The nutrient-sensing mechanisms of the mouse stomach and the ghrelin cell in health and obesity

A thesis submitted by

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Summary

Background: Ghrelin is a gastric hormone with multiple physiological functions, including the stimulation of food intake and adiposity. It is well established that circulating ghrelin levels drop after food intake, however, the mechanisms involved in the reduction of postprandial circulating ghrelin levels are not fully understood. In this regard, the ability of the gastrointestinal tract to detect nutrients is critical in the modulation of gut hormone secretion, with the detection of nutrients performed by specialised nutrient chemosensors located on enteroendocrine cells. However, limited information is available on the nutrient-sensing capabilities of the stomach. This PhD project explored the expression of nutrient chemosensors of the mouse stomach and gastric ghrelin cells in health and obesity. Further, the role of nutrients and nutrient chemosensors in the secretion of gastric ghrelin was also investigated.

Aims:

1) Investigate the expression of nutrient chemosensors in different regions of the mouse stomach, with particular emphasis on co-expression with ghrelin.

2) Assess the role of nutrients and nutrient chemosensors in the secretion of gastric ghrelin.

3) Determine the effect of high-fat diet (HFD)-induced obesity on the expression of nutrient chemosensors in the mouse stomach and the level of co-expression with ghrelin.

Methods and results: The study presented in **Chapter 2** characterised the mRNA expression of a repertoire of nutrient chemosensors (protein: GPR93, CaSR, MGluR4; fatty acids: CD36, FFAR2&4; sweet/umami: T1R3, taste-transduction components (TRPM5, GNAT2&3) in the gastric antrum and corpus of the mouse stomach. In addition, immunofluorescence experiments determined the protein expression of GPR93, T1R3, FFAR4 and CD36 in both gastric regions, and their degree of co-expression with ghrelin. Results from this chapter showed that the majority of nutrient chemosensors presented higher mRNA levels in the antrum than corpus, with a similar regionality observed at the protein level. Moreover, co-expression studies showed that at least 60% of ghrelin-positive cells expressed T1R3 and FFAR4, and over 80% expressed GPR93 and CD36. **Chapter 3** extended this investigation by 1) assessing the secretion of total ghrelin (TG) and acyl ghrelin (AG) in response to a wide range of nutrients (2 and 20 mM D-glucose, 20 mM L-phenylalanine, 5% protein hydrolysate (peptone), 5% D-mannitol, 2 mM α-linolenic acid and 5% fat emulsion (Intralipid)), and 2) determining the role of FFAR4 and CD36 in the α-linolenic acid and 5% intralipid-dependent secretion of TG and AG. Results from **Chapter 3** showed that TG and AG secretion from the mouse stomach was modulated in a nutrient-specific manner. Glucose and mannitol did not affect TG and AG secretion. Peptone stimulated TG and AG secretion, while intralipid simultaneously reduced TG and stimulated AG secretion. L-phenylalanine and α-linolenic acid reduced AG release, without changing TG release. Moreover, the modulation of TG and AG secretion by α-linolenic acid and intralipid was independent of FFAR4 and CD36 activation.

Chapter 4 determined the mRNA expression of gastric nutrient chemosensors (i.e. same repertoire investigated in **Chapter 2**) from lean and HFD-induced obese mice. Outcomes from this study showed that the mRNA expression of most gastric nutrient chemosensors was unchanged in HFD-induced obesity, except for a region-specific increase of antral CaSR mRNA levels. Accordingly, immunofluorescence studies explored protein expression of CaSR in the mouse stomach and co-expression with gastric ghrelin cells. The protein expression of CaSR was region-specific with positive cells in the antrum only. Additionally, there was a high co-expression with antral ghrelin cells (≈ 80% co-localisation). Moreover, the density of CaSR-positive cells and co-expression with ghrelin were comparable in lean and HFD-induced obese mice.

Conclusions: The stomach and gastric ghrelin cells express the cellular machinery for the detection of sweet compounds, proteins and lipids. The gastric secretion of TG and AG was modulated by proteins and lipids. However, the lipid-dependent secretion of TG and AG did not involve FFAR4 and CD36 activation. Furthermore, HFD-induced obesity did not alter the expression of most targets investigated, with the exception of an antral-specific increase in CaSR mRNA expression. However, there was no change in the density of CaSR-positive cells or the level of co-expression with ghrelin. This study provided extensive information on the nutrient-sensing ability of the mouse stomach and gastric ghrelin cells. Additional research is needed to further define the functional connections between gastric nutrient chemosensors and ghrelin secretion in health and obesity.

Declaration of originality

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Publications arising from this thesis

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Conference proceedings

ORAL PRESENTATIONS:

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The poster above received the Best Poster Award (PhD researcher) at The South Australian Health and Medical Research Institute (SAHMRI) Research showcase (2018), Adelaide, Australia.

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Additional co-authored publications and conference proceedings

The following publications resulted from collaborations. However, they are not part of this PhD thesis.

Li H, Buisman-Pijlman FTA, **Nunez-Salces M**, Christie S, Frisby CL, Inserra A, Hatzinikolas G, Lewis MD, Karitas S, Wong ML, Page AJ. Chronic stress induces hypersensitivity of murine gastric vagal afferents. *Neurogastroenterol Motil.* 2019;31(12):e13669.

Kaur H, Muhlhausler BS, Sim PS, Page A, Li H, **Nunez-Salces M**, Clarke GS, Huang L, Wilson RL, Veldhuis JD, Chen C, Roberts CT, Gatford KL. Pregnancy, but not dietary octanoic acid supplementation, stimulates the ghrelin-pituitary growth hormone axis in mice. *J Endocrinol.* 2020;245(2):327-342.

Christie S, O'Reilly R, Li H, **Nunez-Salces M**, Wittert G, Page AJ. Modulatory effect of methanandamide on gastric vagal afferent satiety signals depends on nutritional status. *J Physiol.* 2020;598(11):2169-2182.

The following conference proceedings resulted from training undergraduate students.

Plakhova N, Shah K, Li H, **Nunez-Salces M**, Gatford K, Page AJ, Muhlhausler BS, Lin PS-L. A novel strategy to prevent intrauterine growth restriction (2017). 11th annual Florey International Postgraduate Research Conference, Adelaide, Australia.

Nor MS, Isaacs NJ, Pezos N, **Nunez-Salces M**, Li H, Kaur H, Muhlhausler B, Roberts CT, Gatford KL, Young RL, Page AJ. Adaptations of gastrointestinal sensors, transporters and hormones during pregnancy (2018). The South Australian Health and Medical Research Institute (SAHMRI) Research Showcase, Adelaide, Australia.

Del Rosario C, Clarke G, **Nunez-Salces M**, Li H, Gatford K, Page AJ. The effect of dietary octanoic acid supplementation and pregnancy on gastric ghrelin and GOAT expression (2018). The University of Adelaide Undergraduate Research Conference, Adelaide, Australia

Clarke G, Del Rosario C, Li H, Kaur H, **Nunez-Salces M**, Gatford K, Page AJ. The effect of dietary octanoic acid supplementation and pregnancy on gastric ghrelin and GOAT expression (2019). Australian Society for Medical Research Week, Adelaide, Australia.

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List of abbreviations

AG	Acyl ghrelin
AgRP	Agouti-related peptide
AH 7614	4-Methyl-N-9H-xanthen-9-yl-benzenesulfonamide (selective free fatty acid receptor 4 antagonist)
ANOVA	Analysis of variance
ARC	Arcuate nucleus
Arg	Arginine
B2M	Beta-2 microglobulin
BAs	Bile acids
BMI	Body mass index
BSA	Bovine serum albumin
Calhex-23	4-Chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl) ethyl]amino]cyclohexyl]-benzamide hydrochloride (negative allosteric modulator of calcium-sensing receptor)
CaSR	Calcium-sensing receptor
CCK	Cholecystokinin
CCK-1	Cholecystokinin A receptor
CD36	Cluster of differentiation 36 (fatty acid translocase)
CFMB	(S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-yl)butamide (free fatty acid receptor 2 agonist)
CNS	Central nervous system
C-terminal	Carboxyl-terminus of amino acid chain

DAG	Des-acyl ghrelin
DB	Denatonium benzoate
DMSO	Dimethyl sulfoxide
EBWL	Excess body weight loss
ELISA	Enzyme-linked immunosorbent assay
FAT	Fatty acid translocase (cluster of differentiation 36)
FFAR	Free fatty acid receptor
FFAR1	Free fatty acid receptor 1 (G protein-coupled receptor 40)
FFAR2	Free fatty acid receptor 2 (G protein-coupled receptor 43)
$FFAR2^{-/-}$	Free fatty acid receptor 2 knockout
FFAR3	Free fatty acid receptor 3 (G protein-coupled receptor 41)
$FFAR3^{-/-}$	Free fatty acid receptor 3 knockout
FFAR4	Free fatty acid receptor 4 (G protein-coupled receptor 120)
FIJI	Fiji Is Just ImageJ
GABA	Gamma-aminobutyric acid
GB	Glandular base
GHSR1a	Growth hormone secretagogue receptor 1a
GI	Gastrointestinal
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GLP-2	Glucagon-like peptide-2
GLUT2	Glucose transporter 2
GM	Gut microbiome

GN	Glandular neck
GOAT	Ghrelin O-acyltransferase
GPR	G protein-coupled receptor
GPR120	G protein-coupled receptor 120 (free fatty acid receptor 4)
GPR40	G protein-coupled receptor 40 (free fatty acid receptor 1)
GPR43	G protein-coupled receptor 43 (free fatty acid receptor 2)
GPR6A	G protein-coupled receptor 6A
GPR93	G-protein coupled receptor 93
GW-9508	4-[[(3-Phenoxyphenyl)methyl]amino]benzenepropanoic acid (free fatty acid receptor 4 agonist)
GWAS	Genome-wide association studies
HFD	High-fat diet
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
i.v.	Intravenous
IP	Intraperitoneal
LAGB	Laparoscopic adjustable gastric banding
LCFA	Long-chain fatty acid
LEAP2	Liver-enriched antimicrobial peptide 2
L-Phe	L-Phenylalanine
LS	Luminal surface
MCFA	Medium-chain fatty acid
MCT	Medium-chain triglyceride
MEDICA16	3,3,14,14-Tetramethylhexadecanedioic acid (free fatty acid receptor 1 agonist)
mGluR4	Metabotropic glutamate receptor 4

mRNA	Messenger ribonucleic acid
NPS 2143	2-Chloro-6-[(2R)-3-[[1,1-dimethyl-2-(2-naphthalenyl) ethyl]amino-2-hydroxypropoxy]benzonitrile hydrochloride (selective calcium-sensing negative allosteric modulator)
NPY	Neuropeptide Y
OECD	The Organisation for Economic Co-operation and Development
P/D1 cells	Human ghrelin cells
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PC1/3	Prohormone convertase 1/3
Pept1	Peptide transporter 1
PFA	Paraformaldehyde
POMC	Proopiomelanocortin
PPIA	Peptidylprolyl isomerase A
Pro	Proline
PVN	Paraventricular nucleus
PWS	Prader-Willi syndrome
PYY	Peptide YY; peptide tyrosine tyrosine
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RYGB	Roux-en-Y gastric bypass
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
SG	Sleeve gastrectomy

SGLT1	Sodium-dependent glucose cotransporter 1
SLD	Standard laboratory diet
SNP	Single nucleotide polymorphism
SS	Somatostatin
SSO	Sulfosuccinimidyl oleate sodium (irreversible inhibitor of the cluster of differentiation 36)
SSTR1, 2, 3	Somatostatin receptor type 1, 2, 3
T1R1, 2, 3	Taste receptor type 1 member 1, 2, 3
T2D	Type 2 diabetes
T2R	Taste receptor type 2
T2R5, 10, 126	Taste receptor type 2 member 5, 10, 126
TC LPA5 4	5-(3-Chloro-4-cyclohexylphenyl)-1-(3-methoxyphenyl)-1H-pyrazole-3-carboxylic acid (G protein-coupled receptor 93 antagonist)
TG	Total ghrelin
VA	Vagal afferent
VTA	Ventral tegmental area
WHO	The World Health Organisation
X/A-like cells	Rodent ghrelin cells
α-gust	Alpha-gustducin
$\alpha\text{-gust}^{-/-}$	Alpha-gustducin knockout
αLA	Alpha-linolenic acid
β_1-AR	Beta 1-adrenergic receptor

Chapter 1: Introduction

1.1. OBESITY

1.1.1. Definition and impact

Obesity is a medical condition defined by The World Health Organisation (WHO) as "an abnormal or excessive fat accumulation that may impair health"[1]. Obesity is due to a chronic disruption in energy balance, where energy intake exceeds energy expenditure. It is diagnosed and classified by the body mass index (BMI ≥ 30 kg/m^2), which is the most commonly used scale for describing the weight relative to the height (Table 1.1)[2].

Table 1.1. Body mass index (BMI) classification.

Classification	BMI (kg/m^2)	
Underweight	<18.5	
Normal weight	18.5 - 24.9	
Overweight	25.0 - 29.9	
Obesity	Class I	30.0 - 34.9
	Class II	35.0 - 39.9
	Class III/Severe obesity	≥ 40.0

Source: The World Health Organisation (WHO)[2].

Obesity is a public health issue of epidemic proportions, with the WHO estimating that 13% of adults worldwide were obese in 2016[1]. The situation is even more alarming at a national level, with the Australian Bureau of Statistics reporting that 31% of Australian adults were obese in 2017-2018[3]. Furthermore, the prevalence of obesity is rapidly growing, and it is estimated that 35% of Australian adults will be obese by 2025[4].

Obesity is associated with a number of secondary health complications[5], including insulin resistance, type 2 diabetes, hypertension, cardiovascular diseases, non-alcoholic fatty liver disease, and kidney disease, among many others[5,6]. Additionally, obesity increases the risk of certain cancers, such as breast, prostate and colon, and is also associated with psychological and behavioural problems, including depression and eating disorders[5,6]. As a result, obesity is a life-threatening disease. Indeed, epidemiological studies have demonstrated that there is a positive correlation between BMI and risk of mortality, with BMI ≥ 25 kg/m^2 associated with higher mortality rates compared to normal weight (BMI = 18.5 - 24.9 kg/m^2)[7]. In this context, it is estimated that 2.8 million people die yearly as a consequence of being overweight or obese[8]. Moreover, important economic costs are attributed to obesity, with a recent report from The Organisation for Economic Co-operation and Development (OECD) estimating that each Australian pays an additional AUD $678 in taxes per year, in order to cover health care expenditure and lost labour market output due to overweight and obesity[9].

1.1.2. Causes

Obesity is caused by an energy imbalance generally associated with high consumption of calorie-dense foods and physical inactivity[1]. Dietary factors, such as bigger portion sizes and the increasing availability of highly palatable discretionary foods (i.e. energy-dense, nutrient-poor)[6], have been associated with increased energy consumption. In fact, a recent report assessing the healthiness of Australian supermarkets found that supermarkets heavily promote discretionary food consumption[10]. Furthermore, the modern sedentary

lifestyle is a key factor in the development of obesity, with the 2017-18 National Health Survey reporting that about half of Australians are insufficiently active[3]. These examples emphasise the critical role of the current "obesogenic environment" in the development of obesity[6]. On the other hand, there is a genetic component which contributes to the regulation of body weight. For example, genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) and pools of genes that are associated with obesity-related traits, such as high body weight and percentage of body fat[11-13]. However, development of obesity due to genetic defects is rare. For instance, only a few dozen cases of congenital mutation in the *ob* gene have been reported worldwide[14]. This mutation in the *ob* gene causes deficiency in the adipokine leptin[15] that has an essential role in the long-term modulation of energy homeostasis. As a result, leptin deficiency results in hyperphagia and development of obesity[16]. Another example, of genetic-derived obesity, is the Prader-Willi syndrome (PWS) with a prevalence of 1 in 10,000 to 30,000 people worldwide[17]. PWS is commonly caused by the lack of expression of the paternal chromosome 15q11-q13[18]. This chromosome abnormality causes a medical condition that is characterised by distinctive body features such as hypopigmentation, narrow forehead, almond-shaped eyes, triangular mouth and small hands and feet[18]. Moreover, during childhood (2-3 years old), most of PWS individuals develop severe hyperphagia and obesity that has been associated with elevated circulating levels of the stomach-derived orexigenic hormone ghrelin[18].

In summary, obesity is a multifactorial disease caused by environmental and genetic factors, and therefore, challenging to treat.

1.1.3. Current options for the treatment of obesity

1.1.3.1. Lifestyle interventions

Lifestyle interventions remain the first option for weight management[19]. These interventions reduce body weight by reducing food intake and/or increasing physical activity[19]. These behavioural interventions are characterised by a modest unsustained weight reduction[19]. In fact, it has been reported that two-thirds of individuals undergoing these programs lost <10% of their initial weight after one year, and nearly 50% returned to their original weight within five years[19]. In this context, evidence shows that poor weight loss is a key dropout factor during behavioural interventions[20]. Additionally, poor motivation, time and economic constraints, physical limitations and social pressure are other important reasons for poor adherence[21]. Overall, lifestyle interventions are not sufficiently effective for the treatment of obesity.

1.1.3.2. Pharmacotherapy

Numerous pharmacological options have been developed for the treatment of obesity. However, due to the high occurrence of serious adverse effects, such as psychiatric and cardiovascular events[22], multiple obesity drugs have been withdrawn. This section discusses prominent pharmacological treatments that are currently available.

Phentermine is a sympathomimetic agent that suppresses appetite in the central nervous system via an increase of the hypothalamic release of norepinephrine[23]. The average phentermine-induced weight loss reduction is 3.6 kg after six months of therapy, compared to placebo[24]. However, some serious side effects have been reported for this drug, including heart palpitations, restlessness, dizziness and increased blood pressure[22,24]. Therefore, phentermine is not recommended for individuals with cardiovascular risk[22,24].

Orlistat is a gastrointestinal (GI) lipase inhibitor that reduces the absorption of dietary fat in the small intestine and, therefore, reduces energy input[23]. On average, treatment with orlistat reduces 2.5-3.5 kg of initial body weight after 12 months[24]. However, individuals with orlistat treatment are prone to fat-soluble vitamin deficiencies[25]. Therefore, daily supplements containing vitamins A, D, E and K are advised[25]. Additionally, GI side effects derived from the inhibition of dietary fat absorption, such as faecal incontinence, flatulence, oily spotting and abdominal pain, are commonly reported. As a result, orlistat may not be well tolerated[22].

Liraglutide is part of the relatively new group of gut hormone-based therapies for the treatment of obesity. It is an analogue of the intestinal hormone, glucagon-like peptide-1 (GLP-1), and has a stimulating effect in insulin secretion[26]. Accordingly, liraglutide was initially used to treat type 2 diabetes[27]. However, liraglutide also reduces food intake via appetite centres in the brain and vagus nerve in the gut[23]. Therefore, it is also employed for weight

management[28], with a large clinical trial demonstrating that liraglutide treatment results in a reduction in body weight by approximately 5.6 kg per year, compared to the control group[28]. Similar to other obesity drugs, liraglutide treatment produces side effects, including nausea, vomiting and diarrhoea[28]. However, they are transient and mostly reported during the first 4-8 weeks of treatment[28]. As a result, this GLP-1 analogue is a widely used obesity drug that also improves glycaemic control.

Pharmacological options for the treatment of obesity produce a modest weight loss. Further, they are often associated with specific side effects that can restrict their use. However, the development of gut-hormone based therapies appears to be a promising area for the treatment of obesity and comorbidities, such as type 2 diabetes.

1.1.3.3. Surgical interventions

Bariatric surgery is a weight loss option available for individuals with class III obesity or individuals with class II obesity with undergoing severe health complications, such as type 2 diabetes or cardiovascular disease[29,30]. These surgical interventions are characterised by a significant reduction of body weight caused by a restriction of the gastric capacity, malabsorption of nutrients, or a combination of both approaches[31]. While numerous types of bariatric surgeries are available[32], sleeve gastrectomy (SG), Roux-en-Y gastric bypass (RYGB) and laparoscopic adjustable gastric banding (LAGB) are the most common procedures (Figure 1.1)[29].

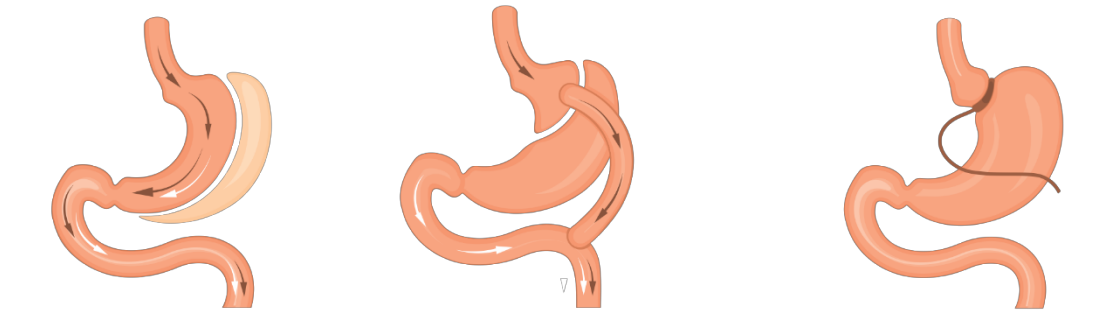

Figure 1.1. Most common bariatric surgery procedures.

SG involves the removal of 75-80% of the stomach greater curvature, which results in a smaller tubular-shaped stomach[30,33]. This is a restrictive surgery that reduces the volume of the stomach and accelerates gastric emtying[33,34]. In comparison, RYGB involves the division of the stomach into two parts and the creation of a small pouch at the level of the proximal stomach. This small pouch is connected to the mid-jejunum, with the gastric contents bypassing the proximal small intestine, which is a key GI region for the absorption of nutrients[30]. Thus, RYGB restricts food ingestion as well as causes nutrient malabsorption[30]. SG and RYGB are highly invasive procedures that may incur serious complications, such as GI leak[30,35]. Moreover, they are associated with vitamin and mineral deficiencies[36]. However, they are also very effective weight-loss surgeries, with an excess body weight loss (EBWL) higher than 60%[35,37].

Laparoscopic adjustable gastric banding (LAGB) involves the placement of a band around the proximal stomach to create a small pouch. As a result, there

is a restriction on the amount of food consumed[30]. The advantage of LAGB is the relative simplicity of the procedure without irreversible changes in the GI tract[31,38]. However, common complications associated with this procedure include pouch dilation and band slippage[31,38]. Moreover, LAGB has a lower weight-loss efficacy than SG and RYGB, with a reported EBWL lower than 50%[35,37].

1.1.3.3.1. Bariatric surgery, gut hormones, bile acids and microbiota

It is currently widely accepted that the effective weight loss associated with bariatric surgery is not simply due to the reduced energy input caused by the restriction of food intake and/or malabsorption of nutrients[30,39]. Indeed, bariatric surgery leads to significant changes in the secretion of gut hormones[39], bile acids[40] and the bacterial makeup of the GI tract[41], which play important metabolic roles in the modulation of energy homestasis[39-41]. While precise gut hormone responses to different types of bariatric surgery are not fully defined[42], a marked decrease in the secretion of the orexigenic gastric hormone, ghrelin, has been reported after SG[43-45]. Furthermore, an increase in the secretion of the anorexigenic intestinal hormone, GLP-1, has been consistently observed after SG and RYGB[45-47] (Table 1.2). The lower circulating levels of ghrelin after SG are explained by the removal of a major part of the stomach, which is the primary source of ghrelin production and secretion[48]. Additionally, the increased circulating levels of GLP-1 after SG and RYGB have been associated with the faster gastric emptying observed after SG[34], and the bypass of the proximal GI tract after RYGB[39]. These changes in GI anatomy lead to an increase in nutrient delivery to the distal small intestine[30,39]. While the density of small intestinal

GLP-1-secreting cells peaks in distal regions of the small intestine[49,50], it is well established that the secretion of GLP-1 is triggered by nutrient stimulation[51]. Therefore, GLP-1 hypersecretion appears to be explained by an increased arrival of nutrients to the distal small intestine[45-47]. Overall, the decrease in orexigenic hormone secretion (e.g. ghrelin) and/or increase in anorexigenic hormone secretion (e.g. GLP-1) have been associated with an increase in satiety and effective weight loss after SG[39,52]. Moreover, the increase in circulating GLP-1 levels appears to play an important role in the weight loss after RYGB[39,52], while the changes in circulating ghrelin levels are less defined with studies showing that circulating ghrelin is reduced[47,53-55], increased[56] or not changed after this surgery[45,46]. Possible explanations for these conflicting findings are technical variations between the surgical procedures[57], such as differences in the volume of the gastric pouch, length of the alimentary limb and vagal denervation. However, studies investigating the association between circulating ghrelin levels and the volume of the gastric pouch[56,58] and vagal denervation[56,59] are discrepant, and the relationship between the length of alimentary limb and ghrelin levels is unknown. Moreover, it is important to notice that circulating ghrelin levels increase under conditions of negative energy balance[60,61]. Therefore, weight loss reduction after RYGB may be another significant factor influencing ghrelin levels after this surgical procedure. However, further research is needed to understand the relationship between weight loss and ghrelin levels in RYGB.

Table 1.2. Effect of SG and RYGB on fasting ghrelin levels and postprandial GLP-1 levels

Bariatric surgery	*N* number	Follow up	Effect on fasting ghrelin levels	Effect on postprandial GLP-1 levels	Ref
SG	11	1 week; 3 months; 1 year	↓ (all time-points)	↑ (all time-points)	62
	14	1 week; 3 months	↓ (all time-points)	↑ (all time-points)	47
	8	6 weeks; 12 weeks	↓ (all time-points)	↑ (all time-points)	46
	15	6 and 12 months	↓ (all time-points)	↑ (all time-points)	63
	18	6 and 18 months	↓ (all time-points)	↑ (all time-points)	45
RYGB	8	6 weeks	↓	-	53
	13	1 week; 3 months	↓ (all time-points)	↑ (all time-points)	47
	6	1.5 years	↓	-	54
	18	2 years	↓	-	55
	12	1 week; 3 months; 1 year	↓ (1 week; 3 months) ↔ (1 year)	↑ (all time-points)	62
	10	6 weeks; 12 weeks	↔ (all time-points)	↑ (all time-points)	46
	23	6 and 18 months	↔ (all time-points)	↑ (all time-points)	45
	15	1, 2, 4 and 6 days; 1, 6 and 12 months	↓ (1, 2, 4 and 6 days) ↑ (1, 6, 12 months)	-	59
	96	6, 12 and 24 months	↑ (all time-points)	-	56

Fasting ghrelin levels and postprandial GLP-1 levels decrease (↓; pink), increase (↑; green) or do not change (↔; grey) after sleeve gastrectomy (SG) and Roux-en-Y gastric bypass (RYGB).

Bile acids (BAs) are conventionally defined as fat emulsifiers that facilitate intestinal fat absorption[64]. However, there is extensive information supporting the role of BAs in the modulation of the metabolism[65]. For instance, it has been demonstrated that BAs stimulate the secretion of glucoregulatory and energy homoeostasis-regulating hormones, such as GLP-1[66,67]. Further, it has been demonstrated that circulating concentrations of BAs are increased after SG[68,69] and RYGB[68-70]. The increase in BAs after these surgical procedures has been associated with improved glucose homeostasis[68-70]. Furthermore, the increase of BAs after RYGB has been positively associated to postprandial peak GLP-1 secretion[70]. Overall, available information indicates that BAs play an important role in the beneficial metabolic effects of bariatric surgery, particularly in the post-surgery improvement of glycaemic control[30].

The GI tract is colonised by a diverse range of microorganisms or gut microbiome (GM) that coexists in symbiosis with the human body[71]. Indeed, the GM is known to prevent overgrowth of pathogenic microorganisms, and contribute to a healthy function of the GI tract[71]. However, available research has identified a low GI microbial diversity[72] and richness[73] (i.e. dysbiosis) in obesity. This microbial dysbiosis seems to be sufficient to produce a higher increment of body fat in germ-free mice receiving caecal microbiota transplants from obese (*ob/ob*) mice, compared to germ-free mice receiving microbiota from lean mice[74]. In this regard, a large body of evidence indicates that bariatric surgery increases diversity[75] and richness[73,76] of the GM, with evidence indicating that post-surgery changes in the GM are associated with the positive metabolic outcomes of these surgeries[41]. For instance, it has been shown that

germ-free mice that underwent microbial faecal transplantation from RYGB-operated mice displayed lower fat mass and body weight than controls receiving transplants from sham-operated mice[77]. Moreover, similar results have been shown in germ-free mice receiving faecal transplants from obese RYGB-operated humans[78]. While extensive research is still needed to fully characterise the human GM and its influence in health and disease, emerging evidence shows that the bacteria colonising the gut play a significant role in the modulation of energy homeostasis.

In conclusion, bariatric surgery is the most effective option for the reduction of body weight. However, it is a highly invasive approach that is recommended in cases of severe obesity only. Nonetheless, the efficacy of bariatric surgery has highlighted the important role of the GI tract in the regulation of food intake and body weight[27,79]. Accordingly, novel approaches for weight management are now focusing on the GI tract.

1.2. THE GI TRACT, FOOD INTAKE AND METABOLISM

The GI tract is a highly specialised organ in charge of the digestion and absorption of ingested foods[64]. However, the GI tract also acts as a sensory system for monitoring ingested nutrients and other food components[80]. While it is known that the central nervous system (CNS) is able to rapidly anticipate food ingestion (i.e. cephalic phase of digestion)[81], it is also known that post-ingestive GI mechanical and chemical signals confirm information to the CNS regarding the amount and nutrient composition of a meal (gastrointestinal phase of digestion)[82]. This crosstalk between the GI tract and CNS plays a major role in the control of GI function and energy homeostasis, predominantly energy/food intake[81,82]. As a result, the GI tract is a target for the modulation of energy homeostasis, with vagal afferents and gut hormones considered promising areas for weight management.

1.2.1. Vagal afferents

Vagal afferent (VA) signalling is a major pathway for the control of GI function and energy intake[83-85]. VAs innervating the stomach and intestine are activated by mechanical and chemical stimuli derived from the arrival of nutrients into the GI tract[86,87]. This information is transmitted to the CNS, where it is processed, eventually leading to the control of GI function and food intake[83-85].

VAs innervating the stomach are predominantly mechanosensitive and influence initial stages of digestion and appetite suppression[88]. There are two types of mechanosensitive VAs in the stomach, tension and mucosal sensitive VAs[89]. Tension sensitive VAs respond to stomach distention[89], and are involved

in the generation of mechanical fullness for the inhibition of food intake[87]. Mucosal VAs respond to tactile contact[89], and are thought to be involved in the detection of food particle size providing negative feedback on the control of gastric emptying[88]. Additionally, it is also known that gastric VA responses can be modulated by gut hormones, such as the orexigenic hormone, ghrelin[89], and the anorexigenic hormone, GLP-1[90]. It has been shown that gastric VAs are positioned in close proximity to gastric ghrelin cells, with electrophysiological studies demonstrating that ghrelin reduces the response of tension-sensitive VAs[89]. Overall, these findings suggest that gastric ghrelin reduces the response of gastric VAs to mechanical stimuli to promote food intake via paracrine mechanisms. In this regard, a recent report demonstrated that intraperitoneal ghrelin injection fails to stimulate food intake in rats that underwent subdiaphragmatic vagotomy compared to sham-operated animals[91]. Findings that demonstrated that VA signalling is required for the orexigenic effect of exogenous ghrelin. However, the same report demonstrated that knockout of the ghrelin receptor (i.e. growth hormone secretagogue receptor 1a (GHSR1a)) in VAs innervating the GI tract increased meal frequency and tended to decrease meal size in rats without affecting cumulative food intake[91]. While these findings demonstrate the role of VAs in the modulation of food intake via ghrelin/GHSR1a signalling, they also emphasise the complexity of this orexigenic system. Additionally, it has been demonstrated that the stomach is highly innervated with VAs expressing the GLP-1 receptor (GLP-1R). While GLP-1 expressing VAs account for most of the mechanosensitive VAs in the gut[86], it has also been demonstrated that the GLP-1R agonists, Liraglutide and Exendin-4, suppress food intake via the GLP-1R expressed in vagal afferents[90].

The VA innervation of the intestine displays peak density in the duodenum and decreases towards the distal intestine[87]. Due to the key role of the intestine in nutrient breakdown and absorption[64], it has been traditionally assumed that intestinal VAs mostly respond to chemical stimulation[86], such as nutrients[92]. However, it has recently been shown that intestinal VAs also respond to mechanical distention to powerfully induce satiation and reduce food intake[87]. In this regard, it has been shown that mechanosensitive VAs expressing the GLP-1R innervate the intestinal muscle, although, their proportion is lower, compared to the GLP-1R neurons innervating the stomach mucosa[86]. On the other hand, VAs expressing the G-protein coupled receptor 65 (GPR65), which respond to a wide range of chemicals, such as serotonin and ingested nutrients[86], are highly expressed in the intestinal villi and are involved in the control of GI motility[86]. Overall, these findings demonstrate the role of intestinal VAs in mechanical and chemical detection of food components.

In summary, gastric and intestinal VAs are activated by mechanical and chemical stimuli, generated by ingested food, and relay this information to the CNS for the modulation of GI function and food intake. Consequently, they are neural targets for the modulation of energy balance in obesity.

1.2.2. Gut hormones and nutrient sensing

The GI tract has a key endocrine role for the secretion of multiple hormones in response to nutritional and energy status[93]. These gut hormones are signalling elements that inform the CNS and peripheral tissues on the availability of nutrients and energy for the modulation of a wide range of physiological

processes, including GI function, food intake and metabolism[94]. Gut hormones are produced and released by enteroendocrine cells that are equipped with receptors for the detection of multiple chemical signals, such as nutrients[80]. It is well established that nutrient digestion products activate these receptors or nutrient chemosensors[80], initiating an intracellular process that modulates the release of gut hormones (Figure 1.2). This includes key gut hormones involved in modulation of GI function and energy balance, such as cholecystokinin, GLP-1, peptide YY and ghrelin. The following sections describe their most prominent effects in GI function, food intake and metabolism, as well as the nutrient-sensing mechanisms modulating their secretion.

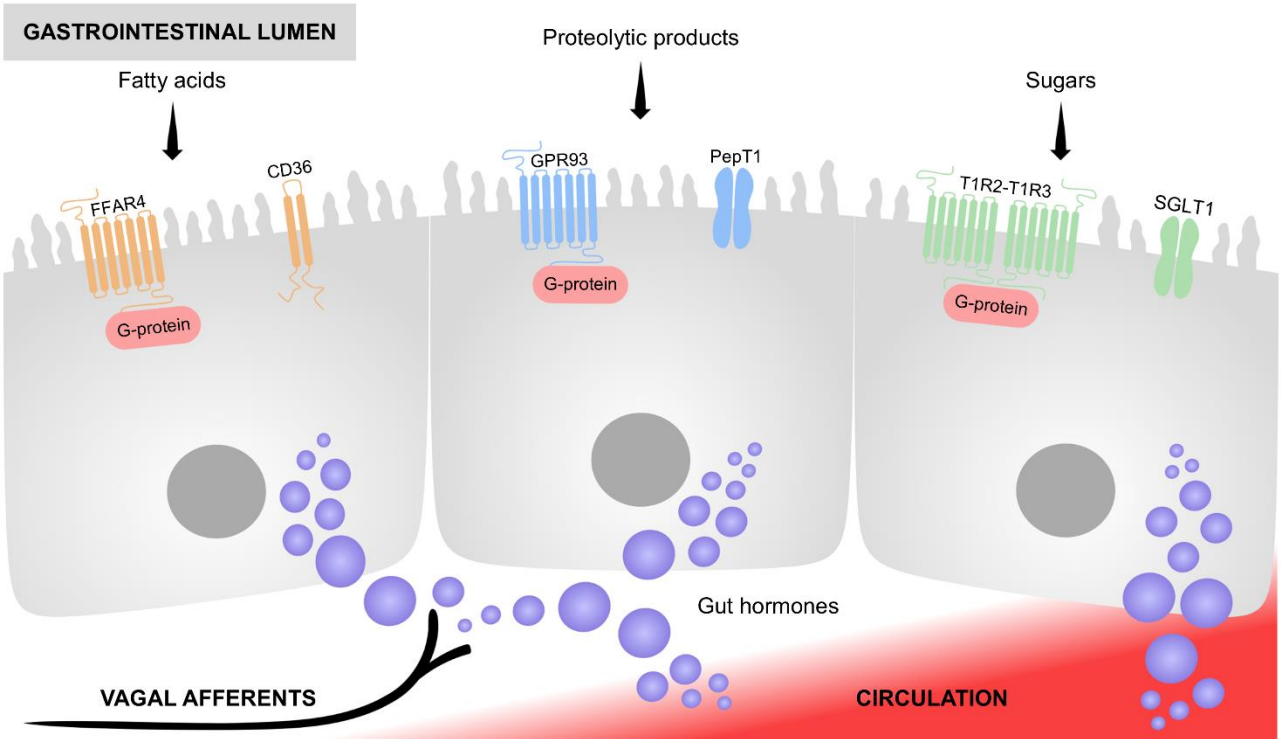

Figure 1.2. Schematic illustration of GI nutrient sensing and gut hormone secretion.

Following food ingestion, nutrient digestion products in the GI lumen activate nutrient chemosensors expressed on enteroendocrine cells. Prominent nutrient chemosensors include G protein-coupled receptors and nutrient transporters that upon activation modulate the secretion of gut hormones. Following secretion, gut hormones may enter the circulation and/or activate vagal afferents for the modulation of GI functions, food intake and metabolism. Fatty acid chemosensors (in orange): Free fatty acid receptor 4 (FFAR4) and cluster of differentiation (CD) 36. Chemosensors for protein digestion products (in blue): G protein-coupled receptor (GPR) 93 and peptide transporter 1 (PepT1). Sugar chemosensors (in green): The heterodimeric sweet-taste receptor formed by taste receptor type 1 (T1R) members 2 and 3, and sodium-glucose cotransporter 1 (SGLT1).

1.2.2.1. Cholecystokinin

Cholecystokinin (CCK) is primarily secreted by small intestinal I-cells[95] in response to food intake[96]. Postprandially, all macronutrients stimulate CCK secretion[97], which is known to be largely mediated by activation of GI nutrient chemosensors. Table 1.3 shows available evidence on the numerous nutrient chemosensors involved in the secretion of CCK and illustrates the powerful effect of nutrient signals in the secretion of gut hormones.

CCK displays important digestive functions, including stimulation of gallbladder contraction[98,99] and pancreatic enzyme secretion[99], as well as the deceleration of gastric emptying[100]. Further, CCK is a well-established satiety signal, with studies in rodents[101] and humans[102] demonstrating that CCK reduces food intake. This anorexigenic effect of CCK predominantly occurs via activation of VAs, with studies in rodents showing that abdominal vagotomy blocks the food intake-suppressing effect of CCK[103]. However, CCK may also modulate satiety directly in the CNS[104].

Table 1.3. Overview of nutrient chemosensors involved in the nutrient-mediated secretion of the gut hormones CCK, GLP-1 and PYY.

Nutrient	Nutrient chemosensor, ligand example	CCK	GLP-1	PYY	Ref
Protein hydrolysates and amino acids	T1R1/T1R3, glutamate	✓CL,R			105
	CaSR, protein hydrolysates and L-phenylalanine	✓R	✓R	✓R	106-108
	GPR93, protein hydrolysates	✓CL			109
	GPR6A, L-ornithine		✓CL		110
Fatty acids	FFAR1, α-linolenic	✓R	✓R		111,112
	FFAR2, propionate		✓R	✓R	113,114
	FFAR3, propionate		✓R		115
	FFAR4, α-linolenic	✓CL	✓CL,R		116,117
	CD36, α-linolenic	✓CL,R	✓CL		118,119
Glucose	T1R2/T1R3, glucose		✓CL,H	✓H	120-122
	SGLT1, glucose		✓CL,R,H	✓R	51,108,123,124
	GLUT2, glucose		✓R,H	✓R	51,108,123,125

The involvement of nutrient chemosensors in the secretion of gut hormones has been demonstrated in a variety of experimental settings, including cell lines (CL) and studies using rodents (R) and humans (H). Other abbreviations: Taste receptor type 1 (T1R) members 1, 2 and 3; calcium-sensing receptor (CaSR); G protein-coupled receptor (GPR) 93 and 6A; free fatty acid receptors (FFAR) 1, 2, 3 and 4; cluster of differentiation (CD) 36; sodium-glucose cotransporter 1 (SGLT1); glucose transporter 2 (GLUT2).

1.2.2.2. GLP-1

GLP-1 is released by intestinal L-cells[49], with high density of GLP-1-immunopositive cells in the distal regions of the small intestine and colon[49,50]. GLP-1 is secreted postprandially, with all macronutrients[126] playing a role in the mobilisation of this hormone. However, it is known that glucose is a powerful signal for the release of GLP-1[127]. The glucose-stimulated secretion of GLP-1 involves the activation of the sweet taste receptor, formed by the dimerisation of taste receptor type 1 (T1R) members 2 and 3 (i.e. T1R2/T1R3)[128]. Additionally, the sodium-glucose cotransporter 1 (SGLT1) and the glucose transporter 2 (GLUT2) play an important role in the glucose-induced secretion of GLP-1[51,123]. Further, the postprandial secretion of GLP-1 involves the activation of numerous protein digestion products and lipid chemosensors (Table 1.3). There are two bioactive forms of GLP-1 in humans, GLP-1 (7-36) amide and GLP-1 (7-37), with GLP-1 (7-36) amide accounting for approximately 80% of the total bioactive GLP-1 in the circulation[129]. Following secretion, GLP-1 influences GI functions, with available research showing the inhibitory effects of this hormone on GI motility[130] and gastric emptying[131]. Remarkably, GLP-1 also stimulates insulin secretion[132] and suppresses food intake[133]. Accordingly, GLP-1-based drugs have been used for the treatment of type 2 diabetes and obesity (see section 1.1.3.2: Pharmacotherapy)[27]. This food intake-suppressing effect occurs via VA[134] and central signalling pathways[135]. In fact, studies in rats have demonstrated that subdiaphragmatic vagotomy is not enough to block the anorexigenic effect of GLP-1[90]. Further, mechanistic studies have shown that GLP-1 activates brain centres involved in the modulation of food intake[135]. Moreover, it has been demonstrated that GLP-1 decreases fat storage in white

adipose tissue[136] and increases brown adipose tissue thermogenesis[137]. These effects on lipid metabolism are independent of the anorexigenic action of GLP-1 and are mediated centrally[136,137]. Overall, GLP-1 influences energy balance by regulating food intake as well as metabolism.

1.2.2.3. PYY

PYY is primarily secreted by intestinal L-cells, with the peak density of PYY-immunopositive cells observed in the colon[50]. Following food intake, luminal nutrients[96] play an essential role in the stimulation of PYY secretion. Considering that GLP-1 and PYY are secreted by L-cells[138], it is reasonable to assume that their secretion is controlled by the same mechanisms. However, PYY protein expression is higher in more distal L-cells subpopulations compared to GLP-1[50,139]. Additionally, PYY secretion is strongly stimulated by lipids[140], while GLP-1 is powerfully stimulated by glucose[127,128]. In this context, an elegant microscopy study demonstrated that human, pig, rat and mouse PYY and GLP-1 are mostly contained in different storage vesicles in L-cells[138]. Taken together, these findings suggest that PYY and GLP-1 secretion are controlled by different mechanisms, with available information on the nutrient-sensing mechanisms involved in the secretion of PYY displayed on Table 1.3.

Circulating PYY levels are a mix of the full-length peptide, PYY (1-36), and the truncated form, PYY (3-36)[141]. While both PYY forms slow gastric emptying[142], only PYY (3-36) reduces energy intake[143]. The anorexigenic effect of PYY (3-36) seems to be mediated via both, VAs[144] and central mechanisms[143].

1.2.2.4. Ghrelin

Ghrelin is a gastric hormone primarily secreted by mucosal P/D1 (humans) and X/A-like (rodents) cells[48,145]. It is well established that circulating ghrelin levels increase preprandially and drop postprandially, suggesting a role of ghrelin as a meal-initiating factor[146]. Indeed, ghrelin is the only GI hormone that stimulates food intake[147]. However, it also displays numerous physiological functions, including the stimulation of growth hormone secretion[48] and lipid accumulation[147]. Further, ghrelin influences digestion by stimulating GI motility[148] and gastric acid secretion[149]. Section 1.3 presents a detailed discussion of the physiological functions of ghrelin and the mechanisms controlling the secretion of this gastric hormone.

1.3. THE MODULATION OF GASTRIC GHRELIN SECRETION

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Contribution to the Paper	Conceptualized the review, wrote, edited and approved final version of the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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1.3.1. Abstract

Ghrelin is a gastric hormone with multiple physiological functions, including the stimulation of food intake and adiposity. It is well established that circulating ghrelin levels are closely associated with feeding patterns, rising strongly before a meal, and lowering upon food intake. However, the mechanisms underlying the modulation of ghrelin secretion are not fully understood. This review discusses current knowledge on the neural mechanisms stimulating fasting ghrelin levels, and peripheral mechanisms modulating postprandial ghrelin levels. Moreover, the therapeutic potential of targeting the ghrelin pathway is discussed in the context of the treatment of metabolic conditions associated with chronic excess energy intake, such as obesity, Prader-Willi syndrome and type 2 diabetes.

1.3.2. Introduction

Ghrelin is a gastrointestinal (GI) hormone originally identified in 1999 by Kojima *et al*. as the endogenous ligand of the growth hormone secretagogue receptor 1a (GHSR1a)[48]. In 2000 Tschöp *et al.* reported that ghrelin promotes food intake and body weight[147]. Accordingly, ghrelin was termed the “hunger hormone”. Since these early findings, numerous biological functions of ghrelin have been discovered, including the modulation of glucose[150] and lipid[151] metabolism, as well as the stimulation of gastric acid secretion, motility and emptying[148,149,152]. Consequently, the traditional role of ghrelin as a “hunger hormone” has evolved to the recognition of ghrelin as a pleiotropic hormone[145,153,154].

It is well established that circulating ghrelin levels are highly associated with feeding patterns, with an increase in circulating ghrelin levels before meals[146] and during fasting[155,156], and a reduction upon food intake[146]. However, the mechanisms responsible for these ghrelin responses are not fully understood. This review discusses the most prominent neural mechanisms stimulating ghrelin secretion during fasting, including the stimulation of sympathetic and vagal nerves. Furthermore, peripheral mechanisms governing postprandial ghrelin levels are also detailed, including the role of gastric nutrient-sensing mechanisms, small intestinal factors and hormonal signals. Moreover, the therapeutic potential of targeting circulating ghrelin levels in obesity, type 2 diabetes and Prader-Willi syndrome is discussed.

1.3.3. The ghrelin system

Ghrelin is a 28-amino acid peptide hormone primarily produced by P/D1 (human) and X/A-like (rodent) cells of the stomach[145]. The ghrelin gene encodes a 117-amino acid pre-proghrelin peptide that undergoes a series of processing steps for its maturation and activation[157] (Figure 1.3). Pre-proghrelin is first cleaved to produce proghrelin, which is then cleaved at the C-terminal Pro-Arg site by the enzyme prohormone convertase 1/3 (PC1/3) to produce the mature ghrelin peptide[158]. Ghrelin can also undergo an acylation on its third serine residue. This acyl modification is catalysed by ghrelin O-acyltransferase (GOAT), an essential ghrelin-processing enzyme for the production of acyl ghrelin (AG)[159,160] that binds the GHSR1a. Although GOAT is able to utilise a broad range of fatty acids (C2 to C16) for acylation, octanoic acid is the major acyl donor for the activation of ghrelin[161,162]. Mechanistic studies in the

ghrelinoma PG-1 cell line suggest that ghrelin cells can produce acyl donors by β-oxidation of long-chain fatty acids obtained from the circulation[163]. In addition, increased ingestion of medium-chain fatty acids can stimulate AG levels in humans[164,165] and rodents[166]. Less than 10% of total ghrelin (TG) in the circulation is acylated, while 90% is found as des-acyl ghrelin (DAG)[157,167]. Limited information is available on the biological effects of DAG, although there is growing evidence suggesting that DAG mostly counteracts AG effects[168,169], possibly via GHSR1a-independent pathways, and activation of its own, yet unidentified, receptor[153,154]. The AG:DAG ratio in the circulation is influenced by different deacylating enzymes that transform AG into DAG, including butyrylcholinesterase[170,171] and acyl-protein thioesterase 1[172] in humans, and carboxylesterase in rats[171]. Moreover, AG signalling can be modulated by the liver-enriched antimicrobial peptide 2 (LEAP2), which has been recently designated as the first endogenous non-competitive allosteric antagonist of the GHSR1a. Indeed, Ge *et al.* demonstrated that intraperitoneal administration of LEAP2 potently inhibited ghrelin action in mice, including ghrelin-induced growth hormone release and food intake[173]. This review discusses available knowledge on the modulation of circulating AG and TG levels, and the potential therapeutic use of different members of the ghrelin system in the context of metabolic diseases.

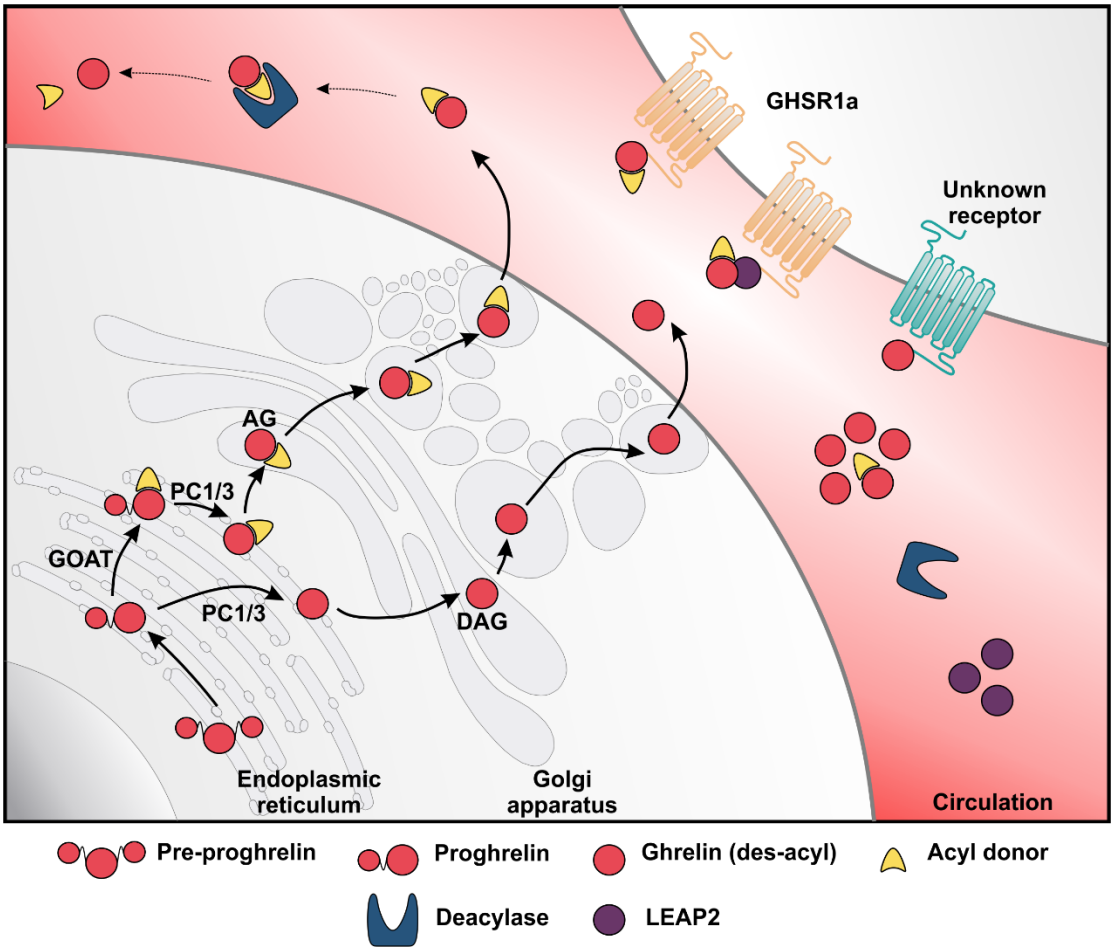

Figure 1.3. Schematic illustration of the post-translational processing of ghrelin.

In the endoplasmic reticulum, pre-proghrelin peptides are cleaved to form proghrelin, which can be subsequently acylated by the enzyme ghrelin O-acyltransferase (GOAT). In a final processing step, the prohormone convertase, PC1/3, cleaves the proghrelin peptides to produce the mature forms of acyl ghrelin (AG) and des-acyl ghrelin (DAG) that are packaged into secretory vesicles by the Golgi apparatus. After secretion, AG activates the growth hormone secretagogue receptor 1a (GHSR1a; orange), whereas DAG may activate its own, yet unidentified, receptor (green). AG signalling can be affected by the activity of deacylases that degrades AG in the circulation, and the liver-expressed antimicrobial peptide 2 (LEAP2), which can bind the GHSR1a to antagonise AG signalling in a non-competitive manner.

1.3.3.1. Ghrelin, food intake and metabolism

Ghrelin is a multifaceted gut hormone with an important role in the modulation of food intake, and lipid and glucose metabolism. To date, ghrelin is the only known GI hormone with an orexigenic role[174,175]. It promotes positive energy balance predominantly via the hypothalamic arcuate nucleus (ARC)[176-178], where it activates neurons secreting orexigenic peptides (i.e. neuropeptide Y (NPY) and agouti-related peptide (AgRP)), that signal to the paraventricular nucleus (PVN) to stimulate food intake, and indirectly inhibits neurons expressing the anorexigenic peptide proopiomelanocortin (POMC) via gamma-aminobutyric acid (GABA) inputs from NPY/AgRP neurons[176,178]. Ghrelin also stimulates food intake in other central networks, including dopaminergic neurons of the ventral tegmental area (VTA) that are associated with reward-based eating behaviour[179]. In addition, ghrelin stimulates energy intake via peripheral pathways. For example, it has been reported that ghrelin reduces the mechanosensitivity of tension-sensitive gastric vagal afferent endings[89]. As a result, ghrelin may attenuate the perception of gastric fullness to promote food intake. In addition, ghrelin induces adiposity independently of its orexigenic effect[180]. Chronic central[147,180,181] and peripheral[147,182,183] administration of ghrelin promotes adiposity in rodents, with increased gene expression of adipogenic and fat storage-promoting enzymes in white adipose tissue[180,181,183] and decreased expression of fat oxidation enzymes[181]. Additionally, central[181] and peripheral[182] administration of ghrelin in rodents downregulates thermogenesis-related uncoupling proteins in brown adipocytes. Furthermore, ghrelin induces lipid accumulation in the liver[183]. Research has shown that ghrelin influences glucose homeostasis by increasing glycaemia[184], decreasing

pancreatic insulin secretion[185] and plasma insulin levels[186], as well as decreasing insulin sensitivity in humans[187]. Moreover, ghrelin interacts with other gut hormones to modulate energy balance. For instance, studies in rats have shown that intravenous infusion of ghrelin stimulates food intake, while attenuating the food intake-suppressant effects of glucagon-like peptide 1 and peptide YY[188]. Due to the significant influence of ghrelin on food intake, as well as lipid and glucose metabolism, the modulation of ghrelin circulating levels is a promising target in the treatment of a variety of metabolic disorders, including obesity, type 2 diabetes and Prader-Willi syndrome.

1.3.4. Neural stimulation of ghrelin secretion during fasting

Circulating ghrelin levels are increased preprandially[146] and during fasting[155,156]. Findings from numerous reports have demonstrated that the autonomic nervous system is involved in the increase in circulating ghrelin levels during fasting[189-193]. The following sections discuss available literature on stimulation of ghrelin secretion via the sympathetic nervous system and the vagus nerve.

1.3.4.1. The sympathetic nervous system

Ghrelin secretion is readily increased by the sympathetic nervous system. It has been shown, in anaesthetised rats, that plasma TG levels are increased by electrical stimulation of sympathetic nerves[192]. Similarly, chemical sympathetic nerve activation with tyramine increases circulating TG[192]. β_1-adrenergic receptor (β_1-AR) is highly enriched in gastric ghrelin cells in mice[193]. Accordingly, adrenaline, noradrenaline and the β_1-AR agonist, isoproterenol,

strongly increase AG and DAG secretion from rodent gastric mucosal cells and ghrelinoma PG-1 cells[189,190,193,194]. In agreement with these results, Mani *et al.* demonstrated blunted AG and TG ghrelin secretion in mice lacking β_1-AR specifically in ghrelin cells[195]. Moreover, the fasting-induced stimulation of plasma AG and DAG levels in mice is totally blocked by administration of the catecholamine-depleting agent, reserpine, as well as the β_1-AR antagonist, atenolol[190]. Therefore, stimulation of ghrelin secretion during fasting results from activation of sympathetic nerves that release adrenergic agents, which subsequently activate β_1-AR on gastric ghrelin cells[193].

1.3.4.2. The vagus nerve

The vagus nerve is an important pathway for the transmission of information between the GI tract and the central nervous system[88]. Consequently, various reports have assessed the role of the vagus nerve in the control of circulating ghrelin levels[196,197]. Mechanistic studies in the isolated rat stomach have shown that electrical vagal stimulation increases TG secretion[191]. Furthermore, studies in sham-operated and vagotomised rats show that fasting circulating TG levels were reduced after vagotomy, while postprandial TG levels were not affected by vagotomy[196]. Consistent with these findings, a recent study in humans demonstrated that fasting baseline TG levels were lower in vagotomised participants compared to healthy controls, while circulating TG concentrations after meals are equally suppressed in healthy and vagotomised individuals[197]. Therefore, evidence suggests that the vagus nerve plays an important role in increasing ghrelin levels during fasting, but is not involved in the modulation of postprandial ghrelin levels.

1.3.5. Peripheral mechanisms modulating postprandial ghrelin secretion

Circulating ghrelin levels are reduced after food intake[146,198]. Studies in healthy humans demonstrate that mechanical distention of the stomach with water[199] and highly viscous guar solution[200] does not change plasma ghrelin levels. Similarly, intragastric infusions of water under closed-pylorus conditions failed to suppress circulating ghrelin levels in rats[201]. Together, this evidence suggests that food-related mechanical stimuli may not contribute to the postprandial suppression of plasma ghrelin levels. However, abundant evidence indicates that postprandial circulating ghrelin levels are modulated by multiple peripheral signals, including gastric nutrient-sensing mechanisms, small intestinal factors and hormonal signals. This section discusses the extensive repertoire of mechanisms modulating circulating ghrelin levels after food intake, with particular emphasis on the gastrointestinal control of ghrelin secretion.

1.3.5.1. The emerging role of gastric nutrient-sensing mechanisms in ghrelin secretion

Gastric ghrelin cells are located in the glandular base and neck of the stomach, with no direct contact with the gastric lumen[202,203]. Additionally, the gastric mucous-bicarbonate barrier of the stomach provides a shield that minimises lumen-to-mucosa interactions and protects the stomach from self-digestion[204]. Accordingly, gastric luminal events may not play a major role in the modulation of ghrelin secretion. However, there is increasing evidence showing that nutrient sensing, at the level of the gastric ghrelin cells, contributes to the control of ghrelin mobilisation, including mechanistic studies demonstrating that

ghrelin cells are equipped with nutrient sensors (Figure 1.4) that modulate ghrelin secretion (Table 1.4). Therefore, post-absorptive nutrient signals, originating from the blood, may influence gastric ghrelin secretion. The following sections discuss available information on nutrient-sensing mechanisms in the gastric ghrelin cells and the modulation of gastric ghrelin secretion by nutrients and bitter compounds.

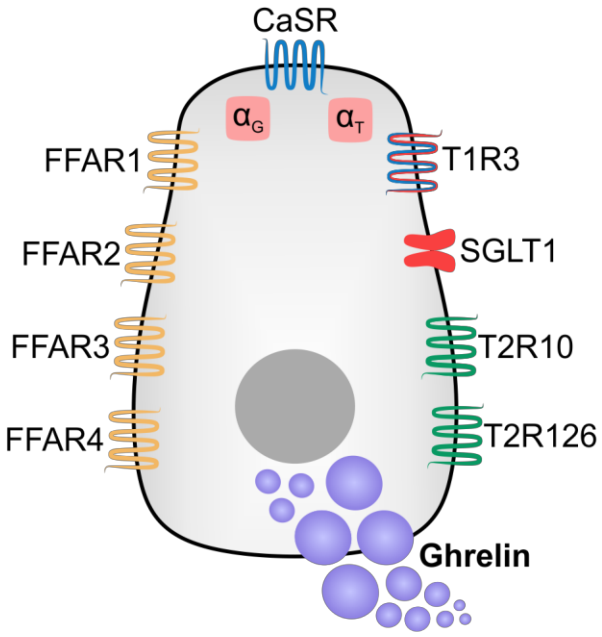

Figure 1.4. Nutrient sensors and taste-transduction components expressed in the gastric ghrelin cells.

Numerous chemosensors for fatty acids (in orange: Free fatty acid receptor (FFAR) 1, 2, 3 and 4), protein digestion products (in blue: Calcium-sensing receptor (CaSR) and taste receptor type 1 (T1R3)), glucose (in red: T1R3 and sodium-dependent glucose cotransporter 1 (SGLT1)) and bitter compounds (in green: taste receptor 2 (T2R) members 10 and 126) are expressed in ghrelin cells. Also, the taste transduction components (in pink), α-gustducin (α_G) and α-transducin (α_T), are highly co-expressed within the gastric ghrelin cell population.

Table 1.4. Overview of mechanistic studies investigating the role of chemosensors for nutrients and bitter compounds in the secretion of ghrelin from the stomach.

Nutrient	Nutrient chemosensor, ligand example	Method for assessing ghrelin secretion	Role on ghrelin secretion	Ref
Protein hydrolysates and amino acids	T1R3, glutamate and alanine	MGN3-1 cells	↑AG	[205]
		Human obese gastric segments	↔TG	[206]
	CaSR, protein hydrolysates and L-phenylalanine	MGN3-1 cells	↑AG	[205]
		Human obese gastric segments	↔TG	[206]
	GPCR6A, arginine	MGN3-1 cells	↑AG	[205]
Fatty acids	FFAR1, α-linolenic (LCFA)	Mice (*in vivo*)	↔TG; ↔AG	[207]
		MGN3-1 cells	↔TG; ↔AG	[207]
	FFAR2, propionate (SCFA)	Wild type and FAR2$^{-/-}$ mice mucosal cultures	↓AG	[193]
	FFAR3, propionate (SCFA)	Wild type and FFAR3$^{-/-}$ mice mucosal cultures	↔AG	[193]
	FFAR4, α-linolenic (LCFA)	Wild type and FFAR4$^{-/-}$ mice mucosal cultures	↓AG	[193]
		Mice (*in vivo*)	↔↓TG; ↓↑AG	[193,207,208]
		SG-1 cells	↓AG	[208]
		MGN3-1 cells	↔TG; ↔↓AG	[207]
Glucose	T1R3, glucose	Human obese mucosal cultures	↓AG	[209]
		MGN3-1 cells	↔AG	[210]
	SGLT1, glucose	Human obese mucosal cultures	↓AG	[209]
		MGN3-1 cells	↔AG	[210]
	GLUT2, glucose	MGN3-1 cells	↔ AG	[210]
Bitter compounds	T2Rs, denatonium benzoate and quinine (bitter compounds)	Human obese mucosal cultures	↑ AG	[209]
		Mice (*in vivo*)	↑TG; ↑AG	[211]
		Humans (*in vivo*)	↓TG; ↔↓AG	[212,213]
Taste transduction components	α-Gustducin, glucose	Wild type and α-Gust$^{-/-}$ mice (*in vivo*)	↔TG; ↔AG	[210]
	α-Gustducin, denatonium benzoate and quinine (bitter compounds)	Wild type and α-Gust$^{-/-}$ mice (*in vivo*)	↔TG; ↑AG	[211]

Total (TG) and acyl (AG) ghrelin secretion are increased (↑), decreased (↓) or not affected (↔) by activation of nutrient-sensing mechanisms. For detailed information on these studies see **sections 1.3.5.1.1-1.3.5.1.4**.

1.3.5.1.1. Protein digestion products

Several human studies have shown that high-protein meals and beverages display a more sustained and, therefore, stronger reduction in postprandial ghrelin levels than other nutrients[198,214-216]. However, evidence from mechanistic studies demonstrated that protein breakdown products act locally to stimulate gastric AG secretion[205,206]. Hormonal secretion studies using MGN3-1 cells[205], full-thickness gastric segments from mice[205] and human gastric mucosal segments[206] show that protein hydrolysates promote AG mobilisation from the stomach. Additionally, the protein hydrolysate and aromatic amino acid sensor, calcium-sensing receptor (CaSR)[206], as well as the taste receptor type 1 member 3 (T1R3)[206,217], that heterodimerises to form the umami receptor, T1R1/T1R3, have been identified in gastric ghrelin cells. Release experiments, using ghrelinoma MGN3-1 cells, demonstrate that protein hydrolysate-induced AG secretion is partially inhibited by calhex-231, a CaSR negative allosteric modulator, and calindol, an antagonist of the G protein coupled-receptor 6A (GPR6A, basic amino acid sensor), which also acts as a CaSR positive allosteric modulator[205]. Moreover, experiments using the same cell line confirmed that numerous amino acids (aromatic: L-phenylalanine, L-tryptophan; branched-chain: L-alanine; sulphur-containing: L-methionine and threonine) increased secretion of AG[205]. This amino acid-mediated release of AG was partially prevented by CaSR inhibition (antagonist: calhex-231) in the case of L-phenylalanine, and by inhibition of T1R3 (antagonist: gurmarin) and CaSR (antagonist: calhex-231) in the case of L-alanine[205]. Therefore, protein hydrolysates and amino acids may stimulate AG secretion via nutrient-sensing receptors, such as CaSR and T1R3. Remarkably, TG secretion from human

gastric mucosal segments is inhibited by protein hydrolysates in a T1R3 and CaSR-independent manner[206]. These findings suggest that secretion of AG and DAG (i.e. comprising more than 90% of TG) are controlled by different mechanisms. Moreover, *in vivo* studies have demonstrated that intragastric administration of protein hydrolysates and L-phenylalanine in rodents decreased circulating levels of TG and AG[205,218]. Accordingly, it may be possible that additional physiological mechanisms, such as the rise in circulating gut hormones levels after protein ingestion[216], inhibit ghrelin secretion and override the local AG stimulating effect of protein hydrolysates.

In summary, extensive research has demonstrated that ingestion of proteins and their digestion products suppress circulating ghrelin levels in humans[198,214,219] and rodents[205,218,220]. In contrast, *in vitro* studies demonstrated that a diverse range of protein digestion products increases the local mobilisation of gastric AG via activation of nutrient chemosensors. Further mechanistic studies are essential to understand the gastric and systemic mechanisms involved in the modulation of TG and AG release by proteins.

1.3.5.1.2. Fatty acids

Intravenous[221] and oral administration[193,200,222] of lipids in humans[200,221] and mice[193,222] have been shown to reduce ghrelin levels in the circulation. While precise mechanisms are largely unknown, a range of free fatty acid receptors (FFARs) are expressed on ghrelin-producing cells of the mouse stomach[193,207]. Therefore, it is plausible that the postprandial inhibition of ghrelin secretion involves fatty acid sensing.

FFAR1 (GPR40) is a chemosensor for long-chain fatty acids (LCFAs). Available research demonstrates co-localisation of DAG and FFAR1 in the mouse stomach[207]. Nonetheless, the FFAR1 agonist, MEDICA16, does not modulate TG and AG secretion in mice and MGN3-1 cells[207]. Similar outcomes have been reported for FFAR3 (GPR41), which is activated by short-chain fatty acids (SCFAs) and is expressed in about 50% of mouse gastric ghrelin cells[193]. Despite this co-expression with ghrelin, gastric mucosal cells from wild type and FFAR3$^{-/-}$ mice present a comparable propionate-induced reduction of AG secretion[193]. Therefore, the modulation of ghrelin release by propionate appears to involve a different chemosensor. In this regard, gene and protein expression experiments have confirmed the presence of the SCFA receptor, FFAR2 (GPR43), in ghrelin-positive cells[193,222]. Functional studies exposing mouse primary gastric mucosal cells to the FFAR2 agonist, (S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-yl)butamide (CFMB), have shown that activation of FFAR2 reduces AG secretion in a dose-dependent manner[193]. In addition, gastric mucosal cells derived from FFAR2$^{-/-}$ mice failed to show propionate-dependent suppression of AG secretion[193]. These findings strongly suggest that FFAR2 modulates ghrelin secretion from gastric ghrelin cells.

FFAR4 (GPR120) is the most enriched LCFA receptor in mouse gastric ghrelin cells[193,222], with numerous reports investigating the role of FFAR4 in the modulation of ghrelin secretion[193,207,208]. Engelstoft *et al*. demonstrated that the FFAR4 agonist, Compound B, suppressed AG release *in vitro*, an effect not observed in experiments using gastric mucosal cells from FFAR4$^{-/-}$ mice[193].

Moreover, mice orally-dosed with Compound B displayed dose-dependent reductions of TG circulating concentrations[193]. However, studies with other FFAR4 agonists have shown conflicting outcomes. *In vivo* studies in mice showed that the FFAR4 agonist, grifolic acid[207], increased AG without affecting circulating TG levels, while the FFAR4 agonist, GW-9508[208], decreased circulating AG levels. Moreover, in ghrelinoma cell lines, GW-9508 and α-linolenic acid reduced AG secretion from SG-1 cells[208], while grifolic acid had no effect on TG and AG secretion from MGN3-1 cells, and α-linolenic acid decreased AG secretion without affecting TG release from these cells[207]. While these conflicting findings could be partially explained by different methodological approaches, it is also known that FFAR4 effects can be coupled to different effectors (i.e. G-proteins[193] and β-arrestins[223]), which add complexity to the study of FFAR4 signalling and may explain the inconsistent results observed in the literature.

Studies in humans[164,165] and rodents[166,207] have shown that gastric AG content and circulating AG levels can be boosted with chronic ingestion (i.e. 1-2 weeks) of medium-chain fatty acids (MCFAs) and medium-chain triglycerides (MCTs). On this subject, Janssen *et al.* demonstrated that the taste-specific G protein, α-gustducin, which is highly co-localised with gastric ghrelin[207], plays a role in the acylation of ghrelin induced by MCFA intake[207]. In this study, wild type and α-gustducin knockout (α-gust$^{-/-}$) mice were given a glyceryl trioctanoate-enriched diet for 2 weeks. This diet specifically increased AG content in the stomach of control mice, but did not change gastric AG levels in α-gust$^{-/-}$ mice. Moreover, the glyceryl trioctanoate-enriched diet also increased ghrelin mRNA

levels in control animals, but not in α-gust$^{-/-}$ mice, and did not affect GOAT mRNA levels in both groups[207]. Taken together, these findings suggest that gastric lipid-sensing is important for the production and acylation of ghrelin.

In conclusion, available evidence strongly supports the involvement of α-gustducin in the acylation of ghrelin, as well as the role of FFAR2 and FFAR4 in the modulation of ghrelin secretion. However, conflicting findings on the role FFAR4 in ghrelin secretion call for further research to clarify the stimulating or suppressant role of FFAR4 in the lipid-mediated secretion of TG and AG.

1.3.5.1.3. Glucose

Gastric ghrelin cells have been shown to secrete ghrelin in a glucose-dependent manner. Sakata *et al.*[194] showed an inverse association between glucose concentration and the release of AG and DAG from primary cultures of gastric mucosal cells: low concentrations of glucose (1 mM) enhanced the release, whereas high concentrations (10 mM) reduced it. These findings suggest that gastric mucosal cells, possibly ghrelin cells, can sense glucose.

The sodium-glucose cotransporter 1 (SGLT1) and T1R3 are expressed in ghrelin cells[206,217]. T1R3 typically heterodimerises (T1R2/T1R3) to form a receptor for the detection of sweet compounds[224]. However, T1R3 has also been described to homodimerise (T1R3/T1R3)[225,226], and respond to high-sucrose concentrations (500 mM) in HEK-293 cells[224], and low glucose levels (16.7 mM) in pancreatic cells[227]. In gastric mucosal cells from obese individuals, the T1R3-specific antagonist, lactisole, and the SGLT1 antagonist, phlorizin,

reversed the reduction in AG secretion in response to 100 mM glucose[209]. This is in contrast to experiments using the gastric MGN3-1 ghrelinoma cell, where a reduction in AG secretion by 200 mM glucose did not involve T1R3, SGLT1 or the glucose transporter 2 (GLUT2)[210]. The G protein, α-gustducin, is part of the small intestinal intracellular cascade for the transduction of sweet stimuli[228]. However, intragastric administration of glucose (4 g/kg) in wild type and α-gustducin knockout mice generated comparable reductions in circulating TG and AG levels, demonstrating that glucose modulates ghrelin secretion in an α-gustducin-independent manner[210]. Furthermore, in gastric mucosal cells from obese individuals, AG release is reduced in the presence of 3-O-methyl-D-glucopyranose, an absorbable but non-metabolisable glucose analogue. This suggests that glucose absorption rather than intracellular glucose metabolism is necessary for the reduction in AG secretion. However, the glycolysis disruptor and glucose analogue, 2-deoxy-D-glucose, has been shown to block the glucose-dependent suppression of AG in mouse primary gastric mucosal cells[194]. Therefore, the modulation of gastric ghrelin secretion by intracellular metabolic pathways remains unclear.

In conclusion, gastric ghrelin cells seem to engage receptor-based sweet sensing and glucose transport mechanisms to deploy ghrelin. Additional studies are needed to further determine the downstream signalling pathways.

1.3.5.1.4. Bitter compounds

Increasing evidence is available on the role of bitter compounds in the modulation of ghrelin secretion. While detection of bitter compounds is

accomplished by a large family of type 2 receptors (T2Rs)[229,230], existing reports show that T2R10[209], T2R126[231] and α-transducin[211], a G protein involved in the detection of bitter compounds[232], are co-localised with ghrelin cells. Further, studies using human gastric mucosal cultures from female and male obese subjects have shown that activation of a variety of bitter receptors, such as T2R10 (agonists: denatonium benzoate (DB), chloroquine and erythromycin A) and T2R5 (agonist: 1,10-phenanthroline) stimulates AG secretion[209]. Similarly, studies in mice show that intragastric administration of a bitter agonist cocktail increased AG secretion in an α-gustducin-dependent manner, while the increase in TG secretion was independent of α-gustducin[211]. Further, this stimulation in AG and TG secretion was accompanied by a transient increase in food intake (30 min after bitter agonists administration), followed by prolonged suppression of food intake (subsequent 4 hours)[211]. Additionally, a few studies in healthy women have shown that intragastric administration of the bitter compound, quinine, influences circulating ghrelin levels, with one study showing a decrease in circulating TG and AG levels[212], while another study showed a reduction in plasma TG without significantly affecting AG[213]. While study design and species differences may explain the different outcomes of these studies, bitter compounds are effective modulators of gastric ghrelin secretion. Further investigations are necessary to understand the signals overriding the gastric stimulation of ghrelin secretion *in vivo*.

1.3.5.2. Small intestinal factors influencing ghrelin secretion

Abundant evidence indicates that the small intestine plays an important role in the postprandial suppression of ghrelin levels. Studies in humans and rodents

have shown an equal reduction in plasma ghrelin levels after the infusion of nutrient solutions in the stomach and duodenum[233-235]. These results indicate that nutrients suppress circulating ghrelin levels despite bypassing the stomach, and that small intestinal luminal cues, are capable of suppressing postprandial ghrelin levels. Indeed, there is evidence to indicate that nutrient signals and hyperosmolarity in the small intestine are involved in the postprandial modulation of ghrelin levels.

1.3.5.2.1. Small intestinal nutrient signals

Small intestinal nutrient signals, including lipids, protein and carbohydrate, are important drivers of postprandial ghrelin suppression. Intraduodenal infusion of long-chain triglyceride emulsion or olive oil in healthy men potently suppresses plasma ghrelin levels[236,237]. This lipid-induced ghrelin suppression is mediated by lipid digestion, since the effect of lipid can be completely abolished in the presence of lipase inhibitor, orlistat[236,237]. Furthermore, intraduodenal infusion of protein (whey hydrolysate) also suppresses plasma ghrelin levels in healthy men[238]. Moreover, the distal small intestine appears to be important for the glucose-dependent reduction in circulating ghrelin levels, with a study in humans showing that ghrelin suppression is only observed when distal regions of the small intestine (i.e. beyond 60 cm post-pylorus) are exposed to glucose[239]. These findings indicate that the food-related signals in the small intestine are involved in the suppression of plasma ghrelin levels.

In addition, studies in healthy humans, investigating the relation between the rate of gastric emptying and circulating ghrelin levels, have shown that the rate

of delivery of nutrients to the small intestine is a determining factor for the degree of postprandial reduction of circulating ghrelin levels[216]. For example, high-protein drinks display a slower gastric emptying and a prolonged inhibition of circulating ghrelin levels compared to drinks containing other nutrients[216]. In contrast, the transient suppression of plasma ghrelin levels after high-carbohydrate meals[198,219] may be associated with the quicker arrival of carbohydrates to the small intestine[240]. Moreover, studies investigating the effect of intraduodenal infusions of lipids and proteins on circulating ghrelin levels, demonstrated that under controlled conditions of nutrient delivery into the small intestine (3 kcal/min), lipids and proteins potently reduced circulating ghrelin levels to equal magnitudes[238].

1.3.5.2.2. Duodenal hyperosmolarity

Studies in rats[241] and humans[242] have demonstrated that duodenal infusions of 5-fold hypertonic saline (≈ 1500 mOsm/L) significantly suppressed plasma TG concentrations. The osmolarity of ingested fluids is monitored by central osmoreceptor neurons and afferent systems[243,244]. However, the hyperosmolarity-mediated suppression of ghrelin secretion seems to be independent of intestinal neural activity, as lidocaine, an intestinal afferent and submucosal neuronal blocker, did not reverse the ghrelin-suppressing effect of hypertonic saline in rats[241]. A recent clinical trial[242], performed in healthy young men, reported that duodenal infusions of 5-fold hypertonic saline increased circulating levels of cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), and suppressed circulating TG levels. As a result, the hyperosmolarity-derived inhibition of TG secretion might be explained by the

elevation of ghrelin-suppressing peptides, including CCK and PYY (see section 1.3.5.3: *Hormonal signals influencing ghrelin secretion*).

In conclusion, luminal small intestinal events including nutrients and osmolarity are important contributing factors for the inhibition of circulating ghrelin levels

1.3.5.3. Hormonal signals influencing ghrelin secretion

Gastric ghrelin secretion is influenced by multiple hormonal signals, including GI hormones[221,245,246], the pancreatic hormone, insulin[247-249], and the adipokine, leptin[250,251]. Regarding GI hormones, somatostatin (SS) is a hormone with profound inhibitory effects on GI secretions[252]. SS is found in various body organs, including the pancreas, small intestine and gastric antrum[252]. Circulating TG[221,253] and AG[253] levels are reduced by SS in humans and rodents. This suppression is likely to occur through the SS receptors, SSTR1, SSTR2 and SSTR3, expressed on ghrelin cells[193].

Gastrin is secreted by G-cells of the distal stomach in response to food intake[254]. Given the common role of gastrin and ghrelin in the stimulation of gastric acid secretion[255], a few studies have examined their secretory interactions[191,255,256]. There is evidence showing that gastrin receptors are expressed on rat ghrelin-immunopositive cells[255]. In this context, it has been reported that peripheral injection of gastrin induced a transient increase in circulating AG levels in vagotomised rats[255]. In contrast, gastrin infusion in the isolated rat stomach reduced the increase in ghrelin secretion in response to vagal electrical stimulation[191]. Therefore, it is possible that gastrin modulates

the vagal stimulation of ghrelin secretion, however, this requires further investigation. Nonetheless, vagotomised individuals display a normal reduction in postprandial circulating ghrelin levels[197], which suggest that gastrin does not have a substantial role in the decrease of ghrelin secretion after food intake. Further, *in vivo* studies in rats have demonstrated that continuous IP infusions of gastrin during two days did not affect circulating ghrelin levels[256]. Overall, more mechanistic and *in vivo* studies are needed to better establish the role of gastrin on ghrelin secretion.

CCK is a satiety hormone primarily secreted from the human proximal small intestine[95] in response to food intake[257]. A clinical study investigating the effects of exogenous CCK on circulating ghrelin levels demonstrated that intravenous (i.v.) administration of CCK reduced circulating TG levels[245]. This effect seems to be mediated via CCK-1 receptors, since, in humans, the CCK-1 receptor antagonist, dexloxiglumide, blocked the inhibition of TG release by the endogenous increase of circulating CCK levels[237].

PYY and GLP-1 are anorexigenic hormones predominantly secreted in the distal small intestine and colon after food intake[49,258]. While PYY i.v. infusions decreased plasma TG levels in healthy and obese individuals[246], i.v. infusions of GLP-1 did not affect plasma TG levels[197,245]. Therefore, PYY, but not GLP-1, is involved in the postprandial reduction of ghrelin levels.

The glucoregulatory and pancreatic hormone, insulin, and ghrelin present a reciprocal pattern of secretion[215,219], with high glycaemia increasing plasma

insulin levels[259] and decreasing TG levels[260]. The acylated form of ghrelin has an important role in the maintenance of physiological glucose levels in periods of calorie restriction (see section 1.3.6.3: *Glucose metabolism and diabetes*). Therefore, a secretory interaction between these glucoregulatory hormones seems reasonable, with most clinical studies reporting an inhibitory effect of insulin on TG release[247-249]. These findings are supported by *ex vivo* release studies, using the isolated rat stomach, which demonstrated a consistent insulin-dependent suppression of TG release[191,251,261]. Furthermore, rat gastric ghrelin cells express insulin receptors[189]. Therefore, the insulin-mediated suppression of ghrelin secretion is likely a direct effect of insulin on gastric ghrelin cells.

Leptin is a hormone mainly produced by white adipose tissue, although it is also found in other tissues, including the stomach[262]. Leptin is a well-established regulator of energy homeostasis, with leptin-deficient (*ob/ob)* mice presenting hyperphagic behaviour, low body energy expenditure and development of obesity[15]. On the other hand, leptin-replacement in mice[15] and humans[16,263] with leptin deficiency leads to a reduction in food intake accompanied by a decrease in body fat. Circulating leptin levels rise after food intake[146]. Despite the complementary nature of leptin and ghrelin, a potential crosstalk between leptin and ghrelin secretion has not been fully established. Mechanistic studies, using isolated perfused rat stomach, have shown that physiological concentrations of leptin reduce TG secretion[250,251]. However, the only clinical study investigating the effect of leptin in circulating ghrelin showed that neither physiological nor pharmacological leptin concentrations affected circulating TG

levels[264]. Accordingly, more studies are necessary to establish the role for leptin in the secretion of ghrelin.

A diverse range of hormonal signals participates in the modulation of postprandial ghrelin levels. Further studies are needed to dissect the individual and cooperative contribution of these hormones in the control of gastric ghrelin release.

1.3.6. Targeting circulating ghrelin levels for the treatment of metabolic disease.

Ghrelin is an orexigenic hormone with an important role in the regulation of lipid and glucose metabolism. Accordingly, there has been considerable interest in targeting the ghrelin pathway for the treatment of metabolic disorders associated with chronic excess energy intake. The following sections discuss relevant literature on current approaches for targeting ghrelin circulating levels for the treatment of obesity, Prader-Willi syndrome and type 2 diabetes, and emphasise the need for a better understanding of the mechanisms regulating ghrelin secretion to establish effective strategies for the treatment of these diseases.

1.3.6.1. Obesity

Obesity is a chronic medical condition of epidemic proportions[1], defined by an increase in body weight (i.e. body mass index ≥ 30 kg/m^2) and often associated with co-morbidities, such as type 2 diabetes and cardiovascular disease[265]. Consequently, obesity is associated with increased mortality[266,267].

Obesity is extremely resistant to behavioural interventions, with most patients regaining weight after lifestyle intervention for weight loss[268]. To date, bariatric surgery is the most effective weight-loss option[31]. However, it is a highly invasive approach with several associated complications, and therefore, not suitable for everyone[31]. On the other hand, pharmacotherapy often presents unacceptable adverse effects, such as neuro-psychiatric issues, cardiovascular toxicity and pulmonary hypertension[22,269]. As a result, gut hormone research for the control of body weight and appetite has exponentially increased in order to provide alternative options for the treatment of obesity. Ghrelin has a well-established role in the stimulation of adiposity and food intake[270,271]. Despite the lower circulating ghrelin levels[272] and central ghrelin resistance[273] observed in obesity, the modulation of the ghrelin pathway is a promising strategy to supplement weight-loss. Metabolic adaptations protect the higher body weight set-point established in obesity and counteract weight loss with a reduction in the resting metabolic rate[268] and changes in circulating levels of energy balance-associated hormones[274]. In this regard, diet-induced weight loss totally reverses the obesity-induced reduction in circulating ghrelin levels[275-277] and restores ghrelin sensitivity[276,278]. This gain in ghrelin function during weight loss promotes the restoration of energy stores leading to weight regain[279]. Under these circumstances, inhibition/antagonism of the AG pathway may restrict rebound weight gain.

Multiple approaches targeting the inhibition of the ghrelin pathway have been investigated in pre-clinical studies. While many studies have demonstrated that food intake remains unaffected in ghrelin knockout mouse models[280-282],

several studies employing the genetic ablation of ghrelin[281,283], GOAT[284] and GHSR1a[283,285], as well as the neutralisation of the hormone via anti-ghrelin antibodies[286] or L-RNA aptamers[287], and the antagonism of GOAT[288] have shown a reduction in body weight[283-288] and fat mass[283-285,287,288]. While this may be associated to the adiposity-promoting effect of ghrelin, which has been demonstrated to be independent of food intake[180], these conflicting findings have also shown a need for a deeper understanding of ghrelin secretion and function.

A growing body of knowledge on the physiology of ghrelin is yielding new strategies for the regulation of food intake and body weight. For instance, emerging knowledge on the deacylated form of ghrelin suggests that DAG acts as a separate hormone that mostly antagonises AG effects, possibly via GHSR1a-independent pathways[289]. Available reports show that administration of DAG in rodents decreases food intake, and blocks the orexigenic action of AG via the hypothalamus[289,290]. Additionally, transgenic mice overexpressing DAG present lower body weight, food intake and fat mass[290]. These findings suggest that an increase in DAG circulating levels could be beneficial for the treatment of obesity. Furthermore, the recent identification of the first endogenous antagonist of the metabolic actions of ghrelin, LEAP2[173], could have significant implications for the treatment of obesity[291,292]. It has been demonstrated that LEAP2 blocks ghrelin-induced activation of hypothalamic NPY neurones[293] and inhibits the orexigenic action of AG in mice[173]. Additionally, studies in humans and mice demonstrate that circulating LEAP2 levels correlate inversely with plasma AG levels[293], with LEAP2 concentrations

being increased in obesity and reduced after weight loss[293]. Therefore, the stimulation of LEAP2 levels during weight loss may be a promising approach for counteracting the weight loss-induced restoration of circulating AG levels[275-277] and sensitivity[276,278].

A deeper understanding of the ghrelin pathway has the potential to provide highly selective and improved strategies for the treatment of obesity and/or maintenance of body weight. Future studies should include exploration of mechanisms modulating AG and DAG circulating levels, and the potential functional interactions between AG, DAG and LEAP2. Moreover, the mechanisms underlying the disrupted ghrelin pathway in obesity, such as the impairment of adrenergic[209,294] and nutrient-sensing pathways modulating ghrelin secretion[206,209], warrant further investigation.

1.3.6.2. Prader–Willi syndrome

Prader-Willi syndrome (PWS) is a complex genetic condition characterised by augmented circulating ghrelin levels and the development of severe obesity[295,296]. In infancy, individuals with PWS present excessive concentrations of DAG that have been associated with anorexia and poor growth[297]. Later in childhood (2-3 year of age), there is a switch to hyperphagia and development of obesity that has been linked to an increase in AG levels and a relative deficit of DAG in PWS children, which persists in adults[298]. Due to the impairment of the ghrelin system in this disease, multiple strategies for the modulation of the AG/DAG ratio during the hyperphagic phase of PWS have been suggested[18,299]. For example, the antagonism of GOAT can reduce

excessive AG production. Similarly, the control of available fatty acids for the acylation of ghrelin may help to downregulate AG concentrations. Moreover, due to the emerging role of DAG counteracting the effect of AG on food intake[289,290], an increase in circulating DAG levels may be beneficial for the modulation of AG/DAG ratio in PWS.

1.3.6.3. Glucose metabolism and diabetes

Ghrelin has a critical role in the regulation of blood glucose levels[150]. During periods of negative energy balance, AG acts as a "survival hormone"[300-302] to maintain physiological blood glucose levels via multiple mechanisms[302], including the stimulation of hepatic gluconeogenesis[303] and the inhibition of insulin secretion from pancreatic β-cells[304]. Indeed, mice that over-express the endogenous antagonist of ghrelin, LEAP2, and undergo 60% calorie restriction have a dramatic decrease in body weight and blood glucose levels (<30 mg/dl). Further, unlike their control counterparts, mice over-expressing LEAP2 became moribund and lethargic and were euthanized[173]. In contrast, in situations of energy abundance, AG plays a detrimental role in glucose homeostasis by reducing glucose-stimulated insulin secretion[186,305] and promoting insulin resistance[306,307]. Consequently, antagonism of AG has attracted attention to improve glycaemic control in type 2 diabetes. Studies in rodents demonstrate that antagonism of GHSR1a[308,309], GOAT[288] and genetic ablation of ghrelin[309] improves glucose tolerance and enhances glucose-induced insulin secretion. On the other hand, a study in a small group of obese diabetic subjects (*n*=8) showed that overnight DAG infusion decreased postprandial glucose levels, improved insulin sensitivity during hyperinsulinemic-euglycemic clamp, and

reduced fasting and postprandial AG levels[310], suggesting that DAG has potential benefits for glycaemic control and the treatment of metabolic disorders with disrupted glucose metabolism, such as type 2 diabetes. However, further studies are necessary to validate these findings. Moreover, further fundamental studies are required to understand the glucoregulatory mechanisms of AG and DAG.

In conclusion, available research strongly indicates that modulation of circulating ghrelin levels, particularly reduction of AG secretion and signalling, may be beneficial in metabolic conditions, such as obesity, PWS and type 2 diabetes. Nonetheless, emerging research also indicates that ghrelin presents protective effects in cardiovascular disease[311] and stress-related psychiatric disorders[312]. While these topics are beyond the scope of this review, it is important to consider that ghrelin is a hormone with multiple body functions. Therefore, manipulation of circulating ghrelin levels should be prudently considered.

1.3.7. Concluding remarks

Ghrelin is a gastric hormone with multiple therapeutic applications, including the treatment of obesity, PWS and type 2 diabetes. Despite the therapeutic potential of ghrelin, there is limited information on the mechanisms controlling the secretion of ghrelin, which subsequently impact circulating ghrelin levels (Figure 1.5). Neural signals seem to synergistically work to increase circulating ghrelin levels during fasting. On the other hand, peripheral signals mostly reduce postprandial ghrelin levels. Furthermore, it appears that protein

digestion products and bitter compounds stimulate gastric ghrelin secretion from the stomach of humans and rodents, although, *in vivo*, they inhibit circulating ghrelin levels in humans. Considering the numerous mechanisms involved in the modulation of postprandial ghrelin levels, it is plausible that the local stimulating effect of proteins and bitter compounds is abolished by the combined effect of the numerous ghrelin-inhibiting factors after food intake. However, further research is required to understand the mechanisms controlling ghrelin secretion and their crosstalk. Furthermore, there is an increasing number of studies demonstrating the role of gastric nutrient-sensing in the modulation of ghrelin secretion. However, more fundamental research is needed to define the role of an extensive repertoire of nutrients, as well as the nutrient-sensing mechanisms driving their effects on ghrelin secretion. Finally, more human studies are necessary to validate and translate findings from fundamental studies into potential disease treatments. Together, this information will contribute to a better understanding and targeting of the ghrelin system for therapeutic benefits.

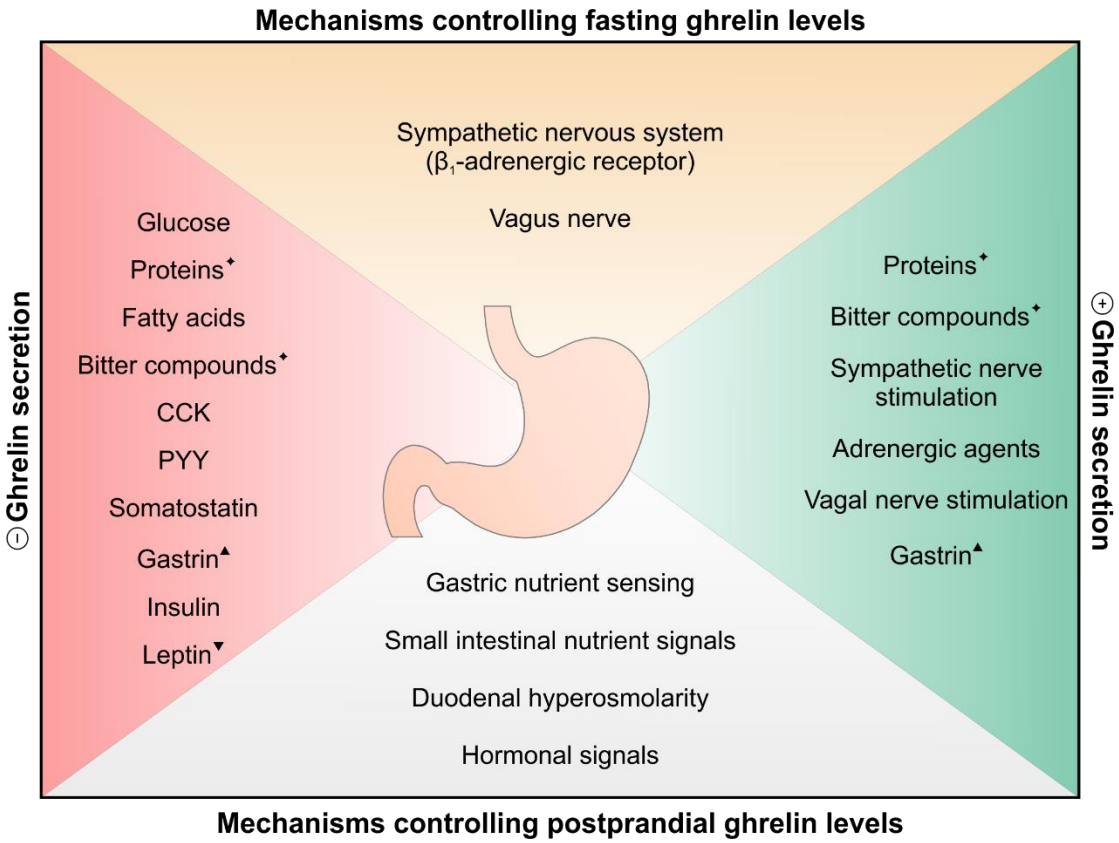

Figure 1.5. Overview of the complex modulation of circulating ghrelin.

Mechanisms controlling fasting ghrelin levels (in orange): The sympathetic β1-adrenergic receptors and the vagus nerve stimulate gastric ghrelin secretion. Mechanisms influencing postprandial ghrelin levels (in grey): Small intestinal factors, including nutrient signals, duodenal hyperosmolarity and GI hormones suppress ghrelin secretion. Upon nutrient absorption, the postprandial rise of insulin levels, as well as gastric nutrient sensing from the circulation, contribute to the suppression of plasma ghrelin levels. Summary of individual factors shown to reduce (in red) or stimulate (in green) ghrelin secretion. ✦ Bitter compounds and proteins inhibit postprandial circulating ghrelin levels, although they have been shown to stimulate ghrelin secretion from the stomach. ▲ Gastrin may reduce, stimulate or not affect ghrelin secretion.▼ The ghrelin-inhibiting effect of leptin has been demonstrated in *ex vivo* settings only.

1.4. AIMS

The GI tract has a key role in the modulation of energy homeostasis, with gut hormones displaying important effects on the control of food intake and metabolism. In this regard, the gastric hormone, ghrelin, has a well-established role in promoting a positive energy balance. Consequently, the modulation of circulating ghrelin levels is an attractive target for the management of body weight in obesity. However, the mechanisms involved in the control of gastric ghrelin secretion are not well understood. While nutrients are known to be powerful signals influencing the secretion of gut hormones, via activation of GI nutrient chemosensors, there is limited information on the effects of nutrients and the role of nutrient chemosensors in the modulation of gastric ghrelin secretion. Further, the nutrient-sensing capability of the stomach and gastric ghrelin cells is not fully defined. In addition, alterations in the expression of gastric nutrient chemosensors may play a role in the obesity-induced reduction of circulating ghrelin levels. However, scarce information is available on the effects of obesity on the expression of gastric nutrient chemosensors. In this context, this PhD thesis aimed to:

1) Investigate the expression of nutrient chemosensors in different regions of the mouse stomach, with particular emphasis on co-expression with ghrelin (**Chapter 2**).

2) Assess the role of nutrients and nutrient chemosensors in the secretion of gastric ghrelin (**Chapter 3**).

3) Determine the effect of high-fat diet (HFD)-induced obesity on the expression of nutrient chemosensors in the mouse stomach and the level of co-expression with ghrelin (**Chapter 4**).

Chapter 2: The nutrient-sensing components of the mouse stomach and the gastric ghrelin cell

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Contribution to the Paper	Designed the study, conducted experiments, analysed and interpreted data, wrote and approved the final version of the manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/07/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Interpreted data, edited and approved the final version of the manuscript.		
Signature		Date	15/07/2020

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Contribution to the Paper	Conceptualised and designed the study, interpreted data, wrote and approved the final version of the manuscript.		
Signature		Date	13/7/20

2.1. ABSTRACT

Background and aim: The ability of the gut to detect nutrients is critical to the regulation of gut hormone secretion, food intake and postprandial blood glucose control. Ingested nutrients are detected by specific gut chemosensors. However, knowledge of these chemosensors has primarily been derived from the intestine, while available information on gastric chemosensors is limited. This study aimed to investigate the nutrient-sensing repertoire of the mouse stomach with particular emphasis on ghrelin cells.

Methods: Quantitative RT-PCR was used to determine mRNA levels of nutrient chemosensors (protein: GPR93, CaSR, mGluR4; fatty acids: CD36, FFAR2&4; sweet/umami taste: T1R3), taste-transduction components (TRPM5, GNAT2&3) and, ghrelin and ghrelin-processing enzymes (PC1/3, GOAT) in the gastric corpus and antrum of adult male C57BL/6 mice. Immunohistochemistry was performed to assess protein expression of chemosensors (GPR93, T1R3, CD36 and FFAR4) and their co-localisation with ghrelin.

Key results: Most nutrient chemosensors had higher mRNA levels in the antrum compared to the corpus, except for CD36, GNAT2, ghrelin and GOAT. Similar regional distribution was observed at the protein level. At least 60% of ghrelin-positive cells expressed T1R3 and FFAR4, and over 80% expressed GPR93 and CD36.

Conclusions and inferences: The cellular mechanisms for the detection of nutrients are expressed in a region-specific manner in the mouse stomach and

gastric ghrelin cells. These gastric nutrient chemosensors may play a role modulating gastrointestinal responses, such as the inhibition of ghrelin secretion following food intake.

Keywords: Gastric ghrelin, nutrients, receptors, stomach.

Graphical abstract:

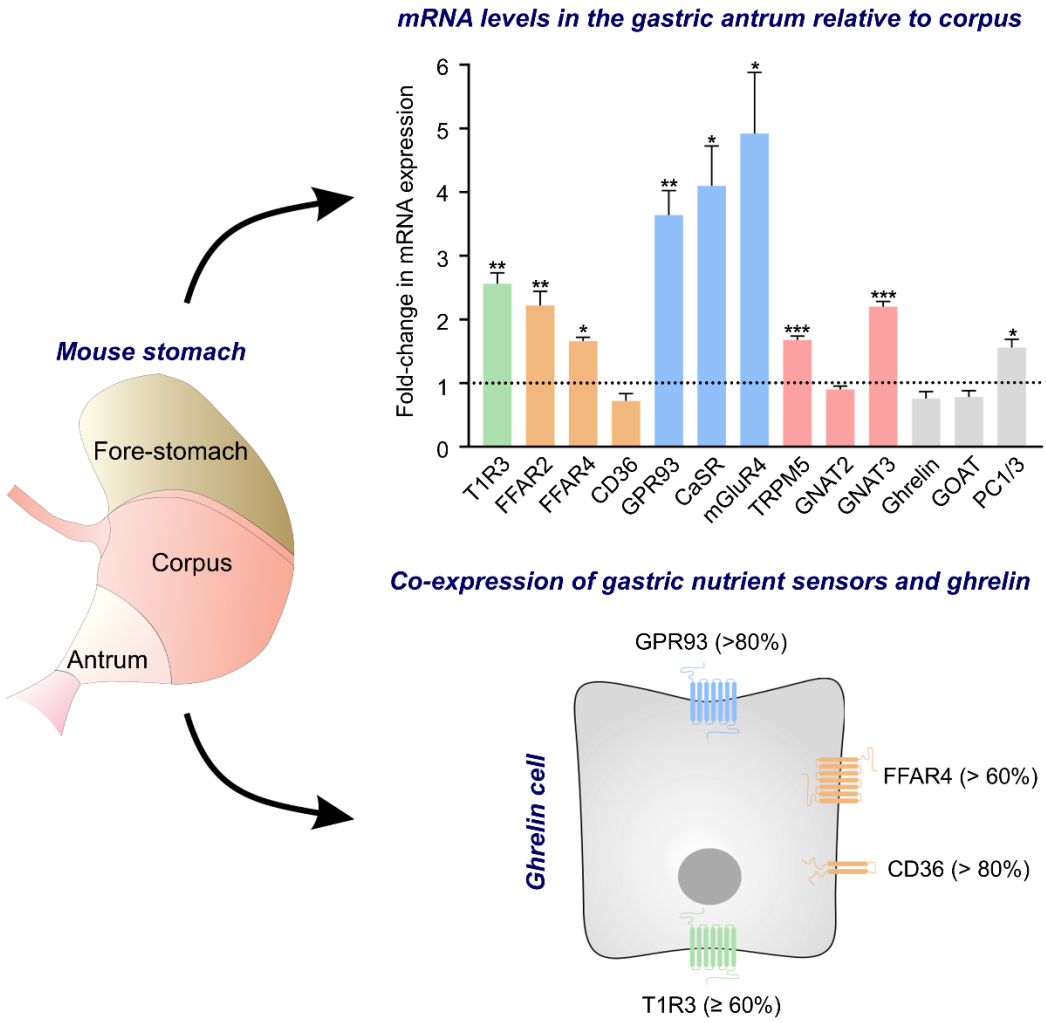


Figure 2.1. Chemosensors in the mouse stomach.

Most chemosensors had higher expression in the antrum versus corpus, except for CD36, GNAT2, ghrelin, and GOAT. Chemosensors for fatty acids (FFAR4, CD36), protein (GPR93) and the sweet and umami taste receptor subunit (T1R3) were co-expressed with ghrelin.

2.2. INTRODUCTION

The gastrointestinal (GI) tract relies on chemical and neural signals to control physiological processes, such as digestion, nutrient assimilation and energy intake[128]. Nutrient chemosensors are essential for the assessment of the composition of luminal content within the GI tract[313,314]. They also play a crucial effector role in triggering gut hormone secretion responsible for the fine-tuning of GI function and feeding behaviour[51,218,315].

An extensive array of nutrient chemosensors has been reported throughout the GI tract[315-317]. Sweet molecules are known to be sensed in the proximal intestine upon activation of the heterodimeric G protein-coupled sweet taste receptor T1R2/T1R3, the G protein, α-gustducin, and taste-specific transient receptor potential cation channel subfamily melastatin member 5 (TRPM5)[228]. Nutrient stimulation of this sweet-taste pathway can trigger the release of GI hormones, including the incretin hormone glucagon-like peptide-1 (GLP-1), and intestinotrophic peptide, glucagon-like peptide-2 (GLP-2)[121,122,318-320]. Products of protein digestion are detected via multiple nutrient chemosensors, including, the peptone sensor, G protein-coupled receptor 93 (GPR93)[109,321], the peptone[107] and aromatic amino acid sensor[218,322], calcium-sensing receptor (CaSR), the metabotropic glutamate receptor type 4 (mGluR4)[323,324], and the heterodimeric umami chemosensor, T1R1/T1R3[323,324]. Furthermore, it is known that the G protein subunits, α-gustducin and α-transducin couple with T1R1/T1R3 for umami detection[325]. The activation of chemosensors for protein digestion products trigger the secretion of GI hormones, including GLP-1[108,326], gastrin[327], cholecystokinin (CCK)[105,109] and peptide tyrosine tyrosine (PYY)[108].

Fatty acid detection occurs upon activation of a variety of free fatty acid receptors (FFARs) and the fatty acid transporter, cluster of differentiation (CD) 36. The short-chain fatty acid (SCFA) receptor, FFAR2, and the long-chain fatty acid (LCFA) receptor, FFAR4, are key fat sensors in the GI tract, and regulate diverse GI functions. For example, FFAR2 has been linked to the detection of SCFAs derived from microbiota fermentation[328], while FFAR4 can modulate GLP-1 secretion[117]. Furthermore, CD36 has been proposed to work in a coordinated cascade with FFAR4 and TRPM5 to detect fatty acids[329,330].

Substantial knowledge is available on the nutrient-sensing capabilities of the intestine, with most chemosensors presenting region-specific expression patterns that are highly associated with distinctive functional characteristics of each intestinal region[315,317]. While little data is available on the nutrient-sensing repertoire of the stomach, it is known that the expression of several bitter taste receptors (T2Rs), known to be highly co-localised with ghrelin[211,231] and involved in the stimulation of ghrelin secretion[209,211], varies in different compartments of the stomach[209,231]. Therefore, the first aim of this study was to investigate the expression of gastric nutrient chemosensors, as well as their regional distribution within the mouse stomach.

Ghrelin is a 28-amino acid anabolic hormone primarily produced by gastric endocrine cells[48,331] that stimulates the release of growth hormone[48] and food intake[174,175], as well as modulates GI function[149,332] and metabolism[168,271,333]. Ghrelin requires a series of processing steps in order to display its biological functions. Proghrelin peptides require a cleavage at the C-terminal Pro-Arg site.

This cleavage is an enzymatic process involving prohormone convertase 1/3 (PC1/3) and is crucial for the production of the mature ghrelin peptide[158]. Moreover, ghrelin can undergo an acylation on its third serine residue. This acyl modification is catalysed by ghrelin O-acyltransferase (GOAT), an essential ghrelin-processing enzyme for the production of the bioactive form of ghrelin[159,160].

The secretion of ghrelin is tightly linked to feeding patterns, with a peak in plasma ghrelin levels prior to meals, followed by an immediate postprandial fall[146]. The mechanisms that control ghrelin release are diverse and include neural[190,192,196], hormonal[245,246,248,334,335] and nutrient-sensing events[198,336]. Regarding the nutrient-related signals, the nutritional composition of meals appears to be a key factor in the inhibition of ghrelin secretion, which is more potently suppressed by dietary proteins than carbohydrates and lipids in humans[198,336]. However, the mechanisms that underlie ghrelin suppression remain to be elucidated. Therefore, the second aim of this study was to explore the expression of nutrient chemosensors of the ghrelin cells in the mouse stomach.

2.3. MATERIALS AND METHODS

2.3.1. Mice

Eight-week-old male C57BL/6 mice were group-housed in a controlled environment (12 hours light/12 hours dark cycle, temperature 22 ± 0.5°C, 40-60% humidity) with *ad libitum* access to drinking water and a standard laboratory chow comprising 24%, 18% and 58% of energy from protein, fat and carbohydrates, respectively (Teklad Rodent Diet 2018, ENVIGO, Wisconsin, USA). All experiments were approved by the Animal Ethics Committees of the South Australian Health & Medical Research Institute and The University of Adelaide, Australia, and were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition).

2.3.2. Tissue collection and preparation

Mice were humanely killed via CO_2 inhalation for gene expression experiments. The stomach and duodenum (0.5-2 cm post-pylorus) were removed and opened. Mucosal scrapings from the glandular regions of the stomach (corpus and antrum) and duodenum were collected, snap-frozen in liquid nitrogen and stored at -80°C until analysis. For immunohistochemistry experiments, mice were anaesthetised by isoflurane inhalation before pentobarbitone injection (0.2 mL ip, 60 mg mL^{-1}). Immediately after, mice were perfused via the left cardiac ventricle with warm heparinised saline (flow speed: 17 mL min^{-1}, 3 min), followed by cold 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PFA-PB, flow speed: 15 mL min^{-1}, 50 mL). The stomach was removed, opened along the greater curvature and rinsed in saline. Tissue was then incubated at room temperature in PFA-PB buffer for 2 hours before cryoprotection in 30%

sucrose-PB solution overnight. Finally, the stomachs were embedded in optimal cutting temperature compound (Tissue-Tek, ProSciTech, QLD, Australia), frozen and stored at -80 °C until sectioning.

2.3.3. Quantitative RT-PCR

Total RNA was extracted from mucosal scrapings of corpus, antrum and duodenum using the PureLink RNA Mini kit (Thermo Fisher Scientific, SA, Australia) according to manufacturer's instructions. Total RNA was quantified by spectrophotometry (260 nm), and purity was estimated by the $A_{260/280}$ ratio.

Quantitative real-time PCR (qRT-PCR) was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, SA, Australia) and EXPRESS One-Step Superscript™ qRT-PCR Kit (Life Technologies, SA, Australia). All primers (Table 2.1) were predesigned TaqMan™-based assays (Thermo Fisher Scientific). A DNase digestion with ezDNase kit (Invitrogen, SA, Australia) was required to eliminate genomic DNA from total RNA samples. qRT-PCR reactions were performed under the following conditions: Reverse transcription 50 °C for 15 min; initial PCR activation 95 °C for 20 sec; PCR cycles 95 °C for 3 sec followed by 60 °C for 30 sec repeated for 40 cycles[337]. β-2 microglobulin (B2M), hypoxanthine-guanine phosphoribosyltransferase (HPRT) and peptidylprolyl isomerase A (PPIA) were chosen as housekeeping genes based on their stability value (0.002) in gastric mucosal scrapings tested by NormFinder (MOMA, Aarhus University Hospital, Denmark)[337,338]. mRNA levels were calculated relative to the averaged cycle threshold (Ct) value of B2M, HPRT and PPIA using the $2^{-\Delta CT}$ method[339]. Each assay was run in triplicate.

Negative controls and no reverse transcriptase controls were carried out by substituting RNA template and reverse transcriptase for RNase-free water, respectively.

Table 2.1. Details of qRT-PCR primers.

Target	Description	TaqMan™ Assay ID
Ghrelin	Gut hormone	Mm00445450_m1
GOAT	Ghrelin-processing enzyme	Mm01200389_m1
PC1/3	Ghrelin-processing enzyme	Mm00479023_m1
T1R3	Sweet and umami taste receptor subunit	Mm00473459_g1
FFAR2 (GPR43)	Short-chain fatty acid receptor	Mm02620654_s1
FFAR4 (GPR120)	Long-chain fatty acid receptor	Mm00725193_m1
CD36 (FAT)	Fatty acid translocase	Mm00432403_m1
GPR93 (GPR92)	Protein hydrolysate receptor	Mm02621109_s1
CaSR	Calcium and aromatic amino acids receptor	Mm00443375_m1
mGluR4	Glutamate receptor	Mm01306128_m1
TRPM5	Taste-specific cation channel	Mm01129032_m1
GNAT2	α-Transducin subunit	Mm01165313_m1
GNAT3	α-Gustducin subunit	Mm00492394_m1
B2M	Housekeeping gene	Mm00437762_m1
HPRT	Housekeeping gene	Mm01545399_m1
PPIA	Housekeeping gene	Mm02342429_g1

2.3.4. Immunohistochemistry

Experimental conditions of immunohistochemistry experiments were adapted from previous reports[316,337]. Longitudinal cryosections of the stomach (10 µm) were air-dried, rinsed three times in PBS (pH 7.4) containing 0.2% Triton X-100 (PBS-TX) (Sigma-Aldrich, NSW, Australia) for 5 min, and blocked for 60 min at room temperature with 10% normal donkey serum (Sigma-Aldrich) dissolved in PBS-TX. Cryosections were then washed three times for 2 min with PBS-TX. In single and dual labelling experiments, primary antibodies were diluted in PBS-TX. Blocked cryosections were then incubated with diluted primary antibodies targeting rabbit anti-ghrelin (1:1600, ab129383, Abcam, VIC, Australia), goat anti-ghrelin (1:800, ab104307, Abcam), goat anti-T1R3 (1:400, sc-22458, Bio-Strategy Laboratory Products, QLD, Australia), rabbit anti-FFAR4 (1:800, NBP1-00858, In Vitro Technologies, VIC, Australia), rabbit anti-CD36 (1:600, NB400-144, In Vitro Technologies) and rabbit anti-GPR93 (1:100, ABT114, Merck, VIC, Australia) overnight at 4 °C. After washing unbound antibody with PBS-TX (3 times, 5 min), appropriate donkey anti-rabbit or anti-goat secondary antibodies conjugated to Alexa Fluor® 488 or 568 (Invitrogen) and dissolved in PBS-TX (1:200) were added to the slides for 60 min at room temperature. Subsequently, cryosections were rinsed with PBS-TX (3 times, 5 min) and mounted with ProLong® Diamond Antifade reagent with DAPI (Invitrogen) and coverslipped. Single label controls were run to confirm no bleed-through of fluorescence under different filters. Slides that omitted the primary antibody served as negative controls. Specific immunolabelling of primary antibodies was previously tested in gustatory cells of taste buds (T1R3[340,341] and FFAR4[342]), liver (CD36[343,344]) and CHEM-1 cells transfected

and non-transfected with GPR93[345]. The specificity of ghrelin antibodies was confirmed in double-labelling experiments in the mouse stomach, with rabbit anti-ghrelin and goat anti-ghrelin primary antibodies co-labelling in 95.2% of ghrelin cells.

2.3.5. Microscopy, photography and cell quantification

Immunofluorescence was visualised using a BX51 epifluorescence microscope (Olympus, SA, Australia). Images were captured using an XM10 monochrome camera (Olympus). Brightness and contrast were adjusted with CellSens Dimensions Imaging Software (Olympus).

FIJI was used to manually count immunopositive cells from 159 µm × 159 µm areas in the glandular base of the stomach (i.e. typical location of gastric ghrelin cells[202,346]). FFAR4 immunopositive cells of the apical surface of the glandular stomach were not counted due to the high density of overlapping cells. Mean values of immunopositive cells for each gastric region were obtained from the average of 5-6 sections. Immunopositive cells with DAPI-stained nuclei were included in counts.

2.3.6. Statistical analysis

Results are expressed as mean ± SEM. Comparisons of mRNA expression were performed using one-way ANOVA with repeated measures followed by Tukey's multiple comparisons tests (GraphPad Prism version 7.02, La Jolla California USA). Differences in the number of immunopositive cells were

compared using paired Student's *t*-test. Statistical significance was defined as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

2.4. RESULTS

2.4.1. The gene expression of gastric nutrient chemosensors and ghrelin-processing enzymes is region-specific

Relative mRNA expression of nutrient chemosensors and ghrelin-processing enzymes in the corpus, antrum and duodenum are shown in Figure 2.2. Additionally, detailed statistics are presented in Supporting information (Table 2.2). Receptors for protein digestion products were expressed at higher levels in the antrum than corpus for GPR93 ($P < 0.01$), CaSR ($P < 0.05$) and mGluR4 ($P < 0.05$) (Figure 2.2A). The expression of GPR93 and mGluR4 was comparable in the antrum and the duodenal reference tissue. In contrast, CaSR expression was 17.3-fold higher in the antrum ($P < 0.05$) and 4.2-fold higher in the corpus than in duodenum ($P < 0.05$).

Transcript levels of the fatty acid transporter, CD36, were 4.1-fold higher in the antrum ($P < 0.05$) and 5.7-fold higher in the corpus ($P < 0.001$) than the duodenum, with no difference between gastric regions (Figure 2.2B). Expression of the SCFA receptor, FFAR2 ($P < 0.01$), and LCFA receptor, FFAR4 ($P < 0.05$), was lower in the corpus than the antrum (Figure 2.2B). In addition, FFAR2 expression was 3.1-fold ($P < 0.01$) higher in the antrum than duodenum, while FFAR4 was expressed similarly in both regions.

The mRNA expression of the taste receptor subunit T1R3 ($P < 0.01$) and TRPM5 ($P < 0.001$), as well as α-gustducin subunit, GNAT3 ($P < 0.001$), was lower in the gastric corpus than the antrum (Figure 2.2C-D). GNAT3 mRNA expression was higher in the antrum (44.5-fold; $P < 0.001$) and corpus (20.9-

fold; $P < 0.001$) than in duodenum. In contrast, expression of the α-transducin subunit, GNAT2, was similar in the corpus and antrum, and 2.1-fold ($P < 0.05$) higher in the corpus than the duodenum (Figure 2.2D). The umami (T1R1) and sweet (T1R2) taste receptor subunits were not detected in either the gastric antrum or corpus.

The mRNA expression of ghrelin and its processing enzymes, PC1/3 and GOAT, was higher in both gastric regions than the duodenum, with the highest PC1/3 expression observed in the antrum compared to corpus ($P < 0.05$) (Figure 2.2E).

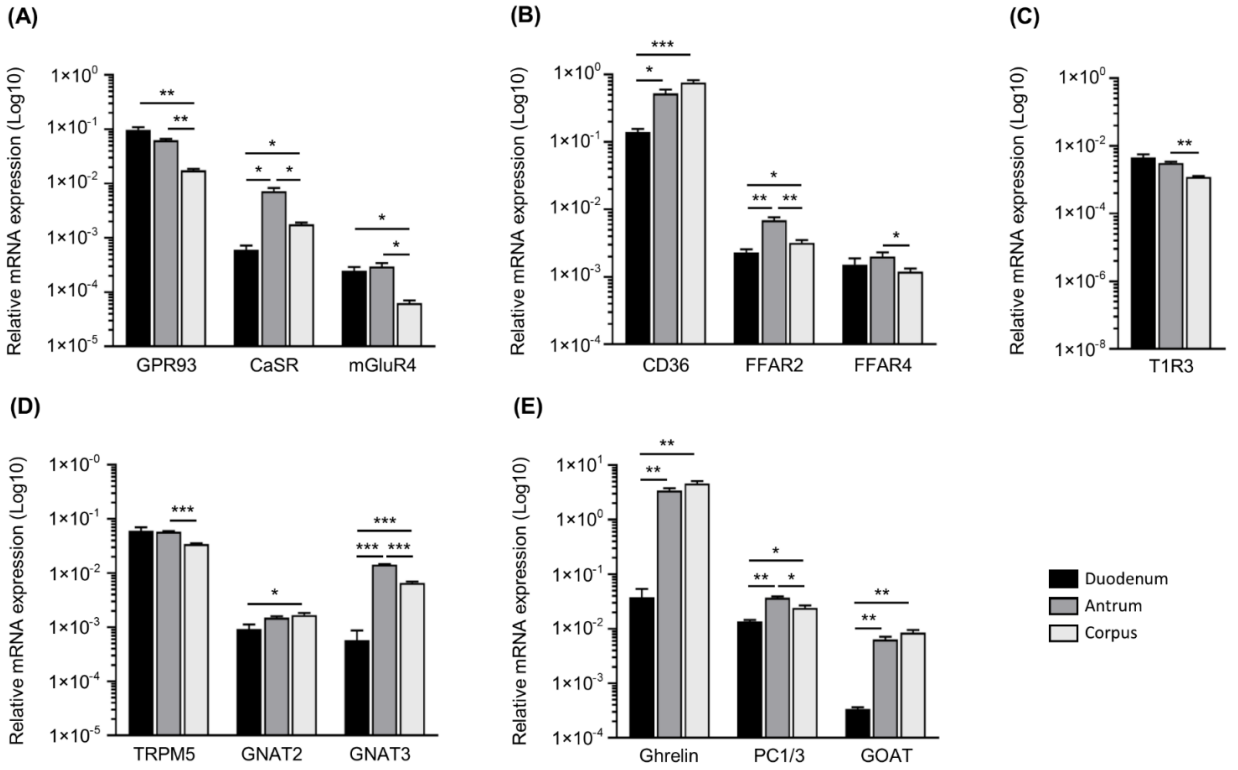


Figure 2.2. Gene expression of nutrient chemosensors, ghrelin and ghrelin-processing enzymes in the duodenum, gastric corpus and antrum.

Relative mRNA expression of nutrient chemosensors for products of (A) protein digestion, (B) fatty acids, (C) sweet and umami taste receptor subunit T1R3, (D) taste-transduction components TRPM5, GNAT2 and GNAT3, (E) and ghrelin and the ghrelin-processing enzymes PC1/3 and GOAT. Expression relative to the average Ct value of the housekeepers PPIA, HPRT and B2M (*n*=5 mice/gastrointestinal region). Data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA with repeated measures followed by Tukey's *post hoc* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

2.4.2. The distinctive protein expression of nutrient chemosensors in the mouse stomach and the ghrelin cells

Gastric mucosal immunofluorescence of GPR93, T1R3, CD36 and FFAR4, as well as their co-expression with ghrelin, are displayed in Figure 2.3-6. In addition, immunofluorescence statistics information is shown in Supporting information (Table 2.3).

2.4.2.1. GPR93

Immunolabelling for GPR93 revealed cells (12.0 ± 0.5 cells/unit area) at the glandular base of the antrum, with no immunolabelling in the corpus (Figure 2.3A). Co-expression experiments for GPR93 and ghrelin indicated that both targets are highly co-localised in the gastric antrum (Figure 2.3B, D), with 86.5 ± 0.9% of ghrelin-positive cells co-expressing GPR93, and 94.8 ± 1.9% of GPR93-positive cells containing ghrelin (Figure 2.3C).

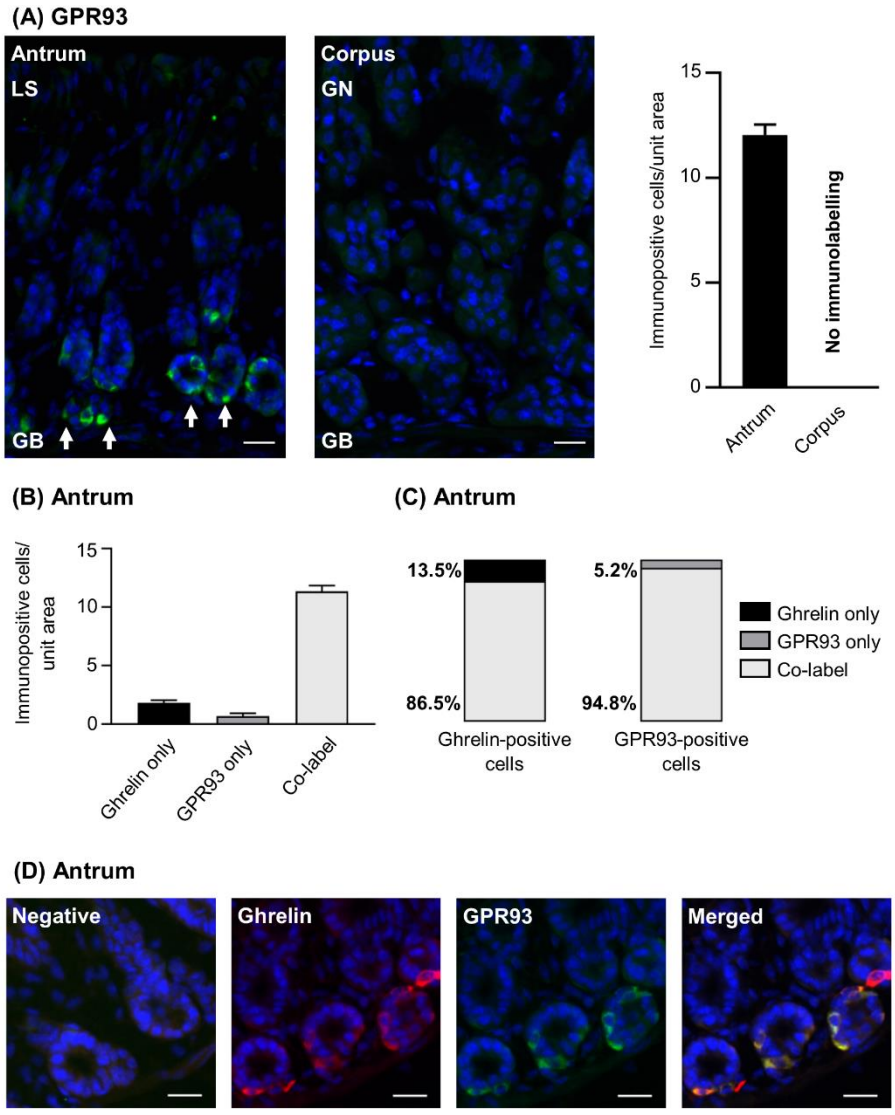


Figure 2.3. Protein expression of GPR93 in the glandular regions of the mouse stomach and co-expression with ghrelin.

(A) GPR93 cells were located in the antral glandular base only. No immunopositive cells were observed in the corpus. (B) Most immunopositive cells co-expressed ghrelin and GPR93. (C) 86.5% of ghrelin-positive cells are positive for GPR93, and 94.8% of GPR93 cells contain ghrelin (D) Representative images of ghrelin and GPR93 co-expression. Cell counts reflect the mean value of 5-6 tissue sections per mouse and gastric region (unit area=159 µm × 159 µm). *n*=5 mice. Abbreviations: LS, luminal surface; GB, glandular base; GN, glandular neck. Scale bars=20µm.

2.4.2.2. T1R3

T1R3 immunopositive cells were equally abundant in the antrum (12.6 ± 0.4 cells/unit area) and corpus (12.6 ± 0.7 cells/unit area). However, differences in T1R3 cell morphology, distribution and immunolabelling density were apparent between gastric regions (Figure 2.4A). Antral T1R3-positive cells presented a rounded shape in the glandular base. In contrast, the T1R3-positive cells of the gastric corpus often presented an elongated morphology, although round-shaped cells were also found in this region. Immunopositive cells for T1R3 in the corpus were located glandular neck and base, and presented lower immunolabelling density than antral T1R3-positive cells. Co-localisation experiments showed an equal number of cells expressing ghrelin and T1R3 in the antrum (6.8 ± 2.4 cells/unit area) and corpus (6.7 ± 1.0 cells/unit area) (Figure 2.4B-C). Accordingly, T1R3 expression in ghrelin cells was similar in both gastric regions, with 63.8 ± 11.0% of antral ghrelin cells and 59.9 ± 4.6% of corpus ghrelin cells co-expressing T1R3. Similarly, 54.4 ± 16.2% of antral T1R3-positive cells and 54.3 ± 7.4% of corpus T1R3-positive cells were positive for ghrelin (Figure 2.4D-E, 2.4F-G).

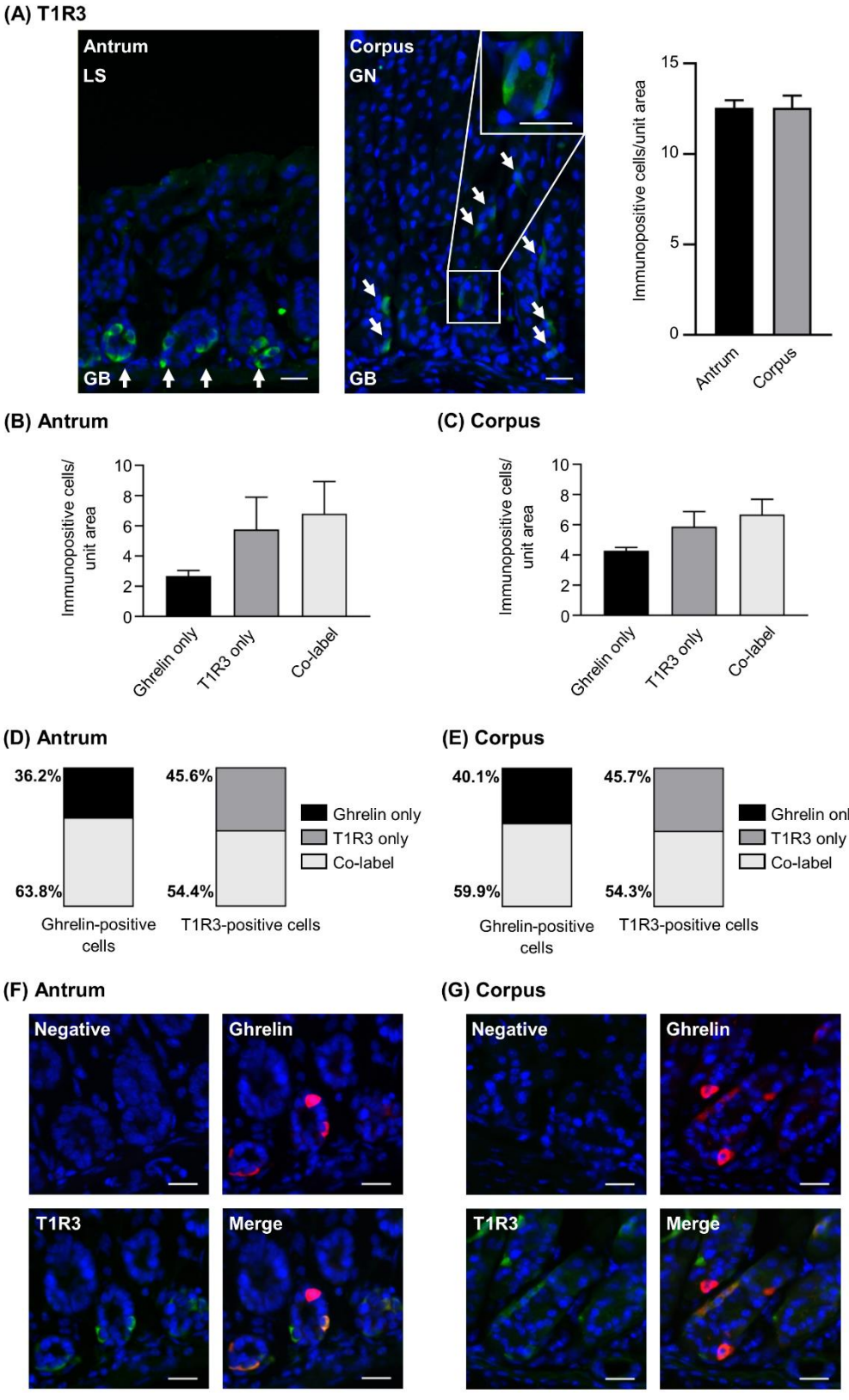
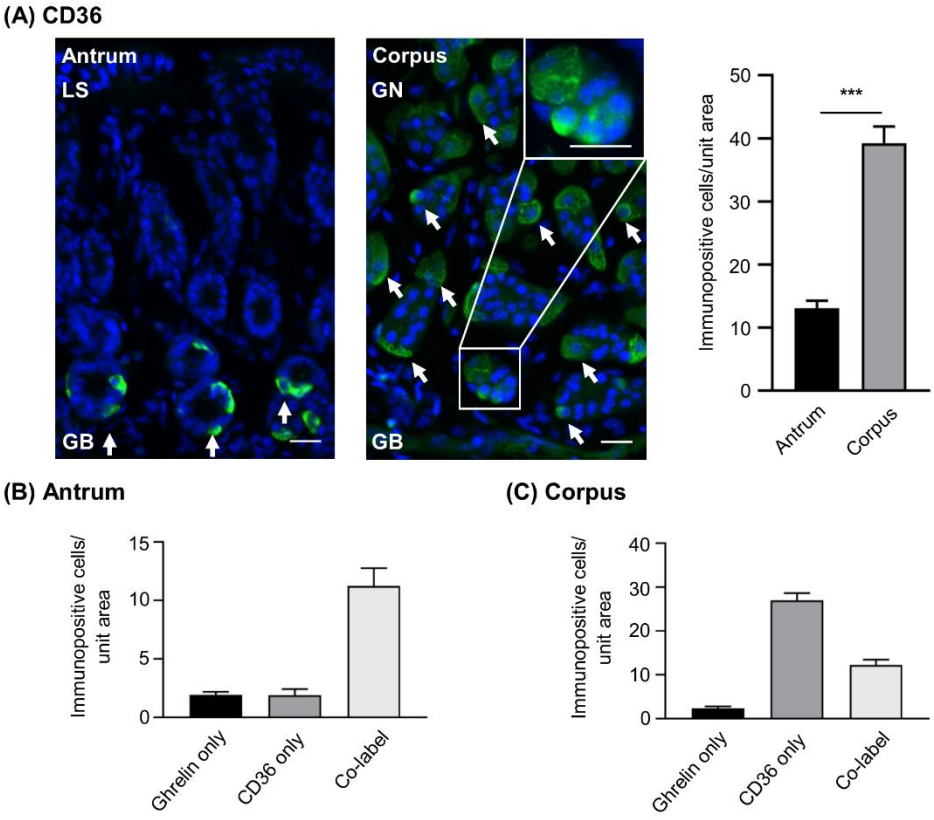

(A) T1R3
Antrum
LS
GB
Corpus
GN
GB
Immunopositive cells/unit area
15
10
5
0
Antrum
Corpus
(B) Antrum
Immunopositive cells/ unit area
10
8
6
4
2
0
Ghrelin only
T1R3 only
Co-label
(C) Corpus
Immunopositive cells/ unit area
10
8
6
4
2
0
Ghrelin only
T1R3 only
Co-label
(D) Antrum
36.2%
63.8%
45.6%
54.4%
Ghrelin only
T1R3 only
Co-label
Ghrelin-positive cells
T1R3-positive cells
(E) Corpus
40.1%
59.9%
45.7%
54.3%
Ghrelin only
T1R3 only
Co-label
Ghrelin-positive cells
T1R3-positive cells
(F) Antrum
Negative
Ghrelin
T1R3
Merge
(G) Corpus
Negative
Ghrelin
T1R3
Merge

Figure 2.4. Protein expression of T1R3 in the glandular regions of the mouse stomach and co-expression with ghrelin.

(A) An equal number of T1R3 cells were identified at the glandular base of the antrum and the glandular neck and base of the corpus, with stronger immunolabelling in antral-located T1R3 cells than corpus-located T1R3 cells. (B-C) Comparable co-labelling values were observed for ghrelin and T1R3 in both gastric regions. (D) In antrum, 63.8% of ghrelin cells co-express T1R3, and 54.4% of T1R3-positive cells contain ghrelin. (E) In corpus, 59.9% of ghrelin cells co-express T1R3, and 54.3% of T1R3-positive cells contain ghrelin. (F-G) Representative images of ghrelin and T1R3 co-expression in gastric antrum and corpus. Cell counts reflect the mean value of 5-6 tissue sections per mouse and gastric region (unit area=159 μm × 159 μm). Data are expressed as mean ± SEM. Statistical significance was determined by paired Student's *t*-test. *n*=5 mice. Abbreviations: LS, luminal surface; GB, glandular base; GN, glandular neck. Scale bars=20μm.

2.4.2.3. CD36

CD36-positive cells were more abundant in the corpus (39.2 ± 2.7 cells/unit area) than antrum (13.1 ± 1.2 cells/unit area; $P < 0.001$) (Figure 2.5A). Also, differences in features of the CD36-positive cells were observed between stomach regions (Figure 2.5A). The antrum contained strongly labelled CD36-positive cells located in the glandular base. While two types of moderately labelled CD36 cells were evident in the corpus: a small round-shaped cell type in the glandular neck and base, and a larger cell type with the prominent appearance of the gastric parietal cells distributed throughout the corpus gland. Dual immunofluorescence experiments displayed a comparable number of cells co-expressing ghrelin and CD36 in the gastric antrum (11.2 ± 1.5 cells/unit area) and corpus (12.2 ± 1.2 cells/unit area) (Figure 2.5B-C). Consistent with these results, 83.9 ± 2.9% of antral ghrelin cells, and 84.6 ± 2.7% of corpus ghrelin cells co-expressed CD36 (Figure 2.5D-E). In contrast, the CD36 cell populations of the antrum and corpus differed in ghrelin content, with 82.9 ± 6.6% of antral CD36-positive cells co-expressing ghrelin (Figure 2.5D,F), while only 32.5 ± 2.0% of corpus CD36-positive cells contained ghrelin (Figure 2.5E,G). These findings are explained by regional differences in CD36 distribution, with a higher CD36 cell density in the corpus (27.0 ± 1.6 cells/unit area) than in the antrum (1.9 ± 0.5 cells/unit area) (Figure 2.5B-C).


(A) CD36
Antrum
LS
GB
Corpus
GN
GB
***
Immunopositive cells/unit area
50
40
30
20
10
0
Antrum
Corpus
(B) Antrum
Immunopositive cells/ unit area
15
10
5
0
Ghrelin only
CD36 only
Co-label
(C) Corpus
Immunopositive cells/ unit area
40
30
20
10
0
Ghrelin only
CD36 only
Co-label
(D) Antrum
16.1%
83.9%
17.1%
82.9%
Ghrelin only
CD36 only
Co-label
Ghrelin-positive cells
CD36-positive cells
(E) Corpus
15.4%
84.6%
67.5%
32.5%
Ghrelin only
CD36 only
Co-label
Ghrelin-positive cells
CD36-positive cells
(F) Antrum
Negative
Ghrelin
CD36
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(G) Corpus
Negative
Ghrelin
CD36
Merge

Figure 2.5. Protein expression of CD36 in the glandular regions of the mouse stomach and co-expression with ghrelin.

(A) Antral CD36 cells were strongly labelled and located in the glandular base only, while CD36 cells in the corpus were widely distributed and moderately immunolabelled. (B-C) Cell counts showed a comparable number of immunopositive cells co-expressing ghrelin and CD36 in the antrum and corpus. However, a large number of CD36-positive cells in the corpus did not contain ghrelin. (D) In the antrum, 83.9% of ghrelin cells are positive for CD36, and 82.9% of CD36-positive cells contain ghrelin. (E) In the corpus, 84.6% of ghrelin cells express CD36, but only 32.5% of CD36-immunopositive cells contain ghrelin. (F-G) Representative images of ghrelin and CD36 co-expression in gastric antrum and corpus. Cell counts show the mean value of 5-6 tissue sections per mouse and gastric region (unit area=159 μm × 159 μm). Data are expressed as mean ± SEM. Statistical significance was determined by paired *t*-test. *n*=5 mice. ***$P < 0.001$. Abbreviations: LS, luminal surface; GB, glandular base; GN, glandular neck. Scale bars=20μm.

2.4.2.4. FFAR4

Immunopositive cells for FFAR4 were found in the glandular base of the antrum (10.1 ± 1.0 cells/unit area) and the luminal surface of the antrum and corpus (Figure 2.6A). FFAR4-apical cells could not be counted due to the high crowding and overlapping that limited the accuracy of the counting. Co-labelling for FFAR4 and ghrelin showed that the majority of ghrelin-positive cells in the antral glandular base expressed FFAR4 (65.9 ± 5.9%) (Figure 2.6B-D). Similarly, the majority of FFAR4-positive cells in the base of the antrum contained ghrelin (69.9 ± 10.1%) (Figure 2.6C). Due to the typical immunolabelling of ghrelin-positive cells in the glandular neck and base of the stomach[202,203], FFAR4-apical cells did not co-express ghrelin.

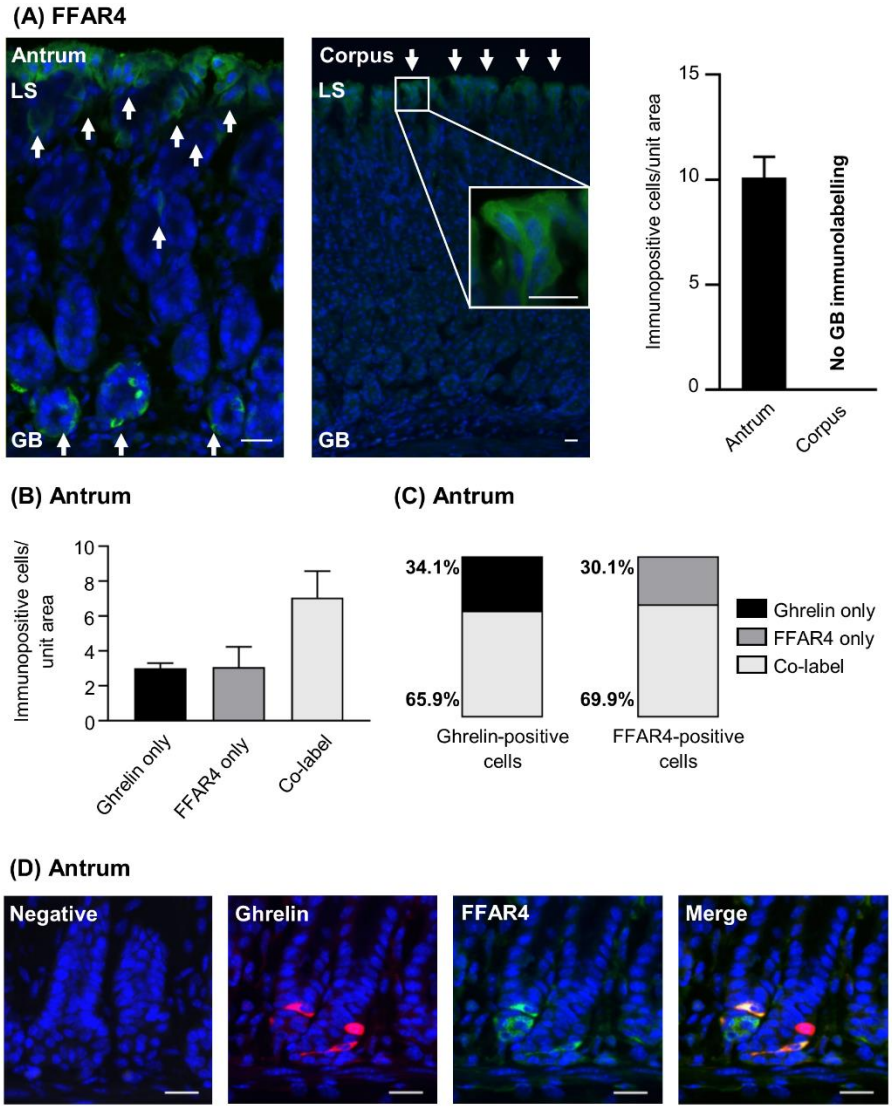

Figure 2.6. Protein expression of FFAR4 in the glandular regions of the mouse stomach and co-expression with ghrelin.

(A) FFAR4 cells were located at the luminal surface and glandular base of the gastric antrum, and were absent from the glandular base of the corpus. FFAR4 apical cells were not counted due to high crowding and overlapping. (B) The majority of immunopositive cells co-expressed ghrelin and FFAR4. (C) 65.9% of ghrelin-positive cells are positive for FFAR4, and 69.9% of FFAR4 cells contain ghrelin. (D) Representative images of ghrelin and FFAR4 co-expression in the gastric antrum. Cell counts show the mean value of 5-6 tissue sections (at the glandular base only) per mouse and gastric region (unit area=159 μm × 159 μm). Data are expressed as mean ± SEM. Statistical significance was determined by paired Student's *t*-test. *n*=6 mice. Abbreviations: LS, luminal surface; GB, glandular base; GN, glandular neck. Scale bars=20μm.

2.5. DISCUSSION

This study investigated the mRNA and protein expression of a range of nutrient chemosensors in the antrum and corpus of the mouse stomach, as well as the nutrient-sensing repertoire of gastric ghrelin cells. Limited information is available on the occurrence of nutrient-sensing components in the stomach, and the current report provides detailed evidence of their region-specific expression within this organ. Additionally, previous studies have characterised the gene expression of nutrient chemosensors in the gastric ghrelin cell[193,222]. This report further establishes the proportion of gastric ghrelin cells equipped with GPR93, T1R3, CD36 and FFAR4. These findings provide comprehensive information about the nutrient-sensing capabilities of the antrum and corpus of the mouse stomach and identify potential nutrient-sensing targets for the modulation of ghrelin secretion.

The expression of nutrient chemosensors along the GI tract is known to be region-specific[210,315,317]. The current study shows that the majority of nutrient-sensing components examined in this report display higher mRNA levels in the antrum than the corpus, and a distinctive immunolabelling pattern between gastric regions. However, there was a comparable mRNA expression of GPR93, mGluR4, FFAR4, T1R3 and TRPM5 in the gastric antrum and the duodenal reference tissue. In terms of the GI signals controlling feeding behaviour and digestion, it is typically assumed that the small intestine primarily responds to the nutrient composition of a meal, while the stomach senses mechanical stimulation[234]. However, the comparable expression of nutrient chemosensors between the antrum and duodenum and the peak expression of

CaSR, FFAR2 and GNAT3 in the antrum supports a possible role of the stomach in the chemical detection of nutrients. Antral CaSR has been associated with the stimulation of gastrin release by protein digestion products[327], which may explain the high mRNA levels of this nutrient sensor in the antrum. In addition, FFAR2 has been shown to be highly co-localised with gastric ghrelin and mediate the SCFA-induced reduction of ghrelin secretion[193]. Furthermore, GNAT2 and GNAT3 are highly expressed within the ghrelin cell population, although only GNAT3 has been associated with the modulation of ghrelin secretion[211]. Taken together, these findings support the nutrient-sensing capability of the stomach. Regarding the mRNA expression of ghrelin and the ghrelin-processing enzymes, the expression of ghrelin and GOAT was higher in the gastric corpus and antrum, in agreement with earlier literature reporting peak expression of ghrelin[331] and GOAT[159] in the stomach, while PC1/3 mRNA levels were highest in the antrum, possibly reflecting its involvement in the processing of antral gastrin[347,348].

GPR93 is a peptone receptor expressed along the GI tract[315,321,349]; however, limited information is available about the expression of GPR93 in the stomach[350,351]. In the current study, GPR93 had antral predominant mRNA expression, while GPR93 immunopositive cells were restricted to the antrum. The absence of GPR93-positive cells in the corpus emphasises the regional expression of this receptor in the stomach and may result from multiple processes[352], such as post-transcriptional and post-translational regulation, that may inhibit the protein expression of GPR93. In addition, previous studies have shown the expression of this nutrient chemosensor in the antral

gastrin[350,351] and somatostatin cells[351,353]. The current study extends this further and, demonstrates that GPR93 is highly co-localised with ghrelin, raising the possibility of the involvement of this receptor in the modulation of ghrelin secretion. Ghrelin release studies using the ghrelinoma MGN3 cell line and gastric mouse segments have shown that acyl ghrelin secretion is stimulated by peptones, an effect that is partially reversed by CaSR antagonism[205]. Accordingly, it is plausible that GPR93 may also participate in this peptone-induced stimulation of acyl ghrelin release. However, intragastric administration of peptones in mice reduces circulating ghrelin levels[205]. Therefore, further research is needed to understand the modulation of ghrelin secretion by peptones, as well as determine the role of GPR93 in the regulation of ghrelin secretion.

T1R3 is a component of the heterodimeric umami (T1R1/T1R3) and sweet (T1R2/T1R3) taste receptors. T1R3 mRNA levels were higher in the antrum than in the corpus. In contrast, T1R3 immunolabelling showed an equal number of positive cells in both gastric regions, albeit with weaker immunolabelling density (i.e. lower protein expression) in the corpus than the antrum. Previous studies have shown expression of T1R3 in gastric ghrelin cells[206,217], and the current study demonstrates that nearly two-thirds gastric ghrelin cells are equipped with T1R3. Considering the absence of T1R1 and T1R2 mRNA in the current studies, and the remarkably low expression of these receptor subunits reported in the mouse stomach in previous studies[217,315], T1R3 may not be able to form the heterodimeric umami and sweet taste receptors in the gastric mucosa. However, available research indicates that T1R3 may be able to form

homodimers (T1R3/T1R3) that are sensitive to glucose[225,226]. Intragastric infusions of glucose in rats under closed- and open-pylorus conditions fail to suppress circulating ghrelin levels when gastric emptying is prevented[201], indicating that the stomach does not detect luminal glucose for the reduction of ghrelin secretion. However, ghrelin release from primary gastric mucosal cells is adjusted upon glucose availability, with low glucose concentrations (1 mM) enhancing, and high concentrations (10 mM) reducing release[194]. These findings suggest that gastric mucosal cells, possibly ghrelin positive cells, are sensitive to circulating blood glucose levels. Therefore, it is plausible that T1R3 homodimers detect circulating blood glucose levels. However, this is speculative and requires further investigation.

CD36 is a fatty acid transporter with multiple proposed functions, including detection[354,355] and absorption[356,357] of LCFAs. The expression of CD36 has been previously reported in different regions of the GI tract, including the stomach[358]. The current study reveals a distinctive expression of this nutrient chemosensor in the mucosa of the gastric antrum and corpus. Although CD36 mRNA levels were comparable in the antrum and corpus, a greater number of CD36-positive cells were found in the corpus compared to the antrum. In contrast, weaker immunostaining (i.e. lower protein expression) was observed in the CD36-positive cells of the corpus. The immunolabelling also revealed the presence of two cell populations positive for CD36 in the corpus. The first cell type matched the prominent appearance of the gastric parietal cells, as well as their region-specific immunolabelling (i.e. corpus). This is consistent with a previous study[359] that has reported mRNA enrichment of CD36 in parietal cells.

However, further immunolabelling experiments exploring the expression of CD36, specifically in parietal cells should be undertaken to confirm these findings. The second CD36 cell subset was highly co-localised with ghrelin in the gastric antrum and corpus. The function of CD36 in gastric ghrelin cells is unknown. However, ghrelin activation requires the addition of medium-chain fatty acids, such as octanoic acid[162]. It has been suggested that fatty acids used for acyl-modification of ghrelin are produced in the ghrelin cells via β-oxidation of LCFAs provided from the circulation[163]. Therefore, it is plausible that CD36 plays an important role in the delivery of LCFAs for the activation of ghrelin. However, this hypothesis requires further investigation.

The LCFA receptor, FFAR4, constitutes another example of the region-specific expression of nutrient chemosensors in the mouse stomach. The gastric antrum presented higher FFAR4 mRNA levels than the corpus. Consistent with these results, the antrum presented FFAR4-positive cells in the luminal surface and glandular base of the mucosa, whereas, the corpus displayed positive cells on the luminal surface only. The expression of FFAR4 within the somatostatin[360] and ghrelin[193,222] cells has been previously reported, with the current study revealing that approximately two-thirds of antral ghrelin-positive cells express FFAR4. While gastric FFAR4 has been shown to participate in the reduction of somatostatin secretion from primary gastric mucosal cells[360], *in vivo* studies investigating the role of FFAR4 in the modulation of ghrelin secretion are conflicting, with FFAR4 agonists either stimulating (grifolic acid: intragastric administration[207]) or inhibiting (compound B: intragastric administration[193]; GW-9508: subcutaneous injection[208]) circulating ghrelin levels in mice. Therefore,

the involvement of this nutrient chemosensor in the secretion of ghrelin remains elusive.

In conclusion, the findings of this study provide evidence of the region-specific expression of nutrient chemosensors in the mouse stomach. Most nutrient chemosensors displayed higher mRNA and immunopositive cells in the gastric antrum compared to the corpus, suggesting a higher nutrient-sensing capability of the antrum that may be important for the modulation of antral-predominant hormone secretion, such as gastrin and somatostatin[361]. Furthermore, GPR93 and FFAR4 were highly expressed within antral ghrelin cells, and T1R3 and CD36 were highly and uniformly co-localised with ghrelin across the antrum and corpus. The significance of GPR93, T1R3, CD36 and FFAR4 in the physiology of the gastric ghrelin cells remains to be determined. Ghrelin-positive cells are located in the glandular base and neck of the stomach[202,361], making it difficult for luminal nutrients to access the nutrient chemosensors. However, it has been shown that luminal L-phenylalanine and peptones can stimulate gastrin release from G-cells via CaSR activation despite the fact that these cells are also located in the base of antral glands[327]. Therefore, it is possible that nutrients modulate ghrelin secretion from the lumen, although ghrelin cells may also respond to post-absorptive nutrient signals from the circulation. Nutrient chemosensors of the gastric ghrelin cells are potential candidates for the control of ghrelin secretion and the modulation of biological functions influenced by ghrelin, like the stimulation of food intake[174,175], adiposity[147,180,181], glycaemia[184,362,363], as well as gastric emptying[332,364]. Therefore, the nutrient-sensing components of the ghrelin cell may present a therapeutical potential

for the treatment of diverse pathologic conditions such as obesity, type 2 diabetes and gastroparesis.

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Competing interests

The authors have no competing interests.

Author contributions

MNS, HL and AP designed the study; MNS conducted experiments and analysed data; MNS and AP wrote the manuscript; All authors were involved in data interpretation and drafting the manuscript.

2.6. SUPPORTING INFORMATION

Table 2.2. qRT-PCR statistics information. Gene expression of nutrient chemosensors, ghrelin and ghrelin-processing enzymes in the duodenum, gastric corpus and antrum.

qRT-PCR Target	Relative mRNA expression			One-way ANOVA
	Duodenum	Antrum	Corpus	
GPR93	$9.79 \times 10^{-2} \pm 1.17 \times 10^{-2}$ a	$6.25 \times 10^{-2} \pm 3.83 \times 10^{-3}$ a	$1.75 \times 10^{-2} \pm 1.09 \times 10^{-3}$ b	$P < 0.01$ $F_{(1.183, 4.732)} = 31.17$
CaSR	$6.05 \times 10^{-4} \pm 1.14 \times 10^{-4}$ a	$7.22 \times 10^{-3} \pm 1.10 \times 10^{-3}$ b	$1.78 \times 10^{-3} \pm 1.31 \times 10^{-4}$ c	$P < 0.01$ $F_{(1.052, 4.207)} = 29.56$
mGluR4	$2.48 \times 10^{-4} \pm 4.27 \times 10^{-5}$ a	$2.98 \times 10^{-4} \pm 4.62 \times 10^{-5}$ a	$6.31 \times 10^{-5} \pm 7.46 \times 10^{-6}$ b	$P < 0.05$ $F_{(1.284, 5.135)} = 9.20$
CD36	$1.41 \times 10^{-1} \pm 1.59 \times 10^{-2}$ a	$5.25 \times 10^{-1} \pm 7.47 \times 10^{-2}$ b	$7.63 \times 10^{-1} \pm 6.10 \times 10^{-2}$ b	$P < 0.01$ $F_{(1.343, 5.372)} = 28.52$
FFAR2	$2.29 \times 10^{-3} \pm 2.79 \times 10^{-4}$ a	$6.92 \times 10^{-3} \pm 7.28 \times 10^{-4}$ b	$3.20 \times 10^{-3} \pm 3.16 \times 10^{-4}$ c	$P < 0.01$ $F_{(1.183, 4.734)} = 47.64$
FFAR4	$1.51 \times 10^{-3} \pm 3.65 \times 10^{-4}$ a	$2.00 \times 10^{-3} \pm 3.00 \times 10^{-4}$ a	$1.20 \times 10^{-3} \pm 1.36 \times 10^{-4}$ b	$P = 0.061$ $F_{(1.481, 5.925)} = 4.95$
T1R3	$4.50 \times 10^{-3} \pm 1.15 \times 10^{-3}$ a	$3.13 \times 10^{-3} \pm 2.93 \times 10^{-4}$ a	$1.22 \times 10^{-3} \pm 7.79 \times 10^{-5}$ b	$P = 0.086$ $F_{(1.046, 4.184)} = 5.01$
TRPM5	$6.06 \times 10^{-2} \pm 9.41 \times 10^{-3}$ a	$5.82 \times 10^{-2} \pm 1.68 \times 10^{-3}$ a	$3.43 \times 10^{-2} \pm 1.14 \times 10^{-3}$ b	$P < 0.05$ $F_{(1.037, 4.146)} = 8.91$
GNAT2	$9.22 \times 10^{-4} \pm 2.02 \times 10^{-4}$ a	$1.50 \times 10^{-3} \pm 9.69 \times 10^{-5}$ a	$1.68 \times 10^{-3} \pm 1.56 \times 10^{-4}$ b	$P < 0.05$ $F_{(1.474, 5.898)} = 8.04$
GNAT3	$5.72 \times 10^{-4} \pm 2.95 \times 10^{-4}$ a	$1.44 \times 10^{-2} \pm 2.78 \times 10^{-4}$ b	$6.60 \times 10^{-3} \pm 3.44 \times 10^{-4}$ c	$P < 0.0001$

				$F_{(1.069, 4.275)} = 541.80$
Ghrelin	$3.77 \times 10^{-2} \pm 1.60 \times 10^{-2}$ a	$3.44 \times 10^{0} \pm 3.34 \times 10^{-1}$ b	$4.62 \times 10^{0} \pm 4.42 \times 10^{-1}$ b	$P < 0.001$ $F_{(1.383, 5.531)} = 48.77$
PC1/3	$1.37 \times 10^{-2} \pm 8.31 \times 10^{-4}$ a	$3.69 \times 10^{-2} \pm 2.39 \times 10^{-3}$ b	$2.43 \times 10^{-2} \pm 2.58 \times 10^{-3}$ c	$P < 0.001$ $F_{(1.915, 7.661)} = 35.31$
GOAT	$3.38 \times 10^{-4} \pm 2.56 \times 10^{-5}$ a	$6.40 \times 10^{-3} \pm 7.51 \times 10^{-4}$ b	$8.53 \times 10^{-3} \pm 9.43 \times 10^{-4}$ b	$P < 0.001$ $F_{(1.626, 6.505)} = 38.57$

Data are expressed as mean ± SEM (*n*=5 mice). Different letters indicate significant differences between groups ($P < 0.05$ determined by one-way ANOVA with repeated measures followed by Tukey's *post hoc* test).

Table 2.3. Immunofluorescence statistics information. Number of immunopositive cells for GPR93, T1R3, CD36 and FFAR4 in the gastric antrum and corpus.

Immunofluorescence target	Immunopositive cells/unit area		Paired *t*-test
	Antrum	Corpus	
GPR93	12.1 ± 0.5	No immunolabelling	N/A
T1R3	12.6 ± 0.4	12.6 ± 0.7	$P > 0.99$ $t = 0$ $df = 4$
CD36	13.1 ± 1.2	39.2 ± 2.7	$P < 0.001$ $t = 13.61$ $df = 4$
FFAR4	10.1 ± 1.0	No immunolabelling in the glandular base*.	N/A

Cell counts show the mean value of 5-6 tissue sections (at the glandular base only) per mouse and gastric region (unit area=159 µm × 159 µm). *FFAR4 apical cells were not counted due to high crowding and overlapping. Data are expressed as mean ± SEM (*n*=5-6 mice). Statistical significance was determined by paired *t*-test.

Chapter 3: The secretion of total and acyl ghrelin from the mouse gastric mucosa: role of nutrients and the lipid chemosensors FFAR4 and CD36

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Name of Principal Author (Candidate)	Maria Nunez-Salces		
Contribution to the Paper	Designed the study, conducted experiments, analysed and interpreted data, wrote and approved the final version of the manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/07/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Hui Li		
Contribution to the Paper	Designed the study, conducted experiments, interpreted data, edited and approved the final version of the manuscript.		
Signature		Date	13//7/2020

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Contribution to the Paper	Conceptualised and designed the study, interpreted data, edited and approved the final version of the manuscript.		
Signature		Date	15/07/2020

Name of Co-Author	Amanda J Page		
Contribution to the Paper	Conceptualised and designed the study, interpreted data, wrote and approved the final version of the manuscript.		
Signature		Date	13/7/20

3.1. ABSTRACT

Background/objective: Ghrelin is a multifunctional orexigenic hormone primarily secreted by the stomach. Circulating ghrelin levels rise before meals and fall promptly after food intake, with the postprandial ghrelin reduction being powerfully influenced by meal macronutrient content. Limited information is available on the control of ghrelin secretion by macronutrients and nutrient chemosensors. Therefore, this study investigated the nutrient-mediated modulation of total ghrelin (TG) and acyl ghrelin (AG) secretion from the mouse stomach, and the role of long-chain fatty acid chemosensors, FFAR4 and CD36, in lipid-mediated modulation of TG and AG release.

Methods: *Ex-vivo* experiments were conducted using gastric mucosa to examine effects of nutrients (2 and 20 mM D-glucose, 20 mM L-phenylalanine, 5% peptone (a water-soluble mixture of oligopeptides and single amino acids), 5% D-mannitol, 2 mM α-linolenic acid and 5% fat emulsion (intralipid)) on TG and AG secretion in mice. The effect of blocking FFAR4 (with AH 7614) and CD36 (with sulfosuccinimidyl oleate sodium) on α-linolenic acid and intralipid-mediated regulation of TG and AG secretion was also assessed.

Results: TG and AG secretion were unaffected by glucose and mannitol. Peptone stimulated the release of TG and AG. In contrast, L-phenylalanine reduced AG secretion only. Intralipid reduced TG secretion and stimulated AG secretion in a FFAR4 and CD36-independent manner. α-linolenic acid reduced AG release, in a FFAR4 and CD36-independent manner, without affecting TG mobilisation.

Conclusion: Ghrelin secretion from the mouse gastric mucosa is modulated in a nutrient-specific manner by proteins and lipids, with TG and AG displaying independent responses to the same stimuli. In addition, the results of this report indicate that FFAR4 and CD36 do not participate in the α-linolenic acid and intralipid-mediated TG and AG secretion.

3.2. INTRODUCTION

Ghrelin is a stomach-derived hormone[48], the natural ligand of the growth hormone secretagogue receptor 1a (GHSR1a), and a multifaceted hormone that stimulates growth hormone release, food intake[174,175], adiposity[147,180,181] and hyperglycaemia[184,362] via GHSR1a activation. In order to bind the GHSR1a and display its biological effects, ghrelin requires acylation on its third serine residue[160], which is catalysed by the enzyme ghrelin O-acyltransferase (GOAT)[159,160]. While only acyl ghrelin (AG) binds the GHSR1a, approximately 90% of total ghrelin (TG) in the circulation is present as des-acyl ghrelin (DAG)[157]. Limited knowledge exists on the biological effects of DAG, although there is growing evidence to suggest that DAG acts as a separate hormone, mostly antagonising the effects of AG, possibly via a GHSR1a-independent pathway[365]. For instance, DAG administration has been shown to decrease food intake[290], and block the orexigenic action of peripherally administered AG in rodents[289]. Due to the effects of AG and DAG, the modulation of their circulating levels has been a target for the development of therapies in the treatment of obesity. It is well established that circulating ghrelin levels rise before meals and fall after food intake[146]. While the preprandial rise of ghrelin appears to be regulated by the autonomic nervous system[189-193], the postprandial reduction of circulating ghrelin levels is strongly regulated by macronutrient content, with a transient decrease in circulating ghrelin in response to dietary carbohydrates, a prolonged inhibition by proteins and weak inhibition by lipids[198,219]. However, minimal information is available on the effects of nutrients on ghrelin secretion, specifically, in the stomach, with mechanistic reports showing that glucose either reduces[210] or does not affect[366]

gastric ghrelin secretion, while lipids and proteins have been shown to decrease[193,207,366] and increase[205,207] ghrelin release. Different methodological approaches and nutrient stimuli may explain these discrepancies. Accordingly, the first aim of the current study was to determine, under the same experimental conditions, the effect of a wide nutrient repertoire, including glucose, mannitol, L-phenylalanine, peptones, α-linolenic acid and intralipid, in the *ex vivo* gastric secretion of TG and AG.

There is increasing evidence supporting the role of nutrient-sensing in the regulation of ghrelin secretion, with several reports showing that ghrelin cells are equipped with the molecular machinery for the detection of nutrients[193,206,207,217,222]. For example, it has been demonstrated that the long-chain fatty acid (LCFA) receptor, free fatty acid receptor 4 (FFAR4), and LCFA chemosensor and transporter, cluster of differentiation (CD) 36, were highly co-localised with gastric ghrelin in the mouse stomach[367]. Mechanistic studies have confirmed the involvement of FFAR4 in the secretion of intestinal hormones, including cholecystokinin (CCK)[116], gastric inhibitory polypeptide (GIP)[368] and glucagon-like peptide (GLP-1)[117], and the involvement of CD36 in the release of CCK and secretin[118]. However, the role of FFAR4 and CD36 in lipid-mediated ghrelin secretion remains unclear. While conflicting findings have been reported about the involvement of FFAR4 in gastric ghrelin secretion[193,207,208], the participation of CD36 has not been investigated. Thus, the second aim of this study was to determine the role of FFAR4 and CD36 in the lipid-mediated secretion of gastric TG and AG.

3.3. MATERIALS AND METHODS

3.3.1. Mice

All experiments were approved by Animal Ethics Committees of the South Australian Health & Medical Research Institute and The University of Adelaide, Australia, and were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). 8-week-old male C57BL/6 mice were group-housed in a controlled environment (12 hours light/12 hours dark cycle, temperature 22 ± 0.5°C, 40-60% humidity) with *ad libitum* access to drinking water and a standard laboratory diet containing 24%, 18% and 58% of energy from protein, fat and carbohydrates, respectively (Teklad Rodent Diet 2018, ENVIGO, Wisconsin, USA).

3.3.2. Chemicals

D-glucose was obtained from Univar, SA Australia. D-mannitol, L-phenylalanine (L-Phe), α-linolenic acid (αLA), sodium octanoate and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich, NSW Australia. Intralipid 20% was obtained from Fresenius Kabi, NSW Australia; and peptone from casein (tryptone) from Merck, VIC Australia. 4-Methyl-N-9H-xanthen-9-yl-benzenesulfonamide (AH 7614; FFAR4 antagonist), 5-(3-Chloro-4-cyclohexylphenyl)-1-(3-methoxyphenyl)-1H-pyrazole-3-carboxylic acid (TC LPA5 4; G protein-coupled receptor 93 (GPR93) antagonist) and NPS 2143 hydrochloride (calcium-sensing receptor (CaSR) negative allosteric modulator) were purchased from Tocris Bioscience, VIC Australia; and sulfosuccinimidyl oleate sodium ((SSO), CD36 inhibitor) from Abcam, VIC Australia.

3.3.3. *Ex vivo* ghrelin secretion experiments

Experimental conditions for *ex vivo* ghrelin secretion experiments using tissue segments were adapted from previous reports[51,194,210]. Available evidence has demonstrated that a high variability and depletion in gastric ghrelin reservoirs is observed in rats[369] and humans[206] that underwent overnight fasting. Therefore, for normalisation and maximisation of gastric ghrelin reservoirs, mice were fasted the day before the experiment at 1700 h, and refed the following morning, from 0800 to 1100 h, prior to being humanely killed via CO_2 inhalation. The stomach was removed, opened along the greater curvature, divided longitudinally into ventral and dorsal tissue specimens and placed in modified Krebs buffer containing (mM): Glucose 20.0 (to suppress ghrelin release during dissection[194]), citric acid 1.0, NaCl 118.1, KCl 4.7, $NaHCO_3$ 25.1, NaH_2PO_4 1.3, $MgSO_4.7H_2O$ 1.2 and $CaCl_2$ 1.5, bubbled with 95% O_2-5% CO_2 at 4°C to prevent metabolic degradation. Tissue specimens were pinned mucosa side up and, under the dissecting microscope, the mucosa was gently separated from the muscle using fine forceps (style 7, polished, Dumont Switzerland). Isolated mucosa from each tissue specimen was weighed and transferred to a 48-well plate for secretion experiments. In this way, mucosal segments from one side of the stomach were incubated in a control solution, while segments from the other side of the stomach were incubated in a nutrient solution (allocation to control or nutrient solution was randomised, 500 µL/well, 1 h incubation at 37°C, 95% O_2-5% CO_2). For experiments with nutrient receptor inhibitors, mucosal segments were pre-incubated in Krebs buffer (37 °C) containing the test antagonist or vehicle (1% dimethyl sulfoxide (DMSO)) for 8-10 min, prior to incubation in nutrient solutions (37°C) containing the test

antagonist or vehicle. After incubation, nutrient solutions were collected, centrifuged (1500 rpm, 3 min) and acidified with HCl (final concentration 0.05N) to minimise AG breakdown and stored at -80 °C until analysis. TG and AG content of supernatants was assessed using commercial ELISA kits according to the manufacturer's instructions (TG: EZRGRT-91K, AG: EZRGRA-90K, Merck).

3.3.4. Nutrient solutions

For experiments exploring the nutrient-mediated secretion of ghrelin, mucosal segments were exposed to 2 and 20 mM D-glucose, 20 mM L-Phe, 5% peptone, 5% D-mannitol, 2 mM αLA or 5% intralipid emulsion. Nutrient concentrations were derived from previous reports investigating the effect of nutrients on ghrelin secretion[194,205,221,366]. Nutrient solutions were prepared in Krebs buffer containing 2 mM D-Glucose (except for the solutions containing 2 and 20 mM D-glucose). For nutrient solutions containing αLA, a 200 mM αLA stock solution (100×) was prepared by mixing equimolar concentrations of αLA and NaOH. On the day of the experiment, 0.33 mM BSA was added to the 2 mM αLA solution to form a BSA-fatty acid conjugate. BSA and NaOH were added to the control solutions for the αLA experiments. For experiments testing the involvement of nutrient chemosensors in the nutrient-mediated secretion of ghrelin, mucosal segments were exposed to inhibitors of FFAR4 (10 μM AH 7614), CD36 (400 μM SSO), GPR93 (10 μM TC LPA5 4) and CaSR (25 μM NPS 2143). The concentration of inhibitors was consistent with previous reports[119,370-372]. All nutrient solutions contained a protease inhibitor cocktail (cOmplete ULTRA Tablets, Mini, EDTA-free; Merck) and 50 μM sodium

octanoate as a source of acyl donors for activation of ghrelin[194]. A stock solution of 10 mM sodium octanoate (200×) was prepared according to a previous report[194]. Briefly, 10 mM sodium octanoate was dissolved in 6.7 mL of 100 mM NaCl, and mixed with 3.3 mL of 10% fatty acid-free BSA. All solutions were adjusted to pH 7.4 and filtered through a Millex-GV sterile syringe filter unit (0.22 μm pore size, Merck). The osmolality of nutrient solutions was measured by SA Pathology, SA Australia and fell within 329-341mOsmol/Kg (see Table 3.1 for details), except for the 5% peptone solution (601 mOsmol/Kg) and the 5% D-mannitol solution (612 mOsmol/Kg).

Table 3.1. Osmolality of nutrient solutions.

Nutrient solution	Osmolality (mOsmol/Kg)
2 mM D-Glucose*	329
2 mM D-Glucose containing NaOH and BSA**	315
20 mM D-Glucose	339
20 mM L-Phe	341
5% Peptone	601
5% D-Mannitol	612
2 mM α-Linolenic acid	332
5% Intralipid	341

*Control solution for most ghrelin secretion experiments. ** Control solution for experiments investigating the effect of α-Linolenic acid on ghrelin secretion.

3.3.5. Statistical analysis

Results are expressed as mean ± SEM. Ghrelin secretion results were normalised to wet weight of tissue specimens. Differences in nutrient-mediated secretion of ghrelin were assessed by unpaired Student's *t*-test. The effect of nutrient chemosensor inhibitors on ghrelin secretion was evaluated using two-

way ANOVA followed by Tukey's multiple comparison tests (GraphPad Prism version 7.02, La Jolla California USA). Statistical significance for unpaired Student's *t*-test and two-way ANOVA is denoted as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Statistical significance for Tukey's multiple comparisons test is indicated as $^{\#}P < 0.05$, $^{\S}P < 0.01$ and $^{\wedge}P < 0.001$.

3.4. RESULTS

3.4.1. The effect of 2 and 20 mM D-Glucose on the gastric secretion of total and acyl ghrelin

TG and AG secretion in response to different glucose environments is shown in Figure 3.1. The secretion of TG (Figure 3.1A) and AG (Figure 3.1B) from gastric mucosal segments was unaffected by 2 and 20 mM glucose.

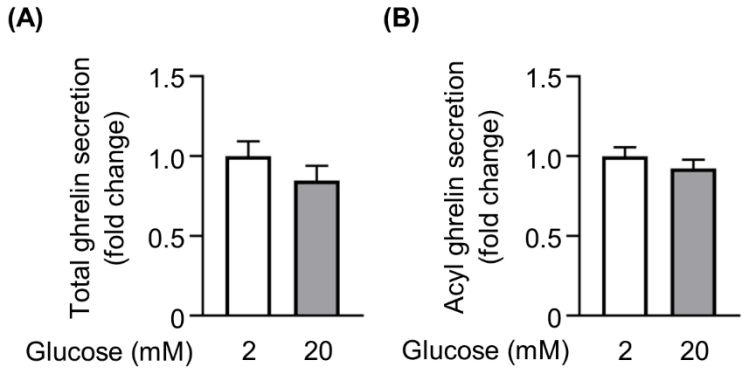

Figure 3.1. Glucose does not affect secretion of TG and AG from mouse gastric mucosal segments.

Levels of TG (A) and AG (B) released by gastric mucosal segments in response to 2 and 20 mM glucose. Data are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's *t*-test. *n*=7 mice.

3.4.2. The effect of protein digestion products and hyperosmolality on the gastric secretion of total and acyl ghrelin

The effects of protein digestion products and hyperosmolality on the secretion of TG and AG are shown in Figure 3.2. A hyperosmolar solution (601 mOsmol/Kg) containing 5% peptone strongly stimulated TG (25.32 ± 2.54-fold; $P < 0.001$; Figure 3.2A) and AG secretion (2.30 ± 0.25-fold; $P < 0.001$; Figure 3.2B) from gastric mucosal segments. An equiosmolar solution containing D-mannitol (612 mOsmol/Kg) did not affect TG and AG release (Figure 3.2C-D). In contrast, 20 mM L-Phe reduced AG secretion (0.51 ± 0.02-fold; $P < 0.01$; Figure 3.2F), but did not change TG secretion (Figure 3.2E). The role of GPR93 (TC LPA5 4) and CaSR (NPS 2143) in acyl ghrelin release could not be assessed due to perturbations of the vehicle (1% DMSO) on the effect of protein digestion products on acyl ghrelin secretion (Supplementary information: Figure 3.5).

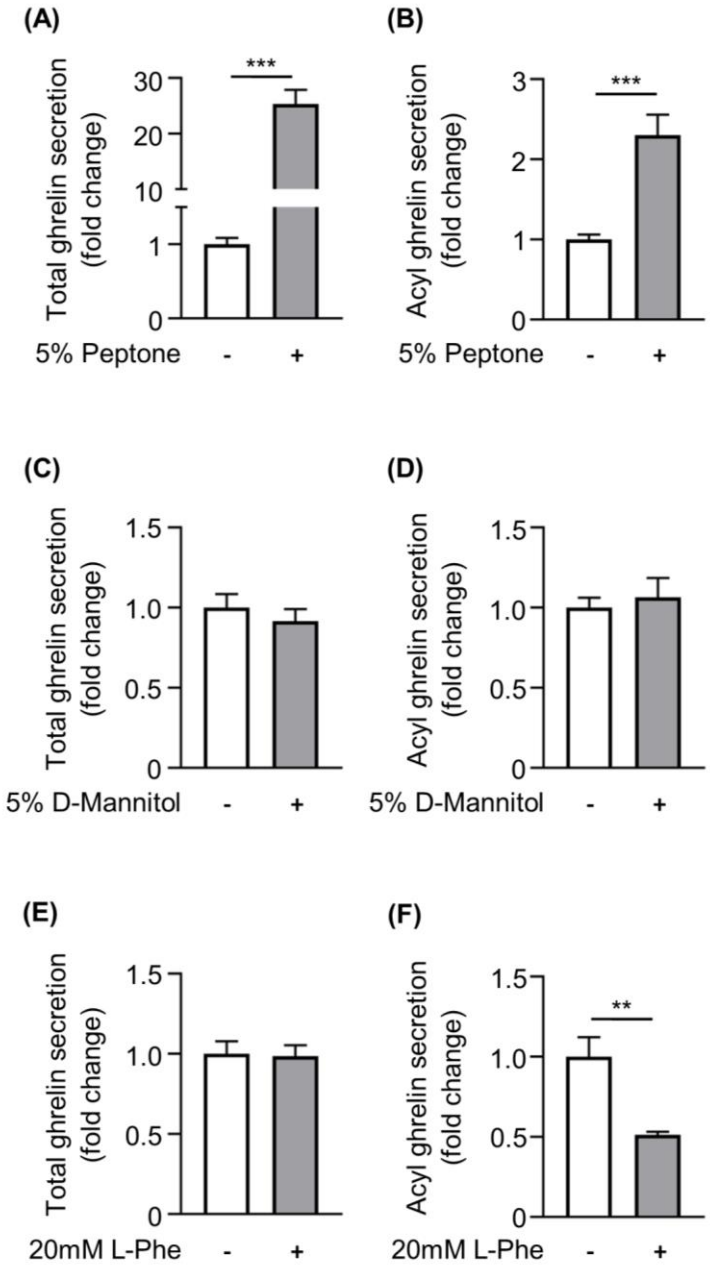

Figure 3.2. The effect of protein digestion products and osmolality on the secretion of TG and AG from mouse gastric mucosal segments.

Secretion of TG (A) and AG (B) was stimulated by 5% peptone. Experiments with equiosmolar solutions of D-mannitol did not affect the secretion of TG (C) or AG (D), indicating that 5% peptone stimulates ghrelin secretion in an osmolality-independent manner. (E-F) 20 mM L-Phe reduced the secretion of AG only. Data are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's *t*-test. *n*=6 mice. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

3.4.3. The effect of lipids on the gastric secretion of total and acyl ghrelin

The effects of 5% intralipid on the secretion of TG and AG by gastric mucosal segments are shown in Figure 3.3. A solution containing 5% intralipid reduced 0.67 ± 0.03-fold TG secretion ($P < 0.01$; Figure 3.3A), but increased 1.74 ± 0.22-fold AG secretion ($P < 0.05$; Figure 3.3B). Experiments investigating the effect of the FFAR4 antagonist (10 μM AH 7614) revealed that TG secretion was unchanged in the presence of AH 7614 (Intralipid: 0.69 ± 0.02-fold vs Intralipid + AH 7614: 0.67 ± 0.06-fold; Figure 3.3C). In addition, the intralipid-mediated stimulation of AG secretion was unchanged in the presence of AH 7614 (Intralipid: 1.20 ± 0.04-fold vs Intralipid + AH 7614: 1.21 ± 0.06-fold; Figure 3.3D). No interaction between the effect of intralipid and the effect of AH 7614 was observed. The CD36 inhibitor, SSO (400μM), did not affect the intralipid-mediated inhibition of TG secretion (intralipid: 0.69 ± 0.02-fold vs intralipid + SSO: 0.63 ± 0.04-fold). However, SSO alone decreased the secretion of TG (0.66 ± 0.05-fold; $P < 0.01$; Figure 3.3E). A significant interaction was observed between the effect of intralipid and the effect of SSO ($P < 0.05$). Furthermore, the increase in AG secretion in response to intralipid was unchanged by SSO (intralipid: 1.20 ± 0.04-fold vs intralipid + SSO: 1.16 ± 0.06-fold, Figure 3.3F), with no interaction observed.

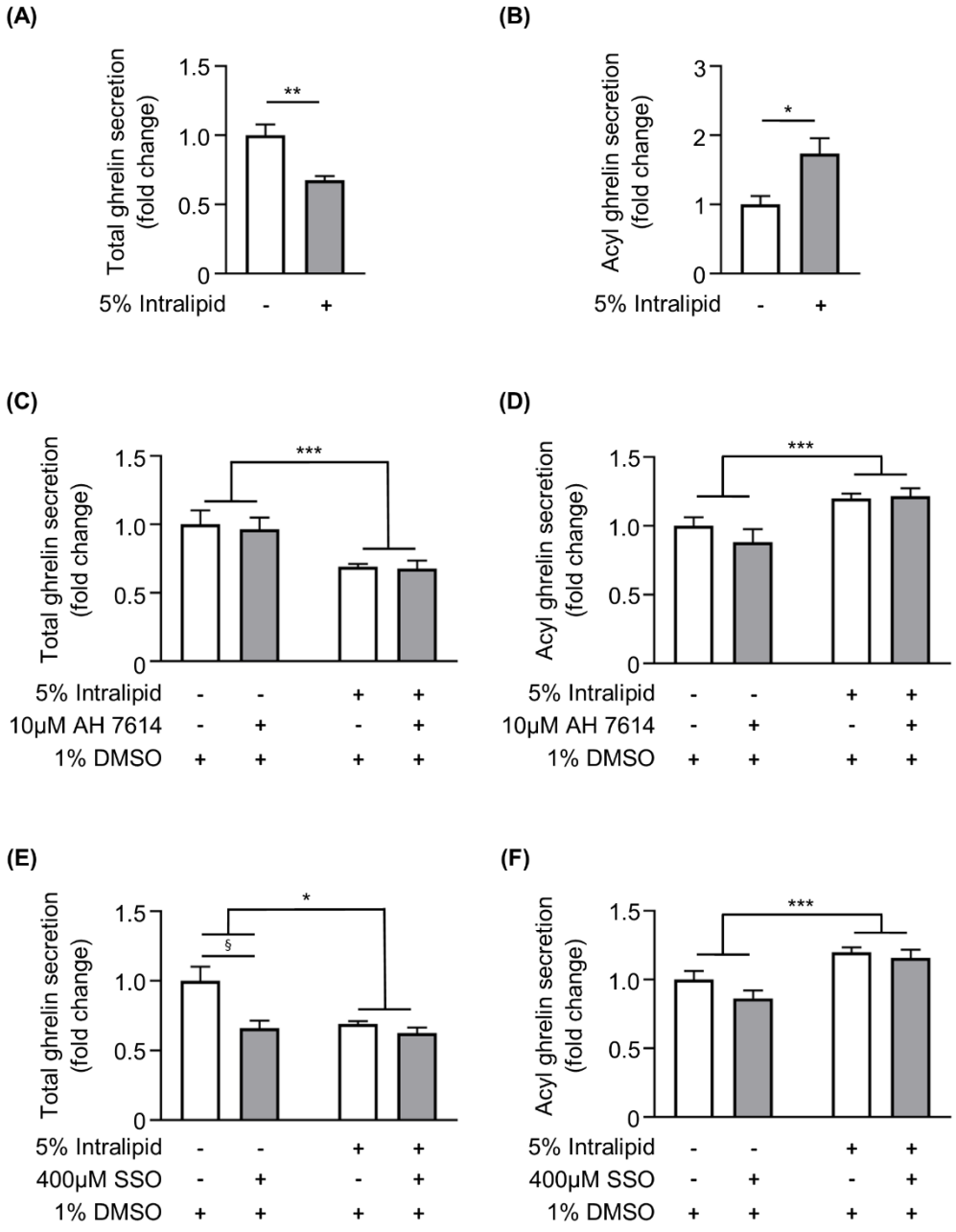

Figure 3.3. The modulation of TG and AG by 5% intralipid from mouse gastric mucosal segments does not involve FFAR4 and CD36.

(A-B) TG secretion from gastric mucosal segments was reduced by intralipid, while AG secretion was augmented. The intralipid-mediated effects on TG (C,E) and AG (D,F) were unchanged in response to antagonists for FFAR4 (AH 7614) and CD36 (SSO). (E) SSO alone decreased TG secretion. Data are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's *t*-test for experiments exploring the effect of nutrients in the secretion of ghrelin, and by two-way ANOVA followed by Tukey's multiple comparisons test for experiments including nutrient chemosensor inhibitors. *n*=6 mice. Statistical significance for unpaired Student's *t*-test and two-way ANOVA is denoted as **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. Statistical significance for Tukey's multiple comparisons test is indicated as §*P* < 0.01.

The effect of 2 mM αLA on gastric ghrelin release is shown in Figure 3.4. The secretion of TG was unaffected by αLA (Figure 3.4A), while AG secretion was reduced (0.68 ± 0.04-fold; $P < 0.05$; Figure 3.4B). In experiments with the FFAR4 antagonist, αLA-induced inhibition of AG secretion was not different in the absence or presence of AH 7614 (0.54 ± 0.04-fold vs 0.46 ± 0.03-fold, respectively; Figure 3.4C). AH 7614 significantly reduced AG concentrations ($P < 0.01$), however, there was no interaction between the effects of αLA and AH 7614. Therefore, αLA and AH 7614 reduced AG secretion, but their effects were independent. In a similar manner, the αLA-mediated inhibition of AG secretion (0.64 ± 0.12-fold) was unchanged by the addition of the CD36 inhibitor, SSO (0.80 ± 0.11-fold; Figure 3.4D).

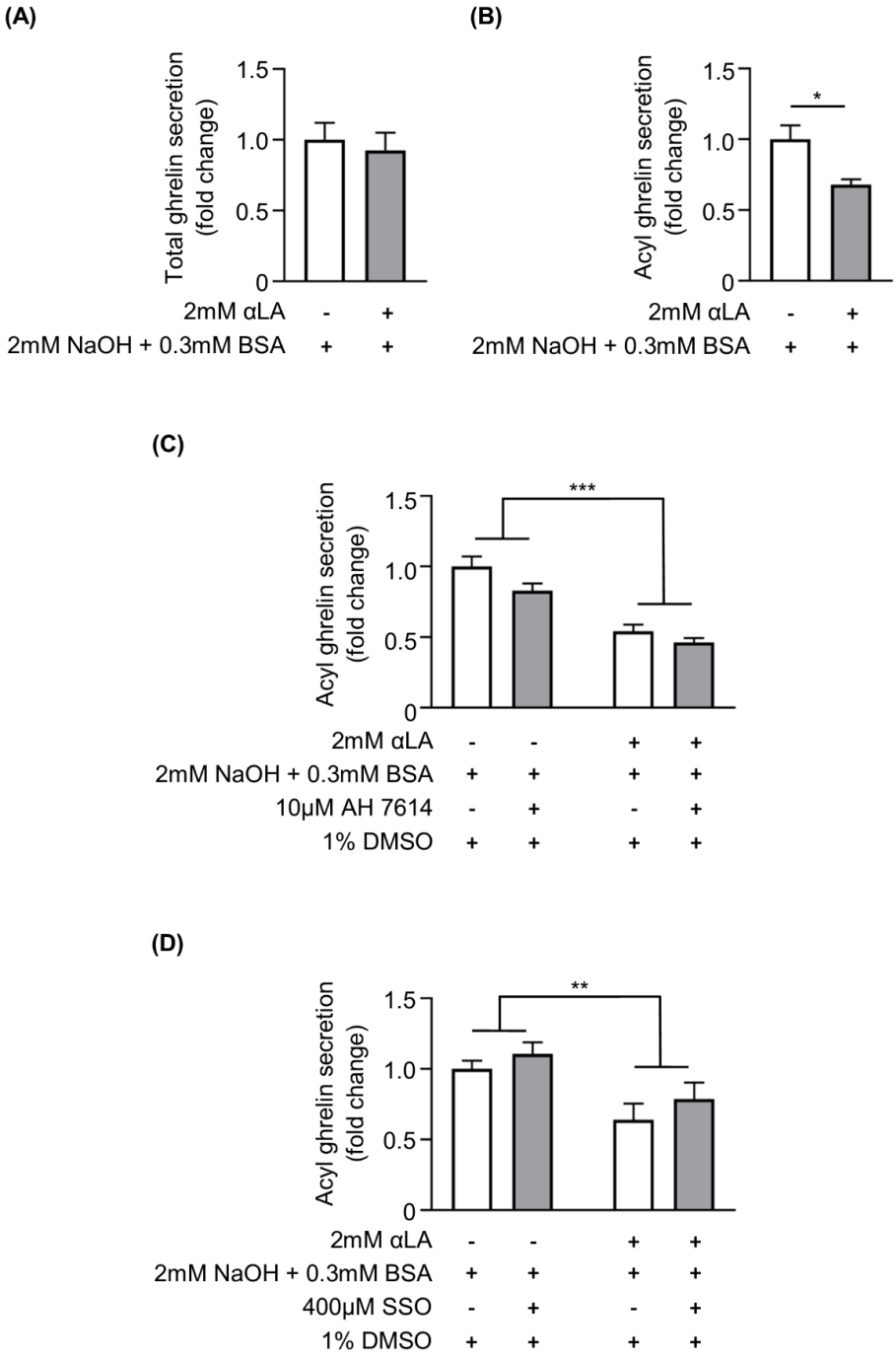

Figure 3.4. The modulation of TG and AG secretion by 20mM αLA from mouse gastric mucosal segments does not involve FFAR4 and CD36.

(A) TG secretion from gastric mucosal segments was unaffected by 20 mM αLA, (B) while AG was reduced. (C) The effects of αLA on AG secretion were unchanged in response to FFAR4 antagonist, AH 7614, which decreased AG secretion independently of αLA. (D) Similarly, the CD36 antagonist, SSO, did not impact the decrease of AG secretion by αLA. Data are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's *t*-test for experiments exploring the effect of nutrients in the secretion of ghrelin, and by two-way ANOVA followed by Tukey's multiple comparisons test for experiments including nutrient chemosensor antagonists. n=6 mice. *P < 0.05, **P < 0.01 and ***P < 0.001.

3.5. DISCUSSION

The current study investigated the nutrient-dependent modulation of ghrelin secretion, as well as the role of major gastric lipid chemosensors in the release of ghrelin. This report shows that TG and AG secretion from the stomach were modulated in a nutrient-specific manner. TG and AG remained unchanged upon exposure to different D-glucose concentrations (i.e. 2 and 20 mM). While peptone stimulated TG and AG secretion, L-Phe did not affect TG and reduced AG secretion. Furthermore, intralipid decreased TG and stimulated AG secretion in a FFAR4 and CD36-independent manner. On the other hand, α-LA reduced AG secretion in a FFAR4 and CD36-independent manner, without affecting TG. Accordingly, this report provides comprehensive information on the modulation of gastric ghrelin secretion by macronutrients and the gastric lipid chemosensors FFAR4 and CD36.

Glucose is a strong stimulus for the release of gastrointestinal (GI) hormones, including GLP-1[51] and serotonin[373]. Previous mechanistic studies have shown that ≥ 200 mM glucose, mimicking intestinal luminal glucose concentrations[51,210,373], are required for GLP-1[51] and serotonin[373] release. In contrast, intragastric infusion of a 25% glucose solution in rats failed to suppress circulating ghrelin levels when gastric emptying was prevented[201], suggesting that gastric luminal glucose has no impact on ghrelin secretion. The current report investigated ghrelin secretion in response to glucose, at concentrations simulating low (2 mM glucose) and high (20 mM glucose) glycaemia in mice[374,375], with no change in TG and AG release. In contrast, Sakata *et al.* showed, using mouse gastric cultured cells, that 1 mM glucose

enhanced, while 10 mM glucose reduced AG and DAG release[194]. Our AG results, however, may be explained by a previous study demonstrating that high glucose concentrations (≥ 50 mM) are necessary to suppress AG secretion from the human gastric mucosa[209]. This suppression was dependent on the activation of the taste receptor type 1 member 3 (T1R3) and the sodium/glucose cotransporter 1 (SGLT1)[209]. However, studies in mice have demonstrated that, although 200 mM glucose decreases TG and AG secretion from gastric segments, intragastric administration of glucose (4 g/Kg body weight) does not affect gastric ghrelin reservoirs, but boosts duodenal ghrelin content[210]. This suggests that glucose-mediated inhibition of circulating ghrelin is due to inhibition of small intestinal, rather than gastric, ghrelin release[210]. However, this requires further investigations.

Consumption of proteins and amino acids strongly suppresses plasma ghrelin levels in humans[198,214,219] and rodents[205,218,220]. Nonetheless, increasing evidence has shown that protein digestion products promote ghrelin secretion within the stomach[205,206]. The current study demonstrates that 5% peptone potently stimulates TG and AG secretion. This is consistent with studies demonstrating peptone-induced stimulation of AG secretion from mouse full-thickness gastric segments[205] and human mucosal segments[206]. However, in the same study, peptone reduced TG secretion from human mucosal segments[206], a polar difference possibly explained by distinct mechanisms governing AG and DAG secretion[206]. Furthermore, our results suggest species differences between peptone-mediated TG secretion in mice and humans. Duodenal hyperosmolarity affects circulating ghrelin levels in rats[241] and

humans[242]. However, the hyperosmolar control, 5% D-mannitol, did not affect TG and AG secretion in the current study, suggesting that 5% peptone stimulated ghrelin secretion in an osmolarity-independent manner. In contrast to the peptone solution, L-Phe halved AG secretion without affecting TG. This result is aligned with a previous report showing a comparable drop in rat plasma AG after gavage of L-Phe[218]. In contrast, a study by Vancleef *et al.* reported a 37% increase in L-Phe-evoked AG secretion from mouse ghrelinoma (MGN3-1) cells[205]. However, the same study showed that intragastric and intravenous administration of L-Phe decreased plasma AG and TG levels of mice[205]. Together, the balance of evidence, supports an inhibitory effect of L-Phe on gastric ghrelin secretion.

The nutrient-sensing mechanisms underlying ghrelin secretion in response to protein digestion products are not fully understood. To date, T1R3 (component of the umami-taste receptor T1R1/T1R3)[206,217,367], GPR93 (peptone receptor)[367] and CaSR[206] (calcium, aromatic amino acid and peptone receptor) have been shown to co-localise with ghrelin in the human[206] and mouse[217,367] stomach. It has been demonstrated that peptone and L-Phe-dependent effects on ghrelin secretion are independent of T1R1/T1R3 in human fundic segments[206] and MGN3-1 cells[205]. Furthermore, there is a high degree of co-expression (> 85%) of the peptone receptor, GPR93, and ghrelin in the mouse stomach[367]. Therefore, it was logical to assess the role of GPR93 in the peptone-stimulated secretion of ghrelin. However, due to possible interactions of the proteins with the vehicle, DMSO (1%), which has been shown to induce perturbations in the structure[376-378] of proteins and their interactions with the

surrounding water[378], the effect of the GPR93 inhibitor, TC LPA5 4, could not be defined. While DMSO-induced alterations in proteins have been normally investigated at ≥10% DMSO doses[376-378], changes in their structure and/or solubility may explain the perturbation in the peptone-induced secretion of AG. Furthermore, DMSO specifically altered the peptone-evoked stimulation in AG secretion. It is possible this is due to disruption in the acylation of ghrelin (i.e. GOAT activity), however, this requires further investigation. On the other hand, the stimulation of TG secretion, which was not affected by DMSO, remained unchanged by TC LPA5 4 (10 μM), suggesting GPR93 is not be involved in the peptone-evoked secretion of TG (i.e. mainly comprised of DAG).

The study of CaSR-mediated responses is challenging, with multiple ligands engaging distinct intracellular cascades that interact to contribute to cooperative responses[379]. DMSO-related disruption of AG secretion did not allow evaluation of the effect of the CaSR negative allosteric modulator, NPS 2143, on peptone and L-Phe-mediated AG release. However, peptone-induced TG secretion was unaffected by NPS 2143, suggesting that CaSR does not participate in peptone-induced stimulation of TG secretion (i.e. mostly containing DAG). Involvement of CaSR in the secretion of AG has been previously assessed by Engelstoft *et al.* who showed that only supraphysiological concentrations of $CaCl_2$ (40 mM) significantly decreased AG secretion from mouse primary gastric mucosal cells[193]. Similarly, the CaSR positive allosteric modulator, R-568, was shown to reduce AG secretion in the presence of 1.8 mM $CaCl_2$, but stimulated AG secretion at higher $CaCl_2$ concentrations (4 mM)[193]. Although DMSO prevented determination of the role

of the CaSR in peptone and L-Phe mediated AG secretion, it is plausible that, in the current study conditions, with a constant $CaCl_2$ concentration of 1.5 mM, L-Phe, a positive allosteric modulator of CaSR[380], would inhibit AG secretion via activation of this receptor. Moreover, the inhibitory effect of L-Phe, contained in the casein peptone solution, could be overruled due to the presence of other CaSR ligands (i.e. oligopeptides and amino acids). However, this requires further investigation. In summary, protein digestion products generally suppress plasma ghrelin levels in humans[198,214,219] and rodents[205,218,220]. However, the protein-induced mobilisation of gastric ghrelin is less straightforward and requires more research.

Studies in humans[200,221] and rodents[193,222] have shown that plasma ghrelin levels are reduced by oral[193,200,222] and intravenous[221] administration of lipids. Consistent with previous reports[207,381], the current study demonstrated that αLA (2 mM) suppressed gastric AG release only. Further, 5% intralipid simultaneously reduced TG and stimulated AG secretion. The intralipid-mediated reduction of TG levels has been described in studies in humans[221], and gastric mucosal segments from rats[366]. However, no previous report has assessed the effect of intralipid on AG secretion. Ghrelinoma PG-1 cells have been shown to produce acyl-donors for the activation of ghrelin by β-oxidation of LCFAs[163]. The major components of intralipid are LCFAs (52% linoleic acid (C18:2n-6), 22% oleic acid (C18:1n-9), 13% palmitic acid (C16:0), 8% αLA (C18:3n-3), 4% stearic acid (C18:0) and 1% others)[382]. Accordingly, the intralipid-mediated stimulation of AG release may arise due to the abundance of LCFA substrates for the acylation of ghrelin. Moreover, the different patterns

of TG and AG secretion between αLA and intralipid experiments show the complexity of the lipid-mediated secretion of ghrelin.

Previous gene expression studies in mice have identified FFAR4 as the most enriched fatty acid receptor in gastric ghrelin cells[193], with FFAR4 expressed in at least 65% of antral ghrelin cells in mice[367]. Nonetheless, the current study demonstrated that FFAR4 is not involved in αLA and intralipid-mediated secretion of TG and AG. Consistent with these results, Janssen *et al.* reported a lack of effect of the FFAR4 agonist, grifolic acid, on the secretion of TG and AG from MGN3-1 cells, however, oral gavage of grifolic acid increased plasma AG in mice[207]. Further, other reports have demonstrated that the FFAR4 agonists, Compound B[193] and GW-9508[208], reduced plasma AG levels in mice, and inhibited AG secretion from mouse gastric primary mucosal cells and PG-1 cells. Different experimental conditions may partially explain these discrepancies. Moreover, the promiscuous nature of FFAR4[383-385] and the diverse downstream signalling cascades triggered upon FFAR4 activation[193,223] may act as a confounding factor for the study of FFAR4-mediated ghrelin secretion. Therefore, our results suggest that FFAR4 is not involved in αLA or intralipid-mediated modulation of ghrelin secretion. However, more detailed studies are necessary to understand the complexity of FFAR4 signalling.

Immunofluorescence studies in mice have demonstrated that over 85% of gastric ghrelin cells express CD36[367]. Ghrelin cells may use LCFAs to produce acyl donors for the activation of ghrelin[163,386]. Therefore, it is plausible that CD36 participates in the LCFA uptake of gastric ghrelin cells. However, this

study showed that the irreversible CD36 inhibitor, SSO, did not affect the αLA and intralipid-mediated secretion of TG and AG. Remarkably, SSO alone decreased basal TG secretion to an equal magnitude to that observed with the intralipid solution, suggesting that CD36 does have a role in ghrelin mobilisation. Further studies are necessary to understand the modulation of ghrelin secretion by CD36.

In conclusion, TG and AG secretion are modulated in a nutrient-specific manner by lipids and proteins. Gastric ghrelin secretion was reduced or stimulated by nutrients. While food intake typically decreases circulating ghrelin levels[198,219], previous reports have demonstrated that gastric ghrelin content[166,207,387] and circulating ghrelin levels[164,165,387-389] can be increased by dietary supplementation with lipids[164-166,207] and proteins[387-389]. Furthermore, this report demonstrated that TG and AG do not necessarily follow the same patterns of mobilisation, and suggest that they are regulated by different mechanisms. The outcomes from this study also indicate that FFAR4 and CD36 do not participate in αLA and intralipid-mediated secretion of TG and AG in mice. The results from this report display the complex regulation of gastric ghrelin release by nutrients, and in adding new knowledge, emphasise the need for further mechanistic insight into the nutrient-specific pathways that control ghrelin secretion. In this regard, future studies using knockout models of nutrient chemosensors will help to better understand the physiological relevance of FFAR4 and CD36 in the modulation of gastric ghrelin secretion, which subsequently impacts circulating ghrelin levels.

3.6. SUPPLEMENTARY INFORMATION

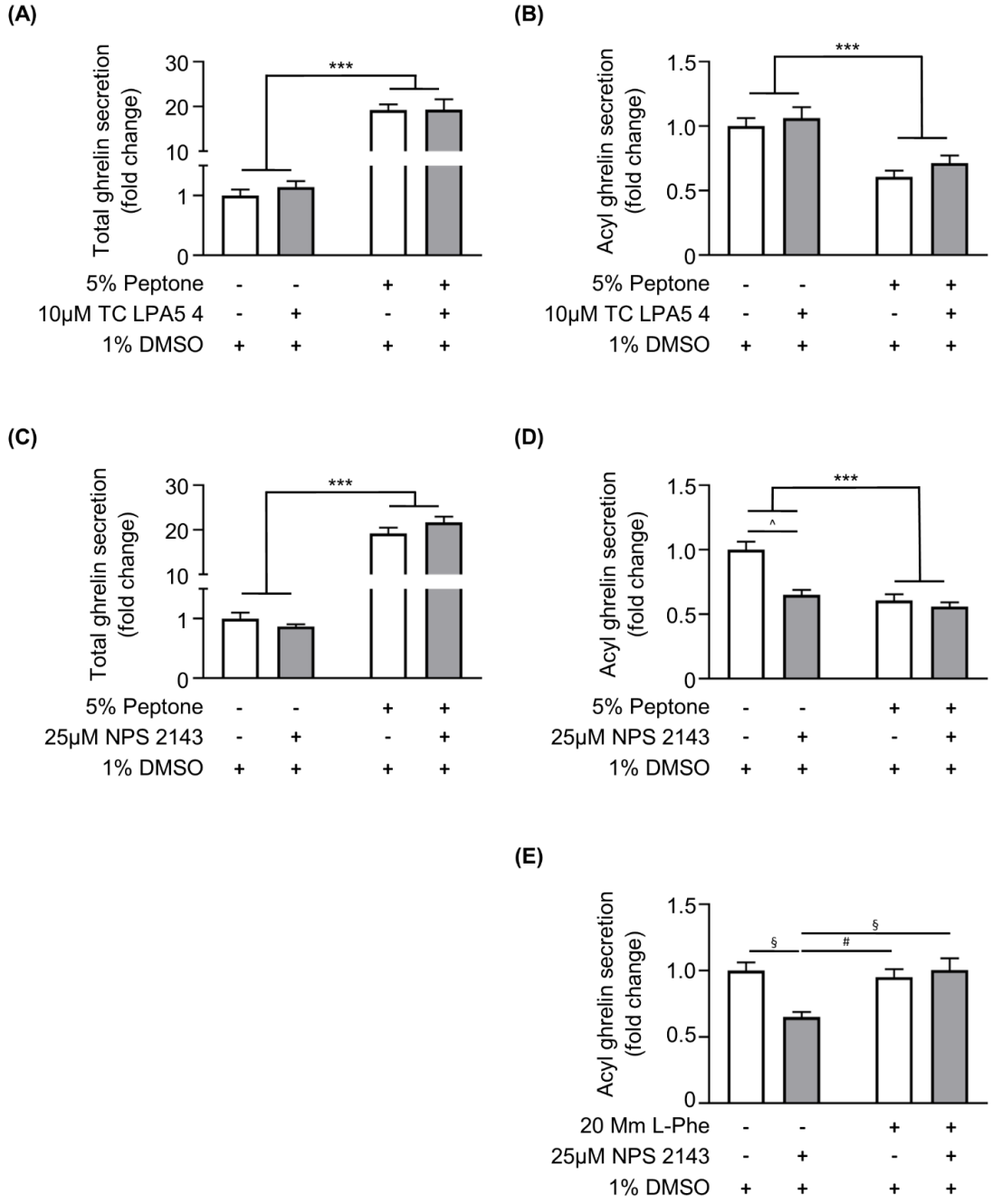

Figure 3.5. The effects of antagonists of GPR93 (TC LPA5 4), CaSR (NPS 2143) and 1% DMSO on protein-dependent secretion of TG and AG from gastric mucosal segments.

(A) A 19.18 ± 1.29-fold increase of TG secretion by peptone was unaltered by the addition of TC LPA5 4 (19.28 ± 2.30-fold increase). (C) Similarly, the stimulation of TG secretion by peptone was unaffected by the addition of NPS 2143 (21.66 ± 1.29-fold increase). (B, D) However, the peptone-mediated stimulation of AG secretion was altered by 1% DMSO, causing a reduction in AG concentration (0.61 ± 0.05-fold) compared to the control. (E) The reduction in AG secretion by L-Phe was abolished by 1% DMSO. (D, E) The CaSR antagonist, NPS 2143, suppressed the basal secretion of AG (0.65 ± 0.04-fold), resulting in significant interactions between effects of peptone and NPS 2143 ($P < 0.001$), and the effects of L-Phe and NPS 2143 ($P < 0.01$). Data are expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test. n=6 mice. Statistical significance for two-way ANOVA is denoted as $^{***}P < 0.001$. Statistical significance for Tukey's multiple comparisons test is indicated as $^{\#}P < 0.05$, $^{\S}P < 0.01$ and $^{\wedge}P < 0.001$.

Chapter 4: The effect of high-fat diet-induced obesity on the expression of nutrient chemosensors in the mouse stomach and the gastric ghrelin cell

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Contribution to the Paper	Designed the study, conducted experiments, analysed and interpreted data, wrote and approved the final version of the manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/07/2020

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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4.1. ABSTRACT

The stomach is the primary source of the orexigenic and adiposity-promoting hormone, ghrelin. There is emerging evidence on nutrient mediated modulation of gastric ghrelin secretion. However, limited information is available on gastric nutrient-sensing mechanisms in high-fat diet (HFD)-induced obesity. This study investigated the impact of HFD-induced obesity on the expression of nutrient chemosensors in the mouse stomach, particularly ghrelin cells. Male C57BL/6 mice were fed either standard laboratory diet (SLD) or HFD for 12 weeks. Expression of ghrelin, enzymes involved in ghrelin production (PC1/3, GOAT) and nutrient chemosensors (CD36, FFAR2&4, GPR93, CaSR, mGluR4 and T1R3) was determined by quantitative RT-PCR in the mouse corpus and antrum. Immunohistochemistry assessed the protein expression of CaSR and ghrelin in the corpus and antrum. Antral mRNA levels of CaSR and PC1/3 were increased in HFD compared to SLD mice, while mRNA levels of all other nutrient chemosensors examined remained unchanged. CaSR immunolabelling was observed in the gastric antrum only. Nearly 80% of antral ghrelin cells expressed CaSR, with similar cell density and co-expression in SLD and HFD mice. In conclusion, HFD-induced obesity increased CaSR mRNA expression in mouse antrum. However, the high antral co-expression of CaSR and ghrelin was unaltered in HFD compared to SLD mice.

Keywords: Ghrelin; stomach; nutrient sensing; CaSR; obesity; high-fat diet

4.2. INTRODUCTION

Obesity is a global health issue characterised by an excessive body weight (i.e. body mass index (BMI) ≥ 30 kg/m^2) and accompanying metabolic disorders, such as type 2 diabetes and cardiovascular disease[1]. Obesity is primarily caused by a chronic energy imbalance, often resulting from increased consumption of energy-dense foods that are rich in fat and sugars[1]. Accordingly, a better understanding of the mechanisms controlling food intake is essential for the development of strategies for the treatment of obesity. In this context, research focused on gastrointestinal hormones that modulate appetite and energy metabolism has exponentially increased.

Ghrelin is a gastrointestinal hormone with an important role in body energy homeostasis, stimulating food intake and adiposity[270,271]. Ghrelin is primarily produced by P/D1 (human) and X/A-like (rodent) cells of the stomach[145], and requires a number of enzymatic steps to become biologically active. Initially, cleavage of pre-proghrelin produces proghrelin, which subsequently undergoes another cleavage step at the Pro-Arg site on the C-terminal region, by the enzyme prohormone convertase 1/3 (PC1/3), to produce the mature ghrelin peptide[154,158]. In addition, acylation of ghrelin, on its third serine residue, by the enzyme ghrelin O-acyltransferase (GOAT), results in the production of acyl ghrelin[154,159,160], which can bind the ghrelin receptor, growth hormone secretagogue receptor 1a (GHSR1a)[154,390]. Acyl ghrelin accounts for less than 10% of the circulating levels of total ghrelin, while 90% is found as des-acyl ghrelin[157,167,391].

It is well established that circulating ghrelin levels fall after food intake[146,197,198]. Multiple peripheral mechanisms are involved in the postprandial reduction in circulating ghrelin levels, including digestive breakdown of nutrients[236] and the increase in circulating levels of ghrelin-inhibiting hormones (e.g. cholecystokinin[245] and insulin[247,248]). Furthermore, there is emerging evidence to indicate that gastric nutrient sensing is an important contributing factor in the control of ghrelin secretion. Previous studies have demonstrated that numerous nutrient chemosensors are highly expressed in gastric ghrelin cells, including receptors for bitter (taste receptors type 2; T2Rs[211,231]), sweet and umami compounds (taste receptor 1 member 3; T1R3[217,367]), short-chain fatty acids (free fatty acid receptor (FFAR) 2[193]), long-chain fatty acids (FFAR4[193,367] and cluster of differentiation (CD) 36[367]), and protein digestion products (calcium-sensing receptor (CaSR[193,206]) and G protein-coupled receptor 93 (GPR93[367])). Many of these nutrient chemosensors were found to be involved in ghrelin secretion. Bitter compounds stimulated ghrelin secretion, via T2R5 and T2R10 activation, in gastric segments and cultures from humans[209]. In contrast, glucose reduced ghrelin release, via T1R3 activation, in gastric cultures from obese patients[209]. Similarly, FFAR2 agonists reduced ghrelin secretion from mouse gastric cultures[193]. However, the role of FFAR4 and CaSR in ghrelin release remains elusive, with reports showing that they can stimulate[193,205,207], reduce[193,208] or not affect[206,207] ghrelin secretion.

Disturbances in the ghrelin system, such as reduced circulating ghrelin levels[272,275,392], central ghrelin resistance[273], and inability of ghrelin to stimulate food intake[278], have been reported in obesity. However, there is limited

knowledge available on the mechanisms that control ghrelin secretion in obesity. Regarding the role of nutrients as mediators of ghrelin secretion, previous reports have shown that obesity impairs ghrelin secretion in response to bitter and sweet stimuli in the small intestine[209] as well as gastric ghrelin secretion mediated by protein hydrolysates[206]. The aim of the current study was to determine, using a HFD-induced obese mouse model[393,394], obesity-induced changes in mRNA and protein expression of gastric nutrient chemosensors, with a particular emphasis on the degree of co-expression with gastric ghrelin cells.

4.3. MATERIALS AND METHODS

4.3.1. Study design and ethics

Male C57BL/6 mice (7-weeks old) were group-housed with littermates for the duration of the experiment in a facility with light (12 hour light/dark cycle), temperature (22 ± 0.5°C) and humidity (40-60%) controlled environment. After one-week acclimatisation, mice were randomly assigned to 12 weeks of either a SLD or HFD, with *ad libitum* access to their respective diet and drinking water. The SLD contained 18%, 24% and 58% energy from fat, protein and carbohydrates (Teklad Rodent Diet 2018, ENVIGO, Wisconsin, USA), and the HFD contained 60%, 20% and 20% energy from fat (lard), protein and carbohydrates, respectively (adapted from Research Diets Inc., New Brunswick, USA). Body weight of animals was determined weekly. All experiments were approved by the South Australian Health & Medical Research Institute Animal Ethics Committee (SAM232, 01 December 2016).

4.3.2. Procedure for tissue collection

For gene expression experiments, mice were anaesthetised by isoflurane inhalation (3% isoflurane in 1.5% oxygen) and exsanguinated for blood glucose measurement using a glucose meter (Accu-Chek, NSW, Australia). The gonadal fat pad was excised and weighed. After removal of the stomach, mucosal scrapings of the corpus and antrum (i.e. glandular regions) were collected and snap-frozen in liquid nitrogen prior to storage (-80°C) until required. For the immunohistochemistry experiments, mice were anaesthetised by isoflurane inhalation. Once fully anaesthetised, mice were given an IP injection of pentobarbitone (0.2 mL, 60 mg mL^{-1}) immediately prior to

transcardial perfusion of warm heparinised saline (flow rate: 17 mL min^{-1}, 3 min) followed by cold 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PFA-PB, flow rate: 15 mL min^{-1}, 50 mL). The stomach was removed, opened along the greater curvature and incubated in PFA-PB buffer at room temperature for 2 hours. After fixation, the stomach was placed in 30% sucrose-PB solution overnight for cryoprotection, prior to positioning and embedding in optimal cutting temperature compound (Tissue-Tek, ProSciTech, QLD, Australia) to enable longitudinal cryosectioning of the stomach. After processing, the tissue was stored at -80°C until required for sectioning.

4.3.3. Measurement of chemosensor mRNA and protein levels in the mouse stomach and degree of co-expression with ghrelin

4.3.3.1. Quantitative RT-PCR

The detailed protocol for gene expression experiments has been previously reported[367]. In brief, a PureLink RNA Mini kit (Thermo Fisher Scientific, SA, Australia) was used to extract total RNA from the corpus and antrum mucosa, according to the manufacturer's instructions. Spectrophotometry (260 nm) was used to quantify total RNA, and the $A_{260/280}$ ratio was used as an estimation of purity.

An EXPRESS One-Step Superscript™ qRT-PCR Kit (Life Technologies, SA, Australia) and 7500 Fast Real-Time PCR system (Applied Biosystems, SA, Australia) was used for the quantitative real-time PCR (qRT-PCR) experiments. Predesigned TaqMan™-based assays (Thermo Fisher Scientific (Table 4.1)) for ghrelin, ghrelin-processing enzymes, chemosensors for fatty acids and

proteins, as well as the sweet/umami receptor subunit T1R3 were used, with each assay run in triplicate. Three housekeeping genes, namely β-2 microglobulin (B2M), hypoxanthine-guanine phosphoribosyltransferase (HPRT) and peptidylprolyl isomerase A (PPIA), were used based on their averaged stability value (0.001) determined by NormFinder (Department of Molecular Medicine (MOMA), Aarhus University Hospital, Denmark). A further DNase digestion with ezDNase kit (Invitrogen, SA, Australia) was performed to eliminate genomic DNA from total RNA samples. Negative controls were performed substituting RNA template with RNase-free water. Minus-reverse transcriptase controls were performed substituting reverse transcriptase with RNase-free water. Relative mRNA expression was calculated using the $2^{-\Delta CT}$ method[339].

Table 4.1. Details of qRT-PCR primers.

Target	Description	TaqMan™ Assay ID
Ghrelin	Gastrointestinal hormone	Mm00445450_m1
GOAT	Ghrelin-processing enzyme	Mm01200389_m1
PC1/3	Ghrelin-processing enzyme	Mm00479023_m1
FFAR2 (GPR43)	Short-chain fatty acid receptor	Mm02620654_s1
FFAR4 (GPR120)	Long-chain fatty acid receptor	Mm00725193_m1
CD36 (FAT)	Fatty acid translocase	Mm00432403_m1
GPR93 (GPR92)	Protein hydrolysate receptor	Mm02621109_s1
CaSR	Calcium and aromatic amino acid and protein hydrolysate receptor	Mm00443375_m1
mGluR4	Glutamate receptor	Mm01306128_m1
T1R3	Sweet and umami taste receptor subunit	Mm00473459_g1
B2M	Housekeeping gene	Mm00437762_m1
HPRT	Housekeeping gene	Mm01545399_m1
PPIA	Housekeeping gene	Mm02342429_g1

4.3.3.2. Immunohistochemistry

The experimental conditions for the immunohistochemistry experiments were adapted from a previous report[367]. Stomach cryosections (10 µm) were air-dried prior to three 5 min rinses in PBS (pH 7.4) containing 0.2% Triton X-100 (PBS-TX; Sigma-Aldrich, NSW, Australia). The tissue was then blocked for 60 min at room temperature with PBS-TX containing 10% normal donkey serum (Sigma-Aldrich). This was followed by three washes with PBS-TX for 2 min. Goat anti-ghrelin (1:800, ab104307, Abcam, VIC, Australia) and rabbit anti-CaSR (1:100, NBP2-38622, Novus Biologicals, CO, USA) antibodies were diluted in PBS-TX, and cryosections underwent overnight incubation at 4 °C. This was followed by a PBS-TX wash (3 times, 5 min) to remove unbound antibody. The cryosections were then incubated for 60 min at room temperature with donkey anti-rabbit conjugated to Alexa Fluor® 488 or donkey anti-goat conjugated to Alexa Fluor® 568 (Invitrogen) dissolved in PBS-TX (1:200). The cryosections were then rinsed with PBS-TX (3 times, 5 min) prior to mounting, using ProLong® Diamond Antifade reagent with DAPI (Invitrogen), and coverslipping. Single label controls were run to confirm no bleed-through of fluorescence under different filters. No immunofluorescence was detected in slides where the primary antibody was omitted. The specificity of the ghrelin antibody was confirmed in double-labelling experiments in the mouse stomach, with goat anti-ghrelin immunoreactive cells displaying 95.2% co-localisation with a second rabbit anti-ghrelin antibody (1:1600, ab129383, Abcam). Specific immunolabelling of the CaSR antibody was previously tested in the parathyroid gland and kidney[395].

4.3.3.3. Microscopy, imaging and cell quantification

Immunolabelling was visualised using a BX51 epifluorescence microscope (Olympus, SA, Australia) equipped with narrow filters for Alexa Fluor® 488 and 568. An XM10 monochrome camera (Olympus) was used to acquire the images. CellSens Dimensions Imaging Software (Olympus) was used to adjust the brightness and contrast.

Cell counts were performed in 5-6 non-consecutive sections per gastric region and animal. Immunopositive cells were manually counted in a 159 µm × 159 µm square area at the base of the glandular region (i.e. location of gastric ghrelin[202] and CaSR[396] immunopositive cells), using the software FIJI[397].

4.3.4. Statistical analysis

Data are expressed as mean ± SEM. All statistical analysis was performed using GraphPad Prism software version 7.02 (La Jolla California, USA). Differences in gonadal fat mass, blood glucose levels and the density of CaSR-immunopositive cells in gastric antrum between SLD and HFD groups were evaluated by unpaired Student's *t*-test. Differences in weekly body weight, mRNA expression, density of ghrelin-immunopositive cells, and percentage of co-expression of ghrelin and CaSR were determined by two-way ANOVA followed by Sidak *post hoc* test. Statistical significance for unpaired Student's *t*-test and two-way ANOVA is defined as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Statistical significance for Sidak *post hoc* test is indicated as $^{\#}p < 0.05$, $^{\S}p < 0.01$ and $^{\wedge}p < 0.001$.

4.4. RESULTS

4.4.1. Metabolic parameters in SLD and HFD mice

Weekly weight, gonadal fat mass and blood glucose levels are illustrated in Figure 4.1. HFD-fed mice weighed significantly more than SLD-fed mice by the end of the experiment (Figure 4.1a; HFD: 48.1 ± 1.6 g vs SLD: 36.2 ± 0.7 g; diet effect, $p < 0.001$; time effect, $p < 0.001$; interaction, $p < 0.001$). Gonadal fat pad mass was significantly increased in HFD mice compared to SLD mice after 12 weeks of diet (HFD: 2.5 ± 0.2 g vs SLD: 0.9 ± 0.05 g; $p < 0.001$; Figure 4.1b). Furthermore, *ad libitum* fed blood glucose levels at week 12 were significantly higher in HFD mice compared to SLD mice (HFD: 13.8 ± 0.8 mM vs SLD: 10.9 ± 0.5 mM; $p < 0.05$; Figure 4.1c).

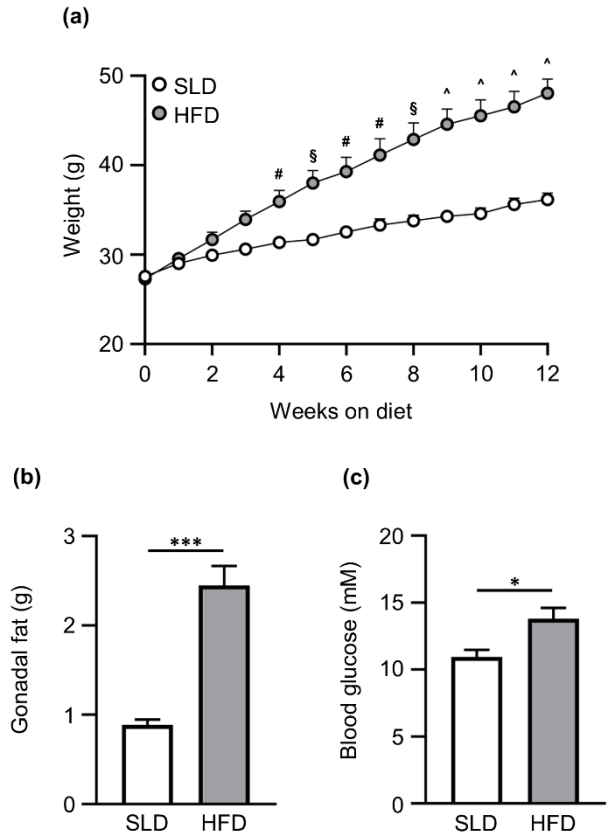

Figure 4.1. Metabolic parameters of C57BL/6 mice on a standard laboratory diet (SLD) or high-fat diet (HFD) for 12 weeks.

(a) HFD mice (n = 14) gained significantly more weight than SLD-fed mice (n = 14). HFD mice presented higher (b) fat mass and (c) blood glucose levels than SLD mice after 12 weeks in the diet (n=7/group). Data are expressed as mean ± SEM. Two-way ANOVA followed by Sidak *post hoc* test was used to determine the differences in body weight, and unpaired Student's *t*-test was used to assess differences in gonadal fat mass and blood glucose levels. Statistical significance for two-way ANOVA and unpaired Student's *t*-test is defined as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Statistical significance for Sidak *post hoc* test is denoted as $^{\#}p < 0.05$, $^{\S}p < 0.01$ and $^{\wedge}p < 0.001$.

4.4.2. Gastric nutrient chemosensors, ghrelin and ghrelin-processing enzymes mRNA expression in SLD and HFD mice

Relative mRNA levels of nutrient chemosensors, ghrelin and enzymes involved in ghrelin production in the corpus and antrum of SLD and HFD-mice are shown in Figure 4.2. Transcript levels of ghrelin (Figure 4.2a) and GOAT (Figure 4.2b) were higher in corpus compared to antrum (region effect, $p < 0.01$); however, they remained unchanged by HFD. In contrast, mRNA expression of PC1/3 (Figure 4.2c) was higher in the antrum compared to the corpus (region effect, $p < 0.001$). Further, there was a diet effect ($p < 0.05$) and an interaction ($p < 0.05$) due to an increase of PC1/3 expression in the gastric antrum of HFD mice (1.5-fold higher, $p < 0.01$).

The mRNA expression of fatty acid receptors FFAR2 (Figure 4.1d) and FFAR4 (Figure 4.1e) was higher in the antrum than corpus (region effect for both receptors, $p < 0.001$), however, there was no diet effect. In contrast, the expression of the fatty acid transporter, CD36 (Figure 4.1f), was lower in the antrum compared to the corpus (region effect, $p < 0.001$), with no difference between SLD and HFD mice.

The mRNA expression of receptors for protein digestion products was higher in the antrum compared to the corpus for GPR93 (region effect, $p < 0.001$; Figure 4.2g), CaSR (region effect, $p < 0.001$; Figure 4.2h) and mGluR4 (region effect, $p < 0.001$; Figure 4.2i). Although, there was no diet effect on mRNA transcript levels of GPR93 and mGluR4, there was a diet effect ($p < 0.01$) and interaction

($p < 0.05$) due to the increase in antral CaSR in HFD mice (1.6-fold higher; $p < 0.01$).

The mRNA levels of T1R3 (sweet and umami receptor subunit) were higher in the antrum compared to the corpus (Figure 4.2j; region effect, $p < 0.001$), with no significant effect of HFD in the expression of this chemosensor.

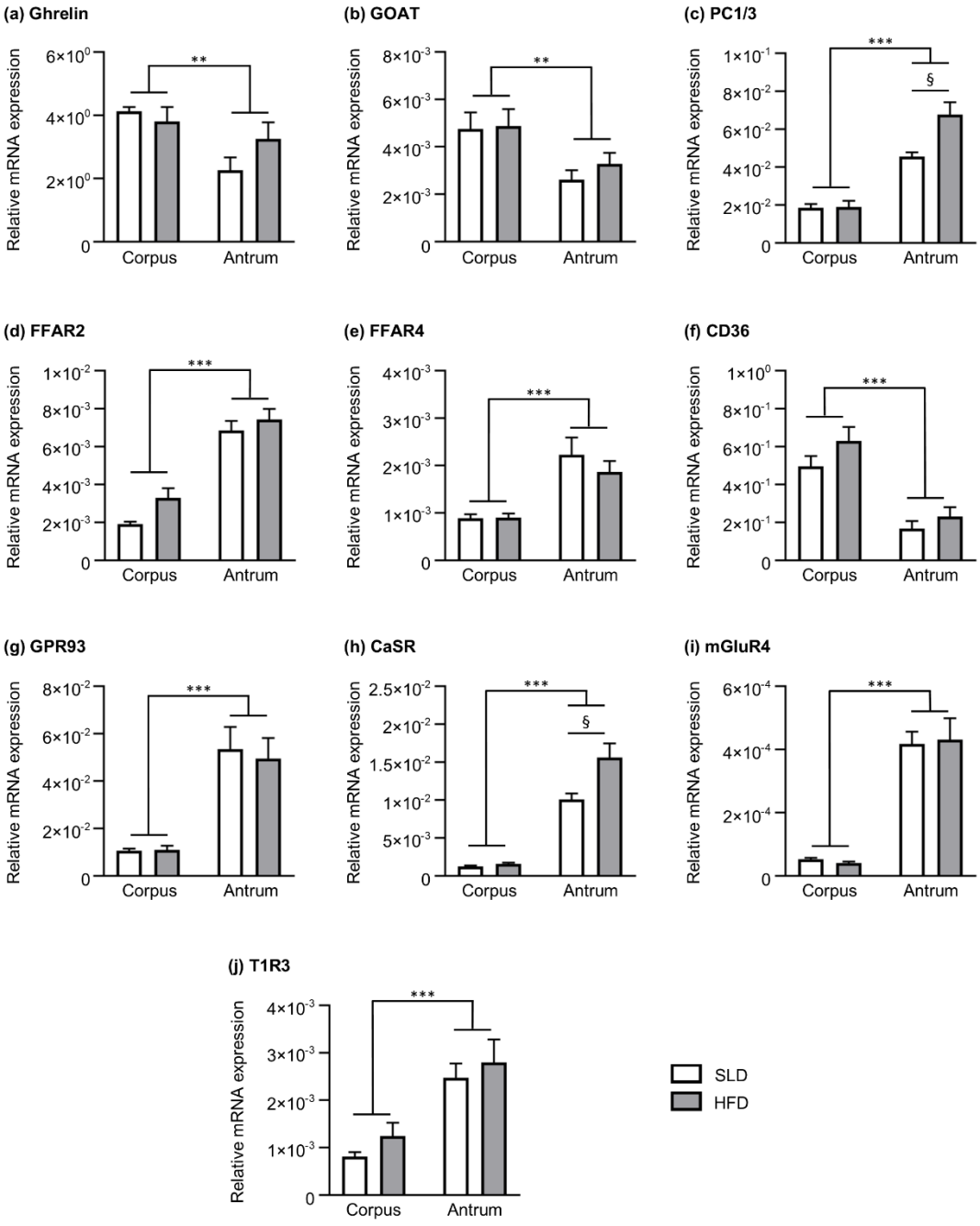
(a) Ghrelin
(b) GOAT
(c) PC1/3
(d) FFAR2
(e) FFAR4
(f) CD36
(g) GPR93
(h) CaSR
(i) mGluR4
(j) T1R3
Relative mRNA expression
Corpus
Antrum
SLD
HFD
**
***
§

Figure 4.2. Nutrient chemosensors, ghrelin and ghrelin-processing enzymes relative mRNA expression in the corpus and antrum of 12-week standard laboratory diet (SLD, *n* = 5) or high-fat diet (HFD, *n* = 5-7) fed mice.

Relative mRNA expression of (a) ghrelin, the ghrelin-processing enzymes (b) GOAT and (c) PC1/3, the nutrient chemosensors for the detection of (d-f) fatty acids, (g-i) protein digestion products and (j) sweet and umami taste. The expression is relative to the average housekeeper values for PPIA, HPRT and B2M. Data are expressed as mean ± SEM. Differences in gene expression were determined by two-way ANOVA followed by Sidak *post hoc* test. Statistical significance for two-way ANOVA is defined as $^{**}p < 0.01$ and $^{***}p < 0.001$. Statistical significance for Sidak *post hoc* test is denoted as $^{\S}p < 0.01$.

4.4.3. The density of ghrelin and CaSR immunopositive cells in SLD and HFD mice

Immunofluorescence studies were performed to assess the HFD-induced changes in the density of ghrelin immunopositive cells. Further, due to the obesity-induced increase in antral CaSR mRNA expression, the density of CaSR immunopositive cells was also determined. The number of ghrelin-positive cells was higher in the antrum (SLD antrum: 18.0 ± 0.8 cells/unit area; HFD antrum: 19.2 ± 1.2 cells/unit area) compared to the corpus (SLD corpus: 11.5 ± 1.1 cells/unit area; HFD corpus: 12.3 ± 0.7 cells/unit area; $p < 0.001$; Figure 4.3a); a finding that was associated with the higher density of ghrelin cells/unit area in the glandular base of the antrum compared to the corpus (see Supplementary information: Figure 4.5). Furthermore, the density of ghrelin-positive cells in both gastric regions remained stable in SLD and HFD conditions. Immunolabelling for CaSR was observed in the gastric antrum only, with no change in the density of CaSR-positive cells in HFD compared to SLD mice (SLD antrum: 15.8 ± 1.1 cells/unit area vs HFD antrum: 16.2 ± 1.2 cells/unit area; Figure 4.3b).

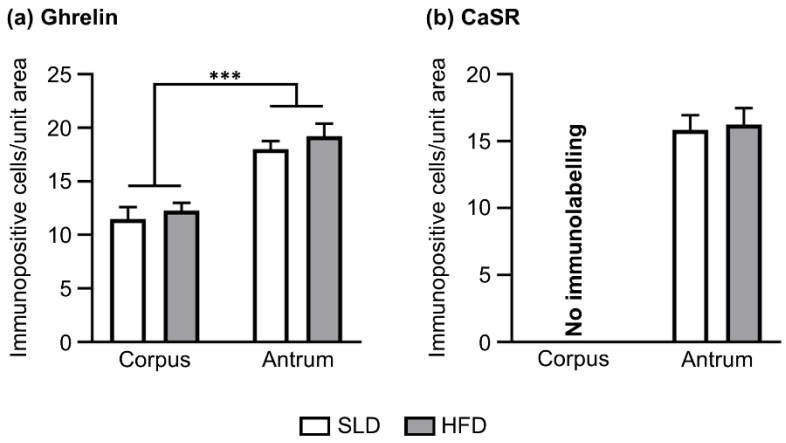

Figure 4.3. Density of ghrelin and CaSR immunopositive cells in the glandular base of gastric corpus and antrum of mice fed standard laboratory diet (SLD, *n* = 5) or high-fat diet (HFD, *n* = 5) for 12 weeks.

(a) Cell counts showed a higher density of ghrelin cells in the glandular base of the gastric antrum than corpus. A comparable number of ghrelin cells was observed in the corpus of SLD and HFD mice. Similarly, comparable numbers of ghrelin cells were found in the antrum of SLD and HFD mice. (b) CaSR cells were located in the antrum only, with no immunolabelling observed in the corpus. A comparable number of CaSR cells were observed in the antrum of SLD and HFD mice. Cell counts are the mean value of 5-6 non-adjacent tissue sections per mouse and gastric region (unit area = 159 µm × 159 µm). Two-way ANOVA followed by Sidak *post hoc* test was used to assess the differences in the number of ghrelin cells, and unpaired Student's *t*-test was used to assess differences in the number of CaSR cells. ***$p < 0.001$.

4.4.4. Co-expression of ghrelin and CaSR in the gastric antrum of SLD and HFD mice

Dual immunofluorescence experiments of ghrelin and CaSR were performed to determine the expression of CaSR within ghrelin cells of the gastric antrum of SLD and HFD mice. The majority of gastric antral immunopositive cells were found to co-express ghrelin and CaSR in SLD (14.4 ± 1.0 cells/unit area) and HFD mice (15.0 ± 1.0 cells/unit area; Figure 4.4a, 4.4d-e). Accordingly, nearly 80% of ghrelin immunopositive cells in the glandular base of antrum expressed CaSR, with no difference between SLD (79.8 ± 2.8% co-localisation) and HFD mice (78.7 ± 3.4% co-localisation; Figure 4.4b). Over 90% of CaSR immunopositive cells contained ghrelin, irrespectively of the type of diet (SLD: 91.2 ± 0.8% co-localisation vs HFD: 93.2 ± 2.0% co-localisation; Figure 4.4c).

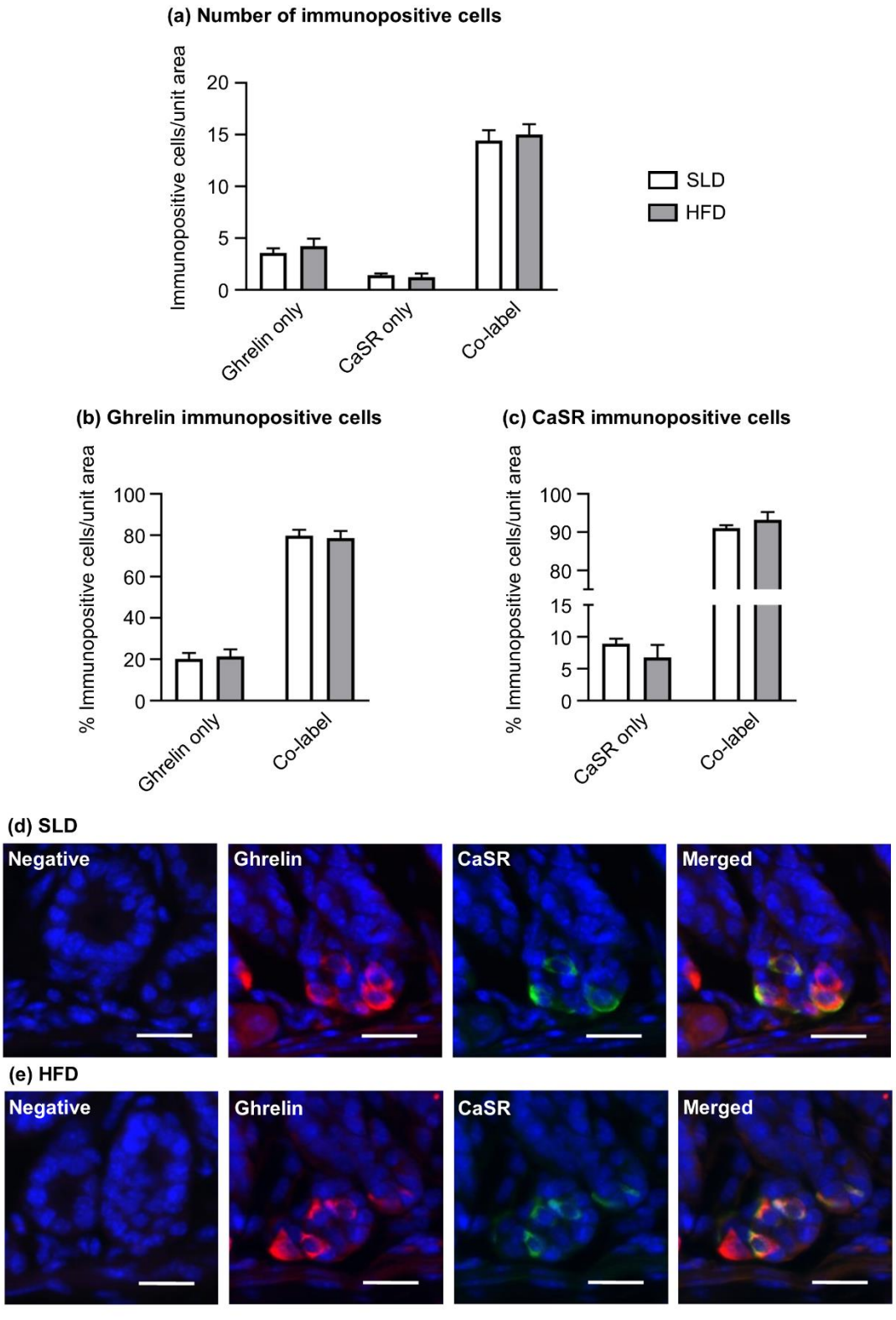
(a) Number of immunopositive cells
Immunopositive cells/unit area
20
15
10
5
0
Ghrelin only
CaSR only
Co-label
SLD
HFD
(b) Ghrelin immunopositive cells
% Immunopositive cells/unit area
100
80
60
40
20
0
Ghrelin only
Co-label
(c) CaSR immunopositive cells
% Immunopositive cells/unit area
100
90
80
15
10
5
0
CaSR only
Co-label
(d) SLD
Negative
Ghrelin
CaSR
Merged
(e) HFD
Negative
Ghrelin
CaSR
Merged

Figure 4.4. Co-expression of ghrelin and CaSR in the antrum of mice fed standard laboratory diet (SLD, *n* = 5) and high-fat diet (HFD, *n* = 5) for 12 weeks.

(a) Most immunopositive cells co-expressed ghrelin and CaSR. (b) Approximately 80% of ghrelin immunopositive cells expressed CaSR in SLD and HFD-fed mice, (c) and over 90% of CaSR immunopositive cells contained ghrelin, independently of the diet. (d-e) Images of ghrelin and CaSR co-expression in the gastric antrum of SLD and HFD mice. Cell counts are the mean counts of 5-6 tissue sections per mouse and gastric region (unit area = 159 µm × 159 µm). Data are expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by Sidak *post hoc* test.

4.5. DISCUSSION

The current study found that HFD-induced obesity did not alter the mRNA expression of ghrelin and a wide range of nutrient chemosensors in the mouse stomach. However, antral CaSR mRNA levels were augmented after chronic HFD feeding. Mechanistic studies have shown the involvement of CaSR in the modulation of acyl ghrelin secretion[193,205]. Therefore, this report further investigated the changes in the number of CaSR and ghrelin immunopositive cells, as well as their co-expression in HFD-induced obesity, and showed that HFD-induced obesity did not affect the density of CaSR and ghrelin immunopositive cells in the mouse stomach. Furthermore, CaSR was highly expressed within the antral ghrelin cell population of lean and HFD-induced obese mice in comparable proportions.

The expression of nutrient receptors in the mouse stomach is known to be regional, with a previous report showing that a large repertoire of gastric nutrient chemosensors are highly expressed in the antrum, compared to the corpus[367]. The current study confirmed these findings, showing that most chemosensors presented higher mRNA expression in the antrum compared to the corpus, with the exception of CD36. The higher expression of CD36 in the gastric corpus may be explained by the enrichment of this fatty acid transporter in corpus-predominant parietal[359,367] and ghrelin cells[367]. Furthermore, this report extends previous knowledge on the expression of gastric nutrient chemosensors, by defining changes in their mRNA levels in a well-established diet-induced obese mouse model[393,394] with elevated body weight, fat mass and blood glucose levels. HFD mice presented an increased mRNA expression of PC1/3 and

CaSR compared to SLD mice, specifically in the gastric antrum. PC1/3 is responsible for catalysing the conversion of proghrelin into mature ghrelin[158,398], however, this enzyme is also involved in the proteolytic cleavage of multiple prohormones, including prosomatostatin[398,399] and progastrin[348,398]. Ghrelin levels are known to be decreased in obesity[260,293,294], while the density of somatostatin-producing cells remains unchanged in the gastric mucosa of obese mice[294], and plasma gastrin levels are increased in HFD mice[254]. Therefore, it is possible that the increase of PC1/3 mRNA expression in the gastric antrum is responsible for the increased plasma gastrin levels[254], rather than the reduced ghrelin levels observed in HFD mice[294]. However, studies are required to elucidate the effect of increased PC1/3 on gastrin and ghrelin secretion in HFD-induced obesity.

It is well established that circulating ghrelin levels are reduced in obesity[260,293,294]. The current study shows no significant difference in ghrelin mRNA levels and density of ghrelin immunopositive cells in HFD-induced obese mice compared to control mice. Previous reports have shown similar findings in lean and obese humans[209,400], as well as SLD and HFD rats[401]. Additionally, it has been demonstrated in human and rodent studies that, the obesity-dependent reduction of ghrelin secretion, occurs despite unchanged[209], decreased[273] and increased[294] ghrelin mRNA levels and density of ghrelin cells. This suggests that these fluctuating parameters may not be a determining factor for the reduction of ghrelin secretion in obesity. Moreover, most reports have shown a stable expression of GOAT in obesity[393,402,403]. Consistent with these

findings, the current study shows that GOAT mRNA levels remained unchanged in HFD-induced obese mice compared to SLD mice.

The mRNA expression of the CaSR is known to peak in the antrum of humans[206] and mice[367,396], in comparison to other gastric regions. In agreement with previous studies[367,396], this report demonstrated higher CaSR mRNA levels in the antrum compared to the corpus, and an antral-specific immunolabelling. There is minimal information available on the expression of gastric CaSR in obesity, with the current report showing an increase in antral CaSR mRNA expression in HFD-induced obese mice compared to controls. In contrast, a previous report, using gastric tissue from obese humans, showed a region-specific downregulation of antral CaSR mRNA levels[206]. It is known that gene expression of nutrient chemosensors is modulated by fasting and feeding[404,405], diet[406-408] and grade of obesity[409]. While the current study showed CaSR mRNA expression from *ad libitum* fed mice, the human study presented CaSR mRNA levels from fasted obese individuals and organ donors[206]. Consequently, the contrasting results may reflect differences in feeding state or simply species variations. Moreover, these findings demonstrate a region-specific alteration in the human and murine antral CaSR mRNA expression under chronic excess energy intake, and call for further research to determine if changes in CaSR in HFD-induced obesity are associated with antral impairment of gastric hormone secretion, such as ghrelin.

CaSR is a chemosensor for calcium[410], aromatic amino acids[410] and protein hydrolysates[107], which when activated stimulates the secretion of multiple gut

hormones, including gastrin[327], cholecystokinin[322] and glucagon-like peptide 1[107]. However, the role of CaSR in ghrelin secretion is less established, with reports showing that activation of CaSR results in an increase[193,205] or decrease[193] in acyl ghrelin secretion. For example, mechanistic experiments using mouse gastric mucosal cells have shown that only supraphysiological concentrations of the CaSR agonist, $CaCl_2$ (40 mM), significantly decrease acyl ghrelin release[193]. Similarly, the CaSR positive allosteric modulator, R-568, decreased acyl ghrelin secretion in the presence of 1.8 mM $CaCl_2$ in the cell culture media. However, this effect was shifted to stimulation when R-568 was tested under higher $CaCl_2$ concentrations that were initially ineffective in the mobilisation of acyl ghrelin secretion (4 mM)[193]. These results illustrate the complex nature of CaSR, which has different ligand binding sites[379,411] that, upon activation, are able to couple numerous G proteins and downstream pathways that interact to contribute to cooperative responses[379,412], plausibly modulating the decrease or increase of acyl ghrelin secretion. Previous reports have demonstrated that peptones stimulate acyl ghrelin secretion from gastric mucosal segments from humans[206] and mice[205]. In contrast, peptones down-regulate total ghrelin secretion (i.e. containing mostly des-acyl ghrelin) from human gastric mucosal segments[206]. Mechanistic studies using the ghrelinoma MGN3-1 cell line have shown that the peptone-induced secretion of acyl ghrelin is partially reversed by the CaSR negative allosteric modulator, calhex-23[205]. However, the peptone-induced inhibition of total ghrelin secretion from human mucosal segments is unchanged by the negative allosteric modulator, NPS-2143[206]. Overall, these findings suggest that acyl and des-acyl ghrelin secretion are independently modulated, with CaSR possibly only participating in the

control of acyl ghrelin secretion. However, *in vivo* studies are necessary to confirm the role of this chemosensor in acyl ghrelin secretion. Supporting this possibility, the current report established a high degree of ghrelin and CaSR co-expression in the antrum of the mouse stomach, which suggests a direct role of CaSR in the control of ghrelin secretion from antral ghrelin cells. Furthermore, the number of CaSR immunopositive cells and co-expression with ghrelin were comparable in lean and HFD-induced obese mice. Therefore, the characteristic reduction in ghrelin secretion in obesity[260,293,294] is not due to changes in the number of CaSR immunopositive cells and/or the degree of co-expression of ghrelin and CaSR. However, as a limitation of the current study, quantitative protein expression of CaSR was not measured. Therefore, whether the protein level of CaSR is altered in HFD-induced obesity and responsible for the reduction in ghrelin secretion in obesity requires further investigation. Moreover, species differences in the expression of nutrient chemosensors are possible. Accordingly, a careful comparison between rodent and human studies is necessary.

4.6. CONCLUSIONS

This study provided information on the expression of nutrient chemosensors and ghrelin in a diet-induced obese mouse model. Most nutrient chemosensors investigated in this report had a comparable mRNA expression in lean and HFD-induced obese mice. Similarly, ghrelin mRNA expression and density of ghrelin immunopositive cells remained unchanged in HFD mice, indicating that reduced circulating levels of ghrelin in obesity are caused by a reduction in ghrelin protein production and/or secretion. Gastric nutrient chemosensors are important contributors to the modulation of ghrelin secretion, which is critical for the regulation of food intake and energy homeostasis. Available research is contradictory and indicates that CaSR is a signalling mechanism for the reduction[193] and stimulation[193,205] of acyl ghrelin secretion. This report established a high co-expression of CaSR and ghrelin in the gastric antrum, suggesting a direct role of this chemosensor in the antral secretion of acyl ghrelin. Circulating ghrelin levels are reduced in obesity, and it is possible that the increase in antral CaSR mRNA in HFD-induced obesity may impact acyl ghrelin secretion. However, further research is needed to confirm translation at the protein level, and how this may affect gastric ghrelin secretion in obesity.

Author Contributions: M.N.S., H.L. and A.P. designed the study; M.N.S. conducted experiments and analysed data; S.C. provided samples for gene expression studies. M.N.S. and A.P. wrote the manuscript; All authors contributed to data interpretation and the drafting of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

4.7. SUPPLEMENTARY INFORMATION

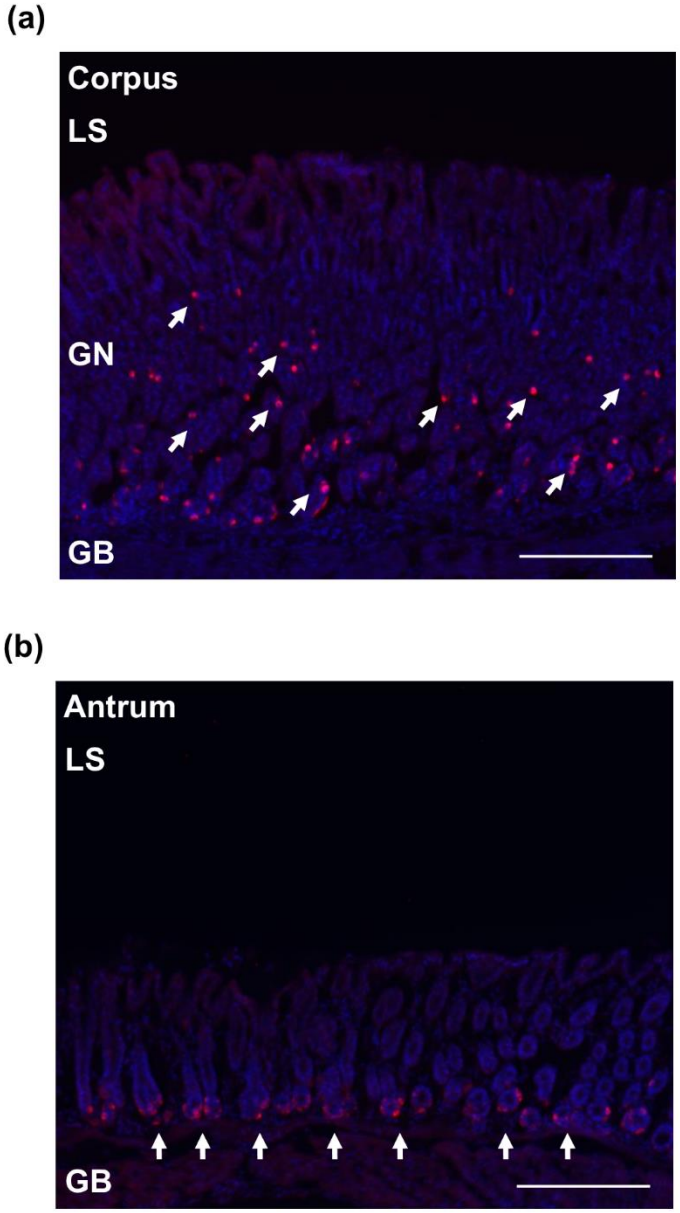

Figure 4.5. The gastric corpus presents a higher number of ghrelin immunopositive cells compared to the antrum.

(a) While the corpus presents numerous ghrelin cells uniformly distributed throughout the glandular base and neck of the tissue, (b) the gastric antrum presents a high density of ghrelin cells located in the glandular base of the tissue only. Abbreviations: LS, luminal side; GN, glandular neck; GB, glandular base. Scale bars = 200μm.

Chapter 5: General conclusions

This thesis has examined the transcriptional and protein expression of nutrient chemosensors for sweet compounds, protein digestion products and fatty acids in the mouse stomach and gastric ghrelin cell. Furthermore, the effect of an extensive array of nutrients, including glucose, peptones, L-Phe, intralipid fat emulsion and αLA, on the secretion of ghrelin was determined in experiments using mouse gastric mucosa. Similarly, the role of the LCFA chemosensors, FFAR4 and CD36, in the αLA and intralipid-dependent modulation of ghrelin secretion from mouse gastric mucosa was investigated. Moreover, studies in an obese mouse model examined the effect of HFD-induced obesity on the transcriptional expression of a repertoire of gastric nutrient chemosensors, as well as the protein expression of the chemosensor for calcium, aromatic amino acids and peptones, CaSR. Prominent findings reported in this thesis are illustrated in Figure 5.1.

5.1. THE NUTRIENT-SENSING CAPABILITY OF THE MOUSE STOMACH

The study presented in **Chapter 2** investigated the mRNA expression of gastric chemosensors for protein digestion products (GPR93, CaSR, mGluR4), fatty acids (CD36, FFAR2 and FFAR4), sweet and umami taste (T1R3), and taste-specific components of downstream cascades (TRPM5, GNAT2 and GNAT3) in the corpus and antrum of the mouse stomach. These murine gastric regions are comparable to their human counterparts in terms of anatomy[413], ghrelin expression[414], and nutrient-sensing phenotype of endocrine cells[351]. In this regard, Haid *et al.* demonstrated that murine and human antral endocrine cells present a comparable expression of chemosensors for protein digestion products[351]. Furthermore, immunolabelling experiments characterised the protein expression of T1R3, GPR93, CD36 and FFAR4 in the mouse stomach, and their co-expression with ghrelin. It was shown that gastric nutrient chemosensors displayed a regional expression, with most targets presenting higher mRNA levels in the antrum than in the corpus. Furthermore, immunolabelling experiments demonstrated that most nutrient chemosensors had a higher immunolabelling density in the gastric antrum than corpus. In addition, the distinctive immunolabelling pattern of each nutrient chemosensor may be indicative of their specific gastric functions. For instance, the antral-specific immunolabelling of GPR93 indicates that detection of peptones is important for antral-specific functions. In this regard, it is possible that GPR93 is involved in the secretion of antral-predominant hormones, such as gastrin[327,350]. Moreover, T1R3, GPR93, FFAR4 and CD36 were highly co-localised with ghrelin. Findings that strongly suggested a specific role for these chemosensors in gastric ghrelin cells.

The study presented in **Chapter 3** determined the gastric mucosal modulation of TG and AG secretion by multiple nutrients (2 and 20 mM glucose, 5% mannitol, 5% peptone, 20 mM L-Phe, 5% intralipid fat emulsion and 2 mM αLA). Furthermore, expanding on the findings from **Chapter 2**, the role of FFAR4 and CD36 in the intralipid and αLA-dependent secretion of TG and AG was investigated. Experiments assessing the role of nutrients in the secretion of ghrelin showed that TG and AG were unaffected by different glucose concentrations, strongly stimulated by peptones, while L-Phe reduced AG secretion only. α-LA decreased AG only, while intralipid simultaneously inhibited TG and stimulated AG secretion. These findings demonstrated that gastric secretion of TG and AG is regulated in a nutrient-specific manner. Further, the hypertonic mannitol solution did not affect TG and AG secretion, indicating that hyperosmolarity is not a major player for the modulation of gastric ghrelin secretion. This contrasts to the effect of hyperosmolarity in the small intestine[241], where hypertonic solutions suppress circulating ghrelin levels, possibly via stimulation of other gut hormones that display ghrelin-inhibiting effects, such as CCK[242]. Additionally, it was shown that nutrient mixtures, such as intralipid, produced different ghrelin responses than single nutrients, such as α-LA. In this regard, a previous report has shown that different fatty acids generate distinct secretory ghrelin responses from MGN3-1 cells, with octanoic acid increasing AG secretion and α-LA reducing it[207]. Therefore, it is possible that the intralipid-evoked secretion of AG resulted from a cooperative effect of the LCFA mixture contained in the intralipid solution. Further, it is also possible that the abundance of acyl donors (i.e. fatty acids) contained in the intralipid solution was responsible for the increase in AG secretion. In this regard, it has

been shown that ghrelin cells use β-oxidation to process LCFAs into MCFAs for the acylation of ghrelin[163].

Results from the secretion studies investigating the role of the LCFA chemosensors, FFAR4 and CD36, in the intralipid and αLA-dependent secretion of TG and AG showed that FFAR4 and CD36 inhibitors did not reverse the effects of the nutrients. These findings indicated that, despite the high co-expression of FFAR4 and CD36 with gastric ghrelin, these LCFA chemosensors do not mediate the effects of intralipid and αLA in the secretion of TG and AG. As discussed in **Chapter 1**, many conflicting findings have been reported on the role of FFAR4 in the secretion of ghrelin. While different methodological approaches could partly explain the conflicting results, emerging research is revealing the complex pharmacology[379,385,411,412,415] of G-protein coupled receptors (GPCRs), such as FFAR4. Indeed, nutrient-associated GPCRs often present a promiscuous nature[385,416] as well as different binding sites[411,417]. Therefore, upon activation, different downstream cascades may be triggered[225,379,412,418], which can also interact to generate distinctive cellular responses[225,379,417,418]. Additionally, GPCRs can form oligomeric complexes and signal from multiple cellular compartments[415], which makes GPCR signalling much more complex than conventionally anticipated. In this regard, scarce information is available on FFAR4 signalling[384], as well as limited availability of chemical probes (i.e. agonists and antagonists) for the study of FFAR4 pharmacology[384]. Results from **Chapter 3** demonstrated that intralipid and αLA-dependent secretion of ghrelin was not reversed by the only available FFAR4 antagonist[384]. However, the development of more FFAR4

chemical probes will allow future studies to further characterise the FFAR4 signalling pathways and better define the role of this LCFA receptor in the secretion of ghrelin. Moreover, regarding CD36, it was observed that a widely used CD36 inhibitor[119,354,419] alone reduced TG secretion to the same level as that observed in the presence of the intralipid solution, which indicates that CD36 might participate in the basal secretion of TG. Future studies specifically investigating the role of CD36 in ghrelin secretion are needed to further investigate and corroborate this finding.

Previous research in gut nutrient sensing has been primarily focused on the small intestine, while it has been typically assumed that the stomach responds to the mechanical stimulation generated by the ingestion of food[420]. Findings from **Chapters 2** and **3** add extensive evidence on the nutrient-sensing ability of the mouse stomach. Overall, these studies have demonstrated that a variety of nutrient chemosensors are expressed in the mouse stomach and gastric ghrelin cells. It has also been shown that the stomach responds to chemical/nutrient stimulation for the modulation of ghrelin secretion. However, further studies are necessary to identify the specific nutrient-sensing pathways mediating these effects. As discussed in **Chapter 1**, the postprandial regulation of ghrelin levels is not well understood due to the multifactorial modulation of ghrelin secretion. The ghrelin secretion studies presented in this PhD thesis, minimise systemic inputs, such as the ghrelin-inhibiting effect of insulin[247]. However, it should be noted that our secretion studies reflect the regulation of ghrelin secretion by the investigated nutrients, and possibly, by other gastric hormones, such as gastrin, which is known to be secreted in response to

nutrients[327] and may also influence ghrelin secretion[255]. Similarly, gastric nutrient chemosensors that were expressed in a variety of gastric cell populations, such as CD36, may also influence ghrelin secretion indirectly. Moreover, findings from previous studies[206,207] and this thesis have demonstrated that TG (i.e. mostly comprising DAG) and AG do not necessarily follow the same patterns of secretion. These findings add an extra layer of complexity into the study and interpretation of ghrelin secretion. It is possible that differences in the secretion of TG and AG reflect GOAT activity. In this regard, future studies are needed to determine how different nutrients affect the activity of this enzyme. However, given that AG and DAG may have different functional effects in the body[290], it is also possible that the release of AG and DAG is regulated by independent pathways.

5.2. THE EFFECT OF HFD-INDUCED OBESITY ON THE EXPRESSION OF NUTRIENT CHEMOSENSORS IN THE MOUSE STOMACH AND GASTRIC GHRELIN CELLS

Chapter 4 extended on the findings presented in **Chapter 2**, by investigating the effect of HFD-induced obesity on the expression of nutrient chemosensors in the mouse stomach. Therefore, mRNA expression studies investigated the same set of gastric nutrient chemosensors, with the exception of taste-transduction components, in the corpus and antrum of lean and HFD-induced obese mice. In addition, the gastric immunolabelling of CaSR and co-expression with ghrelin in SLD and HFD mice were also assessed. Consistent with **Chapter 2**, regional patterns of mRNA expression were maintained, with most nutrient chemosensors presenting higher mRNA transcript levels in the antrum than corpus. Additionally, it was observed that some non-significant regional differences in mRNA expression from **Chapter 2** reached statistical significance in **Chapter 4**. This was the case for CD36, ghrelin and GOAT, which all displayed significantly higher expression in the gastric corpus compared to antrum in **Chapter 4**. While this might partly be explained by differences in statistical analysis (i.e. one-way ANOVA (**Chapter 2**) vs two-way ANOVA (**Chapter 4**)), it is also possible that regional mRNA expression of some targets, such as CD36, become more defined in older mice (i.e. 8-week old (**Chapter 2**) vs 20-week old (**Chapter 4**)). Furthermore, in relation to the effect of HFD-induced obesity on mRNA expression of nutrient chemosensors, no changes were observed, with the exception of an antral-specific increase in CaSR. Accordingly, immunolabelling experiments focused on this chemosensor. CaSR immunopositive cells were observed in the gastric antrum

only. Findings that are consistent with previous reports[396], and emphasise the regional and distinctive protein expression of gastric nutrient chemosensors. Moreover, CaSR was highly co-expressed with antral ghrelin. However, HFD-induced obesity did not affect the density of antral CaSR immunopositive cells and degree of co-expression with ghrelin.

CaSR participates in the peptone-induced stimulation of AG secretion in MGN3-1 cells[205]. Additionally, it is known that peptone-evoked secretion of AG from gastric mucosal segments of obese individuals is impaired compared to lean individuals, whereas the peptone-dependent reduction in TG secretion is maintained[206]. These findings suggest that TG and AG secretion are controlled via different pathways. Further, although CaSR seems to be involved in AG secretion[205], mechanistic experiments have demonstrated that the peptone-dependent reduction in TG secretion is independent of CaSR activation[206]. Nonetheless, it is unknown whether CaSR disruption is responsible for the lack of effect of peptone on AG secretion in obesity. In this regard, the results from this chapter indicate that changes in the density of antral CaSR immunopositive cells and co-expression with antral ghrelin are not responsible for the alterations in AG secretion in obesity. However, it is possible that the increase in antral CaSR mRNA levels, observed in HFD mice, leads to an increase in CaSR protein levels, which may affect the release of AG. Therefore, further studies are necessary to determine CaSR protein levels and how this affects ghrelin secretion in obesity. Nonetheless, evidence for the involvement of CaSR in AG secretion[193,205] and the high co-expression of CaSR with ghrelin in the current studies suggests a direct effect of this chemosensor on ghrelin cells.

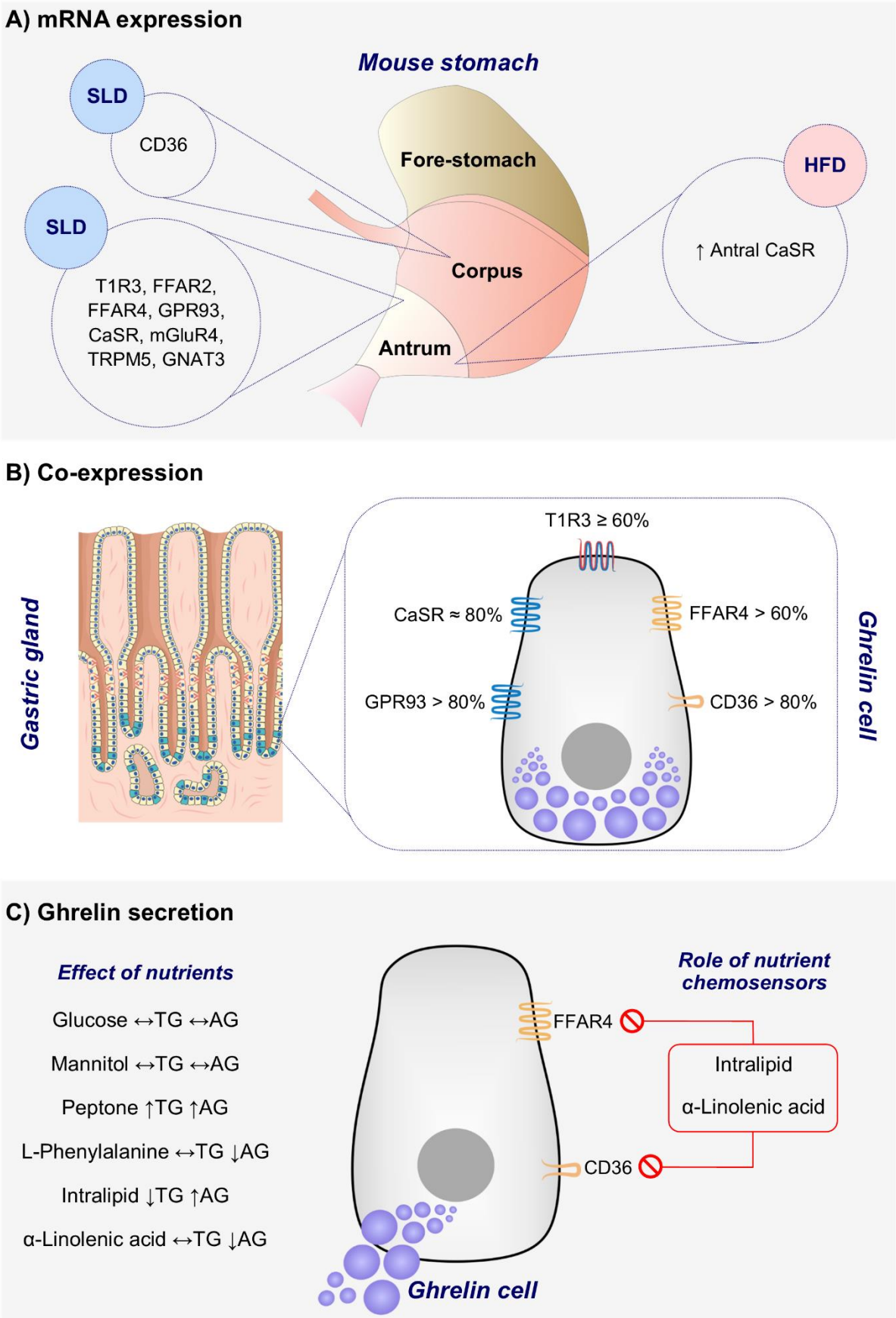
A) mRNA expression
Mouse stomach
SLD
CD36
SLD
T1R3, FFAR2, FFAR4, GPR93, CaSR, mGluR4, TRPM5, GNAT3
Fore-stomach
Corpus
Antrum
HFD
↑ Antral CaSR
B) Co-expression
Gastric gland
T1R3 ≥ 60%
CaSR ≈ 80%
FFAR4 > 60%
GPR93 > 80%
CD36 > 80%
Ghrelin cell
C) Ghrelin secretion
Effect of nutrients
Glucose ↔TG ↔AG
Mannitol ↔TG ↔AG
Peptone ↑TG ↑AG
L-Phenylalanine ↔TG ↓AG
Intralipid ↓TG ↑AG
α-Linolenic acid ↔TG ↓AG
Role of nutrient chemosensors
FFAR4
Intralipid
α-Linolenic acid
CD36
Ghrelin cell

Figure 5.1. Overview of prominent findings reported in this thesis.

(A) The mRNA expression of nutrient chemosensors in the mouse stomach is regional. Most of nutrient chemosensors investigated in this thesis displayed a higher mRNA expression in the gastric antrum. Additionally, it was observed that HFD-induced obesity specifically increased the mRNA expression of antral CaSR. (B) Ghrelin cells from the mouse stomach expressed chemosensors for the detection of all macronutrients. Gastric ghrelin cells presented a high degree of co-localisation with chemosensors for calcium and amino acids (CaSR), protein hydrolysates (CaSR and GPR93), sweet and umami taste (T1R3), and long-chain fatty acids (FFAR4 and CD36). (C) Ghrelin secretion from the mouse stomach is modulated by nutrients. Different nutrients, including peptones, L-phenylalanine, intralipid and α-linolenic acid, display a distinctive effect on total ghrelin (TG) and acyl ghrelin (AG) secretion. The effect of α-linolenic acid and intralipid on TG and AG secretion does not involve FFAR4 and CD36 activation.

5.3. CONSIDERATIONS AND LIMITATIONS

The studies contained in this thesis determined the expression of gastric nutrient chemosensors using qRT-PCR and immunofluorescence (i.e. semi-quantitative protein expression). While immunofluorescence experiments were required to determine the expression of nutrient chemosensors within the gastric ghrelin cell population, Western blot (i.e. quantitative protein expression) would be necessary to quantify and confirm the translation from mRNA transcript into protein, particularly in the case of the increase in antral CaSR mRNA in HFD-induced obesity (**Chapter 4**).

Circulating ghrelin levels are typically reduced after food intake[146]. However, findings from **Chapter 3** and previous reports[205-207] demonstrated that nutrients can also stimulate gastric ghrelin secretion. Mouse gastric mucosal segments were used to study gastric ghrelin secretion, with this approach chosen to minimise systemic inputs that add complexity to the study of gastric ghrelin secretion, such as the ghrelin-inhibiting effect of insulin[247]. Accordingly, it should be noticed that this *ex vivo* gastric mucosal model specifically reflects the local modulation of gastric ghrelin secretion. Therefore, results from these studies should be carefully interpreted in the context of an isolated model of the mouse stomach.

As limitation of **Chapter 3**, it was observed that the vehicle, 1% DMSO, disturbed the effects of protein digestion products (i.e. L-Phe and peptones) on AG secretion. The effect of DMSO on AG secretion mediated by protein digestion products was discussed in **Chapter 3**, and may be partly explained

by DMSO-induced alterations in the solubility and/or structure of the proteins and amino acids[376-378]. However, given the specific effect of DMSO on AG secretion, it is also possible that an alteration in GOAT activity could be involved in this effect. It should also be noted that 1% DMSO does not affect the viability of GI cell cultures during incubation periods of 1-24 hours[421,422]. Nonetheless, the effects of DMSO on ghrelin secretion should be considered in future studies.

The studies contained in this thesis were performed in mouse models. Therefore, species differences in the expression and function of nutrient chemosensors are possible. Gene expression studies from **Chapter 4** showed an antral-specific increase in CaSR mRNA levels in HFD mice, while previous studies, using tissue from obese humans, showed an antral-specific decrease in CaSR[206]. While conflicting results may be explained by numerous factors, such as differences in feeding states[404,405,423], diet[406-408] and grade of obesity[409,424] between the human and the mouse studies, it is possible that there are also species variations.

5.4. FUTURE STUDIES

Future investigations, using human samples, are needed to corroborate the findings from this thesis. Additionally, *in vivo* studies are necessary to interpret the results from mechanistic ghrelin secretion studies from a physiological perspective. On this subject, studies using knockout models will elucidate the relevance of GI nutrient chemosensors in energy homeostasis. Furthermore, it is important to note that knockout models of the ghrelin system (i.e. ghrelin, GHSR1a, GOAT) do not show strong feeding behavioural changes[281,283,285]. However, as discussed in **Chapter 1**, they often display reduced body fat and weight[281,283-285]. Further, ghrelin has an essential function in maintaining blood glucose levels[173]. Therefore, future *in vivo* studies will help to understand the relationship between nutrient sensing, circulating ghrelin levels, and energy and glucose homeostasis.

In terms of gastric ghrelin secretion, possible differences in the secretion of AG and DAG call for detailed measurement and interpretation of future ghrelin secretion studies, as well as careful quantification of GOAT expression and/or activity. Moreover, as discussed in **Chapter 1**, abundant evidence indicates that nutrient-sensing signals from the gastric lumen do not play a major role in the modulation of ghrelin secretion. For instance, gastric and small intestinal infusion of nutrients generates a comparable reduction in circulating ghrelin levels[233-235], indicating that ghrelin secretion is readily suppressed despite bypassing the stomach. Additionally, gastric ghrelin cells have no direct contact with the gastric lumen[202,203]. However, findings from **Chapter 2**, demonstrated luminal protein expression of FFAR4, suggesting that the stomach may be able

to detect luminal LCFAs, which could indirectly affect ghrelin secretion. Whether nutrient signals modulate ghrelin secretion from the lumen or blood circulation requires more detailed investigation. In this regard, ussing chamber experiments, with either luminal or basolateral nutrient stimulation, would provide important information on the postprandial control of ghrelin secretion.

5.5. CONCLUSION

Limited information is available on the nutrient signals contributing to the regulation of gastric ghrelin secretion. This thesis provided substantial information on the nutrient-sensing capability of the mouse stomach and gastric ghrelin cells in health and obesity, as well as the effect of an extensive range of nutrients on the modulation of gastric ghrelin secretion. The studies presented in this thesis provide new knowledge for a better understanding on the mechanisms controlling ghrelin secretion, which are necessary to establish effective strategies for the treatment of energy homeostasis disorders, such as obesity.

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