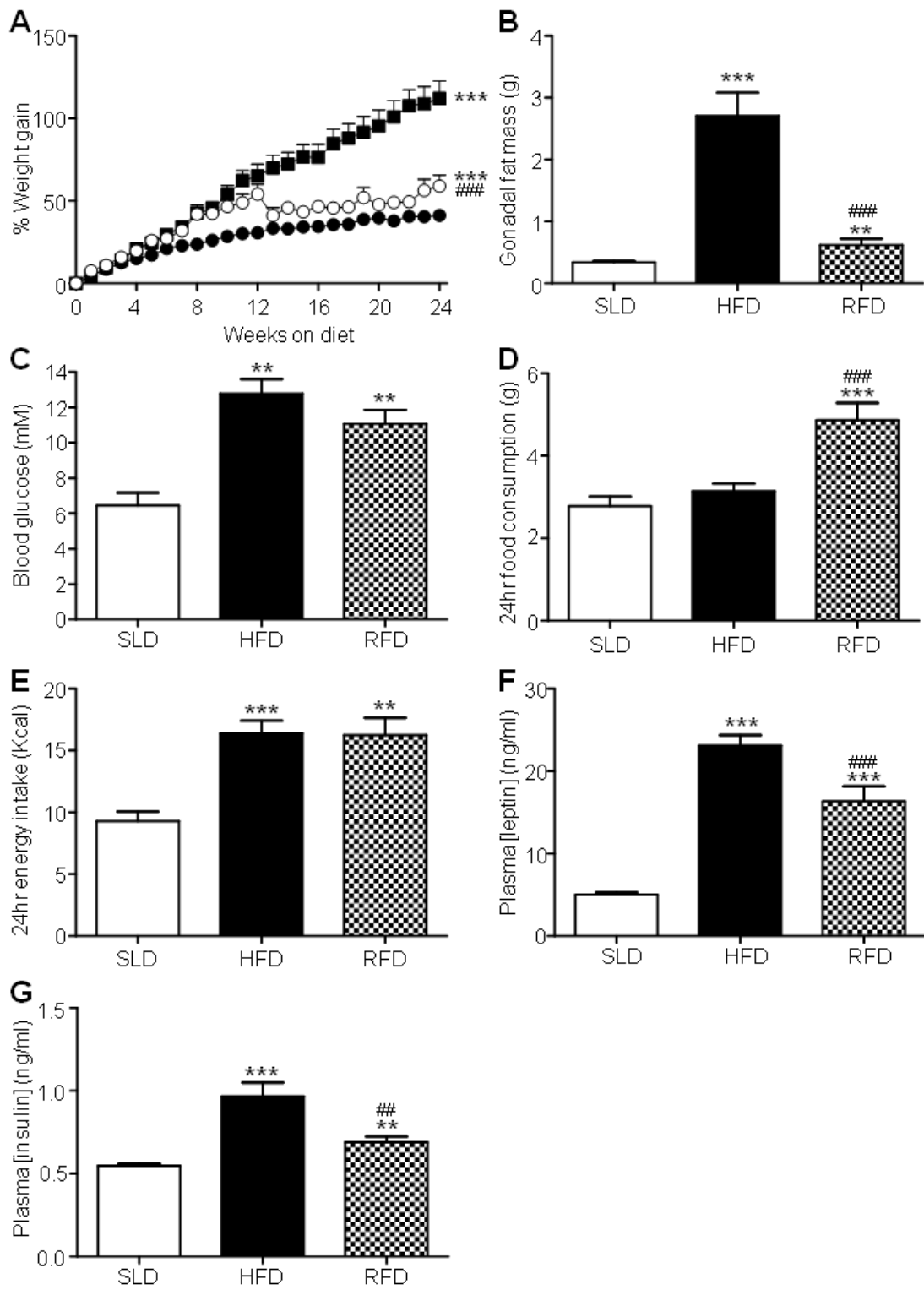


Figure 5.1: Diet dependent changes to mouse body parameters. (A) The weight gain of mice that were either fed a chow diet for 24 weeks (SLD, N=16) (●), a high fat diet for 24 weeks (HFD, N= 16) (■) or a HFD for 12 weeks followed by 12 weeks on a chow diet (RFD, N=14) (○). The gonadal fat deposit weight (B), blood glucose levels (C), 24 hour food consumption (D), 24 hour energy consumption (E), plasma leptin (F) and insulin (G) concentration of SLD, HFD & RFD mice at the end of the 24 week diet regime. ***p<0.001 vs. SLD, ** p<0.01 vs. SLD, ### p<0.001 vs. HFD, ## p<0.01 vs. HFD.

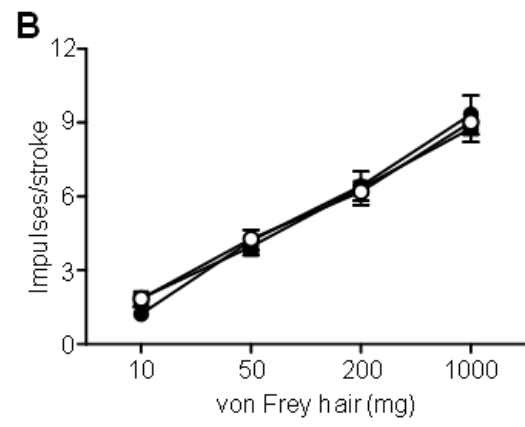
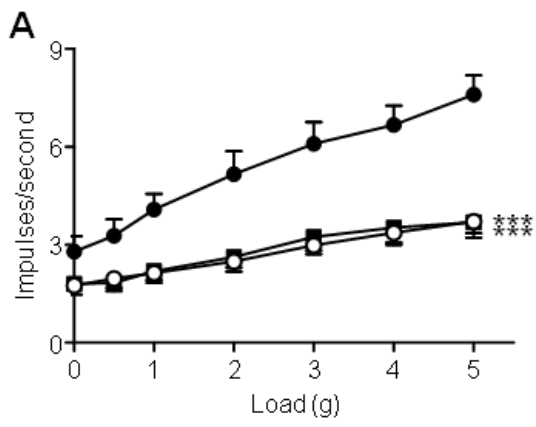


Figure 5.2: Selective high fat diet suppression of gastric tension receptor mechanosensitivity is maintained after chow diet feeding. Stimulus response functions of tension-sensitive (A) and mucosal (B) gastric vagal afferents from mice fed a SLD (●, A: n=16 B: n=18), HFD (■, A: n=25, B: n=29) or RFD (○ A: n=18, B: n=22). *** p<0.001 vs. SLD.

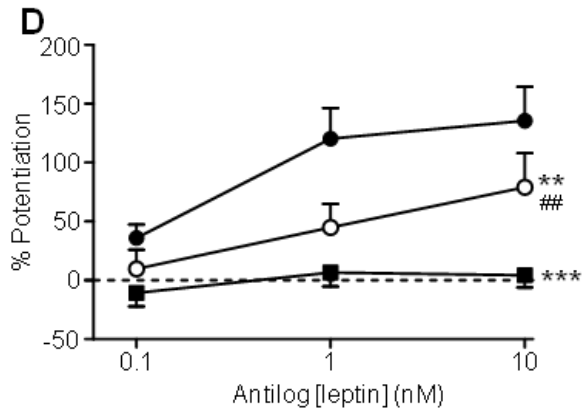
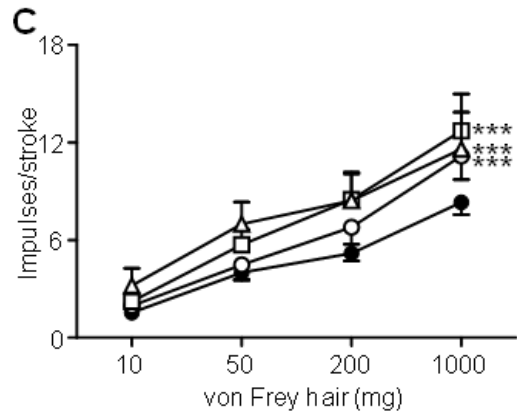
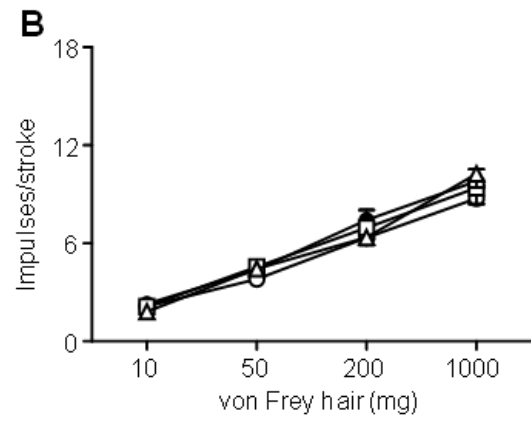
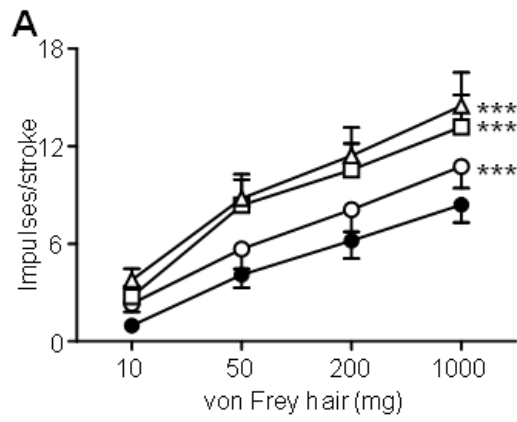


Figure 5.3: Mucosal receptor sensitivity to leptin is partially restored upon reverting to a chow diet. The responses of gastric mucosal receptors to stroking with calibrated von Frey hairs (10-1000mg) in the absence (●) and presence of leptin 0.1 (○), 1 (□) and 10nM (Δ) from mice fed either a SLD for 24 weeks (A, N=8), HFD for 24 weeks (B, N=9) or a HFD for 12 weeks followed by 12 weeks of SLD (C, N=6).*** p<0.001 vs. afferents prior to leptin exposure. (D) The effect of leptin on the response to mucosal stroking with a 50mg von Frey hair in SLD (●), HFD (■) and RFD (○) mice. Diet significantly modulated the potentiating ability of leptin. *** p<0.001 vs. SLD, ** p<0.01 vs. SLD, ## p<0.01 vs. HFD. Original recordings of a mucosal receptor in response to 50mg stroking before (i) and after addition of leptin 10nM (ii) from SLD (E), HFD (F) and RFD (G) mice.

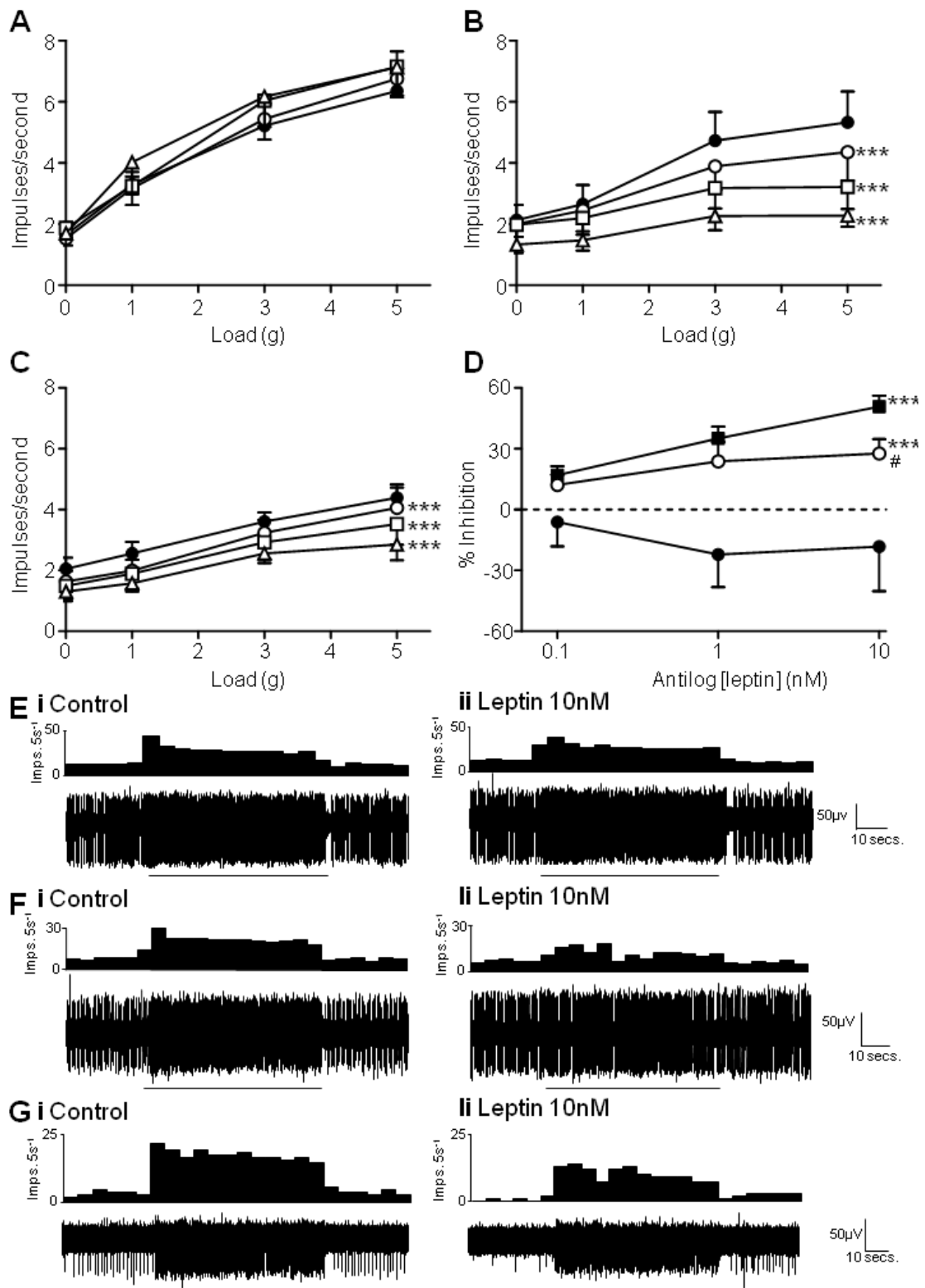


Figure 5.4: Tension receptor inhibition by leptin is reduced by subsequent chow feeding. The responses of gastric tension receptors to circular stretch (1-5g) in the absence (●) and presence of leptin 0.1 (○), 1 (□) and 10nM (Δ) from mice fed either a SLD for 24 weeks (A, N=8), HFD for 24 weeks (B, N=9) or a HFD for 12 weeks followed by 12 weeks of SLD (C, N=10). *** $p < 0.001$ vs. afferents prior to leptin exposure. (D) The effect of leptin on the response to 3g circular tension in SLD (●), HFD (■) and RFD (○) mice. Diet significantly modulated the inhibiting ability of leptin. *** $p < 0.001$ vs. SLD, # $p < 0.05$ vs. HFD. Original recordings of a tension receptor in response to 3g tension before (i) and after addition of leptin 10nM (ii) from SLD (E), HFD (F) and RFD (G) mice.

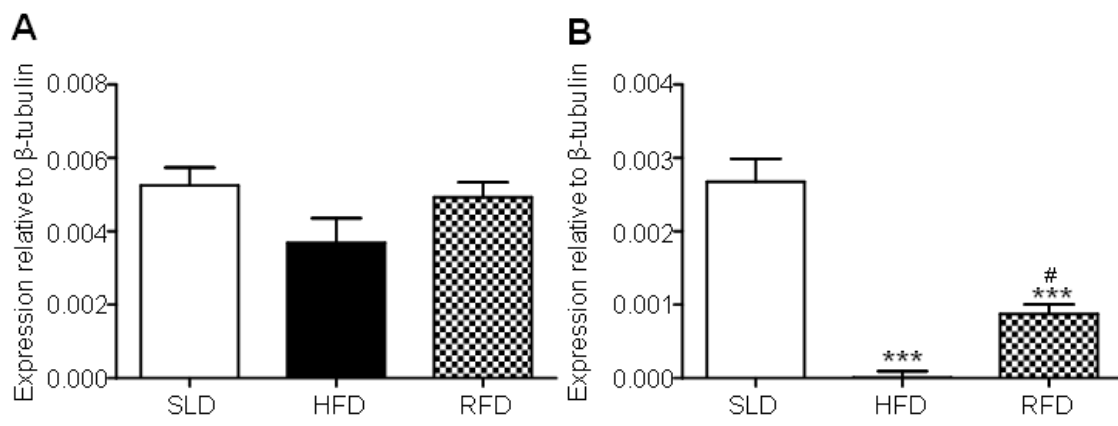


Figure 5.5: Diet induced changes in leptin receptor mRNA expression in nodose ganglia and mucosal afferent cell bodies. Leptin receptor mRNA in whole nodose ganglia was no different in any of the groups of mice (all groups N=8). In mucosal traced afferents leptin receptor mRNA was reduced in afferents from HFD and RFD mice (N=4, all groups). *** $p < 0.001$ vs. SLD, # $p < 0.05$ vs. HFD.

CHAPTER 6: CONCLUSIONS

This thesis highlights specific mechanisms that may underlie peripheral satiety signalling from the molecular basis of receptor regulation through to changes observed in live animals in terms of feeding behaviour. As a whole these studies demonstrate that the endogenous gastric peptides, leptin and ghrelin, have distinct effects on specific populations of mechanosensitive gastric vagal afferents, which are consistent with their previously observed general effects on feeding and vagal afferent modulation [301, 326, 355, 548]. Previously, it has been established that the expression profile of appetite regulatory peptides and their receptors, on rodent vagal afferents, is dependent on fed status [368, 369, 424, 452, 453, 460, 461]. This thesis now demonstrates that the ability of mechanosensitive vagal afferents to respond to mechanical stimuli and the endogenous peptides leptin and ghrelin is also dependent on fed status.

In lean mice the effect of ghrelin on vagal afferent mechanosensitivity is consistent with previous observations [301, 326, 355]. The opposing effects of leptin and ghrelin on gastric vagal afferent mechanosensitivity in lean mice may play a role in their respective anorexigenic and orexigenic effects. However, the maintenance of an obese state depends upon a sustained increase in food intake, which would require a shift in food intake signals away from anorexigenic and towards orexigenic. Consistent with this idea is the orexigenic effect of ghrelin potentially being amplified by gaining an inhibitory effect on mucosal afferents, which is not seen in lean mice, and leptin losing its potentiating and possibly anorexigenic effect and gaining an inhibitory and potentially orexigenic effect. The potential dysfunction of peripheral satiety

signalling observed in the electrophysiological data was complimented by equally dramatic changes in terms of receptor transcript regulation with gut peptides, ghrelin and NPW causing decreases in anorexigenic leptin and CCK receptor in vagal afferent cell bodies from obese mice, which could cause a further suppression of anorexigenic signals and increase food consumption. It is important to remember that this study only examined mRNA levels which, as demonstrated previously, does not necessarily translate to changes in the amount of functional protein at the cell surface [549]. However, these adaptations may permit increased food intake to occur to protect a new higher level of adiposity, if reflected at the functional protein level.

The electrophysiological studies revealed that the changes observed in obesity in terms of baseline mechanosensitivity and the effects leptin and ghrelin on vagal afferents were the same as observed in acutely food restricted mice. This appears counterintuitive as the two states are fundamentally opposite, one is a lack of food availability and energy stores, the other is an excess of energy stores in the presence of food availability. However, this may point to a potential mechanism to increase food intake to the higher level necessary to maintain the obese state just as a food deprived animal would want to consume extra food to restore any lost adiposity.

Whilst it is yet to be determined whether the observed changes in leptin and ghrelin on gastric vagal afferents in obesity causes increased orexigenic signalling, there is some evidence which suggests that ghrelin stimulates a greater increase in food intake in obese humans compared to lean ones [360],

which would be consistent with increased orexigenic potency of ghrelin in obesity. Furthermore, obesity is associated with an increased hedonic hunger [550], which as discussed earlier are modulated by peripheral neuronal signals (via the NTS and ARC), as well as directly by ghrelin and leptin. It remains to be determined if, similar to gastric vagal afferents, there are altered reward centre responses to leptin and ghrelin in obesity. There are also changes to food patterns associated with obesity including changes to meal size and frequency. Both the change in tension receptor mechanosensitivity and effect of leptin and ghrelin on the gastric vagal afferents from obese mice suggests meal size may be increased, which is consistent with meal pattern analysis in rats [431, 487] and humans [551].

Leptin resistance is believed to occur in the majority of obese individuals [552]. Mouse studies have revealed that this is probably, at least in part, caused by an inability of leptin to activate neurons. This has been demonstrated both centrally, in the ARC [553] and peripherally in the vagus [336]. This thesis confirms the presence of leptin resistance in vagal afferents, but in terms of gastric mechanosensitive vagal afferents, leptin resistance only occurred within mucosal afferents. Tension sensitive gastric vagal afferents did not exhibit leptin resistance in obese mice, but instead leptin exhibited an inhibitory effect on tension receptor mechanosensitivity. This suggests that leptin resistance occurs in a very specific manner in which only subpopulations of nerves are affected. This also suggests that more refined approaches need to be taken to treat obesity. In this instance, trying to increase leptin signalling in obesity through the activation of the leptin

receptor could lead to a reduction in response to gastric distension, which could potentially cause a further unwanted increase in food intake. A more detailed analysis of the role leptin plays in appetite regulation and the plasticity of this system in obesity is needed before the therapeutic potential of this system can be fully determined. Downstream second messenger molecules or effector channels of leptin receptors may offer more suitable and effective therapeutic targets for obesity. In this case inhibiting BKCa channels (which may be responsible for the inhibitory action of leptin) or activating TRPC1 channels (which may be responsible for the potentiating effect of leptin) may prove to be better targets than leptin and its receptor in the treatment of obesity. However, both these channels have functional purposes in a variety of tissue types and locations including smooth muscle [554] and the heart [555]. This suggests that they would need to be specifically targeted in the vagal afferents innervating the stomach to avoid the systemic side effects that have plagued previous pharmacological approaches [556].

Whilst plenty of information exists regarding obesity induced dysfunction of appetite regulatory systems, there is a lack of detailed studies looking at the plasticity of these changes. Weight regain in obese people trying to lose weight is exceptionally common [557-560]. This suggests that after attaining the obese state adaptations occur that favour the maintenance of this obese state [78]. This thesis has identified that the mechanosensitivity of gastric tension receptors is reduced in obesity. This would decrease gastric distension induced satiety signalling and may serve to potentiate or maintain the obese state. In addition, it has been established that these alterations are

not readily reversible. Whilst the only intervention performed was changing the diet this may provide a suitable comparison for the common human weight loss experience. It demonstrates that simply changing dietary composition may not be enough to cause sustained weight loss as persisting obesity induced adaptations, that decrease the satiety signal, may lead to increased food intake and subsequent weight gain even in the absence of an obesogenic diet [78, 558]. This facet of the thesis will need further expansion including looking for the presence of a theoretical point of no return, a point in the development of obesity where the observed obesity induced adaptations may become permanent as previously suggested [561, 562]. Furthermore, other interventions need to be explored including pair feeding to determine whether it is the diet or the adiposity that causes these adaptations. More precise meal analysis needs to be undertaken, as currently the food intake data featured in this thesis used a rather crude method of measurement. The approach used, in this thesis, made it impossible to determine specific eating parameters such as meal size, number, duration, inter-meal duration as well as energy expenditure, all of which are vitally important when looking at weight control, as there are substantially different effects of consuming the same number of calories in a different pattern [563, 564].

Given that currently the most effective treatment for obesity is bariatric surgery, with the roux-en-y gastric bypass (RYGP) being the gold standard of weight loss procedures; this thesis highlights mechanisms that may underlie its efficacy in weight loss. The nature of the gastrotomy in a RYGP means that the bypassed section of the stomach would be left without vagal innervation.

The gastric pouch on the other hand would still retain its innervation meaning that vagal satiety signals could still be transmitted. There would likely be an enhanced response to distension of the stomach by an isovolumetric meal given the substantially reduced size of the gastric pouch, which could overcome the reduced sensitivity of the gastric tension receptors to trigger early satiety. RYGB patients also often exhibit reduced ghrelin secretion [565], which is proposed to be caused by the removal of autonomic input to the ghrelin containing cells of the stomach (the vast majority of which are in the bypassed portion of the stomach) [566]. It would be interesting and worthwhile to examine the responsiveness of gastric vagal afferents to mechanical stimuli, as well as leptin and ghrelin, in a mouse model of the RYGB to determine whether the effects documented in this thesis are reversed or merely counteracted.

Whilst it is well accepted that gastric vagal afferents are involved in the control of food intake [567, 568], they are also involved in the regulation of wider gastrointestinal function [210, 467]. This is highlighted by the fact that they are targeted in the treatment of multiple gastrointestinal disorders including functional dyspepsia and gastro-oesophageal reflux disease [284, 285, 438, 569-572]. It is not known whether there are discrete and identifiable subpopulations of vagal afferents that are responsible for initiating different feedback signals. Thus whilst the studies within this thesis suggest that in obesity leptin and ghrelin may act to mediate an increase in food intake it is possible that their effects on gastric vagal afferents are in fact mediating changes in other functions. Therefore, it is imperative that future studies

determine the behavioural effect of modulating specific populations of gastric vagal mechanosensitive afferents.

Regardless of the limitations of these studies they have identified novel and new effects of previously studied molecules in a common disease. The results highlight possible new specific targets for obesity treatments, as well as providing potential mechanisms and explanations for adaptations that favour weight gain/maintenance when weight loss is desirable, further adding to the ever expanding understanding of obesity.

REFERENCES

- [1] Nguyen T, Lau DCW. The Obesity Epidemic and Its Impact on Hypertension. *Canadian Journal of Cardiology*. 2012;28:326-33.
- [2] Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ, et al. 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. *The Lancet*. 2011;377:557-67.
- [3] Wang YC, McPherson K, Marsh T, Gortmaker SL, Brown M. Health and economic burden of the projected obesity trends in the USA and the UK. *The Lancet*. 2011;378:815-25.
- [4] Ogunbode AM, Ladipo M, Ajayi IO, Fatiregun AA. Obesity: an emerging disease. *Nigerian journal of clinical practice*. 2011;14:390-4.
- [5] Popkin BM. The Nutrition Transition and Obesity in the Developing World. *The Journal of Nutrition*. 2001;131:871S-3S.
- [6] Caballero B. Introduction. *The Journal of Nutrition*. 2001;131:866S-70S.
- [7] Jones COH, White NG. Adiposity in Aboriginal people from Arnhem Land, Australia: variation in degree and distribution associated with age, sex and lifestyle. *Annals of Human Biology*. 1994;21:207-27.
- [8] McGarvey ST. Obesity in Samoans and a perspective on its etiology in Polynesians. *The American Journal of Clinical Nutrition*. 1991;53:1586S-94S.
- [9] Bhatnagar D, Anand IS, Durrington PN, Patel DJ, Wander GS, Mackness MI, et al. Coronary risk factors in people from the Indian subcontinent living in west London and their siblings in India. *Lancet*. 1995;345:405-9.
- [10] Popkin BM, Keyou G, Zhai F, Guo X, Ma H, Zohoori N. The nutrition transition in China: a cross-sectional analysis. *European journal of clinical nutrition*. 1993;47:333-46.
- [11] Murphy NJ, Schraer CD, Thiele MC, Boyko EJ, Bulkow LR, Doty BJ, et al. Dietary Change and Obesity Associated with Glucose Intolerance in Alaska Natives. *Journal of the American Dietetic Association*. 1995;95:676-82.
- [12] Curb JD, Marcus EB. Body fat and obesity in Japanese Americans. *The American Journal of Clinical Nutrition*. 1991;53:1552S-5S.
- [13] Ravussin E, Valencia ME, Esparza J, Bennett PH, Schulz LO. Effects of a Traditional Lifestyle on Obesity in Pima Indians. *Diabetes Care*. 1994;17:1067-74.

- [14] Young LR, Nestle M. The Contribution of Expanding Portion Sizes to the US Obesity Epidemic. *American Journal of Public Health*. 2002;92:246-9.
- [15] Rolls BJ, Roe LS, Meengs JS, Wall DE. Increasing the portion size of a sandwich increases energy intake. *Journal of the American Dietetic Association*. 2004;104:367-72.
- [16] Rolls BJ, Morris EL, Roe LS. Portion size of food affects energy intake in normal-weight and overweight men and women. *The American Journal of Clinical Nutrition*. 2002;76:1207-13.
- [17] Rolls BJ, Engell D, Birch LL. Serving Portion Size Influences 5-Year-Old but Not 3-Year-Old Children's Food Intakes. *Journal of the American Dietetic Association*. 2000;100:232-4.
- [18] Young LR, Nestle M. Expanding portion sizes in the US marketplace: Implications for nutrition counseling. *Journal of the American Dietetic Association*. 2003;103:231-4.
- [19] Nielsen S PBM. PAtterns and trends in food portion sizes, 1977-1998. *JAMA : the journal of the American Medical Association*. 2003;289:450-3.
- [20] Rozin P, Kabnick K, Pete E, Fischler C, Shields C. The Ecology of Eating: Smaller Portion Sizes in France Than in the United States Help Explain the French Paradox. *Psychological Science*. 2003;14:450-4.
- [21] Schneider H, Dietrich ES, Venetz WP. Trends and stabilization up to 2022 in overweight and obesity in Switzerland, comparison to France, UK, US and Australia. *Int J Environ Res Public Health*. 2010;7:460-72.
- [22] Keast DR, Fulgoni VL, 3rd, Nicklas TA, O'Neil CE. Food sources of energy and nutrients among children in the United States: National Health and Nutrition Examination Survey 2003-2006. *Nutrients*. 2013;5:283-301.
- [23] Duffey KJ, Popkin BM. Energy Density, Portion Size, and Eating Occasions: Contributions to Increased Energy Intake in the United States, 1977-2006. *PLoS Med*. 2011;8:e1001050.
- [24] Bell EA, Castellanos VH, Pelkman CL, Thorwart ML, Rolls BJ. Energy density of foods affects energy intake in normal-weight women. *The American Journal of Clinical Nutrition*. 1998;67:412-20.
- [25] Kral TV, Roe LS, Rolls BJ. Combined effects of energy density and portion size on energy intake in women. *The American Journal of Clinical Nutrition*. 2004;79:962-8.
- [26] Milanski M, Arruda AP, Coope A, Ignacio-Souza LM, Nunez CE, Roman EA, et al. Inhibition of Hypothalamic Inflammation Reverses Diet-Induced Insulin Resistance in the Liver. *Diabetes*. 2012;61:1455-62.
- [27] Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, et al. Saturated Fatty Acids Produce an Inflammatory Response Predominantly

through the Activation of TLR4 Signaling in Hypothalamus: Implications for the Pathogenesis of Obesity. *The Journal of Neuroscience*. 2009;29:359-70.

[28] De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, et al. Consumption of a Fat-Rich Diet Activates a Proinflammatory Response and Induces Insulin Resistance in the Hypothalamus. *Endocrinology*. 2005;146:4192-9.

[29] Johnstone AM, Murison SD, Duncan JS, Rance KA, Speakman JR. Factors influencing variation in basal metabolic rate include fat-free mass, fat mass, age, and circulating thyroxine but not sex, circulating leptin, or triiodothyronine. *The American Journal of Clinical Nutrition*. 2005;82:941-8.

[30] Astrup A, Gøtzsche PC, van de Werken K, Ranneries C, Toubro S, Raben A, et al. Meta-analysis of resting metabolic rate in formerly obese subjects. *The American Journal of Clinical Nutrition*. 1999;69:1117-22.

[31] Astrup A, Buemann B, Toubro S, Ranneries C, Raben A. Low resting metabolic rate in subjects predisposed to obesity: a role for thyroid status. *The American Journal of Clinical Nutrition*. 1996;63:879-83.

[32] Vosselman MJ, van Marken Lichtenbelt WD, Schrauwen P. Energy dissipation in brown adipose tissue: From mice to men. *Molecular and cellular endocrinology*. 2013.

[33] Tremblay A, Royer MM, Chaput JP, Doucet E. Adaptive thermogenesis can make a difference in the ability of obese individuals to lose body weight. *International journal of obesity (2005)*. 2012.

[34] French SA, Story M, Jeffery RW. Environmental Influences on Eating and Physical Activity. *Annual Review of Public Health*. 2001;22:309-35.

[35] Webber J. Energy balance in obesity. *Proceedings of the Nutrition Society*. 2003;62:539-43.

[36] Sharma S, Kavuru M. Sleep and Metabolism: An Overview. *International Journal of Endocrinology*. 2010;2010.

[37] López-García E, Faubel R, León-Muñoz L, Zuluaga MC, Banegas JR, Rodríguez-Artalejo F. Sleep duration, general and abdominal obesity, and weight change among the older adult population of Spain. *The American Journal of Clinical Nutrition*. 2008;87:310-6.

[38] Watanabe M, Kikuchi H, Tanaka K, Takahashi M. Association of short sleep duration with weight gain and obesity at 1-year follow-up: a large-scale prospective study. *Sleep*. 2010;33:161-7.

[39] Nedeltcheva AV, Kilkus JM, Imperial J, Schoeller DA, Penev PD. Insufficient Sleep Undermines Dietary Efforts to Reduce Adiposity. *Annals of Internal Medicine*. 2010;153:435-41.

- [40] Booth JN, Bromley LE, Darukhanavala AP, Whitmore HR, Imperial JG, Penev PD. Reduced physical activity in adults at risk for type 2 diabetes who curtail their sleep. *Obesity (Silver Spring, Md)*. 2012;20:278-84.
- [41] St-Onge M-P, Roberts AL, Chen J, Kelleman M, O'Keeffe M, RoyChoudhury A, et al. Short sleep duration increases energy intakes but does not change energy expenditure in normal-weight individuals. *The American Journal of Clinical Nutrition*. 2011;94:410-6.
- [42] Nedeltcheva AV, Kilkus JM, Imperial J, Kasza K, Schoeller DA, Penev PD. Sleep curtailment is accompanied by increased intake of calories from snacks. *The American Journal of Clinical Nutrition*. 2009;89:126-33.
- [43] Schmid SM, Hallschmid M, Jauch-Chara K, Wilms B, Benedict C, Lehnert H, et al. Short-term sleep loss decreases physical activity under free-living conditions but does not increase food intake under time-deprived laboratory conditions in healthy men. *The American Journal of Clinical Nutrition*. 2009;90:1476-82.
- [44] Brondel L, Romer MA, Nougues PM, Touyarou P, Davenne D. Acute partial sleep deprivation increases food intake in healthy men. *The American Journal of Clinical Nutrition*. 2010;91:1550-9.
- [45] Spiegel K, Tasali E, Penev P, Cauter EV. Brief Communication: Sleep Curtailment in Healthy Young Men Is Associated with Decreased Leptin Levels, Elevated Ghrelin Levels, and Increased Hunger and Appetite. *Annals of Internal Medicine*. 2004;141:846-50.
- [46] Magee CA, Huang X-F, Iverson DC, Caputi P. Acute sleep restriction alters neuroendocrine hormones and appetite in healthy male adults. *Sleep and Biological Rhythms*. 2009;7:125-7.
- [47] Spiegel K, Leproult R, Van Cauter E. Impact of sleep debt on metabolic and endocrine function. *The Lancet*. 1999;354:1435-9.
- [48] Buxton OM, Pavlova M, Reid EW, Wang W, Simonson DC, Adler GK. Sleep Restriction for 1 Week Reduces Insulin Sensitivity in Healthy Men. *Diabetes*. 2010;59:2126-33.
- [49] Nedeltcheva AV, Kessler L, Imperial J, Penev PD. Exposure to Recurrent Sleep Restriction in the Setting of High Caloric Intake and Physical Inactivity Results in Increased Insulin Resistance and Reduced Glucose Tolerance. *Journal of Clinical Endocrinology & Metabolism*. 2009;94:3242-50.
- [50] Yaggi HK, Araujo AB, McKinlay JB. Sleep Duration as a Risk Factor for the Development of Type 2 Diabetes. *Diabetes Care*. 2006;29:657-61.
- [51] Müssig K, Remer T, Maser-Gluth C. Brief review: Glucocorticoid excretion in obesity. *The Journal of Steroid Biochemistry and Molecular Biology*. 2010;121:589-93.

- [52] Pi-Sunyer FX. The obesity epidemic: pathophysiology and consequences of obesity. *Obesity research*. 2002;10 Suppl 2:97S-104S.
- [53] Herrera B, Lindgren C. The Genetics of Obesity. *Curr Diab Rep*. 2010;10:498-505.
- [54] Bouchard C, Tremblay A, Després J-P, Nadeau A, Lupien PJ, Thériault G, et al. The Response to Long-Term Overfeeding in Identical Twins. *New England Journal of Medicine*. 1990;322:1477-82.
- [55] Stunkard AJ, Sørensen TIA, Hanis C, Teasdale TW, Chakraborty R, Schull WJ, et al. An Adoption Study of Human Obesity. *New England Journal of Medicine*. 1986;314:193-8.
- [56] 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.
- [57] Drel VR, Mashtalir N, Ilnytska O, Shin J, Li F, Lyzogubov VV, et al. The Leptin-Deficient (ob/ob) Mouse: A New Animal Model of Peripheral Neuropathy of Type 2 Diabetes and Obesity. *Diabetes*. 2006;55:3335-43.
- [58] Lowell BB, V SS, Hamann A, Lawitts JA, Himms-Hagen J, Boyer BB, et al. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature*. 1993;366:740-2.
- [59] Strobel A, Issad T, Camoin L, Ozata M, Strosberg AD. A leptin missense mutation associated with hypogonadism and morbid obesity. *Nature genetics*. 1998;18:213-5.
- [60] Montague CT, Farooqi IS, Whitehead IP, Soos MA, Rau H, Wareham NJ, et al. Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature*. 1997;387:903-8.
- [61] Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, et al. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature*. 1998;392:398-401.
- [62] Krude H, Grüters A. Implications of Proopiomelanocortin (POMC) Mutations in Humans: The POMC Deficiency Syndrome. *Trends in Endocrinology & Metabolism*. 2000;11:15-22.
- [63] Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S. A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nature genetics*. 1998;20:111-2.
- [64] Scheermeyer E. Prader-Willi syndrome Care of adults in general practice. *Australian Family Physician*. 2013;42:51-4.
- [65] Katsanis N, Lupski JR, Beales PL. Exploring the molecular basis of Bardet–Biedl syndrome. *Human Molecular Genetics*. 2001;10:2293-9.

- [66] Bettini V, Maffei P, Pagano C, Romano S, Milan G, Favaretto F, et al. The progression from obesity to type 2 diabetes in Alström syndrome. *Pediatric Diabetes*. 2012;13:59-67.
- [67] Goldstone AP, Beales PL. Genetic obesity syndromes. *Frontiers of hormone research*. 2008;36:37-60.
- [68] Wadden TA, Frey DL. A multicenter evaluation of a proprietary weight loss program for the treatment of marked obesity: A five-year follow-up. *International Journal of Eating Disorders*. 1997;22:203-12.
- [69] Simpson SJ, Raubenheimer D. Obesity: the protein leverage hypothesis. *Obesity Reviews*. 2005;6:133-42.
- [70] Griffioen-Roose S, Mars M, Siebelink E, Finlayson G, Tomé D, de Graaf C. Protein status elicits compensatory changes in food intake and food preferences. *The American Journal of Clinical Nutrition*. 2012;95:32-8.
- [71] Gosby AK, Conigrave AD, Lau NS, Iglesias MA, Hall RM, Jebb SA, et al. Testing Protein Leverage in Lean Humans: A Randomised Controlled Experimental Study. *PloS one*. 2011;6:e25929.
- [72] de Castro JM. Behavioral genetics of food intake regulation in free-living humans. *Nutrition*. 1999;15:550-4.
- [73] Kennedy GC. The Role of Depot Fat in the Hypothalamic Control of Food Intake in the Rat. *Proceedings of the Royal Society of London Series B, Biological Sciences*. 1953;140:578-92.
- [74] Mrosovsky N. Body fat: What is regulated? *Physiology & behavior*. 1986;38:407-14.
- [75] Keesey RE, Hirvonen MD. Body Weight Set-Points: Determination and Adjustment. *The Journal of Nutrition*. 1997;127:1875S-83S.
- [76] Hu FB, Chen C, Wang B, Stampfer MJ, Xu X. Leptin concentrations in relation to overall adiposity, fat distribution, and blood pressure in a rural Chinese population. *Int J Obes Relat Metab Disord*. 2001;25:121.
- [77] Gray DS, Fislser JS, Bray GA. Effects of repeated weight loss and regain on body composition in obese rats. *The American Journal of Clinical Nutrition*. 1988;47:393-9.
- [78] Sumithran P, Prendergast LA, Delbridge E, Purcell K, Shulkes A, Kriketos A, et al. Long-Term Persistence of Hormonal Adaptations to Weight Loss. *New England Journal of Medicine*. 2011;365:1597-604.
- [79] Crujeiras AB, Goyenechea E, Abete I, Lage M, Carreira MC, Martínez JA, et al. Weight Regain after a Diet-Induced Loss Is Predicted by Higher Baseline Leptin and Lower Ghrelin Plasma Levels. *Journal of Clinical Endocrinology & Metabolism*. 2010;95:5037-44.

- [80] Holm JC, Gomborg M, Ward L, Ibsen KK, Gammeltoft S, Sørensen TIA, et al. Longitudinal Analysis of Leptin Variation during Weight Regain after Weight Loss in Obese Children. *Obesity Facts*. 2009;2:243-8.
- [81] Mars M, de Graaf C, de Groot CP, van Rossum CT, Kok FJ. Fasting leptin and appetite responses induced by a 4-day 65%-energy-restricted diet. *International journal of obesity (2005)*. 2006;30:122-8.
- [82] Wyrwicka W, Dobrzecka C. Relationship between feeding and satiation centers of the hypothalamus. *Science*. 1960;132:805-6.
- [83] Hollifield G, Parson W. Studies of the Satiety Response in Mice. *The Journal of Clinical Investigation*. 1957;36:1638-41.
- [84] Li A-J, Dinh TT, Ritter S. Hyperphagia and obesity produced by arcuate injection of NPY-saporin do not require upregulation of lateral hypothalamic orexigenic peptide genes. *Peptides*. 2008;29:1732-9.
- [85] Baldwin BA. Neural and hormonal mechanisms regulating food intake. *The Proceedings of the Nutrition Society*. 1985;44:303-11.
- [86] Bellinger LL, Bernardis LL. The dorsomedial hypothalamic nucleus and its role in ingestive behavior and body weight regulation: Lessons learned from lesioning studies. *Physiology & behavior*. 2002;76:431-42.
- [87] Anand BK, Brobeck JR. Localization of a "Feeding Center" in the Hypothalamus of the Rat. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY)*. 1951;77:323-5.
- [88] Debons AF, Silver L, Cronkite EP, Johnson HA, Brecher G, Tenzer D, et al. Localization of gold in mouse brain in relation to gold thioglucose obesity. *The American journal of physiology*. 1962;202:743-50.
- [89] Peruzzo B, Pastor FE, Blázquez JL, Schöbitz K, Peláez B, Amat P, et al. A second look at the barriers of the medial basal hypothalamus. *Experimental Brain Research*. 2000;132:10-26.
- [90] Nonaka N, Shioda S, Niehoff ML, Banks WA. Characterization of Blood-Brain Barrier Permeability to PYY3-36 in the Mouse. *Journal of Pharmacology and Experimental Therapeutics*. 2003;306:948-53.
- [91] Kastin A, Akerstrom V, Pan W. Interactions of glucagon-like peptide-1 (GLP-1) with the blood-brain barrier. *Journal of Molecular Neuroscience*. 2002;18:7-14.
- [92] Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM. Leptin enters the brain by a saturable system independent of insulin. *Peptides*. 1996;17:305-11.
- [93] Woods SC, Seeley RJ, Porte D, Schwartz MW. Signals That Regulate Food Intake and Energy Homeostasis. *Science*. 1998;280:1378-83.

- [94] Marsh DJ, Hollopeter G, Kafer KE, Palmiter RD. Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nature medicine*. 1998;4:718-21.
- [95] Clark JT, Kalra PS, Crowley WR, Kalra SP. Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. *Endocrinology*. 1984;115:427-9.
- [96] Légrádi G, Lechan RM. The Arcuate Nucleus Is the Major Source for Neuropeptide Y-Innervation of Thyrotropin-Releasing Hormone Neurons in the Hypothalamic Paraventricular Nucleus. *Endocrinology*. 1998;139:3262-70.
- [97] Bai FL, Yamano M, Shiotani Y, Emson PC, Smith AD, Powell JF, et al. An arcuate-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. *Brain Research*. 1985;331:172-5.
- [98] Csiffáry A, Görcs TJ, Palkovits M. Neuropeptide Y innervation of ACTH-immunoreactive neurons in the arcuate nucleus of rats: a correlated light and electron microscopic double immunolabeling study. *Brain Research*. 1990;506:215-22.
- [99] Cowley MA, Smart JL, Rubinstein M, Cerdán MG, Diano S, Horvath TL, et al. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature*. 2001;411:480-4.
- [100] Ellacott KLJ, Halatchev IG, Cone RD. Characterization of Leptin-Responsive Neurons in the Caudal Brainstem. *Endocrinology*. 2006;147:3190-5.
- [101] Pritchard L, Turnbull A, White A. Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. *Journal of Endocrinology*. 2002;172:411-21.
- [102] Rossi M, Kim MS, Morgan DGA, Small CJ, Edwards CMB, Sunter D, et al. A C-Terminal Fragment of Agouti-Related Protein Increases Feeding and Antagonizes the Effect of Alpha-Melanocyte Stimulating Hormone in Vivo. *Endocrinology*. 1998;139:4428-31.
- [103] Chen YY, Pelletier G. Demonstration of contacts between proopiomelanocortin neurons in the rat hypothalamus. *Neuroscience Letters*. 1983;43:271-6.
- [104] Eskay RL, Giraud P, Oliver C, Brown-Stein MJ. Distribution of alpha-melanocyte-stimulating hormone in the rat brain: evidence that alpha-MSH-containing cells in the arcuate region send projections to extrahypothalamic areas. *Brain Res*. 1979;178:55-67.
- [105] Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, et al. The Distribution and Mechanism of Action of Ghrelin in the CNS Demonstrates a Novel Hypothalamic Circuit Regulating Energy Homeostasis. *Neuron*. 2003;37:649-61.

- [106] Date Y, Shimbara T, Koda S, Toshinai K, Ida T, Murakami N, et al. Peripheral ghrelin transmits orexigenic signals through the noradrenergic pathway from the hindbrain to the hypothalamus. *Cell metabolism*. 2006;4:323-31.
- [107] Xu L, Gao S, Guo F, Sun X. Effect of motilin on gastric distension sensitive neurons in arcuate nucleus and gastric motility in rat. *Neurogastroenterology & Motility*. 2011;23:265-e121.
- [108] Jeanningros R. Modulation of lateral hypothalamic single unit activity by gastric and intestinal distension. *Journal of the autonomic nervous system*. 1984;11:1-11.
- [109] Zhu JN, Guo CL, Li HZ, Wang JJ. Dorsomedial hypothalamic nucleus neurons integrate important peripheral feeding-related signals in rats. *Journal of neuroscience research*. 2007;85:3193-204.
- [110] Ueta Y, Kannan H, Yamashita H. Gastric afferents to the paraventricular nucleus in the rat. *Experimental Brain Research*. 1991;84:487-94.
- [111] Mazda T, Yamamoto H, Fujimura M, Fujimiya M. Gastric distension-induced release of 5-HT stimulates c-fos expression in specific brain nuclei via 5-HT₃ receptors in conscious rats. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2004;287:G228-G35.
- [112] Singru PS, Wittmann G, Farkas E, Zséli G, Fekete C, Lechan RM. Refeeding-Activated Glutamatergic Neurons in the Hypothalamic Paraventricular Nucleus (PVN) Mediate Effects of Melanocortin Signaling in the Nucleus Tractus Solitarius (NTS). *Endocrinology*. 2012;153:3804-14.
- [113] Zheng H, Patterson LM, Phifer CB, Berthoud H-R. Brain stem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2005;289:R247-R58.
- [114] Barsh GS, Schwartz MW. Genetic approaches to studying energy balance: perception and integration. *Nature reviews Genetics*. 2002;3:589-600.
- [115] Yoshida M, Yokoo H, Mizoguchi K, Kawahara H, Tsuda A, Nishikawa T, et al. Eating and drinking cause increased dopamine release in the nucleus accumbens and ventral tegmental area in the rat: Measurement by in vivo microdialysis. *Neuroscience Letters*. 1992;139:73-6.
- [116] Dahan L, Astier B, Vautrelle N, Urbain N, Kocsis B, Chouvet G. Prominent burst firing of dopaminergic neurons in the ventral tegmental area during paradoxical sleep. *Neuropsychopharmacology*. 2007;32:1232-41.
- [117] Nishino H, Ono T, Muramoto K, Fukuda M, Sasaki K. Neuronal activity in the ventral tegmental area (VTA) during motivated bar press feeding in the monkey. *Brain Research*. 1987;413:302-13.

- [118] Wise RA. Addictive drugs and brain stimulation reward. Annual review of neuroscience. 1996;19:319-40.
- [119] Stratford TR, Kelley AE. Evidence of a Functional Relationship between the Nucleus Accumbens Shell and Lateral Hypothalamus Subserving the Control of Feeding Behavior. The Journal of Neuroscience. 1999;19:11040-8.
- [120] Alhadeff AL, Rupprecht LE, Hayes MR. GLP-1 Neurons in the Nucleus of the Solitary Tract Project Directly to the Ventral Tegmental Area and Nucleus Accumbens to Control for Food Intake. Endocrinology. 2012;153:647-58.
- [121] Manta S, El Mansari M, Debonnel G, Blier P. Electrophysiological and neurochemical effects of long-term vagus nerve stimulation on the rat monoaminergic systems. The International Journal of Neuropsychopharmacology. 2013;16:459-70.
- [122] Ziomber A, Thor P, Krygowska-Wajs A, Zalecki T, Moskala M, Romanska I, et al. Chronic impairment of the vagus nerve function leads to inhibition of dopamine but not serotonin neurons in rat brain structures. Pharmacological reports : PR. 2012;64:1359-67.
- [123] Tomasi D, Wang G-J, Wang R, Backus W, Geliebter A, Telang F, et al. Association of Body Mass and Brain Activation during Gastric Distention: Implications for Obesity. PloS one. 2009;4:e6847.
- [124] Epstein LH, Leddy JJ, Temple JL, Faith MS. Food reinforcement and eating: a multilevel analysis. Psychological bulletin. 2007;133:884-906.
- [125] Oswald KD, Murdaugh DL, King VL, Boggiano MM. Motivation for palatable food despite consequences in an animal model of binge eating. International Journal of Eating Disorders. 2011;44:203-11.
- [126] Goldfield GS, Lumb AB, Colapinto CK. Relative reinforcing value of energy-dense snack foods in overweight and obese adults. Canadian journal of dietetic practice and research : a publication of Dietitians of Canada = Revue canadienne de la pratique et de la recherche en dietetique : une publication des Dietetistes du Canada. 2011;72:170-4.
- [127] Stice E, Spoor S, Bohon C, Veldhuizen MG, Small DM. Relation of reward from food intake and anticipated food intake to obesity: a functional magnetic resonance imaging study. Journal of abnormal psychology. 2008;117:924-35.
- [128] Geiger BM, Behr GG, Frank LE, Caldera-Siu AD, Beinfeld MC, Kokkotou EG, et al. Evidence for defective mesolimbic dopamine exocytosis in obesity-prone rats. The FASEB Journal. 2008;22:2740-6.
- [129] Volkow ND, Wang G-J, Telang F, Fowler JS, Thanos PK, Logan J, et al. Low dopamine striatal D2 receptors are associated with prefrontal metabolism in obese subjects: Possible contributing factors. NeuroImage. 2008;42:1537-43.

- [130] Papp M, Bal A. Separation of the motivational and motor consequences of 6-hydroxydopamine lesions of the mesolimbic or nigrostriatal system in rats. *Behavioural brain research*. 1987;23:221-9.
- [131] Berridge KC, Valenstein ES. What psychological process mediates feeding evoked by electrical stimulation of the lateral hypothalamus? *Behavioral neuroscience*. 1991;105:3-14.
- [132] Davis JF, Tracy AL, Schurdak JD, Tschop MH, Lipton JW, Clegg DJ, et al. Exposure to elevated levels of dietary fat attenuates psychostimulant reward and mesolimbic dopamine turnover in the rat. *Behavioral neuroscience*. 2008;122:1257-63.
- [133] Zhou Q-Y, Palmiter RD. Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic. *Cell*. 1995;83:1197-209.
- [134] Kobayashi M, Iaccarino C, Saiardi A, Heidt V, Bozzi Y, Picetti R, et al. Simultaneous absence of dopamine D1 and D2 receptor-mediated signaling is lethal in mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:11465-70.
- [135] Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK. Expression of ghrelin receptor mRNA in the rat and the mouse brain. *The Journal of Comparative Neurology*. 2006;494:528-48.
- [136] Jerlhag E, Janson AC, Waters S, Engel JA. Concomitant Release of Ventral Tegmental Acetylcholine and Accumbal Dopamine by Ghrelin in Rats. *PloS one*. 2012;7:e49557.
- [137] Jerlhag E, Egecioglu E, Landgren S, Salomé N, Heilig M, Moechars D, et al. Requirement of central ghrelin signaling for alcohol reward. *Proceedings of the National Academy of Sciences*. 2009;106:11318-23.
- [138] Jerlhag E, Egecioglu E, Dickson S, Engel J. Ghrelin receptor antagonism attenuates cocaine- and amphetamine-induced locomotor stimulation, accumbal dopamine release, and conditioned place preference. *Psychopharmacology*. 2010;211:415-22.
- [139] Egecioglu E, Jerlhag E, Salomé N, Skibicka KP, Haage D, Bohlooly-Y M, et al. Ghrelin increases intake of rewarding food in rodents. *Addiction Biology*. 2010;15:304-11.
- [140] Naleid AM, Grace MK, Cummings DE, Levine AS. Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. *Peptides*. 2005;26:2274-9.
- [141] Dickson SL, Hrabovszky E, Hansson C, Jerlhag E, Alvarez-Crespo M, Skibicka KP, et al. Blockade of central nicotine acetylcholine receptor signaling attenuate ghrelin-induced food intake in rodents. *Neuroscience*. 2010;171:1180-6.

- [142] Szczypka MS, Rainey MA, Palmiter RD. Dopamine is required for hyperphagia in Lep(ob/ob) mice. *Nature genetics*. 2000;25:102-4.
- [143] Figlewicz DP, Higgins MS, Ng-Evans SB, Havel PJ. Leptin reverses sucrose-conditioned place preference in food-restricted rats. *Physiology & behavior*. 2001;73:229-34.
- [144] Hommel JD, Trinko R, Sears RM, Georgescu D, Liu Z-W, Gao X-B, et al. Leptin Receptor Signaling in Midbrain Dopamine Neurons Regulates Feeding. *Neuron*. 2006;51:801-10.
- [145] Krügel U, Schraft T, Kittner H, Kiess W, Illes P. Basal and feeding-evoked dopamine release in the rat nucleus accumbens is depressed by leptin. *European Journal of Pharmacology*. 2003;482:185-7.
- [146] Flier JS. Obesity Wars: Molecular Progress Confronts an Expanding Epidemic. *Cell*. 2004;116:337-50.
- [147] Ferris WF, Crowther NJ. Once fat was fat and that was that: our changing perspectives on adipose tissue. *Cardiovascular journal of Africa*. 2011;22:147-54.
- [148] Kershaw EE, Flier JS. Adipose Tissue as an Endocrine Organ. *Journal of Clinical Endocrinology & Metabolism*. 2004;89:2548-56.
- [149] Maffei M, Fei H, Lee GH, Dani C, Leroy P, Zhang Y, et al. Increased expression in adipocytes of ob RNA in mice with lesions of the hypothalamus and with mutations at the db locus. *Proceedings of the National Academy of Sciences*. 1995;92:6957-60.
- [150] Ahima RS, Lazar MA. Adipokines and the Peripheral and Neural Control of Energy Balance. *Molecular Endocrinology*. 2008;22:1023-31.
- [151] Mercer JG. Regulation of appetite and body weight in seasonal mammals. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*. 1998;119:295-303.
- [152] Otis JP, Raybould HE, Carey HV. Cholecystokinin activation of central satiety centers changes seasonally in a mammalian hibernator. *General and Comparative Endocrinology*. 2011;171:269-74.
- [153] Prentice AM, Whitehead RG, Roberts SB, Paul AA. Long-term energy balance in child-bearing Gambian women. *The American Journal of Clinical Nutrition*. 1981;34:2790-9.
- [154] Sabbağ Ç. Seasonal BMI Changes of Rural Women Living in Anatolia. *International Journal of Environmental Research and Public Health*. 2012;9:1159-70.
- [155] Velkoska E, Morris MJ, Burns P, Weisinger RS. Leptin reduces food intake but does not alter weight regain following food deprivation in the rat.

International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2003;27:48-54.

[156] Hope PJ, Wittert GA, Horowitz M, Morley JE. Feeding patterns of *S. crassicaudata* (Marsupialia:Dasyuridae): role of gender, photoperiod, and fat stores. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 1997;272:R78-R83.

[157] Benatti F, Solis M, Artioli G, Montag E, Painelli V, Saito F, et al. Liposuction Induces a Compensatory Increase of Visceral Fat Which Is Effectively Counteracted by Physical Activity: A Randomized Trial. *Journal of Clinical Endocrinology & Metabolism*. 2012;97:2388-95.

[158] Löfgren P, Andersson I, Adolfsson B, Leijonhufvud BM, Hertel K, Hoffstedt J, et al. Long-term prospective and controlled studies demonstrate adipose tissue hypercellularity and relative leptin deficiency in the postobese state. *Journal of Clinical Endocrinology and Metabolism*. 2005;90:6207-13.

[159] Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, et al. Dynamics of fat cell turnover in humans. *Nature*. 2008;453:783-7.

[160] Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between Adipocyte Size and Adipokine Expression and Secretion. *Journal of Clinical Endocrinology & Metabolism*. 2007;92:1023-33.

[161] Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of Lipid Research*. 2005;46:2347-55.

[162] Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. *The American Journal of Clinical Nutrition*. 2006;83:461S-5S.

[163] Lumeng CN, DelProposto JB, Westcott DJ, Saltiel AR. Phenotypic Switching of Adipose Tissue Macrophages With Obesity Is Generated by Spatiotemporal Differences in Macrophage Subtypes. *Diabetes*. 2008;57:3239-46.

[164] Shin MJ, Hyun YJ, Kim OY, Kim JY, Jang Y, Lee JH. Weight loss effect on inflammation and LDL oxidation in metabolically healthy but obese (MHO) individuals: Low inflammation and LDL oxidation in MHO women. *International Journal of Obesity*. 2006;30:1529-34.

[165] Koppaka S, Kehlenbrink S, Carey M, Li W, Sanchez E, Lee D-E, et al. Reduced Adipose Tissue Macrophage Content Is Associated With Improved Insulin Sensitivity in Thiazolidinedione-Treated Diabetic Humans. *Diabetes*. 2013.

- [166] Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of Clinical Investigation*. 2003;112:1821-30.
- [167] Zhang HH, Huang J, Düvel K, Boback B, Wu S, Squillace RM, et al. Insulin Stimulates Adipogenesis through the Akt-TSC2-mTORC1 Pathway. *PLoS one*. 2009;4:e6189.
- [168] Gathercole LL, Morgan SA, Bujalska IJ, Hauton D, Stewart PM, Tomlinson JW. Regulation of Lipogenesis by Glucocorticoids and Insulin in Human Adipose Tissue. *PLoS one*. 2011;6:e26223.
- [169] Choi SM, Tucker DF, Gross DN, Easton RM, DiPilato LM, Dean AS, et al. Insulin Regulates Adipocyte Lipolysis via an Akt-Independent Signaling Pathway. *Molecular and Cellular Biology*. 2010;30:5009-20.
- [170] Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG, et al. Visceral and Subcutaneous Adipose Tissue Volumes Are Cross-Sectionally Related to Markers of Inflammation and Oxidative Stress: The Framingham Heart Study. *Circulation*. 2007;116:1234-41.
- [171] Fain JN, Buehrer B, Bahouth SW, Tichansky DS, Madan AK. Comparison of messenger RNA distribution for 60 proteins in fat cells vs the nonfat cells of human omental adipose tissue. *Metabolism*. 2008;57:1005-15.
- [172] Rao C, Rana SS, Lal A, Kumar M, Behera A, Dahiya D, et al. Large gastric lipoma presenting with GI bleeding. *Gastrointestinal Endoscopy*. 2013;77:512-3.
- [173] John BJ, Abulafi AM, Poullis A, Mendall MA. Chronic subclinical bowel inflammation may explain increased risk of colorectal cancer in obese people. *Gut*. 2007;56:1034-5.
- [174] Speakman JR, Levitsky DA, Allison DB, Bray MS, de Castro JM, Clegg DJ, et al. Set points, settling points and some alternative models: theoretical options to understand how genes and environments combine to regulate body adiposity. *Disease models & mechanisms*. 2011;4:733-45.
- [175] Villanova N, Azpiroz F, Malagelada JR. Gastrogastric reflexes regulating gastric tone and their relationship to perception. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 1997;273:G464-G9.
- [176] Cannon WB, Lieb CW. The receptive relaxation of the stomach. *American Journal of Physiology*. 1911;29:267-73.
- [177] Schwizer W, Steingötter A, Fox M, Zur T, Thumshirn M, Bösigler P, et al. Non-invasive measurement of gastric accommodation in humans. *Gut*. 2002;51:i59-i62.
- [178] Tack J, Piessevaux H, Coulie B, Caenepeel P, Janssens J. Role of impaired gastric accommodation to a meal in functional dyspepsia. *Gastroenterology*. 1998;115:1346-52.

- [179] Takahashi T, Owyang C. Characterization of vagal pathways mediating gastric accommodation reflex in rats. *The Journal of Physiology*. 1997;504:479-88.
- [180] Mearadji, Straathof, Lamers, Masclee. Effect of gastrin on proximal gastric motor function in humans. *Neurogastroenterology & Motility*. 1999;11:449-55.
- [181] Sanders KM, Koh SD, Ro S, Ward SM. Regulation of gastrointestinal motility--insights from smooth muscle biology. *Nature reviews Gastroenterology & hepatology*. 2012;9:633-45.
- [182] Kito Y. The functional role of intramuscular interstitial cells of Cajal in the stomach. *Journal of Smooth Muscle Research*. 2011;47:47-53.
- [183] Janssen P, Vanden Berghe P, Verschueren S, Lehmann A, Depoortere I, Tack J. Review article: the role of gastric motility in the control of food intake. *Alimentary pharmacology & therapeutics*. 2011;33:880-94.
- [184] Croom WJ, Bull LS, Taylor IL. Regulation of Pancreatic Exocrine Secretion in Ruminants: A Review. *The Journal of Nutrition*. 1992;122:191-202.
- [185] Furness JB, Kunze WAA, Clerc N. II. The intestine as a sensory organ: neural, endocrine, and immune responses. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 1999;277:G922-G8.
- [186] Nakamura E, Hasumura M, San Gabriel A, Uneyama H, Torii K. New frontiers in gut nutrient sensor research: luminal glutamate-sensing cells in rat gastric mucosa. *Journal of pharmacological sciences*. 2010;112:13-8.
- [187] Meyer JH, Tabrizi Y, DiMaso N, Hlinka M, Raybould HE. Length of intestinal contact on nutrient-driven satiety. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 1998;275:R1308-R19.
- [188] Powley TL, Phillips RJ. Gastric satiation is volumetric, intestinal satiation is nutritive. *Physiol Behav*. 2004;82:69-74.
- [189] Maljaars PWJ, Peters HPF, Mela DJ, Masclee AAM. Ileal brake: A sensible food target for appetite control. A review. *Physiology & behavior*. 2008;95:271-81.
- [190] Zittel TT, De Giorgio R, Sternini C, Raybould HE. Fos protein expression in the nucleus of the solitary tract in response to intestinal nutrients in awake rats. *Brain Research*. 1994;663:266-70.
- [191] Polak JM, Bloom SR, Rayford PL, Pearse AGE, Buchan AMJ, Thompson JC. Identification of Cholecystokinin-Secreting Cells. *The Lancet*. 1975;306:1016-8.

- [192] Sharara AI, Bouras EP, Misukonis MA, Liddle RA. Evidence for indirect dietary regulation of cholecystokinin release in rats. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 1993;265:G107-G12.
- [193] Cummings DE, Overduin J. Gastrointestinal regulation of food intake. *The Journal of Clinical Investigation*. 2007;117:13-23.
- [194] Rehfeld JF. Immunochemical studies on cholecystokinin. II. Distribution and molecular heterogeneity in the central nervous system and small intestine of man and hog. *Journal of Biological Chemistry*. 1978;253:4022-30.
- [195] Lo C-M, Ma L, Zhang DM, Lee R, Qin A, Liu M, et al. Mechanism of the induction of brain c-Fos-positive neurons by lipid absorption. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2007;292:R268-R73.
- [196] Schwartz GJ, Moran TH. CCK Elicits and Modulates Vagal Afferent Activity Arising from Gastric and Duodenal Sites. *Annals of the New York Academy of Sciences*. 1994;713:121-8.
- [197] Raybould HE, Tache Y. Cholecystokinin inhibits gastric motility and emptying via a capsaicin-sensitive vagal pathway in rats. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 1988;255:G242-G6.
- [198] Raybould H, Gayton R, Dockray G. Mechanisms of action of peripherally administered cholecystokinin octapeptide on brain stem neurons in the rat. *The Journal of Neuroscience*. 1988;8:3018-24.
- [199] Blouet C, Schwartz GJ. Duodenal Lipid Sensing Activates Vagal Afferents to Regulate Non-Shivering Brown Fat Thermogenesis in Rats. *PloS one*. 2012;7:e51898.
- [200] Sutherland K, Young RL, Cooper NJ, Horowitz M, Blackshaw LA. Phenotypic characterization of taste cells of the mouse small intestine. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2007;292:G1420-G8.
- [201] Jang H-J, Kokrashvili Z, Theodorakis MJ, Carlson OD, Kim B-J, Zhou J, et al. Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1. *Proceedings of the National Academy of Sciences*. 2007;104:15069-74.
- [202] Liou AP, Lu X, Sei Y, Zhao X, Pechhold S, Carrero RJ, et al. The G-Protein-Coupled Receptor GPR40 Directly Mediates Long-Chain Fatty Acid-Induced Secretion of Cholecystokinin. *Gastroenterology*. 2011;140:903-12.e4.
- [203] Margolskee RF, Dyer J, Kokrashvili Z, Salmon KSH, Ilegems E, Daly K, et al. T1R3 and gustducin in gut sense sugars to regulate expression of Na⁺-glucose cotransporter 1. *Proceedings of the National Academy of Sciences*. 2007;104:15075-80.

- [204] Glatzle J, Darcel N, Rechs AJ, Kalogeris TJ, Tso P, Raybould HE. Apolipoprotein A-IV stimulates duodenal vagal afferent activity to inhibit gastric motility via a CCK1 pathway. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2004;287:R354-R9.
- [205] Feinle C, Grundy D, Read NW. Effects of duodenal nutrients on sensory and motor responses of the human stomach to distension. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 1997;273:G721-G6.
- [206] Barbera R, Feinle C, Read N. Nutrient-specific modulation of gastric mechanosensitivity in patients with functional dyspepsia. *Digestive diseases and sciences*. 1995;40:1636-41.
- [207] Caldarella MP, Azpiroz F, Malagelada J-R. Selective effects of nutrients on gut sensitivity and reflexes. *Gut*. 2007;56:37-42.
- [208] Phillips RJ, Powley TL. Gastric volume rather than nutrient content inhibits food intake. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 1996;271:R766-R9.
- [209] Deutsch JA, Wang ML. The Stomach as a Site for Rapid Nutrient Reinforcement Sensors. *Science*. 1977;195:89-90.
- [210] Deutsch JA, Young WG, Kalogeris TJ. The stomach signals satiety. *Science*. 1978;201:165-7.
- [211] Deutsch JA, Gonzalez MF. Gastric nutrient content signals satiety. *Behavioral and Neural Biology*. 1980;30:113-6.
- [212] Janssen P, Verschueren S, Tack J. Intra-gastric pressure as a determinant of food intake. *Neurogastroenterology & Motility*. 2012;24:612-e268.
- [213] Höfer D, Püschel B, Drenckhahn D. Taste receptor-like cells in the rat gut identified by expression of alpha-gustducin. *Proceedings of the National Academy of Sciences*. 1996;93:6631-4.
- [214] Hass N, Schwarzenbacher K, Breer H. T1R3 is expressed in brush cells and ghrelin-producing cells of murine stomach. *Cell and tissue research*. 2010;339:493-504.
- [215] Haid D, Widmayer P, Breer H. Nutrient sensing receptors in gastric endocrine cells. *J Mol Hist*. 2011;42:355-64.
- [216] Janssen S, Laermans J, Verhulst P-J, Thijs T, Tack J, Depoortere I. Bitter taste receptors and α -gustducin regulate the secretion of ghrelin with functional effects on food intake and gastric emptying. *Proceedings of the National Academy of Sciences*. 2011;108:2094-9.
- [217] Nakamura E, Hasumura M, Uneyama H, Torii K. Luminal Amino Acid-Sensing Cells in Gastric Mucosa. *Digestion*. 2011;83:13-8.

- [218] Widmayer P, Kuper M, Kramer M, Konigsrainer A, Breer H. Altered expression of gustatory-signaling elements in gastric tissue of morbidly obese patients. *International journal of obesity (2005)*. 2012;36:1353-9.
- [219] Powley TL. Vagal input to the enteric nervous system. *Gut*. 2000;47:iv30-iv2.
- [220] Schubert ML. Gastric secretion. *Current opinion in gastroenterology*. 2002;18:639-49.
- [221] Toumi F, Neunlist M, Cassagnau E, Parois S, Laboisie CL, Galmiche JP, et al. Human submucosal neurones regulate intestinal epithelial cell proliferation: evidence from a novel co-culture model. *Neurogastroenterology & Motility*. 2003;15:239-42.
- [222] Genton L, Kudsk KA. Interactions between the enteric nervous system and the immune system: role of neuropeptides and nutrition. *The American Journal of Surgery*. 2003;186:253-8.
- [223] Waterman SA, Costa M. The role of enteric inhibitory motoneurons in peristalsis in the isolated guinea-pig small intestine. *The Journal of Physiology*. 1994;477:459-68.
- [224] De Giorgio R, Guerrini S, Barbara G, Cremon C, Stanghellini V, Corinaldesi R. New insights into human enteric neuropathies. *Neurogastroenterology & Motility*. 2004;16:143-7.
- [225] Näslund E, Ehrström M, Ma J, Hellström PM, Kirchgessner AL. Localization and effects of orexin on fasting motility in the rat duodenum. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2002;282:G470-G9.
- [226] Sayegh AI, Reeve JR, Jr., Lampley ST, Hart B, Gulley S, Esdaile AR, et al. Role for the enteric nervous system in the regulation of satiety via cholecystokinin-8. *Journal of the American Veterinary Medical Association*. 2005;226:1809-16.
- [227] Sayegh AI, Ritter RC. CCK-A receptor activation induces Fos expression in myenteric neurons of rat small intestine. *Regulatory Peptides*. 2000;88:75-81.
- [228] Schutte IWM, M.A. Akkermans L, Kroese ABA. CCKA and CCKB receptor subtypes both mediate the effects of CCK-8 on myenteric neurons in the guinea-pig ileum. *Journal of the autonomic nervous system*. 1997;67:51-9.
- [229] Sayegh AI, Ritter RC. Vagus nerve participates in CCK-induced Fos expression in hindbrain but not myenteric plexus. *Brain Research*. 2000;878:155-62.
- [230] Kennedy AL, Mawe GM. Duodenal Sensory Neurons Project to Sphincter of Oddi Ganglia in Guinea Pig. *The Journal of Neuroscience*. 1998;18:8065-73.

- [231] Glatzle J, Sternini C, Robin C, Zittel TT, Wong H, Reeve Jr. § JR, et al. Expression of 5-HT₃ receptors in the rat gastrointestinal tract. *Gastroenterology*. 2002;123:217-26.
- [232] Gershon MD, Tack J. The Serotonin Signaling System: From Basic Understanding To Drug Development for Functional GI Disorders. *Gastroenterology*. 2007;132:397-414.
- [233] Raybould HE, Glatzle J, Robin C, Meyer JH, Phan T, Wong H, et al. Expression of 5-HT₃ receptors by extrinsic duodenal afferents contribute to intestinal inhibition of gastric emptying. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2003;284:G367-G72.
- [234] Roosen L, Boesmans W, Dondeyne M, Depoortere I, Tack J, Vanden Berghe P. Specific hunger- and satiety-induced tuning of guinea pig enteric nerve activity. *The Journal of Physiology*. 2012;590:4321-33.
- [235] Grabauskas G, Zhou S-Y, Lu Y, Song I, Owyang C. Essential Elements for Glucosensing by Gastric Vagal Afferents: Immunocytochemistry and Electrophysiology Studies in the Rat. *Endocrinology*. 2013;154:296-307.
- [236] Liu M-t, Seino S, Kirchgessner AL. Identification and Characterization of Glucoreponsive Neurons in the Enteric Nervous System. *The Journal of Neuroscience*. 1999;19:10305-17.
- [237] MacGregor IL, Gueller R, Watts HD, Meyer JH. The effect of acute hyperglycemia on gastric emptying in man. *Gastroenterology*. 1976;70:190-6.
- [238] Covasa M, Grahn J, Ritter RC. Reduced hindbrain and enteric neuronal response to intestinal oleate in rats maintained on high-fat diet. *Autonomic Neuroscience*. 2000;84:8-18.
- [239] Grhkn T, J. Dockray G. Characterization of the peptidergic afferent innervation of the stomach in the rat, mouse and guinea-pig. *Neuroscience*. 1988;25:181-93.
- [240] El Ouazzani T, Mei N. Sensory innervation of the gastro-intestinal junction: new electrophysiological, histological and histochemical data. *Comptes rendus des seances de la Societe de biologie et de ses filiales*. 1978;172:283-8.
- [241] Grundy D. Neuroanatomy of visceral nociception: vagal and splanchnic afferent. *Gut*. 2002;51:i2-i5.
- [242] Zittel TT, Lloyd KCK, Rothenhöfer I, Wong H, Walsh JH, Raybould HE. Calcitonin gene-related peptide and spinal afferents partly mediate postoperative colonic ileus in the rat. *Surgery*. 1998;123:518-27.
- [243] Holzer HH, Raybould HE. Vagal and splanchnic sensory pathways mediate inhibition of gastric motility induced by duodenal distension. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 1992;262:G603-G8.

- [244] Severi C, Jensen RT, Erspamer V, D'Arpino L, Coy DH, Torsoli A, et al. Different receptors mediate the action of bombesin-related peptides on gastric smooth muscle cells. *The American journal of physiology*. 1991;260:G683-90.
- [245] Wada E, Wray S, Key S, Battey J. Comparison of gene expression for two distinct bombesin receptor subtypes in postnatal rat central nervous system. *Molecular and Cellular Neuroscience*. 1992;3:446-60.
- [246] Delle Fave G, Kohn A, De Magistris L, Annibale B, Bruzzone R, Sparvoli C, et al. Effects of bombesin on gastrin and gastric acid secretion in patients with duodenal ulcer. *Gut*. 1983;24:231-5.
- [247] Lieveise RJ, Jansen JB, van de Zwan A, Samson L, Masclee AA, Rovati LC, et al. Bombesin reduces food intake in lean man by a cholecystokinin-independent mechanism. *The Journal of clinical endocrinology and metabolism*. 1993;76:1495-8.
- [248] Denbow DM. Centrally and peripherally administered bombesin decreases food intake in turkeys. *Peptides*. 1989;10:275-9.
- [249] Stuckey JA, Gibbs J, Smith GP. Neural disconnection of gut from brain blocks bombesin-induced satiety. *Peptides*. 1985;6:1249-52.
- [250] Jia YD, Chen X, Tang M, Jiang ZY. Expression of growth hormone secretagogue receptor type 1a in visceral vagal and spinal afferent pathways. *Sheng li xue bao : [Acta physiologica Sinica]*. 2008;60:149-55.
- [251] Ghilardi J, Allen C, Vigna S, McVey D, Mantyh P. Trigeminal and dorsal root ganglion neurons express CCK receptor binding sites in the rat, rabbit, and monkey: possible site of opiate- CCK analgesic interactions. *The Journal of Neuroscience*. 1992;12:4854-66.
- [252] Zittel TT, Reddy SN, Plourde V, Raybould HE. Role of spinal afferents and calcitonin gene-related peptide in the postoperative gastric ileus in anesthetized rats. *Annals of surgery*. 1994;219:79-87.
- [253] Raybould HE. Visceral perception: sensory transduction in visceral afferents and nutrients. *Gut*. 2002;51 Suppl 1:i11-4.
- [254] Li S, Maude-Griffin R, Sun Y, Starkebaum W, Chen JZ. Food Intake and Body Weight Responses to Intermittent vs. Continuous Gastric Electrical Stimulation in Diet-Induced Obese Rats. *Obesity surgery*. 2013;23:71-9.
- [255] Qin C, Chen JD, Zhang J, Foreman RD. Modulatory effects and afferent pathways of gastric electrical stimulation on rat thoracic spinal neurons receiving input from the stomach. *Neuroscience research*. 2007;57:29-39.
- [256] Jones RCW, Xu L, Gebhart GF. The Mechanosensitivity of Mouse Colon Afferent Fibers and Their Sensitization by Inflammatory Mediators Require Transient Receptor Potential Vanilloid 1 and Acid-Sensing Ion Channel 3. *The Journal of Neuroscience*. 2005;25:10981-9.

- [257] Khayutin VM, Baraz LA, Lukoshkova EV, Sonina RS, Chernilovskaya PE. Chemosensitive Spinal Afferents: Thresholds of Specific and Nociceptive Reflexes as Compared with Thresholds of Excitation for Receptors and Axons. In: Iggo A, Ilyinsky OB, editors. Progress in Brain Research: Elsevier; 1976. p. 293-306.
- [258] Brierley SM, Jones lii RCW, Gebhart GF, Blackshaw LA. Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology*. 2004;127:166-78.
- [259] Hughes PA, Brierley SM, Blackshaw LA. Post-inflammatory modification of colonic afferent mechanosensitivity. *Clinical and experimental pharmacology & physiology*. 2009;36:1034-40.
- [260] Ozaki N, Gebhart GF. Characterization of mechanosensitive splanchnic nerve afferent fibers innervating the rat stomach. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2001;281:G1449-G59.
- [261] Qin C, Chandler MJ, Miller KE, Foreman RD. Responses and afferent pathways of C1–C2 spinal neurons to gastric distension in rats. *Autonomic Neuroscience*. 2003;104:128-36.
- [262] Berthoud H-R, Kressel M, Raybould H, Neuhuber W. Vagal sensors in the rat duodenal mucosa: distribution and structure as revealed by in vivo Dil-tracing. *Anatomy and Embryology*. 1995;191:203-12.
- [263] Torvik A. Afferent connections to the sensory trigeminal nuclei, the nucleus of the solitary tract and adjacent structures. An experimental study in the rat. *The Journal of Comparative Neurology*. 1956;106:51-141.
- [264] Kim GR. The Facial Nerve and Gustatory Function. *Yonsei Med J*. 1971;12:49-54.
- [265] Li ET, Anderson GH. A role for vagus nerve in regulation of protein and carbohydrate intake. *American Journal of Physiology - Endocrinology And Metabolism*. 1984;247:E815-E21.
- [266] Treesukosol Y, Blonde GD, Jiang E, Gonzalez D, Smith JC, Spector AC. Necessity of the glossopharyngeal nerve in the maintenance of normal intake and ingestive bout size of corn oil by rats. *American journal of physiology Regulatory, integrative and comparative physiology*. 2010;299:R1050-8.
- [267] Cunningham JT, Mifflin SW, Gould GG, Frazer A. Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by Vagal nerve stimulation. *Neuropsychopharmacology*. 2008;33:1884-95.
- [268] 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.

- [269] Hornby PJ. II. Excitatory amino acid receptors in the brain-gut axis. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2001;280:G1055-G60.
- [270] Browning KN, Travagli RA. Plasticity of vagal brainstem circuits in the control of gastric function. *Neurogastroenterology & Motility*. 2010;22:1154-63.
- [271] Zhou S-Y, Lu Y-X, Yao H, Owyang C. Spatial organization of neurons in the dorsal motor nucleus of the vagus synapsing with intragastric cholinergic and nitric oxide/VIP neurons in the rat. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2008;294:G1201-G9.
- [272] Travagli RA, Hermann GE, Browning KN, Rogers RC. Brainstem circuits regulating gastric function. *Annual review of physiology*. 2006;68:279-305.
- [273] Stearns AT, Balakrishnan A, Radmanesh A, Ashley SW, Rhoads DB, Tavakkolizadeh A. Relative Contributions of Afferent Vagal Fibers to Resistance to Diet-Induced Obesity. *Digestive diseases and sciences*. 2012;57:1281-90.
- [274] Opsahl CA, Powley TL. Body weight and gastric acid secretion in rats with subdiaphragmatic vagotomy and lateral hypothalamic lesions. *J Comp Physiol Psychol*. 1977;91:1284-96.
- [275] Kanno H, Kiyama T, Fujita I, Kato S, Uchida E, Tajiri T. Vagotomy Suppresses Body Weight Gain in a Rat Model of Gastric Banding. *Digestive Surgery*. 2010;27:302-6.
- [276] Andrews PL, Rothwell NJ, Stock MJ. Effects of subdiaphragmatic vagotomy on energy balance and thermogenesis in the rat. *J Physiol*. 1985;362:1-12.
- [277] Czaja K, Burns GA, Ritter RC. Capsaicin-induced neuronal death and proliferation of the primary sensory neurons located in the nodose ganglia of adult rats. *Neuroscience*. 2008;154:621-30.
- [278] Browning KN, Babic T, Holmes GM, Swartz E, Travagli RA. A critical re-evaluation of the specificity of action of perivagal capsaicin. *J Physiol*. 2013;591:1563-80.
- [279] Ritter RC. Gastrointestinal mechanisms of satiation for food. *Physiology & behavior*. 2004;81:249-73.
- [280] Takasugi S, Ueda T, Kurata Y, Kodama M, Ezaki H, Fujh K. Neural and humoral factors influence gastric receptive relaxation in dogs. *The Japanese Journal of Surgery*. 1982;12:208-13.
- [281] Tanaka T, Kendrick ML, Zyromski NJ, Meile T, Sarr MG. Vagal innervation modulates motor pattern but not initiation of canine gastric migrating motor complex. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2001;281:G283-G92.

- [282] Clark CG, Lewin MR, Stagg BH, Wyllie JH. Effect of proximal gastric vagotomy on gastric acid secretion and plasma gastrin. *Gut*. 1973;14:293-9.
- [283] Lorenz DN. Effects of gastric filling and vagotomy on ingestion, nipple attachment, and weight gain by suckling rats. *Developmental Psychobiology*. 1983;16:469-83.
- [284] Laskiewicz J, Krolczyk G, Zurowski D, 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.
- [285] Toouli J, Collins J, Wray N, Billington C, Knudson M, Pulling C, et al. Vagal blocking for obesity control. *Obes Surg*. 2007;17:1043-.
- [286] Berthoud H-R, Lynn PA, Blackshaw LA. Vagal and spinal mechanosensors in the rat stomach and colon have multiple receptive fields. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2001;280:R1371-R81.
- [287] Page AJ, Blackshaw LA. An in vitro study of the properties of vagal afferent fibres innervating the ferret oesophagus and stomach. *J Physiol*. 1998;512:907-16.
- [288] Phillips RJ, Powley TL. Tension and stretch receptors in gastrointestinal smooth muscle: re-evaluating vagal mechanoreceptor electrophysiology. *Brain Research Reviews*. 2000;34:1-26.
- [289] Phillips RJ, Powley TL. Gastric volume detection after selective vagotomies in rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 1998;274:R1626-R38.
- [290] Berthoud H-R, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. *Autonomic Neuroscience*. 2000;85:1-17.
- [291] Zagorodnyuk VP, Chen BN, Brookes SJH. Intraganglionic laminar endings are mechano-transduction sites of vagal tension receptors in the guinea-pig stomach. *J Physiol*. 2001;534:255-68.
- [292] Neuhuber WL, Kressel M, Stark A, Berthoud HR. Vagal efferent and afferent innervation of the rat esophagus as demonstrated by anterograde Dil and DiA tracing: focus on myenteric ganglia. *Journal of the autonomic nervous system*. 1998;70:92-102.
- [293] Berthoud HR, Patterson LM, Neumann F, Neuhuber WL. Distribution and structure of vagal afferent intraganglionic laminar endings (IGLEs) in the rat gastrointestinal tract. *Anatomy and Embryology*. 1997;195:183-91.
- [294] Berthoud HR, Powley TL. Vagal Afferent Innervation of the Rat Fundic Stomach: Morphological Characterization of the Gastric Tension Receptor. *J Comp Neurol*. 1992;319:261-76.

- [295] Wang FB, Powley TL. Topographic inventories of vagal afferents in gastrointestinal muscle. *The Journal of Comparative Neurology*. 2000;421:302-24.
- [296] Berthoud HR. Vagal and hormonal gut–brain communication: from satiation to satisfaction. *J Neurogastroenterol Motil*. 2008;20:64-72.
- [297] Becker JM, Kelly KA. Antral control of canine gastric emptying of solids. *Am J Physiol Gastrointest Liver Physiol*. 1983;8:334-8.
- [298] Page AJ, O'Donnell T, Cooper NJ, Young RL, Blackshaw LA. Nitric Oxide as an Endogenous Peripheral Modulator of Visceral Sensory Neuronal Function. *J Neurosci*. 2009;29:7246-55.
- [299] Page AJ, Blackshaw LA. GABA_B Receptors Inhibit Mechanosensitivity of Primary Afferent Endings. *J Neurosci*. 1999;19:8597–602.
- [300] Page AJ, Slattery JA, O'Donnell TA, Cooper NJ, Young RL, Blackshaw LA. Modulation of gastro-oesophageal vagal afferents by galanin in mouse and ferret. *J Physiol*. 2005;563:809-19.
- [301] Page AJ, Slattery JA, Milte C, Laker R, O'Donnell T, Dorian C, et al. Ghrelin selectively reduces mechanosensitivity of upper gastrointestinal vagal afferents. *Am J Physiol Gastrointest Liver Physiol*. 2007;292:G1376 –G84.
- [302] Dockray GJ, Varro A, Dimaline R. Gastric endocrine cells: gene expression, processing, and targeting of active products. *Physiological Reviews*. 1996;76:767-98.
- [303] Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, et al. Stomach Is a Major Source of Circulating Ghrelin, and Feeding State Determines Plasma Ghrelin-Like Immunoreactivity Levels in Humans. *J Clin Endocrinol Metab*. 2001;86:4753-8.
- [304] Bado A, Levasseur S, Attoub S, Kermorgant S, Laigneau J, Bortoluzzi MI, et al. The stomach is a source of leptin. *Nature*. 1998;394:790- 3.
- [305] Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: Evidence for diet-induced resistance to leptin action. *Nature medicine*. 1995;1:1311-4.
- [306] Kanoski SE, Hayes MR, Greenwald HS, Fortin SM, Gianessi CA, Gilbert JR, et al. Hippocampal Leptin Signaling Reduces Food Intake and Modulates Food-Related Memory Processing. *Neuropsychopharmacology*. 2011;36:1859-70.
- [307] Morton GJ. Hypothalamic leptin regulation of energy homeostasis and glucose metabolism. *The Journal of Physiology*. 2007;583:437-43.
- [308] Morrison CD, Morton GJ, Niswender KD, Gelling RW, Schwartz MW. Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism

that requires phosphatidylinositol 3-OH-kinase signaling. *Am J Physiol Endocrinol Metab.* 2005;289:1051-7.

[309] Wang T, Hartzell DL, Rose BS, Flatt WP, Hulsey MG, Menon NK, et al. Metabolic Responses to Intracerebroventricular Leptin and Restricted Feeding. *Physiology & behavior.* 1998;65:839-48.

[310] Doring H, Schwarzer K, Nuesslein-Hildesheim B, Schmidt I. Leptin selectively increases energy expenditure of food-restricted lean mice. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity.* 1998;22:83-8.

[311] Scarpace PJ, Matheny M, Pollock BH, Tumer N. Leptin increases uncoupling protein expression and energy expenditure. *American Journal of Physiology - Endocrinology And Metabolism.* 1997;273:E226-E30.

[312] Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, et al. Abnormal splicing of the leptin receptor in diabetic mice. *Nature.* 1996;379:632-5.

[313] Cammisotto PG, Gingras ED, Renaud C, Levy E, Bendayan M. Secretion of soluble leptin receptors by exocrine and endocrine cells of the gastric mucosa. *Am J Physiol Gastrointest Liver Physiol.* 2005;290:G242-G9.

[314] Huang L, Wang Z, Li C. Modulation of Circulating Leptin Levels by Its Soluble Receptor. *Journal of Biological Chemistry.* 2001;276:6343-9.

[315] Brabant G, Horn R, von zur Mühlen A, Mayr B, Wurster U, Heidenreich F, et al. Free and protein bound leptin are distinct and independently controlled factors in energy regulation. *Diabetologia.* 2000;43:438-42.

[316] Devos R, Guisez Y, Van der Heyden J, White DW, Kalai M, Fountoulakis M, et al. Ligand-independent Dimerization of the Extracellular Domain of the Leptin Receptor and Determination of the Stoichiometry of Leptin Binding. *Journal of Biological Chemistry.* 1997;272:18304-10.

[317] White DW, Tartaglia LA. Evidence for ligand-independent homo-oligomerization of leptin receptor (OB-R) isoforms: A proposed mechanism permitting productive long-form signaling in the presence of excess short-form expression. *Journal of Cellular Biochemistry.* 1999;73:278-88.

[318] Jiang L, Li Z, Rui L. Leptin Stimulates Both JAK2-dependent and JAK2-independent Signaling Pathways. *Journal of Biological Chemistry.* 2008;283:28066-73.

[319] Bendinelli P, Maroni P, Pecori Giralardi F, Piccoletti R. Leptin activates Stat3, Stat1 and AP-1 in mouse adipose tissue. *Molecular and cellular endocrinology.* 2000;168:11-20.

[320] Darnell JE. STATs and Gene Regulation. *Science.* 1997;277:1630-5.

- [321] Dunn SL, Björnholm M, Bates SH, Chen Z, Seifert M, Myers MG. Feedback Inhibition of Leptin Receptor/Jak2 Signaling via Tyr1138 of the Leptin Receptor and Suppressor of Cytokine Signaling 3. *Molecular Endocrinology*. 2005;19:925-38.
- [322] Harvey J, McKenna F, Herson PS, Spanswick D, Ashford ML. Leptin activates ATP-sensitive potassium channels in the rat insulin-secreting cell line, CRI-G1. *J Physiol*. 1997;504:527-35.
- [323] Yang M, Wang F, Wang J, Wu W, Hu Z, Cheng J, et al. PI3K integrates the effects of insulin and leptin on large-conductance Ca²⁺-activated K⁺ channels in neuropeptide Y neurons of the hypothalamic arcuate nucleus. *Am J Physiol Endocrinol Metab*. 2010;298:193-201.
- [324] Qiu J, Fang Y, Rønnekleiv OK, Kelly MJ. Leptin Excites Proopiomelanocortin Neurons via Activation of TRPC Channels. *J Neurosci*. 2010;30:1560-5.
- [325] Hill JW, Williams KW, Ye C, Luo J, Balthasar N, Coppari R, et al. Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice. *The Journal of Clinical Investigation*. 2008;118:1796-805.
- [326] Peters JH, McKay BM, Simasko SM, Ritter RC. Leptin-induced satiation mediated by abdominal vagal afferents. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:879-84.
- [327] Sobhani I, Bado A, Vissuzaine C, Buyse M, Kermorgant S, Laigneau J-P, et al. Leptin secretion and leptin receptor in the human stomach. *Gut*. 2000;47:178-83.
- [328] Sobhani I, Buyse M, Goiot H, Weber N, Laigneau JP, Henin D, et al. Vagal stimulation rapidly increases leptin secretion in human stomach. *Gastroenterology*. 2002;122:259-63.
- [329] Cinti S, De Matteis R, Ceresi E, Pico C, Oliver J, Oliver P, et al. Leptin in the human stomach. *Gut*. 2001;49:155.
- [330] Attoub S, Lev Asseur S, Buyse M, Goiot H, Laigneau J-P, Moizo L, et al. Physiological Role of Cholecystokinin B/Gastrin Receptor in Leptin Secretion. *Endocrinology*. 1999;140:4406-10.
- [331] Cammisotto PG, Bukowiecki LJ, Deshaies Y, Bendayan M. Leptin biosynthetic pathway in white adipocytes. *Biochemistry and Cell Biology*. 2006;84:207-14.
- [332] Covasa M. CCK- and leptin-induced vagal afferent activation: a model for organ-specific endocrine modulation of visceral sensory information. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2006;290:R1542-R3.

- [333] Peters JH, Karpel AB, Ritter RC, Simasko SM. Cooperative Activation of Cultured Vagal Afferent Neurons by Leptin and Cholecystokinin. *Endocrinology*. 2004;145:3652-7.
- [334] Peters JH, Ritter RC, Simasko SM. Leptin and CCK selectively activate vagal afferent neurons innervating the stomach and duodenum. *Am J Physiol Regul Integr Comp Physiol*. 2006;290:1544-9.
- [335] de Lartigue G, Barbier de la Serre C, Espero E, Lee J, Raybould HE. Leptin resistance in vagal afferent neurons inhibits cholecystokinin signaling and satiation in diet induced obese rats. *PLoS one*. 2012;7:e32967.
- [336] de Lartigue G, Barbier de la Serre C, Espero E, Lee J, Raybould HE. Diet-induced obesity leads to the development of leptin resistance in vagal afferent neurons. *Am J Physiol Endocrinol Metab*. 2011;301:E187-E95.
- [337] Tanaka H, Yoshida T, Miyamoto N, Motoike T, Kurosu H, Shibata K, et al. Characterization of a family of endogenous neuropeptide ligands for the G protein coupled receptors GPR7 and GPR8. *PNAS*. 2003;100:6251-6.
- [338] Levine AS, Winsky-Sommerer R, Huitron-Resendiz S, Grace MK, de Lecea L. Injection of neuropeptide W into paraventricular nucleus of hypothalamus increases food intake. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R1727-R32.
- [339] Shimomura Y, Harada M, Goto M, Sugo T, Matsumoto Y, Abe M, et al. Identification of Neuropeptide W as the Endogenous Ligand for Orphan G-protein-coupled Receptors GPR7 and GPR8. *Journal of Biological Chemistry*. 2002;277:35826-32.
- [340] Mondal MS, Yamaguchi H, Date Y, Toshinai K, Kawagoe T, Tsuruta T, et al. Neuropeptide W is present in antral G cells of rat, mouse, and human stomach. *J Endocrinol*. 2006;188:49-57.
- [341] Caminos JE, Bravo SB, García-Rendueles MER, Ruth González C, Garcés MF, Cepeda LA, et al. Expression of neuropeptide W in rat stomach mucosa: Regulation by nutritional status, glucocorticoids and thyroid hormones. *Regulatory Peptides*. 2008;146:106-11.
- [342] Ishii M, Fei H, Friedman JM. Targeted disruption of GPR7, the endogenous receptor for neuropeptides B and W, leads to metabolic defects and adult-onset obesity. *Proc Natl Acad Sci USA*. 2003;100:10540-5.
- [343] Li H, Kentish S, O'Donnell TA, Wittert G, Blackshaw LA, Page AJ. Modulatory Effect of Npw on Mechanosensitivity of Vagal Afferents in Obesity. *Gastroenterology*. 2011;140:S-34.
- [344] Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. Ghrelin, a Novel Growth Hormone-Releasing Acylated Peptide, Is Synthesized in a Distinct Endocrine Cell Type in the Gastrointestinal Tracts of Rats and Humans. *Endocrinology*. 2000;141:4255-61.

- [345] Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, et al. The Novel Hypothalamic Peptide Ghrelin Stimulates Food Intake and Growth Hormone Secretion. *Endocrinology*. 2000;141:4325-8.
- [346] Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the Acyltransferase that Octanoylates Ghrelin, an Appetite-Stimulating Peptide Hormone. *Cell*. 2008;132:387-96.
- [347] Howard AD, Feighner SD, Cully DF, Arena JP, Liberatore PA, Rosenblum CI, et al. A Receptor in Pituitary and Hypothalamus That Functions in Growth Hormone Release. *Science*. 1996;273:974-7.
- [348] Petersenn S, Rasch AC, Penschorn M, Beil FU, Schulte HM. Genomic Structure and Transcriptional Regulation of the Human Growth Hormone Secretagogue Receptor. *Endocrinology*. 2001;142:2649-59.
- [349] Holst B, Cygankiewicz A, Jensen TH, Ankersen M, Schwartz TW. High Constitutive Signaling of the Ghrelin Receptor—Identification of a Potent Inverse Agonist. *Molecular Endocrinology*. 2003;17:2201-10.
- [350] Damian M, Marie J, Leyris J-P, Fehrentz J-A, Verdier P, Martinez J, et al. High constitutive activity is an intrinsic feature of the ghrelin receptor protein. *J Biol Chem*. 2011.
- [351] Holst B, Holliday ND, Bach A, Elling CE, Cox HM, Schwartz TW. Common Structural Basis for Constitutive Activity of the Ghrelin Receptor Family. *Journal of Biological Chemistry*. 2004;279:53806-17.
- [352] Willesen MG, Kristensen P, Rømer J. Co-Localization of Growth Hormone Secretagogue Receptor and NPY mRNA in the Arcuate Nucleus of the Rat. *Neuroendocrinology*. 1999;70:306-16.
- [353] Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ. Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. *Am J Physiol Gastrointest Liver Physiol*. 2006;290:G1289-G97.
- [354] Murray CDR, Booth CE, Bulmer DCE, Kamm MA, Emmanuel AV, Winchester WJ. Ghrelin augments afferent response to distension in rat isolated jejunum. *Neurogastroenterology & Motility*. 2006;18:1112-20.
- [355] Date Y, Murakami N, Toshinai K, Matsukura S, Nijijima A, Matsuo H, et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology*. 2002;123:1120-8.
- [356] Arnold M, Mura A, Langhans W, Geary N. Gut Vagal Afferents Are Not Necessary for the Eating-Stimulatory Effect of Intraperitoneally Injected Ghrelin in the Rat. *The Journal of Neuroscience*. 2006;26:11052-60.
- [357] Jones RB, McKie S, Astbury N, Little TJ, Tivey S, Lassman DJ, et al. Functional neuroimaging demonstrates that ghrelin inhibits the central nervous system response to ingested lipid. *Gut*. 2012.

- [358] Perez-Tilve D, Heppner K, Kirchner H, Lockie SH, Woods SC, Smiley DL, et al. Ghrelin-induced adiposity is independent of orexigenic effects. *The FASEB Journal*. 2011;25:2814-22.
- [359] Briggs DI, Enriori PJ, Lemus MB, Cowley MA, Andrews ZB. Diet-Induced Obesity Causes Ghrelin Resistance in Arcuate NPY/AgRP Neurons. *Endocrinology*. 2010;151:4745-55.
- [360] Druce MR, Wren AM, Park AJ, Milton JE, Patterson M, Frost G, et al. Ghrelin increases food intake in obese as well as lean subjects. *International journal of obesity (2005)*. 2005;29:1130-6.
- [361] Liou AP, Sei Y, Zhao X, Feng J, Lu X, Thomas C, et al. The extracellular calcium-sensing receptor is required for cholecystokinin secretion in response to l-phenylalanine in acutely isolated intestinal I cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2011;300:G538-G46.
- [362] McLaughlin J, Grazia Luca M, Jones MN, D'Amato M, Dockray GJ, Thompson DG. Fatty acid chain length determines cholecystokinin secretion and effect on human gastric motility. *Gastroenterology*. 1999;116:46-53.
- [363] Feltrin KL, Little TJ, Meyer JH, Horowitz M, Smout AJ, Wishart J, et al. Effects of intraduodenal fatty acids on appetite, antropyloroduodenal motility, and plasma CCK and GLP-1 in humans vary with their chain length. *American journal of physiology Regulatory, integrative and comparative physiology*. 2004;287:R524-33.
- [364] Brennan IM, Little TJ, Feltrin KL, Smout AJ, Wishart JM, Horowitz M, et al. Dose-dependent effects of cholecystokinin-8 on antropyloroduodenal motility, gastrointestinal hormones, appetite, and energy intake in healthy men. *American journal of physiology Endocrinology and metabolism*. 2008;295:E1487-94.
- [365] Sullivan CN, Raboin SJ, Gulley S, Sinzobahamvya NT, Green GM, Reeve JR, Jr., et al. Endogenous cholecystokinin reduces food intake and increases Fos-like immunoreactivity in the dorsal vagal complex but not in the myenteric plexus by CCK1 receptor in the adult rat. *American journal of physiology Regulatory, integrative and comparative physiology*. 2007;292:R1071-80.
- [366] Beglinger C, Degen L, Matzinger D, D'Amato M, Drewe J. Loxiglumide, a CCK-A receptor antagonist, stimulates calorie intake and hunger feelings in humans. *American journal of physiology Regulatory, integrative and comparative physiology*. 2001;280:R1149-54.
- [367] Moran TH, Ameglio PJ, Schwartz GJ, McHugh PR. Blockade of type A, not type B, CCK receptors attenuates satiety actions of exogenous and endogenous CCK. *The American journal of physiology*. 1992;262:R46-50.

- [368] Burdyga G, Lal S, Varro A, Dimaline R, Thompson DG, Dockray GJ. Expression of Cannabinoid CB1 Receptors by Vagal Afferent Neurons Is Inhibited by Cholecystokinin. *The Journal of Neuroscience*. 2004;24:2708-15.
- [369] Paulino G, Barbier de la Serre C, Knotts TA, Oort PJ, Newman JW, Adams SH, et al. Increased expression of receptors for orexigenic factors in nodose ganglion of diet-induced obese rats. *Am J Physiol Endocrinol Metab*. 2009;296:898-903.
- [370] Duca FA, Swartz TD, Sakar Y, Covasa M. Decreased intestinal nutrient response in diet-induced obese rats: role of gut peptides and nutrient receptors. *International journal of obesity (2005)*. 2013;37:375-81.
- [371] de Lartigue G, Dimaline R, Varro A, Raybould H, De La Serre CB, Dockray GJ. Cocaine- and Amphetamine-Regulated Transcript Mediates the Actions of Cholecystokinin on Rat Vagal Afferent Neurons. *Gastroenterology*. 2010;138:1479-90.
- [372] Burdyga G, Lal S, Spiller D, Jiang W, Thompson D, Attwood S, et al. Localization of orexin-1 receptors to vagal afferent neurons in the rat and humans. *Gastroenterology*. 2003;124:129-39.
- [373] Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ. Expression of cannabinoid CB1 receptors by vagal afferent neurons: kinetics and role in influencing neurochemical phenotype. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2010;299:G63-G9.
- [374] Lee J, Martin E, Paulino G, de Lartigue G, Raybould HE. Effect of ghrelin receptor antagonist on meal patterns in cholecystokinin type 1 receptor null mice. *Physiology & behavior*. 2011;103:181-7.
- [375] Lo CM, King A, Samuelson LC, Kindel TL, Rider T, Jandacek RJ, et al. Cholecystokinin knockout mice are resistant to high-fat diet-induced obesity. *Gastroenterology*. 2010;138:1997-2005.
- [376] West DB, Fey D, Woods SC. Cholecystokinin persistently suppresses meal size but not food intake in free-feeding rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 1984;246:R776-R87.
- [377] Donovan MJ, Paulino G, Raybould HE. CCK1 receptor is essential for normal meal patterning in mice fed high fat diet. *Physiol Behav*. 2007;92:969-74.
- [378] Lateef DM, Washington MC, Sayegh AI. The short term satiety peptide cholecystokinin reduces meal size and prolongs intermeal interval. *Peptides*. 2011;32:1289-95.
- [379] Washington MC, Coggeshall J, Sayegh AI. Cholecystokinin-33 inhibits meal size and prolongs the subsequent intermeal interval. *Peptides*. 2011;32:971-7.

- [380] Covasa M, Ritter RC. Rats maintained on high-fat diets exhibit reduced satiety in response to CCK and bombesin. *Peptides*. 1998;19:1407-15.
- [381] Daly DM, Park SJ, Valinsky WC, Beyak MJ. Impaired intestinal afferent nerve satiety signalling and vagal afferent excitability in diet induced obesity in the mouse. *The Journal of Physiology*. 2011;589:2857-70.
- [382] Lee YC, Brubaker PL, Drucker DJ. Developmental and Tissue-Specific Regulation of Proglucagon Gene Expression. *Endocrinology*. 1990;127:2217-22.
- [383] Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V. Glucagon-like peptide-1(7–36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. *Journal of Endocrinology*. 1993;138:159-66.
- [384] Cordier-Bussat M, Bernard C, Levenez F, Klages N, Laser-Ritz B, Philippe J, et al. Peptones stimulate both the secretion of the incretin hormone glucagon-like peptide 1 and the transcription of the proglucagon gene. *Diabetes*. 1998;47:1038-45.
- [385] Eissele R, GÖKe R, Willemer S, Harthus HP, Vermeer H, Arnold R, et al. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. *European Journal of Clinical Investigation*. 1992;22:283-91.
- [386] Bryant MG, Bloom SR, Polak JM, Hobbs S, Domschke W, Domschke S, et al. Measurement of gut hormonal peptides in biopsies from human stomach and proximal small intestine. *Gut*. 1983;24:114-9.
- [387] Schirra J, Katschinski M, Weidmann C, Schafer T, Wank U, Arnold R, et al. Gastric emptying and release of incretin hormones after glucose ingestion in humans. *J Clin Invest*. 1996;97:92-103.
- [388] Little TJ, Doran S, Meyer JH, Smout AJPM, O'Donovan DG, Wu K-L, et al. The release of GLP-1 and ghrelin, but not GIP and CCK, by glucose is dependent upon the length of small intestine exposed. *American Journal of Physiology - Endocrinology And Metabolism*. 2006;291:E647-E55.
- [389] Gutzwiller J-P, Göke B, Drewe J, Hildebrand P, Ketterer S, Handschin D, et al. Glucagon-like peptide-1: a potent regulator of food intake in humans. *Gut*. 1999;44:81-6.
- [390] Fridolf T, Ahren B. GLP-1(7-36) amide stimulates insulin secretion in rat islets: studies on the mode of action. *Diabetes research (Edinburgh, Scotland)*. 1991;16:185-91.
- [391] Drucker DJ. The biology of incretin hormones. *Cell metabolism*. 2006;3:153-65.
- [392] Delgado-Aros S, Kim D-Y, Burton DD, Thomforde GM, Stephens D, Brinkmann BH, et al. Effect of GLP-1 on gastric volume, emptying, maximum

volume ingested, and postprandial symptoms in humans. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2002;282:G424-G31.

[393] Reimann F. Molecular mechanisms underlying nutrient detection by incretin-secreting cells. *International dairy journal / published in association with the International Dairy Federation*. 2010;20:236-42.

[394] Gaisano GG, J. Park S, Daly DM, Beyak MJ. Glucagon-like peptide-1 inhibits voltage-gated potassium currents in mouse nodose ganglion neurons. *Neurogastroenterology & Motility*. 2010;22:470-e111.

[395] Hayes MR, Kanoski SE, De Jonghe BC, Lechner TM, Alhadeff AL, Fortin SM, et al. The common hepatic branch of the vagus is not required to mediate the glycemic and food intake suppressive effects of glucagon-like-peptide-1. *American journal of physiology Regulatory, integrative and comparative physiology*. 2011;301:R1479-85.

[396] Rüttimann EB, Arnold M, Hillebrand JJ, Geary N, Langhans W. Intrameal Hepatic Portal and Intraperitoneal Infusions of Glucagon-Like Peptide-1 Reduce Spontaneous Meal Size in the Rat via Different Mechanisms. *Endocrinology*. 2009;150:1174-81.

[397] Bucinskaite V, Tolessa T, Pedersen J, Rydqvist B, Zerihun L, Holst JJ, et al. Receptor-mediated activation of gastric vagal afferents by glucagon-like peptide-1 in the rat. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 2009;21:978-e78.

[398] Nakagawa A, Satake H, Nakabayashi H, Nishizawa M, Furuya K, Nakano S, et al. Receptor gene expression of glucagon-like peptide-1, but not glucose-dependent insulinotropic polypeptide, in rat nodose ganglion cells. *Autonomic Neuroscience*. 2004;110:36-43.

[399] Washington MC, Raboin SJ, Thompson W, Larsen CJ, Sayegh AI. Exenatide reduces food intake and activates the enteric nervous system of the gastrointestinal tract and the dorsal vagal complex of the hindbrain in the rat by a GLP-1 receptor. *Brain Research*. 2010;1344:124-33.

[400] Vilsbøll T, Agersø H, Krarup T, Holst JJ. Similar Elimination Rates of Glucagon-Like Peptide-1 in Obese Type 2 Diabetic Patients and Healthy Subjects. *Journal of Clinical Endocrinology & Metabolism*. 2003;88:220-4.

[401] Punjabi M, Arnold M, Geary N, Langhans W, Pacheco-López G. Peripheral Glucagon-like Peptide-1 (GLP-1) and Satiating Behavior. *Physiology & Behavior*. 2011;105:71-6.

[402] Merchenthaler I, Lane M, Shughrue P. Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. *The Journal of Comparative Neurology*. 1999;403:261-80.

[403] Llewellyn-Smith IJ, Reimann F, Gribble FM, Trapp S. Preproglucagon neurons project widely to autonomic control areas in the mouse brain. *Neuroscience*. 2011;180:111-21.

- [404] Williams DL, Baskin DG, Schwartz MW. Evidence that Intestinal Glucagon-Like Peptide-1 Plays a Physiological Role in Satiety. *Endocrinology*. 2009;150:1680-7.
- [405] Anini Y, Brubaker PL. Role of Leptin in the Regulation of Glucagon-Like Peptide-1 Secretion. *Diabetes*. 2003;52:252-9.
- [406] Ranganath LR, Beety JM, Morgan LM, Wright JW, Howland R, Marks V. Attenuated GLP-1 secretion in obesity: cause or consequence? *Gut*. 1996;38:916-9.
- [407] Feinle C, Chapman IM, Wishart J, Horowitz M. Plasma glucagon-like peptide-1 (GLP-1) responses to duodenal fat and glucose infusions in lean and obese men. *Peptides*. 2002;23:1491-5.
- [408] Naslund E, Barkeling B, King N, Gutniak M, Blundell JE, Holst JJ, et al. Energy intake and appetite are suppressed by glucagon-like peptide-1 (GLP-1) in obese men. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*. 1999;23:304-11.
- [409] Shyangdan DS, Royle P, Clar C, Sharma P, Waugh N, Snaith A. Glucagon-like peptide analogues for type 2 diabetes mellitus. *Cochrane database of systematic reviews (Online)*. 2011:CD006423.
- [410] Jelsing J, Vrang N, Hansen G, Raun K, Tang-Christensen M, Bjerre Knudsen L. Liraglutide: short-lived effect on gastric emptying—long lasting effects on body weight. *Diabetes, Obesity and Metabolism*. 2012;14:531-8.
- [411] Karra E, Chandarana K, Batterham RL. The role of peptide YY in appetite regulation and obesity. *The Journal of Physiology*. 2009;587:19-25.
- [412] Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR. Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology*. 1985;89:1070-7.
- [413] P. L. H. van Battum WPMH, J. M. J. I. Salemans, J. H. C. Kuijpers, F. M. Nagengast, J. B. M. J. Jansen. Impaired Release of Peptide YY in Patients with Proctocolectomy and Ileal Pouch-Anal Anastomosis. *Scandinavian Journal of Gastroenterology*. 1999;34:404-8.
- [414] Imamura M, Nakajima H, Mikami Y, Yamauchi H. Morphological and Immunohistochemical Changes in Intestinal Mucosa and PYY Release Following Total Colectomy with Ileal Pouch-Anal Anastomosis in Dogs. *Digestive diseases and sciences*. 1999;44:1000-7.
- [415] Greeley GH, Jr., Jeng YJ, Gomez G, Hashimoto T, Hill FL, Kern K, et al. Evidence for regulation of peptide-YY release by the proximal gut. *Endocrinology*. 1989;124:1438-43.
- [416] Pilichiewicz AN, Papadopoulos P, Brennan IM, Little TJ, Meyer JH, Wishart JM, et al. Load-dependent effects of duodenal lipid on

antropyloroduodenal motility, plasma CCK and PYY, and energy intake in healthy men. *American journal of physiology Regulatory, integrative and comparative physiology*. 2007;293:R2170-8.

[417] Batterham RL, Heffron H, Kapoor S, Chivers JE, Chandarana K, Herzog H, et al. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. *Cell metabolism*. 2006;4:223-33.

[418] MacIntosh CG, Andrews JM, Jones KL, Wishart JM, Morris HA, Jansen JB, et al. Effects of age on concentrations of plasma cholecystokinin, glucagon-like peptide 1, and peptide YY and their relation to appetite and pyloric motility. *The American Journal of Clinical Nutrition*. 1999;69:999-1006.

[419] Grandt D, Schimiczek M, Beglinger C, Layer P, Goebell H, Eysselein VE, et al. Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1-36 and PYY 3-36. *Regul Pept*. 1994;51:151-9.

[420] Mentlein R, Dahms P, Grandt D, Kruger R. Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV. *Regul Pept*. 1993;49:133-44.

[421] Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature*. 2002;418:650-4.

[422] Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K, et al. The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats. *Endocrinology*. 2005;146:2369-75.

[423] Abbott CR, Monteiro M, Small CJ, Sajedi A, Smith KL, Parkinson JR, et al. The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. *Brain Res*. 2005;1044:127-31.

[424] Burdyga G, de Lartigue G, Raybould HE, Morris R, Dimaline R, Varro A, et al. Cholecystokinin Regulates Expression of Y2 Receptors in Vagal Afferent Neurons Serving the Stomach. *J Neurosci*. 2008;28:11583-92.

[425] Broberger C, Landry M, Wong H, Walsh JN, Hokfelt T. Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus. *Neuroendocrinology*. 1997;66:393-408.

[426] Scott V, Kimura N, Stark JA, Luckman SM. Intravenous Peptide YY3-36 and Y2 Receptor Antagonism in the Rat: Effects on Feeding Behaviour. *Journal of Neuroendocrinology*. 2005;17:452-7.

[427] Naveilhan P, Hassani H, Canals JM, Ekstrand AJ, Larefalk A, Chhajlani V, et al. Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor. *Nature medicine*. 1999;5:1188-93.

- [428] le Roux CW, Batterham RL, Aylwin SJB, Patterson M, Borg CM, Wynne KJ, et al. Attenuated Peptide YY Release in Obese Subjects Is Associated with Reduced Satiety. *Endocrinology*. 2006;147:3-8.
- [429] Rahardjo GL, Huang X-F, Tan YY, Deng C. Decreased Plasma Peptide YY Accompanied by Elevated Peptide YY and Y2 Receptor Binding Densities in the Medulla Oblongata of Diet-Induced Obese Mice. *Endocrinology*. 2007;148:4704-10.
- [430] Ma Y, Bertone ER, Stanek EJ, Reed GW, Hebert JR, Cohen NL, et al. Association between Eating Patterns and Obesity in a Free-living US Adult Population. *American Journal of Epidemiology*. 2003;158:85-92.
- [431] Furnes M, Zhao C-M, Chen D. 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. *Obesity surgery*. 2009;19:1430-8.
- [432] Geliebter A. Gastric distension and gastric capacity in relation to food intake in humans. *Physiol Behav*. 1988;44:665-8.
- [433] Donovan MJ, Paulino G, Raybould HE. Activation of hindbrain neurons in response to gastrointestinal lipid is attenuated by high fat, high energy diets in mice prone to diet-induced obesity. *Brain Research*. 2009;1248:136-40.
- [434] Bray GA. Afferent signals regulating food intake. *Proceedings of the Nutrition Society*. 2000;59:373-84.
- [435] Deutsch JA. Dietary control and the stomach. *Progress in Neurobiology*. 1983;20:313-32.
- [436] Page AJ, Martin CM, Blackshaw LA. Vagal mechanoreceptors and chemoreceptors in mouse stomach and esophagus. *J Neurophysiol*. 2002;87:2095-103.
- [437] Iggo A. Tension receptors in the stomach and the urinary bladder. *The Journal of Physiology*. 1955;128:593-607.
- [438] Andrews PLR, Sanger GJ. Abdominal vagal afferent neurones: an important target for the treatment of gastrointestinal dysfunction. *Current Opinion in Pharmacology*. 2002;2:650-6.
- [439] Tuleu C, Andrieux C, Boy P, Chaumeil JC. Gastrointestinal transit of pellets in rats: effect of size and density. *International Journal of Pharmaceutics*. 1999;180:123-31.
- [440] McIntyre A, Vincent RM, Perkins AC, Spiller RC. Effect of bran, ispaghula, and inert plastic particles on gastric emptying and small bowel transit in humans: the role of physical factors. *Gut*. 1997;40:223-7.
- [441] Moesgaard SG, Ahrén B, Carr RD, Gram DX, Brand CL, Sundler F. Effects of high-fat feeding and fasting on ghrelin expression in the mouse stomach. *Regul Pept*. 2004;120:261-7.

- [442] Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, et al. The Tissue Distribution of the mRNA of Ghrelin and Subtypes of Its Receptor, GHS-R, in Humans. *Journal of Clinical Endocrinology & Metabolism*. 2002;87:2988.
- [443] Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and Des-acyl Ghrelin: Two Major Forms of Rat Ghrelin Peptide in Gastrointestinal Tissue. *Biochemical and Biophysical Research Communications*. 2000;279:909-13.
- [444] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*. 1999;402:656-60.
- [445] Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, et al. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology*. 2001;120:337-45.
- [446] Druce MR, Neary NM, Small CJ, Milton J, Monteiro M, Patterson M, et al. Subcutaneous administration of ghrelin stimulates energy intake in healthy lean human volunteers. *Int J Obes (Lond)*. 2006;30:293-6.
- [447] Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. *Nature*. 2001;409:194-8.
- [448] Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature*. 2000;407:908-13.
- [449] Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin Enhances Appetite and Increases Food Intake in Humans. *Journal of Clinical Endocrinology & Metabolism*. 2001;86:5992.
- [450] Sakata I, Yamazaki M, Inoue K, Hayashi Y., Kangawa K, Sakai T. Growth hormone secretagogue receptor expression in the cells of the stomach-projected afferent nerve in the rat nodose ganglion. *Neurosci Lett*. 2003;342:183-6.
- [451] Dockray GJ, Burdyga G. Plasticity in vagal afferent neurones during feeding and fasting: mechanisms and significance*. *Acta Physiologica*. 2011;201:313-21.
- [452] Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ. Feeding-dependent depression of melanin-concentrating hormone and melanin-concentrating hormone receptor-1 expression in vagal afferent neurones. *Neuroscience*. 2006;137:1405-15.
- [453] Nefti W, Chaumontet C, Fromentin G, Tome´ D, Darcel N. A high-fat diet attenuates the central response to within-meal satiation signals and modifies the receptor expression of vagal afferents in mice. *Am J Physiol Regul Integr Comp Physiol*. 2009;296:1681-6.

- [454] Slattery JA, Page AJ, Dorian CL, Brierley SM, Blackshaw LA. Potentiation of mouse vagal afferent mechanosensitivity by ionotropic and metabotropic glutamate receptors. *The Journal of Physiology*. 2006;577:295-306.
- [455] Hughes PA, Brierley SM, Young RL, Blackshaw LA. Localization and comparative analysis of acid-sensing ion channel (ASIC1, 2, and 3) mRNA expression in mouse colonic sensory neurons within thoracolumbar dorsal root ganglia. *J Comp Neurol*. 2007;500:863-75.
- [456] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*. 2002;30:e36.
- [457] Gardiner JV, Campbell D, Patterson M, Kent A, Ghatei MA, Bloom SR, et al. The Hyperphagic Effect of Ghrelin Is Inhibited in Mice by a Diet High in Fat. *Gastroenterology*. 2010;138:2468-76.e1.
- [458] Williams RM, Berthoud HR, Stead RH. Vagal afferent nerve fibres contact mast cells in rat small intestinal mucosa. *Neuroimmunomodulation*. 1997;4:266-70.
- [459] Kim DY, Camilleri M, Murray JA, Stephens DA, Levine JA, Burton DD. Is there a role for gastric accommodation and satiety in asymptomatic obese people? *Obesity research*. 2001;9:655-61.
- [460] de Lartigue G, Dimaline R, Varro A, Dockray GJ. Cocaine- and Amphetamine-Regulated Transcript: Stimulation of Expression in Rat Vagal Afferent Neurons by Cholecystokinin and Suppression by Ghrelin. *J Neurosci*. 2007;27:2876-82.
- [461] Zeeni N, Chaumontet C, Moyses E, Fromentin G, Tardivel C, Tome D, et al. A positive change in energy balance modulates TrkB expression in the hypothalamus and nodose ganglia of rats. *Brain Research*. 2009;1289:49-55.
- [462] Ahren B, Mansson S, Gingerich RL, Havel PJ. Regulation of plasma leptin in mice: influence of age, high-fat diet, and fasting. *Am J Physiol Regul Integr Comp Physiol*. 1997;273:113-20.
- [463] Falkén Y, Hellström PM, Sanger GJ, Dewit O, Dukes G, Grybäck P, et al. Actions of prolonged ghrelin infusion on gastrointestinal transit and glucose homeostasis in humans. *Neurogastroenterology & Motility*. 2010;22:e192-e200.
- [464] Sato M, Nakahara K, Miyazato M, Kangawa K, Murakami N. Regulation of GH secretagogue receptor gene expression in the rat nodose ganglion. *J Endocrinol*. 2007;194:41-6.
- [465] Yannakoulia M, Yiannakouris N, Blüher S, Matalas A-L, Klimis-Zacas D, Mantzoros CS. Body Fat Mass and Macronutrient Intake in Relation to Circulating Soluble Leptin Receptor, Free Leptin Index, Adiponectin, and

Resistin Concentrations in Healthy Humans. *Journal of Clinical Endocrinology & Metabolism*. 2003;88:1730-6.

[466] Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, et al. Serum Immunoreactive-Leptin Concentrations in Normal-Weight and Obese Humans. *New England Journal of Medicine*. 1996;334:292-5.

[467] Owyang C, Heldsinger A. Vagal control of satiety and hormonal regulation of appetite. *Journal of neurogastroenterology and motility*. 2011;17:338-48.

[468] Mix H, Widjaja A, Jandl O, Cornberg M, Kaul A, Göke M, et al. Expression of leptin and leptin receptor isoforms in the human stomach. *Gut*. 2000;47:481-6.

[469] Burdyga G, Spiller D, Morris R, Lal S, Thompson DG, Saeed S, et al. Expression of the Leptin Receptor in Rat and Human Nodose Ganglion Neurones. *Neuroscience*. 2002;109:339-57.

[470] Buyse M, Ovesjö M-L, Goïot H, Guilmeau S, Péranzi G, Moizo L, et al. Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve. *Eur J Neurosci*. 2001;14:64-72.

[471] Wang G, Tomasi D, Backus W, Wang R, Telang F, Geliebter A, et al. Gastric distention activates satiety circuitry in the human brain. *Neuroimage*. 2008;39:1824 – 31.

[472] Blackshaw LA, Grundy D, Scratcherd T. Vagal afferent discharge from gastric mechanoreceptors during contraction and relaxation of the ferret corpus. *Journal of the autonomic nervous system*. 1987;18:19-24.

[473] Kentish S, Li H, Philp LK, O'Donnell TA, Isaacs NJ, Young RL, et al. Diet-induced adaptation of vagal afferent function. *J Physiol*. 2012;590:209-21.

[474] Liu Z, Qiu YH, Li B, Ma SH, Peng YP. Neuroprotection of interleukin-6 against NMDA-induced apoptosis and its signal-transduction mechanisms. *Neurotoxicity research*. 2011;19:484-95.

[475] Shyu KG, Wang BW, Chen WJ, Kuan P, Hung CR. Mechanism of the inhibitory effect of atorvastatin on endoglin expression induced by transforming growth factor-beta1 in cultured cardiac fibroblasts. *European journal of heart failure*. 2010;12:219-26.

[476] Williams JL, Jr., Malik KU. Forskolin stimulates prostaglandin synthesis in rabbit heart by a mechanism that requires calcium and is independent of cyclic AMP. *Circulation research*. 1990;67:1247-56.

[477] Rogers RC, Hermann GE. Mechanisms of action of CCK to activate central vagal afferent terminals. *Peptides*. 2008;29:1716-25.

- [478] Womack MD, Khodakhah K. Characterization of large conductance Ca²⁺-activated K⁺ channels in cerebellar Purkinje neurons. *The European journal of neuroscience*. 2002;16:1214-22.
- [479] Lievremont JP, Bird GS, Putney JW, Jr. Mechanism of inhibition of TRPC cation channels by 2-aminoethoxydiphenylborane. *Molecular pharmacology*. 2005;68:758-62.
- [480] Kressel M. Tyramide amplification allows anterograde tracing by horseradish peroxidase-conjugated lectins in conjunction with simultaneous immunohistochemistry. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 1998;46:527-33.
- [481] Zhao AZ, Huan J, Gupta S, Pal R, Sahu A. A phosphatidylinositol 3-kinase-phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. *Nat Neurosci*. 2002;5:727-8.
- [482] Zhao H, Sprunger LK, Simasko SM. Expression of transient receptor potential channels and two-pore potassium channels in subtypes of vagal afferent neurons in rat. *Am J Physiol Gastrointest Liver Physiol*. 2009;298:G212-G21.
- [483] de Lartigue G, Lur G, Dimaline R, Varro A, Raybould H, Dockray GJ. EGR1 is a target for cooperative interactions between cholecystokinin and leptin, and inhibition by ghrelin, in vagal afferent neurons. *Endocrinology*. 2010;151:3589-99.
- [484] Barrachina MD, Martinez V, Wang L, Wei JY, Tache Y. Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci U S A*. 1997;94:10455-60.
- [485] Peiser C, Springer J, Groneberg DA, McGregor GP, Fischer A, Lang RE. Leptin receptor expression in nodose ganglion cells projecting to the rat gastric fundus. *Neurosci Lett*. 2002;320:41-4.
- [486] de Lartigue G, de La Serre CB, Raybould HE. Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. *Physiology & Behavior*. 2011;105:100-5.
- [487] Farley C, Cook JA, Spar BD, Austin TM, Kowalski TJ. Meal Pattern Analysis of Diet-Induced Obesity in Susceptible and Resistant Rats. *Obesity*. 2003;11:845-51.
- [488] Dietrich A, Kalwa H, Storch U, Schnitzler MM, Salanova B, Pinkenburg O, et al. Pressure-induced and store-operated cation influx in vascular smooth muscle cells is independent of TRPC1. *Pflugers Arch*. 2007;455:465-77.
- [489] Yada Y, Nagao S, Okano Y, Nozawa Y. Inhibition by cyclic AMP of guanine nucleotide-induced activation of phosphoinositide-specific phospholipase C in human platelets. *FEBS Lett*. 1989;242:368-72.

- [490] Campbell MD, Subramaniam S, Kotlikoff MI, Williamson JR, Fluharty SJ. Cyclic AMP Inhibits Inositol Polyphosphate Production and Calcium Mobilization in Neuroblastoma x Glioma NG1 08-15 Cells. *Mol Pharmacol*. 1990;38:282-8.
- [491] Williams KW, Zsombok A, Smith BN. Rapid Inhibition of Neurons in the Dorsal Motor Nucleus of the Vagus by Leptin. *Endocrinology*. 2007;148:1868-81.
- [492] Shanley LJ, Irving AJ, Rae MG, Ashford MLJ, Harvey J. Leptin inhibits rat hippocampal neurons via activation of large conductance calciumactivated K⁺ channels. *Nat Neurosci*. 2002;5:299-300.
- [493] Faipoux R, Tomé D, Gougis S, Darcel N, Fromentin G. Proteins Activate Satiety-Related Neuronal Pathways in the Brainstem and Hypothalamus of Rats. *J Nutr*. 2008;138:1172-8.
- [494] Mönnikes H, Lauer G, Arnold R. Peripheral administration of cholecystokinin activates c-fos expression in the locus coeruleus/subcoeruleus nucleus, dorsal vagal complex and paraventricular nucleus via capsaicin-sensitive vagal afferents and CCK-A receptors in the rat. *Brain Res*. 1997;770:277-88.
- [495] Peters JH, Simasko SM, Ritter RC. Modulation of vagal afferent excitation and reduction of food intake by leptin and cholecystokinin. *Physiol Behav*. 2006;89:477-85.
- [496] Kentish SJ, O'Donnell TA, Isaacs NJ, Young RL, Li H, Harrington AM, et al. Gastric vagal afferent modulation by leptin is influenced by food intake status. *J Physiol*. 2013;591:1921-34.
- [497] Levin F, Edholm T, Schmidt PT, Gryback P, Jacobsson H, Degerblad M, et al. Ghrelin Stimulates Gastric Emptying and Hunger in Normal-Weight Humans. *J Clin Endocrinol Metab*. 2006;91:3296-302.
- [498] Date Y, Mondal MS, Kageyama H, Ghamari-Langroudi M, Takenoya F, Yamaguchi H, et al. Neuropeptide W: An Anorectic Peptide Regulated by Leptin and Metabolic State. *Endocrinology*. 2010;151:2200-10.
- [499] Li H, Kentish S, O'Donnell T, Wittert G, Blackshaw A, Page A. The role of gastric neuropeptide W is regulated by food intake. *Obes Res Clin Pract*. 2012;6 (Sup. 1):51.
- [500] Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, et al. Plasma Ghrelin Levels in Lean and Obese Humans and the Effect of Glucose on Ghrelin Secretion. *J Clin Endocrinol Metab*. 2002;87:240-4.
- [501] Kalra SP, Ueno N, Kalra PS. Stimulation of Appetite by Ghrelin Is Regulated by Leptin Restraint: Peripheral and Central Sites of Action. *J Nutr*. 2005;135:1331-5.

- [502] Guilmeau S, Buyse M, Tsocas A, Laigneau JP, Bado A. Duodenal Leptin Stimulates Cholecystokinin Secretion: Evidence of a Positive Leptin-Cholecystokinin Feedback Loop. *Diabetes*. 2003;52:1664-72.
- [503] Dixit VD, Schaffer EM, Pyle RS, Collins GD, Sakthivel SK, Palaniappan R, et al. Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells. *J Clin Invest*. 2004;114:57-66.
- [504] Briggs DI, Lemus MB, Kua E, Andrews ZB. Diet-Induced Obesity Attenuates Fasting-Induced Hyperphagia. *Journal of Neuroendocrinology*. 2011;23:620-6.
- [505] Bivona BJ, Park S, Harrison-Bernard LM. Glomerular filtration rate determinations in conscious type II diabetic mice. *American Journal of Physiology - Renal Physiology*. 2011;300:F618-F25.
- [506] Benomar Y, Roy A, Aubourg A, Djiane J, Taouis M. Cross down-regulation of leptin and insulin receptor expression and signalling in a human neuronal cell line. *Biochem J*. 2005;388:929-39.
- [507] Wu M-H, Chuang P-C, Chen H-M, Lin C-C, Tsai S-J. Increased leptin expression in endometriosis cells is associated with endometrial stromal cell proliferation and leptin gene up-regulation. *Mol Hum Reprod*. 2002;8:456-64.
- [508] Huang XF, Xin X, McLennan P, Storlien L. Role of fat amount and type in ameliorating diet-induced obesity: insights at the level of hypothalamic arcuate nucleus leptin receptor, neuropeptide Y and pro-opiomelanocortin mRNA expression. *Diabetes Obes Metab*. 2004;6:35-44.
- [509] Huang X-F, Han M, Storlien LH. The level of NPY receptor mRNA expression in diet-induced obese and resistant mice. *Brain Res Mol Brain Res*. 2003;115:21-8.
- [510] Lin S, Storlien LH, Huang X-F. Leptin receptor, NPY, POMC mRNA expression in the diet-induced obese mouse brain. *Brain Res*. 2000;875:89-95.
- [511] Mitchell SE, Nogueiras R, Morris A, Tovar S, Grant C, Cruickshank M, et al. Leptin receptor gene expression and number in the brain are regulated by leptin level and nutritional status. *J Physiol*. 2009;587:3573-85.
- [512] Bjørnbæk C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a Potential Mediator of Central Leptin Resistance. *Molecular Cell*. 1998;1:619-25.
- [513] Wilsey J, Scarpace P. Caloric restriction reverses the deficits in leptin receptor protein and leptin signaling capacity associated with diet-induced obesity: role of leptin in the regulation of hypothalamic long-form leptin receptor expression. *J Endocrinol*. 2004;181:297-306.

- [514] Gamber KM, Huo L, Ha S, Hairston JE, Greeley S, Bjørnbæk C. Over-Expression of Leptin Receptors in Hypothalamic POMC Neurons Increases Susceptibility to Diet-Induced Obesity. *PloS one*. 2012;7:e30485.
- [515] Cammisotto PG, Bendayan M, Levy E. Regulation of leptin receptor expression in human polarized Caco-2/15 cells. *Endocr Metab Immune Disord Drug Targets*. 2012;12:57-70.
- [516] Frühbeck G. Intracellular signalling pathways activated by leptin. *Biochem J*. 2006;393:7-20.
- [517] Gaigé S, Abysique A, Bouvier M. Effects of leptin on cat intestinal vagal mechanoreceptors. *J Physiol*. 2002;543:679-89.
- [518] Savastano DM, Covasa M. Adaptation to a High-Fat Diet Leads to Hyperphagia and Diminished Sensitivity to Cholecystokinin in Rats. *J Nutr*. 2005;135:1953-9.
- [519] Levin BE, Dunn-Meynell AA, Ricci MR, Cummings DE. Abnormalities of leptin and ghrelin regulation in obesity-prone juvenile rats. *Am J Physiol Endocrinol Metab*. 2003;285:949-57.
- [520] Luque RM, Huang ZH, Shah B, Mazzone T, Kineman RD. Effects of leptin replacement on hypothalamic-pituitary growth hormone axis function and circulating ghrelin levels in ob/ob mice. *Am J Physiol Endocrinol Metab*. 2007;292:E891-E9.
- [521] Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A Preprandial Rise in Plasma Ghrelin Levels Suggests a Role in Meal Initiation in Humans. *Diabetes*. 2001;50:1714-9.
- [522] Ueno N, Asakawa A, Inui A. Blunted metabolic response to fasting in obese mice. *Endocr*. 2007;32:192-6.
- [523] Perreault M, Istrate N, Wang L, Nichols AJ, Tozzo E, Stricker-Krongrad A. Resistance to the orexigenic effect of ghrelin in dietary-induced obesity in mice: reversal upon weight loss. *Int J Obes Relat Metab Disord*. 2004;28:879-85.
- [524] Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, et al. Ghrelin Causes Hyperphagia and Obesity in Rats. *Diabetes*. 2001;50:2540-7.
- [525] Toshinai K, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, et al. Upregulation of Ghrelin Expression in the Stomach upon Fasting, Insulin-Induced Hypoglycemia, and Leptin Administration. *Biochem Biophys Res Commun*. 2001;281:1220-5.
- [526] Klok MD, Jakobsdottir S, Drent ML. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obesity Reviews*. 2007;8:21-34.

- [527] Guo Z-F, Zheng X, Qin Y-W, Hu J-Q, Chen S-P, Zhang Z. Circulating Preprandial Ghrelin to Obestatin Ratio Is Increased in Human Obesity. *J Clin Endocrinol Metab.* 2007;92:1875-80.
- [528] Sánchez J, Oliver P, Picó C, Palou A. Diurnal rhythms of leptin and ghrelin in the systemic circulation and in the gastric mucosa are related to food intake in rats. *Pflugers Arch - Eur J Physiol.* 2004;448:500-6.
- [529] Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, et al. Characterization of Peripheral Circadian Clocks in Adipose Tissues. *Diabetes.* 2006;55:962-70.
- [530] Wing RR, Phelan S. Long-term weight loss maintenance. *The American Journal of Clinical Nutrition.* 2005;82:222S-5S.
- [531] Skender ML, Goodrick GK, Del Junco DJ, Reeves RS, Darnell L, Gotto Jr AM, et al. Comparison of 2-Year Weight Loss Trends in Behavioral Treatments of Obesity: Diet, Exercise, and Combination Interventions. *Journal of the American Dietetic Association.* 1996;96:342-6.
- [532] Tsai AG, Wadden TA. Systematic Review: An Evaluation of Major Commercial Weight Loss Programs in the United States. *Annals of Internal Medicine.* 2005;142:56-66.
- [533] Woods SC, Porte D, Bobbioni E, Ionescu E, Sauter JF, Rohner-Jeanrenaud F, et al. Insulin: its relationship to the central nervous system and to the control of food intake and body weight. *The American Journal of Clinical Nutrition.* 1985;42:1063-71.
- [534] Stanley S, Wynne K, McGowan B, Bloom S. Hormonal Regulation of Food Intake. *Physiological Reviews.* 2005;85:1131-58.
- [535] Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM, et al. Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. *FASEB J.* 2004;18:439-56.
- [536] Havel PJ. Peripheral Signals Conveying Metabolic Information to the Brain: Short-Term and Long-Term Regulation of Food Intake and Energy Homeostasis. *Experimental Biology and Medicine.* 2001;226:963-77.
- [537] Meyer JH, Hlinka M, Tabrizi Y, DiMaso N, Raybould HE. Chemical specificities and intestinal distributions of nutrient-driven satiety. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology.* 1998;275:R1293-R307.
- [538] D'Alessio D. Intestinal Hormones and Regulation of Satiety: The Case for CCK, GLP-1, PYY, and Apo A-IV. *Journal of Parenteral and Enteral Nutrition.* 2008;32:567-8.
- [539] Read NW. Role of gastrointestinal factors in hunger and satiety in man. *The Proceedings of the Nutrition Society.* 1992;51:7-11.

- [540] Rentsch J, Levens N, Chiesi M. Recombinant OB-Gene Product Reduces Food-Intake in Fasted Mice. *Biochemical and Biophysical Research Communications*. 1995;214:131-6.
- [541] Satoh N, Ogawa Y, Katsuura G, Hayase M, Tsuji T, Imagawa K, et al. The arcuate nucleus as a primary site of satiety effect of leptin in rats. *Neuroscience Letters*. 1997;224:149-52.
- [542] Lemonnier D, Suquet JP, Aubert R, De Gasquet P, Pequignot E. Metabolism of the mouse made obese by a high-fat diet. *Diabete & metabolisme*. 1975;1:77-85.
- [543] Ravussin Y, Gutman R, Diano S, Shanabrough M, Borok E, Sarman B, et al. Effects of chronic weight perturbation on energy homeostasis and brain structure in mice. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2011;300:R1352-R62.
- [544] Levin BE, Dunn-Meynell AA. Defense of body weight against chronic caloric restriction in obesity-prone and -resistant rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2000;278:R231-R7.
- [545] Apolzan JW, Harris RBS. Rapid onset and reversal of peripheral and central leptin resistance in rats offered chow, sucrose solution, and lard. *Appetite*. 2013;60:65-73.
- [546] Gatta B, Nunes ML, Bailacq-Auder C, Etchechoury L, Collet D, Tabarin A. Is bariatric surgery really inefficient in hypothalamic obesity? *Clinical Endocrinology*. 2013;78:636-8.
- [547] Inge TH, Pfluger P, Zeller M, Rose SR, Burget L, Sundararajan S, et al. Gastric bypass surgery for treatment of hypothalamic obesity after craniopharyngioma therapy. *Nature clinical practice Endocrinology & metabolism*. 2007;3:606-9.
- [548] Yuan C, Attele AS, Wu JA, Zhang L, Shi ZQ. Peripheral gastric leptin modulates brain stem neuronal activity in neonates. *Am J Physiol Gastrointest Liver Physiol*. 1999;277:626-30.
- [549] Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature reviews Genetics*. 2012;13:227-32.
- [550] Schultes B, Ernst B, Wilms B, Thurnheer M, Hallschmid M. Hedonic hunger is increased in severely obese patients and is reduced after gastric bypass surgery. *The American Journal of Clinical Nutrition*. 2010;92:277-83.
- [551] Rodrigues AGM, Proença RPdC, Calvo MCM, Fiates GMR. Overweight/obesity is associated with food choices related to rice and beans, colors of salads, and portion size among consumers at a restaurant serving buffet-by-weight in Brazil. *Appetite*. 2012;59:305-11.

- [552] Friedman JM. Leptin at 14 y of age: an ongoing story. *The American Journal of Clinical Nutrition*. 2009;89:973S-9S.
- [553] Enriori PJ, Evans AE, Sinnayah P, Jobst EE, Tonelli-Lemos L, Billes SK, et al. Diet-Induced Obesity Causes Severe but Reversible Leptin Resistance in Arcuate Melanocortin Neurons. *Cell Metab*. 2007;5:181-94.
- [554] Yang Y, Li P-Y, Cheng J, Mao L, Wen J, Tan X-Q, et al. Function of BKCa Channels Is Reduced in Human Vascular Smooth Muscle Cells From Han Chinese Patients With Hypertension. *Hypertension*. 2013;61:519-25.
- [555] Seth M, Zhang Z-S, Mao L, Graham V, Burch J, Stiber J, et al. TRPC1 Channels Are Critical for Hypertrophic Signaling in the Heart. *Circulation research*. 2009;105:1023-30.
- [556] Ioannides-Demos LL, Piccenna L, McNeil JJ. Pharmacotherapies for Obesity: Past, Current, and Future Therapies. *Journal of Obesity*. 2011;2011.
- [557] Pasmán WJ, Saris WH, Westerterp-Plantenga MS. Predictors of weight maintenance. *Obesity research*. 1999;7:43-50.
- [558] Doucet E, Imbeault P, St-Pierre S, Almeras N, Mauriege P, Richard D, et al. Appetite after weight loss by energy restriction and a low-fat diet-exercise follow-up. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*. 2000;24:906-14.
- [559] Perri MG. The Maintenance of Treatment Effects in the Long-Term Management of Obesity. *Clinical Psychology: Science and Practice*. 1998;5:526-43.
- [560] Wadden TA, Butryn ML, Byrne KJ. Efficacy of lifestyle modification for long-term weight control. *Obesity research*. 2004;12 Suppl:151S-62S.
- [561] Llado I, Proenza AM, Serra F, Palou A, Pons A. Dietary-induced permanent changes in brown and white adipose tissue composition in rats. *Int J Obes*. 1991;15:415-9.
- [562] Liu C, Elmquist JK. Tipping the scales early: probing the long-term effects of obesity. *The Journal of Clinical Investigation*. 2012;122:3840-2.
- [563] Fuse Y, Hirao A, Kuroda H, Otsuka M, Tahara Y, Shibata S. Differential roles of breakfast only (one meal per day) and a bigger breakfast with a small dinner (two meals per day) in mice fed a high-fat diet with regard to induced obesity and lipid metabolism. *Journal of Circadian Rhythms*. 2012;10:4.
- [564] Faibry P, Tepperman J. Meal Frequency - A Possible Factor in Human Pathology. *The American Journal of Clinical Nutrition*. 1970;23:1059-68.
- [565] Stylopoulos N, Davis P, Pettit JD, Rattner DW, Kaplan LM. Changes in serum ghrelin predict weight loss after Roux-en-Y gastric bypass in rats. *Surgical endoscopy*. 2005;19:942-6.

[566] Cummings DE, Overduin J, Foster-Schubert KE. Gastric Bypass for Obesity: Mechanisms of Weight Loss and Diabetes Resolution. *Journal of Clinical Endocrinology & Metabolism*. 2004;89:2608-15.

[567] Schwartz GJ. The role of gastrointestinal vagal afferents in the control of food intake: current prospects. *Nutrition*. 2000;16:866-73.

[568] Berthoud H-R. The vagus nerve, food intake and obesity. *Regulatory Peptides*. 2008;149:15-25.

[569] Hausken T, Svebak S, Wilhelmsen I, Haug TT, Olafsen K, Pettersson E, et al. Low vagal tone and antral dysmotility in patients with functional dyspepsia. *Psychosomatic Medicine*. 1993;55:12-22.

[570] Holtmann G, Goebell H, Jockenhoevel F, Talley NJ. Altered vagal and intestinal mechanosensory function in chronic unexplained dyspepsia. *Gut*. 1998;42:501-6.

[571] Hong D, Kamath M, Wang S, Tabet J, Tougas G, Anvari M. Assessment of the afferent vagal nerve in patients with gastroesophageal reflux. *Surgical Endoscopy And Other Interventional Techniques*. 2002;16:1042-5.

[572] Mizrahi M, Ben Ya'acov A, Ilan Y. Gastric stimulation for weight loss. *World journal of gastroenterology : WJG*. 2012;18:2309-19.